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Identification of *Bacteroides thetaiotaomicron* on the Basis of an Unexpected Specific Amplicon of Universal 16S Ribosomal DNA PCR

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We applied a set of commonly used universal primers (primers RW01 and DG74) to amplify partial fragments of 16S ribosomal DNA for bacterial identification and found an unexpected amplicon (547 bp), in addition to the expected 362-bp product, in samples containing *Bacteroides thetaiotaomicron*. It was demonstrated that the internal sequence (508 bp, excluding the primers) of the 547-bp amplicon was identical to the genomic sequence from nucleotide positions 165800 to 166307 of *B. thetaiotaomicron* type strain VPI-5482 by a BLAST search of the sequences in the GenBank database. The existence of this unexpected yet specific amplicon strongly indicated the presence of *B. thetaiotaomicron* in the sample, and it was found that it could be used to discriminate *B. thetaiotaomicron* from closely related species. Another set of PCR primers specific for *B. thetaiotaomicron* was developed on the basis of the sequence of this 547-bp genomic fragment. Both PCR-based assays showed the same sensitivity (88%) and specificity (100%).

Identification of pathogens in specimens usually depends on culture and various phenotypic tests. The phenotypic characteristics of clinical isolates, which are used for conventional identification, are not always typical and may lead to ambiguous results (2). Many new methods based on molecular techniques for the rapid and direct identification of microorganisms in specimens have been developed. Direct sequencing of the PCR-amplified bacterial 16S ribosomal DNA (rDNA) is one of these new molecular identification methods, and it has been widely used (4, 7, 13, 15). The 16S rRNA genes of almost all common bacterial pathogens have been sequenced and are highly conserved among these bacteria. Consequently, the 16S rRNA gene is commonly selected as the target for universal PCR that amplifies partial or nearly full-length 16S rDNA sequences in almost all bacterial pathogens. Many sets of primers have been designed to amplify different regions of 16S rDNA and have been shown to have various sensitivities and specificities. A universal PCR with primers RW01 and DG74A was developed, and it was demonstrated to be a sensitive screening method for detection of bacterial pathogens in cerebrospinal fluid (4). This set of universal primers amplified a portion of 16S rDNA (369 bp in *Escherichia coli*) (4), and it was applied to the identification of bacterial pathogens in clinical specimens. However, we found that an unexpected amplicon (547 bp), in addition to the expected 362-bp product, constantly appeared in samples containing *Bacteroides thetaiotaomicron.*

B. fragilis and *B. thetaiotaomicron* are clinically important because they are the first and second most common agents of infections caused by anaerobic gram-negative bacilli, respectively (1, 3, 8). They are opportunistic pathogens commonly associated with peritonitis, septicemia, and wound infections (3, 8). Although *B. thetaiotaomicron* is part of the indigenous microflora of the gastrointestinal tract, it may cause serious infections such as intra-abdominal sepsis and bacteremia (1, 3, 8). *B. thetaiotaomicron* is resistant to many antimicrobial agents; for example, it has been reported to be more resistant than *B. fragilis* to cephalosporins and clindamycin (1, 9, 10, 12). Therefore, rapid and accurate identification of *B. thetaiotaomicron* in clinical specimens is important, and it may help physicians make rational, rapid choices with respect to antibiotic therapy. In order to establish if we could identify *B. thetaiotaomicron* on the basis of the detection of this additional PCR product, we performed the tests described here.

Bacterial strains. Eight reference strains of *Bacteroides* species (*B. fragilis* ATCC 25285, *B. fragilis* ATCC 29762, *B. thetaiotaomicron* ATCC 29741, *B. caccae* ATCC 43185, *B. ovatus* ATCC 8483, *B. distasonis* ATCC 8503, *B. vulgatus* ATCC 8482, and *B. uniformis* ATCC 8492) were obtained from the American Type Culture Collection (ATCC; Manassas, Va.) A total of 43 clinical isolates initially identified as *B. fragilis* ($n = 7$), *B. caccae* ($n = 7$), *B. thetaiotaomicron* ($n = 16$), *B. uniformis* ($n = 5$), *B. vulgatus* ($n = 5$), and *B. distasonis* ($n = 3$) by conventional methods were included in this study. All the clinical isolates were collected from the Bacteriology Laboratory, National Taiwan University Hospital, a 2,000-bed teaching hospital in northern Taiwan.

***Bacteroides* species identification.** Clinical *Bacteroides* species isolates were initially identified by the Presumpto Plates method of the Centers for Disease Control and Prevention and sugar fermentation. Subsequently, PCR was performed with primers 8FPL (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 (5′-GGTTACCTTGTTACGACTT-3′) to amplify the

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TABLE 1. Discrepancies between phenotypic and molecular identifications

Isolate	Phenotypic identification	PCR product size (bp)[a]	Best match by 16S rDNA sequencing[b]
NTUH-5708	*B. thetaiotaomicron*	362	*B. ovatus*
NTUH-4338	*B. thetaiotaomicron*	362	*B. distasonis*
NTUH-2392	*B. thetaiotaomicron*	362	*B. ovatus*
NTUH-5545	*B. caccae*	362 + 547	*B. thetaiotaomicron*
NTUH-2956	*B. uniformis*	362 + 547	*B. thetaiotaomicron*
NTUH-3508	*B. uniformis*	362 + 547	*B. thetaiotaomicron*

[a] PCR was performed with primers RW01 and DG74 at an annealing temperature of 50°C.

[b] The nucleotide sequences of 16S rDNA were determined from the amplification products by PCR with primers of 8FPL and 1492.

nearly complete length of 16S rDNA in the bacteria. The amplification products obtained by PCR of the nearly complete length of 16S rDNA were sequenced, and the sequences were compared to known 16S rDNA sequences in the GenBank database of the National Center for Biotechnology Information by using the BLAST algorithm. The species with the best match were regarded as pathogenic species in the samples. Three isolates initially identified as *B. thetaiotaomicron* on the basis of phenotypic tests were reidentified by 16S rDNA sequencing as *B. ovatus* (two isolates) and *B. distasonis* (one isolate) (Table 1). Another three isolates initially identified as *B. uniformis* (two isolates) and *B. caccae* (one isolate) by phenotypic tests were reidentified as *B. thetaiotaomicron* by 16S rDNA sequencing (Table 1).

Amplification of partial 16S rDNA using different annealing temperatures. DNA was prepared from the bacteria by using a DNA isolation kit (Puregene; Gentra Systems, Inc., Minneapolis, Minn.), according to the instructions of the manufacturer. Amplification of partial 16S rDNA was performed with the universal primers RW01 (5′-AACTGGAGGAAGGTGGGGAT-3′) and DG74 (5′-AGGAGGTGATCCAACCGCA-3′). The annealing temperature in the original PCR protocol was 55°C (4). We used annealing temperatures of 50, 55, and 60°C in this study. The amplification reaction mixtures contained 50 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.), 200 μM (each) deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP; Perkin-Elmer), 50 pmol of each of the primers, and 2 μl of the DNA sample. The PCR was carried out in a DNA thermal cycler (MJ Research Inc., Watertown, Mass.) with 30 cycles of denaturation (94°C, 1 min), annealing (50, 55, or 60°C, 1 min), and extension (72°C, 1 min), followed by a final extension step (72°C, 7 min). The amplification products were subsequently subjected to gel electrophoresis (FMC BioProducts, Rockland, Maine), stained with ethidium bromide, and photographed under UV light. In addition to the expected 362-bp amplicon, an unexpected band of 547 bp was noted in the *B. thetaiotaomicron* ATCC 29741 strain but not in the other *Bacteroides* species. This unexpected amplification product appeared when the annealing temperature was set to 50 or 55°C (Fig. 1A and B) and disappeared when the annealing temperature was elevated to 60°C (Fig. 1C). We then performed this PCR with DNA from clinical isolates of *Bacteroides* species at an annealing temperature of 50°C. While certain nonspecific faint bands of various sizes were noted for the other *Bacteroides* species, they were not of the same size as the 547-bp amplicon found for *B. thetaiotaomicron* (Fig. 1D). PCR with primers RW01 and DG74 revealed a single 362-bp band for each of the three isolates reidentified as *Bacteroides* other than *B. thetaiotaomicron* and revealed two bands (362 and 547 bp) in each of the three specimens found on subsequent analysis to contain *B. thetaiotaomicron* (Table 1).

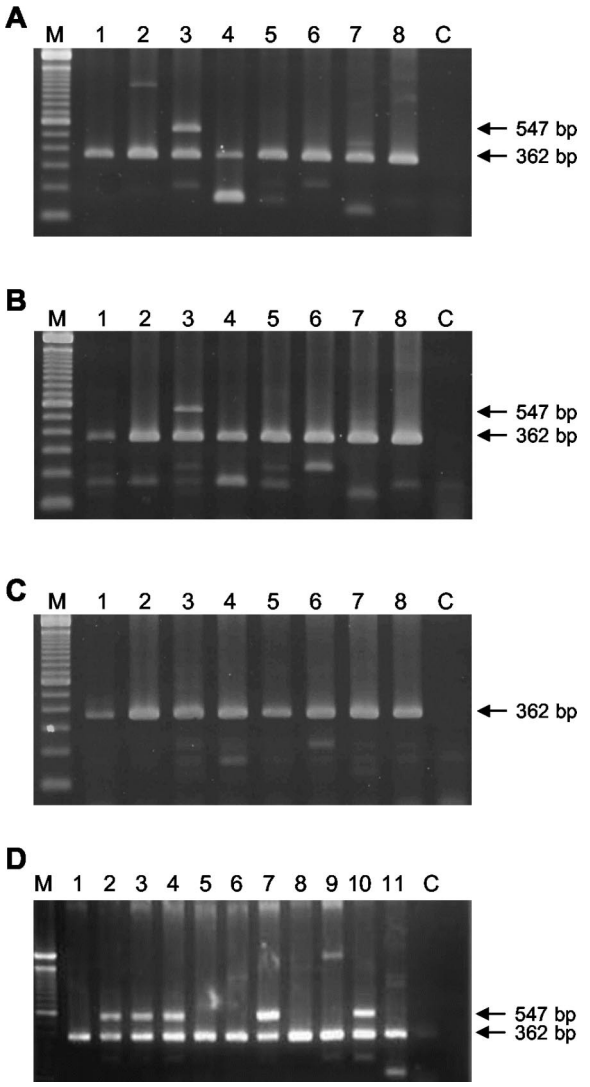

FIG. 1. Gel electrophoresis of partial 16S rDNA PCR products from *Bacteroides* species. PCR was performed with universal primers RW01 and DG74 at various annealing temperatures: 50°C (A), 55°C (B), and 60°C (C). Lanes M, DNA size markers (100-bp ladder); lanes 1, *B. fragilis* ATCC 25285; lanes 2, *B. fragilis* ATCC 29762; lanes 3, *B. thetaiotaomicron* ATCC 29741; lanes 4, *B. caccae* ATCC 43185; lanes 5, *B. ovatus* ATCC; lanes 6, *B. distasonis* ATCC 8503; lanes 7, *B. vulgatus* ATCC 8482; lanes 8, *B. uniformis* ATCC 8492; lanes C, negative control (no DNA). (D) Gel electrophoresis of partial 16S rDNA PCR products from clinical isolates. PCR was performed at annealing temperature of 50°C. Lanes 2, 3, 4, 6, 7, and 10, *B. thetaiotaomicron* isolates; lane 1, *B. fragilis*; lanes 5 and 9, *B. ovatus*; lanes 8 and 11, *B. uniformis*. Identification of the species of the clinical isolates was based on the 16S rDNA sequences. The isolate in lane 6 was identified as *B. thetaiotaomicron*, but only the 362-bp product was noted.

TABLE 2. Alignment of the sequences of PCR primers RW01 and DG74 with those of *E. coli* 16S rDNA, *B. thetaiotaomicron* 16S rDNA, and the *B. thetaiotaomicron* genome

Primer or DNA type	Sequence (5′ to 3′)[a]	Nucleotide positions
RW01	AACTGGAGGAAGGTGGGGAT	1172–1191[b]
16S rDNA	g-tgt---------------	4984595–4984614[c]
Genome	---a--t-----c--c----	165780–165799[c]
DG74	AGGAGGTGATCCAACCGCA	1540–1522 (reverse)[b]
16S rDNA	--------t----g-----	4984956–4984938 (reverse)[c]
Genome	cccc-agcc----------	166326–166308 (reverse)[c]

[a] Lowercase letters indicate nucleotide changes; hyphens indicate no nucleotide change.
[b] Based on the 16S rDNA sequence of *E. coli* (GenBank accession number E05133).
[c] Based on the complete genome sequence of *B. thetaiotaomicron* (GenBank accession number AE016926 or NC_004663).

Sequence analysis of the unexpected 547-bp amplified product from *B. thetaiotaomicron*. The PCR products from four clinical isolates of *B. thetaiotaomicron* were separated by gel electrophoresis. The 362- and 547-bp bands were excised from the gel and purified. The purified DNA fragments were subsequently sequenced on a sequencing system (model 377; Applied Biosystems, Foster City, Calif.) with a *Taq* BigDye-Deoxy Terminator cycle sequencing kit (Applied Biosystems), according to the instructions of the manufacturer. The internal sequence (323 bp, excluding the primers) of the 362-bp band in each isolate was shown to be identical to a fragment of the *B. thetaiotaomicron* 16S rDNA gene, as expected, while the internal sequence (508 bp) of the 547-bp product was 100% identical to the genomic sequence from positions 165800 to 166307 of *B. thetaiotaomicron* type strain VPI-5482 (ATCC 29148) on the basis of a search against the sequences in the GenBank database with the BLAST program (Table 2). The complete genome sequence of *B. thetaiotaomicron* has recently been published (16). According to the sequence data (GenBank accession number AE016926), the fragment from positions 165800 to 166307 contains the partial sequence predicted to encode a hypothetical protein (complement, positions 165668 to 166102) and a putative permease (complement, positions 166150 to 167325). The complete genome contains five copies of the 16S rRNA gene. We aligned the sequences of the 16S rDNA universal primers (primers RW01 and DG74) to the genomic sequence of *B. thetaiotaomicron* and found that the 15 nucleotides at the 3′ end of primer RW01 were 100% identical to a region of the 16S rDNA gene (Table 2). Apart from the 16S rDNA gene, the primer sequence was also similar (identical at 16 of 20 nucleotides) to the sequence of a genomic fragment of *B. thetaiotaomicron* (Table 2). The sequence of reverse primer DG74 was found to be similar to a region of the 16S rDNA gene (identical at 17 of 19 nucleotides) and a genomic site (identical at 10 nucleotides at the 3′ end) not related to the 16S rDNA gene. Accordingly, the 547-bp amplification product was caused by cross-reaction of the 16S rDNA primers (primers RW01 and DG74) with sequences other than the 16S rDNA in the genome, and it was specific to *B. thetaiotaomicron.*

Identification of *B. thetaiotaomicron* species on the basis of the 547-bp amplicon. Although the 547-bp amplicon was not the expected product of the PCR with universal primers RW01 and DG74, it was consistently present in PCRs with *B. thetaiotaomicron* isolates and may have practical value in differentiating *B. thetaiotaomicron* from phenotypically similar species, such as *B. uniformis* and *B. ovatus*. This 547-bp amplicon was clearly seen for 14 of 16 clinical isolates of *B. thetaiotaomicron* but not for any of the other *Bacteroides* species (Fig. 1D). *B. thetaiotaomicron*, *B. uniformis*, and *B. ovatus* are all indole-positive species and are closely genetically related to each other (6, 14). We have applied this PCR to a wide range of other common bacterial pathogens found in clinical specimens and detected only the expected ~370-bp band in PCRs with most of the isolates. Although certain nonspecific bands of various sizes, in addition to the ~370-bp band, were noted for some species other than *B. thetaiotaomicron*, they were faint and not of the same size as the 547-bp amplicon. Thus, the identification of *B. thetaiotaomicron* on the basis of detection of this 547-bp band had a sensitivity of 88% (14 of 16 isolates) and a specificity of 100%.

***B. thetaiotaomicron*-specific PCR.** In order to increase the sensitivity of the PCR for the identification of *B. thetaiotaomicron*, PCR primers based on this 547-bp genomic target (positions 165780 to 166326; GenBank accession number AE016926) were designed. This new set of primers, primers 5′-AACAGGTGGAAGCTGCGGA-3′ (corresponding to nucleotide positions 165780 to 165798) and 5′-AGCCTCCAAC CGCATCAA-3′ (corresponding to nucleotide positions 166321 to 166304), was used to amplify a 542-bp fragment of *B. thetaiotaomicron*. The PCR was carried out in a DNA thermal cycler (MJ Research, Inc.) with 35 cycles of denaturation (94°C, 1 min), annealing (60°C, 30 s), and extension (72°C, 1 min), followed by a final extension step (72°C, 7 min). This PCR was positive for the *B. thetaiotaomicron* ATCC 29741 strain and all the *B. thetaiotaomicron* strains ($n = 14$) which showed the 547-bp band by PCR with primers RW01 and DG74 (Fig. 2). The two *B. thetaiotaomicron* clinical isolates which failed to show the 547-bp band by PCR with primers RW01 and DG74 did not show the expected 542-bp band by PCR with this new set of primers. We have used several other sets of primers based on this 547-bp genomic target (positions 165780 to 166326; GenBank accession number AE016926) to amplify this region of DNA in these two clinical isolates, but all failed to show the corresponding fragment. It seems possible that the region from nucleotides 165780 to 166326 is missing in these two isolates. The 542-bp PCR products were not detected when bacterial DNA other than that of *B. thetaiotaomicron* was used as the template (Fig. 2). Although the newly designed primers do not increase the sensitivity of the PCR for the identification of *B. thetaiotaomicron*, they do increase the specificity of the PCR assay because the additional products originally observed in Fig. 1 (by PCR with universal primers RW01 and DG74) were no longer detected in Fig. 2 (by PCR with the newly designed specific primers).

Phenotypic identification versus molecular identification. Identification of *Bacteroides* species by conventional biochemical identification often requires 3 to 7 days, and the results are sometimes inconclusive (14). Moreover, the automated methods commonly used for the identification of *B. thetaiotaomicron* from clinical isolates are not always reliable (2). This

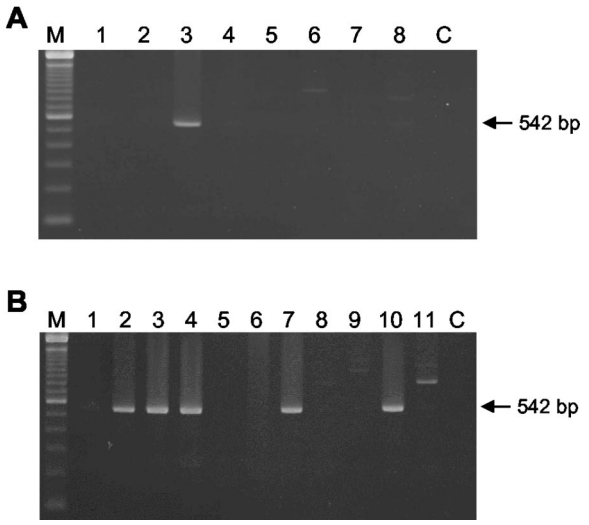

FIG. 2. *B. thetaiotaomicron*-specific PCR amplification. Specific amplification of the 542-bp DNA fragment was detected only in *B. thetaiotaomicron* isolates. Lanes M, 100-bp DNA ladder. (A) Lane 1, *B. fragilis* ATCC 25285; lane 2, *B. fragilis* ATCC 29762; lane 3, *B. thetaiotaomicron* ATCC 29741; lane 4, *B. caccae* ATCC 43185; lane 5, *B. ovatus* ATCC; lane 6, *B. distasonis* ATCC 8503; lane 7, *B. vulgatus* ATCC 8482; lane 8, *B. uniformis* ATCC 8492; lane C, negative control (no DNA). (B) Lanes 2, 3, 4, 6, 7, and 10, *B. thetaiotaomicron* isolates. Lane 1, *B. fragilis*; lanes 5 and 9, *B. ovatus*; lanes 8 and 11, *B. uniformis*; lane C, negative control (no DNA).

problem is caused by the similarity of the phenotypic and biochemical characteristics of closely related species. For the identification of *B. thetaiotaomicron*, genotypic methods such as 16S rDNA PCR (this study) or PCR hybridization (other studies [5, 11]) are more specific and rapid than conventional methods.

In this study, we found an unexpected yet specific amplification of a fragment by PCR caused by cross-reaction of the universal primers (primers RW01 and DG74) with genomic sites of *B. thetaiotaomicron* other than 16S rDNA. The existence of this 547-bp PCR product strongly indicated the presence of *B. thetaiotaomicron* in the samples, and this could subsequently be confirmed by sequence analysis. PCR primers specific for *B. thetaiotaomicron* were developed on the basis of the sequence of this 547-bp genomic fragment. However, the sensitivity and specificity of the new PCR remained the same as those of the original PCR. In conclusion, the PCR-based assays described in this study can be used to discriminate *B. thetaiotaomicron* from the closely related *Bacteroides* species.

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