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Detection, isolation and characterization of *Fusobacterium gastrosuis* sp. nov. colonizing the stomach of pigs

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ABSTRACT

Nine strains of a novel *Fusobacterium* sp. were isolated from the stomach of 6–8 months old and adult pigs. The isolates were obligately anaerobic, although they endured 2 h exposure to air. Phylogenetic analysis based on *16S rRNA* and *gyrase B* genes demonstrated that the isolates showed high sequence similarity with *Fusobacterium mortiferum*, *Fusobacterium ulcerans*, *Fusobacterium varium*, *Fusobacterium russii* and *Fusobacterium necrogenes*, but formed a distinct lineage in the genus *Fusobacterium*. Comparative analysis of the genome of the type strain of this novel *Fusobacterium* sp. confirmed that it is different from other recognized *Fusobacterium* spp. DNA-DNA hybridization, fingerprinting and genomic %GC determination further supported the conclusion that the isolates belong to a new, distinct species. The isolates were also distinguishable from these and other *Fusobacterium* spp. by phenotypical characterization. The strains produced indole and exhibited proline arylamidase and glutamic acid decarboxylase activity. They did not hydrolyse esculin, did not exhibit pyroglutamic acid arylamidase, valine arylamidase, α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate or α-glucosidase activity nor produced acid from cellobiose, glucose, lactose, mannitol, mannose, maltose, raffinose, saccharose, salicin or trehalose. The major fatty acids were C16:0 and C18:1ω9c. The name *Fusobacterium gastrosuis* sp. nov. is proposed for the novel isolates with the type strain CDW1(T) (=DSM 101753(T) = LMG 29236(T)). We also demonstrated that *Clostridium rectum* and *mortiferum Fusobacterium* represent the same species, with nomenclatural priority for the latter.

Introduction

Fusobacteria have been described as anaerobic, non-motile, non-sporulating, fastidious Gram-negative rods that produce butyric acid as major end product of their metabolism [15,28]. The genus currently consists of 15 recognized species [8,11,13,17]. Although Fusobacteria are normal constituents of the oropharyngeal, gastrointestinal and genital microbiota, they are the second most frequently isolated anaerobic microbial group from clinical samples of both human and animal origin, especially from cases of pyonecrotic infections [38]. Considering their fastidious nature, this reported detection frequency still may be an underestimation of the true frequency [21]. In human patients *Fusobacterium* spp. have been described to play a role in gingivitis and dental plaque formation [3,7,16,27,31], whereas in pigs they are associated with lameness and facial skin necrosis [6,19,46]. In cattle and sheep they are involved in necrotic laryngitis and footrot [19,25,37]. In horses they may be associated with necrotic oral and lower respiratory tract diseases as well as intra-abdominal abscesses [19].

Ulceration of the non-glandular *pars oesophagea* of the stomach is very common in pigs and can lead to discomfort, pain, decreased daily weight gain and even sudden death. *Helicobacter* (*H.*) *suis*, *Lactobacillus* spp. and *Bacillus* spp. have all been suggested to play a role in the development of gastric ulceration. Nevertheless, the exact etiology of this disease still is a matter of debate and is clearly multifactorial [18,21,24]. Results of a recent metagenomic analysis of the gastric microbiota of 20 pigs of 6–8 months old showed that an unidentified *Fusobacterium* sp. was abundantly present, representing up to 20% of the gastric microbial community. Compared to *H. suis*-negative animals, higher numbers of this *Fusobacterium* sp. were detected in *H. suis*-infected animals (unpublished results).

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The main aim of the present study was to isolate and characterize this putative new *Fusobacterium* sp. This is required to enable further research into its possible pathogenic significance and role in the pathogenesis of ulceration of the non-glandular part of the stomach in pigs. Therefore, *Fusobacterium* isolates obtained from porcine stomachs were characterized phenotypically and genotypically. The new *Fusobacterium* sp. showed the highest sequence similarity with *Fusobacterium* (*F.*) *mortiferum*, *Fusobacterium russii*, *Fusobacterium ulcerans*, *Fusobacterium varium*, *Fusobacterium necrogenes* and, surprisingly, *Clostridium* (*C.*) *rectum.* Although clostridia are Gram-positive bacteria capable of producing endospores, in previous studies it was described that *C. rectum* is closely related to *Fusobacterium* spp. [4,10,26]. The second aim of the present study was, therefore, to try and solve this inconsistency in the classification of *C. rectum*.

Material and methods

Isolation from porcine stomachs

Thirty five stomachs of 6–8 months old pigs and 25 stomachs of adult sows were collected over a period of 8 months from different slaughterhouses in Flanders, Belgium. The stomachs were transported immediately to the laboratory and stored at 4 °C until further examination within 2 h. The stomachs were opened along the greater curvature one at the time and rinsed with sterile tap water. Swabs were taken from each stomach region (*pars oesophagea*, cardia, fundus and antrum), streaked on Columbia agar plates® (Oxoid, Basingstoke, United Kingdom) supplemented with 5% defibrinated sheep blood® (E&O laboratories, Bonnybridge, Scotland), 100 mg/L Neomycin®, 5 mg/L Vancomycin® and 1 mg/L Erythromycin® (Sigma–Aldrich, Saint Louis, Missouri), and incubated anaerobically for 3 days at 37 °C. Based on previous descriptions of the colony morphology of *Fusobacterium* spp. [5,38,40], colonies of interest were purified on Columbia agar plates® supplemented with 5% defibrinated sheep blood® and incubated anaerobically for 3 days at 37 °C. A Gram-staining was performed on purified cultures. When this staining revealed Gram-negative rods, several colonies were suspended in 200 μL of an in-house bacterial preservation medium [21] for storage at −70 °C. In order to identify the bacterium at species level, DNA was extracted using PrepMan Ultra Sample Preparation Reagent® (Life Technologies, Carlsbad, California) according to the manufacturer's instructions. The near complete *16S rRNA* gene was amplified with αβ-NOT and ωMB primers [2] and sequenced by GATC Biotech, Supremerun sequencing® (Constance, Germany). The obtained sequences were analyzed with Vector NTI® (Life Technologies, Carlsbad, California). Finally, a comparison was made between the 16S rRNA sequences of the isolates and the previously detected *Fusobacterium* sp. (pig gastric microbiota metagenomic analysis, unpublished results). Colonies were considered as the putative new *Fusobacterium* sp. of interest when at least 99% identity was obtained.

Determination of species with high sequence similarities

A consensus sequence of the *16S rRNA* gene of the 9 isolates was obtained using the BioEdit Sequence Alignment Editor and ClustalIW Multiple Alignment® tools (Ibis Biosciences, California, United States) in order to identify potential nucleotide differences between the isolates. The 16S rRNA sequences of the 9 isolates were blasted using EZ taxon database of EZBioCloud® (ChunLab, Korea) and the species showing the highest sequence similarities were selected for further characterization.

The following type strains of *F. mortiferum*, *F. russii*, *F. ulcerans*, *F. varium*, *F. necrogenes* and *C. rectum* were obtained from the Culture Collection of University of Göteborg (CCUG) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) bacterial culture collection: CCUG 14475^T, CCUG 45924, CCUG 50053^T, CCUG 4858^T, CCUG 4949^T and DSM 1295^T, respectively. In order to perform the characterization tests, the organisms were grown on Columbia agar plates® supplemented with 5% defibrinated sheep blood® for 3 days at 37 °C.

Genotypic characterization

Both *16S rRNA* and *gyrase B* (*gyrB*) genes were selected for phylogenetic analysis. The *16S rRNA* gene of the 9 isolates and the species showing high sequence similarities, were amplified and sequenced as described above. The *gyrB* gene was amplified using UP-1 and UP-2r primers as described previously [44], except that 35 cycles were used with an annealing temperature of 57 °C. A consensus sequence of the 9 isolates was also created for the *gyrB* gene, as described above for the *16S rRNA* gene, to determine the sequence similarity. The sequences of both genes were compared with those in the NCBI database using the BLAST search tool. The available *16S rRNA* and *gyrB* gene sequences of the type strains of all recognized *Fusobacterium* spp., were selected for phylogenetic analysis. A multiple alignment was performed using MUSCLE® (EMBL-EBI, Cambridge, United Kingdom) with Gblocks as alignment curation. A phylogenetic tree was created using PhyML® (ATGC, Montpellier, France) with the maximum likelihood method and a bootstrap value of 1000 to estimate the robustness of the topology of the tree. Finally, the 16S rRNA and gyrB trees were visualized using TreeDyn® (GEMI Bioinformatics, Montpellier, France). Maximum parsimony and neighbour-joining algorithm based trees were compared with the maximum likelihood based tree in order to determine the closest phylogenetic neighbours and conserved roots in a reliable way. Finally, gyrB derived amino-acid trees were constructed and compared to the nucleotide based trees.

Isolate CDW1 was chosen as type strain. The genomic DNA G + C content of this strain and *F. mortiferum*, *F. necrogenes* and *C. rectum* were determined [9] and DNA–DNA hybridizations were performed [14]. Repetitive sequence-based PCR fingerprinting with the $(GTG)_5$ primer [39] was also performed to confirm the non-clonal nature of the 9 isolates.

In order to sequence the genome of isolate CDW1, it was cultivated on Columbia agar plates® supplemented with 5% defibrinated sheep blood® and incubated anaerobically for 3 days at 37 °C. Subsequently, genomic DNA was extracted using the Gentra Puregene Yeast/Bact. Kit® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The genome was sequenced using SMRT® Technology PacBio RS II (GATC Biotech®, Constance, Germany), with an average genome coverage of 100 and a run mode of 240-min movie. Gene finding and automatic annotation were performed using the Rapid Annotation Subsystems Technology (RAST) server [1,34]. The available annotated draft and complete genomes of different *Fusobacterium* spp. were obtained from the NCBI database and selected for further phylogenetic comparison. After analyzing these annotated genome assemblies, pangenomes were created using the rapid large-scale prokaryote pan genome analysis (Roary) tool [35]. Briefly, the annotated proteins from all isolates were used for a BLASTP all-versus-all sequence similarity search. From the BLASTP output, groups of orthologous proteins were predicted using the Orthagogue and MCL software [12]. Orthologous groups with exactly one representative protein from each of the input strains were considered to be part of the *Fusobacterium* core genome. This obtained core genome alignment was then used for phylogenetic tree construction using PhyML® (ATGC, Montpellier, France) with maximum likelihood method and a bootstrap value of