Identification of two genetic groups in *Bacteroides fragilis* by multilocus enzyme electrophoresis: distribution of antibiotic resistance (*cfiA*, *cepA*) and enterotoxin (*bft*) encoding genes

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Ninety-three *Bacteroides fragilis* strains of different origin were analysed by multilocus enzyme electrophoresis (MLEE). Fourteen of the 15 genetic loci analysed were polymorphic, whilst nucleoside phosphorylase was monomorphic. There was a mean of six alleles per locus and a mean genetic diversity of 0.393. Cluster analysis identified 90 electrophoretic types (ETs) separated into two major phylogenetic divisions at a genetic distance of 0.70. Division I consisted of 81 ETs carrying the endogenous class A β -lactamase gene *cepA*, whereas division II comprised 9 ETs carrying the class B β -lactamase gene *cfiA*, but not *cepA*. The presence of these two genes was assessed by PCR and the expression of the *cfiA* gene was investigated by determining the level of resistance to the antibiotic imipenem. MLEE showed a smaller genetic distance among the genotypes of the imipenem-resistant than among the imipenem-susceptible strains. No other particular cluster was observed. The enterotoxin gene (*bft*) was detected by PCR: DNA sequencing of the products obtained showed that the different *bft* alleles (*bft-1*, *bft-2* and *bft-3*) were scattered randomly throughout the phylogenetic tree. No association between distinct clones and clinical manifestations (sepsis, abscesses, diarrhoea), geographical origin or host origin (human or animal) could be found.

Keywords: *Bacteroides fragilis*, multilocus enzyme electrophoresis, *cepA*, *cfiA*, enterotoxin

INTRODUCTION

Species of the genus *Bacteroides* are found in the normal human and animal gastrointestinal flora. Occasionally, these Gram-negative, anaerobic bacilli can cause human invasive infections, predominantly bacteraemia, or intra-abdominal and wound infections after surgery of the gastrointestinal or urogenital tract. These micro-organisms carry genes encoding products involved in pathogenicity, such as enzymes synthesizing a capsular polysaccharide which inhibits phagocytosis and induces abscess formation (Tzianabos *et al.*, 1994), fimbriae and

pili promoting adherence to epithelial cells and mucus, neuraminidase, and an enterotoxin (Akimoto *et al.*, 1994; Brook & Mihal, 1991; Brook *et al.*, 1992; Duimstra *et al.*, 1991; Kleivdal & Hofstad, 1995; Moncrief *et al.*, 1995; Myers *et al.*, 1989; Russo *et al.*, 1990; Sack *et al.*, 1992; Smith & Callihan, 1992).

Previous studies have suggested a role of enterotoxin-producing *Bacteroides fragilis* (ETBF) in diarrhoeal disease of children (Sack *et al.*, 1994; San Joaquin *et al.*, 1995). However, the high proportion of healthy carriers (Pantosti *et al.*, 1994, 1997a) and the frequent association of ETBF with bacteraemia (Kato *et al.*, 1996), and thus not exclusively with diarrhoeal disease, make it difficult to define the enteric pathogenic importance of this micro-organism. Franco *et al.* (1997) and Chung *et al.* (1999) have shown that the metalloprotease enterotoxin encoded by the *bft* gene alters the morphology of intestinal epithelial cells and may thus contribute to the

Abbreviations: ET, electrophoretic type; ETBF, enterotoxin-producing *Bacteroides fragilis*; MLEE, multilocus enzyme electrophoresis (abbreviations for the enzymes studied by MLEE are defined in Methods).

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virulence of *B. fragilis* (Franco *et al.*, 1997). Recent studies have shown the existence of more than one allele of the *bft* gene [*bft*-1, *bft*-2 (Franco *et al.*, 1997), *bft*-3 (Kato *et al.*, 2000) and *bft*-Korea (Chung *et al.*, 1999)], and the localization of this gene on the fragilysin pathogenicity islet. However, although Scotto-D'Abusco *et al.* (1998) suggested a higher association of *bft*-2 with diarrhoea in children, little has been done to investigate a preferential correlation between one specific *bft* allele and a clinical manifestation.

DNA-DNA hybridization experiments have shown two DNA-homology groups of *B. fragilis* (I and II), with about 80 % of strains isolated in clinical studies assigned to homology group I (Johnson, 1978). Similarly, two genotypically distinct *B. fragilis* groups have been identified on the basis of ribotyping, restriction fragment length polymorphism (RFLP), PCR-generated fingerprinting, insertion sequence (IS) content and 16S rRNA sequence alignments (Moraes *et al.*, 1999; Kleivdal & Hofstad, 1995; Podglajen *et al.*, 1995; Ruimy *et al.*, 1996; Smith & Callihan, 1992). One group was characterized by the presence of the *cfiA* gene (encoding a metallo- β -lactamase of Ambler's class B) and the absence of the *cepA* gene (encoding a β -lactamase of Ambler's class A). Three specific insertion sequence elements, IS1186, IS942 and IS4351, providing the promoter region for the *cfiA* gene, were shown to be confined to this group (Podglajen *et al.*, 1995). The second group was characterized by the absence of the *cfiA* gene and of the associated insertion sequences, the frequent presence of the *cepA* gene, and a higher genetic heterogeneity (Podglajen *et al.*, 1995; Ruimy *et al.*, 1996). By including strains obtained from Johnson (1978) in their 16S rRNA sequence comparison, Ruimy *et al.* (1996) showed that the two groups described above could be related to the DNA homology groups II and I, respectively. Despite these findings, there is as yet no information dealing with the genetic relationship between invasive and noninvasive strains, human and animal strains and strains of different geographical origin.

Multilocus enzyme electrophoresis (MLEE) estimates the overall genetic relatedness among strains by indicating allele variation in a random sample of chromosomally encoded metabolic housekeeping enzymes (Selander *et al.*, 1986). This technique has already been extensively used in our laboratory for the study of other bacterial species (Aeschbacher & Piffaretti, 1989; Balmelli & Piffaretti, 1996; Boerlin *et al.*, 1992, 1991; Boerlin & Piffaretti, 1995; Piffaretti *et al.*, 1989).

In the present study, we used MLEE to analyse the genetic diversity and the population structure of *B. fragilis*. This may be relevant to identify genotypes, clones or cryptic genospecies nonrandomly associated with clinical manifestations, antibiotic resistance, enterotoxin production or geographical origin.

METHODS

Bacterial strains. Ninety-three *Bacteroides fragilis* strains, one *Bacteroides ovatus*, one *Bacteroides uniformis*, three

Bacteroides vulgatus and one *Porphyromonas gingivalis*, originating from different countries, were investigated (Table 1). This collection included human invasive isolates from our region (the Southern part of Switzerland) and strains isolated from patients' stools, invasive and noninvasive strains from different geographical regions (Switzerland, France, Norway, the United States and Japan), strains producing enterotoxin, invasive isolates from animals, and one reference strain of *B. fragilis*. Strains were routinely identified by the presence of catalase activity and by API 20A (BioMérieux). In isolates showing a genetic distance higher than 0.60 by MLEE, the identification was further confirmed by 16S rRNA sequencing.

Culture conditions and specimen storage. Strains were grown on Columbia blood agar plates enriched with vitamin K₁. Cultures were incubated for 36 h at 37 °C in an anaerobic chamber (Scholzen) containing an atmosphere of 5% CO₂, 10% H₂, 85% N₂. Bacteria harvested from one plate were suspended in skim milk (Difco) and stored. One loopful was suspended in 200 μ l water for DNA extraction. For MLEE, bacteria from two plates were harvested into 1.5 ml phosphate-buffered saline (NaCl 137 mM, KCl 2.6 mM, Na₂HPO₄ 4.3 mM, KH₂PO₄ 1.8 mM), pH 7.2 (PBS). All suspensions were stored at -80 °C.

Antibiotic susceptibility testing. Minimal inhibitory concentrations (MICs) of imipenem were determined on Brucella agar plates supplemented with 5% blood, using the E-test (AB Biodisk) according to the manufacturer's instructions. Strains for which the MIC was >16 μ g ml⁻¹ were considered resistant to imipenem (Nagy *et al.*, 1995).

Enzyme extraction. This was performed according to Loos *et al.* (1993) with slight modifications. Briefly, the *B. fragilis* suspensions in PBS were thawed and kept on ice during all manipulations to preserve the enzyme activity. The cells were lysed for 15 min in 50 mM n-octyl β -D-glucopyranoside (Sigma) with constant stirring. The suspensions were then sonicated twice for 15 s (small tip setting 6.5% duty cycle, sonifier model MSE; N. Ziwy & Cie) for complete cell disruption and to decrease viscosity. Finally, the lysates were centrifuged at 20000 g at 4 °C for 20 min and the supernatants were stored in 200 μ l aliquots at -80 °C.

Enzyme electrophoresis. Bacterial lysates were thawed and subjected to gel electrophoresis under nondenaturing conditions in 10% starch gels (Connaught Laboratories, Fisher Scientific) as described by Selander *et al.* (1986) and Boerlin & Piffaretti (1995). Out of 27 different enzymes tested with different electrophoretic buffers, 15 could be reliably used for MLEE of *B. fragilis*: nucleoside phosphorylase (NSP), phosphoglucose isomerase (PGI), guanosine deaminase (GDA), glutamate dehydrogenase, NAD-dependent (GDH), glucose-6-phosphate dehydrogenase (G6P), malate dehydrogenase (MDH), hexokinase (HEX), phosphoglucomutase (PGM), alanine-phenylalanine peptidase (AF), phenylalanine-proline peptidase (FP), with buffer system F (Tris/maleate, pH 8.2) described by Selander (1986); catalase (CAT), with buffer system B (Tris/citrate, pH 6.3/6.7; Selander *et al.*, 1986); indophenol oxidase (IPO), with buffer system C (borate, pH 8.2/Tris/citrate, pH 8.7; Selander *et al.*, 1986); and esterase (EST), α -galactosidase (α GAL), α -glucosidase (α GLUS), with buffer system A (Tris/citrate, pH 8; Selander *et al.*, 1986). Enzyme staining was performed according to Selander *et al.* (1986). Specific staining procedures for α GAL, α GLUS and CAT were performed using the method of Harris & Hopkinson (1976).

Another 12 enzymes were tested but not retained because of a lack of activity or uninterpretable results.

Table 1. *B. fragilis* strains

Isolate	ET	Biological origin	Geographical origin	Genotypic characterization	MIC of imipenem ($\mu\text{g ml}^{-1}$)	Source*
Division I						
3	1	Ulcer	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	0.064	IBS
V15 2735	2	Blood	Switzerland, Zürich	<i>cepA</i>	0.047	R. Zbinden
D3251	3	Blood, cat	Switzerland, Bern	<i>cepA</i>	0.094	J. Nicolet
D1851	4	Abdominal liquid, cat	Switzerland, Bern	<i>cepA</i>	0.047	J. Nicolet
GAI20273	5	Blood	Japan, Gifu	<i>cepA</i>	0.047	N. Kato
F59D	6	Stool from patient 59	Switzerland, Ticino	<i>cepA</i>	0.19	IBS
GAI97331	7	Stool	Japan, Gifu	<i>cepA</i>	0.047	N. Kato
129	8	Decubitus	Switzerland, Ticino	<i>cepA</i>	0.023	IBS
25458	9	Blood	Norway, Bergen	<i>cepA</i>	0.047	T. Hofstad
47	10	Appendicitis with abscess	Switzerland, Ticino	<i>cepA</i>	0.047	IBS
AIP638 ^b	11	Unknown	France, Paris	<i>cepA</i>	0.032	Podglajen <i>et al.</i> (1995)
F120D	12	Stool from patient 120	Switzerland, Ticino	<i>cepA</i>	0.064	IBS
GAI97330	13	Stool	Japan, Gifu	<i>cepA</i>	0.25	N. Kato
70	14	Intestinal fistula	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	0.047	IBS
GAI20239	15	Pus	Japan, Gifu	<i>cepA</i>	0.19	N. Kato
1253-92	16	Stool, from healthy volunteer	Norway, Bergen	<i>cepA</i> , <i>bft-2</i>	0.064	T. Hofstad
1332	17	Sigmoid pelvic abscess	France, Paris	<i>cepA</i>	0.094	M. Sebal
31959	18	Blood	Norway, Bergen	<i>cepA</i>	0.047	T. Hofstad
30285	19	Blood	Norway, Bergen	<i>cepA</i>	0.125	T. Hofstad
VO5 6252	20	Fistula abscess rectum	Switzerland, Zürich	<i>cepA</i>	0.047	R. Zbinden
F70B	21	Stool from patient 70	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	0.064	IBS
VO5 1019	22	Syringe abscess, pus	Switzerland, Zürich	<i>cepA</i>	0.125	R. Zbinden
BLU 1696	23	Blood	Switzerland, Zürich	<i>cepA</i>	0.19	R. Zbinden
F126A	24	Stool from patient 126	Switzerland, Ticino	<i>cepA</i>	0.75	IBS
F126C	25	Stool from patient 126	Switzerland, Ticino	<i>cepA</i>	0.75	IBS
F126E	25	Stool from patient 126	Switzerland, Ticino	<i>cepA</i>	0.75	IBS
126	26	Femur wound abscess	Switzerland, Ticino	<i>cepA</i>	0.094	IBS
GAI20246	27	Blood	Japan, Gifu	<i>cepA</i> , <i>bft-1</i>	0.125	N. Kato
118	28	Wound abscess after appendectomy	Switzerland, Ticino	<i>cepA</i>	0.064	IBS
F118A	29	Stool from patient 118	Switzerland, Ticino	<i>cepA</i>	0.094	IBS
L186	30	Peritoneal liquid	Switzerland, Lausanne	<i>cepA</i>	0.047	J. Bille
1320	31	Blood	France, Paris	<i>cepA</i>	0.064	M. Sebal
134	32	Unknown	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	0.75	IBS
1475	32	Blood	France, Paris	<i>cepA</i> , <i>bft-2</i>	0.094	M. Sebal
680	33	Digestive tract	France, Paris	<i>cepA</i> , <i>bft-2</i>	0.38	M. Sebal
GAI20261	34	Ascites	Japan, Gifu	<i>cepA</i> , <i>bft-1</i>	0.25	N. Kato
685	35	Caecum abscess	France, Paris	<i>cepA</i> , <i>bft-1</i>	0.25	M. Sebal
136	36	Coproculture, Crohn's disease	France, Paris	<i>cepA</i> , <i>bft-2</i>	0.064	M. Sebal
GAI97077	37	Stool	Japan, Gifu	<i>cepA</i> , <i>bft-1</i>	0.064	N. Kato
L185	38	Blood	Switzerland, Lausanne	<i>cepA</i>	0.25	J. Bille
GAI20241	39	Periproptic abscess	Japan, Gifu	<i>cepA</i>	0.064	N. Kato
D2383	40	Secretion from stomach puncture, cat	Switzerland, Bern	<i>cepA</i>	0.064	J. Nicolet
1286	41	Blood	France, Paris	<i>cepA</i>	0.094	M. Sebal
GAI 20240	42	Pus	Japan, Gifu	<i>cepA</i> , <i>bft-3†</i>	0.064	N. Kato
656	43	Coproculture, digestive lymphoma	France, Paris	<i>cepA</i> , <i>bft-1</i>	0.064	M. Sebal
646	44	Digestive tract	France, Paris	<i>cepA</i> , <i>bft-1</i>	0.25	M. Sebal
L362	45	Blood	Switzerland, Lausanne	<i>cepA</i>	0.125	J. Bille

Table 1 (cont.)

Isolate	ET	Biological origin	Geographical origin	Genotypic characterization	MIC of imipenem ($\mu\text{g ml}^{-1}$)	Source*
925	46	Mesenteric ganglion	France, Paris	<i>cepA</i> , <i>bft-1</i>	0.094	M. Sebald
5.28	47	Unknown	Switzerland, Ticino	<i>cepA</i>	0.064	IBS
V15 2715	48	Wound after appendectomy	Switzerland, Zürich	<i>cepA</i>	0.064	R. Zbinden
F95A	49	Stool	Switzerland, Ticino	<i>cepA</i>	0.064	IBS
VO8 12993	50	Blood	Switzerland, Zürich	<i>cepA</i>	0.047	R. Zbinden
80	51	Swab of infected haematoma with cutaneous fistula (deep wound, pus)	Switzerland, Ticino	<i>cepA</i>	0.064	IBS
1402	52	Blood	France, Paris	<i>cepA</i>	0.094	M. Sebald
D2187	53	Anal abscess, cat	Switzerland, Bern	<i>cepA</i>	0.064	J. Nicolet
V12 6453	54	Abdomen	Switzerland, Zürich	<i>cepA</i>	0.047	R. Zbinden
93	55	Blood, patient 80	Switzerland, Ticino	<i>cepA</i>	0.047	IBS
1301	56	Bone infection	France, Paris	<i>cepA</i>	0.38	M. Sebald
1511	57	Vagina	France, Paris	<i>cepA</i> , <i>bft-1</i>	0.094	M. Sebald
GAI96448	58	Stool	Japan, Gifu	<i>cepA</i> , <i>bft-1</i>	0.19	N. Kato
20	59	Gynaecological pus	Switzerland, Ticino	<i>cepA</i>	0.064	IBS
L754	60	Blood	Switzerland, Lausanne	<i>cepA</i>	0.064	J. Bille
69	61	Bartholinitis	Switzerland, Ticino	<i>cepA</i> , <i>bft-2</i>	0.032	IBS
123	62	Unknown	Switzerland, Ticino	<i>cepA</i> , <i>bft-2</i>	0.064	IBS
1208	63	LCR-meningitis	France, Paris	<i>cepA</i> , <i>bft-1</i>	0.064	M. Sebald
1237	64	Blood	France, Paris	<i>cepA</i>	0.033	M. Sebald
59	65	Intestinal tumour	Switzerland, Ticino	<i>cepA</i>	0.19	IBS
F59G	66	Stool from patient 59	Switzerland, Ticino	<i>cepA</i>	0.50	IBS
1077-92	67	Stool from healthy volunteer	Norway, Bergen	<i>cepA</i> , <i>bft-2</i>	0.25	T. Hofstad
1269	68	Blood	France, Paris	<i>cepA</i>	0.094	M. Sebald
DSM 1396 (= ATCC 23745)	69	Reference strain; clinical origin, pleural fluid	Unknown	<i>cepA</i>	0.064	DSM
64	70	Blood (septicaemia after sacral decubitus)	Switzerland, Ticino	<i>cepA</i> , <i>bft-2</i>	0.38	IBS
F64D	71	Stool from patient 64	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	0.25	IBS
120	72	Inguinal abscess	Switzerland, Ticino	<i>cepA</i>	0.032	IBS
F64B	73	Stool from patient 64	Switzerland, Ticino	<i>cepA</i> , <i>bft-2</i>	0.125	IBS
F120B	74	Stool from patient 120	Switzerland, Ticino	<i>cepA</i>	0.047	IBS
96	75	Blood	Switzerland, Ticino	<i>cepA</i>	0.047	IBS
F64E	76	Stool from patient 64	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	0.25	IBS
135	77	Unknown	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	0.19	IBS
1343	78	Blood	France, Paris	<i>cepA</i> , <i>bft-1</i>	0.064	M. Sebald
L93	79	Blood	Switzerland, Lausanne	<i>cepA</i>	0.5	J. Bille
F70A	80	Stool from patient 70	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	0.047	IBS
25	81	Blood	Switzerland, Ticino	<i>cepA</i>	0.047	IBS
Division II						
1429	82	Eschar	France, Paris	<i>cfiA</i> , IS1186	> 32	M. Sebald
BFR271R	83	Unknown	France, Paris	<i>cfiA</i> , IS1186	> 32	E. Collatz
BFR81R	84	Unknown	France, Paris	<i>cfiA</i> , IS1186	> 32	E. Collatz
TAL2480	85	Unknown	United States	<i>cfiA</i> , IS942	> 32	Thompson & Malamy (1990)
TAL3636	85	Unknown	United States	<i>cfiA</i> , IS942	> 32	Thompson & Malamy (1990)

Table 1 (cont.)

Isolate	ET	Biological origin	Geographical origin	Genotypic characterization	MIC of imipenem ($\mu\text{g ml}^{-1}$)	Source*
VPI2393	86	Unknown	France, Paris	<i>cfiA</i>	3.0	Johnson (1978)
VPI3392	87	Unknown	France, Paris	<i>cfiA</i>	1.5	Johnson (1978)
1297	88	Appendicitis pus	France, Paris	<i>cfiA</i>	1.5	M. Sebal
28794	89	Blood	Norway, Bergen	<i>cfiA</i>	4.0	T. Hofstad
127	90	Unknown	Switzerland, Ticino	<i>cfiA</i>	1.5	IBS
Outgroup†						
D3433	91	Lung abscess, hamster	Switzerland, Bern		0.064	J. Nicolet
ZIB3071	92	Periodontal pocket	Switzerland, Basel		0.023	J. Meyer
F120C	93	Stool from patient 120	Switzerland, Ticino		0.25	IBS
L138	94	Peritoneal liquid	Switzerland, Lausanne		0.50	J. Bille
F120A	95	Stool from patient 120	Switzerland, Ticino		0.75	IBS
23	96	Blood	Switzerland, Ticino		0.094	IBS

* IBS, Istituto Cantonale Batteriosierologico, Lugano; R. Zbinden, Institut für medizinische Mikrobiologie Universität Zürich, Zürich, Switzerland; J. Nicolet, Institute for Veterinary Bacteriology, University of Bern, Bern, Switzerland; N. Kato, Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Gifu, Japan; T. Hofstad, Department of Microbiology and Immunology, The Gade Institute, University of Bergen, Bergen, Norway; M. Sebal, Unité des Anaérobies, Institut Pasteur, Paris, France; J. Bille, Laboratoire de bactériologie médicale, CHUV, Lausanne, Switzerland; E. Collatz, LRMA, Université Paris VI, Paris France; J. Meyer, Zahnärztliches Institut der Universität Basel, Switzerland.

† *bft-3* in the table means 'potential *bft-3*', since it probably corresponds to *bft-3* described by N. Kato at the 2nd World Congress of Anaerobic Bacteria and Infections, 1998 (N. Kato, personal communication; Kato *et al.*, 2000).

‡ Outgroup strains were: D3433, *B. uniformis*; ZIB3071, *P. gingivalis*; F120C, *B. ovatus*; L138, F120A and 23, *B. vulgatus*.

DNA extraction and PCR. Samples (50 μl) of the bacterial suspensions in water were thawed and DNA was extracted with a commercial ion-exchange resin (InstaGene matrix; Bio-Rad) according to the manufacturer's instructions. PCR was performed in a total volume of 50 μl containing: 10 μl of the DNA extract, 10 mM Tris/HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl_2 , 200 μM of each dNTP, 0.01% gelatin type A (Sigma), 0.5 μM of each primer, and 1 unit *Taq* polymerase (Boehringer Mannheim); the reactions were overlaid with paraffin oil (Merck) to prevent evaporation.

All 93 *B. fragilis* strains and also the outgroup strains were subjected to the PCR experiments described hereafter to detect enterotoxin-encoding genes and genes encoding antibiotic resistance. A 294 bp fragment of the *bft* gene was amplified by PCR according to Pantosti *et al.* (1997a) with the primers *BF-1* (5'-GACGGTGTATGTGATTGTCTGAGAGA-3') and *BF-2* (5'-ATCCCTAAGATTTTATTATCCCAAGTA-3'). A thermal profile of 94 °C for 60 s, 52 °C for 60 s and 72 °C for 60 s was repeated for 35 cycles. These primers allowed the amplification of the *bft-1* and *bft-2* alleles of the enterotoxin-encoding gene. To avoid false negative results due to mutations in the primer regions, a second primer pair was developed in our laboratory: *BF-3* (5'-GTTAGTGGCCAG-ATGCAGG-3') and *BF-4* (5'-TAGTTCGTGTGCCATCA-CCC-3'). To amplify the corresponding 341 bp fragment of *bft*, a 35-cycle PCR with the following thermal profile was performed: 94 °C for 60 s, 50 °C for 60 s and 72 °C for 90 s. A third PCR, detecting *bft-1*, *bft-2* and also the third *bft* allele (*bft-3*), was performed with primers provided by N. Kato (N. Kato, personal communication; Kato *et al.*, 2000).

Based on DNA sequence alignment of the published *cfiA* (Khushi *et al.*, 1996; Tally & Jacobus, 1983; Thompson &

Malamy, 1990) and *cepA* (Rasmussen *et al.*, 1990) genes, four primers corresponding to relatively conserved regions of the coding sequences were designed: *cfiA-1* (5'-ATGGTACCT-TCCAACGGG-3') and *cfiA-2* (5'-CACGATATTGTCTGGT-CGC-3'), and *cepA-1* (5'-TTTCTGCTATGTCCTGCC-3') and *cepA-2* (5'-ATCTTTCACGAAGACGGC-3'). The fragment amplified by *cfiA-1* and *cfiA-2* was 353 bp long. The *cfiA* PCR consisted of 35 cycles with a thermal profile of 94 °C for 60 s, 56 °C for 60 s and 72 °C for 60 s. Primers *cepA-1* and *cepA-2* amplified a 780 bp fragment in a PCR of 35 cycles, with the following thermal profile: 94 °C for 60 s, 52 °C for 60 s and 72 °C for 60 s. Furthermore, based on the published sequences (Podglajen *et al.*, 1994; Rasmussen *et al.*, 1990; Thompson & Malamy, 1990), primers were designed for the insertion sequences IS1186 and IS942. The forward primers were 5'-TCCTCAATACATGAGCCGC-3' for IS942, and 5'-TGACCTACAACATCTTCCG-3' for IS1186. The reverse primer was the same for the detection of both insertion sequences. This primer was designed on position 165–186 of the *cfiA* gene: *cfiA-3* (5'-GGTTGTTGATAACAATCATC-CC-3'). PCR of 35 cycles was performed using the following conditions: 94 °C for 60 s, 50 °C for 60 s and 72 °C for 90 s.

Isolates showing a genetic distance from other strains higher than 0.60 by MLEE were confirmed to be *B. fragilis* using 16S rRNA amplification and sequencing. 16S rRNA PCR was performed with universal primers UNI16RNA-L (5'-ATTC-TAGAGTTTGATCATGGCTCA-3'; *E. coli* coordinates 3–26) and UNI16RNA-R (5'-ATGGTACCGTGTGACGGC-GGTGTGTA-3'; *E. coli* coordinates 1419–1393), amplifying a product of roughly 1400 bp (a kind gift from J. Frey, University of Bern). The thermal profile consisted of 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 60 s. The PCR

Table 2. Allelic profile at 15 enzyme loci for 93 *B. fragilis* isolates

ET	No. of alleles at the following enzyme loci:														
	NSP	PGI	GDA	GD1	HEX	G6P	MDH	PGM	α GAL	α GLUS	EST	IPO	AF	FP	CAT
1	2	4	5	2	5	5	4	6	3	5	4	3	7	1	5
2	2	4	5	2	5	5	4	6	3	4	4	3	7	1	5
3	2	4	5	2	5	5	4	6	3	3	4	3	7	2	5
4	2	4	5	2	5	5	4	6	0	5	4	3	7	2	5
5	2	4	5	2	5	5	4	6	5	3	4	3	7	5	5
6	2	4	6	2	5	5	4	6	3	5	4	3	7	2	5
7	2	4	6	2	5	5	4	6	3	4	4	3	7	2	5
8	2	4	6	2	5	5	4	6	3	8	4	3	7	17	5
9	2	4	6	2	5	4	4	6	3	5	4	3	7	2	5
10	2	4	6	2	5	7	4	6	3	3	4	3	7	1	5
11	2	4	6	2	5	5	4	6	2	3	4	3	7	1	5
12	2	4	6	2	5	5	4	6	3	6	4	3	6	1	5
13	2	4	6	2	5	5	4	6	3	4	4	3	6	1	5
14	2	4	6	2	5	5	4	6	3	6	4	3	6	3	5
15	2	4	6	2	5	5	4	6	3	4	4	3	6	18	5
16	2	4	6	2	5	5	4	6	3	5	4	3	6	2	5
17	2	4	6	2	5	5	4	6	3	9	4	3	6	2	5
18	2	4	6	2	5	5	4	6	3	8	4	3	6	5	5
19	2	4	6	2	5	5	4	6	7	6	4	3	6	8	5
20	2	4	5	2	5	5	4	6	10	1	4	3	6	11	5
21	2	4	6	2	5	5	4	6	5	6	4	4	6	1	5
22	2	4	6	2	5	5	4	6	5	6	4	3	7	3	5
23	2	4	6	2	5	5	4	6	8	2	4	3	0	11	5
24	2	4	6	5	5	5	4	6	3	7	4	3	0	2	5
25	2	4	6	5	5	5	4	6	3	7	4	3	6	2	5
26	2	4	6	5	5	5	4	6	3	5	4	3	7	1	5
27	2	4	6	6	5	5	4	6	10	2	4	3	7	11	5
28	2	4	6	2	5	6	4	7	3	5	4	3	7	1	5
29	2	4	6	2	5	5	4	7	3	5	4	3	7	1	5
30	2	4	6	2	5	6	4	6	10	4	4	3	1	6	5
31	2	4	6	2	5	6	4	6	3	3	4	3	1	2	5
32	2	4	6	2	5	5	2	6	5	3	4	3	6	1	5
33	2	4	6	2	5	5	2	6	5	3	4	3	6	2	5
34	2	4	6	2	5	5	2	6	5	3	4	3	6	5	5
35	2	4	6	2	5	5	2	6	5	4	4	3	6	9	5
36	2	4	6	2	5	5	2	6	3	7	4	3	6	5	5
37	2	4	6	2	5	5	2	6	3	3	4	3	6	5	5
38	2	4	6	2	5	5	3	6	3	1	4	3	6	5	5
39	2	4	6	2	5	5	3	6	3	3	4	3	6	2	5
40	2	4	6	2	5	6	4	6	3	4	4	3	6	1	5
41	2	4	6	2	5	3	4	6	3	7	4	3	6	1	5
42	2	4	6	2	5	9	4	6	3	4	4	3	6	2	5
43	2	4	6	2	5	7	2	6	3	9	4	3	6	1	5
44	2	4	6	2	5	6	2	6	3	4	4	3	6	14	5
45	2	4	6	2	4	5	4	6	3	6	4	3	6	2	5
46	2	4	6	2	0	5	4	6	3	4	4	3	6	1	5
47	2	4	6	2	5	5	4	6	3	4	4	3	7	2	4
48	2	4	6	2	5	5	4	6	3	3	4	3	7	2	4
49	2	4	6	2	5	5	4	6	4	3	4	3	6	14	4
50	2	4	6	2	5	5	4	6	3	3	4	3	0	2	6

Table 2 (cont.)

ET	No. of alleles at the following enzyme loci:														
	NSP	PGI	GDA	GD1	HEX	G6P	MDH	PGM	α GAL	α GLUS	EST	IPO	AF	FP	CAT
51	2	4	5	2	5	8	4	6	3	6	4	3	6	2	5
52	2	4	5	2	5	3	4	6	3	7	4	3	6	2	5
53	2	4	5	2	5	5	4	6	3	2	4	3	6	2	5
54	2	4	5	2	5	5	4	6	3	7	4	3	6	2	5
55	2	4	5	2	5	5	4	6	3	11	4	3	6	2	5
56	2	4	5	2	5	5	4	6	3	4	4	3	6	1	5
57	2	4	5	2	5	5	4	6	3	4	4	3	6	12	5
58	2	4	5	2	5	5	4	6	3	4	4	3	9	1	5
59	2	4	5	2	5	6	4	6	6	6	4	3	6	1	5
60	2	4	5	2	5	5	4	2	4	6	4	3	6	2	5
61	2	4	5	2	5	6	3	2	3	5	4	3	1	2	5
62	2	4	5	2	5	5	3	2	5	4	4	3	1	2	5
63	2	4	5	2	5	5	3	6	3	4	4	3	1	1	5
64	2	4	5	2	5	2	3	6	5	7	4	3	6	2	5
65	2	4	6	2	5	5	4	2	3	5	2	3	6	12	5
66	2	4	6	2	5	5	4	2	3	5	2	3	6	2	5
67	2	4	6	2	5	5	4	6	5	6	2	3	6	7	5
68	2	4	6	2	5	5	4	6	3	6	2	3	6	2	5
69	2	4	5	2	5	5	4	6	4	6	2	3	6	1	5
70	2	4	6	2	5	7	4	6	3	5	4	4	6	1	5
71	2	4	6	2	5	6	4	6	3	5	4	4	6	1	5
72	2	4	6	2	5	5	4	6	3	5	4	4	6	1	5
73	2	4	6	2	5	5	4	6	3	1	4	4	6	1	5
74	2	4	6	8	5	5	4	6	5	6	3	3	7	3	5
75	2	4	6	2	5	5	4	6	6	6	4	4	6	2	5
76	2	4	6	2	5	6	4	6	3	1	3	4	6	1	5
77	2	4	6	7	5	5	2	6	5	3	4	3	6	1	5
78	2	0	6	2	5	5	2	6	5	3	4	3	6	0	5
79	2	4	6	7	5	5	5	6	10	1	4	3	8	11	5
80	2	4	6	8	5	5	4	6	5	6	3	3	7	3	5
81	2	4	5	2	5	7	3	6	5	4	2	3	7	5	5
82	2	2	6	6	2	9	10	2	10	4	6	3	6	9	1
83	2	2	6	6	2	9	10	5	10	4	6	3	6	9	1
84	2	2	6	6	2	9	10	1	10	4	6	3	6	9	1
85	2	2	4	6	2	9	10	5	10	4	6	3	6	4	1
86	2	2	6	6	2	5	10	4	10	3	6	3	6	4	1
87	2	4	5	4	2	5	10	5	10	3	6	3	7	4	1
88	2	2	4	6	5	9	10	2	10	4	6	3	6	9	1
89	2	2	4	3	2	9	10	4	2	4	4	3	7	4	1
90	2	4	6	2	1	9	10	5	10	0	6	3	9	13	3
91	3	1	0	3	0	11	4	1	10	4	9	1	3	1	5
92	5	7	0	1	0	0	1	0	0	0	9	1	4	16	5
93	1	2	2	10	1	12	9	4	0	0	8	7	5	10	5
94	4	5	1	4	0	1	7	9	9	10	7	6	2	6	0
95	4	6	1	4	0	1	7	9	11	10	7	6	2	6	0
96	4	5	1	0	0	1	7	9	11	10	7	5	2	6	0

product was identified as *B. fragilis* 16S rRNA by a BLAST sequence similarity search in GenBank.

DNA sequencing and alignment. PCR products for direct sequencing were prepared with the QIAquick PCR Purification Kit (Qiagen) and sequenced with the ABI Prism dRhodamine dye terminator Cycle Sequencing Ready Re-

action Kit (dRhodamine terminator; Perkin-Elmer Applied Biosystems).

Sequence data were analysed by pairwise sequence alignment and by multi-alignment with the Lasergene program Megalign (DNASTAR). Phylogenetic analysis was performed using MEGA (Molecular Evolutionary Genetics Analysis 1.01; Kumar *et al.*, 1993).

The DNA sequences determined in this work are in the GenBank/EMBL/DDBJ databases under accession numbers AF197508–AF197534.

Statistical analysis. Statistical analysis of the MLEE data was performed using a computer program designed by Whittam *et al.* (1983). Briefly, the genetic diversity (h) for each enzyme locus among ETs was calculated as $h = (1 - \sum x_i^2) / [n / (n - 1)]$, where x_i is the frequency of the i th allele and n is the number of ETs. The genetic distance between ETs was expressed as the proportion of loci at which dissimilar alleles occurred, with the contribution of each locus inversely weighted by the genetic diversity (h) at the locus. The ET clustering and the generation of a dendrogram according to genetic distances were determined with the average-linkage method from a matrix of coefficients of pairwise genetic distances (Selander *et al.*, 1986). Variance comparison of the distribution of allele mismatches was done by method B of Brown *et al.* (1980).

RESULTS

Genetic and genotypic diversity

Fourteen of the 15 enzymes analysed in the population of 93 *B. fragilis* isolates were polymorphic. The exception was NSP, which was monomorphic. The mean number of alleles per locus varied from 1 (NSP) to 16 (FP), with an overall mean of 6 (see Table 3). Genetic diversities among ETs at each locus ranged from 0 (NSP) to 0.805 (FP), with a mean genetic diversity per locus of 0.393 (Table 3). The 93 *B. fragilis* strains presented 90 distinct ETs (Tables 1 and 2). The *Bacteroides* strains that were not *B. fragilis* (3 *B. vulgatus*, 1 *B. ovatus*, 1 *B. uniformis*), and the *P. gingivalis* strain, had very distinct allele profiles (Table 2). The five bacterial species studied presented clearly distinct NSP electromorphs.

Genetic relationships among ETs

Cluster analysis of the 90 ETs of *B. fragilis* by the average-linkage method identified two major divisions separated at a genetic distance of 0.70 (Fig. 1). Division I consisted of 81 ETs (83 strains) and division II consisted of 9 ETs (10 strains). The mean genetic diversity per locus of ETs in divisions I and II was 0.314 and 0.356, respectively (Table 3). Division-specific alleles were found for ten enzyme loci (CAT, α GLUS, EST, HEX, IPO, GDA, FP, PGM, MDH, PGI; Table 2).

16S rRNA sequencing combined with a BLAST sequence similarity search in GenBank confirmed that isolates corresponding to ETs 89 and 90 really were *B. fragilis*: 97–99% similarities with other *B. fragilis* 16S rRNA present in the database were observed for both isolates. These isolates were analysed because of their high genetic distances (higher than 0.60) from ETs of other *B. fragilis* isolates of division II.

Table 3. Number of alleles and genetic diversity per enzyme locus for 93 *B. fragilis* isolates

Enzyme locus	No. of alleles	h (genetic diversity per locus)		
		Div. I	Div. II	Total
NSP	1	0.000	0.000	0.000
PGI	3	0.025	0.389	0.166
GDA	3	0.389	0.639	0.427
GD1	6	0.165	0.417	0.299
HEX	5	0.049	0.417	0.206
G6P	8	0.403	0.389	0.479
MDH	5	0.377	0.000	0.487
PGM	6	0.163	0.778	0.318
α GAL	9	0.539	0.222	0.612
α GLUS	11	0.854	0.556	0.845
EST	4	0.184	0.222	0.315
IPO	2	0.160	0.000	0.145
AF	6	0.544	0.417	0.531
FP	16	0.764	0.667	0.805
CAT	5	0.096	0.222	0.262
Mean	6	0.314	0.356	0.393

Population structure and β -lactam resistance

All *B. fragilis* strains of division I had the gene *cepA* but not *cfiA*, whereas the division II organisms had only the *cfiA* gene. Division I included a strain (AIP638) previously described as belonging to the *cepA*-positive/*cfiA*-negative group (Ruimy *et al.*, 1996), whereas division II comprised different strains from the DNA homology group II and the *cfiA*-positive/*cepA*-negative group (VPI2393, VPI3392, TAL3636, Bfr81R, Bfr 271, TAL2480; Johnson, 1978; Podglajen *et al.*, 1995; Ruimy *et al.*, 1996).

Five of the ten isolates of division II expressed imipenem resistance (MICs $\geq 32 \mu\text{g ml}^{-1}$), the other five isolates of division II, and all the isolates of division I, were susceptible to this antibiotic (Table 1). In division II, the five isolates expressing the resistance contained either IS1186 or IS942, whereas the other five susceptible isolates did not contain either insertion sequence (Table 1).

Source of the isolates

The human strains analysed were isolated from patients with different clinical manifestations (Table 1). No

Fig. 1. Genetic relationships among 93 *B. fragilis* strains, five strains of other *Bacteroides* species (*B. ovatus*, *B. uniformis* and *B. vulgatus*) and one *P. gingivalis* strain, based on electrophoretically demonstrable allelic variations at 15 enzyme loci. The dendrogram was generated by using the average-linkage method of clustering and a matrix of pairwise coefficients of genetic distance. Presence of the *cepA* and the *cfiA* gene was assessed by PCR (indicated by $\times$ in the figure). The presence of three alleles of *bft* (indicated by *bft-1*, *bft-2* and *bft-3* in the figure) was assessed by PCR. Lack of a category for a given marker (*bft*, *cepA* or *cfiA*) indicates that it was not present. Capital letters near the clinical origin of the isolates indicate their geographical origin (CH, Switzerland; F, France; J, Japan; N, Norway). Isolates coming from the same patient (pat.) are indicated by the patient's number beside their origin.

Fig. 2. Distribution of ETBF. (a) Dendrogram generated by the CLUSTAL method, based on the alignment of 189 bp of the *B. fragilis* enterotoxin-encoding gene. Three clusters corresponding to the alleles *bft-1*, *bft-2* and *bft-3* were observed. Sequence 086-5443-2-2 (GenBank accession number U90931; Franco *et al.*, 1997) was included in the analysis to show which group was *bft-2*. (b) Dendrogram including only ETBF, generated by MLEE.

particular cluster was uniquely associated with specific presentations (Fig. 1). For example, the 23 *B. fragilis* cultured from blood were assigned to ETs in both divisions I and II. A separate analysis of these strains showed a genetic diversity of 0.371, comparable to that of the whole *B. fragilis* population studied (data not shown).

Faecal isolates were examined from six patients from whom clinical isolates were obtained (Table 1): these faecal isolates all clustered in division I but they did not always belong to the same ET as the invasive strains isolated from the same patient.

Isolates obtained from Norway, Japan, Europe and the United States were not represented by distinct ETs or clusters of ETs, and were distributed throughout the population (Fig. 1).

ETs representing the four animal strains included in this study did not form a separate cluster and were randomly distributed among the ETs representing the human strains.

Distribution of enterotoxigenic *B. fragilis*

Of the 93 *B. fragilis* strains investigated, 29 were identified as ETBF by PCR assays targeting the *bft* gene. The first PCR used for this gene (primers BF-1/BF-2;

Pantosti *et al.*, 1997a) detected *bft-1* and *bft-2*. The second PCR used here (primers BF-3/BF-4) detected one additional *bft* allele (*bft-3*). This observation was supported by a third PCR with primers obtained from N. Kato, which detect *bft-1*, *bft-2* and *bft-3*. Strains of all the ETs in division II lacked the *bft* gene, whereas genotypes carrying the enterotoxin gene were widely distributed in division I (Fig. 1). A common sequence of 189 bp present in all the amplicons generated by the three pairs of primers used was aligned and compared. Sequence alignment showed two ETBF subgroups characterized by either the *bft-1* or the *bft-2* gene described by Franco *et al.* (1997; Fig. 2a), and a single isolate (GAI 20240, originating from Japan) with a different *bft* sequence. We aligned this *bft* sequence with *bft-3* from N. Kato (GenBank accession no. AB026624) and with *bft-Korea* (Chung *et al.*, 1999) and discovered that these three sequences were identical. No apparent association between the presence of one of the *bft* alleles and specific clinical manifestation was observed. Furthermore, from the patient presenting the invasive isolate 64, three faecal isolates were obtained: isolates F64D and F64E carried *bft-1*, and isolates 64 and F64B carried *bft-2*.

All the sequences of *bft-1* were identical (Fig. 2a). In the *bft-2* group all the sequences except one were also

identical with one another but different from *bft-1*. The exception was the sequence of isolate 1475 (ET 32), which had a difference at two bases compared to the other 294 bp *bft-2* amplicons: this sequence was however identical to that published by Franco *et al.* (1997). The 294 bp amplicons of the *bft-1* and *bft-2* alleles obtained with primers *BF-1*/*BF-2* differed at 14 nucleotides, 7 of which resulted in amino acid replacements (data not shown). Based on the 189 bp sequenced from the 341 bp amplicon obtained with primers *BF-3* and *BF-4*, 20 point mutations were observed between *bft-1* and the potential *bft-3*, and 8 point mutations between *bft-2* and the potential *bft-3*. The presence of the three *bft* alleles could not be associated with any specific genomic cluster evidenced by MLEE (Figs 1 and 2b).

Gene linkage disequilibrium

Linkage disequilibrium (nonrandom association of alleles over chromosomal loci) was tested by the method of Whittam *et al.* (1983). The ratio of the observed variance of allele mismatches over the 15 loci to the variance expected in the case of random distribution of alleles was 3.050 for the whole *B. fragilis* population, and 1.079 and 3.384 for divisions I and II, respectively.

DISCUSSION

Genetic structure of the *B. fragilis* population: two main divisions

Using MLEE, 93 isolates of *B. fragilis* analysed at 15 enzyme loci were assigned to 90 ETs distributed into two primary genomic divisions (divisions I and II) separated at a genetic distance of 0.70 (Fig. 1). The ETs of the *B. vulgatus*, *B. ovatus*, *B. uniformis* and *P. gingivalis* isolates included in this study were clearly separated at genetic distances greater than 0.9 from the *B. fragilis* genotypes, thus confirming their identity as distinct species and the validity of the MLEE method in delineating distant genomic groups. ETs 89 and 90 were separated at a higher genetic distance (higher than 0.62) from the other ETs of division II. Since a genetic distance of 0.7 is suggestive of a new genospecies, 16S rRNA sequencing was performed on these isolates, confirming that they were indeed *B. fragilis*.

Using a PCR assay developed in our laboratory, the 83 strains of division I were shown to contain the *cepA* gene (encoding an Ambler's class A β -lactamase) but not *cfiA* (encoding an Ambler's class B β -lactamase), whereas the 10 strains of division II contained the *cfiA* gene but not *cepA*. After exclusion of the strains obtained from laboratories specifically studying these genes, the proportion of isolates encoding *cfiA* was similar to the proportion of DNA homology group II strains obtained in previous studies (Johnson, 1978). *cepA* and *cfiA* have been shown to be related to DNA homology groups I and II, respectively (Ruimy *et al.*, 1996). The finding in our division II of two strains of the DNA homology group II (Table 1), including the type strain (VPI2393; Johnson, 1978), and of isolate 28794, a strain

with a ribotype profile similar to that of VPI2393 (Kleivdal & Hofstad, 1995), strongly suggests that this division corresponds to the DNA homology group II described by Johnson (1978).

Thus, our results delineate two major subdivisions within the species *B. fragilis* – one associated with the *cfiA* gene and the other, more heterogeneous, with the *cepA* gene – and are in agreement with those obtained by DNA–DNA hybridization (Johnson, 1978), ribotyping (Kleivdal & Hofstad, 1995; Smith & Callihan, 1992; Leszczynski *et al.*, 1997), AP-PCR (Moraes *et al.*, 1999), insertion sequence content (Podglajen *et al.*, 1995) as well as 16S rRNA sequence alignment (Ruimy *et al.*, 1996).

Genetic structure of divisions I and II

The genetic diversity observed in the *B. fragilis* sample (0.393) was comparable to the genetic diversity observed in many other bacterial populations (Smith & Callihan, 1992; Go *et al.*, 1996; Selander *et al.*, 1986; Boerlin *et al.*, 1991, 1992; Boerlin & Piffaretti, 1995). This value and the ratio of the observed to the expected variance (3.050) suggest a nonrandom association of the alleles over the chromosome and thus a clonal structure over the whole population. However, the two divisions considered separately show important differences.

Division I presented a large number of ETs separated by low genetic distances (0.018–0.401) and a genetic diversity of 0.314. The ratio of the observed to the expected variances of division I was close to one (1.079), suggesting a random association of the genes over the chromosome. The lack of linkage disequilibrium (non-random association of the alleles over the chromosome) suggests a higher recombination rate, and thus a nonclonal structure of the *B. fragilis* population in division I. This hypothesis is supported by the observation that isolates from different geographical regions did not share the same genotype (with only one exception, ET 32 representing isolate 1475, from Paris, and isolate 134, from Switzerland). Furthermore, in the same geographical region, all isolates belonged to different ETs, supporting the hypothesis of a high recombination rate. Such a high recombination rate is in agreement with the observed heterogeneity of capsular polysaccharides in *B. fragilis* (Pantosti *et al.*, 1995).

In contrast, division II presented a different population structure, with a high value of the ratio of the variances (3.384), suggesting a lower recombination rate.

Podglajen *et al.* (1995) proposed that *B. fragilis* strains carrying *cfiA* form a novel species. These authors observed heterogeneous ribotype profiles in the *cepA*-positive group and highly homogeneous profiles in the *cfiA*-positive group. The two divisions observed here also showed a different population structure, with a nonclonal evolution in division I and a rather clonal evolution in division II. The genetic distance of 0.6–0.7, which is empirically considered to be the limit differentiating two species (Boerlin *et al.*, 1991), further

supports the hypothesis of the presence of two genospecies. Moreover, the MLEE data are consistent with previous DNA–DNA hybridization data, suggesting the existence of two genospecies: Johnson (1978) indeed observed a DNA relatedness of 65–70% between the two DNA homology groups and a ΔT_m ranging from 7.2 to 9.5 °C (Johnson, 1978). A DNA relatedness of less than 70% and a ΔT_m of more than 5 °C is normally considered as the threshold to distinguish bacterial species. Since DNA–DNA hybridization is the reference technique used for species delineation (Wayne *et al.*, 1987, 1996), this method should be performed on our populations to confirm that the *cfiA* subgroup corresponds to a different genospecies.

Degree of expression of the *cfiA* gene

The susceptibility assays of the strains of division II to the antibiotic imipenem showed that the gene was expressed and conferred resistance (MIC > 32 µg ml⁻¹; Table 1) in five isolates carrying *cfiA*. These five resistant isolates formed a closely related cluster of strains (ETs 82–85) within division II. Furthermore, all isolates expressing the imipenem resistance contained at least one insertion sequence (IS1186 or IS942; Table 1), whereas the isolates not expressing the resistance did not contain such insertion sequences. These results are in agreement with those of Podglajen *et al.* (1995), showing that imipenem susceptibility in division II is not due to the lack of the *cfiA* gene but to the absence of a precise insertion sequence element providing the promoter region for the expression of this gene immediately upstream of the gene.

Genetic structure in relation to enterotoxin production

Twenty-nine of the 93 *B. fragilis* isolates of the collection were identified as ETBF by PCR experiments designed to detect the metalloprotease-enterotoxin-encoding gene. Excluding those isolates obtained from laboratories specifically working on ETBF (Sears *et al.*, 1995; Pantosti *et al.*, 1997b; Kato *et al.*, 2000), this represents 18% of the population which was analysed. A similar proportion was found by Pantosti *et al.* (1994, 1997a) in Italy. Previous experiments showed complete agreement between *in vitro* cytotoxicity assays and the results of the PCR experiments amplifying *bft-1*, *bft-2* and *bft-3* (the three alleles of the *bft* gene), suggesting that if the gene is present it is also expressed (Pantosti *et al.*, 1997a; Chung *et al.*, 1999). False negative results due to possible primer mutations or to the presence of a further *bft* allele are not likely since three different PCRs with different primers were performed.

The ETs marking the strains carrying *bft* were distributed in division I, but were not present in division II. Furthermore, they were not associated with particular clinical symptoms, nor clustered in a specific genetic group, which is not surprising in a nonclonal population like that of division I. Recent studies suggested a role for

ETBF in bacteraemia (Kato *et al.*, 1996). However, out of 23 strains isolated from blood cultures only 4 carried the *bft* gene. This finding, together with the existence of a high number of healthy carriers (Pantosti *et al.*, 1994, 1997b), does not support an association between ETBF and bacteraemia.

The data obtained using MLEE are in agreement with previous results based on antigen analysis (Myers & Shoop, 1987), RFLP (Smith & Callihan, 1992) and ribotyping (Leszkczynski *et al.*, 1997), showing no particular feature that would place the ETBF strains within a distinct subgroup of *B. fragilis*. Recently, the *bft* gene has been localized on a pathogenicity islet (Moncrief *et al.*, 1998; Franco *et al.*, 1999). Pathogenicity islets, like the pathogenicity islands, are transmitted by horizontal gene transfer between bacterial populations. The mobility of these unstable DNA elements and the observation that the *bft* gene is found only in division I, which presents an apparently higher recombination rate, could explain the observed distribution of the enterotoxin gene in the *B. fragilis* population, independently from the presence of a particular cluster of genotypes.

The alignment of the *bft* gene DNA sequences of the 29 ETBF strains showed three distinct alleles in the population studied. By sequence alignment and PCR performed with primers obtained from N. Kato, the *B. fragilis* strains were shown to harbour only one of these three alleles, confirming that the different *bft* alleles do not coexist (Franco *et al.*, 1997). The distribution of the sequences in the two clusters was independent of the host origin or clinical manifestation (Table 1). *bft-3* may represent the only exception, since this allele was absent from European and American isolates, supporting the idea of Chung *et al.* (1999) that the third *bft* allele may have originated by a different molecular evolution specific to Asian *B. fragilis*. An interesting finding was the high degree of sequence conservation: the 294 bp amplicon showed no mutations within each allele and 14 point mutations between *bft-1* and *bft-2* (except 1475, showing two point mutations with respect to the other *bft-2* sequences). This conservation should be further investigated, since the primers used have not been designed to amplify a highly conserved DNA region, such as the zinc-binding site. Also *bft-3* seems to be highly conserved and allows a similar observation as for *bft-1* and *bft-2*: an alignment of *bft-3* (N. Kato, GenBank accession number AB026624), *bft-Korea* (Chung *et al.*, 1999) and the amplicon obtained from GAI20240 showed no mutation between the three sequences.

Genetic structure in relation to clinical manifestation

The number of ETs observed, the absence of ETs represented by a large number of isolates and the absence of particular genotypes associated with distinct clinical manifestations showed that in *B. fragilis* populations, pathogenicity (invasiveness) is not caused by a few specific genomic types or clones. No particular clusters of *B. fragilis* were implicated in bacteraemia, abscesses or other clinical manifestations.

Genetic structure of the *B. fragilis* population colonizing patients

Invasive *B. fragilis* seem not to present a genomic group distinct from those of the intestinal flora and patients could be colonized by isolates of different genotypes, e.g. ET 21, 13 and 76 from patient 64.

Genetic structure of *B. fragilis* in different hosts

ETs representing *B. fragilis* isolated from animals did not form a separate cluster and were randomly distributed among the ETs representing the human strains. However, only four animal strains were included in the analysis of *B. fragilis* and they may not be representative.

Conclusion

The observations inferred from MLEE are based on a panel of genes representing the chromosome as a whole and not on a single gene or a unique region of the genome. The data obtained strongly correlated with those provided by other techniques (DNA–DNA hybridization, ribotyping, RFLP, AP-PCR and 16S rRNA), allowing the differentiation of *B. fragilis* into two subgroups associated with different antibiotic resistance traits (*cepA* and *cfiA*). Three alleles of an enterotoxin-encoding gene (*bft-1*, *bft-2* and *bft-3*) were identified in the population. The production of the enterotoxin does not, however, seem to be related to a particular genomic group and seems not to represent a distinctive feature for invasive strains. No definite clones of *B. fragilis* are associated with specific clinical manifestations or geographical origin. Patients are thus infected by *B. fragilis* belonging to a wide variety of genomic types, supporting the hypothesis that *B. fragilis* is largely opportunistic or that invasive bacteria acquire by horizontal gene transfer a gene encoding an as yet unidentified virulence factor.

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