CHARACTERISATION OF CRYPTIC PLASMIDS IN CLINICAL ISOLATES OF *BACTEROIDES FRAGILIS*

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SUMMARY. A screening method for plasmids in the fragilis group of *Bacteroides* spp. was developed, taking account of the lysozyme resistance of these species; 26 strains, 24 of them *B. fragilis*, were investigated by this method. Eleven strains contained plasmids and up to three different plasmids were found in individual strains. The plasmids belonged to five different size classes of mol. wt (10^6) 2.8, 3.5, 3.6, 4.2 and 19. Plasmids of equal size showed homology; no homology was found between plasmids of different sizes. Plasmids of equal size showed identical restriction patterns with 17 restriction endonucleases. Restriction maps were constructed for the five classes of plasmid.

INTRODUCTION

The *Bacteroides fragilis* group represents a significant part of the human intestinal microflora and these organisms are also of clinical importance as a cause of purulent infections in man (for reviews see Dowell and Lombard, 1981; Werner, 1981). However, it is not known whether or not the bacterial strains that cause such infections differ from symbiotic intestinal strains by the possession of specific pathogenicity factors.

Such factors are required for other clinically-important bacterial species and are frequently encoded on plasmids (for reviews see Cabello and Timmis, 1979; Elwell and Shipley, 1980). Functions connected with pathogenicity have not yet been detected on bacteroides plasmids. The only functions of clinical relevance identified in a few cases on plasmids are antibiotic resistances (Mancini and Behme, 1977; Guiney and Davis, 1978; Privitera *et al.*, 1979 and 1981; Tally *et al.*, 1979, 1981 and 1982; Welch *et al.*, 1979; Palchaudhuri and Bawdon, 1980; Magot *et al.*, 1981; Malamy and Tally, 1981; Rashtchian and Booth, 1981; Rotimi, *et al.* 1981).

A prerequisite for the identification of plasmid-coded pathogenicity factors is the determination of the plasmid distribution pattern in the bacterial species. Therefore, several publications concerning the plasmid content of different *Bacteroides* spp. have appeared. Stiffler *et al.* (1974) first reported the occurrence of plasmids in *B. fragilis*. More recently, comprehensive surveys have been performed on the plasmid content of different species of *Bacteroides* by Mays and Johnson (1979), Wallace *et al.* (1981) and

Callihan *et al.* (1983). In these investigations, mostly small cryptic plasmids have been found in *c.* 40% of all strains either from intestinal or from clinical origin.

We have extended these surveys to compare American with European *Bacteroides* strains. Because *B. fragilis* is found more frequently in clinical isolates than other *Bacteroides* spp. (Dowell and Lombard, 1981; Werner, 1981), we screened 24 strains of this species for their plasmid content. Plasmids obtained by the screening procedure were characterised by restriction mapping and tested for homology by hybridisation.

MATERIALS AND METHODS

Bacterial strains. All strains of *Bacteroides* were clinical isolates from suppurative infections in man. They were isolated at the Institutes of Medical Microbiology of the Universities of Bonn (strains 19610, 23262, 23553, 24102, 25400, 25407, 25448, 25989, 26023, 26458, 26732, 1034, 1249, 1430, 1492, 1560, 2016, 2028, 2769 and 2869), Marburg (strains 1396, 16553, and 16547) and Mainz (strains 2457, 3292 and 4430). The isolates were identified by standard biochemical methods (Holdeman and Moore, 1974). Stock cultures were maintained on Wilkins-Chalgren Agar (Oxoid) at 4°C and passaged twice a month through Thioglycollate Medium U.S.P. (Oxoid). Bacteria were cultivated in defined medium (Varel *et al.*, 1974) without using an anaerobic jar. Agar plates were incubated anaerobically in anaerobic jars (Oxoid). *B. fragilis* TM4003, which carries the clindamycin-resistance plasmid pBFTM10, was obtained from Dr F. P. Tally, Boston, MA. *Escherichia coli* reference strains PRC304 and PRC194, which carry the resistance plasmids RSF1030 and R1 respectively, the colicin-producing strains PRC-595 (ColE3), PRC628(ColA-CA31) and the colicin-sensitive strains PRC072 and JA300 were obtained from Dr E. Lederberg (Plasmid Reference Center, Stanford University, Stanford, CA). *E. coli* strains were grown in Standard Nutrient Broth (Merck) and stored on Standard-I Nutrient Agar (Merck) at 4°C.

Buffers and solutions were prepared as follows: TB buffer: 90mM Tris-HCl, pH8.3, 2.5 mM EDTA, 90mM boric acid. TE buffer: 50mM Tris-HCl, pH8, 1mM EDTA. SDS solution: 50mM Tris-HCl, pH8.0, sodium dodecyl sulphate (SDS) 15%. Alkaline SDS solution: SDS 3% adjusted to pH12.6 with 2M NaOH. Stop mixture: 50mM EDTA, 5mM Tris-HCl, pH 8.0. Restriction buffer: 10mM Tris, pH7.7, 6mM MgCl₂, 0.2mM EDTA, β -mercaptoethanol 0.1%, bovine serum albumin 0.1 μ g/ml, in 0.85% NaCl according to the specifications of the suppliers.

Plasmid screening procedure. Bacteria from 1-ml overnight cultures were harvested by centrifugation and suspended in 100 μ l of TE buffer. Then 100 μ l of alkaline SDS solution was added. The mixture was incubated at 85°C for 15 min, cooled to 20°C and kept at room temperature for 5 min before 100 μ l of 3M sodium acetate solution, pH 4.5, was added; the preparation was kept on ice for another 5 min. Cell debris and chromosomal DNA were then removed by centrifugation at 8000 *g* for 5 min at room temperature. The supernate was mixed with an equal volume of phenol:chloroform (1:1, v:v). DNA in the aqueous phase was precipitated with two volumes of ethanol, then dissolved in 20 μ l of TE buffer and subjected to electrophoresis.

Large scale plasmid preparation. Bacteria from 1.5 L of culture medium were harvested by centrifugation and washed with TE buffer. The *c.* 8 g wet weight of bacteria thus obtained was suspended in 40 ml of sucrose 25% in 50mM Tris solution, pH8; 40 ml of 250mM EDTA solution was added and the suspension was stirred for 10 min. Then 50 ml of 15% SDS solution was added, the mixture heated to 70°C and gently shaken at this temperature for 15 min, after which 50 ml of 5M NaCl solution were added and the mixture was kept on ice for 30 min. Precipitate was removed by centrifugation at 10 000 *g* for 10 min at 4°C. RNAase was added to the supernate to give a final concentration of 10 μ g/ml and the mixture was incubated at 37°C for 1.5 h. DNA was precipitated from the solution by adding polyethylene glycol (56 ml of PEG 6000 40% in TE buffer) and holding the mixture for 30 min on ice. The precipitate was collected by centrifugation at 10 000 *g* for 10 min at 4°C and was redissolved in 20 ml of 50mM Tris, pH 8. The solution was heated to 70°C and 20 ml of prewarmed alkaline SDS solution was added. After 10 min, the mixture was cooled on ice and 20 ml of 3M sodium acetate (pH4.8) was added. After 30 min, the precipitate was removed by centrifugation (as above) and the supernate was treated with 60 ml of

phenol:chloroform (1:1, v:v). From the aqueous phase, the DNA was precipitated with two volumes of ethanol and redissolved in 300 μ l of TE buffer. Final purification was achieved by RbTCA centrifugation as described by Zöllner *et al.* (1983).

Agarose-gel electrophoresis. DNA was subjected to horizontal slab-gel electrophoresis in 0.7% agarose at room temperature for 4 h (150 V, constant voltage; *c.* 40 mA). After electrophoresis, gels were stained with ethidium bromide (0.5 μ g/ml in TB buffer) for 30 min, examined with a Chromato-vue transilluminator (Ultra-violet Products, USA) and photographed through a yellow filter on Polaroid Land Film 667.

Isolation of plasmid DNA from agarose gels. Plasmids from bacterial strains that contained two or more plasmids were separated by electrophoresis in low-gelling agarose gels (Sigma). Bands containing the plasmids were cut from the gels and DNA was extracted according to the methods of Maniatis *et al.* (1982).

Radioactive labelling of plasmid DNA. Radioactively labelled plasmid DNA was prepared by nick translation according to the methods of Rigby *et al.* (1977). DNAase I, *E. coli* polymerase I and deoxynucleosidetriphosphates were obtained from Boehringer Mannheim; 32 P dTTP (800 Ci/mm) was obtained from Amersham.

Hybridisation of plasmid DNA. Transfer of DNA from agarose gels to nitrocellulose filters (BRL) and hybridisation with radioactive plasmid DNA was done as described by Maniatis *et al.* (1982). Autoradiography was done overnight on Kodak X-Omat AR film at -70°C .

Restriction-endonuclease digestion. Plasmid digestion was performed as described by Maniatis *et al.* (1982). Restriction enzymes were obtained from BRL (USA), Miles (USA) and Boehringer (Germany) and used as recommended by the suppliers. Approximately 0.1–0.2 μ g of DNA in 14 μ l of restriction buffer were mixed with 5 units of restriction endonuclease and incubated at 37°C for 60 min. Reactions were stopped by adding 6 μ l of stop mixture and 10 μ l of bromophenol blue solution; then the solution was heated to 65°C for 5 min. DNA fragments were separated by gel electrophoresis in 0.9% agarose at 5 V/cm. Size analysis of restriction fragments was performed by a computer programme of Schaffer and Sedereff (1981) with a phage lambda DNA *Hind* III digest as internal standard. For partial digestions, the reactions were stopped after 1, 2, 5, 10, 20, 60 or 120 min. For simultaneous digestions with two restriction endonucleases, the enzyme requiring the lower salt concentration was added first. After the initial incubation period, the salt concentration was adjusted by adding the appropriate amount of 0.7M NaCl solution. Then the second enzyme was added and the sample reincubated.

Electronmicroscopy. A purified preparation of pZW1709 DNA, which had been stored at 4°C to enrich the open circular form, was used in electronmicroscopy studies for homoduplex analysis. DNA was denatured by heating at 95°C for 1.5 min, allowed to reanneal for 1, 5 or 30 min and then was spread according to the formamide technique of Sharp *et al.* (1973). Plasmids RSF1030 and R1 served as references.

Bacteriocin production and antibiotic resistance. Bacteriocin production was tested according to the method of Riley and Mee (1981). Antibiotic resistance was determined by the agar-dilution method (Nasu *et al.*, 1981) on Wilkins-Chalgren Agar. Penicillin, tetracycline and erythromycin (Merck, BRD), clindamycin (Hoechst, BRD), and ampicillin, kanamycin, streptomycin, chloramphenicol and rifampicin (Serva, BRD) were tested at doubling concentrations from 0.025 to 100 mg/L.

Curing. Bacteria were cultivated in Thioglycollate Medium (Oxoid) in the presence of ethidium bromide 15 μ g/ml. After dilution they were spread on Wilkins-Chalgren Agar. Single colonies were picked from the plates, reincubated in 1.5 ml of thioglycollate medium and analysed for their plasmid content as described above.

RESULTS

Plasmid screening and isolation procedures

Control experiments with *E. coli* standard plasmids confirmed that plasmids up to a mol. wt of at least 60×10^6 could be isolated by treatment of cells with alkaline SDS

TABLE I
Plasmid-containing Bacteroides strains

Strain no.		Number of plasmids	Mol. wt (10 ⁶)	Designation
<i>B. fragilis</i>	24102	1	2.8	pZW 206
	1249	1	3.6	pZW 411
	2028	1	3.6	pZW 408
	2869	1	3.6	pZW 407
	25400	1	3.6	pZW 405
	1560	1	19	pZW 1709
	1492	2	3.5	pZW 310
			3.6	pZW 410
			2.8	pZW 212
	16553	2	3.6	pZW 412
			2.8	pZW 201
			3.6	pZW 401
<i>Bacteroides</i> sp.	4430	1	4.2	pZW 501
			2.8	pZW 202
			3.6	pZW 404
<i>B. distasonis</i>	2457	1		

solution at 85°C for 15 min. This method was used for plasmid screening in *Bacteroides* strains.

Plasmid content of B. fragilis strains

Nine of the 24 strains of *B. fragilis* carried one to three plasmids, which fell into five size classes (table I). Two strains of species other than *B. fragilis* also harboured plasmids of sizes already found in the *B. fragilis* strains.

Endonuclease digestion maps

Eight plasmids that occurred singly were digested with 17 restriction endonucleases (table II). The two plasmids from strain 16553 and the three from strain 3292 were separately isolated from agarose gels and digested with the same enzymes. Plasmids of identical size gave identical restriction patterns with all the enzymes tested.

Because of the small difference in their sizes, the two plasmids from strain 1492 could not be separately isolated from the gel and were jointly digested with the 17 enzymes. The restriction patterns always gave the fragments expected for the plasmids of mol. wt 3.6×10^6 as well as other fragments. The fragments of the plasmid of mol. wt 3.5×10^6 were obtained by subtraction.

The different fragments of the five size classes are listed in table II. Restriction maps were constructed from partial digestions or from double digestions with appropriate enzymes. The maps are depicted in fig. 1.

Screening homology of plasmids

Southern blots containing the plasmids of the eleven plasmid-carrying strains were prepared. Plasmids pZW206, pZW408 and pZW502, mol. wt (10⁶) 2.8, 3.6 and 4.2 respectively, were labelled by nick translation and hybridised against the blots. Only

TABLE II
List of fragments produced by endonuclease digestion of plasmids from *B. fragilis*

Enzyme	Length of fragments (in base pairs) from plasmids of mol. wt (10^6)				
	2.8	3.5	3.6	4.2	19
<i>AluI</i>	*	*	*	*	*
<i>AvaI</i>	—	5300	—	—	28900
<i>AvaII</i>	—	—	5500	—	1100
<i>BamHI</i>	—	—	—	—	30000
<i>BglII</i>	—	—	—	—	30000
					23000
					7000
<i>DdeI</i>	1720	2400	3000	*	*
	720	1150	2500		
	660	950			
	580	600			
	560	200			
<i>EcoRI</i>	—	—	—	3600	25400
				2800	4600
<i>HaeIII</i>	—	—	4650	—	—
			850		
<i>HindIII</i>	—	3200	—	6400	27200
		2100			2800
<i>HpaII</i>	*	5300	*	*	*
<i>PstI</i>	—	—	5500	—	—
<i>PvuII</i>	—	—	—	—	—†
<i>SacI</i>	4200	3900	5500	—	30000
		1100			
		300			
<i>SalI</i>	—	—	—	—	—†
<i>SmaI</i>	—	5300	—	—	—
<i>SphI</i>	—	—	—	—	—†
<i>XhoI</i>	—	—	—	6400	—

* More than 10 fragments, size not determined.

† Activity of enzyme was tested with pBR322 DNA.

hybridisation with plasmids of the same size was observed. The two plasmids of strain 1492 were labelled together and hybridised against a blot. In comparison to the test with pZW408, only one additional band was observed indicating hybridisation of the plasmid of mol. wt 3.5×10^6 with itself. From these results we concluded that all the small plasmids of different sizes belonged to different homology classes.

The large plasmid pZW1709 did not react with the radioactive probes and, therefore, belonged to a separate homology class. Finally, the plasmid pBFTM10 from strain TM4003, which contains a resistance gene for clindamycin, was used as a probe with our plasmids; none showed any hybridisation with pBFTM10.

From these results and from the results of the restriction analysis we assume, as a working hypothesis, that plasmids of the same size are identical.

Homoduplex analysis

Inverted repeats indicating the occurrence of transposon-like structures in a plasmid can be identified by homoduplex analysis. Optimal experimental conditions for the demonstration of homoduplexes by electronmicroscopy were determined with the transposon-containing plasmids RSF1030 and R1. With these plasmids, homodup-

FIG. 1.—Restriction maps of the large and the four small plasmids isolated from the *B. fragilis* strains.

lices could be demonstrated in high yield, but no homoduplex could be shown with pZW1709. The small plasmids were not included in this analysis, because occurrence of transposons in plasmids of such size seemed improbable.

Bacteriocin production and antibiotic resistance

All plasmid-carrying strains and the plasmid-free strains 25989, 23553, 25407 and 16547 were tested for bacteriocin production against each other. No inhibition of growth was observed. The assay procedure was tested with colicin-producing *E. coli* strains PRC595(ColE3) and PRC628(ColA-CA31) against the colicin-sensitive strains PRC072 and JA300 and the expected inhibition was observed. Colicin ColE3 even

showed weak inhibition of *Bacteroides* strain 23553. We conclude, therefore, that our plasmids do not code for bacteriocin production.

The same 15 *Bacteroides* strains were tested for resistance to nine antibiotics (see *Materials and methods*). No correlation between plasmid content and antibiotic resistance was observed.

Stability of plasmid content

Because no selection marker for any of the plasmids is known yet, curing experiments were difficult to perform. We cultivated the strains 2028, 2869 and 3292 for 48 h in the presence of subinhibitory concentrations of ethidium bromide as proposed by Rotimi and Duerden (1982) and analysed approximately 200 single colonies from each strain for their plasmid content. No loss of plasmids was observed. Additionally, strain 2896 was subcultured 20 times successively in the presence of ethidium bromide; after each subcultivation 20 colonies were analysed, but no loss of plasmids was observed. Furthermore, the plasmid-carrying strains were passaged approximately twice a month for a period of 2 years without loss of plasmids.

DISCUSSION

B. fragilis cells normally are resistant to digestion by lysozyme (Werner, 1981). Even lysis by neutral SDS at elevated temperatures (Hansen and Olsen, 1978) or by alkaline SDS at lower temperature (Birnboim and Doly, 1979) gives unsatisfactory results. However, hot alkaline SDS treatment (Kado and Liu, 1981) produces an acceptable yield of plasmids; a slight modification of this procedure, namely incubation at 85°C for 15 min, additionally leads to a significant loss of RNA. This method proved to be a good compromise between yield and purity.

Of the 24 strains of *B. fragilis* investigated, nine strains (37%) contained up to three different plasmids. Mostly small plasmids were found, ranging in size from 2.8×10^6 to 3.6×10^6 mol. wt. Only one strain contained a larger plasmid of mol. wt 19×10^6 . Five size classes of plasmids were found with no apparent homology between different classes. Plasmids belonging to the same class, however, showed sequence homology and gave identical restriction patterns with the 17 endonucleases tested. They were, therefore, tentatively considered to be identical. Two of the plasmids were also found in *Bacteroides* strains of species other than *B. fragilis*. The same plasmids were found in strains from different locations in Germany.

These observations resemble the results of recent investigations by Mays and Johnson (1979), Wallace *et al.* (1981) and Callihan *et al.* (1983) of clinical and intestinal isolates of *B. fragilis*. These authors found small plasmids belonging to several homology classes in about 40% of all strains investigated. Occurrence of more than one plasmid in a strain was fairly common and apparently-identical plasmids were observed in different species of *Bacteroides*. Large plasmids were found less frequently. From the plasmid sizes reported by Wallace *et al.* (1981) and from the sizes and the restriction patterns reported by Callihan *et al.* (1983) we conclude that the plasmids isolated by these groups were different from the plasmids found in our investigation. Our plasmids are also nonhomologous to the well characterised bacteroides resistance plasmid pBFTM10.

Summarising the results of this and of the previous investigations, we have to consider it to be a well established fact that a rather high number of different, nonhomologous and compatible small plasmids exist and that a high proportion of American as well as European *B. fragilis* strains contain such plasmids. The question arises, which biological functions do these plasmids encode? Because the plasmids occur in both intestinal and clinical strains, we agree with Callihan *et al.* (1983) that the plasmids probably do not code for pathogenicity factors. The only functions identified so far on small bacteroides plasmids are antibiotic resistance (Palchaudhuri and Bowdon, 1980; Rashtchia and Booth, 1981); furthermore, a correlation between one small plasmid and bacteriocin production has been observed (Mays and Johnson, 1979). We found that our plasmids did not code for these functions.

The plasmid content of the *Bacteroides* strains in this investigation was remarkably stable. While Rotimi and Duerden (1982) reported curing rates of up to 100% for resistance plasmids in *Bacteroides* spp, we must conclude from our data that curing rates for three of the small plasmids are < 1%. This may indicate that these plasmids are of biological importance for the corresponding strains.

The conspicuous feature of the plasmid pattern of *Bacteroides* strains is, on one hand, the similarity in size and frequency of occurrence, and on the other hand, the nonhomology of the small plasmids. Because of the nonhomology, it is difficult to envisage a similar function for all of them. It is possible that the apparent similarity of the occurrence pattern is accidental and that each plasmid may code for a totally different function. The occurrence of more than one plasmid in one strain, which is observed rather frequently, suggests the latter to be more probable. The elucidation of the biological role of small plasmids in *Bacteroides* spp. will certainly help to understand the biology of this clinically important microorganism and will be subject of further investigations.

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