Bacteroides caecicola sp. nov. and Bacteroides gallinaceum sp. nov., isolated from the caecum of an Indonesian chicken

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Six strains of anaerobic bacteria, C13EG70 T, C13EG118, C13EG186 T, C13GAMG5, C13GAMG28 and C13GAMG40, were isolated from the caecum of a healthy chicken bred in Bogor, Indonesia. Phylogenetic analysis showed the isolates were separated into two groups. Group I (C13EG70 T and C13EG118) showed nearly identical 16S rRNA gene sequences (99.9 % sequence similarity). Group II (C13EG186 T, C13GAMG5, C13GAMG28 and C13GAMG40) showed nearly identical 16S rRNA gene sequences (>99.4 % sequence similarity). The isolates showed low 16S rRNA gene sequence similarities to recognized species of the genus Bacteroides. High gene sequence similarities were found between type strains (C13EG70 T and C13EG186 T) and Bacteroides salanitronis JCM 13657 T (87.9, 91.5 %, respectively). Physiological, biochemical and genotypic characteristics demonstrated that these strains could be separated from the type strain of B. salanitronis. It is concluded that Group I and Group II represent novel species. Two novel species of the genus Bacteroides are proposed as Bacteroides caecicola sp. nov. (type strain C13EG70 T = LIPI12-4-Ck732 T = JSAT12-4-Ck732 T = InaCC B449 T = NBRC 110958 T) and Bacteroides gallinaceum sp. nov. (type strain C13EG186 T = LIPI12-4-Ck844 T = JSAT12-4-Ck844 T = InaCC B451 T = NBRC 110963 T).

Gastrointestinal microbes are important factors that influence the health and growth of the host animal. Chickens have a pair of caeca and they are attached at the junction between the small and large intestines. The caecum contains many kinds of micro-organisms capable of breaking down fibrous materials there (Thomas & Skadhauge, 1988) and seems to play an important role in the digestive system.

A number of studies on the microbiota of the chicken caecum have already been reported. Culture-independent approaches based on 16S rRNA gene sequences have clarified the bacterial composition in the chicken caecum (Lan et al., 2002; Zhu et al., 2002; Lu et al., 2003). In addition, metagenomic sequencing has been carried out to analyse the microbiota of the chicken caecum (Qu et al., 2008). These studies show that species in the phylum Firmicutes are the most dominant group followed by species in the phylum Bacteroidetes; however, most of the sequences obtained in these studies are <96 % identical to the closest relatives in the public databases.

Bacteroides barnesiae, Bacteroides salanitronis and Bacteroides gallinarum were isolated from chicken caecum (Lan et al., 2006). The former two form a phylogenetic subcluster together with closely related species. This subcluster has different characteristics from the other subcluster in the genus Bacteroides. For instance, almost all the species in the genus Bacteroides can grow in the presence of 20 % (w/v) bile but some species isolated from...
chicken caecum cannot grow in the presence of bile (Song et al., 2011). These results suggest that more bile-sensitive species of the genus Bacteroides inhabit the gastrointestinal than expected previously.

During a study of the diversity of anaerobic bacteria in the caecum of broiler chickens bred in Bogor, Indonesia, we isolated six strains from the caecum of a chicken in February 2013. These isolates formed two phylogenetic clusters (Group I and Group II) in the genus Bacteroides and their closest relative was B. salanitronis. This paper addresses the taxonomic status of the six isolates and proposes two novel species of the genus Bacteroides.

Six strains (C13EG70T, C13EG118, C13EG186T, C13GAMG5, C13GAMG28 and C13GAMG40) were isolated from the caecum of a healthy chicken (90 days) bred in Bogor, Indonesia. Three strains, C13EG70T, C13EG118 and C13EG186T, were isolated on Eggerth Gagnon (EG) agar plates (Merck) supplemented with 5 % (v/v) horse blood and the other strains were isolated on GAM plates (Nissui) supplemented with 1.0 % (w/v) Gelrite (Wako). Culture was performed in an anaerobic jar (Hirayama Manufacturing) filled with Anaerocult (Merck) at 37 °C for 3 days. After isolation, the isolates were usually cultured on EG agar plates at 37 °C for 2 days in an anaerobic jar filled with 100 % CO₂.

Extraction of bacterial DNA was performed by using a Qiagen Genomic-tip 100/G kit (Qiagen) following the manufacturer’s instructions. The DNA was used as a template for 16S rRNA gene and partial hsp60 (groEL) amplification. The 16S rRNA and partial hsp60 (groEL) gene sequences of the isolates were determined according to the method described previously (Sakamoto et al., 2002, 2010). The closest recognized relatives of the novel isolates were determined by searching 16S rRNA gene sequences in the EMBL/GenBank/DDBJ databases. These sequences were aligned using CLUSTAL X (version 2.1) (Larkin et al., 2007). A distance matrix was obtained by the two-parameter method of Kimura (1980). The neighbour-joining method was used to reconstruct a phylogenetic tree (Saitou & Nei, 1987). The robustness of the individual branches of the tree was established by bootstrapping with 1000 replicates (Felsenstein, 1985). Maximum-likelihood analysis was also performed using version 3.0 of the PhyML program (Guindon & Gascuel, 2003) with the optimal model of nucleotide substitution estimated by jModelTest version 2.1.4 (Darriba et al. 2012) using the Akaike information criterion; the models selected were TIM3 +I+G for the 16S rRNA gene and GTR +I+G for hsp60 (groEL). To differentiate the isolates, an automated Riboprinter microbial characterization system (Qualicon) was used for ribotyping according to the method described by Kitahara et al. (2010), using BioNumerics version 2.5 software (Applied Maths). The DNA G+C contents of the isolates and their phylogenetic relatives were determined according to the methods described by Tamaoka & Komagata (1984) and Kitahara et al. (2001).

Physiological and biochemical characteristics were determined by using an API 20A anaerobic test kit and API Rapid ID 32A enzymatic test kit in duplicate according to the methods recommended by the manufacturer (bioMérieux). In addition, CM-cellulose, pectin, starch and xylan were used to test the ability to decompose polysaccharide. Tolerance to NaCl was examined in GAM containing 1.0–5.0 % (w/v) NaCl after incubation for 2 days at 37 °C. Bile resistance was tested by growing bacteria on Bacteroides bile aesculin agar plates (Shah, 1992). Aesculin reaction was detected as a dark brown to black area surrounding colonies. The optimum temperature (10–45 °C) and pH (pH 4.0–8.0) for growth were tested by using PYG broth; pH was adjusted to each rage with HCl or NaOH solution. Fermentation metabolic end-products were prepared as described by Holdeman et al. (1977) and analysed as previously described (Sakamoto et al., 2004, 2005). Fatty acid methyl esters (FAMEs) were prepared from cells grown on EG agar supplemented with 5 % (v/v) horse blood, incubated for 48 h at 37 °C, as described by Sakamoto & Ohkuma (2013). Cellular fatty acid profiles were determined using version 2.99B of the Sherlock Microbial Identification System (MIDI) and version 3.80 of the BHIBLA database. Peaks were automatically integrated, fatty acids were identified by equivalent chain-length, and the percentage of the total peak area represented by each acid was calculated. MIDI calibration mixture 1 (comprising FAMEs of straight-chain saturated fatty acids from nine to 20 carbons in length and five hydroxy acids) was used for external calibration. The cellular fatty acid profiles of most established members of the genus Bacteroides have been determined (Miyagawa et al., 1979; Mayberry et al., 1982) and these were reviewed for the classification of the genus Bacteroides published by Shah & Collins (1983). Isoprenoid quinones were extracted and analysed using HPLC as described previously (Komagata & Suzuki, 1987; Sakamoto et al., 2004, 2005).

Approximately 1450 bp sequence of the 16S rRNA gene was determined for each of the six isolates. For the phylogenetic analysis, 1395 bp sequence of each isolate was used. The phylogenic tree based on the 16S rRNA gene sequence is shown in Figs 1 and S1 (available in the online Supplementary Material). The six isolates were located in the genus Bacteroides. The strains in each group shared nearly identical 16S rRNA gene sequences (>99.4 % sequence similarity). They showed low similarities of 16S rRNA gene sequence to known species of the genus Bacteroides. The sequences of strains C13EG70T and C13EG186T showed 87.9 % and 91.5 % sequence similarity with B. salanitronis JCM 13657T as the most closely related recognized species, respectively. In addition, the sequence similarity of the 16S rRNA gene was low (89.9 %) between Group I and Group II. The partial sequence (558 bp) of the hsp60 (groEL) gene was determined for each of the six strains. The phylogenetic tree based on the hsp60 gene (Figs 2 and S2) confirmed the close relatedness of Group I and Group II to each other.
as well as to B. salanitronis. In each of Group I and Group II, the strains showed nearly identical sequence of hsp60 gene (>99.5 %). Strain C13EG70\(^T\) (Group I) showed 88.2 % sequence similarity of the hsp60 gene with B. salanitronis JCM 13657\(^T\), whereas strain C13EG186\(^T\) (Group II) shared very high (99.3 %) sequence similarity with B. salanitronis JCM 13657\(^T\). Although the hsp60 gene has been applied to phylogenetic analysis as a useful phylogenetic marker (Sakamoto & Okkuma, 2010, 2011), it was recently reported that hsp60 could not differentiate some species (Sakamoto et al., 2014). DNA–DNA relatedness between the Group I strains and B. salanitronis JCM 13657\(^T\) was low (<14.6 %). The two strains in Group I showed a high level of relatedness (100 %). The Group II strains also showed low levels of DNA–DNA relatedness (<31.8 %) with B. salanitronis JCM 13657\(^T\) and the four strains in Group II showed high levels of relatedness (>79.9 %) with one another. The ribotyping analysis further supported the differentiation of Group I, Group II and B. salanitronis as well as the close relatedness of strains in each group (Fig. 3). These results clearly indicated that Group I, Group II and B. salanitronis represented different species from one another. The DNA G+C content of the Group I strains ranged from 45.3 to 45.6 mol% and those of the strains of Group II ranged from 45.3 to 45.8 mol%. In comparison, the DNA G+C content of B. salanitronis JCM 13657\(^T\) was 45.2 mol%.

The cells of the six strains were observed by microscopy (Fig. S3). Cells of the six isolates were Gram-stain-negative, obligately anaerobic, non-spore-forming, non-motile and rod-shaped. On EG agar with supplemented 5 % (v/v) horse blood, cells of Group I (C13EG70\(^T\) and C13EG118) were 0.4–0.8 µm × 0.4–2.0 µm and cells of Group II (C13EG186\(^T\), C13GAMG40, C13GAMG28 and C13GAMG40) were 0.8–1.0 × 0.8–5.0 µm. Cells of all strains occurred either singly or in pairs. On EG agar supplemented with 5 % (v/v) horse blood, colonies of all strains were semi-translucent and white–greyish. Colonies of Group I were 1.0–2.0 mm in diameter and those of Group II were 0.5–1.5 mm in diameter. Colonies of all strains were circular, raised, convex and smooth.

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**Fig. 1.** Phylogenetic relationship of Bacteroides caecicola sp. nov., Bacteroides gallinaceum sp. nov. and closely related species based on the 16S rRNA gene. The tree was reconstructed by the neighbour-joining method. Parabacteroides distasonis JCM 5825\(^T\) was used as an outgroup. Bootstrap percentages above 70 % are given at branching points. Bar, 1 % sequence divergence.
Fig. 2. Phylogenetic relationship of Bacteroides caecicola sp. nov., Bacteroides gallinaceum sp. nov. and closely related species based on the nucleotide sequence of the hsp60 gene. The tree was reconstructed by the neighbour-joining method. Parabacteroides distasonis JCM 5825T was used as an outgroup. Bootstrap percentages above 70% are given at branching points. Bar, 2% sequence divergence.

Fig. 3. Dendrogram illustrating the relatedness of the ribotyping patterns of C13EG186T, C13HAMG40, C13HAMG5, C13HAMG28, C13HAMG40, C13EG118 and B. salanitronis JCM 13657T. The RiboPrinter microbial characterization system (Qualicon) was used for ribotyping. EcoRI was used as a restriction enzyme. Ribotyping patterns were analysed by BioNumerics version 2.5 software (Applied Math) and were compared by Pearson similarity coefficient analysis and the UPGMA algorithm.
All strains decomposed starch and xylan; however, they did not decompose CM-cellulose or pectin. They did not grow on GAM plates supplemented 1–5 % (w/v) NaCl. All the strains were able to hydrolyse aesculin on Bacteroides bile aesculin agar and API 20A. Strains belonging to Group I grew at 20–45 °C (growth at 10 °C was weakly positive) and pH 4.0–8.0. The optimum ranges for growth were 30–37 °C and pH 7.0–8.0. Strains belonging to Group II grew at 37 °C and pH 6.0–7.0. The optimum ranges for growth were 37 °C and pH 7.0. Fermentation metabolic end-products of the isolates were mainly succinic acid and acetic acid. Members of Group I and Group II also produced a small amount of propionic acid and isovaleric acid, respectively. Phenotypic and physiological characteristics of the novel isolates and B. salanitronis JCM 13657T are shown in Table 1 (details are also given in the species description). From the API 20A test, fermentation of salicin and L-rhamnose could be used to distinguish the novel isolates from B. salanitronis JCM 13657T. From the API Rapid ID 32A test, isolates of Group I differed from those of Group II in that they were positive for β-glucuronidase, N-acetyl-β-glucosaminidase, arginine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. Isolates of Group II also differed from B. salanitronis JCM 13657T in that they were positive for α-fucosidase.

The cellular fatty acid composition of the novel isolates and B. salanitronis JCM 13657T were analysed in the present study and are given in Table S1. The major fatty acids of Group I included anteiso-C₁₅ : ₀, C₁₆ : ₀, C₁₆ : ₀ 3-OH and iso-C₁₇ : ₀ 3-OH. The major fatty acids of Group II included anteiso-C₁₅ : ₀, iso-C₁₅ : ₀, C₁₆ : ₀, C₁₆ : ₀ 3-OH and iso-C₁₇ : ₀ 3-OH.

Isoprenoid quinones (principally menaquinones and ubiquinones) are important in the function of respiratory electron transport systems. Furthermore, they are applied as a useful characteristic in bacterial classification and identification (Collins & Jones, 1981). The major menaquinones of the species in the genus Bacteroides are MK-10 and MK-11. The composition of menaquinones in the novel isolates and B. salanitronis JCM 13657T is given in Table 1. The major menaquinones of Group I were MK-10 (36.8–41.5 %) and MK-11 (33.5–34.9 %). Members of Group I possessed a higher proportion of MK-10 than did those of Group II and B. salanitronis JCM 13657T. In contrast, the major menaquinones of Group II were MK-11 (24.6–30.5 %) and MK-12 (47.2–55.0 %). B. salanitronis JCM 13657T possessed a higher proportion of MK-12 than did isolates of Group II, and those of Group I possessed MK-12 at very low levels (<1.0 %).

On the basis of the results demonstrated in this study, the strains of each of Group I and Group II should be regarded as representatives of a novel species in the genus

Table 1. Phenotypic and physiological characteristics of the novel strains and B. salanitronis JCM 13657T

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<th>Characteristic</th>
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<td>Serine arylamidase</td>
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<td>Distinctive major cellular fatty acids</td>
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<td>Predominant menaquinones</td>
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<td>Metabolic end-products</td>
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Strains: 1, Bacteroides caecicola sp. nov. C13EG70T and C13EG118; 2, Bacteroides gallinaceum sp. nov. C13EG186T, C13GAMG5, C13GAMG28 and C13GAMG40; 3, B. salanitronis JCM 13657T. All data were obtained in the present study. +, Positive; –, negative; w, weakly positive.

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Bacteroides. We propose two novel species of the genus Bacteroides: Bacteroides caecicola sp. nov. for Group I and Bacteroides gallinaceum sp. nov. for Group II.

Description of Bacteroides caecicola sp. nov.
Bacteroides caecicola (cae.ci.co.la. N.L. n. caecum (from L. caecum intestine caecum) caecum; L. suff. -cola (from L. n. incola) dweller, inhabitant; N.L. n. caecicola caecum-dweller).

Cells are strictly anaerobic, non-spore-forming, non-motile, Gram-stain-negative rods, 0.4–0.8 μm. Cells occur singly or in pairs. Surface colonies of two strains on EG blood agar plates after 2 days are 1–2 mm in diameter, circular, raised, convex, smooth, semi-translucent and white–greyish. Growth is not observed on GAM containing 1–5 % (w/v) NaCl. Growth is inhibited on medium containing 20 % (w/v) bile. Indole is not produced. Catalase- and urease-negative. Nitrate is not reduced. Gelatin is not liquefied. Aesculin is hydrolysed. Produces acids from glucose, lactose, sucrose, maltose, D-xylene, L-arabinose, D-mannose and raffinose but not from D-mannitol, glycerol, melezitose, D-sorbitol, D-xylose or trehalose. Gellobiose is weakly fermented by strain C13EG118. Starch and xylan are decomposed. Using the API Rapid ID 32A, positive reactions are obtained for 3-glucosidase, 3-galactosidase, 3-glucuronidase, 3-arabinosidase, 3-fucosidase, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase and glutaminyl glutamic acid arylamidase. Negative reactions are obtained for 3-galactosidase-6-phosphate, 3-glucosaminidase, 3-acetylglucosaminidase, 3-galactosidase, 3-glucosidase, 3-arabinosidase, 3-fucosidase, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase, arginine arylamidase, arginine arylamidase, lysine arylamidase, arginine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase. Negative reactions are obtained for 3-glucosaminidase-6-phosphate, 3-glucosidase-6-phosphate, 3-glucosaminidase, 3-acetylglucosaminidase, 3-galactosidase, 3-glucosidase, 3-arabinosidase, 3-fucosidase, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase, arginine arylamidase, arginine arylamidase, lysine arylamidase, arginine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase. Negative reactions are obtained for 3-glucosaminidase-6-phosphate, 3-glucosidase-6-phosphate, 3-glucosaminidase, 3-acetylglucosaminidase, 3-galactosidase, 3-glucosidase, 3-arabinosidase, 3-fucosidase, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase, arginine arylamidase, arginine arylamidase, lysine arylamidase, arginine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase. Cells grow at 37 °C and pH 6.0–7.0. Optimum growth occurs at 37 °C and pH 7.0. The major end-products of fermentation [1 % (w/v) peptone/1 % (w/v) yeast extract/1 % (w/v) glucose broth culture] are succinic acid and acetic acid; a small amount of isovaleric acid is produced. The major cellular fatty acids are iso-C₁₅₋₀, anteiso-C₁₅₋₀, C₁₆₋₀, iso-C₁₇₋₀ 3-0H and iso-C₁₇₋₀ 3-0H. The principal respiratory quinones are menaquinone MK-11 and MK-12. Menaquinones MK-7, MK-8, MK-9 and MK-10 are also present as minor components. The DNA G+C content ranges from 45.3 to 45.8 mol% (type strain, 45.3 mol%).

Type strain C13EG186T (=LIP112-4-Ck844T=JSAT12-4-Ck844T=LIPI12-4-Ck844T=NBRC 110963T) was isolated from the caecum of a chicken in Bogor, Indonesia.

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