Taxonomy/systematics

*Anaerofustis stercorihominis* gen. nov., sp. nov., from human feces

Sydney M. Finegold\textsuperscript{a,b,c,*}, Paul A. Lawson\textsuperscript{d}, Marja-Liisa Vaisanen\textsuperscript{e}, Denise R. Molitoris\textsuperscript{e}, Yuli Song\textsuperscript{e}, Chengxu Liu\textsuperscript{e}, Matthew D. Collins\textsuperscript{d}

\textsuperscript{a} Infectious Diseases Section (111F), VA Medical Center, 11301 Wilshire Boulevard, West Los Angeles, CA, USA
\textsuperscript{b} Department of Medicine, UCLA School of Medicine, USA
\textsuperscript{c} Department of Microbiology, Immunology, and Molecular Genetics, UCLA School of Medicine, USA
\textsuperscript{d} School of Food Biosciences, University of Reading, Reading, UK
\textsuperscript{e} Research Service, VA Medical Center, West Los Angeles, CA, USA

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Abstract

Phenotypic and phylogenetic studies were performed on an unidentified Gram-positive, strictly anaerobic, non-spore-forming, rod-shaped bacterium isolated from human feces. The organism was catalase-negative, resistant to 20% bile, produced acetic and butyric acids as end products of glucose metabolism, and possessed a G+C content of approximately 70 mol%. Comparative 16S rRNA gene sequencing demonstrated that the unidentified bacterium was a member of the *Clostridium* sub-phylum of the Gram-positive bacteria, and formed a loose association with rRNA cluster XV. Sequence divergence values of 12% or greater were observed between the unidentified bacterium and all other recognized species within this and related rRNA clusters. Treeing analysis showed the unknown anaerobe formed a deep line branching near to the base of rRNA cluster XV and phylogenetically represents a hitherto unknown taxon, distinct from *Acetobacterium*, *Eubacterium* sensu stricto, *Pseudoramibacter* and other related organisms. Based on both phylogenetic and phenotypic evidence, it is proposed that the unknown bacterium from feces be classified in a new genus *Anaerofustis*, as *Anaerofustis stercorihominis* sp. nov. The type strain of *Anaerofustis stercorihominis* is ATCC BAA-858\textsuperscript{T} = CCUG 47767\textsuperscript{T}.

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1. Introduction

The human large intestine harbors a highly diverse and complex community of bacteria that play an important role in the health and physiological function of the host [1,2]. The characterization of bacteria which make up the human gastrointestinal micro-biota has improved much in recent years due to the increasing use of molecular methods of identification [3]. Traditionally the identification of gut bacteria has relied almost solely on cultivation studies in conjunction with phenotypic methods of identification. There is now, however, a growing recognition that our current picture of the diversity of species which make up the gut microbiota is very incomplete. In particular, PCR rDNA direct community sequence analyses have shown that the majority of the generated rDNA lineages derive from hitherto undescribed species [4,5]. Some of this novel diversity represents “non-culturable” organisms; present cultivation methods need to be improved to permit recovery of organisms with special growth requirements. Nevertheless, it seems likely that a number have simply eluded taxonomic description because of the limitations of traditional phenotypic methods of identification. Thus culturing techniques, if used in concert with 16S rRNA gene sequencing, can provide a powerful approach for discerning new species diversity within the human gut and feces (e.g., [6–10]). During the course of a study of the fecal flora of autistic children, we have used the aforementioned approach to facilitate the

Abbreviations: CCUG, Culture Collection of the University of Göteborg; Sweden
\textsuperscript{2} The 16S rRNA gene sequence of strain WAL 14563\textsuperscript{T} has been deposited in GenBank under accession number AJ518871.
\textsuperscript{3} Corresponding author. Infectious Diseases Section (111F), VA Medical Center, 11301 Wilshire Boulevard, West Los Angeles, CA, USA. Tel.: +1-310-268-3678; fax: +1-310-268-4928.
E-mail address: sidfinegol@aol.com (S.M. Finegold).

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identification of a hitherto unknown species within the high G+C Clostridium sub-phylum of the Gram-positive bacteria. Based on the presented findings, we describe a new genus and species, Anaerofustis stercorihominis.

2. Materials and methods

2.1. Isolation and cultivation

Strain WAL 14563T was recovered from a stool specimen of a 5 year 11 month old boy with late onset autism before treatment with vancomycin at the Rush Childrens Hospital, Chicago. This was done as part of a study of likely involvement of bowel flora in autism [11]. The entire stool specimen was homogenized using a sterile stainless-steel blender with one to three volumes of peptone (0.05%) added as diluent. An aliquot of the specimen of approximately 1g weight was used to make serial 10-fold dilutions in Pre Reduced Anaerobically Sterilized (PRAS) dilution blanks (Anaerobe Systems, Morgan Hill, CA, USA). Various dilutions (100 μL) were plated onto brucella blood agar (BAP, Anaerobe Systems) and incubated anaerobically at 37°C under nitrogen (86%), hydrogen (7%), and CO2 (7%), (v/v) gas phase.

2.2. Biochemical characterization

The strain was characterized biochemically by using a combination of conventional tests as described previously in the Wadsworth and VPI anaerobic manuals [12,13] and the API ZYM system according to the manufacturer’s instructions (API bioMérieux, Marcy l’Etoile, France). Growth was examined under aerobic, microaerophilic (2% and 6% oxygen), and anaerobic conditions at 37°C at 2–3 day intervals for up to 1 week. Fermentation tests were performed using pre-reduced, anaerobically sterilized (PRAS) peptone–yeast–sugar broth tubes (Anaerobe Systems, Morgan Hill, CA) for metabolic end product (short-chain volatile and non-volatile fatty acids) analysis by gas–liquid chromatography (GLC). All biochemical tests were performed in duplicate.

2.3. Cellular fatty acid composition

Long-chain cellular fatty acids were analysed as previously described [14].

2.4. DNA base composition

The mol% G+C content of DNA was determined by HPLC according to Mesbah et al. [15] except that the methanol content of the chromatographic buffer was decreased to 8% and the temperature was increased to 37°C.

2.5. 16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA genes of the isolate were amplified by PCR using universal primers pA (positions 8 to 28, Escherichia coli numbering) and pH (positions 1542–1522). The amplified product was purified by using a QIAquick PCR purification kit (Quiagen, Inc., Chatsworth, CA and Quiagen, Ltd., Dorking, UK) and directly sequenced using primers directed towards conserved positions of the rRNA gene and dRhodamine terminator cycle sequencing kit (PE Applied Biosystems, Inc., Foster City, CA, USA) and an automatic DNA sequencer (model 377; PE Applied Biosystems). The closest known relatives of the new isolate were determined by performing database searches using the program FASTA [16]. These sequences and those of other known related strains were retrieved from the GenBank and aligned with the newly determined sequence using the program DNATools [17]. The resulting multiple sequence alignment was corrected manually using the program GeneDoc [18] and a phylogenetic tree was constructed according to the neighbor-joining method with the programs DNATools and TREEVIEW [19] and the stability of the groupings was estimated by bootstrap analysis (1000 replications) using the same program.

3. Results and discussion

The isolate originating from the fecal material was strictly anaerobic and consisted of thin rod-shaped organisms, 0.5–1–3 μm which stained Gram-positive; it was present in a count of 4.0×10⁹ per gm feces (dry weight). Spores were not observed on either gram or spore stains. Colonies on Brucella blood agar plates at 48 h were gray, circular, with entire edges, 1.5mm in diameter, and showed light yellow fluorescence under long-wave (366 nm) UV light. The isolates grew well anaerobically but no growth occurred in atmospheres of 2% or 6% oxygen. The organism was catalase negative, lipase-negative, lecithinase-negative, urease-negative, did not reduce nitrate to nitrite, and was indole-negative. Glucose and some other carbohydrates were fermented (all tests performed as per reference 12). Analysis of the acid end products from peptone yeast glucose broth revealed acetic and butyric acids; acetic acid was produced in peptone–yeast extract broth. The isolate grew well in peptone–yeast extract broth supplemented with 1% of fructose, glucose, mannitol, ribose, salicin, sucrose and xylose but grew poorly in peptone–yeast extract broth without supplementation.
and with lactose, maltose and mannose. Acid and gas were produced from fructose. The strain failed to produce acid from lactose, maltose, mannitol, mannose, melezitose, melibiose, ribose, salicin, and sucrose. Reactions for glucose and xylose were either weakly positive or negative. Using the miniaturized API ZYM system, weak positive reactions were observed for esterase C4, esterase lipase C8, acid phosphatase and naphthol-AS-Bi-phosphohydrolase. All other enzyme reactions were negative with this test kit. The unidentified isolate was sensitive to kanamycin (1000 μg) and vancomycin (5 μg) but resistant to colistin sulfate (10 μg) identification disks. The predominant long-chain cellular fatty acids (CFA) produced were C₁₄:₀ (17.0%), C₁₆:₀ (12.5%), C₁₆:₁ cis 9 (7.5%), and C₁₈:₁ cis 9 (21.5%), together with dimethyl acetal (DMA) C₁₈:₁ cis 9 DMA (17.0%) [12,13]. The G+C content of DNA of the strain was determined and found to be 70 mol%. To determine the phylogenetic position of the unidentified isolate, its almost complete 16S rRNA gene was amplified by PCR and sequenced. Sequence database searches showed the unknown bacterium was a member of the *Clostridium* sub-phyllum of the Gram-positive bacteria (data not shown). Based on sequence similarities, the closest described relatives of the unidentified bacterium corresponded to species within rRNA clusters XII, XIII and XV (see Refs. [20,21] for rRNA grouping designations). A tree constructed using the neighbor-joining method showing the phylogenetic relationships of the unknown bacterium is depicted in Fig. 1 and shows the unidentified rod-shaped bacterium is associated with rRNA cluster XV, which includes *Acetobacterium*, *Eubacterium limosum* and close relatives, and *Pseudoramibacter*. The clustering of the unknown fecal isolate at the base of rRNA cluster XV was confirmed by parsimony analysis (data not shown). Sequence divergence values between the unidentified isolate and species of the aforementioned rRNA cluster XV were greater than 12%, indicating their association was somewhat loose.

It is evident from the presented findings that the unidentified Gram-positive staining, asporogenous, rod-shaped organism represents a hitherto unknown line within the *Clostridium* sub-phyllum. Phylogenetically, the bacterium displayed a loose affinity with rRNA cluster XV, forming a distinct line proximal to the periphery of this cluster. The branching of the unidentified bacterium at the base of rRNA cluster XV was statistically significant (100% bootstrap re-sampling value) although sequence divergence considerations showed the affinity between the unknown isolate and other taxa within this cluster was not particularly close. The unknown bacterium displayed approximately 12–14% sequence divergence from species of the genera *Acetobacterium* and *Eubacterium* sensu strícto. By contrast, the inter-generic divergence between *Acetobacterium* and *Eubacterium* sensu strícto is only about 6–8.5%, and divergence values for species within these genera are approximately 5% or less [20]. Similarly the unknown isolate exhibited 12.8% sequence divergence from the genus *Pseudoramibacter*, and the latter shows approximately 9–10% divergence from *Acetobacterium* and *Eubacterium* sensu strícto. These data, together with the very deep branching position of the fecal bacterium, clearly show it represents a hitherto unknown genus within rRNA cluster XV that is separate from *Acetobacterium*, *Eubacterium* sensu strícto and *Pseudoramibacter*. Support for the separation of the fecal bacterium from these genera also comes from phenotypic considerations. The unidentified bacterium can be readily

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**Fig. 1.** Unrooted tree showing the phylogenetic position of *Anaerofustis stercorihominis* sp. nov. within the *Clostridium* sub-phyllum of the Gram-positive bacteria. The tree constructed using the neighbor-joining method was based on a comparison of approximately 1330 nucleotides. Bootstrap values, expressed as a percentage of 1000 replications, are given at branching points. Scale bar = 1% sequence divergence.
distinguished from the genera \textit{Acetobacterium} and \textit{Eubacterium} sensu stricto by its end products of glucose fermentation. The novel fecal bacterium produces acetic and butyric acids whereas \textit{Acetobacterium} produces acetic acid and \textit{Eubacterium} sensu stricto forms acetic, butyric, and lactic acids (some species may, in addition, produce formic acid) [22]. Similarly the unidentified bacterium can be distinguished from \textit{Pseudoramibacter alactolyticus} by its cellular shape and resistance to 20% bile. \textit{Pseudoramibacter alactolyticus} produces rod-shaped cells which occur in pairs resembling flying birds or Chinese characters [20,21]. In addition, \textit{Pseudoramibacter alactolyticus} produces formic, acetic, butyric and capric acids as end products of glucose metabolism [20,21]. Hence, given these phenotypic differences in concert with the relatively long and distinct subline formed by the fecal bacterium within \textit{Clostridium} rRNA cluster XV, we consider it merits assignment to a new genus. We therefore formally propose that the bacterium be classified as a new genus and species, \textit{Anaerofustis stercorihominis} gen. nov., sp. nov. Although only a single strain of \textit{Anaerofustis stercorihominis} is currently known, we consider the formal description of this species together with 16S rRNA gene sequence data to aid its identification, will facilitate its recognition in the laboratory thereby permitting the recovery of additional strains and enabling an evaluation of its numerical significance in feces and other habitats.

3.1. Description of \textit{Anaerofustis} gen. nov.

\textit{Anaerofustis (an.a.e.ro.fus’i.tis). Gr. pref. an without; Gr. masc. n. aer air; L. masc. n. fusit stick; N. L. masc. n. Anaerofustis stick living without air) cells consist of Gram-positive staining, non-spore-forming, rods. Strictly anaerobic and catalase-negative. Resistant to 20% bile. Some carbohydrates are fermented. End products of metabolism from glucose peptone–yeast extract broth are acetic and butyric acids. Esculin is not hydrolysed. Lipase, lecithinase and urease negative. Indole test is negative. Nitrate is not reduced to nitrite. Using the API system, weak positive reactions are obtained for esterase C4, esterase lipase C8, acid phosphatase and naphthol-AS-Bi-phosphohydrolase. Alkaline phosphatase, lipase C14, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glicosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not detected. The predominant long-chain cellular fatty acids are C\textsubscript{14:0}, C\textsubscript{16:0}, C\textsubscript{18:1 cis9} and C\textsubscript{18:1 cis9} DMA, together with other minor straight-chain saturated and monounsaturated fatty acids and DMAs. Resistant to colistin sulfate (10 μg) but sensitive to vancomycin (5 μg) and kanamycin (1000 μg) identification disks. The G+C content of DNA is 70 mol%. Isolated from human feces. Habitat is not known but probably is a member of the human gut microflora. The type strain is ATCC BAA-858\textsuperscript{T} = CCUG 47767\textsuperscript{T}.

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