ANTIMICROBIAL RESISTANCE

recA* and *glnA* sequences separate the *Bacteroides fragilis* population into two genetic divisions associated with the antibiotic resistance genotypes *cepA* and *cfiA

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The sequences of part of the glutamine synthetase-encoding gene (*glnA*) and of the RecA-encoding gene (*recA*) were determined and aligned for 45 *Bacteroides fragilis* isolates from different clinical and geographical origin. The patterns of sequence divergence of *glnA* and *recA* were very similar. The sequences of a 303-bp fraction of *recA* showed 45 nucleotide substitutions, 40 of which allowed the separation of *B. fragilis* into two major divisions, which were not found when the deduced amino acid sequences were considered. The 687-bp sequences analysed for the *glnA* gene showed 112 nucleotide substitutions, 96 of which separated the population into the same two divisions as those described for *recA*. In this case, the deduced amino acid sequences showed this subdivision as well: three of the six observed amino acid substitutions were division-specific. Within the two divisions, both genes presented a high degree of sequence conservation. Each *B. fragilis* division was associated with the presence of a different antibiotic resistance gene: *cepA* encoding a serine- β -lactamase (division I) and *cfiA* encoding a metallo- β -lactamase (division II). No particular clusters associated with geographical or clinical origin, or with the production of an enterotoxin were observed. Sequencing of the *cfiA* gene allowed identification of two different alleles in division II. However, no association of these different *cfiA* alleles with the expression of imipenem resistance was observed. In conclusion, the phylogenetic patterns observed by sequencing *recA* and *glnA* are in agreement with those obtained previously by MLEE (multilocus enzyme electrophoresis). Thus, it appears that the evolution of *recA* and *glnA* genes is similar to that of the whole chromosome of *B. fragilis*. Horizontal gene transfer between divisions I and II seems to be low, at best. However, the results of the present study could not clarify definitively whether divisions I and II should be considered as two different *B. fragilis* genospecies.

Introduction

Bacteroides fragilis is a gram-negative, obligately anaerobic, opportunist pathogen present in the normal gut flora. Occasionally, some strains produce an enterotoxin [1, 2]. Studies with multilocus enzyme electrophoresis (MLEE) [3] and other molecular techniques – particularly 16S rRNA sequencing [4] and DNA–DNA hybridisation [5] – have shown the separation of the population into two groups presenting

an extensive genetic distance one from the other (divisions I and II). Division I was characterised by the frequent presence of the *cepA* gene (encoding a serine- β -lactamase of Ambler's class A) and the absence of the *cfiA* gene (encoding a metallo- β -lactamase of Ambler's class B). Division II was characterised by the presence of the *cfiA* gene, the absence of the *cepA* gene and a higher genetic homogeneity. Three specific insertion sequence elements, IS1186, IS942 and IS4351, providing the promoter region for the *cfiA* gene and leading to the expression of imipenem resistance, were shown to be strictly associated with this group [6, 7].

As MLEE may underestimate the real extent of

nucleotide sequence differences, this study sequenced two different genetic markers, *recA* and *glnA*, with the aim of obtaining a direct measure of nucleotide diversity among *B. fragilis* strains. Recently, DNA sequence typing has provided significant insights into the population structure and evolution of many bacteria [8–10]. To our knowledge, the present study is the first to apply sequencing of housekeeping genes to *B. fragilis* to obtain information on the structure and evolution of its population.

The *recA* gene encodes RecA, a protein which is ubiquitous in eubacteria, playing a crucial role in homologous recombination, DNA repair and SOS response [11]. Specifically, RecA binds stretches of single-stranded DNA, unwinds duplex DNA and finds regions of homology between chromosomes in homologous recombination. In this context RecA is required for synapses and strand transfer. Moreover, it is implicated in proteolysis of the repressor protein LexA and induces activity of more than 15 SOS response genes [11]. The protein RecA is substantially conserved across bacterial genera [12]. Previous studies based on a RecA amino acid sequence alignment [12] and on significant segment pair alignment [11] showed strong concordance with those derived from 16S rRNA sequences [13]. Furthermore, a study in *Neisseria* spp. focusing on the *recA* gene made it possible to obtain important information on the bacterial evolution and population structure [14]. Thus, RecA seems to be a good phylogenetic marker at several taxonomic levels within eubacteria, and the *recA* gene might also be a valuable phylogenetic marker for the identification of genetic subgroups within a defined species.

The second gene (*glnA*), encoding glutamine synthetase (GS), has also been used in the past for phylogenetic studies comparing archaea to bacteria [15, 16]. GS is an important enzyme involved in nitrogen metabolism and ammonia assimilation in many eukaryotes and prokaryotes. It is present in three distinct isoforms: GSI, GSII and GSIII. GSIII has been observed in only a few bacterial species, including *B. fragilis*. The *B. fragilis* GSIII enzyme is very different from the other GS enzymes (GSI and GSII): its coding region is 2187 bp long (compared with the 1050–1410-bp length of the other two isoforms) and it constitutes a hexamer, not a dodecamer holoenzyme [17]. However, all GS isoforms show at least four regions of amino acid homology [17]. Two of these regions (region II and III) were included in the sequence fragment analysed in the present study.

Materials and methods

Bacterial strains

A total of 45 *B. fragilis* strains originating from different countries, one *Bacteroides ovatus*, and one *Bacteroides uniformis* was chosen from the sample

collection, which was previously investigated by MLEE [3]. This collection included human invasive and non-invasive (recovered from patients' stools) strains from different geographical regions (Switzerland, France, Norway, USA and Japan), strains producing enterotoxin, invasive isolates from animals and one reference strain (ATCC 25285) of *B. fragilis* (Table 1). Bacteria were grown, identified and stored as described previously [3]. *B. ovatus* and *B. uniformis* were used as outgroup strains in the *recA* analysis, whereas no outgroup sequences were available for the *glnA* gene.

DNA extraction and PCR

Bacterial suspensions (50 µl) frozen in water were thawed and DNA was extracted with a commercial ion-exchange resin (InstaGene Matrix, BioRad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions. PCR was performed with 10 µl of the DNA extract, 0.5 µM of each primer and 1 unit of *Taq* polymerase (Boehringer Mannheim, Germany) in a total reaction volume of 50 µl (buffer provided by the manufacturer); the reaction mixtures were overlaid with paraffin oil (Merck, Darmstadt, Germany) to prevent evaporation during the amplification, which was performed in a DNA Thermo Cycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

Primers *recA-1* (5'-GACACTGGCCATCCACG-3') and *recA-2* (5'-GCAACTGGTTGATGAAGATACA-3'), amplifying a 367-bp fragment of the *recA* gene, on the basis of the DNA sequence alignment of *B. fragilis* [18], *Bacteroides tetraiotaomicron* [19] and *B. uniformis* were designed (A. J. Cooper and N. B. Schoemaker, GenBank accession no. AF013606). Then 35 cycles of PCR were performed with these primers with the following thermal profile: 94°C for 60 s, 55°C for 60 s, 72°C for 90 s.

Primers *glnA-1* (5'-AGGAATATTTTTTGGTTGACA-3') and *glnA-2* (5'-AACGATTCCTGTGAATGC-3'), amplifying part of the glutamine synthetase encoding gene, on the alignment of the *glnA* sequence of *B. fragilis* [17] and *Butyrivibrio fibrisolvens* were designed [20]. These primers amplified a 780-bp fragment of the *glnA* gene in a PCR of 35 cycles, with the following thermal profile: 94°C for 60 s, 52°C for 60 s, 72°C for 60 s.

To ensure that negative PCR results obtained with *B. ovatus* and *B. uniformis* were not a consequence of mutations in the regions complementary to the primers *glnA-1* and *glnA-2*, a nested PCR was performed according to the protocol of Yamashita *et al.* [21].

A fragment of 353 bp of the *cfiA* gene was amplified with primers *cfiA-1* and *cfiA-2* as described previously [3].

Table 1. *B. fragilis* strains

Isolate No.	MLEE ET*	Biological origin	Geographical origin	Genetic characteristics	Source or reference
Division I					
V15 2735	2	Blood	Switzerland, Zürich	<i>cepA</i>	R. Zbinden
D3251	3	Blood, cat	Switzerland, Bern	<i>cepA</i>	J. Nicolet
GAI20273	5	Blood	Japan, Gifu	<i>cepA</i>	N. Kato
F59D	6	Stool from patient 59	Switzerland, Ticino	<i>cepA</i>	IBS [†]
25458	9	Blood	Norway, Bergen	<i>cepA</i>	T. Hofstad
47	10	Appendicitis with abscess	Switzerland, Ticino	<i>cepA</i>	IBS
AIP638	11	Unknown	France, Paris	<i>cepA</i>	[6]
70	14	Intestinal fistula	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	IBS
1332	17	Sigmoid pelvic abscess	France, Paris	<i>cepA</i>	M. Sebald
31959	18	Blood	Norway, Bergen	<i>cepA</i>	T. Hofstad
30285	19	Blood	Norway, Bergen	<i>cepA</i>	T. Hofstad
F70B	21	Stool from patient 70	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	IBS
F126A	24	Stool from patient 126	Switzerland, Ticino	<i>cepA</i>	IBS
F126C	25	Stool from patient 126	Switzerland, Ticino	<i>cepA</i>	IBS
F126E	25	Stool from patient 126	Switzerland, Ticino	<i>cepA</i>	IBS
126	26	Femur wound abscess	Switzerland, Ticino	<i>cepA</i>	IBS
118	28	Wound abscess after appendectomy	Switzerland, Ticino	<i>cepA</i>	IBS
F118A	29	Stool from patient 118	Switzerland, Ticino	<i>cepA</i>	IBS
134	32	Unknown	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	IBS
136	36	Stool from patient with Crohn's disease	France, Paris	<i>cepA</i> , <i>bft-2</i>	M. Sebald
L362	45	Blood	Switzerland, Vaud	<i>cepA</i>	J. Bille
VO8 12993	50	Blood	Switzerland, Zürich	<i>cepA</i>	R. Zbinden
93	55	Blood, patient 80	Switzerland, Ticino	<i>cepA</i>	IBS
1301	56	Bone infection	France, Paris	<i>cepA</i>	M. Sebald
GAI96448	58	Stool	Japan, Gifu	<i>cepA</i> , <i>bft-1</i>	N. Kato
L754	60	Blood	Switzerland, Vaud	<i>cepA</i>	J. Bille
1237	64	Blood	France, Paris	<i>cepA</i>	M. Sebald
59	65	Intestinal tumour	Switzerland, Ticino	<i>cepA</i>	IBS
F59G	66	Stool from patient 59	Switzerland, Ticino	<i>cepA</i>	IBS
64	70	Blood (septicaemia after sacral decubitus)	Switzerland, Ticino	<i>cepA</i> , <i>bft-2</i>	IBS
120	72	Inguinal abscess	Switzerland, Ticino	<i>cepA</i>	IBS
F64B	73	Stool from patient 64	Switzerland, Ticino	<i>cepA</i> , <i>bft-2</i>	IBS
F120B	74	Stool from patient 120	Switzerland, Ticino	<i>cepA</i>	IBS
F70A	80	Stool from patient 70	Switzerland, Ticino	<i>cepA</i> , <i>bft-1</i>	IBS
ATCC 25285		Reference strain			American Type Culture Collection
Division II					
1429	82	Eschar	France, Paris	<i>cfiA-2</i> , <i>IS1186</i>	M. Sebald
BFR271R	83	Unknown	France, Paris	<i>cfiA-2</i> , <i>IS1186</i>	E. Collatz
BFR81R	84	Unknown	France, Paris	<i>cfiA-2</i> , <i>IS1186</i>	E. Collatz
TAL2480	85	Unknown	USA	<i>cfiA-1</i> , <i>IS942</i>	[28]
TAL3636	85	Unknown	USA	<i>cfiA-1</i> , <i>IS942</i>	[28]
VPI2393	86	Unknown	France, Paris	<i>cfiA-2</i>	[5]
VPI3392	87	Unknown	France, Paris	<i>cfiA-1</i>	[5]
1297	88	Appendicitis	France, Paris	<i>cfiA-1</i>	M. Sebald
28794	89	Blood	Norway, Bergen	<i>cfiA-1</i>	T. Hofstad
127	90	Unknown	Switzerland, Ticino	<i>cfiA-1</i>	IBS
Outgroup [‡]					
D3433	91	Lung abscess, hamster	Switzerland, Bern		J. Nicolet
F120C	93	Stool from patient 120	Switzerland, Ticino		IBS

*The ET numbers are those reported in Gutacker *et al.* [3].

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[‡]Outgroup strains were D3433 (*B. uniformis*) and F120C (*B. ovatus*).

DNA sequencing and alignment

Templates for direct sequencing were prepared by a simple purification step of the PCR products with the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Cycle sequencing reactions were performed with primers *recA-1* and *glnA-1*, respectively, in total volumes of 15 µl with an ABI Prism dRhodamine dye terminator Cycle Sequencing Ready Reaction Kit (dRhodamine terminator; Perkin-Elmer Applied Bio-

systems) on an ABI Prism 310 Genetic Analyser (Perkin-Elmer Applied Biosystems). When doubtful results or discrepancies were observed, sequences in the opposite directions were obtained with the *recA-2* and *glnA-2* primers, respectively. Amplicons of the *cfiA* gene were sequenced with primers *cfiA-1* and *cfiA-2*.

DNA sequence analysis

The *recA*, *glnA* and *cfiA* sequences were handled and

stored with the Lasergene program EditSeq (1994 release; DNASTar, Madison, WI, USA) and aligned separately with Megalign (1994 release, DNASTar). MEGA (Molecular Evolutionary Genetics Analysis 1.01) [22] was used for distance analyses of the *recA* and the *glnA* sequences. Phylogenetic trees were constructed by the neighbour-joining method [23] and the robustness of each node was tested by bootstrap analysis [24], with 500 replications. The numbers of nucleotide substitutions per site were estimated by the Tamura-Nei model [25]. MEGA was also used for estimating percentage base composition and percentage codon usage. Translations to amino acid sequences used the universal genetic code.

Results

Amplification experiments

All the *B. fragilis* strains generated the expected 367-bp *recA* and 780-bp *glnA* amplicons, respectively. The *B. ovatus* and *B. uniformis* isolates produced the *recA*, but not the *glnA* fragments.

Phylogenetic analysis

The neighbour-joining method [23] was used to construct phylogenetic trees from both the *recA* and the *glnA* sequences (Figs. 1 and 2, respectively). The results obtained with both genes allowed the separation of the

Fig. 1. Neighbour-joining tree based on the sequence alignment of a fragment of 303 bp of the *recA* gene, using Tamura-Nei genetic distances. *cepA* and *cfiA* are genes encoding antibiotic resistance. *bft-1* and *bft-2* are two alleles of the enterotoxin encoding gene *bft*. The clinical and geographical origins of the isolates are summarised in Table 1. Only bootstrap values >50% are shown. Genetic distance values are indicated by italic characters where long branches have been cut, for the rest of the dendrogram refer to the bar.

Fig. 2. Neighbour-joining tree based on the sequence alignment of a fragment of 687 bp of the *glnA* gene, using Tamura-Nei genetic distances. *cepA* and *cfiA* are genes encoding antibiotic resistance. *bft-1* and *bft-2* are two alleles of the enterotoxin encoding gene *bft*. The clinical and geographical origin of the isolates are summarised in Table 1. Only bootstrap values >50% are shown. Genetic distance values are indicated by italic characters where long branches have been cut, for the rest of the dendrogram refer to the bar.

B. fragilis population into two divisions, each associated with a different antibiotic resistance trait (70% support in 500 bootstrap replicates in the *recA* tree; as *B. ovatus* and *B. uniformis* did not generate amplicons, no data were available for the *glnA* gene). Division I comprised 35 isolates. PCR experiments showed that all these isolates carried the *cepA* gene encoding a serine β -lactamase, but not the *cfiA* gene encoding a metallo- β -lactamase. Division II comprised 10 isolates carrying *cfiA* but not *cepA*. Within the divisions, the low amount of DNA variability of the sequences (see below) prevented phylogenetic evaluation.

Isolates of the same clinical or geographical origin could not be associated with specific clusters. Isolates encoding the *B. fragilis* *bft* enterotoxin genes (deter-

mined by PCR assays [3]) were all in division I, but they were not associated with a particular subgroup within the division.

Genetic diversity within the recA gene of B. fragilis

The average base-pair composition of the 303-bp *recA* sequences obtained from the 367-bp amplicons was 28.6% A, 24.2% T, 22.6% C and 24.6% G; 45 of 303 nucleotide sites (14.8%) were variable within the 45 *B. fragilis* strains analysed (Table 2). No insertions or deletions were observed. All nucleotide substitutions were silent; thus, they generated no amino acid replacements. Of 45 variable sites, 40 were directly involved in the subdivision of *B. fragilis* into the two

Table 2. Number of variable sites in the *recA* gene and genetic distances (range) calculated by the Tamura-Nei model

Strains	Variable sites (%)	Amino acid substitutions	Genetic distance*
Total <i>B. fragilis</i> population	45 (14.8)	0	0.1452–0.1633
Division I	7 (2.3)	0	0.0000–0.0167
Division II	3 (0.9)	0	0.0000–0.0100
<i>B. fragilis</i> - <i>B. ovatus</i>	57 (18.8)	8	0.1595–0.2263
<i>B. fragilis</i> - <i>B. uniformis</i>	73 (24.1)	8	0.2787–0.2940
<i>B. ovatus</i> - <i>B. uniformis</i>	69 (22.8)	12	0.2777

*The genetic distance ranges reported are those observed between the isolates of division I and those of division II (total *B. fragilis* population), within division I or division II only, and between *B. fragilis* and other *Bacteroides* species, respectively.

divisions described above. Within the divisions themselves only a few nucleotide substitutions occurred: seven within division I and three within division II (Table 2). This generates high genetic distances between the isolates of the two divisions, but not between isolates of the same division. Indeed, Tamura-Nei genetic distance values among *B. fragilis* ranged from 0.1452 to 0.1633 when isolates of division I were compared to those of division II, whereas they ranged from 0 to 0.0167 when comparing isolates within division I and from 0 to 0.0100 when comparing isolates within division II (Table 2). The comparison of the *recA* sequences of *B. fragilis* with those of *B. ovatus* and *B. uniformis* is also shown in Table 2. In all, 25 of 57 nucleotide substitutions observed between *B. fragilis* and *B. ovatus*, and 43 of 73 substitutions observed between *B. fragilis* and *B. uniformis* were in positions different than the nucleotide substitutions observed between the *B. fragilis* divisions.

Genetic diversity within the *glnA* gene of *B. fragilis*

The mean base-pair composition of the 687-bp sequences obtained from the 780-bp amplicons of the *glnA* gene was 28.1% A, 25.0% T, 25.9% C and 21.0% G in both divisions.

For the *glnA* gene, 112 (16.3%) of 687 nucleotide sites were variable within *B. fragilis*, and again, no insertions or deletions were observed; 96 nucleotide substitutions separated the *B. fragilis* population into the two distinct divisions (Table 3). A low mutation rate was observed within the divisions: 10 nucleotide substitutions within division I and 11 within division

II. The Tamura-Nei genetic distance values ranged from 0.1650 to 0.1865 when isolates of the two divisions were compared with each other, from 0 to 0.0088 when comparing isolates within division I and from 0 to 0.0103 when comparing isolates within division II (Table 3).

Six non-silent variable sites were found. Three of these non-silent sites allowed separation of the *B. fragilis* population into two divisions also at the protein level. However, the substituted amino acids had similar properties: S-105-N (both polar uncharged), V-124-I (both non-polar, hydrophobic) and G-215-S (both polar uncharged).

cfiA sequences of division II

The base-pair composition of the *cfiA* amplicons was 21.0% A, 27.5% T, 25.2% C and 26.3% G.

The 332-bp sequences obtained from the 353-bp amplicons of the *cfiA* gene showed seven variable sites. Four nucleotide substitutions (positions 63, 86, 188 and 292) allowed delineation of two groups into the 10 strains carrying the *cfiA* gene (designated *cfiA*-1 and *cfiA*-2, respectively, Fig. 3). No correlation of the *cfiA* alleles with imipenem resistance or with particular clusters in the *recA* and *glnA* sequence trees was found (Figs. 1, 2).

Discussion

The dendrograms generated with both *recA* and *glnA* gene sequence alignments showed the separation of the

Table 3. Number of variable sites in the *B. fragilis glnA* gene and genetic distances (range) calculated by the Tamura-Nei model

Strains	Variable sites (%)	Amino acid substitutions	Genetic distance*
Total <i>B. fragilis</i> population	112 (16.3)	6	0.1650–0.1865
<i>B. fragilis</i> division I	10 (1.5)	3	0.0000–0.0088
<i>B. fragilis</i> division II	11 (1.6)	2	0.0000–0.0103

*The genetic distance ranges reported are those observed between the isolates of division I and those of division II (total *B. fragilis* population), within division I or division II only.

Fig. 3. Neighbour-joining tree, using Tamura-Nei genetic distances, obtained by the alignment of 333 bp of the *cfiA* gene. *cfiA-1* and *cfiA-2* are two alleles.

B. fragilis population into two robust monophyletic divisions, I and II. The consistency of this subdivision was supported by 70% bootstrap replicates in the *recA* tree (Fig. 1). These results are in agreement with those obtained in this laboratory by MLEE [3]: all the strains grouped in division I or II by the enzyme method were found in the corresponding divisions obtained with the sequencing method. Thus, these data support the proposition that sequencing *recA* and *glnA* is reliable for the assessment of bacterial phylogeny, as the evolution of these two genes reflects that of the whole chromosome, at least in *B. fragilis*.

The β -lactamase encoding genes *cepA* and *cfiA* were never found together in the same isolate; moreover, they were associated with distinct genetic divisions – *cepA* was present only in division I and *cfiA* in division II. This apparent mutual exclusion might be explained by the acquisition of these genes in separate and unique events, generating two distinct lineages, which replaced all the others. These genetic entities have remained successively sufficiently isolated one from the other to avoid horizontal exchanges. Alternatively, a yet unknown gene or genetic element associated with these lineages may inhibit the acquisition of other β -lactamase-encoding genes. The notion that *cepA* and *cfiA* are of different origin is supported by their differing G+C mol% content (40% for *cepA* and 51.5% for *cfiA*) [this work, [26, 27]]. However, their genetic localisation has not been clarified yet. A few strains apparently carry the *cfiA* gene on the chromosome [27, 28], although a plasmidic location has also been suggested [29]. No evidence showing an extra-chromosomal location of the *cepA* gene has ever been reported. Further experiments on a larger number of strains are needed to localise *cfiA* and *cepA* on the genome of *B. fragilis* and to understand whether and how these genes have been acquired. Interestingly, the G+C mol% content values determined in this study for *recA* and *glnA* (47.2% and 46.9%, respectively) are different from the values reported for *cepA* and *cfiA*

(40% and 51.5%, respectively), confirming that these genes have been acquired rather recently by lateral transfer.

By sequencing the *cfiA* gene, this study identified two main alleles, designated *cfiA-1* and *cfiA-2* (Table 1 and Figs. 1 and 2). Ruimy *et al.* [4], using a 729-bp internal fragment of the *cfiA* sequence as a probe, demonstrated two types of *cfiA* genes. One type was characterised by a strong hybridisation signal and the other reacted only weakly; this hybridisation difference might have been due to the existence of the two alleles found in the present study.

Although the *cfiA* gene was present in all 10 isolates of division II, only five *B. fragilis* isolates were phenotypically resistant to imipenem [3]. The expression of the resistance was not correlated to either *cfiA* allele, nor with a particular cluster within division II. Thus, the expression of the imipenem resistance depends only on the presence of an appropriate insertion sequence element bringing the necessary promoter sequence [3, 6].

For both genes, the divergence between the two divisions was consistent (*c.* 15% variable sites), whereas the divergence within the divisions ranged from 1 to 2%. Nucleotide variation within the divisions was insufficient to detect phylogenetic relationships at this level, precluding the identification of particular genotypes associated with specific diseases, geographical origin or the production of an enterotoxin (Figs. 1, 2). Nevertheless, enterotoxin-producing *B. fragilis* were found only in division I, as shown previously [3]. This observation supports the hypothesis that horizontal gene transfer between divisions I and II are low, at best.

Due to the genetic distances observed with MLEE (value of 0.7) [3] and to the hybridisation results obtained with a few strains included in the MLEE study (DNA relatedness from 65 to 70% between the two DNA homology groups and a ΔT_m ranging from 7.2 to 9.5° [5]), it can be hypothesised that the two divisions may represent different species. The relatively high genetic distance between the two divisions (0.1452–0.1633 for *recA* and 0.1650–0.1865 for *glnA*) added to the absence of genetic divergence within each of them (range 0 to 0.0167) supports this view. Furthermore, the different codon usage in the *glnA* sequences observed between the divisions (data not shown) gives added weight to this hypothesis. However, in our opinion, the results obtained in this study together with those obtained in this laboratory by MLEE [3] are still insufficient to clarify definitively whether divisions I and II may be considered as two distinct genospecies which have recently diverged, or if they represent two *B. fragilis* groups not yet separated at the species level, but evolving in this direction.

The GenBank accession nos for the sequences reported in this paper are: AF280701-AF280747 for *recA* amplicons, AF280638-AF280700 for *glnA* amplicons and AF275830-AF275839 for *cfiA* amplicons.

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References

1. Franco AA, Cheng RK, Chung G-T, Wu S, Oh H-B, Sears C L. Molecular evolution of the pathogenicity island of enterotoxigenic *Bacteroides fragilis* strains. *J Bacteriol* 1999; **181**: 6623–6633.
2. Kato N, Liu C-X, Kato H *et al.* A new subtype of the metalloprotease toxin gene and the incidence of the three *bft* subtypes among *Bacteroides fragilis* isolates in Japan. *FEMS Microbiol Lett* 2000; **182**: 171–176.
3. Gutacker M, Valsangiacomo C, Piffaretti J-C. Identification of two genetic groups in *Bacteroides fragilis* by multilocus enzyme electrophoresis: distribution of antibiotic resistance (*cfiA*, *cepA*) and enterotoxin (*bft*) encoding genes. *Microbiology* 2000; **146**: 1241–1254.
4. Ruimy R, Podglajen I, Breuil J, Christen R, Collatz E. A recent fixation of *cfiA* genes in a monophyletic cluster of *Bacteroides fragilis* is correlated with the presence of multiple insertion elements. *J Bacteriol* 1996; **178**: 1914–1918.
5. Johnson JL. Taxonomy of *Bacteroides*. I. Deoxyribonucleic acid homologies among *Bacteroides fragilis* and other saccharolytic *Bacteroides* species. *Int J Syst Bacteriol* 1978; **28**: 245–256.
6. 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.
7. Yamazoe K, Kato N, Kato H, Tanaka K, Katagiri Y, Watanabe K. Distribution of the *cfiA* gene among *Bacteroides fragilis* strains in Japan and relatedness of *cfiA* to imipenem resistance. *Antimicrob Agents Chemother* 1999; **43**: 2808–2810.
8. Enright MC, Spratt BG. Multilocus sequence typing. *Trends Microbiol* 1999; **7**: 482–487.
9. Maiden MCJ, Bygraves JA, Feil E *et al.* Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci USA* 1998; **95**: 3140–3145.
10. Reid SD, Herbelin CJ, Bumbaugh AC, Selander RK, Whittam TS. Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature* 2000; **406**: 64–67.
11. Karlin S, Weinstock GM, Brendel V. Bacterial classifications derived from *recA* protein sequence comparisons. *J Bacteriol* 1995; **177**: 6881–6893.
12. Karlin S, Brocchieri L. Evolutionary conservation of *recA* genes in relation to protein structure and function. *J Bacteriol* 1996; **178**: 1881–1894.
13. Lloyd AT, Sharp PM. Evolution of the *recA* gene and the molecular phylogeny of bacteria. *J Mol Evol* 1993; **37**: 399–407.
14. Feil E, Zhou J, Maynard-Smith J, Spratt BG. A comparison of the nucleotide sequences of the *adk* and *recA* genes of pathogenic and commensal *Neisseria* species: evidence for extensive interspecies recombination within *adk*. *J Mol Evol* 1996; **43**: 631–640.
15. Brown JR, Masuchi Y, Robb FT, Doolittle WF. Evolutionary relationships of bacterial and archaeal glutamine synthetase genes. *J Mol Evol* 1994; **38**: 566–576.
16. Kumada Y, Benson DR, Hillemann D *et al.* Evolution of the glutamine synthetase gene, one of the oldest existing and functioning genes. *Proc Natl Acad Sci USA* 1993; **90**: 3009–3013.
17. Hill RT, Parker JR, Goodman HJK, Jones DT, Woods DR. Molecular analysis of a novel glutamine synthetase of the anaerobe *Bacteroides fragilis*. *J Gen Microbiol* 1989; **135**: 3271–3279.
18. Goodman HJ, Woods DR. Molecular analysis of the *Bacteroides fragilis recA* gene. *Gene* 1990; **94**: 77–82.
19. Cooper AJ, Kalinowski AP, Shoemaker NB, Salyers AA. Construction and characterization of a *Bacteroides thetaiotaomicron recA* mutant: transfer of *Bacteroides* integrated conjugative elements is RecA independent. *J Bacteriol* 1997; **179**: 6221–6227.
20. Goodman HJK, Woods DR. Cloning and nucleotide sequence of the *Butyrivibrio fibrisolvens* gene encoding a type III glutamine synthetase. *J Gen Microbiol* 1993; **139**: 1487–1493.
21. Yamashita Y, Kohno S, Koga H, Tomono K, Kaku M. Detection of *Bacteroides fragilis* in clinical specimens by PCR. *J Clin Microbiol* 1994; **32**: 679–683.
22. Kumar S, Tamura K, Nei M. MEGA molecular evolutionary genetics analysis, version 1.01, 1993. Pennsylvania State University, University Park, PA 16802.
23. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987; **4**: 406–425.
24. Felsenstein J. Phylogenies from molecular sequences: inference and reliability. *Annu Rev Genet* 1988; **22**: 521–565.
25. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 1993; **10**: 512–526.
26. Rogers MB, Parker AC, Smith CJ. Cloning and characterization of the endogenous cephalosporinase gene, *cepA*, from *Bacteroides fragilis* reveals a new subgroup of Ambler class A beta-lactamases. *Antimicrob Agents Chemother* 1993; **37**: 2391–2400.
27. Rasmussen BA, Gluzman Y, Tally FP. Cloning and sequencing of the class B beta-lactamase gene (*ccrA*) from *Bacteroides fragilis* TAL3636. *Antimicrob Agents Chemother* 1990; **34**: 1590–1592.
28. Thompson JS, Malamy MH. Sequencing the gene for an imipenem-cefoxitin-hydrolyzing enzyme (*cfiA*) from *Bacteroides fragilis* TAL2480 reveals strong similarity between *cfiA* and *Bacillus cereus* beta-lactamase II. *J Bacteriol* 1990; **172**: 2584–2593.
29. Bandoh K, Watanabe K, Muto Y, Tanaka Y, Kato N, Ueno K. Conjugal transfer of imipenem resistance in *Bacteroides fragilis*. *J Antibiot* 1992; **45**: 542–547.