Anaerobe 14 (2008) 301–309

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clinical microbiology

Phenotypic and genotypic analyses of clinical *Fusobacterium nucleatum* and *Fusobacterium periodonticum* isolates from the human gut

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ARTICLE INFO

Article history:
Received 19 September 2008
Received in revised form
2 December 2008
Accepted 4 December 2008
Available online 16 December 2008

Keywords:
Fusobacterium nucleatum
Fusobacterium periodonticum
Gut
Phylogeny
DGGE

ABSTRACT

Fusobacterium nucleatum is a Gram-negative anaerobic rod that is part of the normal human microflora, and has also been associated with various infections. Bacterial strains belonging to the species are typically heterogeneous in both phenotype and genotype, which can hinder their identification in a clinical setting. The majority of *F. nucleatum* isolates originate from oral sites, however the species is also a resident of the human gastrointestinal tract. The aim of this study was to compare *F. nucleatum* isolates from human intestinal biopsy samples to try and determine whether isolates from this site are divergent from oral isolates. We used a variety of phenotypic and genotypic markers to compare 21 *F. nucleatum* and *Fusobacterium periodonticum* isolates from the GI tract to oral isolates and recognized type strains in order to study heterogeneity within this set. 16S rDNA and *rpoB* gene sequence analysis allowed us to build phylogenetic trees that consistently placed isolates into distinct clusters. 16S rDNA copy number analyses using Denaturing Gradient Gel Electrophoresis (DGGE) demonstrated potential for use as a method to examine clonality amongst species. Phenotypic analyses gave variable results that were generally unhelpful in distinguishing between phylogenetic clusters. Our results suggest that a) *F. periodonticum* isolates are not restricted to the oral niche; b) phenotypic classification is not sufficient to subspeciate isolates; c) heterogeneity within the species is extensive but constrained; and d) *F. nucleatum* isolates from the gut tend to identify with the *animalis* subspecies.

1. Introduction

The genus *Fusobacterium* currently contains 14 species that are in general Gram-negative, anaerobic, non-sporulating inhabitants of the mammalian mouth and digestive tract [1,2]. Of these species, *Fusobacterium nucleatum* is of particular significance as it has been associated with several infections of man, including periodontitis [3], otitis media [4], Lemierre's disease [5] and amniotic fluid infections leading to preterm labour [6].

Most previous studies of *F. nucleatum* clinical isolates have concentrated on oral isolates because the mouth is considered as the primary niche for this species [1]. Of the 5 recognized subspecies of *F. nucleatum*, namely subspp. *nucleatum*, *polymorphum*, *vincentii*, *animalis* and *fusiforme*, it is thought that subspp. *vincentii*, *polymorphum* and *nucleatum* predominate in the oral cavity, along with the closely related species, *Fusobacterium periodonticum* [3,7], although patterns of distribution of *F. nucleatum* appear to be variable and influenced by the health of the host [3]. The distribution of species and subspecies in the GI tract has not previously been studied.

The aim of the work reported here was to examine a collection of strains of *F. nucleatum* and *F. periodonticum* isolated from human intestinal biopsy samples, and corresponding oral strains from the same patient cohort, in order to determine whether strains from the gastrointestinal tract niche differed from those of the oral niche. To do this we profiled isolates using a range of phenotypic and genotypic markers. Additionally we applied Denaturing Gradient Gel Electrophoresis (DGGE) analysis to determine 16S rDNA (16S) gene copy number and heterogeneity amongst our isolates in order to determine if this method might lend itself to a relatively straightforward laboratory-based subspeciation of clinical isolates.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 26 *F. nucleatum* isolates and 7 *F. periodonticum* isolates were investigated. The panel included reference strains for *F. periodonticum* (ATCC 33693^T), *F. nucleatum* subsp. *nucleatum*

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doi:10.1016/j.anaerobe.2008.12.003

(ATCC 25586^T), subsp. *vincentii* (ATCC 49256^T), subsp. *fusiforme* (ATCC 51190^T) and subsp. *polymorphum* (ATCC 10953^T), the origins of which have been described elsewhere [8–10]. The remaining were clinical isolates obtained from oral swabs and intestinal biopsy specimens from both healthy control and inflammatory bowel disease (IBD) patients as part of a clinical investigation of the prevalence of *Fusobacterium* spp. in the human gut that is to be reported elsewhere. Briefly, sterile cotton-tipped applicators were used to swab across the gumline margin of the lingual side of a molar tooth to obtain oral samples, and intestinal biopsy specimens were taken from the same patients as part of a routine endoscopy procedure following an orally administered polyethylene glycol electrolyte solution (PegLyte® Pharmascience) to prepare the bowel by removing stool. Oral swabs and biopsy specimens were taken on the same day, and transferred to the laboratory within an hour of collection. Both oral and intestinal biopsy specimens were spread across FAA agar plates containing josamycin, norfloxacin and vancomycin (JVN plates [11]), and incubated for 4 days at 37 °C under anaerobic conditions in a Ruskinn Bug Box anaerobic chamber under an atmosphere of H_2:CO_2:N_2 10:10:80. Isolates were identified on the basis of their growth on this selective medium and their colony morphology, and their identity was confirmed by use of 16S PCR and sequence analysis (see Section 2.2 below).

Strains were propagated on Fastidious Anaerobe Agar (FAA) (LabM) containing 5% defibrinated sheep blood (MedOx), and stored as frozen stocks at −80 °C in Fastidious Anaerobe Broth (FAB) + glycerol (50% v/v).

2.2. PCR and sequencing

DNA was extracted from all isolates according to the method of Maloy [12], with the modification that Maxtract gel (Qiagen) was used to help separate aqueous/organic phases to improve DNA purification and recovery. PCR amplification of an 800 bp region of 16S was carried out using primers 8F and 907R as described by Ben-Dov et al. [13] with the modification that the T3 and T7 universal primer sequences were added to the 5′ ends of the forward and reverse primers respectively. Amplified sequence was purified using Qiagen PCR purification columns, quantified and sent for sequence analysis (Macrogen, Seoul, Korea). DNA sequences from the *Fusobacterium* isolates were compared to sequences deposited in GenBank (NCBI) using BLASTn, which allowed speciation of the strains according to their closest match in the database.

The gene coding for the RNA polymerase β subunit, *rpoB*, is highly conserved among bacterial species, and has been established as a useful alternative taxonomic marker gene in phylogenetic studies of closely related bacterial species [14]. Primers for the amplification of 500 bp of *rpoB* sequence were designed based on an alignment of the gene sequences of 2 more distantly related species within the genera, namely *Fusobacterium necrophorum* (GenBank accession number DQ336567) and *F. nucleatum* subsp. *nucleatum* ATCC 25586 (GenBank accession number AE009951). Primers were synthesized with T3 or T7 tails to enable direct sequencing from PCR products. Primer names and 5′–3′ sequences were: FrpoBT3F: ATTAACCCTCACTAAAGGAGACATGGAAACAAAGGGGTTG, and FrpoBT7R: AATACGACTCACTATAGGAGWATATTAGATGCTCCATAWGCTT. Primers were used at a concentration of 50 pmol/μL with *Taq* polymerase and 1X Thermopol buffer (2 mM $MgSO_4$) (NEB), using the following cycling conditions: 94 °C 2 min (94 °C 45 s, 54 °C 45 s, 72 °C 30 s) ×30; 72 °C 10 min.

16S and *rpoB* gene sequences were deposited in GenBank under accession numbers EU419201–EU419256, and FJ196707–FJ196712.

2.3. DGGE analysis

Primers HDA1 and HDA2 [15] were used to amplify the V3 region (339–539 bp, *Escherichia coli* numbering) of 16S. PCRs were performed using *Taq* polymerase and 1X Thermopol buffer (2 mM $MgSO_4$) (NEB), using DNA extracted from isolates as above as template. Samples were amplified using the following cycling conditions: 92 °C 2 min (92 °C 1 min, 55 °C 30 s, 72 °C 1 min) ×35; 72 °C 10 min.

DGGE analysis of PCR amplicons was based on a protocol described by Muyzer et al. [16] using the DCode System (Bio-Rad Laboratories, Hercules, CA, USA). Polyacrylamide gels consisted of 8% (v/v) polyacrylamide (37.5:1 acrylamide/bisacrylamide) in 0.5× TAE. Denaturing acrylamide of 100% was defined as 7 M urea and 40% formamide. A gradient of 30–55% was used for separation of generated amplicons. Electrophoresis was performed in 0.5× TAE buffer at a constant temperature of 60 °C, for 5 h at 120 V. Gels were stained with ethidium bromide (Sigma Aldrich), and images were captured using a SynGene G-Box gel documentation system running GeneSnap software (version 6.08.04, Perkin Elmer). Gels were normalized for saturation and distortion using Syngene GeneTools software (version 3.07.01, Perkin Elmer).

2.4. Phylogenetic analysis

Alignments of the sequence data were performed using the ClustalW algorithm (VectorNTI software (Advance 10 version), Invitrogen). Phylogenetic trees corresponding to the alignments were constructed using MEGA4 software (version 4.0) [17]. Evolutionary history was inferred using the neighbour-joining method, and bootstrap analysis of 1000 permutations was used to verify branch points on the trees.

2.5. Biochemical analysis

API Rapid 32A (BioMerieux) test strips were used to infer the biochemical profiles of each isolate according to manufacturer's instructions. Bile resistance was measured by checking growth on modified Bacteroides Bile Esculin (BBE) agar (with no added gentamicin). Freshly prepared plates were inoculated with test strains and incubated anaerobically for 5 days at 37 °C. Growth on this medium indicated resistance to bile at 20% (v/v). Although blackening of BBE agar is indicative of esculin hydrolysis, we also checked for esculin hydrolysis by bile sensitive strains using trypticase soy esculin (TSE) agar (trypticase soy agar, 4% (w/v); esculin, 0.1% (w/v); ferric ammonium citrate, 0.05% (w/v); hemin 0.001% (w/v)). TSE agar was inoculated with test strains and incubated anaerobically for 5 days at 37 °C, and blackening of the media around zones of growth after this time was scored as positive for esculin hydrolysis; a *Bacteroides fragilis* isolate was used as a positive control in this test. Raffinose fermentation, hippurate hydrolysis and alkaline phosphatase activity were confirmed using appropriate test kits (Key Scientific Products, Stamford, Texas).

2.6. Morphological analysis

Isolated *Fusobacterium* spp. strains were grown in TSB_{supp} (trypticase soy broth supplemented with 5 μg/mL of hemin and 1 μg/mL menadione) at 37 °C until stationary phase, harvested by centrifugation at 4000 × *g* for 10 min, and washed once with sterile distilled water before a small sample was smeared onto a microscope slide and examined under phase using a Leica DMIREB2 inverted microscope. Images were captured using an ORCA-ER digital camera; cell length was assessed by taking mean measurements of 24 representative cell lengths using Openlab software