CHARACTERIZATION OF PLASMIDS FROM CLINICAL ISOLATES OF BACTEROIDES

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SYNOPSIS

Nine of the 22 clinical Bacteroides species were found to contain plasmids of sizes varying from 1.5 Mdal to 60 Mdal. All these strains were identified as B. fragilis resistant to mercury, cadmium, zinc, penicillin G, ampicillin and erythromycin. These plasmid-containing strains were also ß-lactamase producers.

Conjugal transfer of ampicillin resistance was detected in 2 strains (PL15 and PL17), of which PL17 also showed a transfer of resistance to mercuric ion. The 2 ampicillin resistant transconjugants showed almost 6-fold increase in their rate of hydrolysis for ampicillin. Plasmid was not detected in the transconjugant with ampicillin resistance. Two plasmids, 1.5 and 9.2 megadaltons, were isolated from the transconjugant that had acquired resistance to both ampicillin and mercuric ion. Even though ampicillin-resistance was transferred after transformation, no plasmids were detected. It was possible that transposons were involved in the transfer of resistance to ampicillin and mercuric ion.

INTRODUCTION

Many intestinal Bacteroides have been found to contain plasmids of various sizes. No known function has been identified for some plasmids isolated from Bacteroides (1, 2, 3). Yet for others, transfer of resistance factor from B. fragilis to other Bacteroides or Escherichia coli have been reported (4, 5, 6, 7, 8). Young and Mayer (9) reported transfer of the gene for ß-lactamase synthesis from a B. fragilis strain to E. coli. Such transfer was accomplished by means of conjugation or transformation. Conjugational transfer was possible only if the donor strain carried a 20 megadalton plasmid in addition to the 3.0 megadalton plasmid that encodes ß-lactamase. Transformation of E. coli HB101 by the 3.0 megadalton plasmid from B. fragilis also brought about transfer of ß-lactamase. Transfer of ampicillin resistance has been reported (9, 10). However, no plasmids were detected in the transconjugants.

In our previous paper (11), we detected plasmids in 9 of our 22 clinical Bacteroides isolates. These 9 species were resistant to cadmium, mercuric and zinc ions and were also resistant to the antibiotics penicillin G, ampicillin and erythromycin.

The aims of the present study were to detect, if any, transfer of antibiotic resistance and resistance to heavy metal ions from Bacteroides to E. coli and to study the possible role of plasmids involved.

MATERIALS AND METHODS

Source of Bacteroides. The 22 clinical Bacteroides species were obtained from the Pathology Laboratory of the Singapore General Hospital.

Growth and Maintenance of Bacteroides. The medium used for growing Bacteroides was Schaedler Agar (SA) (Gibco) supplemented with 10 μg/ml vitamin KI, 5 μg/ml haemin and 5% defibrinated rabbit blood. Each species was maintained and stored at −70° in Schaedler broth foetal calf serum (SBFS) consisting of 50% Schaedler broth and 50% foetal calf serum (Gibco).

Identification. The cultures were incubated in an anaerobic chamber (Forma Scientific) at 35°C in an atmosphere of 85% N_2, 10% H_2 and 5% CO_2.

The Bacteroides species were identified by studying the morphology, aerotolerance, gram-nature, motility, growth in 20% bile and biochemical reactions of the cultures. API 20A was used for biochemical tests and tests for motility, growth in 20% bile and black pigment production were done as outlined by Dowell and Hawkins (12). Red fluorescence was detected under long wavelength UV light (Ultraviolet Products, Inc.). Volatile fatty acids produced by the test cultures were detected by Gas Liquid Chromotography (GLC) as described by Holdeman et al (13).

Antibiotic sensitivity tests. The agar dilution method of Thornsberry and Swenson (14) was employed. The minimum inhibitory concentrations of carbenicillin, ampicillin, tetracycline, chloramphenicol, clindamycin, cefoxitin, erythromycin and penicillin G were determined using the Denley Multipoint Inoculator A400. The range used was from 0.125 μg/ml to 128 μg/ml of antibiotics. E. coli ATCC 25922 and S. aureus ATCC 25923 were used as controls.

Detection of ß-lactamase production. The chromogenic cephalosporin spot test of Montgomery et al (15) was used to determine ß-lactamase production by the Bacteroides species. Colonies grown on SA or Mueller-Hinton Agar (MHA) were picked and applied onto paper disc soaked with the chromogenic cephalosporin solution. A positive result was indicated by a change in colour from yellow to red within 10 minutes. Klebsiella K1 and S. aureus ATCC 25923 were used as positive and negative controls respectively.

Resistance to Heavy Metal Ions. The procedure described by Novick and Roth (16) was used to determine resistance of the Bacteroides to arsenate (0.1 M), cadmium (0.001 M), lead (0.1 M), mercury (0.001 M) and zinc (0.1 M) metal ions. Blank discs (Schleicher and Schüll) of 9 mm diameter were impregnated with 20 ul of the appropriate metal salt solutions. The discs were dried and placed on SA plates swabbed with the test organisms. Results were read after 24 hours incubation. Sensitive strains showed clear zones of inhibition whilst resistant strains grew around the discs.

Quantitative determination of penicillinase activity. Test organisms, grown overnight at 35°C in 20 mls of Brain Heart Infusion (BHI) broth, were harvested, washed with 0.89% saline and resuspended in 1.0 ml 0.05 M phosphate buffer (pH 7.0). The cells were disrupted by sonication to release ß-lactamase and then centrifuged at 12,000 rpm for 20 minutes. Bijou bottles containing 1 ml of penicillin G at a concentration of 10 μg/ml were incubated with ten-fold dilutions of the supernatant at room temperature for 1 hour. The amount of penicillin G left unhydrolysed was measured by bioassay, with a range of antibiotic standards from 1.25 μg/ml to 10 μg/ml. The hydrolysis of the penicillin G by the ß-lactamase extract was calculated from the amount of antibiotics that was hydrolysed (17).

Procedure for plasmid DNA extraction. The method of Kado and Liu (18), with minor modifications was employed. Cells grown in BHI broth (12 mls) were washed with Tris-acetate buffer (40 mM Tris-acetate – 2 mM Na_2 EDTA [pH 7.9]) and pelleted by centrifugation at 9,500 rpm, 4°C for 10 minutes. The cell pellets were resuspended in 150 μl of Tris-acetate buffer in Eppendorf tube (1.5 ml) and 300 μl of lysis buffer (3% SDS in 50 mM Tris-acetate buffer, pH 12.4) was added. Lysis was carried out at 56°C for 45 minutes and 2 volumes of phenol-chloroform (1:1) was added upon complete lysis of the cells. The mixture was centrifuged for 10 minutes using the Eppendorf Microfuge (Model 5413). The upper aqueous layer contained the plasmid DNA and was used for electrophoresis.

Horizontal gel electrophoresis. DNA samples (30 μl) was mixed with 10 μl of tracking dye (0.25%) bromocresol pruple – 50% glycerol in Tris-acetate buffer) on a parafilm. The DNA samples were electrophoresed through 0.7% agarose in Tris-acetate buffer at 80 V for 4–5 hours. The gel was stained overnight in 0.5 μg/ml ethidium bromide solution and photographed with a Canon camera fitted with a macrolens, Wratten 23A/UV filters and Kodak Plus-X pan film.

Bacterial reference strains as molecular weight markers. The reference plasmids that were used as molecular weight markers were kindly supplied by Dr. Esther M. Lederberg of the Plasmid Reference Center, U.S.A. The following plasmid markers were used: pVA517A-H, S-a, RP4, R1 and R62.

Conjugation. The filter mating technique was used (7). The recipient was a plasmidless strain of E. coli K12 J62–1. About 50 μl of overnight cultures of the parental strains were introduced into 2.0 ml of SBFS. After 4–5 hours incubation in the anaerobic chamber (35°C), the donor and recipient cultures were mixed and collected on a 0.45 um Millipore filter (Millipore Corporation). The filter membrane was placed, cell side-up, on a pre-reduced SA plate and incubated anaerobically (35°C) for 24 hours. The cells were harvested from the filter by resuspension in 1 ml BHI broth. Transconjugants were selected by introducing 0.1 ml of appropriately diluted suspension onto MHA antibiotic plate (either containing 10 μg/ml ampicillin or 30 μg/ml tetracycline). Selection for Zn^r and Hg^r transconjugants was done by placing a disc soaked with the heavy metal ion solution onto a MHA plate swabbed with the suspension. The plates were in-

cubated aerobically at 37°C to select out the transconjugants.

Transformation. E. coli HB101 was rendered competent for DNA uptake by heat shock in the presence of divalent Ca^{2+} ions. This procedure was first described for the uptake of phage DNA (19). 1 ml of an overnight culture of HB101 (PY^S, PN^S, Te^S, Hg^S) was inoculated into 100 ml of L-B broth in a 500 ml flask. The culture was shaken in a 37°C water bath to a density of 5 × 10^7 cells/ml. The cells were chilled on ice for 10 minutes, pelleted by centrifugation and resuspended with 50 ml of ice-cold, sterile solution of 50 mM $CaCl_2$ and 10 mM Tris. Cl (pH 8.0). The cell suspension was chilled for a further 15 minutes in an ice-bath and centrifuged at 4,000 g for 5 minutes at 4°C. The cells were then resuspended in 6.7 ml of ice-cold, sterile solution of 50 mM $CaCl_2$ and 10 mM Tris. Cl (pH 8.0). Aliquots of 0.2 ml were dispensed into pre-chilled Eppendorf tubes and the cells were stored at 4°C for 12–24 hours. 20 μl of DNA sample of the transconjugants and donor strains PL15 and PL17, prepared by the Kado and Liu method, were added to 0.2 ml of the E. coli HB101 cell and stored on ice for 30 minutes. The cells were then transferred to a water bath, preheated to 42°C for 2 minutes and 1.0 ml of LB broth was added to each tube. The cells were next incubated at 37°C for 30 minutes (tetracycline selection) or 1 hour (ampicillin selection). Volumes of 0.1 ml cell cultures were spread on ampicillin agar plates while the rest of the transformation mixture was spread on tetracycline plates. The plates were incubated for 16–24 hours at 37°C. Colonies that appeared on ampicillin plates were selected and analysed for plasmid DNA that may have been transferred to E. coli HB101 recipients. The transformants obtained were re-examined by the disc sensitivity test (11).

RESULTS

Of the 22 clinical isolates, 19 were identified as B. fragilis whilst 2 were identified as B. thetaiotaomicron (PL5 and PL19) and only 1 was B. assacharolyticus (PL4) (11).

Only 9 strains were found to contain plasmids (Table 1). Six strains contained only one type of plasmid of molecular size 1.5, 2.2, 2.5 or 60 Mdal. Two strains contained plasmids of 1.5, 2.5 and 48 Mdal whilst 1 strain contained multiple plasmids of molecular sizes 1.5, 3.2, 3.8 and 6.0 Mdal (Table 1 and Figure 1). All the 9 plasmid-containing Bacteroides were B. fragilis. These species were resistant to mercury, cadmium and zinc (Table 2). The MIC values (Table 2) showed that all were resistant to penicillin, ampicillin and erythromycin. All but 2 strains (PL15 and PL18) were resistant to tetracycline. These 9 species were all sensitive to cefoxitin and chloramphenicol. Only PL1 was resistant to carbenicillin while 3 strains (PL1, PL14 and PL17) were resistant to clindamycin.

All the 22 clinical isolates were ß-lactamase-producers (11). Transconjugants selected from ampicillin plates of 2 plasmid-containing strains (PL15 and PL17) were found to show an almost 6-fold increase in its rate of hydrolysis of ampicillin over that of the recipient E. coli K12 J62-1 parent (Table 3). The rate of ampicillin hydrolysis of these transconjugants were twice that of the donor B. fragilis strains (Table 3). The MIC for ampicillin of the recipient E. coli increased 4-fold for TPL15 (PN) and 16-fold for TPL17 (PN).

No transfer of resistance to tetracycline or zinc metal ion was demonstrated. Transfer of resistance to mercuric ion or ampicillin was evident in 2 strains of B. fragilis, PL15 and PL17 (Table 1). PL15 was shown

TABLE 1: FREQUENCY OF TRANSFER OF ANTIBIOTIC OR HEAVY METAL ION RESISTANCE FROM B. FRAGILIS TO E. COLI

Donors		Size of plasmids (Mdal)	Selected Markers	Frequency of transfer of PN^r
Isolate	Species			
PL1	B. fragilis	2.5	PN^r Te^r Hg^r Zn^r	None
PL10	B. fragilis	1.5, 2.5, 48	PN^r Te^r Hg^r Zn^r	None
PL11	B. fragilis	1.5, 2.5, 48	PN^r Te^r Hg^r Zn^r	None
PL12	B. fragilis	60	PN^r Te^r Hg^r Zn^r	None
PL14	B. fragilis	1.5, 3.2, 3.4, 3.8, 6.0	PN^r Te^r Hg^r Zn^r	None
PL15	B. fragilis	60	PN^r Hg^r Zn^r	1 in 2 (PN^r only)
PL17	B. fragilis	1.5	PN^r Te^r Hg^r Zn^r	1 in 300 (PN^r)*
PL18	B. fragilis	60	PN^r Hg^r Zn^r	None
PL21	B. fragilis	2.2	PN^r Te^r Hg^r Zn^r	None

Recipient was E. coli J62-1 (PN^S Te^S Hg^S Zn^S).

*Resistance to mercury was transferred by PL17 to E. coli but the frequency of transfer was not determined.

only to transfer resistance to ampicillin while PL17 was found to transfer resistance to both ampicillin and mercuric ion. No plasmid was detected in the transconjugant derived from the PL15 donor. Two plasmids of molecular sizes 1.5 Mdal and 9.2 Mdal were detected in the transconjugants of the PL17 donor (Figure 1). These were named TPL17 (PN) and TPL17 (Hg).

No plasmids were found in the transformants obtained from DNA-mediated transformation of E. coli HB101 by the plasmids of TPL17 (Hg), TPL17 (PN), PL15 and PL17. Although plasmids were not found in the transformants, transfer of resistance to ampicillin and carbenicillin was detected in HB101. Disc sensitivity test results showed that HB101 had acquired resistance to ampicillin and carbenicillin. However, transformants resistant to mercuric ion could not be isolated.

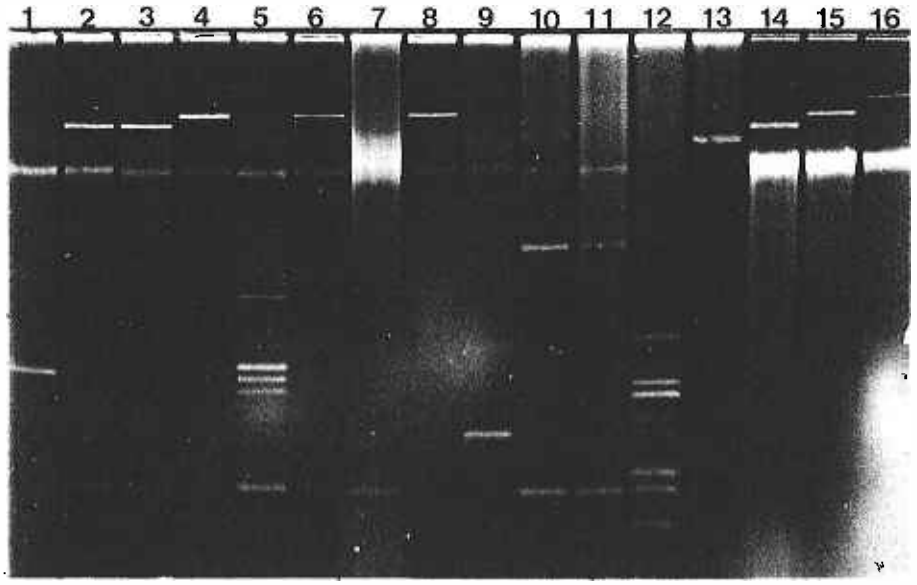

FIGURE 1:
Agarose gel electrophoresis of B. fragilis plasmid DNA and standard molecular weight markers. Lane 1: 2.5 Mdal plasmid of PL1; Lane 2: 1.5, 2.5 and 48 Mdal plasmids of PL10; Lane 3: 1.5, 2.5 and 48 Mdal plasmids of PL11; Lane 4: 60 Mdal plasmid of PL12; Lane 5: 1.5, 3.2, 3.4, 3.8 and 6.0 Mdal plasmids of PL14; Lane 6: 60 Mdal plasmid of PL15; Lane 7: 1.5 Mdal plasmid of PL17; Lane 8: 60 Mdal plasmid of PL18; Lane 9: 2.2 Mdal plasmid of PL21; Lane 10: 1.5 and 9.2 Mdal plasmids of TPL17 (PN); Lane 11: 1.5 and 9.2 Mdal plasmids of TPL17 (Hg); Lane 12: 1.24, 1.51, 1.69, 2.24, 3.03, 3.48, 5.19 Mdal plasmids of pVA517 A-H; Lane 13: 25 Mdal S-a plasmid; Lane 14: 34 Mdal RP4 plasmid; Lane 15: 60 Mdal R1 plasmid and Lane 16: 80 Mdal R62 plasmid.

TABLE 2: RESULTS OF MICs AND RESISTANCE TO HEAVY METAL IONS OF PLASMID-CONTAINING B. FRAGILIS

	Antibiotics (ug/ml)								Heavy Metal Ions				
Isolates	PY	P	PN	FOX	C	DA	E	Te	Cd^{2+} (0.001 M)	Hg^{2+} (0.001 M)	Zn^{2+} (0.1 M)	Pb^{2+} (0.1 M)	ArO_4^- (0.1 M)
PL1	> 128.0	> 128.0	> 128.0	4.0	2.0	8.0	> 128.0	64.0	R	R	R	S	S
PL10	64.0	8.0	32.0	4.0	4.0	4.0	64.0	64.0	R	R	R	R	S
PL11	64.0	8.0	32.0	4.0	4.0	4.0	64.0	64.0	R	R	R	R	S
PL12	16.0	8.0	16.0	4.0	4.0	2.0	64.0	> 128.0	R	R	R	R	S
PL14	64.0	16.0	32.0	4.0	4.0	16.0	> 128.0	> 128.0	R	R	R	R	S
PL15	64.0	16.0	32.0	4.0	4.0	2.0	128.0	2.0	R	R	R	R	S
PL17	32.0	8.0	16.0	4.0	4.0	16.0	128.0	> 128.0	R	R	R	R	S
PL18	32.0	16.0	32.0	4.0	2.0	2.0	128.0	2.0	R	R	R	R	S
PL21	16.0	16.0	16.0	4.0	2.0	0.5	128.0	128.0					
E. coli ATCC 25922	< 0.125	< 0.125	< 0.125	< 0.125	1.0	0.25	2.0	0.25					
S. aureus ATCC 25923	0.5	< 0.125	< 0.25	< 0.125	1.0	0.25	1.0	0.25					

Key: R = resistant; S = sensitive

PY	carbenicillin	C	chloramphenicol
P	penicillin	DA	clindamycin
PN	ampicillin	E	etythromycin
FOX	cefoxitin	Te	tetracycline

TABLE 3: HYDROLYSIS OF AMPICILLIN BY TRANSCONJUGANTS AND PARENTAL STRAINS OF E. COLI AND B. FRAGILIS

Enzyme source	Rate of hydrolysis of ampicillin (ug/ml/enz) extract/hr)	MIC for PN (ug/ml)
Parents: E. coli J62-1	1.2×10^2	4.0
B. fragilis PL15	3.5×10^2	32.0
B. fragilis PL17	4.2×10^2	16.0
Transconjugants*:		
TPL15 (PN)	6.5×10^2	16.0
TPL17 (PN)	7.0×10^2	64.0

*Transconjugants obtained from donor strains PL15 and PL17, selected on ampicillin plates, were designated TPL15 (PN) and TPL17 (PN) respectively. Transconjugant that acquired resistance to mercuric ion was designated TPL17 (Hg).

TABLE 4: COMPARISON OF PLASMIDS DETECTED IN B. FRAGILIS

Strain	Molecular mass (Mdal)	Phenotype	Reference
B. fragilis	2.7, 4.0, 16.0	Unknown	Stiffler et al (1)
	3.0, 23.0	Unknown	Tinnel and Macrina (2)
	2.0, 3.0, 5.0	Non-conjugative	Wallace et al (3)
	27.0	E, DA, Ln	Welch et al (5)
	2.0, 20.0	E, DA	Tally et al (7)
	3.0	ß-lactamase	
	20.0	Conjugative	Young and Mayer (9)
	1.9 to 60	Unknown	Marsh et al (8)
B. fragilis	1.5, 2.2, 2.5, 3.2, 3.4, 3.8, 6.0, 48 and 60	Unknown	Present Study

Key

Ln lincomycin

DISCUSSION

The plasmid DNAs, of molecular sizes varying from 1.5 Mdal to 60 Mdal, isolated from B. fragilis were similar to those found by other investigators (Table 4). The smaller plasmids (<10 Mdal) were non-conjugative with unknown phenotypic expressions. The phenotypes of larger plasmids (16.0 and 23.0 Mdal) were also not known. The results from our study showed similar characteristics where the phenotypic expressions of the plasmids could not be determined.

Plasmids were found to be involved in the transfer of resistance to erythromycin, clindamycin and lincomycin (5, 7). However, plasmids could not be isolated from recipients which had acquired resistance to ampicillin (9, 10). This was also observed in one of the ampicillin resistant transconjugants isolated by us, TPL 15 (PN), in which no plasmid could be detected. The other transconjugants TPL17 (PN) and TPL17 (Hg) were found to contain 2 plasmids, 1.5 and 9.2 Mdal. It could not be confirmed by transformation whether these 2 plasmids carried resistance to ampicillin or mercuric ion. The inability to isolate plasmids from the transconjugants was also observed by Young and Mayer (9) who were unable to detect the 3.0 Mdal plasmid which carried the gene that encoded ß-lactamase. mase.

The transfer of ampicillin resistance from PL15 and PL17 to E. coli K12 J62-1 also resulted in an increase in the rate of hydrolysis for ampicillin in E. coli. This may explain the higher MIC values for ampicillin in the recipient. A similar observation was made by Burt and Woods (10) in which ampicillin-resistant transconjugant of Bacteroides showed a rise in ß-lactamase activity.

We found that there was an increase in the mole-

cular weight of the plasmids in one of the transconjugants, TPL17 (PN). Hedges and Jacob (20) isolated a series of plasmid derivatives which after having acquired the ampicillin-resistant trait from the plasmid RP4 increased in molecular weight. They concluded that a transposable DNA sequence (TnA) carried the information for ampicillin-resistance (TEM ß-lactamase gene). All known TnA elements have a molecular weight of 2.8 to 3.2 Mdal and Heffron et al (21) demonstrated that the TnA is common to naturally occurring plasmids. Based on these results, there was a strong indication that the ampicillin resistance gene in PL17 was carried out on a transposon.

Richmond and Sykes (22) found that the TEM ß-lactamase gene derived from a related RP1 plasmid could integrate into the E. coli chromosome. This could explain why we were unable to isolate any plasmid from TPL15 (PN). Nakazawa and Mitsubashi (23) have isolated a transposon, Tn2011 (12.5 Mdal), which transposed as a unit resistance to ampicillin, streptomycin, sulphonamide and mercuric chloride in Haemophilus influenzae. The 2 plasmids of similar molecular sizes isolated from TPL17 (PN) and TPL17 (Hg) may indicate that a single transposon carrying resistance to ampicilin and mercuric ion was involved. However, from transformation results where only ampicillin resistance was transferred, it would be doubtful that a single transposon was involved in carrying the genes for resistance to ampicillin and mercuric ions.

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