Usefulness of the *hsp60* gene for the identification and classification of Gram-negative anaerobic rods

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The *hsp60* gene sequences were determined for 121 strains of Gram-negative anaerobic rods, including the genera *Bacteroides*, *Barnesiella*, *Butyrivimonas*, *Odoribacter*, *Parabacteroides*, *Paraprevotella*, *Porphyromonas*, *Prevotella* and *Tannerella*. The mean pairwise *hsp60* gene sequence similarity (73.8–97.1 %) between species in each genus, except for the genus *Tannerella* that comprises one species, was significantly less than that of the 16S rRNA gene sequence (88.3–96.3 %). Only pairwise *hsp60* gene sequence similarity (97.1 %) of the genus *Paraprevotella* was higher than that of the 16S rRNA gene sequence (93.8 %). Each genus formed a distinct clade in the phylogenetic analysis of the *hsp60* gene sequence as well as the 16S rRNA gene sequence. The phylogenetic analysis indicated a higher evolutionary rate for the *hsp60* gene sequence than the 16S rRNA gene sequence, especially in the genera *Porphyromonas* and *Prevotella*. This study suggests that the *hsp60* gene is a useful alternative phylogenetic marker for the identification and classification of a broad range of Gram-negative anaerobic rods.

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INTRODUCTION

Gram-negative anaerobic rods that consist of the genera *Bacteroides*, *Porphyromonas*, *Prevotella* (Shah & Collins, 1988, 1989, 1990) are part of the indigenous microbiota of the human and animal gastrointestinal tract, oral cavity etc., but various species in this group are commonly associated with a variety of human and animal infections. The taxonomy of Gram-negative anaerobic rods has undergone significant changes in the past decade and several novel genera have been described (Hardham *et al.*, 2008; Morotomi *et al.*, 2009; Sakamoto & Benno, 2006; Sakamoto *et al.*, 2002, 2007b, 2009b). A large number of novel species have also been proposed in the genera *Bacteroides* and *Prevotella* (Bakir *et al.*, 2006a, b, c; Hayashi *et al.*, 2007a, b; Kitahara *et al.*, 2005; Lan *et al.*, 2006; Sakamoto *et al.*, 2004, 2005a, b, 2007c, 2009a, 2010a). These recent taxonomic changes of Gram-negative anaerobic rods are mainly based on 16S rRNA gene sequence analysis (Shah *et al.*, 2009). It has been reported that 16S rRNA gene sequencing is useful for the identification of isolates of clinically significant *Bacteroides* species (Song *et al.*, 2005). However, accurate identification of an isolate closely related to known species is often difficult with only the 16S rRNA gene analysis and other DNA-based methods such as

DNA–DNA hybridization are required for differentiating closely related species as shown in the case of *Parabacteroides johnsonii* and *Parabacteroides merdae* (Sakamoto *et al.*, 2007a). Because the DNA–DNA hybridization method is time-consuming and laborious, an alternative molecular marker is required.

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 some *Prevotella* species (Sakamoto *et al.*, 2010a). A reference database of the *cpn60* (*hsp60*) gene sequences, cpnDB, has been made freely available via a web interface (<http://cpndb.cbr.nrc.ca/home.php>) (Hill *et al.*, 2004). The potential for the use of the cpnDB has been demonstrated by its use as a tool for the identification of *Campylobacter* spp. and their distinction from phenotypically similar *Helicobacter* and *Arcobacter* spp. (Hill *et al.*, 2006). More recently, the *hsp60* gene has been found to be an alternative phylogenetic marker for the classification of species of the genus *Bacteroides* (Sakamoto *et al.*, 2010b). Except for the genus *Bacteroides*, however, there is little information about whether the *hsp60* gene is a suitable phylogenetic marker for the taxonomy of Gram-negative anaerobic rods. In this study, we have evaluated the usefulness of the *hsp60* gene for the identification and classification of a broad range of Gram-negative anaerobic rods, including the genera *Barnesiella*, *Butyrivimonas*, *Odoribacter*, *Parabacteroides*, *Paraprevotella*, *Porphyromonas*, *Prevotella* and *Tannerella*, as well as *Bacteroides*.

Abbreviation: JCM, Japan Collection of Microorganisms.

The GenBank/EMBL/DDBJ accession numbers for the partial *hsp60* gene sequences of Gram-negative anaerobic rods determined in this study are listed in Table 1.

Tables of sequence similarity data are available as supplementary material with the online version of this paper.

Table 1. Bacterial strains used in this study and their accession numbers in the databases

Genus	Species	Strain	Accession no.		Reference
			<i>hsp60</i> gene	16S rRNA gene	
<i>Bacteroides</i>	<i>B. acidifaciens</i>	JCM 10556 ^T	AB510667	AB510696	Sakamoto <i>et al.</i> (2010b)
	<i>B. barnesiiae</i>	JCM 13652 ^T	AB510668	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. caccae</i>	JCM 9498 ^T	AB510669	AB510697	Sakamoto <i>et al.</i> (2010b)
	<i>B. cellulosilyticus</i>	JCM 15632 ^T	AB510670	AB510698	Sakamoto <i>et al.</i> (2010b)
	' <i>B. chinchillae</i> '	JCM 16497 ^T	AB547549	AB547636	This study
		JCM 16498	AB547550	AB547637	This study
	<i>B. clarus</i>	JCM 16067 ^T	AB547551	AB547638	This study
	<i>B. coagulans</i>	JCM 12528 ^T	AB547552	AB547639	This study
	<i>B. coprocola</i>	JCM 12979 ^T	AB510671	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. coprophilus</i>	JCM 13818 ^T	AB510672	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. coprosuis</i>	JCM 13475 ^T	AB510673	AB510699	Sakamoto <i>et al.</i> (2010b)
	<i>B. dorei</i>	JCM 13471 ^T	AB510674	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. eggerthii</i>	JCM 12986 ^T	AB510675	AB510700	Sakamoto <i>et al.</i> (2010b)
	' <i>B. faecis</i> '	JCM 16478 ^T	AB547553	AB547640	This study
		JCM 16477	AB547554	AB547641	This study
	<i>B. finegoldii</i>	JCM 13345 ^T	AB510676	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. fluxus</i>	JCM 16101 ^T	AB547555	AB547642	This study
	<i>B. fragilis</i>	JCM 11019 ^T	AB510677	AB510701	Sakamoto <i>et al.</i> (2010b)
		JCM 11017	AB542762	AB542764	Sakamoto <i>et al.</i> (2010b)
	<i>B. gallinarum</i>	JCM 13658 ^T	AB510678	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. graminisolvens</i>	JCM 15093 ^T	AB547556	AB547643	This study
	<i>B. helcogenes</i>	JCM 6297 ^T	AB510679	AB510702	Sakamoto <i>et al.</i> (2010b)
	<i>B. intestinalis</i>	JCM 13265 ^T	AB510680	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. massiliensis</i>	JCM 13223 ^T	AB510681	AB510703	Sakamoto <i>et al.</i> (2010b)
	<i>B. nordii</i>	JCM 12987 ^T	AB510682	AB510704	Sakamoto <i>et al.</i> (2010b)
	<i>B. oleiciplenus</i>	JCM 16102 ^T	AB547557	AB547644	This study
	<i>B. ovatus</i>	JCM 5824 ^T	AB510683	AB510705	Sakamoto <i>et al.</i> (2010b)
	' <i>B. paurosaccharolyticus</i> '	JCM 15092 ^T	AB547558	AB547645	This study
	<i>B. plebeius</i>	JCM 12973 ^T	AB510684	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. propionificaciens</i>	JCM 14649 ^T	AB510685	AB510706	Sakamoto <i>et al.</i> (2010b)
		JCM 14650	AB542763	AB542765	Sakamoto <i>et al.</i> (2010b)
	<i>B. pyogenes</i>	JCM 6294 ^T	AB510686	–	Sakamoto <i>et al.</i> (2010b)
	' <i>B. rodentium</i> '	JCM 16496 ^T	AB547559	AB547646	This study
	<i>B. salanitronis</i>	JCM 13657 ^T	AB510687	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. salyersiae</i>	JCM 12988 ^T	AB510688	AB510707	Sakamoto <i>et al.</i> (2010b)
	<i>B. stercoris</i>	JCM 9496 ^T	AB510689	AB510708	Sakamoto <i>et al.</i> (2010b)
	<i>B. suis</i>	JCM 6292 ^T	AB510690	AB510709	Sakamoto <i>et al.</i> (2010b)
	<i>B. tectus</i>	JCM 10003 ^T	AB510691	–	Sakamoto <i>et al.</i> (2010b)
	<i>B. thetaiotaomicron</i>	JCM 5827 ^T	AB510692	AB510710	Sakamoto <i>et al.</i> (2010b)
	<i>B. uniformis</i>	JCM 5828 ^T	AB510693	AB510711	Sakamoto <i>et al.</i> (2010b)
	<i>B. vulgatus</i>	JCM 5826 ^T	AB510694	AB510712	Sakamoto <i>et al.</i> (2010b)
	<i>B. xylanisolvens</i>	JCM 15633 ^T	AB510695	AB510713	Sakamoto <i>et al.</i> (2010b)
<i>Barnesiella</i>	<i>B. intestinihominis</i>	JCM 15079 ^T	AB547560	AB547647	This study
	<i>B. viscericola</i>	JCM 13660 ^T	AB547561	–	This study
<i>Butyrivimonas</i>	<i>B. synergistica</i>	JCM 15148 ^T	AB547562	–	This study
	<i>B. virosa</i>	JCM 15149 ^T	AB547563	–	This study
<i>Odoribacter</i>	<i>O. laneus</i>	JCM 16069 ^T	AB547564	AB547648	This study
	<i>O. splanchnicus</i>	JCM 15291 ^T	AB547565	AB547649	This study
<i>Parabacteroides</i>	<i>P. distasonis</i>	JCM 5825 ^T	AB547566	–	This study
	<i>P. goldsteinii</i>	JCM 13446 ^T	AB547567	AB547650	This study
	<i>P. gordonii</i>	JCM 15724 ^T	AB547568	–	This study
	<i>P. johnsonii</i>	JCM 13406 ^T	AB547569	–	This study
	<i>P. merdae</i>	JCM 9497 ^T	AB547570	–	This study
<i>Paraprevotella</i>	<i>P. clara</i>	JCM 14859 ^T	AB547571	AB547651	This study

Table 1. cont.

Genus	Species	Strain	Accession no.		Reference
			<i>hsp60</i> gene	16S rRNA gene	
<i>Porphyromonas</i>	<i>P. xylaniphila</i>	JCM 14860 ^T	AB547572	AB547652	This study
	<i>P. asaccharolytica</i>	JCM 6326 ^T	AB547573	AB547653	This study
	<i>P. cangingivalis</i>	JCM 15983 ^T	AB547574	AB547654	This study
	<i>P. canoris</i>	JCM 16132 ^T	AB547575	–	This study
	<i>P. cansulci</i>	JCM 13913 ^T	AB547576	AB547655	This study
	<i>P. catoniae</i>	JCM 13863 ^T	AB547577	AB547656	This study
	<i>P. circumdentaria</i>	JCM 13864 ^T	AB547578	AB547657	This study
	<i>P. crevioricanis</i>	JCM 15906 ^T	AB547579	AB547658	This study
	<i>P. endodontalis</i>	JCM 8526 ^T	AB547580	AB547659	This study
	<i>P. gingivalis</i>	JCM 12257 ^T	AB547581	AB547660	This study
		JCM 8525	AB547582	AB547661	This study
	<i>P. gingivicanis</i>	JCM 15907 ^T	AB547583	AB547662	This study
	<i>P. gulae</i>	JCM 13865 ^T	AB547584	AB547663	This study
	<i>P. levii</i>	JCM 13866 ^T	AB547585	AB547664	This study
	<i>P. macacae</i>	JCM 13914 ^T	AB547586	AB547665	This study
	<i>P. salivosa</i>	JCM 15984 ^T	AB547587	AB547666	This study
	<i>P. somerae</i>	JCM 13867 ^T	AB547588	AB547667	This study
	<i>P. uenonis</i>	JCM 13868 ^T	AB547589	AB547668	This study
<i>Prevotella</i>	<i>P. albensis</i>	JCM 12258 ^T	AB547590	AB547669	This study
	<i>P. amnii</i>	JCM 14753 ^T	AB547591	AB547670	This study
	<i>P. aurantiaca</i>	JCM 15754 ^T	AB480536	AB480534	Sakamoto <i>et al.</i> (2010a)
	<i>P. baroniae</i>	JCM 13447 ^T	AB547592	AB547671	This study
	<i>P. bergensis</i>	JCM 13869 ^T	AB547593	AB547672	This study
	<i>P. bivia</i>	JCM 6331 ^T	AB547594	AB547673	This study
		JCM 6332	AB547595	AB547674	This study
	<i>P. buccae</i>	JCM 12245 ^T	AB547596	AB547675	This study
	<i>P. buccalis</i>	JCM 12246 ^T	AB547597	AB547676	This study
	<i>P. copri</i>	JCM 13464 ^T	AB547598	–	This study
	<i>P. corporis</i>	JCM 8529 ^T	AB547599	AB547677	This study
	<i>P. dentalis</i>	JCM 13448 ^T	AB547600	AB547678	This study
	<i>P. dentasini</i>	JCM 15908 ^T	AB547603	AB547681	This study
	<i>P. denticola</i>	JCM 13449 ^T	AB547601	AB547679	This study
		JCM 8528	AB547602	AB547680	This study
	<i>P. disiens</i>	JCM 6334 ^T	AB547604	AB547682	This study
		JCM 6333	AB547605	AB547683	This study
	<i>P. enoeca</i>	JCM 12259 ^T	AB547606	AB547684	This study
	<i>P. falsenii</i>	JCM 15124 ^T	AB480537	–	Sakamoto <i>et al.</i> (2010a)
	<i>P. histicola</i>	JCM 15637 ^T	AB547607	AB547685	This study
	<i>P. intermedia</i>	JCM 12248 ^T	AB547608	AB547686	This study
		JCM 11150 ^T	AB547609	AB547687	This study
	<i>P. loescheii</i>	JCM 12249 ^T	AB547610	AB547688	This study
		JCM 8530 ^T	AB547611	AB547689	This study
	<i>P. maculosa</i>	JCM 15638 ^T	AB547612	AB547690	This study
	<i>P. marshii</i>	JCM 13450 ^T	AB547613	AB547691	This study
	<i>P. melaninogenica</i>	JCM 6325 ^T	AB547615	AB547693	This study
		JCM 6321	AB547616	AB547694	This study
	<i>P. micans</i>	JCM 16134 ^T	AB547614	AB547692	This study
	<i>P. multiformis</i>	JCM 12541 ^T	AB547617	–	This study
	<i>P. multisaccharivorax</i>	JCM 12954 ^T	AB547618	–	This study
	<i>P. nanceiensis</i>	JCM 15639 ^T	AB547619	AB547695	This study
	<i>P. nigrescens</i>	JCM 12250 ^T	AB547620	AB547696	This study
		JCM 6322 ^T	AB547621	AB547697	This study
	<i>P. oralis</i>	JCM 12251 ^T	AB547622	AB547698	This study
		JCM 6330	AB547623	AB547699	This study

Table 1. cont.

Genus	Species	Strain	Accession no.		Reference
			<i>hsp60</i> gene	16S rRNA gene	
<i>Tannerella</i>	<i>P. oris</i>	JCM 12252 ^T	AB547624	AB547700	This study
		JCM 8540 ^T	AB547625	AB547701	This study
	<i>P. oulorum</i>	JCM 14966 ^T	AB547626	AB547702	This study
	<i>P. pallens</i>	JCM 11140 ^T	AB480538	AB547703	Sakamoto <i>et al.</i> (2010a)
	<i>P. paludivivens</i>	JCM 13650 ^T	AB547627	AB547704	This study
		JCM 13651	AB547628	AB547705	This study
	<i>P. pleuritidis</i>	JCM 14110 ^T	AB547629	–	This study
	<i>P. salivae</i>	JCM 12084 ^T	AB547630	–	This study
	<i>P. shahii</i>	JCM 12083 ^T	AB547631	–	This study
	<i>P. stercorea</i>	JCM 13469 ^T	AB547632	–	This study
	<i>P. timonensis</i>	JCM 15640 ^T	AB547633	AB547706	This study
	<i>P. veroralis</i>	JCM 6290 ^T	AB547634	AB547707	This study
	<i>T. forsythia</i>	JCM 10827 ^T	AB547635	AB547708	This study

METHODS

Bacterial strains. A total of 121 strains (106 species from 9 genera) were used in this study and were obtained from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Wako, Japan, and are listed in Table 1. These belonged to the genera *Bacteroides* (42 strains from 38 species), *Barnesiella* (2 strains from 2 species), *Butyrivibrio* (2 strains from 2 species), *Odoribacter* (2 strains from 2 species), *Parabacteroides* (5 strains from 5 species), *Paraprevotella* (2 strains from 2 species), *Porphyromonas* (17 strains from 16 species), *Prevotella* (48 strains from 38 species) and *Tannerella* (1 strain from 1 species). The strains were maintained according to the JCM online catalogue (<http://www.jcm.riken.jp/JCM/catalogue.shtml>). Among the species mentioned, there were some that did not have standing in the nomenclature as the papers were in press for publication (therefore the species names are written in ‘ ’).

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 the previously described 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 GCY 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 H279A and H280A primers (Goh *et al.*, 1997) by addition of the sequences for the commercially available M13 24 bp sequencing primers at the 5′ end (indicated by the underlined nucleotides). Primers were designed to amplify the ‘universal target’ (Hill *et al.*, 2004), that is the region of the *cpn60* gene encoding the 60 kDa chaperonin protein subunits (Cpn60, also known as Hsp60 or GroEL) and 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 dNTP), and 5 pmol each primer. The *hsp60* genes were amplified in a thermocycler TGradient (Biomtra) using the following program: 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 primers 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 with 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 constructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap resampling analysis (Felsenstein, 1985) was performed to estimate the confidence of the tree topologies. A maximum-likelihood tree was also generated by the DNAML program in the PHYLIP software package version 3.69 (Felsenstein, 2005).

Statistical analysis. Statistical analysis was performed using analysis of variance (ANOVA) with R version 2.10.1 (2009; R Development Core Team) (<http://www.R-project.org>). A *P* value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

In this study, 558 bp nucleotide sequences (186 codons) of the *hsp60* gene from 121 strains of Gram-negative anaerobic rods were used for analysis and the sequences were deposited in the DNA Data Bank of Japan (DDBJ) – the assigned accession numbers are listed in Table 1. The intra-species comparisons of the 14 species where more than one strain was represented demonstrated high sequence similarities for both the *hsp60* and the 16S rRNA gene: ‘*Bacteroides chinchillae*’ (100 and 99.7%), ‘*Bacteroides faecis*’ (100 and 100%), *Bacteroides fragilis* (99.8 and 99.7%), *Bacteroides propionificiens* (100 and 100%), *Porphyromonas gingivalis* (98.9 and 99.7%), *Prevotella bivia* (99.1 and 99.9%), *Prevotella denticola* (99.6 and 99.7%), *Prevotella disiens* (99.6 and 99.7%), *Prevotella intermedia* (100 and 99.7%), *Prevotella loescheii* (100 and 100%), *Prevotella melaninogenica* (100 and 100%), *Prevotella nigrescens* (99.3 and 99.7%), *Prevotella oris* (100 and 100%) and *Prevotella paludivivens* (99.3 and 99.9%). These intra-species similarity values (98.9–100%) for the *hsp60* gene were slightly higher than those of *Arcobacter* spp. (96–100%) (Debruyne *et al.*, 2010) and *Campylobacter* spp. (95–100%) (Hill *et al.*, 2006).

Phylogenetic trees based on the *hsp60* and 16S rRNA gene sequences were constructed by the neighbour-joining and maximum-likelihood methods. Whichever algorithm was used, most phylogenetic relationships of the Gram-negative anaerobic rods were stably maintained, except for some minor differences at the deepest branching points (data not shown). Each genus formed a distinct clade in the phylogenetic analyses (Fig. 1). The phylogenetic analyses indicated a higher evolutionary rate for the *hsp60* gene sequence than the 16S rRNA gene sequence. The details of each genus are described below.

The genus *Bacteroides*

Most of the *Bacteroides* strains (31 strains from 29 species) have already been analysed (Sakamoto *et al.*, 2010b). In this study, the *hsp60* gene sequences of 11 strains from 9 species were newly analysed. Sequence similarities among the *Bacteroides* strains ranged from 71.6 to 100 % (mean $\pm$ SEM: 84.3 $\pm$ 0.2 %) in the *hsp60* gene (Table 2, Supplementary Table S1 available with the online journal). *Bacteroides coagulans* JCM 12528^T was excluded because of significantly low sequence similarities (59.6–66.8 %) compared with those of other *Bacteroides* strains. *B. coagulans* is now treated as *incertae sedis* species (Shah *et al.*, 2009). This was supported by 16S rRNA gene sequence analyses; comparative 16S rRNA gene sequence analysis revealed a tighter range of identities (83.5–100 %) (mean $\pm$ SEM: 91.2 $\pm$ 0.1 %), excluding *B. coagulans* (70.9–73.4 %). In the future, a novel genus will be proposed to accommodate *B. coagulans* that is phylogenetically far removed from the genus *Bacteroides* (Fig. 1). The taxonomic problem of three species, *Bacteroides pyogenes*, *Bacteroides suis* and *Bacteroides tectus*, that showed high sequence similarities (99.6–100 % for the *hsp60* gene sequence), has already been resolved. Sakamoto *et al.* (2010b) provided evidence that *B. suis* (Benno *et al.*, 1983) and *B. tectus* (Love *et al.*, 1986) were heterotypic synonyms of *B. pyogenes* (Benno *et al.*, 1983). Consequently, the results of this study reinforce that *hsp60* gene sequence analysis is useful for differentiation of truly distinct species in the genus *Bacteroides*.

The genus *Parabacteroides*

Sequence similarities among *Parabacteroides* spp. ranged from 83.3 to 97.1 % (mean $\pm$ SEM: 87.9 $\pm$ 1.5 %) in the *hsp60* gene (Table 2, Supplementary Table S2 available with the online journal), whereas pairwise 16S rRNA gene sequence similarities ranged from 92.1 to 97.9 % (mean $\pm$ SEM: 94.1 $\pm$ 0.5 %). The genus *Parabacteroides* (Sakamoto & Benno, 2006) was created to accommodate three species that were previously classified in the genus *Bacteroides*, viz. *Parabacteroides distasonis* (previously *Bacteroides distasonis*), *Parabacteroides goldsteinii* (previously *Bacteroides goldsteinii*) and *Parabacteroides merdae* (previously *Bacteroides merdae*). The creation of the genus *Parabacteroides* was based on biochemical and physiological examination and 16S rRNA gene sequence analysis. These three species were

phylogenetically far removed from the genus *Bacteroides*. This phylogenetic position observed in the 16S rRNA gene sequence analysis was again supported by the results of the *hsp60* gene sequence analysis (Fig. 1). Likewise, the relationship between closely related species *Parabacteroides johnsonii* and *Parabacteroides merdae* (97.9 % similarity for the 16S rRNA gene), was also evident based on the *hsp60* gene sequence (95.0 % similarity).

The genus *Porphyromonas*

Sequence similarities among the *Porphyromonas* strains ranged from 67.3 to 100 % (mean $\pm$ SEM: 73.8 $\pm$ 0.5 %) in the *hsp60* gene (Table 2, Supplementary Table S3 available with the online journal), whereas pairwise 16S rRNA gene sequence similarities ranged from 84.6 to 99.9 % (mean $\pm$ SEM: 88.3 $\pm$ 0.3 %). Most *Porphyromonas* species showed less than 80 % similarity in their *hsp60* gene sequences. The mean sequence similarity of the genus *Porphyromonas* (73.8 %) was significantly less than those of the genera *Bacteroides* (84.3 %) and *Parabacteroides* (87.9 %) ($P < 0.001$). This finding indicates that the *hsp60* gene has a high discriminatory power particularly in the genus *Porphyromonas*, except for some species (see below).

The *hsp60* gene sequence similarities between *Porphyromonas gingivalis* JCM 12257^T or JCM 8525 and *Porphyromonas gulae* JCM 13865^T were 99.6 or 98.9 %, respectively. Similarity values for the 16S rRNA gene sequences were also high (98.1 or 98.0 %) as reported by Fournier *et al.* (2001). *Porphyromonas gulae* was formerly known as animal strains of *Porphyromonas gingivalis* that were catalase positive. Although the above two species show high 16S rRNA gene sequence similarity, DNA–DNA hybridization data demonstrated that the animal strains of *Porphyromonas gingivalis* represent a novel species of the genus *Porphyromonas*, *Porphyromonas gulae* sp. nov., that is distinct from *Porphyromonas gingivalis* (Fournier *et al.*, 2001). The separation of *Porphyromonas gingivalis* and *Porphyromonas gulae* into distinct species has been confirmed by 16S–23S rRNA gene internal transcribed spacer data (Conrads *et al.*, 2005). Therefore, *hsp60* gene sequence analysis might be insufficient to differentiate between *Porphyromonas gingivalis* and *Porphyromonas gulae*.

Organisms now known as *Porphyromonas macacae* were first described by Slots & Genco (1980) as a distinct subspecies of *Bacteroides melaninogenicus* associated with monkey periodontal disease. Subsequently, *B. melaninogenicus* subsp. *macacae* was elevated to the species rank as *Bacteroides macacae* (Coykendall *et al.*, 1980). However, organisms now known as *Porphyromonas salivosa* were originally described by Love *et al.* (1987) as an asaccharolytic black-pigmented *Bacteroides* species isolated from soft-tissue infections and normal gingival margins from cats. Then, *Bacteroides salivosa* was reassigned to the genus *Porphyromonas* as *Porphyromonas salivosa* (Love *et al.*, 1992). As DNA–DNA hybridization data showed *B. macacae* and *Porphyromonas salivosa* are members of a single species, *Porphyromonas*

(a) *hsp60* gene sequences

Table 2. Mean percentage sequence similarity of the *hsp60* and 16S rRNA genes within each of major genera

	Percentage similarity	
	<i>hsp60</i> gene	16S rRNA gene
<i>Bacteroides</i>		
Mean $\pm$ SEM	84.3 $\pm$ 0.2	91.2 $\pm$ 0.1
Range	71.6–100	83.5–100
<i>Parabacteroides</i>		
Mean $\pm$ SEM	87.9 $\pm$ 1.5	94.1 $\pm$ 0.5
Range	83.3–97.1	92.1–97.9
<i>Porphyromonas</i>		
Mean $\pm$ SEM	73.8 $\pm$ 0.5	88.3 $\pm$ 0.3
Range	67.3–100	84.6–99.9
<i>Prevotella</i>		
Mean $\pm$ SEM	77.5 $\pm$ 0.1	89.6 $\pm$ 0.1
Range	67.9–94.4	85.6–97.8

salivosa has become a later synonym of *B. macacae* (Love, 1995). Consequently, *B. macacae* has been transferred to the genus *Porphyromonas* as *Porphyromonas macacae*. In this study, according to expectation, the 16S rRNA gene sequence similarity between *Porphyromonas macacae* JCM 13914^T and *Porphyromonas salivosa* JCM 15984^T was significantly high (99.6%); this is in agreement with the report of Paster *et al.* (1994). The similarity value for the *hsp60* gene sequence was 98.2%. From the viewpoint of *hsp60* gene sequence similarity, the relationship between *Porphyromonas macacae* and *Porphyromonas salivosa* is opposite to the relationship between *Porphyromonas gingivalis* and *Porphyromonas gulae* mentioned above.

Finally, *Porphyromonas cansulci* JCM 13913^T showed very high *hsp60* gene sequence similarity level (100%) to *Porphyromonas crevioricanis* JCM 15906^T, as well as a very high 16S rRNA gene sequence similarity level (99.9%). *Porphyromonas crevioricanis* (Hirasawa & Takada, 1994) and *Porphyromonas cansulci* (Collins *et al.*, 1994) appeared in the same issue (vol. 44, no. 4) of the *International Journal of Systematic Bacteriology*. If the level of DNA–DNA hybridization is sufficient to consider *Porphyromonas crevioricanis* and *Porphyromonas cansulci* members of the same species, *Porphyromonas cansulci* is a later synonym of *Porphyromonas crevioricanis* because the name *Porphyromonas crevioricanis* (Hirasawa and Takada 1994) has page priority over the name *Porphyromonas cansulci* (Collins *et al.* 1994). To resolve this issue further studies will be necessary.

The genus *Prevotella*

Sequence similarities among the *Prevotella* strains ranged from 67.9 to 94.4% (mean $\pm$ SEM: 77.5 $\pm$ 0.1%) in the *hsp60* gene (Table 2, Supplementary Table S4 available with the online journal), while pairwise 16S rRNA gene sequence similarities ranged from 85.6 to 97.7% (mean $\pm$ SEM: 89.6 $\pm$ 0.1%). Most *Prevotella* species showed less than 80% similarity in their *hsp60* gene sequences as in the case of the genus

Porphyromonas. The *hsp60* gene sequence of *Prevotella oralis* JCM 6330 was 75.9% identical to that of *P. oralis* JCM 12251^T, but *P. oralis* JCM 6330 shared 100% *hsp60* gene sequence similarity with *Prevotella veroralis* JCM 6290^T. This result was also confirmed using the cpnDB. *P. oralis* JCM 6330 is now catalogued as *Prevotella* sp. JCM 6330. In four cases (*P. intermedia*, *P. loescheii*, *P. nigrescens* and *P. oris*) two type strains per species do exist in the JCM collection (Table 1). The history since isolation is different in each type strain. In *P. loescheii* and *P. oris* intra-species similarity was 100% (*hsp60* gene) and 100% (16S rRNA gene) according to expectation. However, the intra-species similarity of *P. intermedia* and *P. nigrescens* was not 100% (exception for the *hsp60* gene sequence of *P. intermedia*) (Supplementary Table S4 available with the online journal). This finding indicates that the maintenance and propagation of strains in laboratories causes an ongoing microevolution of genotype and phenotype that jeopardizes the reproducibility of research.

Other genera

The *hsp60* gene sequence similarities between the species of the genera *Barnesiella*, *Butyrivibrio*, *Odoribacter* and *Paraprevotella* were 84.7, 83.5, 78.0 and 97.1%, respectively, while the 16S rRNA gene sequence similarities between the species of these genera were 96.3, 94.1, 90.0 and 93.8%, respectively. These data indicated that the *hsp60* gene has a higher discriminatory power than the 16S rRNA gene, including for the above-mentioned four genera. Regarding the genus *Paraprevotella*, however, the *hsp60* gene sequences of two species showed 97.1% similarity although their 16S rRNA gene sequences showed only 93.8% similarity. The analysis of *hsp60* gene sequences of additional strains is necessary.

Conclusion

This study suggests that the *hsp60* gene is an alternative phylogenetic marker for the identification and classification of a broad range of Gram-negative anaerobic rods. The data obtained in this study are also useful for helping in the design of specific primers for PCRs that are capable of assisting in the rapid identification of clinical isolates of Gram-negative anaerobic rods. We are currently planning the analysis of multiple genes (*dnaJ*, *gyrB*, *recA*, *rpoB* etc.) other than *hsp60* gene to generate more useful data.

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