hsp60 and 16S rRNA gene sequence relationships among species of the genus *Bacteroides* with the finding that *Bacteroides suis* and *Bacteroides tectus* are heterotypic synonyms of *Bacteroides pyogenes*

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hsp60 gene sequences were determined for members of the genus *Bacteroides* and sequence similarities were compared with those obtained for the 16S rRNA gene. Among the 29 *Bacteroides* type strains, the mean sequence similarity of the *hsp60* gene (84.5%) was significantly less than that of the 16S rRNA gene (90.7%), indicating a high discriminatory power of the *hsp60* gene. Species of the genus *Bacteroides* were differentiated well by *hsp60* gene sequence analysis, except for *Bacteroides pyogenes* JCM 6294^T, *Bacteroides suis* JCM 6292^T and *Bacteroides tectus* JCM 10003^T. The *hsp60* gene sequence analysis and the levels of DNA–DNA relatedness observed demonstrated that these three type strains are a single species. Consequently, *B. suis* and *B. tectus* are heterotypic synonyms of *B. pyogenes*. This study suggests that the *hsp60* gene is an alternative phylogenetic marker for the classification of species of the genus *Bacteroides*.

INTRODUCTION

Organisms of the genus *Bacteroides* are part of the indigenous microbiota of human and animal gastrointestinal tracts, but different species in this group are commonly associated with a variety of human and animal infections. The genus *Bacteroides* is important in clinical bacteriology. Although species of the genus *Bacteroides* are isolated frequently from hospitalized patients, it can be difficult to identify every clinical isolate to the species level. In the past, because of poor definition of the genus, more than 50 species of the genus *Bacteroides* have been included in this group. The taxonomy of the genus *Bacteroides* has undergone significant changes in the last two decades (Dewhirst *et al.*, 1990; Moore & Moore, 1994; Rautio *et al.*, 2003; Sakamoto *et al.*, 2002, 2007; Sakamoto & Benno, 2006; Shah & Collins, 1988, 1989, 1990). It has also been reported that 16S rRNA gene sequencing is useful for the

identification of isolates of clinically significant species of the genus *Bacteroides* (Song *et al.*, 2005).

To date, a multilocus sequence typing (MLST) approach has been widely used to infer the phylogeny of organisms (Alexandre *et al.*, 2008; Itoh *et al.*, 2006; Shah *et al.*, 2007; Yamamoto & Harayama, 1995). However, only limited information has been accumulated for species of the genus *Bacteroides*. It has been reported that housekeeping gene sequences, e.g. *rpoB*, could be used to clarify interspecies phylogenetic relationships within the genus *Bacteroides* (Ko *et al.*, 2007). The ad hoc committee for the re-evaluation of the species definition in bacteriology has recommended evaluation of protein-coding gene sequence analysis for its applicability to genomically circumscribe the taxon species and differentiate it from neighbouring species detected by 16S rRNA gene sequence analysis (Stackebrandt *et al.*, 2002).

Hsp60, also known as Cpn60 or GroEL, is a member of the heat-shock protein (Hsp) family. The *hsp60* gene has been shown to be more discriminative than the 16S rRNA gene for the identification of *Streptococcus suis* serotypes (Brousseau *et al.*, 2001) and species of the genus *Prevotella* (Sakamoto *et al.*, 2010). The present study was designed to compare two methods, one based on the 16S

The GenBank/EMBL/DDBJ accession numbers for the partial *hsp60* gene sequences of *Bacteroides* strains determined in this study are listed in Table 1.

A table showing sequence similarity based on pairwise comparisons of the *hsp60* and 16S rRNA gene sequences of species of the genus *Bacteroides* is available with the online version of this paper.

rRNA gene and one based on the *hsp60* gene, for the classification and establishment of phylogenetic relationships among species of the genus *Bacteroides*. Consequently, the description of *Bacteroides pyogenes* was emended.

METHODS

Bacterial strains. A total of 29 *Bacteroides* type strains and two reference strains were obtained from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Wako, Japan, and are listed in Table 1. The strains used in the present study were maintained on Eggerth Gagnon (EG) agar (Merck) supplemented

with 5 % (v/v) horse blood for 2 to 5 days at 37 °C in an atmosphere containing 100 % CO₂ [except for *Bacteroides propionificiens* JCM 14649^T, which was grown on modified PY4S agar (Ueki *et al.*, 2008) for 5 to 7 days at 30 °C in an atmosphere containing 10 % CO₂ and 90 % N₂].

API tests. Physiological and biochemical reactions were determined in duplicate with the API 20A anaerobe test kit and the Rapid ID 32A anaerobe identification kit, respectively (bioMérieux), according to the manufacturer's instructions.

Cellular fatty acids. Fatty acid methyl esters (FAMES) were obtained from about 40 mg wet cells by saponification, methylation and extraction using minor modifications (Kuykendall *et al.*, 1988) of the method of Miller (1982). Cellular fatty acid profiles were determined by the MIDI microbial identification system (Microbial ID). Peaks were automatically integrated, fatty acids were identified by equivalent chain-length (ECL), and percentages of the total peak area were calculated. External calibration was done by using MIDI calibration mixture 1 (FAMES of straight-chain saturated fatty acids from nine to 20 carbons in length and five hydroxy acids).

DNA–DNA hybridization. Chromosomal DNA was isolated by previously described methods (Marmur, 1961; Saito & Miura, 1963), with some modifications. The DNA base composition was determined by the HPLC method of Tamaoka & Komagata (1984). The elution solvent was a mixture of 0.02 M NH₄H₂PO₄ and acetonitrile (20:1, v/v). The DNA–DNA relatedness experiment was carried out in microplate wells as described by Ezaki *et al.* (1989). Hybridization was performed at 44 °C for 16 h.

PCR amplification and sequencing. The 16S rRNA gene was analysed as described previously (Sakamoto *et al.*, 2002). The partial *hsp60* gene was amplified by PCR using primers H729 (5'-CGC CAG GGT TTT CCC AGT CAC GAC GAI III GCI GGI GAY GGI ACI ACI AC-3') and H730 (5'-AGC GGA TAA CAA TTT CAC ACA GGA YKI YKI TCI CCR AAI CCI GGI GCI TT-3') (Brousseau *et al.*, 2001); inosine was used to reduce the degeneracy of the sequences (Ohtsuka *et al.*, 1985). These primers were derived from the previously described H279A and H280A primers (Goh *et al.*, 1997) by addition of the sequences for commercially available M13 24 bp sequencing primers (underlined nucleotides). Primers were designed to amplify the 'universal target' (Hill *et al.*, 2004), the region of the *cpn60* gene, encoding 60 kDa chaperonin protein subunits (Cpn60, also known as Hsp60 or GroEL), corresponding to nucleotides 274–828 of the *Escherichia coli cpn60* gene sequence. Amplification reactions were performed in a total volume of 50 µl containing 2.5 µl DNA (50 ng), 1.25 U TaKaRa Ex Taq (Takara Bio), 5 µl 10 × Ex Taq buffer, 4 µl dNTP mixture (2.5 mM each), and 5 pmol each primer. *hsp60* genes were amplified in a Biometra Thermocycler TGradient using the following programme: 94 °C for 5 min, followed by 40 cycles consisting of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, with a final extension period at 72 °C for 10 min. Sequencing of purified PCR products was performed with standard M13 sequencing primers, as indicated in the primer sequences above, and a BigDye Terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems) in an Applied Biosystems 3130xl Genetic Analyzer.

Phylogenetic data analysis. Related sequences were aligned by using the CLUSTAL X 2.0.12 program (Larkin *et al.*, 2007) and corrected by manual inspection. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura, 1980) after gaps and unknown bases were eliminated. The phylogenetic tree was reconstructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap resampling analysis (Felsenstein, 1985) was performed to estimate the confidence of tree topologies. A maximum-likelihood tree was also generated by the DNAML program in the PHYLIP software package v3.69.

Table 1. *Bacteroides* strains investigated and accession numbers of the *hsp60* and 16S rRNA gene sequences determined in this study

Species	Strain	Accession no.	
		<i>hsp60</i> gene	16S rRNA gene
<i>B. acidifaciens</i>	JCM 10556 ^T	AB510667	AB510696
<i>B. barnesiae</i>	JCM 13652 ^T	AB510668	–
<i>B. caccae</i>	JCM 9498 ^T	AB510669	AB510697
<i>B. cellulosilyticus</i>	JCM 15632 ^T	AB510670	AB510698
<i>B. coprocola</i>	JCM 12979 ^T	AB510671	–
<i>B. coprophilus</i>	JCM 13818 ^T	AB510672	–
<i>B. coprosuis</i>	JCM 13475 ^T	AB510673	AB510699
<i>B. dorei</i>	JCM 13471 ^T	AB510674	–
<i>B. eggerthii</i>	JCM 12986 ^T	AB510675	AB510700
<i>B. finegoldii</i>	JCM 13345 ^T	AB510676	–
<i>B. fragilis</i>	JCM 11019 ^T	AB510677	AB510701
	JCM 11017	AB542762	AB542764
<i>B. gallinarum</i>	JCM 13658 ^T	AB510678	–
<i>B. helcogenes</i>	JCM 6297 ^T	AB510679	AB510702
<i>B. intestinalis</i>	JCM 13265 ^T	AB510680	–
<i>B. massiliensis</i>	JCM 13223 ^T	AB510681	AB510703
<i>B. nordii</i>	JCM 12987 ^T	AB510682	AB510704
<i>B. ovatus</i>	JCM 5824 ^T	AB510683	AB510705
<i>B. plebeius</i>	JCM 12973 ^T	AB510684	–
<i>B. propionificiens</i>	JCM 14649 ^T	AB510685	AB510706
	JCM 14650	AB542763	AB542765
<i>B. pyogenes</i>	JCM 6294 ^T	AB510686	–
	ATCC 35418 ^T	–	AB542766
	CCUG 15419 ^T	–	AB542767
	DSM 20611 ^T	–	AB542768
<i>B. salanitronis</i>	JCM 13657 ^T	AB510687	–
<i>B. salyersiae</i>	JCM 12988 ^T	AB510688	AB510707
<i>B. stercoris</i>	JCM 9496 ^T	AB510689	AB510708
<i>B. suis</i>	JCM 6292 ^T	AB510690	AB510709
	ATCC 35419 ^T	–	AB542769
	CCUG 15420 ^T	–	AB542770
	DSM 20612 ^T	–	AB542771
<i>B. tectus</i>	JCM 10003 ^T	AB510691	–
<i>B. thetaiotaomicon</i>	JCM 5827 ^T	AB510692	AB510710
<i>B. uniformis</i>	JCM 5828 ^T	AB510693	AB510711
<i>B. vulgatus</i>	JCM 5826 ^T	AB510694	AB510712
<i>B. xylanisolvens</i>	JCM 15633 ^T	AB510695	AB510713

RESULTS AND DISCUSSION

hsp60 gene sequence analysis

In this study, 558 bp nucleotide sequences (186 codons) of the *hsp60* gene from 29 *Bacteroides* type strains were used for analysis and the sequences were deposited in the DDBJ and assigned accession numbers as listed in Table 1. In addition, intra-species variations were determined for *Bacteroides fragilis* and *B. propionificiens*. High sequence similarities were observed among the *hsp60* genes of *B. fragilis* (99.8 %) and *B. propionificiens* (100 %) as well as among the 16S rRNA genes of *B. fragilis* (99.7 %) and *B. propionificiens* (100 %). Mean *hsp60* gene sequence similarity among the 29 *Bacteroides* type strains was 84.5 %, indicating a divergence of the *hsp60* gene that is greater than that of the 16S rRNA gene (90.7 %) (Table 2). Sequence similarity among the *Bacteroides* strains ranged from 72.9 to 100 % in the *hsp60* gene (see Supplementary Table S1 available in IJSEM Online). On the other hand, a comparative nucleotide sequence analysis of the 16S rRNA gene revealed a tighter range of identities (83.5–100 %); most species of the genus *Bacteroides* showed more than 90 % similarity in their 16S rRNA gene sequences. These results indicated that the base substitution rate of the *hsp60* gene sequence was much faster than that of the 16S rRNA gene sequence. Phylogenetic trees based on the *hsp60* and 16S rRNA gene sequences were reconstructed by the neighbour-joining and maximum-likelihood methods. Whichever algorithm was used, most phylogenetic relationships among species of the genus *Bacteroides* were

stably maintained, except for some minor differences at the deepest branching points (data not shown). The phylogenetic analysis indicated a higher evolutionary rate for the *hsp60* gene sequence than for the 16S rRNA gene sequence (Fig. 1). Phylogenetic analysis of species of the genus *Bacteroides* based on the *hsp60* gene sequence suggested that the *hsp60* gene would be able to differentiate related species, except for *Bacteroides pyogenes* JCM 6294^T, *Bacteroides suis* JCM 6292^T and *Bacteroides tectus* JCM 10003^T. In this study, the *hsp60* gene sequences were used, since the phylogenetic tree obtained from the Hsp60 amino acid sequences showed low resolution (data not shown). Nucleotide substitution of the *hsp60* gene provoked synonymous amino acid substitution.

A range of similarity values of the *hsp60* and 16S rRNA genes were compared for each group of the genus *Bacteroides* to evaluate discrimination powers (Table 2). Among the 29 *Bacteroides* type strains, the mean sequence similarity between *Bacteroides coprosuis* JCM 13475^T and the other strains for the *hsp60* gene (76.5 %) was significantly less than that for the 16S rRNA gene (88.4 %), and the mean sequence similarity between *B. propionificiens* JCM 14649^T and other *Bacteroides* strains was remarkably low (76.0 %). This comparison indicated that the *hsp60* gene has a higher discriminatory power than the 16S rRNA gene, except for the *B. pyogenes* group.

Similarity values for *hsp60* gene sequences among 29 different species of the genus *Staphylococcus* ranged from 74 to 93 % (mean 82 %) (Kwok *et al.*, 1999). In addition, pairwise sequence identity scores based on partial *hsp60* gene sequences among the four *Macrococcus* species tested ranged from 82 to 87 % (mean 83 %) (Kwok & Chow, 2003). These values were in agreement with the present study (range 72.9–100 %, mean 84.5 %). Our results reconfirmed the usefulness of the *hsp60* gene sequence for identification and phylogenetic analysis of micro-organisms.

B. pyogenes group

B. pyogenes and *B. suis* were proposed by Benno *et al.* (1983) and deposited in several public culture collections. It has been reported that *B. pyogenes* and *B. suis* are genomically different species (Benno *et al.*, 1983). In this study, the sequence similarity between *B. pyogenes* JCM 6294^T and *B. suis* JCM 6292^T was 100 % for the *hsp60* gene and 100 % for the 16S rRNA gene. It is, therefore, considered that the depositors might have mistaken the strains. On the other hand, when *B. tectus* was proposed by Love *et al.* (1986) these authors did not use *B. pyogenes* and *B. suis* as reference strains. The similarity value of *B. tectus* JCM 10003^T and *B. pyogenes* JCM 6294^T or *B. suis* JCM 6292^T was 99.6 % for the *hsp60* gene and 98.7 % for the 16S rRNA gene. This finding suggests that *B. tectus*, *B. pyogenes* and *B. suis* may be the same species, with *B. tectus* a later heterotypic synonym of *B. pyogenes* or *B. suis*. To resolve these problems, further studies were performed.

Table 2. Percentage sequence similarity among the *hsp60* and 16S rRNA genes of 29 *Bacteroides* type strains

	Percentage similarity	
	<i>hsp60</i> gene	16S rRNA gene
<i>B. coprosuis</i> – others		
Mean	76.5	88.4
Range	72.9–82.4	83.5–93.5
<i>B. propionificiens</i> – others		
Mean	76.0	88.7
Range	73.3–82.4	83.9–93.5
<i>B. ovatus</i> group*		
Mean	92.3	96.0
Range	89.6–96.6	94.7–97.7
<i>B. pyogenes</i> group†		
Mean	99.7	99.1
Range	99.6–100	98.7–100
All members		
Mean	84.5	90.7
Range	72.9–100	83.5–100

**B. ovatus* group contains *B. ovatus*, *B. xylanisolvens*, *B. acidifaciens*, *B. caccae*, *B. thetaiotaomicron* and *B. finegoldii*.

†*B. pyogenes* group contains *B. pyogenes*, *B. suis* and *B. tectus*.

Fig. 1. Phylogenetic tree based on *hsp60* (a) and 16S rRNA (b) gene sequences showing the relationships among the 29 species of the genus *Bacteroides* recognized at the time of writing. The tree was reconstructed by the neighbour-joining method. Numbers at nodes indicate percentage bootstrap values of 1000 replicates. Bars, 0.02 substitutions per nucleotide position. Accession numbers are given for each strain.

Table 3. Phenotypic and biochemical characteristics of *Bacteroides pyogenes*, *Bacteroides suis* and *Bacteroides tectus*

Strains: 1, *B. pyogenes* JCM 6294^T; 2, *B. suis* JCM 6292^T; 3, *B. tectus* JCM 10003^T. All strains produced acid from glucose, lactose and maltose. All strains failed to produce acid from L-arabinose, D-mannitol, melezitose, D-rhamnose, salicin, trehalose and D-xylose. All strains were negative for indole production, gelatin digestion, aesculin hydrolysis and urease and catalase activities. All strains were positive in Rapid ID 32A tests for β -N-acetylglucosaminidase, alkaline phosphatase, α -fucosidase, β -galactosidase, α -glucosidase, leucyl glycine arylamidase and alanine arylamidase activities. All strains were negative in Rapid ID 32A tests for indole production, nitrate reduction, urease, arginine dihydrolase, α -arabinosidase, α -galactosidase, β -galactosidase 6-phosphate, β -glucuronidase, glutamic acid decarboxylase, arginine arylamidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, phenylalanine arylamidase, proline arylamidase, pyroglutamic acid arylamidase, serine arylamidase and tyrosine arylamidase activities. +, Positive; w, weakly positive; -, negative.

Characteristic	1	2	3
Acid produced from:			
Cellobiose	-	+	-
Glycerol	-	+	-
D-Mannose	+	-	+
Raffinose	-	+	-
D-Sorbitol	-	+	-
Sucrose	+	+	-
Rapid ID 32A tests			
β -Glucosidase	+	+	-
Glutamyl glutamic acid arylamidase	+	+	w
Mannose fermentation	+	+	w
Raffinose fermentation	+	+	w

16S rRNA gene sequences of *B. pyogenes* and *B. suis* type strains obtained from other culture collections (ATCC, CCUG and DSMZ) (Table 1) were analysed and compared with the JCM type strains. The levels of sequence similarity among the eight strains were 100 %. Consequently, other analyses were performed using only the JCM type strains. Physiological and biochemical characteristics of *B. pyogenes* JCM 6294^T, *B. suis* JCM 6292^T and *B. tectus* JCM 10003^T are shown in Table 3. These three strains were phenotypically similar. The cellular fatty acid compositions of *B. pyogenes* JCM 6294^T, *B. suis* JCM 6292^T and *B. tectus* JCM 10003^T were almost the same. The major cellular fatty acid of the above strains was anteiso-15:0 (22–24 %) (Table 4). The DNA G+C contents of *B. pyogenes* JCM 6294^T, *B. suis* JCM 6292^T and *B. tectus* JCM 10003^T were 46.6, 46.7 and 46.9 mol%, respectively. The levels of DNA–DNA relatedness between *B. pyogenes* JCM 6294^T, *B. suis* JCM 6292^T and *B. tectus* JCM 10003^T are shown in Table 5 and demonstrate that these three type strains represent a single species. Consequently, *B. suis* and *B. tectus* are heterotypic synonyms of *B. pyogenes*.

Table 4. Cellular fatty acid compositions of *Bacteroides pyogenes*, *Bacteroides suis* and *Bacteroides tectus*

Strains: 1, *B. pyogenes* JCM 6294^T; 2, *B. suis* JCM 6292^T; 3, *B. tectus* JCM 10003^T. Values are percentages of total fatty acids. tr, Trace amount (<1 %).

Fatty acid	1	2	3
Saturated straight-chain			
14:0	tr	tr	tr
15:0		tr	1.3
16:0	5.2	3.5	6.2
18:0	tr	tr	tr
Unsaturated straight-chain			
18:2 ω 6,9c	1.4	1.3	1.5
18:1 ω 9c	15.9	11.8	16.9
Hydroxy acids			
iso-15:0 3-OH	tr	tr	
15:0 3-OH		tr	tr
16:0 3-OH	6.9	4.4	5.5
iso-17:0 3-OH	17.0	17.7	14.0
anteiso-17:0 3-OH	4.5	3.7	4.9
17:0 3-OH			1.3
Saturated branched-chain			
iso-14:0			tr
iso-15:0	12.5	14.2	10.4
iso-15:0 DMA		tr	
iso-16:0	tr	tr	tr
iso-17:0	1.3	1.3	1.2
anteiso-15:0	21.6	22.9	23.7
anteiso-17:0	8.4	7.5	2.5
Summed features*			
3	tr	5.8	4.5
9	tr	tr	
10	2.1	1.8	2.8

*Summed feature 3 contained an unknown fatty acid of ECL 13.570 and/or iso-15:0 ALDE. Summed feature 9 contained iso-16:0 3-OH and/or an unknown fatty acid of ECL 17.157. Summed feature 10 contained an unknown fatty acid of ECL 17.834 and/or 18:1 ω 11c/9t/6t.

Emended description of *Bacteroides pyogenes* Benno *et al.* 1983

The description is as given by Benno *et al.* (1983) and Love *et al.* (1986) with the following modification: the major cellular fatty acid is anteiso-15:0.

In conclusion, this study suggests that the *hsp60* gene is an alternative phylogenetic marker for the classification of species of the genus *Bacteroides*. Analysis based on the *hsp60* gene sequences in combination with other house-keeping gene sequences may provide a highly reliable identification system for species of the genus *Bacteroides*. In addition, the greater variability of the *hsp60* gene sequences should be useful in designing specific primers for PCRs capable of assisting in the rapid identification of species of the genus *Bacteroides*. We are currently planning the

Table 5. DNA base compositions and levels of DNA–DNA relatedness between *Bacteroides pyogenes*, *Bacteroides suis* and *Bacteroides tectus*

Strain	G + C content (mol%)	DNA relatedness (%) with:		
		1	2	3
1. <i>B. pyogenes</i> JCM 6294 ^T	46.6	100	95	95
2. <i>B. suis</i> JCM 6292 ^T	46.7	71	100	70
3. <i>B. tectus</i> JCM 10003 ^T	46.9	76	71	100

analysis of multiple genes (*dnaJ*, *gyrB*, *recA*, *rpoB*, etc.) other than *hsp60* to generate more useful data.

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