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Characterisation of a 5.5-kb cryptic plasmid present in different isolates of *Bacteroides* spp. originating from Hungary

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The plasmid profiles of 97 *Bacteroides* isolates collected during screening for different pathogenic markers of this genus were investigated. In all, 48% of 69 isolates from infections that belonged to six species harboured low mol. wt plasmids (2.8–11.0 kb). Similar plasmids were also found in 39% of 28 isolates, belonging to eight species, from faeces of healthy persons. The two most frequently obtained types were the 5.5- and the 4.2-kb plasmids, which were present in 70% and 52% of all plasmid-bearing isolates, respectively. Restriction endonuclease analysis revealed that the 5.5-kb plasmids found in the different *Bacteroides* spp. exhibited the same restriction map, with the exception that pBVP61 lacked the *Pst*I recognition site. The two plasmid types (4.2 and 5.5 kb) seem to be most widely distributed among *Bacteroides* isolates independent of the site of isolation and with some differences depending on geographic regions.

Introduction

Species of the genus *Bacteroides* are important constituents of the human and animal intestinal microflora, accounting for *c.* 30% of the total organisms cultured from faeces. Although different species of *Bacteroides* play several important roles in the normal intestinal activities, they are also the most frequently isolated anaerobic bacteria of clinical relevance. They can cause serious infections such as brain, lung, intra-abdominal and intra-pelvic abscesses, peritonitis and sepsis [1], where *B. fragilis* in most cases outnumbers other related species. Phylogenetically, together with *Flavobacterium*, *Porphyromonas* and *Fusobacterium*, they form an ancient and distinct group of eubacteria [2]. A high proportion of them carry low mol. wt plasmids to which no function has yet been assigned [3, 4]. About 26–50% of *Bacteroides* isolates from different species isolated from infections have been shown to exhibit such plasmids. Southern hybridisation has revealed that they are mainly from three groups [5]: class I (1.8 MDa), class II (2.6–4 MDa) and class III (3.7 MDa). The pB8-51 4.2-kb (class II) cryptic *Bacteroides* plasmids were found to be the most prevalent in colonic *Bacteroides* isolates [6]. During an earlier study in this laboratory, 26 of 52 *Bacteroides* isolates from different serious infections, harboured one or more low mol. wt plasmids [4]. The

type found most frequently in this group of isolates was a 3.7-MDa plasmid, which was present in 66% of the isolates containing plasmids. Only one *B. vulgatus* isolate from an intra-abdominal infection harboured the 2.6-MDa plasmid.

The aims of the present study were to compare the plasmid content of *Bacteroides* isolates originating from serious infections and from faeces of healthy persons, to compare the plasmid content of isolates with different pathogenicity determinants and to characterise a 5.5-kb plasmid found most frequently in isolates of different origins.

Materials and methods

Bacterial isolates

Bacteroides spp. involved in infectious processes (69 isolates) were isolated in different laboratories in Hungary; those from the faeces of healthy persons (*n* = 28) were isolated in this laboratory. Bacteria were cultured in brain-heart infusion (BHI) broth and on BHI agar, supplemented with vitamin K1 and cow's blood, in an anaerobic chamber (Sheldon, USA) at 37°C for 48 h. Isolates were identified with the ATB (bioMérieux) system and by conventional methods [7]. These isolates were initially collected to screen for different pathogenicity markers, such as toxin production of *B. fragilis* isolates, the binding of physiological matrix proteins to different *Bacteroides* species, and

evaluation of antibiotic resistance. The isolates from normal faeces were: *B. fragilis* (15), *B. vulgatus* (3), *B. ovatus* (3), *Mitsuokella (B.) multiacidus* (2), *B. eggerthii* (2), *B. stercoris* (1), *B. distasonis* (1) and *B. merdae* (1), while the 69 isolates from different infections comprised *B. fragilis* (51), *B. ovatus* (8), *B. vulgatus* (5), *B. thetaiotaomicron* (2), *B. caccae* (2) and *B. distasonis* (1). *Escherichia coli* strains V/517 and 39R861 with reference mol. wt plasmids, and strain JM107 containing the pUC19 plasmid were propagated in Mueller-Hinton broth at 37°C overnight.

Plasmid detection

Plasmid screening was performed by the alkaline-SDS method of Birnboim and Doly [8]. *E. coli* reference plasmids were isolated by the same method. *Bacteroides* plasmids used for restriction endonuclease analysis were isolated by the method of Zöllner *et al.* [9]. Briefly, cells were cultured in 10 ml of BHI broth for 24 h in an anaerobic environment. After further incubation in the presence of chloramphenicol 170 mg/L for 24 h, the cells were pelleted by centrifugation and then resuspended in 100 µl of 50 mM Tris-HCl buffer, pH 8.0. Samples were heated to 95°C, and 100 µl of SDS 3%, 50 mM Tris/HCl, pH 12.6, buffer were added and incubated at 90°C for 5 min. Samples were placed on ice, 100 µl of 3 M sodium acetate (pH 4.8) were added and the mixtures were held at 0°C for 30 min. The precipitate was removed by centrifugation at 12 000 rpm at 4°C for 12 min. The cleared lysate was purified by extraction with phenol:chloroform (1:1 v:v). The plasmid DNA was precipitated with 2 volumes of cold ethanol and the precipitate was centrifuged at 12 000 rpm at 4°C for 10 min, washed with ethanol 70%, dried and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) 200 µl. Traces of RNA were digested with RNA-ase 0.1 µg/ml at 37°C for 30 min.

Restriction endonuclease analysis

Restriction endonuclease analysis of the isolated plasmid samples was performed with the following enzymes: *Ava* II, *Bam* HI, *Bg* III, *Bsp* RI (*Hae* III isoschizomer), *Hinc* II, *Hind* III, *Pst* I, *Sac* I, *Sac* II, *Sau* 96 I and *Sma* I in volumes of 20 µl, under the conditions recommended by the suppliers. In double digestions, the ionic strength was increased appropriately, or after precipitation of the nucleic acids, the reaction buffer was changed.

Purified plasmids and digestion mixtures were submitted to horizontal slab-gel electrophoresis in agarose 0.8% gels (containing ethidium bromide 0.5 µg/ml) with TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.25) at 3.3 V/cm. The molecular sizes of the plasmids and their fragments were determined from a calibration curve plotting the log₁₀ of the size of molecular marker fragments against relative mobility.

The *Pst* I restriction fragments of phage λ DNA were used as mol. wt standards. Two-dimensional agarose gel electrophoresis was carried out as described by Hintermann *et al.* (with minor modifications) [10]. Polyacrylamide gel electrophoresis was used to reveal suspected small fragments after *Bsp* RI (*Hae* III) endonuclease digestion [11].

Antibiotic susceptibility testing

Antibiotic resistance of the strains was determined by the micro-broth dilution method according to the NCCLS guidelines [12]. Toxin production by *B. fragilis* strains was investigated with HT29 cells as described previously [13]. Binding of extracellular matrix proteins to the surface of strains was investigated with a latex agglutination test [14].

Results

Low mol. wt plasmids (2.8–11 kb) were found in 33 (48%) of the 69 isolates of pathogenic origin (Fig. 1) and 11 (39%) of the 28 isolates of non-pathogenic origin (Tables 1 and 2). In seven isolates from both groups, high mol. wt (>25 kb) plasmid forms were also observed. Altogether 15 isolates (3 faecal isolates and 12 of pathogenic origin) contained more than one plasmid. When a strain harboured more than one plasmid, the exact plasmid profile was determined by two-dimensional agarose gel electrophoresis, which is suitable for the separation and recognition of different forms of each plasmid present. By these methods, the different plasmid types were determined according to their mol. wts (2.8, 3.4, 4.2, 5.0, 5.5, 6.0, 7.6, 9.3, 10.0 and 11.0 kb). A comparison of the prevalence of plasmids in *B. fragilis* isolates of different origins (normal faecal flora and infections) and those belonging to species other than *B. fragilis* revealed no significant differences (33% and 49% versus 46% and 44%, respectively). In isolates from infectious processes, the 5.5-kb plasmid was predominant being present in 24 (35%) isolates, alone or together with other plasmids. It was also found in 5 of 28 isolates from faeces of healthy persons. The second most frequently isolated plasmid, with a mol. wt of 4.2 kb, was more equally distributed among the isolates from the two groups (22 and 29% in isolates from infectious processes and from normal faecal flora, respectively).

To test whether the 5.5-kb plasmids present in different isolates were similar, five *B. fragilis* isolates (K25, P27, P35, P36 and P66) and one *B. vulgatus* isolate (P61) containing only this plasmid were selected. As the alkaline/SDS lysis method used for plasmid screening usually yielded a low amount of plasmid DNA, the large scale plasmid isolation method described by Zöllner *et al.* [9], which gave plasmid DNA in sufficient quantity and purity, was used. Digestion with *Ava* II, *Bsp* RI (*Hae* III isoschi-

Fig. 1. Gel electrophoresis of representative plasmid preparations of *Bacteroides* isolates from infectious processes. Lanes 1–19, plasmid patterns of *Bacteroides* isolates P25, P27, P28, P29, P30, P31, P32, P33, P34, P35, P36, P37, P38, P61, P62, P63, P64, P66 and P67, respectively. The position of the 5.5-kb plasmid is indicated on the left, the position and mol. wt values (kb) of the reference plasmids of *E. coli* strain V/517 are indicated on the right.

Table 1. Plasmid content, antibiotic resistance profile and enterotoxin production of *Bacteroides* isolates from the faeces of healthy persons

Isolate designation	<i>Bacteroides</i> species	Antibiotic resistance pattern*	Enterotoxin production	Plasmids (kb)
F01	<i>B. fragilis</i>	Amp, Cfx, Cln	—	4.2
F03	<i>B. fragilis</i>	Amp, Cfx, Cln, Tet	—	4.2
F09	<i>B. fragilis</i>	Amp	+	49, 33, 10, 4.2, 3.4
F10	<i>B. fragilis</i>	Amp, Cfx, Tet	—	25, 5.5, 4.2, 3.4
F31	<i>B. fragilis</i>	Amp	—	5.5
F13	<i>B. ovatus</i>	Amp, Cfx, Cln, Tet	NT	4.2
F20	<i>B. ovatus</i>	Amp, Cln, Tet	NT	10, 5.5, 4.2
F11	<i>B. eggerthii</i>	Amp, Tet	NT	4.2
F16	<i>B. eggerthii</i>	Amp, Cfx, Tet	NT	4.2
F15	<i>Mitsuokella (B.) multiacida</i>	Amp	NT	5.5
F18	<i>B. vulgatus</i>	Amp, Cln	NT	5.5

NT, not tested.

*The strains were considered resistant if their MIC values fell into the categories moderately resistant or resistant according to NCCLS standards [12] for the following antibiotics: ampicillin (Amp), cefoxitin (Cfx), imipenem (Imi), chloramphenicol (Cam), clindamycin (Cln), tetracycline (Tet), metronidazole (Met).

zomer), *Sac*I and *Sau*96I resulted in the same size fragments for all six plasmids (Table 3, Fig. 2). *Pst*I digested only five of the six plasmids (Table 3). Splitting did not occur with *Bam*HI, *Bgl*II, *Hinc*II, *Hind*III, *Sac*II and *Sma*I in any case (Fig. 3). The relative positions of the recognition sites of cutting by restriction endonucleases on the plasmid molecules were mapped by means of single and double digestions. The restriction fragments obtained by double digestions were identical for the plasmids pBFK25, pBFP27, pBFP35, pBFP36 and pBFP66 from *B. fragilis* isolates. The *Pst*I recognition site was missing from the pBVP61 plasmid harboured by the *B. vulgatus* isolate (Fig. 4). As the *Bsp*RI (*Hae*III) fragments seen on agarose gel were not large enough to fill the entire 5.5-kb plasmid size, smaller fragment(s) were searched for by polyacrylamide gel electrophoresis. Two such fragments were found that may be located between the *Sac*I and *Ava*II sites on the basis of the lacking fragment length in *Bsp*RI (*Hae*III) double digestions (Table 3).

No correlation was observed between plasmid profile and enterotoxin production in *B. fragilis* isolates originating from infectious processes or from the faeces of healthy persons (Tables 1 and 2), nor between plasmid profile and the binding capacity of fibronectin, vitronectin and collagen to different *Bacteroides* isolates from infections (Table 2). All but one strain in both groups were resistant to ampicillin. Clindamycin resistance in both groups was high (45% and 27%, respectively) (Tables 1 and 2) as was tetracycline resistance (54% and 63%, respectively). No correlation between resistance pattern and plasmid profile was apparent.

Discussion

A variety of low mol. wt plasmids is present in many *Bacteroides* strains originating from infections or from the normal faecal flora [3–5, 15]. The prevalence of such plasmids varies according to the method used for

Table 2. Plasmid content of *Bacteroides* isolates from infections and their pathogenicity markers

Strain designation	Bacteroides species	Site of isolation*	Antibiotic resistance pattern†	Enterotoxin production	Binding of cells to extracellular matrix proteins				Plasmids (kb)
					Collagen	Fibronectin	Vitronectin		
K1	<i>B. fragilis</i>	ia.a.	Amp, Tet	+					>50
K6	<i>B. fragilis</i>	ip.a.	Amp, Tet	-					9.0
K8	<i>B. fragilis</i>	ia.a.	Amp, Cln	-					4.2
K22	<i>B. fragilis</i>	ia.a.	Amp, Chl, Cln, Tet	-					5.5, 4.2
K23	<i>B. fragilis</i>	ia.a.	Tet	-	+	++	+/-		4.2
K25	<i>B. fragilis</i>	ia.a.	Amp	-	+++	+++	++		5.5
K30	<i>B. fragilis</i>	blood	Amp, Tet	-					>50, 10, 5.5, 4.2
K48	<i>B. fragilis</i>	io.a.	Amp, Tet	+					5.5
K50	<i>B. fragilis</i>	ia.a.	Amp, Tet	-					5.5
K54	<i>B. fragilis</i>	ip.a.	Amp, Tet	-					5.5, 5.0
K67	<i>B. fragilis</i>	ia.a.	Amp, Tet	-	+	+	-		>50, 5.5, 4.2, 2.8
E2	<i>B. fragilis</i>	ia.a.	Amp	-					5.5, 4.2
E5	<i>B. fragilis</i>	ia.a.	Amp	-					5.5
E7	<i>B. fragilis</i>	ip.a.	Amp	-					5.5, 4.2
P27	<i>B. fragilis</i>	blood	Amp, Tet	-					5.5
P29	<i>B. fragilis</i>	ip.a.	Amp, Cfx, Tet	-					10, 9.3, 5.5
P30	<i>B. fragilis</i>	ip.a.	Amp, Tet	-					7.6, 6.0, 5.5
P35	<i>B. fragilis</i>	ia.a.	Amp, Tet	-	+++	++	+		5.5
P36	<i>B. fragilis</i>	ia.a.	Amp, Cln, Tet	-					5.5
P63	<i>B. fragilis</i>	ia.a.	Amp, Cln	-	++	+	+		>50, 11, 5.5
P66	<i>B. fragilis</i>	ia.a.	Amp, Cln	-					5.5
P67	<i>B. fragilis</i>	blood	Amp, Tet	-					4.2
P68	<i>B. fragilis</i>	ia.a.	Amp, Cfx, Tet	-					4.2
P83	<i>B. fragilis</i>	ip.a.	Amp, Tet	-					4.2
P84	<i>B. fragilis</i>	ia.a.	Amp, Cln	-					5.5, 4.2
K2	<i>B. ovatus</i>	io.a.	Amp, Tet	-					4.2
K4	<i>B. ovatus</i>	ia.a.	Amp, Cfx, Cln	-					4.2
P47	<i>B. ovatus</i>	ia.a.	Amp, Cln, Tet	-					10, 5.5, 4.2
P52	<i>B. vulgatus</i>	io.a.	Amp, Cfx, Tet	-					>50, 11, 10, 6.8, 5.5, 4.2
P61	<i>B. vulgatus</i>	ip.a.	Amp, Tet	-					5.5
P64	<i>B. vulgatus</i>	ip.a.	Amp, Cfx, Cln, Tet	-					5.5
E6	<i>B. caccae</i>	pa.a.	Amp	-					5.5
K51	<i>B. caccae</i>	pa.a.	Amp, Cfx	-					5.5

*ia.a., intra-abdominal abscess; ip.a., intra-pelvic abscess; io.a., intra-oral abscess; pa.a., perianal abscess.

†See footnote to Table 1.

Table 3. Fragments produced by restriction endonuclease digestions of selected 5.5-kb plasmids

Endonuclease	Lengths of fragments (kb) from plasmids of the following isolates					
	<i>B. fragilis</i> K25	<i>B. fragilis</i> P27	<i>B. fragilis</i> P35	<i>B. fragilis</i> P36	<i>B. vulgatus</i> P61	<i>B. fragilis</i> P66
<i>Ava</i> II	5.5	5.5	5.5	5.5	5.5	5.5
<i>Bam</i> HI	—	—	—	—	—	—
<i>Bgl</i> II	—	—	—	—	—	—
<i>Bsp</i> RI (<i>Hae</i> III)	4.2 1.0 0.15 0.12	4.2 1.0 0.15 0.12	4.2 1.0 0.15 0.12	4.2 1.0 0.15 0.12	4.2 1.0 0.15 0.12	4.2 1.0 0.15 0.12
<i>Hinc</i> II	—	—	—	—	—	—
<i>Hind</i> III	—	—	—	—	—	—
<i>Pst</i> I	5.5	5.5	5.5	5.5	—*	5.5
<i>Sac</i> I	5.5	5.5	5.5	5.5	5.5	5.5
<i>Sac</i> II	—	—	—	—	—	—
<i>Sau</i> 96 I	5.5	5.5	5.5	5.5	5.5	5.5
<i>Sma</i> I	—	—	—	—	—	—

—, no digestion.

*The result was confirmed by co-digestion with pUC19 DNA.

Fig. 2. Restriction endonuclease digestion and mapping of four 5.5-kb *Bacteroides* plasmids. Plasmid preparations that contained only a 5.5-kb plasmid were digested with the following endonucleases: *Pst* I (lanes 1–5); *Pst* I + *Ava* II (6–10); *Pst* I + *Sac* I (11–15); *Ava* II + *Sac* I (16–19). Plasmid preparations from the following strains were used: *B. fragilis* P27 (1, 6, 11, 16); *B. fragilis* P35 (2, 7, 12, 17); *B. fragilis* P36 (3, 8, 13); *B. vulgatus* P61 (4, 9, 14, 18); *B. fragilis* P66 (5, 10, 15, 19). *Pst* I restriction fragments of phage λ DNA (20) were used as molecular size marker (sizes in kb of some marker fragments are indicated on the right).

their isolation and the origin of the isolates. With the lysozyme/SDS isolation method, Wallace *et al.* [3] found that 25% of 32 randomly selected clinical *Bacteroides* isolates contained plasmids of three different mol. wts (5.0, 3.0 and 2.0 MDa). Beul *et al.* [15] presumed that *Bacteroides* cells were resistant to lysozyme, and applied the heat/mild alkaline/SDS method to 26 clinical isolates, 24 of which were *B. fragilis*. Eleven isolates (42%) contained one or several (up to three) plasmids of different sizes. Plasmids of similar size showed identical restriction patterns with 17 restriction endonucleases. The most frequently found plasmid size was 3.6-kb. Only one *B. fragilis* isolate contained a 4.2-kb plasmid together with two other plasmids. Callihan *et al.* [5] tested clinical

isolates and isolates from the normal faecal flora. When the high salt precipitation method was used, 15 (44%) (two *B. thetaiotaomicron* and 13 *B. fragilis*) of 34 isolates exhibited plasmids of small size. Three classes of plasmids were identified by Southern hybridisation. Plasmids with a mol. wt of 5.5 kb were found in only two *B. fragilis* isolates. When plasmids were isolated by the lysozyme/SDS/high salt precipitation method, a plasmid of the same size (5.5 kb) was found in 17 *Bacteroides* isolates (*B. fragilis*, *B. thetaiotaomicron* and *B. vulgatus*) among 26 plasmid-bearing clinical isolates in Hungary [4].

The present study confirms the wide distribution (66% of the plasmid-bearing strains) of a 5.5-kb plasmid

Fig. 3. Results of restriction endonuclease treatment of five 5.5-kb plasmids from *Bacteroides* isolates by restriction endonucleases that do not cut these plasmids: *Sac* II (lanes 2–6); *Sma* I (7–11); *Bam* HI (12–16); *Bgl* II (17–21). Lane 1, undigested 5.5-kb plasmid DNA from *B. vulgatus* P61 strain. Plasmid preparations from the following strains were used: *B. fragilis* P27 (lanes 2, 7, 12, 17); *B. fragilis* P35 (3, 8, 13, 18); *B. fragilis* P36 (4, 9, 14, 19); *B. vulgatus* P61 (5, 10, 15, 20); *B. fragilis* P66 (6, 11, 16, 21). Positions of phage λ DNA *Hind* III restriction fragments are indicated on the right.

Fig. 4. Restriction map of the 5.5-kb plasmid pBFP36. No recognition sites were found for *Bam* HI, *Bgl* II, *Hinc* II, *Hind* III, *Sac* II and *Sma* I.

among *Bacteroides* spp., both clinical isolates as well as isolates from faecal samples from healthy persons. The isolates that harboured this plasmid belonged to different species of *Bacteroides*. The homology of six isolated plasmids of this type is suggested by restriction endonuclease analysis. The six plasmids, originating from five *B. fragilis* and one *B. vulgatus* isolates, were identical, except that the *B. vulgatus* plasmid pBVP61 did not have a recognition site for *Pst* I. This could be due to a point mutation in the *Pst* I recognition site or probably methylation of the *Pst* I site. The physical map of the 5.5-kb plasmid in the present study corresponded closely to that obtained by Beul *et al.* [15] for the 5.5-kb plasmid present in

German isolates. These 5.5-kb plasmids of *Bacteroides* spp. may represent a uniform and homologous plasmid type present in strains isolated in different parts of the world.

The second most frequently found plasmid in this study was 4.2 kb in size and was present in 23 of 44 plasmid-bearing isolates. Plasmids of the same mol. wt were found in *Prevotella melaninogenica* [16] and in one *B. fragilis* strain investigated by Beul *et al.* [15]. A 4.2-kb cryptic *Bacteroides* plasmid pB8-51 was also found in several colonic *Bacteroides* spp. in the USA [9] and could be mobilised by *Tc*^r conjugal elements and the conjugal plasmid pBF4, which carry a gene that encodes resistance to clindamycin and erythromycin.

The frequent presence of cryptic, low mol. wt plasmids in *Bacteroides* strains and in other gram-negative anaerobes, such as *Prevotella* spp. irrespective of whether they originate from normal flora or from infections, could not be correlated with any pathogenetic marker. In the present study, the *B. fragilis* isolates tested for the presence of plasmids were also tested for the presence of enterotoxin production, and all *Bacteroides* isolates from infections were tested for binding to matrix proteins such as fibronectin, vitronectin and collagen. No correlation was found.

The high frequency of tetracycline (53–65%) and clindamycin (21–27%) resistance among Hungarian isolates of *Bacteroides* isolates from infectious processes has been observed previously [17]. In the current study, 67% and 45% of 28 *Bacteroides* isolates from normal faeces showed resistance to tetracycline and to clindamycin, respectively.

The occurrence of small, cryptic plasmids with the same molecular size in different *Bacteroides* strains in different parts of the world may be due to their horizontal mobilisation by several different conjugative elements [6, 18, 19], which may promote their spread among normal flora. However, the wide distribution of these plasmids may signal a potential problem if they acquire antibiotic resistance or pathogenicity determinants which may render the treatment of bacteroides infections more difficult.

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