***Bacteroides acidifaciens* sp. nov., isolated from the caecum of mice**

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During studies of the bacterial flora in the intestines of mice, we isolated characteristic strains which lowered the pH of peptone-yeast broth containing Fildes' digest. Based on 16S rRNA sequence comparison, these isolates were considered to belong to the *Bacteroides* cluster in the bacteroides subgroup of the *Cytophaga-Flavobacterium-Bacteroides* phylum, and were divided into two groups. Their phenotypic characteristics, i.e. growth in 20% bile, aesculin hydrolysis, and glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) activity, were the same as those of the '*Bacteroides fragilis* group'. The low level of DNA–DNA hybridization with type strains in the *Bacteroides* cluster confirmed the novel species status of these isolates. It is proposed that these isolates be named *Bacteroides acidifaciens*, the type strain of which is A40^T (= JCM 10556^T).

Keywords: *Bacteroides acidifaciens* sp. nov., mouse intestine, 16S rRNA sequencing, acid production

INTRODUCTION

More than 50 bacteroides species are recognized in the Approved Lists of Bacterial Names (amended edition) (Skerman *et al.*, 1989). These species exhibit a variety of cellular morphologies and are biochemically and physiologically extremely heterogeneous. Therefore, Shah & Collins (1989) proposed on the basis of biochemical and chemical criteria that the genus *Bacteroides* should be restricted to *Bacteroides fragilis* and closely related organisms.

On the other hand, on the basis of the analysis of the 16S rRNA sequence, *Bacteroides* forms the CFB group with *Cytophaga* and *Flavobacterium* (Woese, 1987). The CFB group was divided into the cytophaga subgroup, flavobacterium subgroup, bacteroides subgroup, etc. The bacteroides subgroup was further divided into the *Prevotella* cluster, *Porphyromonas* cluster, *Bacteroides* cluster and two new clusters. The *Bacteroides* cluster was equivalent to the old '*Bacteroides fragilis* group' except for *Bacteroides distasonis* and some other species (Paster *et al.*, 1994).

Abbreviations: CFB, *Cytophaga-Flavobacterium-Bacteroides*; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase.

The DDBJ accession numbers for the 16S rRNA gene sequences of strains A1, A8, A24, A29, A31, A32, A37, A40 and A43 are AB021158, AB021157, AB021159, AB021160, AB021161, AB021162, AB021163, AB021164 and AB021165, respectively.

We isolated a number of characteristic anaerobic bacteria from the mouse caecum. The isolates lowered the pH of peptone-yeast broth with Fildes' digest (PYF) (Kaneuchi *et al.*, 1979), acid fermentation by the isolates could not be evaluated. In this study, we attempted to clarify the genus and the species of these isolates. From the results of 16S rRNA gene sequence and DNA–DNA homology, we propose that these strains should be classified as a new species of the genus *Bacteroides*, *Bacteroides acidifaciens*.

METHODS

Bacterial strains and culture. Bacterial strains were isolated from the caecum of mice bred in our laboratory. Caecal contents were suspended and diluted with anaerobic-buffered (Mitsuoka *et al.*, 1965). Then, each dilution was spread on Eggerth–Gangon (EG) agar (Eiken) and neomycin-brilliant green-taurocholate acid (NBGT) agar and incubated at 37 °C in an anaerobic stainless steel jar filled with an atmosphere of 100% CO₂ by the steel-wool method (Parker, 1955). Strain A1, strain A24, and strains A29, 31, 32, 37, 40 and 43 were isolated from CF#1, NC, and BALB/c, respectively. These isolates predominantly colonized in mouse intestines. All type strains used in this study were obtained from the Japan Collection of Micro-organisms (JCM) and the German Collection of Micro-organisms and Cell Cultures (DSMZ).

Biological and biochemical tests. Morphological and biochemical characteristics were determined by the methods described by Kaneuchi *et al.* (1976, 1979). For preparation

Table 1. Characteristics that differentiate new isolates from phylogenetically closely related bacteroides species

JCM, Japan Collection of Microorganisms; DSM, German Collection of Microorganisms and Cell Cultures. Strains 1–6: 1, *Bacteroides ovatus* JCM 5824^T (= ATCC 8483^T); 2, *Bacteroides thetaiotaomicron* JCM 5827^T (= ATCC 29148^T); 3, *Bacteroides uniformis* JCM 5828^T (= ATCC 8492^T); 4, *Bacteroides eggerthii* DSM 20697^T (= ATCC 27754^T); 5, *Bacteroides vulgatus* JCM 5826^T (= ATCC 8482^T); 6, *Bacteroides fragilis* DSM 2151^T (= ATCC 25285^T). Symbols: +, positive; ++, strongly positive; –, negative. All strains were Gram-negative, and positive for aesculin hydrolysis and NH₃ production; all strains showed excellent growth in 20% bile and had stimulated growth, i.e. more growth than in the PYG control tube.

Characteristic	Strain/isolate:													
	1	2	3	4	5	6	A24	A31	A43	A32	A1	A37	A29	A40
Final pH	5.97	5.98	6.49	6.56	6.77	6.61	5.69	5.60	5.57	5.59	5.59	5.58	5.61	5.58
End product*	SAP	SA	SAP	SAP	SAP	SAP	SA	SA	SA	SA	SA	SA	SA	SA
Starch hydrolysis	+	+	+	+	–	+	–	–	–	+	+	+	+	+
H ₂ S production	+	+	+	–	+	+	–	–	–	–	–	–	–	–
Indole production	+	+	+	+	–	–	–	–	–	+	+	+	–	+
Nitrate reduction	++	–	–	++	–	++	–	–	–	–	–	++	+	–
Gelatin digestion	–	–	–	–	+	–	–	–	–	–	–	–	++	–
G6PDH activity	++	+	+	+	+	++	++	++	++	++	++	++	++	++
6PGDH activity	+	+	+	++	+	+	++	+	+	+	++	++	++	+

* End products: S, Succinic acid; A, Acetic acid; P, Propionic acid; upper-case letters indicate 1 meq (or more) per 100 ml culture; lower-case letters indicate less than 1 meq per 100 ml culture.

of inocula, peptone-yeast extract broth with 4% (v/v) Fildes' digest [peptic digestive of horse blood (Fildes *et al.*, 1936)] (PYF) was used.

End products. Strains were grown for 7 d in PYF with glucose. Fatty acids were analysed by GC according to the methods of Kaneuchi *et al.* (1976, 1979).

G6PDH and 6PGDH enzyme assay. Cells were suspended in 1 ml Tris/HCl (pH 7.8), lysed by sonication at 4 °C and centrifuged at 17000 *g*, 20 min at 4 °C to remove cellular debris. The Bio-Rad protein assay was used to determine the protein concentration. The reaction mixture contained 160 µl cell lysis supernatant, 20 µl NADP (5.4 mM), 20 µl NAD (5.4 mM), and 4 µl glucose 6-phosphate as substrate. The G6PDH activity was measured at 340 nm after the incubation of the reaction mixture at 37 °C for 10 min. The substrate of the 6PGDH assay was changed to 6-phosphogluconic acid and the G6PDH assay was performed (Bailey & Love, 1995).

16S rRNA gene sequencing and phylogenetic analysis. One bacterial colony was scraped from the agar plate and suspended in 50 µl TE buffer, then frozen at –20 °C and heated three times at 100 °C for 5 min each. This raw extracted DNA was used as template DNA for PCR. PCR amplification was performed with a DNA thermal cycler (Takara). PCR mixture contained 10× EX *Taq* buffer (10 µl), a mixture of dNTPs (2.5 mM) (8 µl), 4 µl of each of the primers (20 pM) (8F, 5'-AGA GTT TGA TCM TGG CTC AG; 15R, 5'-AAG GAG GTG ATC CAR CCG CA), 1 µl raw template DNA and 3 U EX *Taq* (Takara). Distilled water was added to make 100 µl, and 50 µl mineral oil was then added. Initially, these mixtures were preheated for 3 min at 72 °C, 35 cycles of amplification in total were performed with template DNA denaturation at 94 °C for 30 s, followed by annealing of primers at 55 °C for 30 s,

and extension of primers at 72 °C for 2 min, and finally postheating for 2 min at 94 °C. The PCR products were separated by electrophoresis in agarose gels and stained with ethidium bromide (1 µg ml^{–1}). PCR products were purified by MicroSpin S-400 HR columns (Pharmacia) according to the manufacturer's instructions. The 16S rRNA gene sequence was determined by direct PCR sequencing using the method of Anzai *et al.* (1997). The 16S rRNA gene sequences of other Gram-negative bacteria were available in GenBank, EMBL and DDBJ. Levels of sequence similarity were calculated and used to produce an unrooted phylogenetic tree by the neighbour-joining method (Saitou & Nei, 1987). The alignment and the stability of relationships were assessed by bootstrapping using CLUSTAL W (Thompson *et al.*, 1994).

Determination of G + C content. High-molecular-mass DNA of bacterial strains was prepared by the procedures of Marmur (1961) with minor modifications. Determination of DNA base composition by reversed-phase HPLC was performed as described by Tamaoka & Komagata (1984).

DNA–DNA hybridization. DNA of bacterial strains was prepared by the procedures of Marmur (1961) with minor modifications. DNA–DNA hybridization was performed as described by Ezaki *et al.* (1989).

RESULTS

Biochemical and biological characterization

All of the strains in this study showed similar colony and cell forms. They were obligately anaerobic Gram-negative rods. The most remarkable characterization of these strains was that the final pH which was less than 5.7 in PYF broth after 7 d incubation. A

Fig. 1. Dendrogram of the eight isolates and their reference strains in the *Bacteroides* cluster and *E. coli* analysed using CLUSTAL W (Thompson *et al.*, 1994). *Prevotella heparinolytica* and *Prevotella zooglyphiformans* were previously classified in the genus *Bacteroides*. GenBank accession numbers are given in parentheses. Bar indicates a genetic distance of 0.01.

comparison of these isolates and type strains for biological characterization are shown in Table 1. G6PDH and 6PGDH activity, end production of succinic and acetic acids, growth stimulation in 20 %

bile acid, aesculin hydrolysis, no production of H_2S and production of NH_3 were common features of all strains. Starch hydrolysis, indole production and nitrate reduction were different among the strains. Only strain A29 digested gelatin.

Analysis of 16S rRNA sequence

Approximately 1500 bp regions of the 16S rRNA gene for these isolates were sequenced. These sequences suggested strong similarity for the species in the *Bacteroides* cluster. A phylogenetic tree of the relationship between the type strains in the *Bacteroides* cluster and the isolates is shown in Fig. 1. The range of 16S rRNA sequence homology between the isolates and type strains in the *Bacteroides* cluster was 91.5–95.3 %.

This tree suggested that the isolates can be divided in two groups, with strains A1, A29, A32, A37 and A40 belonging to group 2 of Fig. 1 and A24, A31 and A43 belonging to group 1 of Fig. 1. 16S rRNA sequence homology between groups 1 and 2 was 98.2 %.

DNA–DNA hybridization

To confirm whether these strains are new species, DNA–DNA hybridization was performed. All isolates showed a low level of homology against the type strains (Table 2). Homology between the isolates was over 80 % (Table 2).

DISCUSSION

In this report, we describe the isolates of a novel *Bacteroides* species predominantly colonized in the mouse caeca. Their characteristics, i.e. obligately anaerobic, Gram-negative, good growth in bile acid, and aesculin hydrolysis, and G6PDH and 6PGDH

Table 2. Percentage DNA–DNA hybridization of the new isolates with biotin-labelled DNA from type strains of some species of the genus *Bacteroides*

Strains 1–7: 1, *B. eggerthii* DSM 20697^T; 2, *B. fragilis* DSM 2151^T; 3, *B. ovatus* JCM 5824^T; 4, *B. thetaiotaomicron* JCM 5827^T; 5, *B. uniformis* JCM 5828^T; 6, *B. vulgatus* JCM 5826^T; 7, *B. distasonis* JCM 5825^T. Data are percentage homology to reference DNA.

Isolate	Labelled DNA from:	Unlabelled DNA from strain/isolate:									
	G + C mol %	1	2	3	4	5	6	7	A31	A40	A37
A24	41.5	8.6	2.0	12.6	6.1	5.9	3.0	3.0	84.7	85.0	83.6
A31	40.8	12.3	8.7	14.7	12.9	7.1	7.8	2.0	100.0	87.7	84.9
A43	39.8	16.6	5.4	7.1	9.4	7.5	11.8	4.1	100.0	102.3	91.4
A32	41.9	9.5	3.1	9.1	7.1	5.8	2.4	4.5	91.2	97.8	95.0
A1	42.2	0.0	0.0	0.0	18.8	0.0	8.0	8.6	84.7	89.2	93.3
A37	39.4	9.0	2.4	7.9	7.8	2.8	1.8	0.0	94.8	92.6	100.0
A29	41.8	8.6	1.9	11.3	5.5	4.6	1.3	1.2	81.6	88.1	82.6
A40	41.4	16.5	0.4	14.6	9.6	11.8	3.7	7.4	104.3	100.0	96.5

activity suggested that these isolates were contained in the '*Bacteroides fragilis* group'. The specific character of these strains was reduction of the pH of PYF broth without carbohydrate.

16S rRNA gene sequence analysis suggested that these isolates belong to a new species in the *Bacteroides* cluster in the bacteroides subgroup. The low level of DNA–DNA hybridization with the type strains of other species in the *Bacteroides* cluster confirmed the novel species status of these isolates.

Inter-individual genetic heterogeneity of these isolates was suggested from 16S rRNA analysis. Starch hydrolysis suggested their heterogeneity. All biological and biochemical characters of strains in group 1 were the same. Strain A29 was slightly different from the other four strains in group 2 in indole production and gelatin digestion. However, homology of the 16S rRNA sequence between group 1 and group 2 was about 98.2% and DNA–DNA homology between the isolates of group 1 and group 2 was over 80%. Therefore, these two groups appeared to be one species (Stackebrandt & Goebel, 1994).

Description of *Bacteroides acidifaciens* sp. nov.

Bacteroides acidifaciens (a.ci.di'fa'ci.ens. M.L. n. *acidum* acid; L. v. *facio* produce; M.L. part. adj. *acidifaciens* acid-producing).

Cells are obligately anaerobic, non-sporeforming, non-motile, Gram-negative rods. Cells grown on EG agar plates are 0.8–1.3 × 1.6–8.0 µm. Colonies on EG agar plates after two days incubation are 1–3 mm in diameter, circular, entire, raised convex, smooth and greyish-coloured. Acid is produced from prereduced anaerobically sterilized peptone-yeast extract broth with Fildes' digest (PYF) without any carbohydrate. Aesculin hydrolysis, NH₃ production and G6PDH and 6PGDH enzyme activities are positive, and H₂S production is negative. The major end products of glucose fermentation are acetic and succinic acids. The G+C content of DNA is 39.4–42.2 mol%. At the molecular level, this species can be distinguished from other *Bacteroides* species by the sequence of the 16S rRNA gene. Strain A40^T is the type strain and has been deposited in the Japan Collection of Microorganisms as JCM 10556^T.

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