Alistipes onderdonkii sp. nov. and *Alistipes shahii* sp. nov., of human origin

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Two groups of previously unknown Gram-negative, strictly anaerobic, pigment-producing, rod-shaped bacteria, which phenotypically and phylogenetically displayed a close association with the recently described species *Alistipes finegoldii*, were characterized using phenotypic and molecular taxonomic methods. A 16S rRNA gene sequence divergence of approximately 3 % between the two unknown bacteria and *A. finegoldii*, as well as distinguishable biochemical characteristics, demonstrates that these organisms are genotypically and phenotypically distinct and that each group represents a previously unknown subline within the genus *Alistipes*. Chromosomal DNA–DNA reassociation studies further confirmed the separateness of the unidentified bacteria and *A. finegoldii*. On the basis of the phenotypic and phylogenetic findings, two novel species, *Alistipes onderdonkii* sp. nov. and *Alistipes shahii* sp. nov., are proposed. The type strains of *A. onderdonkii* and *A. shahii* are WAL 8169^T (=CCUG 48946^T=ATCC BAA-1178^T) and WAL 8301^T (=CCUG 48947^T=ATCC BAA-1179^T), respectively; their DNA G+C contents are 58 and 56 mol%, respectively.

The taxonomy of the genus *Bacteroides* has undergone significant changes in the past few years. A majority of the species previously included in the genus *Bacteroides* has been placed in the genera *Porphyromonas*, *Prevotella* and *Bacteroides sensu stricto* (Shah & Collins, 1988, 1989, 1990). Several other genera have subsequently been described for *Bacteroides*-like species that do not conform to these three major groups. The recent description of such a group, *Alistipes*, included two species, *Bacteroides putredinis* reclassified as *Alistipes putredinis* and *Alistipes finegoldii* as a novel species (Rautio *et al.*, 2003). These species produce succinic acid as the principal metabolic end-product of glucose fermentation and iso-$C_{15:0}$ as their major long-chain cellular fatty acid (CFA) (Rautio *et al.*, 2003).

During studies on bile-resistant, pigment-producing strains from human intestinal sources, later leading to the description of the genus *Alistipes* and the species *A. finegoldii* (Rautio *et al.*, 2003), another group of strains with similar phenotypic characteristics but a 16S rRNA gene sequence divergence of approximately 3 % was observed (Rautio *et al.*, 1997a) but remained uncharacterized. In another project evaluating 16S rRNA gene sequencing for species identification of *Bacteroides fragilis* group isolates (Song *et al.*, 2005), two groups of bile-resistant, pigment-producing organisms resembling *A. finegoldii* were found. Again, 16S rRNA gene sequencing revealed approximately 3 % sequence divergence between the unidentified bacteria and their phylogenetically closest species, *A. finegoldii*. A detailed comparison between these unknown groups of bile-resistant, pigment-producing organisms and *A. finegoldii* was performed. On the basis of the phenotypic and phylogenetic findings presented here, we propose two novel *Alistipes* species; in addition, we describe

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Abbreviation: CFA, cellular fatty acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains WAL 8169^T and WAL 8301^T are AY974071 and AY974072, respectively.

The cellular fatty acid compositions of representative novel strains described in this work are presented in a supplementary table available in IJSEM Online.

phenotypic tests useful in distinguishing between these novel organisms and related taxa.

Altogether, 32 isolates, including the type strains of *A. putredinis* and *A. finegoldii*, from human specimens were included in the present study. All of the strains were cultivated on Brucella blood agar supplemented with 5 % sheep blood, haemin and vitamin K_1, and were incubated at 37 °C under anaerobic conditions. The strains were characterized biochemically by using a combination of conventional tests, as described in the *Wadsworth–KTL Manual* (Jousimies-Somer *et al.*, 2002), plus the API ZYM and API Rapid ID 32A test kits (bioMérieux) and Rosco diagnostic tablets, according to the manufacturers' instructions; tests were performed in duplicate or in triplicate. Fermentation tests were performed using pre-reduced, anaerobically sterilized peptone/yeast extract/sugar broth tubes (Anaerobe Systems). The strains were grown in peptone/yeast extract and peptone/yeast extract/glucose broth for determination of metabolic end-products by GLC. The MICs of metronidazole, clindamycin, penicillin, cefotetan, ertapenem, ampicillin/sulbactam and vancomycin were determined for the seven WAL strains by the Clinical Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) reference agar dilution method (NCCLS, 2001). CFAs were detected with a gas chromatograph (Hewlett Packard) and Microbial Identification System software (MIDI). The isolates were grown on supplemented brain heart infusion agar with blood, and the bacterial mass was harvested directly from the plates because of poor growth in peptone/yeast extract liquid media. The corresponding library (ANAEROBE, version Moore 5.0) was used in successive analyses. The G+C content of the DNA (mol%) was determined by HPLC as described previously (Mesbah *et al.*, 1989) except that the methanol content of the chromatographic buffer was reduced to 8 % and the temperature increased to 37 °C.

The 16S rRNA genes were amplified by PCRs using universal primers 8UA (positions 8–28, *Escherichia coli* numbering) and 1485B (positions 1485–1507) as described previously (Brosius *et al.*, 1978). The amplified product was purified by using the QIAamp PCR purification kit (Qiagen) and directly sequenced with the Big Dye sequencing kit (Biotech Diagnostic) on an ABI 7700 sequencer (Applied Biosystems). The closest known relatives of the isolates were determined by performing database searches using BLAST software (Benson *et al.*, 1997). Almost-complete 16S rRNA gene sequences (>1400 nt) of the isolates and of closely related bacteria were aligned using CLUSTAL W (http://genome.kribb.re.kr). A phylogenetic tree was constructed using the DNA analysis software PAUP*, version 4.0 (Sinauer Associates). The stability of the groupings was estimated by bootstrap analysis (1000 replications) using the same program. DNA–DNA reassociation experiments were carried out according to the spectrophotometric method of De Ley *et al.* (1970).

Non-motile, Gram-negative, rod-shaped bacteria were recovered from specimens of human intestinal origin (Table 1), together with other strict anaerobes (e.g. species of the *B. fragilis* group) and/or aerobes, and often showed heavy growth upon primary isolation. The isolates grew anaerobically on agar media well but very poorly in liquid media. No growth occurred in subcultures when exposed to oxygen. Typically, the isolates produced pigment on laked rabbit blood agar and grew on Bacteroides/bile/aesculin agar, showing tolerance to 20 % bile. All isolates were resistant to special-potency antimicrobial discs containing vancomycin (5 μg), kanamycin (1000 μg) and colistin (10 μg), produced indole and hydrolysed gelatin. The results for lipase and urease production, for arginine hydrolysis and for nitrate reduction were negative. Acid was produced from glucose. Because of the scanty growth in liquid pre-reduced, anaerobically sterilized biochemicals, we failed to demonstrate the fermentation of other carbohydrates reliably. Mannose and raffinose were fermented according to the API Rapid ID 32A system (bioMérieux). Positive enzyme reactions were obtained for alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and *N*-acetyl-β-glucosaminidase with the API ZYM kit, and for α-galactosidase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, leucyl glycine arylamidase, alanine arylamidase and glutamyl glutamate arylamidase with the API Rapid ID 32A kit. The major metabolic end-product was succinic acid; minor amounts of acetic and propionic acids were also produced. The principal long-chain CFA was iso-$C_{15:0}$ (20–53 % of the total CFAs). For the seven WAL strains tested, the MICs with respect to metronidazole, clindamycin, ampicillin/sulbactam and ertapenem were $\leqslant 0{\cdot}5$ μg ml^{-1}, and those with respect to penicillin G were $\leqslant 1{\cdot}0$ μg ml^{-1}; however, resistance to cefotetan (MICs $\geqslant 16$ μg ml^{-1}) and vancomycin (MICs >32 μg ml^{-1}) was shown. Notably, penicillin resistance due to β-lactamase production may be found (Rautio *et al.*, 1997b).

To assess the genealogical affinity between the isolates and their relationships with other taxa, their almost-complete (>1400 nt) 16S rRNA gene sequences were determined. The identification of nine of the 12 isolates previously identified as *A. finegoldii* (Rautio *et al.*, 2003) was confirmed, but one isolate (AHN 19871) did not cluster with *A. finegoldii* or with any of the isolates examined in the present study; the remaining two isolates (AHN 2398 and AHN 19499) clustered with four previously unknown isolates (WAL 8301^T, WAL 11404, WAL 11550 and WAL 12401) (Table 1). Pairwise analysis showed that these six isolates formed a novel group and 15 previously unknown isolates another group where the isolates within each group were phylogenetically closely related to each other (>99 % sequence similarity). Sequence searches of the GenBank and Ribosomal Database Project libraries revealed that these groups represent two hitherto unknown sublines within the genus *Alistipes*, being members of the phylum *Bacteroidetes*.

Table 1. Source and identification for the 32 human strains examined

Strain	Source	Previous identification
***Alistipes onderdonkii* sp. nov.**		
AHN 2149	Appendix tissue	Unknown
AHN 2150	Appendix tissue	Unknown
AHN 2320	Appendix tissue	Unknown
AHN 2357 (=CCUG 46019)	Appendix tissue	Unknown
AHN 2438 (=CCUG 46021)	Appendix tissue	Unknown
AHN 2457	Appendix tissue	Unknown
AHN 2531	Appendix tissue	Unknown
AHN 2747	Appendix tissue	Unknown
AHN 18808	Faeces (from subject with antibiotic-associated diarrhoea)	Unknown
AHN 20284	Faeces (from healthy subject)	Unknown
AHN 20585	Faeces (from healthy subject)	Unknown
AHN 20651	Faeces (from healthy subject)	Unknown
WAL 8169^T (=CCUG 48946^T=ATCC BAA-1178^T)	Abdominal abscess	Unknown
WAL 13320	Urine	Unknown
WAL 16329	Faeces (from subject with autism)	Unknown
***Alistipes shahii* sp. nov.**		
AHN 2398	Appendix tissue	*A. finegoldii*
AHN 19499	Faeces (from healthy subject)	*A. finegoldii*
WAL 8301^T (=CCUG 48947^T=ATCC BAA-1179^T)	Appendix tissue	Unknown
WAL 11404	Intraabdominal fluid	Unknown
WAL 11550	Intraabdominal fluid	Unknown
WAL 12401	Appendix tissue	Unknown
Alistipes finegoldii		
AHN 2319	Appendix tissue	*A. finegoldii*
AHN 2437^T (=CCUG 46020^T)	Appendix tissue	*A. finegoldii*
AHN 2528	Appendix tissue	*A. finegoldii*
AHN 2532	Appendix tissue	*A. finegoldii*
AHN 2583 (=CCUG 46022)	Appendix tissue	*A. finegoldii*
AHN 2740	Appendix tissue	*A. finegoldii*
AHN 2748	Appendix tissue	*A. finegoldii*
AHN 2752	Appendix tissue	*A. finegoldii*
AHN 20601	Faeces (from healthy human subject)	*A. finegoldii*
Alistipes putredinis		
ATCC 29800^T	Faeces	*A. putredinis*
Species unknown		
AHN 19871	Faeces (from healthy subject)	*A. finegoldii**

*Results of 16S rRNA gene sequencing in the present study suggest that this strain does not belong to *A. finegoldii*.

A tree constructed by the maximum-parsimony method (Fig. 1), depicting the phylogenetic affinity of the two novel bacterial groups, as exemplified by strains WAL 8169^T (=CCUG 48946^T) and WAL 8301^T (=CCUG 48947^T), confirmed the placement of the unknown bacteria in the genus *Alistipes*. It is evident from the branching pattern in the tree that the two novel species have a significantly close relationship with *A. finegoldii*. Pairwise comparison based on almost-complete (>1400 nt) 16S rRNA gene sequences revealed approximately 4 % sequence divergence between these novel taxa and approximately 3 % sequence divergence between them and the type strain of their most closely related species with a validly published name, *A. finegoldii*. Furthermore, DNA–DNA reassociation values of 43·5, 49·6 and 33·6 % were observed between AHN 2357 (=CCUG 46019) and CCUG 46020^T (the type strain of *A. finegoldii*), between WAL 8169^T (=CCUG 48946^T) and CCUG 46020^T and between WAL 8301^T (=CCUG 48947^T) and CCUG 46020^T, respectively, thereby confirming that these unidentified bacterial strains represent novel species.

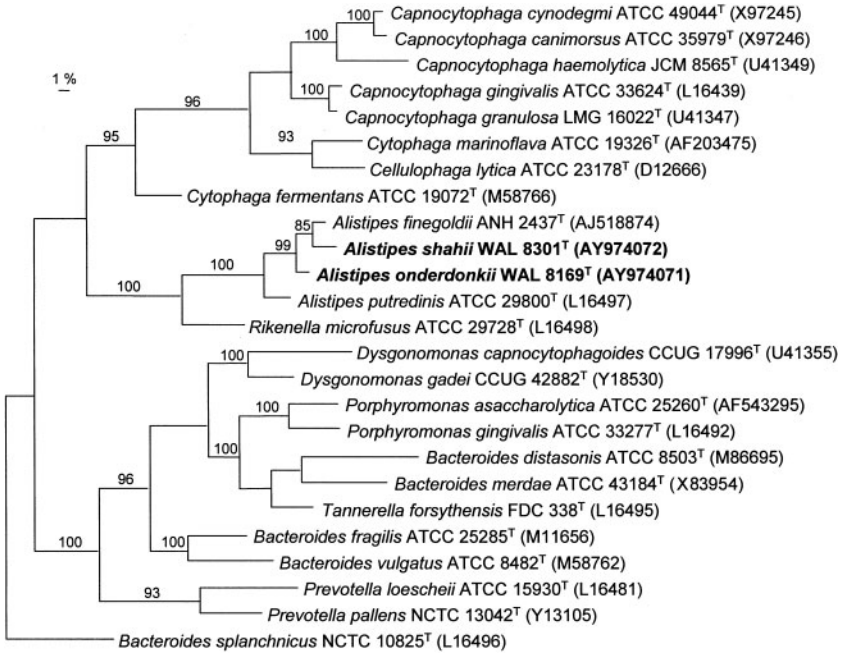

Fig. 1. Unrooted tree showing the phylogenetic relationship of *A. onderdonkii* sp. nov. WAL 8169^T and *Alistipes shahii* sp. nov. WAL 8301^T with respect to related taxa. The tree, constructed using the maximum-parsimony method, was based on a comparison of 16S rRNA gene sequences of approximately 1400 nt. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. Bar, 1 % sequence divergence.

Support for the separation of the unknown bacteria from related bacterial species also comes from their phenotypic characterization. The capacity of the isolates to ferment, to produce pigment and to tolerate bile separate the novel isolates from *A. putredinis* (Rautio *et al.*, 2003). Biochemical characteristics useful for distinguishing *A. finegoldii* and the two novel groups within the genus from each other are based on reactions with the API ZYM kit (Table 2). In contrast to the corresponding API ZYM reactions, the reactions obtained with individual Rosco diagnostic tablets were positive for β-glucosidase and α-fucosidase for most strains. Table 3 presents a list of distinguishing characteristics for the genus *Alistipes* and related genera. The pigment-producing *A. finegoldii* and two novel groups within the genus *Alistipes* can be distinguished readily from pigment-producing species of the genera *Porphyromonas* and *Prevotella* by their resistance to 20 % bile. In addition, the novel isolates are fermentative, unlike the pigment-producing species within the genus *Porphyromonas*. Our data, based on phylogenetic and phenotypic analyses, show that both novel groups represent hitherto unknown sublines within the genus *Alistipes* and can be separated from each other and from other *Alistipes* species.

Description of *Alistipes onderdonkii* sp. nov.

Alistipes onderdonkii (on′der.don′ki.i. N.L. gen. n. *onderdonkii* of Onderdonk, to honour Andrew B. Onderdonk, a contemporary American microbiologist, for his contribution to increased knowledge about intestinal microbiota and anaerobic bacteria).

Table 2. Characteristics that may be useful in the separation of *Alistipes* species

+, Positive; −, negative; V, variable. All four species are positive for α-glucosidase activity.

Characteristic	*A. onderdonkii* (*n*=15)	*A. shahii* (*n*=6)	*A. finegoldii* (*n*=9)	*A. putredinis* (*n*=1)*
Pigment production	+	+	+	−
Growth in 20 % bile	+	+	+	−
Carbohydrate fermentation	+	+	+	−
Enzyme activities†				
α-Chymotrypsinase	V	V	+	−
β-Glucosidase	−	+	−	−
α-Fucosidase	−	+	+	−

*Data are from Rautio *et al.* (2003) and from the present investigation of the type strain.
†Based on reactions obtained using the API ZYM system.

Table 3. Some distinguishing characteristics of *Alistipes* and related genera

Data for *Bacteroides* refer to *Bacteroides sensu stricto*. +, Positive; −, negative; V, variable.

Characteristic	*Alistipes*	*Bacteroides*	*Porphyromonas*	*Prevotella*	*Rikenella*
Pigment production	+⁻*	−	+⁻†	V	−
Growth in 20 % bile	+⁻*	+	−	−	+
Susceptibility to:‡					
Vancomycin	R	R	V	R	R
Kanamycin	R	R	R	V	S
Colistin	R	R	R	V	R
Carbohydrate fermentation	+⁻*	+	−⁺†	+	−
Proteolytic activity	+	−	V	V	V
Major metabolic end-product(s)§	S	A, S	A, B	A, S	P, S
Major CFA	iso-$C_{15:0}$	anteiso-$C_{15:0}$	iso-$C_{15:0}$	anteiso-$C_{15:0}$	iso-$C_{15:0}$
DNA G+C content (mol%)	55–58	40–48	40–55	39–60	60–61

*A. *putredinis* does not produce pigment, is susceptible to bile and is non-fermentative.

†*Porphyromonas catoniae* does not produce pigment and is fermentative.

‡Tested using special-potency antimicrobial identification discs. R, Resistant; S, susceptible; V, variable.

§A, Acetic acid; B, butyric acid; P, phenylacetic acid; S, succinic acid.

Cells are slender rods with rounded ends and are 0·2–0·5 × 0·5–3 μm in size. After incubation for 48 h anaerobically at 37 °C on supplemented Brucella blood agar, colonies are circular, entire, convex, grey, opaque, weakly β-haemolytic and 0·5–0·8 mm in diameter. After 4 days incubation on laked rabbit blood agar, colonies appear black. Under UV light (365 nm), colonies are brown but no fluorescence is observed. Strictly anaerobic. Resistant to 20 % bile. Indole-positive and catalase-negative. Nitrate is not reduced to nitrite. Aesculin hydrolysis is variable. When tested with the API Rapid ID 32A system, mannose and raffinose are fermented, and positive reactions are obtained for α-galactosidase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, indole, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase, α-fucosidase and glutamyl glutamate arylamidase. Variable results for β-glucosidase, α-arabinosidase, glutamate decarboxylase and α-fucosidase (when positive, the reaction is weak). Other reactions are negative. With the API ZYM kit, positive enzyme reactions are obtained for alkaline phosphatase, esterase (weak), esterase lipase (weak), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and *N*-acetyl-β-glucosaminidase. Variable reaction for α-chymotrypsin, but, when positive, the reaction is weak. Negative for lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, β-glucuronidase and α-mannosidase. The principal long-chain fatty acid of the six isolates tested (iso-$C_{15:0}$) varies in content from 23 to 27 % (of total CFAs).

The type strain is WAL 8169^T (=CCUG 48946^T=ATCC BAA-1178^T). Fifteen strains were isolated from human specimens: 14 were of intestinal origin and one was from urine. The habitat is probably the human gut. The DNA G+C content of the type strain is 58 mol%.

Description of *Alistipes shahii* sp. nov.

Alistipes shahii (sha′hi.i. N.L. gen. n. *shahii* of Shah, to honour Haroun N. Shah, a contemporary microbiologist, for his contribution to anaerobic bacteriology).

Cells are slender rods, 0·1–0·2 μm by 0·6–4 μm. After incubation for 48 h anaerobically at 37 °C on supplemented Brucella blood agar, colonies are circular, entire, convex, grey, opaque, weakly β-haemolytic and 0·5–1 mm in diameter. After 4 days incubation on laked rabbit blood agar, colonies appear black. Under UV light (365 nm), colonies are brown but no fluorescence is observed. Strictly anaerobic. Resistant to 20 % bile. Indole-positive and catalase-negative. Nitrate is not reduced to nitrite. Aesculin is hydrolysed. When tested with the API Rapid ID 32A system, mannose and raffinose are fermented, and positive reactions are obtained for α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, indole, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase, α-fucosidase and glutamyl glutamate arylamidase. All other reactions are negative. With the API ZYM system, positive enzyme reactions are obtained for alkaline phosphatase, esterase (weak), esterase lipase (weak), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and *N*-acetyl-β-glucosaminidase. Two strains have very weak reactions to α-chymotrypsin. Negative for lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, β-glucuronidase and α-mannosidase. The content of the principal long-chain fatty acid (iso-$C_{15:0}$) of six tested strains varies from 36 to 43 % (of total CFAs).

The type strain is WAL 8301^T (=CCUG 48947^T=ATCC BAA-1179^T). The six strains were from human specimens of intestinal origin. The habitat is probably the human gut. The DNA G+C content of the type strain is 56 mol%.

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