The Journal of Medical Microbiology

Vol. 14, No. 4

TRANSFERABLE PLASMID-MEDIATED ANTIBIOTIC RESISTANCE IN *BACTEROIDES*

V. O. ROTIMI, B. I. DUERDEN AND S. HAFIZ

*Department of Medical Microbiology, University of Sheffield Medical School, Beech
Hill Road, Sheffield S10 2RX*

SUMMARY. Plasmid-mediated resistance to chloramphenicol (Chl^r), erythromycin (Ery^r), tetracycline (Tet^r) and clindamycin (Clind^r) was transferred from three clinical isolates of *Bacteroides fragilis* and one faecal isolate of *B. thetaiotaomicron* to strains of *B. fragilis*, *B. distasonis* and *Escherichia coli*, and subsequently to *B. fragilis* and *E. coli* second- and third-stage recipients in series. Successful transfer was achieved by membrane-filter and centrifugation techniques that provide stable cell-to-cell contact but not by simple mixed broth culture. Chl^r Ery^r Tet^r and Clind^r Ery^r were transferred at high frequency (1.9×10^{-3} – 1.8×10^{-4}) but Tet^r was transferred at low frequencies (1 – 1.6×10^{-6}). Segregation of resistance markers was observed with selection for Tet^r when donors were Chl^r Ery^r Tet^r and Chl^r Tet^r. All transipients were identical with the parent recipient strains but had the resistance markers of the donor strains. Resistance to antibiotics other than tetracycline was cured by growth with subinhibitory concentrations of aminoacridines and ethidium bromide for 24 h; cure of solitary Tet^r required longer incubation (21 days). Identical plasmid DNA bands were demonstrated by agarose-gel electrophoresis in all the donor and corresponding transipient strains but plasmids were not found in the recipient strains or in strains cured of resistance. Plasmid-mediated transferable antibiotic resistance in *Bacteroides* spp. may compromise the treatment of infections and may provide a reservoir of antibiotic resistance in the intestinal flora.

INTRODUCTION

There has been an increasing number of reports in recent years of the

emergence of strains of the *Bacteroides fragilis* group resistant to antibiotics previously used with success in anaerobic infections. Species in the *B. fragilis* group are innately resistant to the penicillins and aminoglycosides. Some isolates are resistant to erythromycin and tetracycline (Nastro and Finegold, 1972) but they have usually been sensitive to chloramphenicol and clindamycin. Recently Salaki *et al.* (1976) and Imamura *et al.* (1978) reported the isolation of clindamycin-resistant *B. fragilis* strains from man.

The resistance of *Bacteroides* spp. to these antibiotics may be determined by transmissible resistance (R) plasmids that are heterogeneous, autonomously replicating, extrachromosomal units of deoxyribonucleic acid (DNA). Four independent groups of investigators have recently reported transferable drug resistance in *Bacteroides* spp. (Mancini and Behme, 1977; Privitera, Dublanchet and Sebald, 1979; Tally *et al.*, 1979; Welch, Jones and Macrina, 1979).

The present study was undertaken to determine whether antibiotic resistance in the *B. fragilis* group is plasmid mediated and whether genetic determinants for chloramphenicol, erythromycin, tetracycline and clindamycin resistances can be transferred between species of *Bacteroides* and between the *B. fragilis* group and *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains

The donors were three strains of *B. fragilis* designated M2178, resistant to clindamycin (Clind^r) and erythromycin (Ery^r), M490 resistant to chloramphenicol (Chl^r), erythromycin and tetracycline (Tet^r) and W3139 resistant to tetracycline, and *B. thetaiotaomicron* strain C4/3 resistant to tetracycline and chloramphenicol. The strains were isolated from clinical specimens in the Departments of Bacteriology, Royal Hallamshire and Children's Hospitals, Sheffield. The recipient strains were: *B. fragilis* strain O58 resistant to metronidazole (Met^r) isolated from a clinical specimen in the Children's Hospital (Rotimi *et al.*, 1979); *B. distasonis* strain C1/4 isolated from normal human faeces in our laboratory and spontaneously resistant to rifampicin (Rif^r); *B. melaninogenicus* ss. *melaninogenicus* strain W515 and *B. asaccharolyticus* strain W1309 isolated from clinical specimens in the Children's Hospital; *E. coli* K-12 strain J53, plasmid-free, Rif^r, and requiring proline (pro⁻) and methionine (met⁻) for growth was obtained from Professor N. Datta, Royal Postgraduate Medical School, London; *E. coli* K-12 strain CSH₁, plasmid-free, streptomycin resistant (Str^r) and requiring tryptophan (try⁻) and thiamine (thi⁻) for growth was obtained from Dr R. J. Behme, Department of Bacteriology, University Hospital, Ontario, Canada. *B. fragilis* strain 92 containing a plasmid of mol. wt 46×10^6 was obtained from Dr M. Sebald (Institute Pasteur, Paris, France) and two strains of *E. coli* C600 containing plasmids R1 (mol. wt 62×10^6) and RP₄ (mol. wt 38×10^6) respectively were obtained from Dr J. Saunders, Department of Microbiology, University of Liverpool. The strains were identified by standard methods (Cowan, 1974; Duerden *et al.*, 1980).

Bacteriophages

A set of bacteroides phages and their propagating strains were obtained from Dr M. Sebald. Nine phages designated B, C, E, F, K, N, O, ST and W₁ were used as nonselective markers for the identification of bacteroides strains. Phage assays were done by the methods of Booth *et al.* (1979). Sensitivity of test strains to the nine phages was determined by the agar-overlay method

with phage suspensions containing 10^8 plaque forming units (pfu/ml); sensitive strains were indicated as + and resistant strains as —.

Culture media. The liquid medium for all transfer experiments was a modification of the BM medium of Nash (see Deacon, Duerden and Holbrook, 1978) supplemented with menadione (1 $\mu\text{g/ml}$), haemin (5 $\mu\text{g/ml}$) and L-cysteine hydrochloride (2 $\mu\text{g/ml}$). The basic solid media were BM agar (Holbrook, Ogston and Ross, 1978), with a 0.7% soft BM agar overlay for phage typing, MacConkey Agar (Oxoid), blood agar (Oxoid Blood Agar Base No. 2 with 7% defibrinated horse blood), minimum agar supplemented with combinations of amino acids and vitamins (Davis and Mingioli, 1950) and DST Agar (Oxoid) with 5% lysed blood. The selective media were BM agar and MacConkey agar containing the following antibiotics: (i) metronidazole (5 $\mu\text{g/ml}$) plus chloramphenicol (10 $\mu\text{g/ml}$) or tetracycline (8 $\mu\text{g/ml}$) or clindamycin (5 $\mu\text{g/ml}$) or erythromycin (8 $\mu\text{g/ml}$); (ii) rifampicin (15 $\mu\text{g/ml}$) plus chloramphenicol (10 $\mu\text{g/ml}$) or tetracycline (8 $\mu\text{g/ml}$) or clindamycin (5 $\mu\text{g/ml}$) or erythromycin (8 $\mu\text{g/ml}$).

Sensitivity tests

Antimicrobial susceptibility of the bacterial strains was determined initially by the disk-diffusion method with inocula of 10^5 cfu/ml for *Bacteroides* spp. and 10^4 cfu/ml for *E. coli*. The minimum inhibitory concentrations (MICs) were determined by seeding 0.03 ml of a 10^{-2} dilution of overnight cultures of the test strains on to sets of DST-agar plates containing doubling dilutions of filter-sterilised antibiotic solutions (0.25–128 $\mu\text{g/ml}$); the MIC was the lowest concentration that did not permit visible growth of bacteria after incubation for 24 h.

Transfer experiments

Mixed-broth-culture technique. Overnight cultures of donor and recipient strains were diluted by adding 0.1 ml to 9.9 ml of fresh BM broth and incubated anaerobically for 4 h to produce actively growing cells. One ml of donor and 4.5 ml of recipient cultures were then mixed in 4.5 ml of fresh BM broth. Similarly overnight broth cultures of donor (1 ml) and recipient (4.5 ml) were mixed in 4.5 ml of fresh BM broth. The mixtures were incubated anaerobically or aerobically at 37°C for 24 h and then diluted in three serial tenfold dilutions; 0.1 ml of each dilution was plated on the appropriate selective media which were incubated at 37°C in anaerobic or aerobic conditions for up to 5 days.

Membrane-filter technique. The method of Brefort *et al.* (1977) was modified. Cultures of donor and recipient strains in BM broth were incubated anaerobically or aerobically as necessary for 24 h to a density of $c. 5 \times 10^8$ cfu/ml; 3.5 ml of donor and recipient cultures were mixed and filtered through a 0.45- μm Millipore filter (Millipore Corp., Bedford, Mass, USA). The filter was then placed on BM agar and incubated anaerobically at 37°C for 24 h. The bacterial growth was harvested in 1 ml of BM broth and the suspensions plated on selective media that permitted only the growth of transipients. A series of nine tenfold dilutions of the suspensions were made and 0.1 ml of each dilution was plated on media selective for the donor or the transipient cells; the selective media were incubated anaerobically or aerobically at 37°C for up to 5 days. The frequency of transfer was calculated as the number of transipient cells per donor per ml of mating mixture. In experiments with *B. melaninogenicus* and *B. asaccharolyticus* as recipients, mating mixtures were plated on media that would allow growth of the donor and the potential transipients, and selection of transipients was attempted by examination for pigment production.

Centrifugation technique. Five ml of 24-h cultures of donor and recipient strains in BM medium were mixed and centrifuged at 2000 *g* for 10 min. The supernate was decanted and 1 ml of fresh BM broth was layered on top of the cell pellet without shaking so that cell-to-cell contact was not disturbed. After anaerobic incubation for 24 h, 0.1 ml of the mixture was plated on appropriate selective media and incubated anaerobically or aerobically at 37°C for up to 5 days.

In all successful mating experiments, transipients were subcultured twice on selective media before transfer to plain BM agar or MacConkey agar for phenotypic tests.

Control experiments. A series of experiments was undertaken to eliminate the possibility of

transfer by transformation or transduction: (i) 100 µg/ml of type I DNAase (Sigma Chemicals Co., Poole, Dorset) with endonuclease and exonuclease activity was added to mating mixtures and these were also plated on BM selective media containing DNAase 100 µg/ml; (ii) cultures of resistant donor strains in BM medium were filtered through 0.45-µm Millipore filters and the cell-free filtrates were incubated anaerobically and aerobically at 37°C for 24 h with sensitive recipient strains before plating on appropriate selective media; (iii) lysates of resistant donor strains were obtained by ultrasonication at an amplitude of 9 µm for 10 min with a 4-mm probe (MSE, Spenser Street, London, S.W.1) of cells harvested from 24-h cultures in BM medium. These lysates were mixed with suitable recipients and incubated anaerobically at 37°C for 24 h; 0.1 ml of the mixture was plated on selective media and incubated for up to 7 days; (iv) specific phages from the typing set were propagated on donors and transipients with resistance markers and then the phage preparations were added at a concentration of 10⁹ pfu/ml to suitable recipient cultures and incubated anaerobically for 24 h before plating on the appropriate selective media.

Curing experiments

Resistant donor and transipient strains were grown in BM media plus cooked-meat particles with acriflavine, acridine orange or ethidium bromide 16 µg/ml; this was found to be the subinhibitory concentration of each agent in a pilot experiment (Rotimi and Duerden, 1980). The three test media and a control plain broth were seeded with *c.* 5 × 10⁴ cfu/ml of the test strains and the resulting four cultures were subcultured daily on to appropriate selective media for various periods up to 21 days.

Demonstration of plasmid DNA

Plasmid DNA was extracted from the cells by the salt-precipitation method of Guerry, Leblanc and Falkow (1973). The DNA was resuspended in TES buffer and used immediately or stored at -20°C in 25-µl portions. DNA samples were analysed by electrophoresis at 60 mA and 120 V in vertical 0.7% agarose gels (Power Supply, Model 2103, LKB, Addington Road, South Croydon, Surrey). The wells were loaded with 25 µl of the DNA extract mixed with ethidium bromide (5 µg/ml) and 5 µl of bromophenol-blue solution. After electrophoresis, for *c.* 2 h or until the bromophenol blue had reached the bottom of the gel, the plasmid bands were viewed under UV light (Model C-62, Blak-Ray Transilluminator, Ultra-Violet Products Inc., San Gabriel, Ca 91778, USA), and photographed on Polaroid film type 107c with a Polaroid MP-4 Land Camera, model 44-03 (Polaroid Corp., Cambridge, Mass, USA) and a skylight filter No. 1A and a red filter No. 25 (Eastman Kodak Company, Rochester, NY, USA).

RESULTS

Transfer of antibiotic-resistance markers

The bacterial strains used as donors and recipients and their selected and non-selected phenotypic markers are shown in table I. Transfer of antibiotic resistance was not achieved with any of the donors in simple mixed-broth cultures. Successful transfer of antibiotic resistance from donor to recipient strains was obtained by the membrane-filter and the centrifugation methods. Identical results were obtained with the two methods in parallel experiments with three mating mixtures: *B. fragilis* strain M2178 with *B. fragilis* strain O58 or *B. distasonis* strain C1/4, and *B. fragilis* strain M490 with *B. fragilis* strain O58. Subsequently only the centrifugation method was used. The transfer frequencies quoted in the results are those of single experiments and are

TABLE I
Characteristics of test strains

Species	Strain no.	Resistance markers	Unselected markers					
			Φsen	lac	pro	met	try	thi
Donors								
<i>B. fragilis</i>	M2178	Clind, Ery	—	...	...	...	...	...
<i>B. fragilis</i>	M490	Chl, Ery, Tet	+	...	...	...	...	...
<i>B. fragilis</i>	W3139	Tet	—	...	...	...	...	...
<i>B. thetaiotaomicron</i>	C4/3	Chl, Tet	—	...	...	...	...	...
Recipients								
<i>B. fragilis</i>	O58	Met	+	...	...	...	...	...
<i>B. distasonis</i>	C1/4	Rif	—	...	...	...	...	...
<i>B. melaninogenicus</i>								
ss. <i>melaninogenicus</i>	W515	...	—	...	...	...	...	...
<i>B. asaccharolyticus</i>	W1309	...	—	...	...	...	...	...
<i>E. coli</i> K-12	J53	Rif	...	+	+	+	—	—
<i>E. coli</i> K-12	CSH ₁	Sm	...	—	—	—	+	+

Clind = clindamycin, Ery = erythromycin, Chl = chloramphenicol, Tet = tetracycline, Met = metronidazole, Rif = rifampicin, Sm = streptomycin.

Φsen = phage sensitivity, lac = lactose fermentation, pro = proline, met = methionine, try = tryptophan, thi = thiamine requirement.

representative of the very similar results obtained in three or four experiments with each mating pair.

Bacteroides to *Bacteroides* transfer. Antibiotic-resistance markers were transferred consistently from donor strains *B. fragilis* M2178, M490 and W3139 to recipient strains *B. fragilis* O58 and *B. distasonis* C1/4. The resultant transipients are shown in table II. Mating mixtures of *B. fragilis* strain M2178 Clind^r Ery^r with *B. fragilis* strain O58 or *B. distasonis* strain C1/4 yielded transipients that were Clind^r Ery^r but were otherwise phenotypically identical with the parent recipient strains. Clind^r and Ery^r were generally

TABLE II
Transfer of antibiotic resistance from *Bacteroides* spp. to other *Bacteroides* spp. and to *E. coli*

Donor strain	Recipient strain	Resistance markers transferred	Frequency of transfer*
<i>B. fragilis</i> M2178	<i>B. fragilis</i> O58	Clind Ery	1.8×10^{-4}
	<i>B. distasonis</i> C1/4	Clind Ery	7.8×10^{-5}
<i>B. fragilis</i> M490	<i>B. fragilis</i> O58	Chl Ery Tet/Tet	1.9×10^{-3}
	<i>E. coli</i> J53	Chl Tet/Tet	2.4×10^{-4}
	<i>E. coli</i> CSH ₁	Chl Tet/Tet	1.7×10^{-4}
	<i>B. fragilis</i> O58	Chl Tet/Tet	1.5×10^{-3}
<i>B. thetaiotaomicron</i> C4/3	<i>E. coli</i> J53	Chl Tet/Tet	2.2×10^{-6}
	<i>E. coli</i> CSH ₁	Chl Tet/Tet	1.3×10^{-6}
	<i>B. fragilis</i> O58	Tet	1.6×10^{-6}
	<i>E. coli</i> J53	Tet	1.1×10^{-6}
<i>B. fragilis</i> W3139	<i>E. coli</i> CSH ₁	Tet	1.0×10^{-6}

Abbreviations as in table I.

* Number of transipients per donor cell per ml of mating mixture.

transferred together to *B. fragilis* strain O58 at a high frequency of 1.8×10^{-4} whereas transfer to *B. distasonis* strain C1/4 was at a slightly lower frequency of 7.8×10^{-5} . Segregation of Clind^r and Ery^r was not observed in transcipts selected for either Clind^r or Ery^r; transcipts derived from the M2178–O58 mating mixtures were always Met^r Clind^r Ery^r and those from the M2178–C1/4 mating mixtures were Rif^r Clind^r Ery^r.

Resistance was transferred readily from *B. fragilis* strain M490 Chl^r Ery^r Tet^r to *B. fragilis* strain O58 at a consistently high frequency of 1.9×10^{-3} . There was no segregation when transcipts were selected for either Chl^r or Ery^r on plates containing metronidazole plus chloramphenicol or erythromycin; all transcipts were Met^r Chl^r Ery^r Tet^r. However, there was segregation of resistance markers to yield Met^r Chl^r Ery^r Tet^r and Met^r Chl^s Ery^s Tet^r transcipts when selection was for Tet^r; the majority of these transcipts were Met^r Chl^s Ery^s Tet^r. The frequency of transfer when transcipts were selected for Tet^r was generally lower ($2-7.8 \times 10^{-5}$) than when Chl^r or Ery^r was selected.

Bacteroides to E. coli transfer. The Chl^r Tet^r resistance markers were transferred from *B. fragilis* strain M490 to *E. coli* K12 strains J53 and CSH₁ at a fairly high frequency ($1.7-2.4 \times 10^{-4}$) and the same pattern of segregation occurred as with transfer from strain M490 to *B. fragilis* strain O58: with selection for Chl^r, all transcipts were Chl^r Tet^r, whereas with selection for Tet^r a mixture of Chl^s Tet^r and Chl^r Tet^r transcipts were obtained, with Chl^s Tet^r strains predominant. Similarly, the Chl^r Tet^r resistance markers were transferred from *B. thetaiotaomicron* strain C4/3 to both *E. coli* recipients, with the same pattern of segregation, but at a lower frequency of $1-2 \times 10^{-6}$. The solitary Tet^r marker was also transferred from *B. fragilis* strain W3139 to both *E. coli* recipients at a low frequency (1×10^{-6}). The transfer of the Clind^r Ery^r markers to *E. coli* K12 could not be assessed because the two recipient strains were resistant to erythromycin and clindamycin. However, transfer of the Ery^r marker from *B. fragilis* strain M490 Chl^r Ery^r Tet^r to *E. coli* was inferred from the results of a subsequent mating experiment in which resistance was transferred from an *E. coli* transcipt carrying the Chl^r Tet^r marker from strain M490 to *B. fragilis* strain O58 (see below). The resultant *B. fragilis* transcipt had the Chl^r Ery^r Tet^r markers.

Serial transfer of resistance. In another series of experiments, resistance markers were transferred from the first-stage transcipts (above) to sensitive *E. coli* K12 recipients and from them back into sensitive *B. fragilis* strain O58 recipients. For example, the resistance markers from *B. fragilis* strain M490 Chl^r Ery^r Tet^r were transferred to *B. fragilis* strain O58, then from this O58 Chl^r Ery^r Tet^r transcipt to *E. coli* K12 strain CSH₁; from CSH₁ Chl^r Tet^r (Ery^r) the resistance markers were transferred to *E. coli* K12 strain J53 (Rif^r) and from this Chl^r Tet^r (Ery^r) transcipt back to a sensitive *B. fragilis* strain O58 recipient; all three markers were expressed in the final transcipt.

When the transcipts were characterised by conventional methods and by the use of the unselected markers, all were phenotypically identical with their parent recipient strains except that they had now acquired the resistance

TABLE III
Characteristics of transcient strains

Strain no.	Number of colonies tested	Species	Resistance markers							Unselected markers					
			Clin	Ery	Chl	Tet	Met	Rif	Sm	Φsen	lac	pro	met	try	thi
O58/M2178	10	<i>B. fragilis</i>	R	R	...	...	R	S	...	+	...	...	...	...	...
C1/4/M2178	12	<i>B. distasonis</i>	R	R	...	...	S	R	...	—	...	...	...	...	...
O58/M490	10	<i>B. fragilis</i>	S	R	R	R	R	S	...	+	...	...	...	...	...
O58/W3139	5	<i>B. fragilis</i>	S	S	S	R	R	S	...	+	...	...	...	...	...
O58/C4/3	6	<i>B. fragilis</i>	S	S	R	R	R	S	...	+	...	...	...	...	...
J53/M490	10	<i>E. coli</i>	...	...	R	R	...	R	S	...	+	+	+	—	—
CSH ₁ /M490	10	<i>E. coli</i>	...	...	R	R	...	S	R	...	—	—	—	+	+
J53/C4/3	10	<i>E. coli</i>	...	...	R	R	...	R	S	...	+	+	+	—	—
CSH ₁ /C4/3	10	<i>E. coli</i>	...	...	R	R	...	S	R	...	—	—	—	+	+
J53/W3139	15	<i>E. coli</i>	...	...	S	R	...	R	S	...	+	+	+	—	—
CSH ₁ /W3139	15	<i>E. coli</i>	...	...	S	R	...	S	R	...	—	—	—	+	+

R = resistance, S = sensitive; other abbreviations as in table I.

markers of the donors. The characteristics of the transcipts are shown in table III. All those derived from *B. fragilis* strain O58 were Met^r and sensitive to the same seven phages as the parent. Transcipts derived from *E. coli* K12 strain K53 (Rif^r) fermented lactose and required proline and methionine and those derived from *E. coli* K12 strain CSH₁ did not ferment lactose and required tryptophan and thiamine.

TABLE IV
Minimum inhibitory concentrations for donor, recipient and transcient strains

Species	Strain no.	Minimum inhibitory concentration (μg/ml) of						
		Chl	Tet	Ery	Clind	Met	Rif	Sm
Donors								
<i>B. fragilis</i>	M2178	1	2	64	64	...	...	...
<i>B. fragilis</i>	M490	32	> 128	64	< 0.25	...	...	...
<i>B. thetaiotaomicron</i>	C4/3	64	32	1	1	...	...	...
<i>B. fragilis</i>	W3139	< 0.25	> 128	0.5	1	...	...	...
Recipients								
<i>B. fragilis</i>	O58	1	2	0.5	< 0.25	32	...	...
<i>B. distasonis</i>	C1/4	1	1	0.5	0.5	0.5	> 128	...
<i>E. coli</i>	J53	0.5	1	32	32	...	> 128	...
<i>E. coli</i>	CSH ₁	1	2	32	32	...	...	64
Transcipts								
<i>B. fragilis</i>	O58/M2178	1	1	64	64	32	...	...
<i>B. fragilis</i>	O58/M490	32	64	64	0.25	32	...	...
<i>B. fragilis</i>	O58/C4/3	64	64	0.5	0.5	32	...	...
<i>B. fragilis</i>	O58/W3139	1	128	0.5	0.5	32	...	...
<i>B. distasonis</i>	C1/4/M2178	1	1	64	32	...	> 128	...
<i>E. coli</i>	J53/M490	64	64	32	32	...	> 128	...
<i>E. coli</i>	CSH ₁ /M490	64	128	32	32	...	...	64
<i>E. coli</i>	J53/C4/3	64	32	32	16	...	> 128	...
<i>E. coli</i>	J53/W3139	1	128	32	32	...	> 128	...

Abbreviations as in table I.

Minimum inhibitory concentrations. The MICs of the test antibiotics for the donor, recipient and transcient strains are shown in table IV. In each case the MICs for the donor and the transcipients that had acquired resistance from it were the same.

Curing of antibiotic resistance

Resistance to erythromycin and clindamycin was eliminated *en bloc* from *B. fragilis* strain M2178 and transcient strain *B. fragilis* O58/M2178 after growth in liquid media containing subinhibitory concentrations (16 µg/ml) of acriflavine, acridine orange or ethidium bromide for 24 h. Similarly the Chl^r Ery r marker was readily eliminated from *B. fragilis* strain M490 and the Chl^r marker from *B. thetaiotaomicron* strain C4/3 but elimination of the Tet r marker from these donor strains required longer incubation. Tet r was eliminated only after incubation for 18–21 days with acriflavine or ethidium bromide; it was not eliminated by incubation with acridine orange. However, Tet r was eliminated *en bloc* with the other resistance markers from the transcient strains *B. fragilis* O58/M490, *E. coli* J53/M490, *E. coli* CSH₁/M490 and *B. fragilis* O58/C4/3 by all three agents after incubation for only 24 h. Tet r was not eliminated from donor strain *B. fragilis* W3139 by any of the agents after incubation for 21 days but Tet r was eliminated from transcipients *B. fragilis* O58/W3139 and *E. coli* J53/W3139 after this period. The cured strains did not revert to resistant variants after culture on non-selective media or in plain BM broth.

Demonstration of plasmid DNA

Plasmid DNA was demonstrated in the donor and transcient strains by vertical gel electrophoresis in 0.7% agarose. The distances migrated by plasmid bands from the donor strains were compared with the distances migrated by plasmids of known molecular weights extracted from *E. coli* and *B. fragilis* reference strains. Plasmids from strains M2178 and C4/3 moved the same distance as the plasmid from *B. fragilis* strain 92 (mol. wt 46×10^6). The plasmid from *B. fragilis* strain M490 moved more slowly to a point between the plasmid from *B. fragilis* strain 92 and plasmid R1 from *E. coli* strain C600 (mol. wt 62×10^6). The plasmid from *B. fragilis* strain W3139 migrated the same distance as plasmid RP4 (mol. wt 38×10^6) from *E. coli* strain C600.

Plasmid bands were not demonstrated in extracts from any of the recipient strains. Plasmids were found in all transcient strains that had received resistance markers *en bloc* and the plasmids were identical with those of the donor strains in electrophoretic mobility. The plasmids from transcipients in which segregation of resistance markers had occurred requires further investigation. Plasmid bands were absent from extracts obtained from donor and transcient strains cured of their resistance markers.

Control experiments

The addition of type I DNAase 100 µg/ml to the three mating mixtures of

B. fragilis strain M2178 with *B. distasonis* strain C1/4 and *B. fragilis* strain O58, and *B. fragilis* strain M490 with strain O58 did not prevent the transfer of the resistance markers to the recipients with the same frequency as in the experiments described above. The same recipient strains did not acquire resistance markers after incubation with cell-free filtrates of the donor strains or with ultrasonic lysates of the donor strains. Similarly, we failed to demonstrate transduction of resistance markers by incubation of the recipient strains with phage preparations propagated on donor strain M490 and transcient strain O58/M490.

DISCUSSION

The increasing prevalence of plasmid-mediated transferable antibiotic resistance and associated pathogenicity factors has serious implications (Falkow, 1975). The *B. fragilis* group of gram-negative anaerobic bacilli form a major part of the normal intestinal flora and are also significant causes of clinical infection, especially after abdominal surgery or as a complication of intestinal pathology.

Privitera *et al.* (1979a), Tally *et al.* (1979) and Welch *et al.* (1979) demonstrated that resistance to clindamycin, erythromycin and tetracycline could be transferred between species in the *B. fragilis* group. The results of the present study confirm and extend these findings. Resistance to clindamycin, erythromycin, chloramphenicol and tetracycline was transferred between different strains of *B. fragilis*, between different species in the *B. fragilis* group such as *B. fragilis*, *B. distasonis* and *B. thetaiotaomicron*, and between the *B. fragilis* group and *E. coli*. The failure to transfer resistance to *B. melaninogenicus* and *B. asaccharolyticus* may reflect the unfortunate choice of poor recipient strains; this requires further investigation.

Segregation of resistance markers is common in enterobacteria (Watanabe and Lyang, 1962). Brefort *et al.* (1977) also showed segregation of Tet^r markers in *Clostridium perfringens*. Segregation of the Tet^r marker with loss of chloramphenicol and erythromycin resistances was common in the present study when transipients were selected for tetracycline resistance. Segregation may arise when donor cells carry two or more independent plasmids which segregate during cell division or during conjugation. Alternatively, a single plasmid may segregate or may lose markers as a result of transposition or insertion activity. These possibilities were not investigated but only a single plasmid band was observed in extracts of donor cells, and segregant plasmids with the Chl^r Ery^r but not Tet^r markers were not detected. The characteristics of Tet^r markers are often different from those of other R plasmids. Privitera, Sebald and Fayolle (1979) were unable to transfer solitary Tet^r markers without induction of resistance by prior exposure to subinhibitory levels of tetracycline, but in the present study a solitary Tet^r marker was transferred between different species of the *B. fragilis* group and *E. coli* without prior induction although transfer was at a low frequency.

The transfer of genetic material between unrelated species of bacteria may

be prevented by the activity of restriction enzymes in the recipient (Saunders, 1978). Burt and Woods (1976) were able to transfer antibiotic resistance markers from *E. coli* to *B. fragilis* only after heating the recipient at 50°C to destroy the restriction enzymes; the markers remained unstable and could not be transferred to other strains. Mancini and Behme (1977), however, demonstrated the transfer of multiple antibiotic resistance from *B. fragilis* to *E. coli* without heating. In the present study, resistance markers were transferred from *B. fragilis* to *E. coli* and thence to other *E. coli* and *B. fragilis* strains in series without heating.

Genetic information can be transferred between bacteria by conjugation, transduction or transformation. Transfer of antibiotic resistance in facultative gram-negative bacteria is by conjugation but transfer of R plasmids in *Staphylococcus aureus* is by transduction (Falkow, 1975). The results obtained in the present studies show that resistance to clindamycin, erythromycin, chloramphenicol and tetracycline in the *Bacteroides* spp. studied was plasmid mediated and that resistance markers were transferred by conjugation. There was no evidence of transformation by free DNA; transfer occurred in the presence of added DNAase and the extracellular DNAase produced by *B. fragilis* (Rudek and Haque, 1976). Moreover, resistance was not acquired by competent recipients from cell-free lysates of resistant donor strains. Attempts to transfer resistance markers by transduction were not successful. Treatment of sensitive recipient strains with phages propagated on resistant donor and transcient strains shown to harbour R plasmids did not yield any resistant transductants. Transfer was achieved only by methods that provided cell-to-cell contact. Our inability to demonstrate transfer in simple mixed liquid cultures and the successful transfer of resistance by the membrane-filter and centrifugation methods indicate that the formation of stable mating pairs may require external support. Stabilisation of mating pairs on membrane or glass surfaces would be necessary if the sex pili were particularly fragile or if conjugation required some alteration of the cell surface through contact with another surface.

The demonstration of plasmid DNA by agarose-gel electrophoresis confirms that antibiotic resistance in the bacteroides strains studied was plasmid mediated. Plasmid DNA was present in all the resistant donor and transcient strains but not in the sensitive recipients. The plasmid DNA bands obtained from the donors and the transcipients derived from them were identical. A variety of plasmids have been described in *Bacteroides* spp.; initially they were described as "cryptic" (Stiffler, Keller and Traub, 1974). Tally *et al.* (1979) and Welch *et al.* (1979) identified plasmids responsible for transferable resistance to clindamycin and erythromycin (Clind^r Ery^r). Although these plasmids had identical resistance properties, the plasmids were of different sizes, *i.e.*, mol. wt 20×10^6 (Tally *et al.*, 1979) and 27×10^6 (Welch *et al.*, 1979). The plasmid responsible for clindamycin and erythromycin resistance in the present study was different from both; it had the same electrophoretic mobility as the plasmid from *B. fragilis* strain 92 (mol. wt 46×10^6) which was also responsible for clindamycin and erythromycin

resistance (Dr M. Sébald, personal communication). The relationship of these plasmids to those found in enterobacteria and other facultative bacteria has yet to be determined.

The results of this study have shown that antibiotic resistance in *Bacteroides* spp. in the intestinal flora may be transferred to a variety of opportunist pathogens especially under selective pressures such as the use of prophylactic antibiotics in colonic surgery. Clarke *et al.* (1977) suggested erythromycin for prophylaxis but Tally *et al.* (1979) have shown that erythromycin selects for clindamycin resistance and the results of the present study and those of Privitera *et al.* (1979a) showed that Ery^r and Clind^r markers were transferred together. The transfer of clindamycin resistance is important; clindamycin is one of the antibiotics of choice for the treatment of bacteroides infections, and the spread of clindamycin resistance may limit the choice of antibiotic therapy for serious infections. Similarly the use of long-term tetracycline therapy for acne vulgaris selects for multiply resistant faecal organisms (Datta *et al.*, 1971; Møller *et al.*, 1977).

The presence in the *B. fragilis* group of R plasmids that may be transferred between members of the group and between the *B. fragilis* group and other intestinal commensals such as *E. coli* may compromise the treatment of individual bacteroides infections and may also provide a reservoir of antibiotic resistance in the intestinal flora.

We thank Professor M. G. McEntegart for his encouragement. V.O.R. is grateful to the Wellcome Trust for financial support. We thank May and Baker Ltd, Dagenham, Essex (metronidazole), Parke, Davis and Co., Pontypool, Gwent (chloramphenicol), Abbot Laboratories Ltd, Queensborough, Kent (erythromycin) and Upjohn Ltd, Crawley, Sussex (clindamycin) for providing the antibiotic powders.

REFERENCES

- BOOTH, S. J., VAN TASSELL, R. L., JOHNSON, J. L. AND WILKINS, T. D. 1979. Bacteriophages of bacteroides. *Rev. infect. Dis.*, **1**, 325.
- BREFORT, G., MAGOT, M., IONESCO, H. AND SEBALD, M. 1977. Characterisation and transferability of *Clostridium perfringens* plasmids. *Plasmid*, **1**, 52.
- BURT, S. J. AND WOODS, D. R. 1976. R factor transfer to obligate anaerobes from *Escherichia coli*. *J. gen. Microbiol.*, **93**, 405.
- CLARKE, J. S., CONDON, R. E., BARTLETT, J. G., GORBACH, S. L., NICHOLS, R. L. AND OCHI, S. 1977. Pre-operative oral antibiotics reduce septic complications of colon operations: results of prospective, randomized, double-blind clinical study. *Ann. Surg.*, **186**, 251.
- COWAN, S. T. 1974. Cowan and Steel's manual for the identification of medical bacteria, 2nd ed., Cambridge University Press, Cambridge.
- DATTA, N., FAIERS, M. C., REEVES, D. S., BRUMFITT, W., ØRSKOV, F. AND ØRSKOV, I. 1971. R factors in *Escherichia coli* in faeces after oral chemotherapy in general practice. *Lancet*, **1**, 312.
- DAVIES, B. D. AND MINGIOLI, E. S. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. *J. Bact.*, **60**, 17.
- DEACON, A. G., DUERDEN, B. I. AND HOLBROOK, W. P. 1978. Gas-liquid chromatographic analysis of metabolic products in the identification of Bacteroidaceae of clinical interest. *J. med. Microbiol.*, **11**, 81.
- DUERDEN, B. I., COLLEE, J. G., BROWN, R., DEACON, A. G. AND HOLBROOK, W. P. 1980. A

- scheme for the identification of clinical isolates of gram-negative anaerobic bacilli by conventional bacteriological tests. *J. med. Microbiol.*, **13**, 231.
- FALKOW, S. 1975. Infectious multiple drug resistance, Pion, London.
- GUERRY, P., LEBLANC, D. J. AND FALKOW, S. 1973. General method for the isolation of plasmid deoxyribonucleic acid. *J. Bact.*, **116**, 1064.
- HOLBROOK, W. P., OGSTON, S. A. AND ROSS, P. W. 1978. A method for the isolation of *Bacteroides melaninogenicus* from the human mouth. *J. med. Microbiol.*, **11**, 203.
- IMAMURA, H., WATANABE, K., EZAKI, T., SAWA, K., MOCHIZUKI, I., KOBAYASHI, T., KOUBATA, S., NINOMIYA, K., UENO, K. AND SUZUKI, S. 1978. Appearance of clindamycin-resistant *Bacteroides fragilis* in faeces of patients administered clindamycin. In Current chemotherapy, Proceedings of 10th international congress of chemotherapy, vol. I, edited by W. Siegenthaler and R. Lüthy, American Society for Microbiology, Washington, DC, p. 304.
- MANCINI, C. AND BEHME, R. J. 1977. Transfer of multiple antibiotic resistance from *Bacteroides fragilis* to *Escherichia coli*. *J. infect. Dis.*, **136**, 597.
- MØLLER, J. K., BAK, A. L., STENDERUP, A., ZACHARIAE, H. AND AFZELIUS, H. 1977. Changing patterns of plasmid-mediated drug resistance during tetracycline therapy. *Antimicrob. Agents Chemother.*, **11**, 388.
- NASTRO, L. J. AND FINEGOLD, S. M. 1972. Bactericidal activity of five antimicrobial agents against *Bacteroides fragilis*. *J. infect. Dis.*, **126**, 104.
- PRIVITERA, G., DUBLANCHET, A. AND SEBALD, M. 1979a. Transfer of multiple antibiotic resistance between subspecies of *Bacteroides fragilis*. *J. infect. Dis.*, **139**, 97.
- PRIVITERA, G., SEBALD, M. AND FAYOLLE, F. 1979b. Common regulatory mechanism of expression and conjugative ability of a tetracycline resistance plasmid in *Bacteroides fragilis*. *Nature, Lond.*, **278**, 657.
- ROTIMI, V. O. AND DUERDEN, B. I. 1980. Curing of antibiotic resistance in *Bacteroides* species by aminoacridines and ethidium bromide. *Afr. J. med. Sci.*, **8**, 1.
- ROTIMI, V. O., DUERDEN, B. I., EDE, V. AND MACKINNON, A. E. 1979. Metronidazole-resistant *Bacteroides* from untreated patient. *Lancet*, **1**, 833.
- RUDEK, W. AND HAQUE, R. U. 1976. Extracellular enzymes of the genus *Bacteroides*. *J. clin. Microbiol.*, **4**, 458.
- SALAKI, J. S., BLACK, R., TALLY, F. P. AND KISLAK, J. W. 1976. *Bacteroides fragilis* resistant to the administration of clindamycin. *Am. J. Med.*, **60**, 426.
- SAUNDERS, J. R. 1978. Anaerobes and transferable drug resistance. *Nature, Lond.*, **274**, 113.
- STIFFLER, P. W., KELLER, R. AND TRAUB, N. 1974. Isolation and characterization of several cryptic plasmids from clinical isolates of *Bacteroides fragilis*. *J. infect. Dis.*, **130**, 544.
- TALLY, F. P., SNYDMAN, D. R., GORBACH, S. L. AND MALAMY, M. H. 1979. Plasmid-mediated transferable resistance to clindamycin and erythromycin in *Bacteroides fragilis*. *J. infect. Dis.*, **139**, 83.
- WATANABE, T. AND LYANG, K. W. 1962. Episome-mediated transfer of drug resistance in enterobacteriaceae. V. Spontaneous segregation and recombination of resistance factors in *Salmonella typhimurium*. *J. Bact.*, **84**, 422.
- WELCH, R. A., JONES, K. R. AND MACRINA, F. L. 1979. Transferable lincosamide-macrolide resistance in *Bacteroides*. *Plasmid*, **2**, 261.