Anaerobe 19 (2013) 58–61

Contents lists available at SciVerse ScienceDirect

Anaerobe

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

Molecular biology, genetics and biotechnology

Application of DNA sequence analysis based on five different conserved genes (16S rDNA, *rpoB*, *gdh*, *est* and *pgm*) for intra-species discrimination of *Bacteroides fragilis*

Karla Rodrigues Miranda [a,*], Felipe Piedade Gonçalves Neves [b], Joaquim dos Santos-Filho [a], Geraldo Renato de Paula [c], Leandro Araújo Lobo [a], Walter Martin Roland Oelemann [a], Regina Maria Cavalcanti Pilotto Domingues [a]

[a] *Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Brazil*
[b] *Departamento de Microbiologia e Parasitologia, Instituto Biomédico, Universidade Federal Fluminense, Brazil*
[c] *Departamento de Tecnologia Farmacêutica, Faculdade de Farmácia, Universidade Federal Fluminense, Brazil*

ARTICLE INFO

Article history:
Received 11 September 2012
Received in revised form
13 November 2012
Accepted 19 November 2012
Available online 27 November 2012

Keywords:
Bacteroides fragilis
Phylogenetic analysis
Systematics
Genetic diversity

ABSTRACT

In the past few years, many studies revealed a remarkable genetic variability in *Bacteroides fragilis* species, and the existence of two divisions was proposed according to presence or absence of the *cfiA* (metallo-β-lactamase/carbapenemase) gene. The aim of this study was to evaluate the use of DNA sequence analysis for glutamate dehydrogenase (*gdh*), phosphoglucomutase (*pgm*) and esterase (*est*) metabolic genes, in comparison to RNA polymerase β subunit (*rpoB*) and 16S ribosomal RNA (*rrs*) gene sequencing, to identify the presence of these two groups in seventeen *B. fragilis* strains. Based on phylogenetic trees, only the *est* gene sequences generated a classification similar to *rrs*- and *rpoB*-genes. On the other hand, the genes *pgm* and *gdh* did not allow the discrimination of these divisions. The *est* gene sequence can be suggested as an additional tool for differentiation of the two groups in *B. fragilis*, providing highly reproducible and reliable data in *B. fragilis* taxonomy.

1. Introduction

Bacteroides fragilis is the opportunistic anaerobe most frequently isolated from human infections [1]. In the past few years, many studies have revealed a remarkable genetic variability in *B. fragilis* species [2,3].

The presence of two distinctive groups or divisions within *B. fragilis* was revealed by employing different techniques, including DNA–DNA hybridization [4], sequence analyses [5], Multilocus Enzyme Electrophoresis (MLEE) [3], ribotyping, restriction fragment length polymorphism (RFLP), PCR-generated fingerprinting, insertion sequence (IS) analysis and other techniques [2,6,7]. Division I possesses the *cepA* gene encoding a serine-beta-lactamase of Amber's class A, and division II possesses the *cfiA* gene that encodes a metallo-beta-lactamase of Amber's class B [2,3].

In 2011, Nagy et al. [8] described the usefulness of Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in differentiate *B. fragilis* strains, which harbor the *cfiA* gene from those that do not. MALDI-TOF MS clearly distinguished strains belonging to *B. fragilis* division I (*cfiA* negative) from division II (*cfiA* positive).

Concerning changes in susceptibility over time, in particular among the carbapenems, the periodic monitoring of the susceptibility patterns of anaerobic bacteria is desirable. The development of methods that help to identify *B. fragilis* strains, specially monitoring the presence of resistance genes, could be useful in empiric treatment protocols and save the more potent drugs for treatment of more severe infections caused by resistant bacteria [9,10]. Conserved genes have been widely used in phylogenetic analysis mainly because of their function as molecular chronometers. Gene sequence-based identification of bacteria at species level may require sequencing of the entire gene, but even then, in some cases, phylogenetically closely related bacterial species cannot be differentiated [11]. Assessment of the RNA gene sequences in phylogenetic analyses at the genus level has been helpful, but at the species level the usefulness of this approach is in discussion [12]. A range of gene sequences have been employed and were shown to be more

* Corresponding author. Laboratório de Biologia de Anaeróbios, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Av Carlos Chagas Filho, 373, CEP 21941-902 Rio de Janeiro, Brazil. Tel.: +55 21 2560 8344x136; fax: +55 21 2560 8028.
E-mail addresses: karlarodr@yahoo.com.br, karlarodr@micro.ufrj.br (K.R. Miranda).

1075-9964/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.anaerobe.2012.11.005

effective than RNA analysis, since the latter is highly conserved and has limited discriminatory power compared to other genes.

rpoB encodes the beta-subunit of RNA polymerase and has been described as a feasible target for phylogenetic relationship studies due to its higher mutation rate when compared to *rrs*, the gene encoding the 16S ribosomal RNA [5]. Missense mutations in *rpoB* are related to rifampicin resistance in some bacteria. This gene has been also used for identification of bacteria [13].

Glutamate dehydrogenases (GDH) are chromosomally encoded metabolic housekeeping enzymes that catalyze the reversible oxidative deamination of glutamate to α-ketoglutarate and ammonia [14,15]. Phosphoglucomutase (PGM) is an evolutionarily conserved protein that catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate. This enzyme is essential for both glycolysis and gluconeogenesis, and presents a common catalytic mechanism in diverse cells [16,17]. Esterase (EST) belongs to the hormone-sensitive lipase family and hydrolyzes glycerol esters of both short- and long-chain fatty acids [18].

In the present study, we used DNA sequence analysis with a set of different genes as a new approach to investigate intra-species phylogenetic relationships. Taking advantage of these data, we investigated the usefulness of *rrs*, *rpoB*, *est* (esterase), *pgm* (phosphoglucomutase), and *gdh* (glutamate dehydrogenase) genes as targets.

2. Material and methods

2.1. Bacterial strains and DNA preparation

A total of 127 strains of *B. fragilis* strains were used in this study, including the reference strain ATCC 25285. With exception of the reference strain, all isolates were obtained either from patients with diverse clinical presentations, or from gastrointestinal and genital microbiota colonization. Species identification was performed by using the API 20A identification kit (bioMérieux), in accordance with the manufacturer's instructions. Template DNA was obtained by guanidium thiocyanate lysis according to Pitcher et al. [19].

2.2. PCR amplification

PCR was performed in a final volume of 50 μL containing 50 ng template DNA, 20 pmoles of each primer (Table 1), 1 U of *Taq* DNA polymerase, 250 μM of each dNTP, 50 mM Tris–HCl (pH 8.3), 40 mM KCl and 1.5 mM $MgCl_2$. The reaction comprised 35 cycles of 30 s at 94 °C, 30 s at 45 °C–57 °C (see Table) and 1 min at 72 °C, with a final extension step of 72 °C for 5 min, performed in a Model 9700 Thermocycler (Perkin–Elmer Cetus, Warrington, UK). PCR products were visualized on agarose 1% w/v gels stained with ethidium bromide.

2.3. DNA sequencing

PCR products were purified with Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare), according to the manufacturer's recommendations. Products were analyzed using an automated MegaBACE™1000 (GE Healthcare, USA) and DYEnamic ET Dye Terminator Cycle Sequencing Kit (Thermo Sequenase™ II DNA Polymerase). The resulting sequences were manually edited with the BioEdit Sequence Alignment Editor version 7.0.1 and analyzed by Blast software (www.ncbi.nlm.nih.gov/blast) against sequences deposited in GenBank.

2.4. Sequence analysis

rpoB, *est*, *pgm*, *gdh* and *rrs* gene sequences of the strains were edited and aligned using the Bioedit software. The gene sequences were used to evaluate intra-species similarities and for the purpose of phylogenetic reconstruction. Phylogenetic relationships were inferred from the DNA sequences using the Neighbor-Joining, Maximum Parsimony and Maximum likelihood methods in Mega 4.2, with *p*-distance. Neighbor Joining (1000 bootstraps), Maximum Parsimony (1000 bootstraps) and Maximum likelihood (100 bootstraps) were employed. Outgroup strains included in this study were: *Bacteroides thetaiotaomicron* VPI5482, *Bacteroides vulgatus* ATCC8482, *Bacteroides xylanisolvens* XB1, *Bacteroides stercoris* ATCC43183. The ingroup strain *B. fragilis* MBK12 was also included in phylogenetic analysis.

3. Results

In this work, the presence of genetic markers associated with antimicrobial susceptibility in *B. fragilis*, namely the genes *cfiA* and *cepA* were assayed by PCR. A total of 127 strains of *B. fragilis* isolated from different sources were tested. Among those, 111 were *cfiA*-negative/*cepA*-positive (division I), including ATCC 25285, and 16 were *cfiA*-positive/*cepA*-negative (division II). Taking advantage of these preliminary results, we decided to analyze two groups consisting of *cfiA* positive and *cfiA* negative *B. fragilis* strains. After

Table 1
Gene fragments and primers used in this study.

Gene[a]	Primers sequences (5′ to 3′)	Tm[b]	Position 5′	Amplicon size	References	Sequence dissimilarities[c]
cepA	cepA1: TTT CTG CTA TGT CCT GCC C cepA2: ATC TTT CAC GAA GAC GGC	50 °C	472–491 1234–1251	780 pb	[3]	–
cfiA	cfiA1: CCA TGC TTT TCC CTG TCG CAG cfiA2: GGG CTA TGG CTT TGA AGT GC	52 °C	557–582 1266–1285	747 pb	[2]	–
rrs	U968f: AAC GCG AAG AAC CTT AC L1401r: CCG TGT GTA CAA GAC CC	45 °C	828–845 1211–1228	463 pb	[21]	1.3–3.4%
rpoB	rpobF: CTG AGA AGC GTA AAA AAG AG rpobR: GTA GAT ATA AAG CAC AGC CTC	51 °C	125–144 964–984	859 pb	NC003228; this study	5.7–6.6%
est	estF: GTA GTG ACC CCT GAT GTT GC estR: CAT GAT CTA TCT TGC GGC CC	57 °C	121–140 692–721	601 pb	NC003228; this study	11.8–12.8%
pgm	pgmF: CGA GTT TGG TAC GGG CGG AC pgmR: CCG TCC TTA ATC ATC TGC TC	55 °C	150–169 796–815	682 pb	NC003228; this study	–
gdh	gdhF: GCA TTC CAG ACA GAG TAT AC gdhR: GGA GAT CAG GCA TAC TTT AC	53 °C	152–170 683–702	551 pb	NC003228; this study	–

[a] Genes *cepA* (cephalosporinase A), *cfiA* (metallo-β-lactamase/carbapenemase), *rrs* (rDNA 16S), *rpoB* (RNA polymerase β subunit), *est* (esterase), *pgm* (phosphoglucomutase), *gdh* (glutamate dehydrogenase).
[b] Tm: melting temperature.
[c] Sequence dissimilarity between divisions I and II.