CHARACTERISATION OF A β -LACTAMASE OBTAINED FROM A STRAIN OF *BACTEROIDES FRAGILIS* RESISTANT TO β -LACTAM ANTIBIOTICS

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WHEN the antibiotic resistance patterns of ten clinical isolates of *Bacteroides fragilis* were determined it was noted that one strain was exceptionally resistant to β -lactam antibiotics. This highly resistant strain produced a β -lactamase, which was absent from the other moderately resistant strains. It was therefore investigated further because there has been no detailed study of β -lactamases from *Bacteroides* spp.

Bacteroides fragilis and related species form the predominant flora of the human large intestine (Drasar, Shiner and McLeod, 1969). They are usually opportunist pathogens, and infection frequently occurs by autoinfection from the gastro-intestinal tract (Marcoux *et al.*, 1970; Felner and Dowell, 1971) or from the female genital tract (Gelb and Seligman, 1970; Ledger, Sweet and Headington, 1971). *B. fragilis* is moderately or very resistant to many of the common antibiotics (Bodner, Koenig and Goodman, 1970; Kislak, 1972). This organism also provides a large reservoir of antibiotic-resistant organisms in the body and might constitute a source of resistance for other organisms if the genes concerned were transferable. Because resistance transfer has not yet been reported for *Bacteroides* spp., an attempt was made to show resistance transfer from *B. fragilis* to *Escherichia coli* and vice versa, and also from clinical isolates of *B. fragilis* to genetically labelled laboratory strains of this organism.

METHODS

Organisms. Ten isolates of *B. fragilis* were obtained from clinical material at the Bristol Royal Infirmary. Isolate no. 1 was obtained from the blood of a patient who had just completed a 7-day course of ampicillin; the others were from wounds, abscesses and blood.

Suitable laboratory strains of *E. coli*, were used in antibiotic-resistance-transfer studies, some containing appropriate repressed or derepressed R factors, with or without chromosomally determined rifampicin or nalidixic acid resistance.

Media. All culture plates were prepared with Oxoid DST Agar (Oxoid Ltd, London S.E.1) supplemented with saponin-lysed oxalated horse blood (5 per cent.). Antibiotics were incorporated into the medium by the addition of appropriate amounts of sterile solutions of rifampicin, ampicillin, cephaloridine, chloramphenicol, streptomycin, tetracycline, and sulphathiazole, and sterile suspensions of nalidixic acid. A cysteine-serum broth (Hofstad and Kristoffersen, 1971) was used for liquid cultures.

Carbohydrate-fermentation reactions were studied in a medium containing Bactopeptone (Difco Laboratories, Detroit), 1 per cent.; sodium chloride 0.5 per cent.; horse serum,

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10 per cent.; Difco Bacto Agar, 0.2 per cent.; pH adjusted to 7.6. Gelatinase activity was determined with formalised charcoal-gelatin disks (Kohn, 1953) in nutrient broth supplemented with 10 per cent. horse serum. The ability to produce hydrogen sulphide was tested in a medium containing cysteine (Difco, 1964) by means of lead acetate papers.

Methods of anaerobic culture. Plate cultures of *B. fragilis* were incubated at 37°C in an atmosphere of hydrogen (98 per cent.) and carbon dioxide (2 per cent.) in anaerobic jars containing Deoxo Capsules (Baird & Tatlock Ltd, Chadwell Heath, Essex). Cultures of *B. fragilis* for β -lactamase studies was obtained by growth in cysteine-serum broth medium (100-ml portions in 250-ml bottles). The use of a heavy inoculum (c. 5×10^7 organisms per ml) obviated the need for specific anaerobic methods. The bottles were gently rotated at intervals during incubation at 37°C for 30 hr and yielded cultures containing about 10^9 viable organisms per ml.

Induction of β -lactamase activity was attempted in this liquid medium with three of the isolates of *B. fragilis* by the addition of ampicillin at an initial concentration of about one-half the minimum inhibitory concentration after growth had continued for 24 hr. During further incubation for 6 hr the number of viable organisms increased three- to four-fold.

Antibiotic-sensitivity testing. The sensitivity of *E. coli* and *B. fragilis* strains to some antibiotics was determined by subculture on to series of plates of medium into which an antibiotic had been incorporated at c. two-fold dilution steps. Each culture was tested at a range of bacterial-cell densities, so that "single cell" antibiotic inhibitory concentrations could be determined. Sensitivity tests with other antibiotics were performed with antibiotics incorporated into 6-mm-diameter filter-paper disks placed on a "lawn" inoculum on lysed blood agar plates.

Treatment of organisms for β -lactamase studies. Cultures of *B. fragilis* in the liquid medium were centrifuged, and the pellet of organisms was resuspended in 0.1M-phosphate buffer, pH 5.9, centrifuged again and dispersed in phosphate buffer as a suspension containing 10 mg dry weight of organisms per ml (about thirty times the density of the original culture). The concentrated bacterial suspension was treated for 5 min. at 4°C in an ultrasonic disintegrator (Dawe Instruments Ltd, Beckenham, Kent) set at 3 amp and 25,000 Hz. The disintegrated material was centrifuged at 35,000g at 4°C for 30 min. The supernate constituted the β -lactamase preparation and the precipitated cell-debris possessed no measurable β -lactamase activity.

Assay of β -lactamase. Rates of hydrolysis of penicillins were determined at 30°C by the micro-iodometric method of Sykes and Nordström (1972). The hydrolysis of cephaloridine was also studied at 37°C by a spectrophotometric method (O'Callaghan, Muggleton and Ross, 1969). β -Lactamase activity has been expressed in International Units (IU). One IU of enzyme will hydrolyse 1 mole of β -lactam antibiotic per min. at 30°C and pH 5.9.

Inhibition of β -lactamase. Inhibition of the hydrolysis of a 10^{-4} -M solution of cephaloridine by 10^{-4} -M solutions of substrate analogues was studied by the spectrophotometric method (O'Callaghan *et al.*, 1968).

Antibiotic resistance transfer. All organisms were grown in cysteine broth medium. Appropriate pairs of potential donor and recipient organisms were usually mixed in a 1 to 1 ratio and incubated for 24 hr under conditions that permitted further growth. In each individual experiment about 10^{10} total viable organisms were streaked on to blood agar plates containing suitable antibiotics. The plates were incubated aerobically or anaerobically as appropriate. The antibiotic resistance pattern of any transipients was confirmed on subculture.

RESULTS AND DISCUSSION

Characteristics of isolates of B. fragilis

All ten organisms were strictly anaerobic, non-motile, non-pigmented Gram-negative bacilli of similar morphological and colonial appearance and

produced α -haemolysis on horse-blood agar in 2 to 3 days. All failed to produce indole, to hydrolyse gelatin in 7 days, or to form hydrogen sulphide in 3 days. In peptone-serum-glucose medium, all produced acid with a trace of gas; the final pH of the medium always lay between 4.5 and 4.8. All produced acid only from sucrose and none acidified mannitol. Little or no acid, and no gas, was formed in media containing either lactose or maltose.

These results suggest that the isolates were all of the same species and although the classification of *Bacteroides* spp. is unsatisfactory (Spiers, 1971) the observed characteristics closely fit those of *B. fragilis* (Breed, Murray and Smith, 1957).

TABLE
Minimum inhibitory concentration of cephaloridine and ampicillin for
ten isolates of *Bacteroides fragilis*

<i>B. fragilis</i> isolate number	Minimum inhibitory concentration (μ g per ml)* of	
	cephaloridine	ampicillin
1	250	100
2	20	5
3	4	2.5
4	25	5
5, 6, 7	25	7
8, 9	25	8
10	10	3

* Means of triplicate determinations.

When tested by the filter-paper disk technique, all isolates were completely resistant to kanamycin (1000 μ g) and benzyl-penicillin (1.5 μ g), very resistant to colistin (10 μ g) and neomycin (1000 μ g), and sensitive to erythromycin (5 μ g) and rifamide (15 μ g). This characteristic sensitivity pattern confirms that all isolates were strains of *B. fragilis* (Sutter and Finegold, 1971).

Sensitivity to various antibiotics. In addition to the antibiotic-disk tests described above, which were primarily intended to aid identification, other antibiotics were tested to see which might be useful in resistance-transfer experiments. The observed minimum inhibitory concentrations (MICs) were: chloramphenicol 1 to 5 μ g per ml; tetracycline 1 to 5 μ g per ml; sulphathiazole 5 to 20 μ g per ml; and streptomycin more than 100 μ g per ml. Unfortunately there was no marked difference in the antibiotic sensitivities of the various isolates except to β -lactam antibiotics. Because the β -lactam antibiotics were chosen for detailed resistance-transfer and enzymic studies the MICs of cephaloridine and ampicillin for individual strains of *B. fragilis* are shown in the table. Sensitivities were determined at approximately doubling dilutions between 1 and 500 μ g per ml; there was excellent agreement between triplicate determinations.

Studies on the β -lactamase of B. fragilis

Holt and Stewart (1964) found neither β -lactamase nor amidase activity in eight unspecified strains of *Bacteroides*, but Garrod (1955) suspected penicillinase activity in 2 of 8 isolates of *B. fragilis*. Furthermore, Pincus, Veto and Braude (1968) found that 5 of 18 isolates of *B. fragilis* destroyed benzyl-penicillin and 2 of 18 destroyed ampicillin in a microbiological test system, and that the ability to destroy these two penicillins tended to be associated with a high degree of resistance to these antibiotics. They also showed by chromatographic examination of the hydrolysis products of benzyl-penicillin, ampicillin, and oxacillin that penicillin destruction was due to a β -lactamase without any detectable amidase activity.

The β -lactamase activities (per mg dry weight of organisms) of the supernate after sonic disintegration of isolate no. 1 were: 130×10^{-4} IU against cephaloridine; 83×10^{-4} IU against benzyl-penicillin and 15×10^{-4} IU against ampicillin. In contrast, the corresponding preparations from isolates 2 to 10, which were relatively sensitive to β -lactam antibiotics, contained no detectable β -lactamase activity (less than 4×10^{-4} IU) against any of those substrates.

Whole-cell suspensions and cell debris after sonic disintegration of all the isolates showed no detectable β -lactamase activity. Growth in media supplemented with ampicillin did not increase the level of β -lactamase activity of any of the strains that were tested. Cephalosporinase activity of the soluble β -lactamase preparation from *B. fragilis* isolate no. 1, was inhibited by carbenicillin, cloxacillin, and ampicillin but not by ampicillin or benzyl-penicillin.

The production of only a small amount of β -lactamase is sufficient to confer a high degree of penicillin resistance upon the aerobic Gram-negative bacilli (Percival, Brumfitt and de Louvois, 1963). The behaviour of *B. fragilis* appears to be analogous to that of the aerobic Gram-negative bacilli in that a moderate resistance to β -lactam antibiotics may be increased considerably by the acquisition of β -lactamase activity.

Attempts to transfer antibiotic resistance to and from B. fragilis

No detectable transfer of ampicillin resistance occurred (transfer frequency less than 2×10^{-10} per donor organism) from *B. fragilis* isolate no. 1 to four rifampicin-resistant mutants of this organism. Likewise there was no detectable transfer of streptomycin resistance from three strains of *B. fragilis* to two strains of *E. coli* or of various repressed or de-repressed R factors from strains of *E. coli* to four strains of *B. fragilis*. Transfer of repressed and de-repressed R factors occurred freely between two pairs of suitable strains of *E. coli* in the test system despite the anaerobic conditions.

SUMMARY

The minimum inhibitory concentration of six antibiotics for ten clinical isolates of *Bacteroides fragilis* was determined. One of the isolates, which was unusually resistant to ampicillin and cephaloridine, possessed an intracellular

β -lactamase. This enzyme, which was more active against cephaloridine than against the penicillins, was characterised. No β -lactamase activity could be detected in any of the moderately resistant strains, or in whole-cell preparations, or culture filtrates. The production of β -lactamase was not induced in resistant or sensitive strains by growth in media containing ampicillin.

The above evidence supports the view that strains of *B. fragilis* that are highly resistant to β -lactam antibiotics owe this property to the presence of an intracellular β -lactamase.

Attempts to transfer antibiotic resistance from *B. fragilis* to *Escherichia coli* and vice versa, and from one strain of *B. fragilis* to another, were unsuccessful.

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