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Identification of *Abiotrophia adiacens* and *Abiotrophia defectiva* by 16S rRNA Gene PCR and Restriction Fragment Length Polymorphism Analysis

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***Abiotrophia adiacens* and *Abiotrophia defectiva*, previously referred to as nutritionally variant streptococci, *Streptococcus adjacens* and *Streptococcus defectivus*, respectively, are causes of infective endocarditis. We describe a method of identifying these two species and also of distinguishing them from 15 other major etiological pathogens of infective endocarditis by means of 16S rRNA gene PCR amplification followed by restriction fragment length polymorphism analysis (PCR-RFLP). The 16S rRNA genes were successfully amplified with a set of universal primers from all 17 species of bacteria examined, including viridans group streptococci. The RFLP patterns of *A. adiacens* and *A. defectiva* obtained by *Hae*III or *Msp*I digestion were readily distinguished from each other and from those of other bacteria. When PCR analysis was performed with the supernatant of a suspension of a boiled colony, the 16S rRNA genes of 80 of 82 isolates (97%) of *A. adiacens* and all isolates (11 of 11) of *A. defectiva* were amplified. The *Hae*III RFLP patterns of the isolates were the same as those of the corresponding type strains, although 28% of *A. adiacens* isolates revealed intraspecies polymorphism. The detection limit of this method was 0.1 pg of genomic DNA, as assessed by using the digoxigenin-labeling DNA detection system. Thus, the PCR-RFLP analysis that we developed is applicable for the routine detection of *Abiotrophia* from clinical specimens.**

The transfer of *Streptococcus adjacens* and *Streptococcus defectivus* to the new genus *Abiotrophia* as *Abiotrophia adiacens* and *Abiotrophia defectiva*, respectively, has recently been proposed on the basis of their 16S rRNA gene sequences and phenotypic characteristics (13). These species were first described by Frenkel and Hirsch (9) as new types of viridans group streptococci and colonize the oral cavity and the intestinal and genitourinary tracts as normal flora (10, 19). Like other viridans group streptococci, *Abiotrophia* species cause sepsis and bacteremia and are also one of the major pathogens of infective endocarditis, accounting for 5 to 6% of the occurrences this disease (5, 18). Furthermore, although the causative microorganism is not identified in approximately 15% of the cases, *Abiotrophia* species are likely to be responsible for many of the incidences of culture-negative endocarditis (18). In Japan, only eight cases of infective endocarditis caused by *Abiotrophia* have been reported so far (20). Blood culture-negative cases of this disease accounted for 16% of the Japanese total during the period from 1988 to 1992 (21).

Abiotrophia species have preferentially been referred to as nutritionally variant streptococci because of their fastidious nutritional requirements for growth. They hardly grow in the ordinary growth media for streptococci, such as sheep blood agar based on Trypticase soy medium, and require a supplement of L-cysteine (9) or pyridoxal (6). In the absence of the supplement, a streak of *Staphylococcus aureus* or *Staphylococcus epidermidis* provides suitable culture conditions, where *Abiotrophia* species grow as satellite colonies adjacent to the helper *Staphylococcus* species (15). In addition to this fastidiousness, aberrant morphological characteristics (cocci, filament formation, and bulbous swellings) and a changeable Gram-staining nature cause further difficulties in their identification (2, 4).

Data on the 16S rRNA gene sequences of bacteria have accumulated, and these data greatly contribute to the studies of bacterial phylogeny. On the basis of the sequence similarity of conserved regions around both the 5′ and 3′ ends of the genes, several sets of universal primers have been designed for amplification of most of the 16S rRNA genes from eubacteria (25). In addition, restriction fragment length polymorphism analysis (RFLP) following 16S rDNA PCR amplification (PCR-RFLP) has been successfully used for the identification of methanogens (11). The PCR-based assay is of great advantage in identifying some expected bacterial pathogens whose specific genes have not been cloned to identify the species or pathogens which are extremely fastidious or highly pathogenic. Thus, the 16S rRNA gene PCR-RFLP analysis seems very useful for the diagnosis of acute and subacute infectious endocarditis and bacteremia caused by *Abiotrophia* species. In this study, we developed a highly sensitive, nonradioactive 16S rRNA gene PCR-RFLP analysis method that clearly identifies *A. adiacens* and *A. defectiva* and distinguishes these from the other organisms that are major causes of infective endocarditis.

MATERIALS AND METHODS

Bacterial strains. *A. adiacens* ATCC 49175^T and *A. defectiva* ATCC 49176^T were used. *A. adiacens* 4096-P, isolated from a patient with infective endocarditis in 1984 in Japan, was a generous gift from H. Enari (Kyokuto Co., Tokyo, Japan). The following type and reference strains were also used to compare the restriction patterns of the 16S rRNA genes: *Streptococcus sanguis* ATCC 10556^T, *Streptococcus oralis* NCTC 11427^T, *Streptococcus gordonii* ATCC 10558^T, *Streptococcus mitis* NCTC 12261^T, *Streptococcus salivarius* subsp. *salivarius* ATCC 7073^T, *Streptococcus bovis* NCFB 597^T, *Streptococcus mutans* ATCC 25175^T, *Streptococcus sobrinus* ATCC 33478^T, *Streptococcus pyogenes* ATCC 12344^T, *Streptococcus pneumoniae* NCTC 7465^T, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 14990^T, *Enterococcus faecalis* NCTC 775^T, *Haemophilus influenzae* ID 984, and *Escherichia coli* ATCC 25922.

Culture conditions. The *Abiotrophia* strains and the other type and reference strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented or not supplemented with 10 μg of pyridoxal hydrochloride (Wako

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Pure Chemicals, Tokyo, Japan) per ml and 200 μg of L-cysteine (Kanto Chemicals, Tokyo, Japan) per ml, respectively, at 35°C for 20 h. *Abiotrophia* and viridans group streptococci were cultured under anaerobic conditions.

Preparation of genomic DNA for PCR analysis. (i) DNA preparation. Bacterial cells from a 1.5-ml culture were harvested, washed with ice-cold TE (50 mM Tris-HCl [pH 8.0], 1 mM EDTA), and then suspended in 100 μl of lysis buffer (10 mM Tris-HCl [pH 7.5], 5 mM EDTA, 25% [wt/vol] sucrose). Lysozyme (Seikagaku Kogyo, Tokyo, Japan) and mutanolysin (Sigma Chemical, St. Louis, Mo.) were added to final concentrations of 0.5 mg/ml and 10 U/ml, respectively. After incubation at 37°C for 15 min, the suspension was centrifuged and the cell pellet was resuspended in 100 μl of 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 0.5% sodium dodecyl sulfate, and 4 μg of RNase A per ml. After 15 min at 60°C, phenol saturated with 0.5 M Tris-HCl (pH 8.0) containing 1 mM EDTA and 0.5% sodium dodecyl sulfate was added, and the sample was incubated at 60°C for 10 min with vortexing every minute. The lysate was extracted twice with phenol and then once with chloroform, and the DNA was precipitated with ethanol. In order to determine the detection limit, DNA samples were further purified with a Qiaex II gel (Qiagen, Hilden, Germany), according to the manufacturer's procedure, to eliminate contaminated RNA. The DNA concentration was determined spectrophotometrically by measuring the A_{260}. The purified DNA was stored at −40°C until use.

(ii) DNA samples from *Abiotrophia* isolates. Several colonies on Columbia agar base (Becton Dickinson and Co., Cockeysville, Md.) supplemented with 5% sheep blood, 10 μg of pyridoxal per ml, and 200 μg of L-cysteine per ml were suspended in 100 μl of autoclaved distilled water. The suspensions were then heated at 100°C for 10 min and centrifuged at 20,000 × *g* for 10 min. The supernatant was used as a bacterial cell lysate for the PCR amplification.

PCR amplification of the 16S rRNA gene. The primers 5′-AGAGTTTGATCC TGGCTCAG-3′, corresponding to *E. coli* 16S rRNA gene positions 8 to 27, and 5′-GGCTACCTTGTTACGACTT-3′, corresponding to *E. coli* 16S rRNA gene positions 1492 to 1510 (25), were used. PCR was carried out in a 40-μl volume of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 0.64 μM (each) primer, and 1 U of *Taq* DNA polymerase (Pharmacia, Uppsala, Sweden). The reaction was performed for 35 cycles with a 9600 DNA thermal cycler (Perkin-Elmer Cetus, Foster City, Calif.), and the following cycling profile was used. The first DNA denaturation step was at 94°C for 2 min, and then the PCR cycles were 30 s of denaturation at 94°C, 30 s of annealing at 50°C, and 30 s of extension at 72°C. Finally, the reaction mixture was kept at 72°C for 6 min. To achieve a highly sensitive, nonradioactive means of detection, 8.0 μM digoxigenin-11-dUTP (DIG-dUTP Boehringer Mannheim, Mannheim, Germany) was included in the reaction mixture. Then the PCR was performed as described above, except that the annealing temperature was lowered to 44°C. An aliquot (10 μl) of the PCR products was separated on a 0.9% agarose gel prepared with 0.5× TBE (66 mM Tris-borate, 5 mM EDTA [pH 8.3]) in the presence of 0.5 μg of ethidium bromide per ml.

RFLP analysis. Aliquots (10 μl) of the PCR products were digested with 4 U of either *Hae*III or *Msp*I (New England Biolabs, Beverly, Mass.). After 1.5 h at 37°C, the samples were separated on a 1.8% agarose gel in the presence of 0.5 μg of ethidium bromide per ml, and the results were processed with Polaroid 665 film. Alternatively, DIG-labeled DNA fragments were transferred from an agarose gel to a nylon membrane (Hybond-N^+; Amersham, Arlington Heights, Ill.) with 20× SSC (3 M NaCl plus 0.3 M sodium citrate [pH 7.0]) at room temperature for 1.5 h, and then the membrane was washed twice with 2× SSC. DIG-labeled DNA fragments were detected immunologically with alkaline phosphatase-conjugated anti-DIG antibody and CDP-Star by the manufacturer's protocol. The results were processed with Hyperfilm MP (Amersham) or with a phosphascreen CM (Bio-Rad Laboratories, Hercules, Calif.) and were analyzed with a Molecular Imager and Molecular Analyst software (Bio-Rad).

DNA sequence data. The GenBank and DDBJ accession numbers of the 16S rRNA genes used in this study are as follows: *A. adiacens*, D50540; *A. defectiva*, D50541; *S. sanguis*, X53653; *S. oralis*, X58308; *S. gordonii*, D38483; *S. mitis*, D38482; *S. salivarius*, X58320; *S. bovis*, X58317; *S. mutans*, X58303; *S. sobrinus*, X58307; *S. pyogenes*, X59029; *S. pneumoniae*, X58312; *S. aureus*, X68412; *S. epidermidis*, L37605; *Enterococcus* sp., X76177; *H. influenzae*, M35019; and *E. coli*, J01859. Since the 5′ and 3′ ends of the genes have not been entirely cloned in some species, the nucleotide length of the *Hae*III or *Msp*I restriction fragments containing either end was estimated by alignment of the respective sequences to that of *S. sanguis* (26), the complete 16S rRNA gene sequence of which has been processed by using the Genetyx multialignment software program (Genetyx, Tokyo, Japan).

RESULTS

PCR-RFLP analysis of the major bacterial pathogens of infective endocarditis. A PCR amplification was performed with a pair of primers reported to be suitable for the amplification of the 16S rRNA genes from most eubacteria (25). Genomic DNAs purified from the type strains of two *Abiotrophia* species and 15 type and reference strains of etiologic microorganisms of infective endocarditis, including viridans group streptococci, *S. pyogenes*, *S. pneumoniae*, staphylococci, *E. faecalis*, *H. influenzae*, and *E. coli*, were used as templates for the amplification. As indicated in Fig. 1a, one major PCR product of approximately 1.5 kb in length was detected, and the amplification efficiency was comparable among all samples. These fragments correspond to nearly the entire part of each 16S rRNA gene.

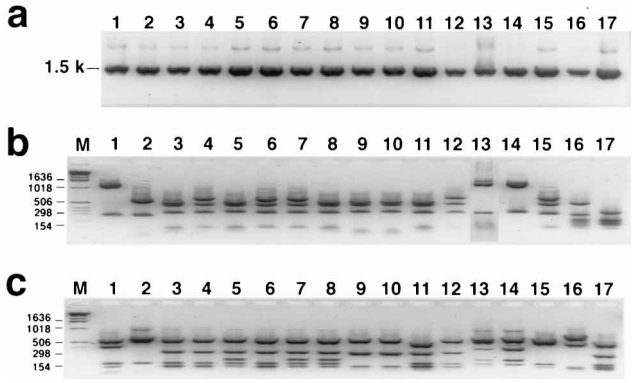

FIG. 1. PCR-RFLP analysis of the 16S rRNA genes. (a) The 16S rRNA gene was amplified from purified genomic DNA of the type or reference strains. PCR products were separated on a 0.9% agarose gel. An aliquot of the PCR products was cleaved with *Hae*III (b) and *Msp*I (c) and was then separated on a 1.8% agarose gel. Lanes: M, size marker (1-kb ladder; Gibco-Bethesda Research Laboratories); 1, *A. adiacens* ATCC 49175; 2, *A. defectiva* ATCC 49176; 3, *S. sanguis* ATCC 10556; 4, *S. oralis* ATCC NCTC 11427; 5, *S. gordonii* ATCC 10558; 6, *S. mitis* NCTC 12261; 7, *S. salivarius* subsp. *salivarius* ATCC 7073; 8, *S. bovis* NCFB 597; 9, *S. mutans* ATCC 25175; 10, *S. sobrinus* ATCC 33478; 11, *S. pyogenes* ATCC 12344; 12, *S. pneumoniae* NCTC 7465; 13, *S. aureus* ATCC 25923; 14, *S. epidermidis* ATCC 14990; 15, *E. faecalis* NCTC 775; 16, *H. influenzae* ID 984; 17, *E. coli* ATCC 25922. Numbers to the left of panels b and c are in base pairs; 1.5 k, 1.5 kbp.

The RFLP patterns of the PCR products were then examined following digestion with the appropriate restriction enzyme (Fig. 1b and c). The *Hae*III RFLP profiles of *A. adiacens* (Fig. 1b, lane 1) and *A. defectiva* (Fig. 1b, lane 2) were obviously different, as were the *Msp*I RFLP profiles (fig. 1c, lanes 1 and 2, respectively). Furthermore, these two species profiles could be distinguished from the RFLP profiles of the other species examined. In some cases, however, differences were not obvious when only the *Hae*III digestion profiles were compared, e.g., lanes 1 and 13 of Fig. 1a. In such cases, an additional analysis by *Msp*I digestion could afford the reliable identification. Among the 15 species excluding the *Abiotrophia* species examined (Fig. 1), the restriction patterns of *S. aureus* (lane 13) and *S. epidermidis* (lane 14) and those of gram-negative bacilli such as *H. influenzae* (lane 16) and *E. coli* (lane 17) were quite distinguishable. On the other hand, as a reflection of close phylogenetic lineages, the fragment patterns of the RFLP profiles of the remaining species (Fig. 1), i.e., viridans group streptococci (lanes 3 to 10), *S. pyogenes* (lane 11), *S. pneumoniae* (lane 12), and *E. faecalis* (lane 15), rather resembled each other, with three major apparently characteristic bands observed in the *Hae*III digestion and three to five shown in the *Msp*I digestion. However, as indicated in Fig. 1, *S. sanguis* (lane 3), *S. salivarius* (lane 7), *S. pyogenes* (lane 11), and *E. faecalis* (lane 15) were identifiable by consideration of both the *Hae*III and the *Msp*I restriction profiles. Among the viridans group streptococci (Fig. 1), two distantly related lineages, *S. oralis* (lane 4), *S. mitis* (lane 6), and *S. pneumoniae* (lane 12) representing one lineage and *S. mutans* (lane 9) and *S. sobrinus* (lane 10) representing another (12), were distinguishable by

TABLE 1. DNA fragment sizes of the 16S rRNA gene PCR products cleaved with *Hae*III

Strain	Estimated size (bp)	Deduced size (bp)[a]
A. adiacens ATCC 49175^T	1,160, 270	[1,168],[b] 276
A. defectiva ATCC 49176^T	560, 270	[599], 564, 270
S. sanguis ATCC 10556^T	510, 460, 310, 110	505, 455, 309, [109]
S. oralis NCTC 11427^T	600, 460, 310, 110	598, 455, [315], [110]
S. gordonii ATCC 10558^T	510, 460, 320, 110	598, 457, 317, [106]
S. mitis NCTC 12261^T	600, 460, 320, 110	598, 457, 263, [116]
S. salivarius subsp. *salivarius* ATCC 7073^T	600, 460, 320, 110	597, 456, [315], [110]
S. bovis NCFB 597^T	510, 460, 320, 110	505, 457, [315], [109]
S. mutans ATCC 25175^T	510, 460, 320, 90	505, 456, [315], [109]
S. sobrinus ATCC 33478^T	510, 460, 320, 90	598, 456, [271], [109]
S. pyogenes ATCC 12344^T	510, 460, 320, 110	505, 456, [315], [109]
S. pneumoniae NCTC 7465^T	600, 470, 320, 110	598, 456, [315], [109]
S. aureus ATCC 25923	1,200, 310	1,203, 310
S. epidermidis ATCC 14990	1,200, 310	1,189, 310
E. faecalis NCTC 775^T	600, 460, 320, 110	598, 455, [315], 110
H. influenzae ID 984	470, 280, 230, 210, 180	476, 278, 224, 206, 204, 180
E. coli ATCC 25922	320, 210, 180, 110	317, 210, 204, 180, 166, 161, 123

[a] Deduced sizes of fragments of less than 99 bp are not provided.
[b] Deduced fragments containing either uncloned 5′ or 3′ ends are given in brackets.

their RFLP profiles. *S. gordonii* ATCC 10558 (Fig. 1, lane 5) and *S. bovis* NCFB 597 (Fig. 1, lane 8) were not distinguishable by *Hae*III and *Msp*I digestions.

The lengths of the restriction fragments obtained by *Hae*III or *Msp*I digestion were calculated by measuring their relative mobilities on an agarose gel. These data are summarized in Tables 1 and 2, with the restricted DNA fragment lengths deduced from the DNA sequences of the corresponding 16S rRNA genes from the databases. The estimated fragment lengths were sufficiently in accordance with the deduced DNA fragment lengths. Although we noticed some disagreements, these were possibly explained by one or two ambiguous nucleotides at the expected restriction sites (discussed below). In the cases of *Abiotrophia* species, the estimated sizes of ones DNA fragments were fully consistent with the expected deduced from the sequences.

Isolation of *Abiotrophia* species from oral specimens. *A. adiacens* and *A. defectiva* were isolated from specimens of saliva and dental plaque from 93 healthy dental students. First, colonies demonstrating bacteriolytic activity on heat-killed *Micrococcus luteus* ATCC 9341 on double-layer nutrient agar plates were isolated as described by Pompei et al. (17). Then these microorganisms were subcultured on a Todd-Hewitt agar plate with a streak of *S. aureus* ATCC 25923 to examine satellitism. Ninety-two strains were finally isolated. All isolates were typical gram-positive cocci in chains and produced pyrrolidonyl arylamidase and aminopeptidase. Isolates that exhibited positive reactions for α- and β-galactosidases and for trehalose and starch fermentation but negative ones for inulin fermentation were classified as *A. defectiva*. *A. adiacens* isolates were negative for α- and β-galactosidases and for trehalose and starch fermentation. These characteristics are consistent with those of each of the *Abiotrophia* species described previously (4, 13). The species classification was also confirmed with randomly selected isolates (10 isolates of *A. adiacens* and 5 isolates of *A. defectiva*) by DNA-DNA hybridization, in which DNAs from these isolates demonstrated more than 70% similarity with DNAs from the respective type strains (data not shown). As a result, 81 strains of *A. adiacens* (81 of 93 strains; a colonization frequency of 87.1%) and 11 strains of *A. defec-*

TABLE 2. DNA fragment sizes of the 16S rRNA gene PCR products cleaved with *Msp*I

Strain	Estimated size (bp)	Deduced size (bp)[a]
A. adiacens ATCC 49175^T	530, 410, 165, 120	537, 407, 164, [116][b]
A. defectiva ATCC 49176^T	560, 165	606, 564, 163, [111]
S. sanguis ATCC 10556^T	560, 320, 165, 120	555, 316, 163, 125, 120
S. oralis NCTC 12427^T	560, 320, 165, 120	[561], 315, 163, 125, 120, [114]
S. gordonii ATCC 10558^T	560, 320, 210, 165, 120	564, 317, 211, 163, 125
S. mitis NCTC 12261^T	550, 320, 165, 120	553, 317, 163, 125, 120, [119]
S. salivarius subsp. *salivarius* ATCC 7073^T	560, 320, 210, 165, 120	[561], 316, 211, 163, 125, [113]
S. bovis NCFB 597^T	560, 320, 210, 165, 120	[562], 316, 211, 163, 125, [112]
S. mutans ATCC 25175^T	560, 300, 120	[562], 303, 288, 120, [113]
S. sobrinus ATCC 33478^T	560, 310, 120	[562], 316, 163, 125, 120, [112]
S. pyogenes ATCC 12344^T	460, 300, 165, 120	[562], 303, 163, 125, 120, [112]
S. pneumoniae NCTC 7465^T	560, 320, 165, 120	[562], 316, 163, 125, 120, [112]
S. aureus ATCC 25923	600, 390, 210, 165	608, 388, 211, 156
S. epidermidis ATCC 14990	600, 390, 210, 165	608, 388, 211, 156
Enterococcus faecalis NCTC 775^T	540, 480, 165, 120	536, 478, 163, 127
Haemophilus influenzae ID 984	690, 500, 125, 110	687, 497, 127, 110
Escherichia coli ATCC 25922	500, 280, 160, 140, 125	496, 280, 162, 137, 126, 110, 106

[a] Deduced sizes of fragments of less than 99 bp are not provided.
[b] Deduced fragments containing either uncloned 5′ or 3′ ends are given in brackets.

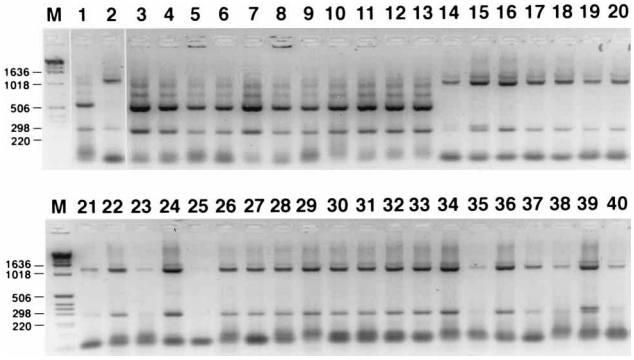

FIG. 2. RFLP analysis of *Abiotrophia* isolates with *Hae*III. Bacterial cell lysates of *A. adiacens* and *A. defectiva* isolates were subjected to 16S rRNA gene PCR-RFLP analysis with *Hae*III. Lanes: M, size marker; 1, *A. defectiva* ATCC 49176; 2, *A. adiacens* ATCC 49175; 3 to 13, *A. defectiva* isolates; 14 to 20, and 22 to 40, *A. adiacens* isolates; 21, *A. adiacens* 4096-P. The results for the 55 other *A. adiacens* isolates are not shown. Numbers to the left are in base pairs.

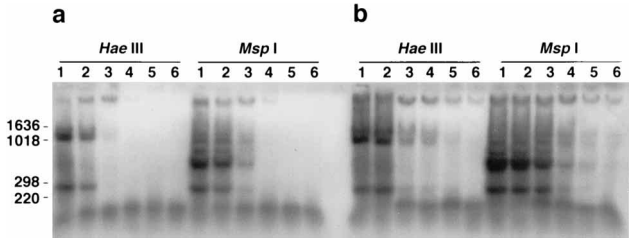

FIG. 3. Detection limit of genomic DNA in 16S rRNA gene PCR-RFLP analysis with DIG-labeling system. Purified genomic DNAs of *A. adiacens* 4096-P (a) and *A. adiacens* ATCC 49175 (b) were sequentially diluted, and the resultant DNAs were subjected to PCR amplification in the presence of DIG-dUTP. After *Hae*III cleavage, the DIG-labeled DNA fragments were blotted onto a Hybond-N^+ membrane filter and visualized with alkaline phosphatase-conjugated anti DIG-antibody and CDP-Star. The amounts of DNA were 10 pg (lane 1), 1 pg (lane 2), 0.1 pg (lane 3), 0.01 pg (lane 4), 0.01 pg (lane 5), and 0.001 pg (lane 6). Numbers to the left are in base pairs.

tiva (11 of 93; 11.8%) were isolated. The ratio of volunteers carrying either *A. adiacens* or *A. defectiva* was 97.8% (91 of 93) and the ratio of volunteers carrying both species was 1.1% (1 of 93), indicating a notably high frequency of colonization of *Abiotrophia* species in the human oral cavity.

PCR-RFLP analysis of *Abiotrophia* isolates. PCR-RFLP analysis was carried out with 82 isolates of *A. adiacens*, including the isolates from the dental students and strain 4096-P derived from a Japanese patient with infective endocarditis, and 11 isolates of *A. defectiva*. In order to obtain the results promptly, PCR was performed with the bacterial cell lysate obtained from the heat-lysed bacterial cell suspension. The 1.5-kb fragments were amplified from 80 of 82 isolates (97.6%) of *A. adiacens* and from all 11 isolates (100%) of *A. defectiva*. Since a second trial of PCR failed with two isolates of *A. adiacens* from the oral cavity for unknown reasons, the 16S rRNA gene fragments of the corresponding isolates were obtained by use of the purified genomic DNA as a PCR template. The RFLP analysis demonstrated that all phenotypically identified isolates gave the expected RFLP profiles by *Hae*III digestion (Fig. 2) and *Msp*I digestion (data not shown). Thus, these results indicated that the 16S rRNA gene PCR-RFLP was a quite efficient and reliable method for identifying *Abiotrophia* species. Furthermore, it is noticeable that 23 of 82 strains (28%) of *A. adiacens* exhibited doublets of 276 and 310 bp on the agarose gel electrophoresis gels.

Detection limit of 16S rRNA gene PCR-RFLP analysis by nonradioactive DIG detection system. A highly sensitive nonradioactive system for detecting DNA is of great advantage for performing 16S rRNA gene PCR-RFLP analysis in the clinical microbiology laboratory. Therefore, we investigated the limit of detection of template DNA with the DIG detection system. Purified genomic DNAs of *A. adiacens* 4096-P (Fig. 3a), *A. adiacens* ATCC 49175 (Fig. 3b), and *A. defectiva* ATCC 49176 (data not shown) were sequentially diluted 10 times, and the resultant DNAs were subjected to PCR amplification in the presence of DIG-dUTP. In all instances we were able to detect 0.1 pg (approximately 30 genomic equivalents) of input DNA, and in most cases we could detect 0.01 pg of genome DNA.

DISCUSSION

In this paper, we demonstrated that *A. adiacens* and *A. defectiva* could readily be identified by 16S rRNA gene PCR-RFLP analysis. It takes approximately 4 h to accomplish all of the procedures involved in this type of analysis, including DNA preparation, PCR, and RFLP analysis. Thus, this method is more rapid and cheaper than conventional phenotypic identification methods. The 16S rRNA gene PCR-RFLP analysis could also be applicable in estimating the causative microorganism of infective endocarditis from clinical specimens, such as blood.

The restriction sites of *Hae*III and *Msp*I deduced from the DNA sequences of the 16S rRNA genes of *Abiotrophia* species and viridans group streptococci are illustrated in Fig. 4. Two *Hae*III restriction sites corresponding approximately to positions 309 and 337 (*S. sanguis* numbering, which is also used below) are conserved among these 10 species. One *Hae*III site at about position 270 is common in two species of *Abiotrophia*. Although this site is deduced only in *S. mitis* but not in other viridans group streptococci, the RFLP profile suggested that the site also does not exist in the gene of *S. mitis* NCTC 12261^T (Fig. 1 and Table 1). One additional *Hae*III site exists at position 902 in *A. defectiva* but not in *A. adiacens*, providing clearly distinguishable RFLP profiles for the two species. The site at position 935 is conserved among all viridans group streptococci except *S. bovis*. The existence of deduced restriction sites at positions 842 and 1390 was confirmed in the corresponding three type strains (*S. sanguis* ATCC 10556, *S. bovis* NCFB 597, and *S. sobrinus* ATCC 33478) and four type strains (*S. oralis* NCTC 11427, *S. gordonii* ATCC 10558, *S. mitis* NCTC 12261, and *S. sobrinus* ATCC 33478), respectively.

In the case of *Msp*I sites, three conserved sites exist in both

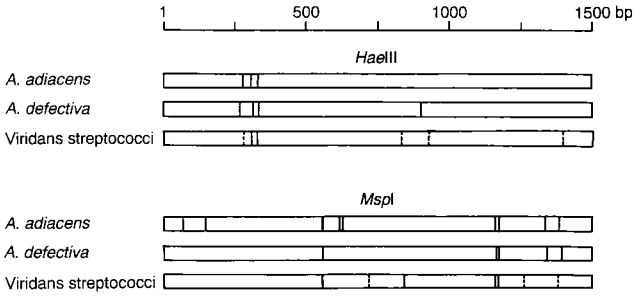

FIG. 4. Schematic illustrations of the *Hae*III and *Msp*I sites of the 16S rRNA genes of the genus *Abiotrophia* and viridans group streptococci. The conserved sites among all bacteria, the conserved site in viridans group streptococci, and the genus- or species-specific sites in *Abiotrophia* are represented as solid lines. The sites that exist in several species of viridans group streptococci are represented as broken lines.

Abiotrophia and viridans group streptococci at positions 555, 1159, and 1170. Additionally, two specific sites for the genus *Abiotrophia* exist at about positions 1340 and 1390. The 16S rRNA gene of *A. adiacens* possesses four more *Msp*I sites at positions 70, 149, 615, and 624. Among the viridans group streptococci, the site at about position 843 is fully conserved, and the sites at positions 718 and 1381 exist in all species except *S. mutans* and *S. bovis*, respectively. The site at position 1261 was observed in six of the type strains examined, but it was not observed in *S. gordonii* ATCC 10558 or *S. salivarius* subsp. *salivarius* ATCC 7073.

Analysis of all 95 *Abiotrophia* strains including the type strains and 93 isolates revealed that 16S rRNA gene PCR-RFLP analysis with either *Hae*III or *Msp*I provided considerable accuracy in classifying *A. adiacens* and *A. defectiva*. Furthermore, the two-step isolation method described by Pompei et al. (17), based on the particular characteristics of *Abiotrophia*, i.e., bacteriolytic activity and satellitism, seems to be a sufficient method for the isolation of these bacteria from human experimental and clinical specimens. When we examined 30 potential colonies that had sizes and shapes similar to those of *Abiotrophia* species but that did not exhibit bacteriolytic activity on heat-killed *M. luteus* on double-layer agar plates, none of these strains exhibited either satellitism or a requirement for pyridoxal or L-cysteine in successive investigations (23). Taken together, it was suggested that bacterial isolates presumed to be members of the genus *Abiotrophia*, because of the bacteriolytic activity and satellitism, could be conveniently identified to the species level by 16S rRNA gene PCR-RFLP analysis without subsequent biochemical tests.

It is particularly important to reexamine the colonization frequencies of these microorganisms in normal human flora, because we unexpectedly observed that the colonization frequency was high in the oral cavity. The 87% figure for *A. adiacens* was comparable to those for *S. sanguis* and *S. salivarius* and even higher than those for *S. oralis*, *S. mitis*, *S. gordonii*, *S. mutans*, and *S. sobrinus* (22). In addition, the two *Abiotrophia* species hardly appeared compatible, because only 1 of 92 volunteers carried both species, and the rest of the volunteers carried only one or the other species. The apparently lower colonization frequency of *A. defectiva* (11.8%) compared with that of *A. adiacens* (87.1%) was also of interest. Since these two species are isolated from patients with infective endocarditis with similar frequencies (19), some particular characteristics of *A. defectiva* may be involved in causing the disease. It has recently been reported that *A. defectiva* produces neuraminidase and α-fucosidase, while *A. adiacens* produces only neuraminidase, and that the substrate specificities of proteases are different in the two species (1).

The 16S rRNA gene PCR-RFLP analysis also revealed the intraspecies genetic polymorphism of *A. adiacens*. This finding may be applicable to molecular epidemiology. As can easily be speculated from the observations that there are six 16S rRNA operons in *S. aureus* (24) and seven in *E. coli* (14), *A. adiacens* probably possess plural operons of that gene. Our results suggested that the restriction site at position 277 is not always conserved among the repetitive genes in *A. adiacens*. Of 81 strains of *A. adiacens*, 16 (19.8%) were negative for inulin fermentation and the rest (80.2%) were positive (23a). However, this biochemical heterogeneity did not apparently correlate with the genetic polymorphism.

Assays based on the PCR technique have recently been developed for the rapid and sensitive detection of human pathogens in clinical specimens (8, 16). The detection limit of the target DNA by PCR has become smaller through the improvement of reaction and detection methodologies. Theoretically, even one copy of the gene in a specimen can be amplified by PCR. It has been reported that 8 CFU of *S. pneumoniae*/ml of whole blood was successfully detected by PCR (27). Under these circumstances, 16S rRNA gene PCR-RFLP analysis has a great advantage in the identification of microorganisms in which the species-specific genes have not been cloned. Furthermore, especially in cases like infective endocarditis, which is caused by both gram-positive cocci and gram-negative bacilli, a rapid and precise diagnosis of bacterial pathogens could greatly help in determining the appropriate antibiotic therapy. The clinical severity of infective endocarditis is closely correlated to the delay in bacteriological diagnosis (3, 7). We think that 16S rRNA gene PCR-RFLP analysis can be a useful diagnostic method.

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