MOLECULAR CHARACTERISATION OF BACTERIA

Use of rep-PCR to define genetic relatedness among *Bacteroides fragilis* strains

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Bacteroides fragilis, a component of the normal flora and an important anaerobic pathogen in non-intestinal endogenous infections, has recently been associated with enteric diseases. In this study, 41 *B. fragilis* strains were analysed in relation to their genetic diversity. This collection included two reference strains (ATCC 23745 and 25285), 20 isolates from non-intestinal infections, six from intestinal infections, five from intestinal microflora and eight from an aquatic environment. The fingerprints were generated by using two repetitive sequences (REP and ERIC) as primers to PCR (rep-PCR). A dendrogram was obtained with the Taxotron® Program. Three clusters (threshold genotypes I, II and III) were observed when the genetic distance was 0.30. These results confirm previous data found regarding the genotypical diversity of *B. fragilis*.

Introduction

Bacteroides fragilis, an obligatory anaerobic bacterial component of the normal flora, is an important pathogen in endogenous infections such as intra-abdominal abscess and septicaemia [1, 2]. In recent years, several studies have focused on the involvement of *B. fragilis* in infections located in the mucosa, causing, for example, diarrhoea [3, 4].

In infectious diseases where the probable aetiological agent is a member of the normal flora (as is *B. fragilis*), simple species identification is not useful for distinguishing between infection and colonisation. In this context, clinical microbiologists have attempted to develop procedures that provide more detailed intra-species delineation.

Several methods for detecting phenotypic or genotypic variation have been used to characterise bacterial isolates and to discriminate strains that are potential pathogens from commensal strains; however, only a few approaches have been applied to *B. fragilis*. Serotyping demonstrated a wide heterogeneity of capsular antigens among *B. fragilis* strains [5]. Genotypic methods, such

as rRNA restriction fragment length polymorphism (RFLP) [6], restriction endonuclease analysis (REA) [7], ribotyping [7, 8] and arbitrary primed PCR (AP-PCR) [9], have also been used to study *B. fragilis* strains and a great diversity has been found. However, if different *B. fragilis* strains are associated with different diseases, many questions remain unanswered.

PCR-based DNA fingerprint methods have been used for molecular typing of several bacterial pathogens and have been cited as techniques that are universally applicable, rapid and with considerable discriminatory power [10, 11]. PCR utilising interspersed repetitive elements as primers for the amplification of regions between neighbouring repetitive elements has been used (REP-PCR, ERIC-PCR and BOX-PCR) and has been collectively designated as rep-PCR [12–14].

This report describes the use of rep-PCR to assay *B. fragilis* strains from different sources (non-intestinal infections, intestinal infections, intestinal microflora and aquatic environment) to evaluate the biological and genotypic relatedness among them.

Materials and methods

Bacterial strains and growth conditions

A total of 41 *B. fragilis* strains was examined, including two type strains (ATCC 25285 and ATCC

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23745) obtained from the American Type Culture Collection, Rockville MD, USA. Thirty-one strains were isolated from different individuals (non-intestinal infections, 20; intestinal infections, 6; intestinal microflora, 5) and eight strains were isolated from an aquatic environment in Rio de Janeiro in the period 1980–1997 (Table 1). Bacterial identification was by conventional bacteriological tests based on bile resistance, catalase production, a negative indole test and fermentation of sugars [15]. All strains were maintained in our culture collection, and were activated by growth in pre-reduced anaerobic sterilised brain-heart infusion broth (BHI-PRAS) when required and then cultured in the same medium for 18 h at 37°C as a source of bacterial suspensions for the following analyses.

DNA template preparation

Cells from broth cultures (5 ml) in the logarithmic phase were harvested by centrifugation at 13 000 g for 1 min. The individual cell pellets were stored at –20°C without additional treatment until DNA isolation. Extraction and purification of chromosomal DNA was performed according to a modification of the procedure of Smith et al. [16], with the addition of RNAase treatment [17]. The concentration of DNA in the samples was determined by spectrophotometry with the GenQuant apparatus (Pharmacia).

Primers

Primer sequences corresponding to REP – REP1R-I (5'-IIIICGICGICATCIGGC-3') and REP2-I (5'-ICGI CTTATCIGGCCTAC-3') – and ERIC – ERIC1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGAAGTGGGGTGAGCG-3') [14] – were used to evaluate the amplicon profiles by PCR.

Amplification conditions

Fifty ng of DNA template were used in each reaction. The rep-PCR amplification was performed in a 25- μ l volume containing 80 pmol each of two opposing primers, 1.25 mM deoxynucleoside triphosphates and 2.5 U of Taq polymerase. Temperature cycling was controlled in a model PTC-100-60 cyler (Peltin-Effect Cycling, Watertown, USA) programmed as follows:

one initial cycle at 95°C for 7 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 44° or 52°C for 1 min with REP and ERIC primers, respectively, and extension at 65°C for 2 min with a single final extension cycle at 65°C for 15 min and a final soak at 4°C. PCR mixtures were overlaid with 25 μ l of mineral oil (M3516; Sigma).

On completion of the PCR programme, the samples were compared by electrophoresis of 10- μ l samples in agarose 1.5% gels in Tris-acetate buffer (0.04 M Tris-acetate, 0.002 M EDTA, pH 8.5) stained with ethidium bromide and photographed on a UV light transilluminator by the Polaroid MP4 system. A standard 1-kb DNA ladder (Gibco BRL, Burlington, Canada) was included.

rep-PCR products analysis

Comparisons between the fingerprints were performed as described previously [18]. Briefly, computer-assisted analysis of the scanned fingerprint was performed with the Taxotron® package, a set of programs for molecular systematics [19]. Only major bands were considered and band intensity was not used as a criterion. The derived molecular sizes (in bp) were used to compute distance (D) based upon band sharing as the complement of the Dice coefficient [20]: $D = 1 - (2n_{xy}/n_x + n_y)$; where n_{xy} is the number of bands in each strain; n_x and n_y are numbers of total bands scored for each individual. In the pairwise comparison to match co-migrating fragment positions between pairs of rep-PCR, a match was recorded if the normalised molecular size of the first amplicon was within a window of 3% of the molecular size of the second amplicon. A matrix combining all the distances was generated from which cluster analysis was performed by the unweighted pair-group method with mathematic averages (UPGMA) and a dendrogram was constructed.

Results

The reproducibility of both methods was tested with DNA preparations made from separate cultures on different days and identical and reproducible strain-

Table 1. *B. fragilis* strains analysed

Strains	Source
ATCC 23745, ATCC 25285	American Type Culture Collection (ATCC) Rockville, MD, USA
016M, 022H, 023A	Otitis media
1031, 1033*, 1048-a, 1058-1, 1304-2, 1034	Intra-abdominal infections
1032, 1037, 1039, 1081, 1241	Bacteraemia
MC2, MC3, 048203, MC1, 1292, 1077	Soft tissue infections
077225-2†, 078819-3†, 283 2.1†, 20656.2.1†, FD 10B1, 079298-3†	Human intestinal infection
FF10, FF14, FF15, F36-6, F43-6	Human intestinal microflora
AA1, AA3, AA7, AA10*, AA41-1, AA42-1, AA42-2, AAa	Aquatic environment

*The *cfiA* gene was detected in strains AA10 and 1033, but not in the other strains (unpublished observations).

†Strains given by Dr Lyle Myers, Montana University, EUA.

specific patterns were obtained. Informative and reproducible fingerprints were obtained with 2.5 U of Taq polymerase. Higher concentrations of Taq polymerase resulted in a higher background without additional prominent bands (data not shown). A concentration of 50 ng in each reaction was used throughout this study.

To investigate the genetic relationship among the 41 *B. fragilis* strains, the profiles of each sample were determined by rep-PCR with REP and ERIC primers. In both methods, amplification reactions generated informative arrays of bands composed of a minimum of three and a maximum of 16 bands with M_r s ranging from 100 to c. 2200 bp.

Amplification of the 41 *B. fragilis* isolates with REP primer resulted in three-to-seven amplicons located between the 200-bp and 2200-bp markers of the DNA ladder (Fig. 1). ERIC primers revealed 11–16 amplicons ranging from 100 bp to 2200 bp (Fig. 2).

The combined data from the two consensus sequences (REP and ERIC) were compiled and a dendrogram was generated (Fig. 3). When a distance of 0.30 was used as a threshold, three clusters (hereafter referred to as genotypes I, II and III) were generated. Genotype I included 37 strains of different origins and it was subdivided into two other groups, genotype IA and IB, with a genetic distance of 0.22. Genotype IA included 18 strains, of which four were isolated from intra-abdominal infections, two from intestinal microflora,

Fig. 1. rep-PCR fingerprints of *B. fragilis* isolates obtained with primer REP. Lane M, 1-kb DNA ladder; 1, enterotoxigenic strain 20656.2.1 (genotype IA); 2, non-enterotoxigenic strain 077225-2 (genotype IA); 3, ATCC 25285 (genotype IB); 4, ATCC 23745 (genotype IB); 5, strain AA1 (genotype II); 6, strain 1033 (genotype III); 7, strain FD 10 B1 (genotype III). The *cfiA* gene was detected in strain 1033.

Fig. 2. rep-PCR fingerprints of *B. fragilis* isolates obtained with primer ERIC. Lane M, 1-kb DNA ladder; 1, enterotoxigenic strain 20656.2.1 (genotype IA); 2, non-enterotoxigenic strain 077225-2 (genotype IB); 3, ATCC 25285 (genotype IB); 4, ATCC 23745 (genotype IB); 5, strain AA1 (genotype II); 6, strain 1033 (genotype III); 7, strain FD 10 B1 (genotype III). The *cfiA* gene was detected in strain 1033.

five from intestinal infections, one from otitis media and six from an aquatic environment. Genotype IB included 19 strains, of which 14 were isolated from non-intestinal infections (five from bacteraemia, four from intra-abdominal infection, three from soft tissue infection and two from otitis media), three from intestinal microflora and the two reference strains. Genotype II consisted of two strains from an aquatic environment (AAa and AA1) and genotype III consisted of two strains, of which one was isolated from intra-abdominal infection (1033) and one from intestinal infection (FD 10B1). The average genetic distances among these groups were as follows: 0.31 between groups I and II and 0.48 between groups I, II and III.

Discussion

This study demonstrated that repetitive extragenic sequences such as REP and ERIC are present in the genome of diverse *B. fragilis* strains, confirming and extending the suggestion made by other authors that these sequences are virtually ubiquitous [14, 21]. The results also suggest that ERIC-like sequences are more widely distributed in the *B. fragilis* genome than REP-like sequences. How this fact can benefit *B. fragilis* has not yet been determined. However, their presence and

Fig. 3. Dendrogram generated by combining the rep-PCR fingerprints obtained with each of the two primers REP and ERIC. The genetic relatedness of 41 *B. fragilis* isolates can be resolved at a distance of 0.30 as three clusters (I, II and III) defining three genotypes. The *cfiA* gene was detected in strains AA10 and 1033, but not in the other strains (unpublished observations). Strains 077225-2, 078819-3, 283.2.1 (non-enterotoxigenic) and 20656.2.1, 079298-3 (enterotoxigenic) were given by Dr L. Myers, Montana University, EUA.

widespread distribution in both prokaryotic and eukaryotic genomes strongly suggests that they are important to the structure and evolution of genomes [14, 21, 22]. In spite of the fact that the precise functions of repetitive sequences in prokaryotic genomes are still obscure, their presence can be used both in applications and molecular genetic manipulations. In recent years, some studies have demonstrated that the REP- and ERIC-PCR protocols, referred to collectively as rep-PCR, are particularly suitable for the rapid molecular characterisation of bacterial species.

This molecular approach has been accepted as a valuable and simple alternative to other typing methods. In the present study, reproducible fingerprint profiles of a particular *B. fragilis* strain could be generated from DNA isolated at different times from the same colony, from different colonies of the same strain, or from serial cultures of the same strain. This suggests that DNA fingerprinting generated by rep-PCR

constitutes a powerful tool for studying *B. fragilis*, as well as other bacterial pathogens.

As *B. fragilis* isolates were obtained from diverse biological origins, the samples can be regarded as representative of the natural population of the taxon. The diversity of the taxon *B. fragilis* has already been demonstrated by genotypic and phenotypic methods. Other studies, using REA and RFLP [6, 7] and AP-PCR [9], clearly demonstrated a wide genetic diversity within the species *B. fragilis*, consisting of nearly as many genotypes as there were isolates. In the present study, the use of a computer-assisted system to analyse the results confirmed a reasonable *B. fragilis* diversity by rep-PCR.

The polymorphism obtained with ERIC and REP allowed the 41 *B. fragilis* isolates to be distributed into a dendrogram in three genotypes (genotype I, II and III) based upon a calculated genetic distance (Fig.

3). Genotype I constituted the most heterogeneous cluster in relation to the biological origin and represented the majority of the strains in this study. The strains of this cluster, isolated from different types of clinical sample (faeces, blood, suppuration, etc.) and from aquatic environments, showed an overall similarity of 70%. Cluster IA was formed by the majority of the strains obtained from infectious processes in the intestinal mucosa area, i.e., strains from human intra-abdominal infections and intestinal microflora or from aquatic environment – except for strain 016M which was isolated from a case of otitis media. Two enterotoxigenic strains (079298-3 and 20656.2.1) and three non-enterotoxigenic strains (077225-5, 283.2.1 and 078819-3; kindly given by Dr L. Myers, Montana University, EUA), were included in this group. The strains in this cluster showed genetic similarity of c. 80%. It appears that it was not possible to observe distinct groups of Ent⁺ and Ent⁻ strains by means of rep-PCR methods. On the other hand, cluster IB consisted of the majority of strains isolated from infectious processes far from the intestinal mucosa area (bacteraemias, otitis media, etc.), which showed genetic similarity of 85%. Based on this fact, different types could cohabit in intestinal microflora and be responsible for causing infectious processes. Strain 016M, isolated from otitis media and grouped in cluster IA, could have originated from intestinal microflora by contact with contaminated objects or hands, thus allowing colonisation of the ear. The relationship between mucosal nasopharynx microflora and otitis media caused by *B. fragilis* has not been confirmed yet.

Genotype II, composed of two strains isolated from an aquatic environment, showed genetic similarity of 79%. The possibility of contamination with animal faeces could explain this finding. However, complementary studies would be needed to determine whether there is truly a relationship between strains of *B. fragilis* isolated from human intestinal microflora and strains from animal intestinal microflora.

Genotype III showed more genetic distance in this study; it grouped the strains into a genetic distance of 0.5. However, the genetic similarity between the strains in this group was up to 80%. Could these strains constitute a new genospecies? Several studies have shown rep-PCR to have a good correlation with pulsed-field gel electrophoresis (PFGE) results. In spite of these facts, the strains are phenotypically very homogeneous [23]. Other studies are needed to confirm if important phenotypic traits should be superimposed upon the polymorphism results to justify species designations [24].

Two β -lactamases, the endogenous cephalosporinase CepA and the carbapenemase CfiA, appear to be confined to the species *B. fragilis*. Furthermore, as suggested by Podglajen et al. [8], cfiA-positive strains may represent a novel species. The use of PCR with

specific primers has shown that the cfiA gene exists in strains AA10 (isolated from an aquatic environment) and 1033 (isolated from an intra-abdominal infection) (unpublished observations). However, these strains could not be found in the same genotype based on the results obtained by combined data from the two primers.

In spite of the wide diversity of the strains studied, including human (clinical and non-clinical) or environmental sources and geographic origin, it was not possible to detect an obvious correlation between a given genotype and the specific disease it can cause or healthy state of the host, or even the polluted environment where it can be found. Nevertheless, the PCR fingerprint techniques used here proved to be relatively simple to perform and reliable for *B. fragilis* genetic diversity studies.

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References

1. Gorbach SL, Bartlett JG. Anaerobic infectious. *N Engl Med* 1974; 290: 1177–1184.
2. Polk BF, Kasper DL. *Bacteroides fragilis* subspecies in clinical isolates. *Ann Intern Med* 1977; 86: 569–571.
3. Myers LL, Shoop DS, Stackhouse LL et al. Isolation of enterotoxigenic *Bacteroides fragilis* from humans with diarrhea. *J Clin Microbiol* 1987; 25: 2330–2333.
4. Sack BS, Albert MJ, Alam K, Neogi PKB, Akbar MS. Isolation of enterotoxigenic *Bacteroides fragilis* from Bangladeshi children with diarrhea: a controlled study. *J Clin Microbiol* 1994; 32: 9600–963.
5. Pantosti A, Colangeli R, Tzianabos AO, Kasper DL. Monoclonal antibodies to detect capsular diversity among *Bacteroides fragilis* isolates. *J Clin Microbiol* 1995; 33: 2647–2652.
6. Smith CJ, Callihan DR. Analysis of rRNA restriction fragment length polymorphisms from *Bacteroides* spp. and *Bacteroides fragilis* isolates associated with diarrhea in humans and animals. *J Clin Microbiol* 1992; 30: 806–812.
7. Kleivdal H, Hofstad T. Chromosomal restriction endonuclease analysis and ribotyping of *Bacteroides fragilis*. *APMIS* 1995; 103: 180–184.
8. Podglajen I, Breuil J, Casin I, Collatz E. Genotypic identification of two groups within the species *Bacteroides fragilis* by ribotyping and by analysis of PCR-generated fragment patterns and insertion sequence content. *J Bacteriol* 1995; 177: 5270–5275.
9. Moraes SR, Gonçalves RB, Mouton C, Seldin L, Ferreira MCS, Domingues RMCP. *Bacteroides fragilis* isolates compared by AP-PCR. *Res Microbiol* 1999; 150: 257–263.
10. Lin AW, Usera MA, Barrett TJ, Goldsby RA. Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella enteritidis*. *J Clin Microbiol* 1996; 34: 870–876.
11. Ménard C, Mouton C. Clonal diversity of the taxon *Porphyromonas gingivalis* assessed by random amplified polymorphic DNA fingerprinting. *Infect Immun* 1995; 63: 2522–2531.
12. de Bruijn FJ. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl Environ Microbiol* 1992; 58: 2180–2187.
13. Dimri GP, Rudd KE, Morgan MK, Bayat H, Ames GF-L. Physical mapping of repetitive extragenic palindromic sequences in *Escherichia coli* and phylogenetic distribution

- among *Escherichia coli* strains and other enteric bacteria. *J Bacteriol* 1992; 174: 4583–4593.
14. Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 1991; 19: 6823–6831.
 15. Summanen P, Baron EJ, Citron DM, Strong C, Wexler HM, Finegold SM. *Wadsworth Anaerobic Bacteriology Manual*. Belmont, CA, Star Publishing Company. 1993.
 16. Smith G, Socransky S, Smith C. Rapid method for the purification of DNA from subgingival microorganisms. *Oral Microbiol Immunol* 1989; 4: 47–51.
 17. Wilson K. Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Struhl K (eds) *Current protocols in molecular biology*. New York, Greene Pub. Associates and Wiley-Interscience. 1991: 2.4.1–2.4.2.
 18. Paquet C, Mouton C. RAPD fingerprint for the distinction of *Prevotella intermedia sensu stricto* from *Prevotella nigrescens*. *Anaerobe* 1997; 3: 271–278.
 19. Grimont PAD. *Taxotron package: User's manual*. Paris, Institute Pasteur. 1994.
 20. Dice LR. Measures of the amount of ecologic association between species. *Ecology* 1945; 26: 297–302.
 21. Louws FJ, Fulbright DW, Stephens CT, de Bruijn FJ. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl Environ Microbiol* 1994; 60: 2286–2295.
 22. Lupski JR, Weinstock GM. Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J Bacteriol* 1992; 174: 4525–4529.
 23. Olive DM, Bean P. Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol* 1999; 37: 1661–1669.
 24. Johnson JL. Specific strains of *Bacteroides* species in human fecal flora as measured by deoxyribonucleic acid homology. *Appl Environ Microbiol* 1980; 39: 407–413.