Anita C. Parker. THE CLONING AND CHARACTERIZATION OF A CEFOXITIN RESISTANCE DETERMINANT IN <u>BACTEROIDES VULGATUS</u> CLA341. (Under the direction of Dr. C. Jeffery Smith) Department of Biology, April 1992.

A clinical isolate of *Bacteroides vulgatus*, strain CLA 341, was resistant to tetracycline (Tc) and ß-lactam antibiotics including cefoxitin (Cf) a drug usually effective in the treatment of *Bacteroides* infections. The objectives of this study were to: (1) identify and characterize the mechanism of Cf resistance; (2) determine if the Cf resistant determinant was transmissible from *B. vulgatus* to other *Bacteroides*; (3) clone the cefoxitin resistance determinant. Cf resistance was shown to be mediated by a ß-lactamase that transferred to other *Bacteroides* by a conjugation-like event when donor cells were pretreated with Tc. Progeny were resistant to Cf, Tc, or both drugs, however, only those resistant to both drugs could become conjugative donors in secondary matings. The structural gene of the ß-lactamse was cloned from genomic libraries in *Escherichia coli*. The library clone containing the Cf resistance gene (*cfx*A) was detected after transfer to *B. fragilis*. Southern analysis of DNA from Cf and Cf/Tc resistant progeny revealed *cfx*A was encoded on chromosomal and not plasmid DNA. Generally, the gene inserted into the chromosome in one preferred site, however, single and multiple insertions at alternate sites were also observed. These results suggest that *cfx*A is located on a transposable element.

THE CLONING AND CHARACTERIZATION OF A CEFOXITIN RESISTANCE DETERMINANT IN *BACTEROIDES VULGATUS* CLA341

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

Anita C. Parker

April 1992

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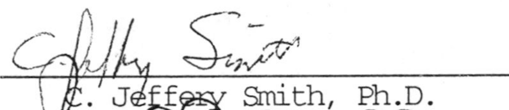

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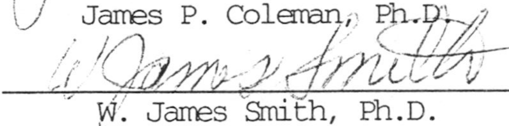

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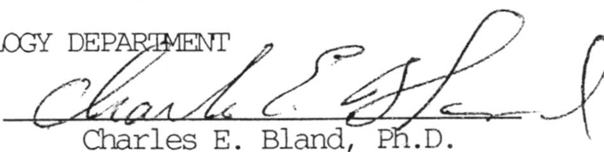

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ACKNOWLEDGEMENTS

I would like to sincerely thank my thesis director, Dr. Jeffery Smith, for his guidance, support, and assistance toward the completion of this thesis. Grateful acknowledgement is made to my committee members Dr. Wendell Allen, Dr. James Coleman, and Dr. James Smith for their advice on this project and reading of the thesis. Special thanks is given to my family for their sacrifices and for the encouragement given to help me complete this work.

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INTRODUCTION

The genus *Bacteroides* is composed of a large group of gram-negative, non-sporeforming, non-motile bacilli. These organisms are obligate anaerobes, however many species are aerotolerant and can survive exposure to oxygen for short periods of time. The *Bacteroides* are found as indigenous flora of the oral cavity, the upper respiratory tract, and the intestinal and genital tracts of animals and humans. The human intestinal *Bacteroides* or the *B. fragilis* group which includes former subspecies *B. fragilis, B. vulgatus, B. distonis, B. thetaiotaomicron, B. ovatus*, and *B. uniformis* are of particular interest for two reasons. First, these bacteria are the most prominent species of the human colon, representing up to 1-3 x 10^{10} organisms per gram dry weight of feces and accounting for approximately thirty percent of the total cultivatable flora (Moore and Holdeman, 1974). In the colon, the *B. fragilis* group organisms play an important role in the colon ecosystem, carrying out a variety of metabolic reactions. The intestinal *Bacteroides* are responsible for much of the digestion of complex polysaccharides, such as plant cell wall carbohydrates and mucins that are not digestible by the human host (Salyers et al., 1977). These microbes also are involved in the transformation of bile salts and other steroids (Draser and Hill, 1974). *B.*

fragilis has been shown to produce enzymes that reduce the mutagenicity of several nitro compounds (Kinouchi and Ohnishe, 1983). It has not been proven however if these enzymes enable *B. fragilis* to protect man from nitro-containing toxins. The second reason that these organisms are of interest is that they are important clinically as opportunistic pathogens. Causing sixty percent of all anaerobic infections in man, they are the most frequently isolated anaerobe from clinical samples of infections of the abdominal cavity, blood stream, brain, lung, and female genital tract (Finegold, 1977). Usually, ß-lactam antibiotics are the drugs of choice in the treatment of anaerobic infections but unfortunately, clinical isolates of *B. fragilis* resistant to these antibiotics are being isolated more frequently (Garcia-Rodriquez and Garcia-Sanchez, 1990).

ß-lactam antibiotics are distinguished by the presence of a four member nitrogen containing ß-lactam ring. There are four groups of ß-lactam drugs each classified by the groups or structures adjoining the ß-lactam ring. It is these adjoining groups or structures that determine the antibacterial or pharmacological properties for a particular ß-lactam drug. The penicillins and cephalosporins have been in clinical use for many years while the monobactams and carbapenems have been introduced recently (Shafran, 1990). All ß-lactam antibiotics function by entering the bacteria cell wall and binding covalently to specific receptors on

the cytoplasmic membrane called penicillin binding proteins (PBPs). The PBPs normally function as transpeptidases catalyzing the crosslinking of peptidoglycan units giving strength and rigidity to the cell wall. ß-lactams act as substrate analogs of the normal transpeptidation substrate, D-alanyl-D-alanine, combining with the transpeptidase, and inversibibly inactivate it. When ß-lactams are bound to PBPs, cell wall synthesis can not be completed. This eventually leads to cell lysis and death unless the cells are in a medium of iso-osmolarity (Tripper, 1986).

All of the members of the *B. fragilis* group are generally resistant to penicillins and to the older cephalosporins due to the production of ß-lactamases (Olsson et al., 1979; Tajima et al., 1983). ß-lactamases are enzymes that hydrolyze the amide bond of the ß-lactam ring of ß-lactam antibiotics to form inactive compounds. ß-lactamases have been found in nearly all bacteria species examined and they have been described with different characteristics. The criteria used in distinguishing individual ß-lactamases are the substrate and inhibitor profiles, genetic origin (encoded chromosomally or on a plasmid), control of synthesis (constitutive or induced), and physical characteristics such as molecular weight and isoelectric point (Bush, 1989).

The most common types of ß-lactamase possessed by members of the *B. fragilis* group have been characterized as chromosomally encoded cephalosporinases because they hydrolyze

cephalosporins at a faster rate than penicillins (Anderson and Sykes, 1973). The production of these ß-lactamases is correlated to a high level of resistance to ß-lactam drugs and these ß-lactamases are not inducible, that is, the amount of ß-lactamase produced does not increase when the bacteria are grown in an appropriate substrate such as benzylpencillin or cephaloridine (Olsson et al., 1976). The ß-lactamases of the *B. fragilis* group are closely associated with the cell envelope and their release by osmotic shock treatments indicates they are probably located in the periplasmic space (Britz and Wilkerson, 1978). Inhibitors of these enzymes include cloxicillin, p-chloromercuribenzoate, clavulanic acid, sulbactam, cefoxitin, and imipenem (Nord, 1986; Cuchural et al., 1986a). In isoelectric focusing experiments, the *B. fragilis* group ß-lactamases focus in the acid range with isoelectric points ranging from 4.0 to 5.6 with evidence for species specific ß-lactamases (Dornbush et al., 1980; Timewell et al., 1981b). The molecular weights of the enzymes range from 29,000 to 40,000 (Nord, 1986).

In contrast to the more typical enzymes from *B. fragilis*, other novel ß-lactamases have been found. A *B. fragilis* ß-lactamase that has penicillinase rather than cephalosporinase activity has been reported. The penicillinase had a reported molecular weight of 41,000 and an isoelectric point of 6.9 (Sato et al., 1982). Three *B. fragilis* strains have

been reported that degrade the highly ß-lactamase resistant drug imipenem and most other ß-lactam antibiotics. Two of the *B. fragilis* imipenem resistant ß-lactamase genes have been cloned and sequenced. These genes, *ccr*A from strain TAL3636 (Rasmussen et al.,1990) and *cfi*A from strain TAL2480 (Thompson and Malamy, 1990), are almost identical in DNA sequence and show greater than thirty-two percent similarity with the zinc requiring *Bacillus cereus* enzyme (Hussain et al., 1985). The ß-lactamases encoded by the *ccr*A and *cfi*A genes are completely inhibited in the presence of EDTA and are not inhibited by clavulanic acid. A third imipemen resistant enzyme produced by *B. distasonis* strain TAL7860 shows no homology to the *cfi*A gene (Thompson and Malamy, 1990). The *B. distasonis* enzyme is not inhibited by EDTA and is sensitive to clavulanic acid suggesting it is different from these ß-lactamases (Hurlburt et al., 1990). *Bacteroides* enzymes capable of degrading cefoxitin and not imipenem have also been described but these enzymes have not been cloned or fully characterized (Cuchural et al., 1983; Eley and Greenwood, 1986).

Cefoxitin is a cephamycin derivative that has been shown to be highly active *in vitro* against most *B. fragilis* group organisms (Cuchural et al., 1984). The cephamycins and the cephalosporins are similar in structure in that they have fused to the ß-lactam ring a six-member dihydrothiazine ring instead of a five-member thiazolidine ring that is pre-

sent in the penicillins. The cephamycins differ from the cephalosporins by the presence of an alpha-methoxyl group in the seven position of the cephem nucleus. Cefoxitin is the cephalorsporin of choice in the treatment of infections caused by *B. fragilis* group organisms. This exceptional activity of cefoxitin has been attributed, in part, to its resistance to hydrolysis by the common *B. fragilis* group ß-lactamases (Neu, 1983).

Until recently cefoxitin resistant strains of the *B. fragilis* group have appeared sporadically and in low frequency. However, resistance is becoming more common in some medical centers. Tufts-New England Medical Center saw a statistically significant increase in the percentage of cefoxitin resistant *B. fragilis* group organisms from fourteen percent in 1981 to thirty percent in 1982 (Cuchural et al., 1984). In a study at the University Clinical Hospital in Spain highly resistant strains of *B. fragilis* group organisms have been isolated every year since 1985. The frequency of cefoxitin resistant strains rose from two percent in 1979 to nineteen percent in 1986 as use of the drug increased (Garcia-Rodriquez and Garcia-Sanchez, 1990). Cefoxitin resistant *B. fragilis* group organisms pose serious therapeutic problems because cefoxitin resistance is often associated with resistance to other antibiotics. In a study of clinical cefoxitin resistant isolates and cefoxitin susceptible *B. fragilis* strains, the cefoxitin resistant

strains were innately more resistant to the penicillins such as amoxycillin, ampicillin, ticarcillin, and piperacillin (Barry and Fuchs, 1991). Cefoxitin resistance has also been found to occur with resistance to the cepholsporins moxalactam, cefotaxime, cefoperazone, and the non-ß-lactam antibiotic clindamycin, the drug of choice for treating *Bacteroides* infections (Bieluch et al., 1987). Usually resistance to cefoxitin has been attributed to failure of the drug to penetrate through the outer membrane or to alterations to the PBPs (Piddock and Wise, 1987; Wexler and Holebran, 1990). However a study of one cefoxitin resistant strain, TAL3636, showed that limited outer membrane permeability and hydrolysis of cefoxitin by a novel ß-lactamase played a role in the mechanism of resistance in this organism (Cuchural et al., 1986b).

Conjugal transfer elements in the *B. fragilis* group have been shown to influence the transfer frequencies of drug resistances. Tetracycline resistance is often associated with conjugal transfer elements. These tetracycline resistance transfer elements or tet elements are thought to be located on the chromosome because no plasmid encoding tetracycline resistance has been found (Salyers et al., 1987). In some systems tetracycline resistance transfer can be increased by incubating the donors with subinhibitory concentrations of tetracycline prior to mating (Privitera et al.,1979; Smith et al.,1982), while in others, transfer is

constitutive (Mays et al., 1982; Valentine et al., 1988) suggesting that the tetracycline elements in different strains are not identical. The inducible tetracycline resistance elements have been shown to increase the transfer of autonomous plasmids (Valentine et al., 1988). Shoemaker and Salyers (1988) have reported that a conjugal tet element mediates the excision of plasmid-like forms from the chromosome of *B. uniformis*. The mechanism of tetracycline resistance and the effect of tetracycline induction on transfer is unknown. It has been proposed that pretreatment of donor cells containing the tet element with tetracycline induces the generation of the transfer machinery as well as activating the transposition of the tet element from the donor chromosome. Other resistance determinates that are adjacent to the tet element can be transferred to recipients along with the tet element at the site where it integrates into the bacteria chromosome (Malamy and Tally, 1981). Such a proposed mechanism resembles the model of the conjugal transposon described in the streptococci. The prototype, Tn*916*, is 15 kb in size, carries tetracycline resistance and mediates its own conjugal transfer. Tn*916* can transpose to conjugative and non-conjugative plasmids or directly to the chromosome of a recipient (Clewell and Gawron-Burke, 1986).

Both plasmid and non-plasmid transfer of ß-lactamase mediated drug resistance has been documented in *B. fragilis* group organisms. Ampicillin resistance transfer between *B.*

fragilis species could be observed when the donor strains were first mated with a strain of *B. fragilis* that carried a transferable tetracycline resistance element. Transfer frequencies were increased by exposing the donor to subinhibitory levels of tetracycline. The progeny that were both tetracycline and ampicillin resistant were able to transfer ampicillin resistance to another *B. fragilis* strain. The original donor, TMP14, contained two plasmids, neither of which were detected in the ampicillin resistant progeny (Butler et al., 1980). The ampicillin resistance marker in TMP14 could also be mobilized after introduction of pBFTM10, a plasmid containing a transfer factor (Malamy and Tally, 1981). The mechanism of ampicillin resistance transfer by the tetracycline resistance element or pBFTM10 has not been determined. A different enzyme with penicillinase rather than cephalosporinase activity has been shown to co-transfer with tetracycline resistance between strains of *B. fragilis* and *B. vulgatus* (Sato et al., 1982). Plasmid mediated transfer ß-lactam drug resistance has also been reported to occur between *B. fragilis* species. The transfer of ß-lactam antibiotic resistance was separate from transfer of a tetracycline resistance element and the ß-lactamase produced was a cephalosporinase (Yamaoka et al., 1990).

Transferable cefoxitin resistance was first reported for a *B. thetaiotaomicron* (Raschtchian et al.,1982). Plasmid DNA was not involved in this transfer, cefoxitin resistance

was self-mobilizing, and exposure of the donor to tetracycline had no effect on transfer frequencies. The cefoxitin resistant progeny were unable to act as conjugal donors. In another report, cefoxitin resistant progeny were able to retransfer cefoxitin resistance. These progeny had a substantial increase in ß-lactamase activity and a new ß-lactamase protein. Transfer frequencies of cefoxitin resistance could be increased by introduction of a tetracycline resistance transfer element and no plasmid DNA was involved. Transfer of cefoxitin was shown to occur by a conjugation-like process (Cuchural et al., 1986). Much of the biochemical basis of conjugation in *Bacteroides* remains unknown. The number of genes and the location of the genes involved in conjugal transfer has not been determined. What is known about conjugation in the *B. fragilis* group is that transfer requires cell-to-cell contact on a solid matrix; it has not been observed in broth cultures. Sex pili have not been associated with this type of conjugation. Transfer frequencies are generally low (10^{-4} to 10^{-6} transconjugants per donor) but higher frequencies can occur between *Bacteroides* of the same species (Welch et al., 1979). Although the conjugal transfer of cefoxitin resistance has not been associated with detectable plasmid DNA, there has been no definitive evidence supporting a chromosomal location.

The goal of this research is to examine the role of ß-lactamase in mediating cefoxitin resistance in *B. vulgatus*

strain CLA341. Preliminary studies of this organism have shown that it produces a high level of ß-lactamase and this study will characterize the enzyme and determine if it is capable of degrading cefoxitin. The determinant encoding cefoxitin resistance will be cloned and the cellular location of the element established. The ability of CLA341 to transfer its cefoxitin resistance to other *Bacteroides* species will be determined and thus establish a mechanism for its dissemination. CLA341 is also resistant to tetracycline and the role of this resistance in the transfer of cefoxitin resistance will be explored. Because there is a possibility that CLA341 possesses a conjugal tetracycline resistance element, matings will be performed with and without tetracycline pretreatment to determine if cefoxitin resistance transfer is affected.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The relevant phenotypes of the bacterial strains and plasmids used in this study are listed in Table 1. CLA341 was obtained from Dr. Peter Applbaum of Hershey Medical Center, Hershey PA. *Escherichia coli* strains were grown on L-broth containing the following (per liter): tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g. *Bacteroides* spp. were grown on either brain heart infusion broth supplemented (BHIS) with hemin (5 mg/L), menadione (1 mg/L), L-cysteine (1 g/L), and $NaHCO_3$ (2 g/L), or Yeast Extract Glucose broth (TYG) which contained the following per liter: tryptone, 20 g; yeast extract, 1 g; glucose, 5 g; cysteine, 5 g; salts solution "A" (Holdeman et al., 1977), 40 ml; hemin, 5 mg; menadione, 1 g; and $NaHCO_3$, 2 g. Media were solidified with 1.5% agar when required. *Bacteroides* cultures were grown at 37 °C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) containing an atmosphere of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen. *E. coli* strains were grown under aerobic conditions at 37°C.

The following antibiotics were added when required at the indicated concentrations (µg/ml) unless otherwise stated in the text: Rf, 20; Tc, 2; Ap, 50; Sp, 50; Cf, 20; Cc, 3; and Gn, 25. All antibiotics were obtained from Sigma Chemi-

Table 1. Bacterial Strains and plasmids

Strain or plasmid	Relevant phenotypes[a]	Source or reference
B. vulgatus		
CLA341	Cf^r, Cc^r, Tc^r, Rha^+	P. Applebaum
B. fragilis		
638	Rf^r, Gn^r	Privitera et al. (1979)
V479-1	Tc^r, Cc^r	Welch and Macrina (1981)
IB220[b]	Cc^r derivative of 638	Smith and Spiegel (1987)
IB245	Cf^r derivative of 638	This study
IB245.1	Tc^r derivative of IB245	This study
IB246	Cf^r, Tc^r derivative of 638	This study
B. uniformis		
V528	Rf^r, Gn^r, $Cello^+$	Welch et al. (1979)
IB247	Cf^r derivative of V528	This study
E. coli		
HB101	*recA*	Maniatis et al. (1982)
DH5-alpha	*recA*, *lac-*	Gibco/BRL, Inc.
Plasmids[c]		
pJST61	Cc^r, Ap^r, *oriT*	Thompson and Malamy (1990)
pFD288	Cc^r, Sp^r, *oriT*	Smith (1989)
pJST61.cfx	Cf^r, Cc^r, Ap^r, *oriT*	This study
pFD351	Cf^r, Cc^r, Sp^r, *oriT*	This study
pBI191	Cc^r	Smith (1985a)
RK231	Tra^+, Tc^r, Kn^r	Guiney et al. (1984)

[a]Abbreviations: Cf^r, cefoxitin resistance; Cc^r, clindamycin resistance; Tc^r, tetracycline resistance; Rf^r, riampicin resistance; Gn^r, gentimycin resistance; Kn^r, kanamycin resistance; Ap^r, ampicillin resistance; Sp^r, spectinomycin resistance; Rha^+, capable of using rhamnose as the sole carbon source; $Cello^+$, capable of using cellobiose as the sole carbon source; Tra^+, capable of self-transfer.

[b]Cc^r in this strain is not transmissible.

[c]In the shuttle cloning vectors pJST61, pJST61.cfx, pFD351, and pFD288, Cc^r functions only in *Bacteroides* spp. The determinants for Ap^r and Sp^r are expressed only in *E. coli*.

cal Co. (St. Louis, MO) except for Cf (Merck, Sharpe and Dohme, Rahway, NJ), Cc (The Upjohn Co., Kalamazoo, MI), and Gn (United States Biochemical, Cleveland, OH). The chemical 5-Bromo-4-chloro-3-indolyl-ß-D-galactoside [X-gal; (Gibco/ BRL, Gaithersburg, MD)] was added when required to a final concentration of 50 µg/ml.

Antibiotic Sensitivity Testing

The minimal inhibitory concentration (MIC) was determined by the agar dilution method of the National Committee for Clinical Laboratory Standards (1987). Cultures to be tested were streaked onto BHIS plates and incubated for 16 to 24 hr. Five to fifteen colonies were inoculated into 5 ml thioglycollate medium supplemented with hemin (5 µg/ml), menadione (0.1 µg/ml) and $NaHCO_3$ (1 mg/ml) so that a turbidity equivalent to the 0.5 McFarland standard was achieved. This resulted in a final inoculum density of $1x10^5$ to $1x10^6$ colony forming units per spot. The inoculum was applied to Wilkins-Chalgren agar plates containing antibiotics and control agar plates with a Steers replicator (Steers et al., 1959). The MIC was defined as the lowest concentration of antibiotic producing no growth, a barely visible haze, or one discrete colony after 48 hr incubation.

Mating Procedures

Filter matings of *Bacteroides* spp. were performed as previously described by Mays et al. (1982). Cells were grown overnight in BHIS then diluted 1:40 and grown to 3-5 x 10^8 cells/ml in BHIS broth. Donor and recipient cells were mixed in a ratio of 0.5 to 1.0 in a 1.5 ml microcentrifuge tube. The cells were centrifuged and suspended in 50 µl of BHIS broth. The mating mixtures were placed on sterile type HA, 25 mm diameter, 0.45 µm pore size filters (Millipore Corp., Bedford, MA) on BHIS agar plates and incubated anaerobically for 16-18 hr. Filters were then aseptically transferred to 17 x 100 mm polypropylene tubes and 1 ml BHIS broth was added to each tube. Tubes were vigorously agitated on a Vortex mixer for 1 min to wash cells from the membrane filters. Appropriate dilutions of the cell suspensions were plated on BHIS plates containing antibiotics. Controls of the donor and recipient cells were done with each mating. In pretreatment experiments, donors were grown overnight in Tc or Cc then subcultured into BHIS containing no antibiotics before mating. Verification of progeny was accomplished by scoring colonies in defined medium [per liter: Mineral solution 3B (Varel and Bryant, 1974), 50 ml; tryptone, 1 g; yeast extract, 1 g; hemin, 5 mg; menadione, 1 mg; L-methionine, 20 mg, casamino acids, 1 g; carbon source, 3 g; cysteine, 1 g; $NaHCO_3$, 2 g] to test for the ability to ferment the sugar rhamnose or cellobiose as the sole carbon source.

To characterize the mechanism of Cf^r transfer, four experiments were performed. The donor, CLA341, was pre-treated with Tc in all these experiments: (1) DNase (Sigma Chemical Co.) at 100 µg per ml was added to the mating mixture and to the mating plates; (2) sterile donor filtrates were prepared by centrifuging the donor cells, then filtering the supernatant through a 0.20 µm pore size filter; (3) donor and recipient strains were placed on separate filters and placed cell side up one on top of another on the mating plate. These filters were then treated as one unit for the remainder of the mating procedure; (4) liquid broth matings were performed by adding 50 µl of both the donor and recipient cells to 5 ml BHIS broth and incubating overnight. The culture was centrifuged, suspended in 1 ml BHIS and plated on selective media.

Transfer of plasmids from *E. coli* to *Bacteroides* was done by triparental filter matings (Shoemaker et al., 1986). The *E. coli* containing the CLA341 library, the helper *E. coli* containing RK231, and the recipient, *B. fragilis* 638 were mixed in a ratio of 0.5 ml: 0.5 ml: 1 ml. Triparental mating plates were incubated aerobically for 18 hr and then plated on selective BHIS media and incubated anaerobically 24 to 48 hr.

Transformation of *Bacteroides*

Transformation by electroporation was carried out according to Smith and co-workers (1990) with a BioRad Gene Pulser (BioRad Laboratories, Richmond, CA). An overnight culture of *B. fragilis* 638 was diluted 1:40 in 5 ml BHIS and grown to 3-5 x 10^8 cells/ml. Cultures were centrifuged at 3,000 x g for 10 min at 4°C and washed in an equal volume of cold electroporation buffer (10% glycerol, 1 mM Mg/Cl_2, filtered sterilized) and suspended in 1/100 volume of electroporation buffer. A portion (0.1 ml) of the cell suspension was placed in a sterile, prechilled, 0.2 cm gap electrode cuvette. Plasmid DNA (1 µg) in TE buffer (10 mM Tris, 1 mM EDTA, pH 7) was added to the cell suspension and mixed by tapping the cuvette lightly. The cell suspension was pulsed for 5 msec at 1.75 kV (8.75 kV/cm). Plasmid pBI191 (Cc^r) was used as a control. Immediately after pulsing, the sample was placed in 1 ml BHIS and allowed to grow overnight. Cells pulsed with CLA341 plasmids were plated on BHIS with Cf and cells pulsed with pBI191 on BHIS with Cc.

Transformation of *B. fragilis* 638 with CLA341 plasmid DNA also was attempted using the polyethylene glycol (PEG) method (Smith, 1985a). Briefly, a 5 ml culture of *B. fragilis* 638 in BHIS with 20 mM $MgCl_2$ (BHIS-Mg) was incubated overnight. A 1:40 dilution (0.75 ml into 30 ml) of the overnight culture was then grown to 7-9 x 10^8 cells/ml in BHIS-Mg.

The culture was harvested by centrifugation at room temperature for 5 min at 4,000 x g and washed in an equal volume of Standard Transformation Buffer [STB; (0.5 M sorbitol, 0.02 M $MgCl_2$, 0.1 M potassium acetate, pH 5.4]. The cell pellet was then suspended in STB at one tenth the original culture volume and transferred in 200 µl aliquots to sterile 1.5 ml microcentrifuge tubes. The cell suspension was allowed to stand at room temperature for 10 min. Next, 5 µl (1 µg) of CLA341 plasmid DNA in TE buffer was added and mixed. After the mixture was allowed to stand at room temperature for 10 min, 200 µl of 50% (wt./vol.) PEG solution [in 20 mM 2 (N-morpholino) ethanesulfonic acid (pH 4.5), filter sterilized] was added. The cell mixture was mixed well, and the contents allowed to stand for 5 min. The tubes were centrifuged for 1 min and the supernatant poured off. The pellet was suspended in 1 ml of prewarmed BHIS-Mg and incubated overnight before plating on BHIS with Cf.

Preparation and Analysis of DNA

Crude lysates of *E. coli* containing pJST61 were prepared by a modified method of Birnboim and Doly (1979). A 400 ml overnight culture was harvested at 4,000 x g at 4°C for 20 min. The pellet was suspended in 8 ml of lysis buffer (50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA, 5 mg/ml lysozyme) and allowed to stand 5 min. Then 16 ml of freshly

prepared alkaline solution (0.2 N NaOH, 1% sodium dodecyl sulfate or SDS) was added to the cells and the tube was mixed and placed on ice for 5 min. Twelve ml of cold acetate solution (60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid, 28 ml H_2O) was added to precipitate protein, chromosomal DNA, and high molecular weight RNA. The solution was allowed to stand on ice for 10 min, then centrifuged at 10,000 x g at 4°C for 20 min. The supernatant was removed and extracted with an equal volume of Tris-saturated phenol and chloroform (1:1). The solution was mixed by vigorous shaking and centrifuged for 30 min at 4,000 x g at 4°C. The aqueous phase was transferred to a clean centrifuge bottle and the DNA was precipitated with two volumes of cold ethanol at -20°C for 30 min. The DNA was harvested at 4,000 x g, 4°C for 30 min. The ethanol was poured off and the nucleic acid pellet allowed to drain completely. The pellet was suspended in 5 ml High Salt TES (25 mM Tris pH 8, 5 mM EDTA, 250 mM NaCl).

Chromosomal DNA from *Bacteroides* was prepared by the method of Smith and Spiegel (1987). A 40 ml culture was harvested at 4,000 x g at 4°C for 20 min. The cell pellet was suspended in 2 ml of 25% sucrose in TE buffer, 6 ml of TE buffer, 3 ml of 0.25 M EDTA, and 1.2 ml lysozyme solution (10 mg lysozyme per ml in TE buffer). Tube contents were mixed and allowed to stand at room temperature for 30 min. Then 0.6 ml of 10% SDS in TE buffer was added and the tubes

mixed by several inversions. When the cells were lysed, the DNA was sheared by vigorous mixing on a Vortex mixer for one or two min. An equal volume of Tris-saturated phenol and chloroform/isoamyl alcohol (24:1) was added and tube contents mixed by shaking. The mixture was centrifuged at 10,000 x g for 20 min at 4°C and the aqueous layer placed in a clean centrifuge tube. The DNA was precipitated by adding one tenth volume 5 M potassium acetate and two volumes of cold ethanol and incubation at -20°C for 30 min. The DNA was harvested at 12,000 x g for 20 min at 4°C and the ethanol poured off. The pellet was suspended in 5 ml High Salt TES. Plasmid and chromosomal DNA was purified from crude lysates during centrifugation in cesium chloride-ethidium bromide gradients (Wilson, 1987).

Routine screening of *E. coli* and *Bacteroides* transformants were performed by the previously described Birnboim and Doly method except culture volumes were 1.5 ml and reagent volume were adjusted accordingly. DNA samples were analyzed by agarose gel electrophoresis with horizontal slab gels in Tris borate (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8) or Tris acetate (10 mM Tris, 1 mM EDTA, pH 8) buffers and developed with 0.5 µg/ml ethidium bromide (Voytas, 1987). Restriction endonuclease digestions were performed as suggested by the suppliers.

The method of Southern (1975) was used to transfer DNA from agarose gels to nitrocellulose membranes. The trans-

ferred DNA was bound to nitrocellulose with a UV crosslinker (Stratalinker, Stratagene, LaJolla, CA). The nitrocellulose membranes were then preincubated in a hybridization buffer of 4 x Denhardts solution (1 x Denhardts is 0.02% bovine serum albumin, 0.02% polypyrrolidone, and 0.02% Ficoll 400), 3 x SSC (1 X SSC is 0.15 M NaCl and 0.015 M trisodium citrate, pH 7), and 10 µg/ml whole yeast RNA at 67°C for 4 hr. DNA fragments used for probes were extracted from agarose gels with glass milk (BIO 101, LaJolla, CA) and then radiolabelled with alpha-^{32}P dCTP using a random primer kit (Pharmicia LKB Biotechnology, Piscataway, NJ). The ^{32}P labelled probe and sheared salmon sperm DNA (100 ug/ml final volume) were denatured in a 100°C water bath for 5 min then quick cooled in an ice water bath. The filters were hybridized with probes in a heat sealable bag with hybridization buffer (.05 ml per cm^2 of nitrocellulose) overnight at 67°C. To remove background, blots were washed at high stringency in 0.1 x SSC and 0.1% SDS at 52°C for 30 min three times and then in 0.1 x SSC for 30 min at the same temperature three times (Thayer, 1979). After air drying, blots were exposed to XAR-2 X-ray film (Eastman Kodak Co., Rochester, NY) for 16 to 24 hr.

Cloning and Subcloning Strategy

Purified chromosomal DNA from CLA341 was partially restricted with the restriction endonuclease *Sau*3AI (0.02 units/g) for 15 min at 37°C. The partially cleaved products were size fractionated on sucrose gradients of 10 to 40% [wt./vol.; (Weis and Quertermous, 1987)]. The size of the collected DNA fractions was determined by electrophoresing 15 µl samples of the gradient in 0.6% agarose gel at 110 V. DNA fragments of 6 to 15 Kb were pooled, ethanol precipitated, and centrifuged at 12,000 x g for 20 min, and the pellet was then dissolved in 100 µl of sterile TE buffer. The fragments were ligated into the unique *Bgl*II site of pJST61 using an insert to vector ratio of 3:1. *E. coli* HB 101 was transformed with the ligated DNA mixture by electroporation (Dower et al., 1988) with selection on ampicillin. Transformed *E. coli* were pooled by flooding the plates with 1 ml L-broth. These pools containing the pJST61-CLA 341 libraries were brought up to a volume of 5 ml of L-broth and grown at 37° for 1 hr. Aliquots were then mated with *B. fragilis* strain 638 and *E. coli* EC182 in triparental filter matings with selection on TYG plates containing Cf, Gn, Rf, and Cc. The region of the cloned fragment containing the Cf resistance determinant was localized by subcloning fragments into pFD288. *E. coli* strains were transformed and selected on Sp. Subclones were then mobilized into *B. fragilis* strain 638 to test for Cf resistance.

Preparation and Analysis of ß-lactamase

Ten ml overnight cultures were inoculated into 200 ml BHIS broth and grown to 1 x 10^9 cells/ml. Cultures were harvested at 4,000 x g for 15 min at 4°C, washed in 50 ml 20 mM phosphate buffer pH 7, and then suspended in 3 ml of the same buffer. Cells were disrupted by two passages through a French Pressure Cell (SLM Instruments, Urbana, IL) at 12,000 lb/in^2 and cellular debris were removed by centrifugation at 12,000 x g for 30 min at 4°C. The extracts were either used fresh or were stored at -70°C. ß-lactamase activity was measured using the chromogenic cephalosporin, nitrocefin (O'Callaghan et al., 1972). The enzyme reaction was carried out in a final volume of 1 ml containing 20 mM phosphate buffer, pH 7 with 100 nmoles nitrocefin, and enyzme extract. Reaction mixtures were temperature equilibrated for 2 min prior to adding cell extract. Assays were monitored at 482 nm for 5 min at 37°C on a Beckman model DU 65 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). One unit of activity was defined as the amount of cell extract that could degrade one micromole of nitrocefin per min.

Substrate Profile and Effects of Inhibitors

Substrate profiles were determined by the hydrolysis of various ß-lactam antibiotics in crude extracts. Reactions were measured at 37°C at the following wavelengths: cephaloridine, 260 nm; cefotaxime, 267 nm; cephalothin, 260 nm; ampicillin, 235 nm; benzylpenicillin, 233 nm; cefuroxime, 265 nm; and cefoperazone, 250 nm (Cuchural et al., 1986). Reactions were carried out in a final volume of 1 ml containing 20 mM phosphate buffer, pH 7. crude cell extract, and 10 µl of substrate. V_{max} and K_m values for cephalosporinase activity were derived from linear regression analysis of Lineweaver-Burke plots of the initial hydrolysis rates obtained at different substrate concentrations that ranged between 25 µM and 500 µM. Inhibitors tested were iodine, ethylenediaminetetraacetic acid (EDTA), p-chloromercuribenzoate (PCMB), dithiothreitol (DTT), and 1, 10-phenanthroline at 100 µM and clavulanic acid, and sulbactam at 10 µM. For the inhibition assay, crude cell extract in 20 mM phosphate buffer was incubated with inhibitor for 5 min at 22°C. Nitrocefin was added to a final concentration of 100 nmoles in a reaction mixture of 1 ml and immediately assayed.

Cefoxitin Inactivation Assay

Bacteroides were assayed for the ability to inactivate Cf by a bioassay modified from Wexler and Halebian (1990). The indicator strain, *E. coli* DH5-alpha, was grown overnight in L-broth. The culture was diluted to Abs_{550} of 0.7 and 1.5 ml was added to 18 ml of melted L-agar cooled to 50°C. The cell/agar mixture was poured into a 150 mm diameter petri dish and wells of 6 mm diameter were cut. Ten microliters of 0.2 mg/ml Cf and 20 µl of crude cell extract or 20 mM phosphate buffer, pH 7 were added to each well. Plates were incubated 18 hr at 37°C and the zones of inhibition measured.

Protein Determination

Protein content was determined by the method of Lowry and co-workers (1951). Cell extracts were appropriately diluted and added to tubes containing water to a volume of 1.2 ml. Then 6 ml of freshly prepared alkaline copper solution (98 ml of 2% $NaCO_3$ in 0.1 N NaOH, 1 ml of 1% $CuSO_4$ $5H_2O$ and 1 ml of 2% sodium tartrate) was added, and the tubes mixed and allowed to stand for 10 min. Then 0.3 ml of Folin-Ciocalteu reagent (Fisher Scientific Co., Pittsburgh, PA) was added and the tubes immediately mixed. After 30 min, absorbance was read at 500 nm. Protein content was determined by comparison of known standards of bovine albumin.

Isoelectric Focusing

Isoelectric focusing methods were adapted from those described by Robertson et al. (1989) using an SE 250 vertical miniature slab gel system (Hoeffer Scientific Instruments, San Francisco, CA). Gels (80 x 70 x 0.75 mm) were cast from the following mixture: 8.3 ml water; 2.8 ml 30% T, 2.7% C acrylamide mixture (30% acrylamide, 2.7% N,N', methylene bis-acrylamide); 3 ml glycerol; and 0.83 ml ampholytes (pH 3-10, Sigma Chemical Co.). After degassing, gels were polymerized by the addition of 62.5 µl of 10% ammonium persulfate and 29 µl N,N,N',N' tetramethylethylenediamine (TEMED). Gels were cast with a 10 well comb in place and allowed to polymerize for 1 hr. In some instances, 2% Triton X-100 was added to the gel before polymerization. The cathode electrolyte, 25 mM NaOH and the anode electrolyte, 20 mM acetic acid were cooled to 4°C prior to use. After the gel was polymerized, the comb was removed and the wells rinsed and then filled with the cathode electrolyte. ß-lactamase preparations were mixed with an equal volume of sample buffer (26% glycerol and 5% ampholytes, pH 3-10). Electrofocusing was done at 22°C for 1.5 hr at 200 V and then increased to 400 V for an additional 1.5 hr with the gel temperature maintained by a circulating water bath. When Triton X-100 was added to the gel, ß-lactamase samples were incubated in 2% Triton X-100 for 20 min at room temperature before electrofocusing. After electrofocusing was complete, the gel was rinsed with dis-

tilled water and the pH gradient measured with a flat bottom pH electrode. The ß-lactamase bands were detected by overlaying the gel with 0.8% agarose containing 50 µg/ml nitrocefin and observing the development of red bands (Gates et al., 1986).

Molecular Weight Determinations

The detection of ß-lactamase activity by SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight. A discontinuous buffer system (Laemmli, 1970) was modified as follows for use with the minigel system described above. The separating gel was made up of the following: 6 ml H_2O, 5 ml 30% T, 2.7% C acrylamide mixture, 3.75 ml running gel buffer (1.5 M Tris, pH 8.8), 0.15 ml 10% SDS and polymerized by 75 µl 10% ammonium sulfate and 5 µl TEMED. The separating gel was layered with 200 µl water saturated butanol and allowed to polymerize 1 hr. The water saturated butanol was poured off and the gel rinsed with distilled water. The gel was then tilted to allow the remaining liquid to drain. The stacking gel was prepared from 6 ml H_2O, 2.5 ml stacking gel buffer (0.5 M Tris, pH 6.8), 1.34 ml 30% T, 2.7%C acrylamide mixture, 0.1 ml 10% SDS, 60 µl 10% ammonium persulfate, and 5 µl TEMED. The polymerized separating gel was rinsed with 1 ml of the stacking gel solution and then the stacking gel solution was

poured on top of the polymerized separating gel. A 10 well comb was inserted and the stacking gel allowed to polymerize. After 1 hr the comb was removed and the wells rinsed and filled with tank buffer (0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS). ß-lactamase extracts were incubated with an equal volume of sample buffer (2% SDS, 5% ß-mercaptoethanol, 50 mM Tris) with 1 µl 0.5% bromophenol blue as the tracking dye at 100°C for 90 sec. The gel was run at 22°C at 20 mAmp constant current. Gels were renatured by the method of Foweraker et al. (1990) by soaking the gel in 5 mM phosphate buffer, pH 6 for 1 hr with one change in buffer, and then soaking in 5 mM phosphate buffer pH 7 for 10 min at 37°C. ß-lactamase activity was detected with nitrocefin as described above and the relative migration measured. The gel was then transferred to protein fixing solution (Sigma Chemical Co.) for 1 hr, stained with Brilliant Blue G (Sigma Chemical Co.) for 1 hr, and then destained with 10% acetic acid and 25% methanol and rinsing with 25% methanol. The relative mobilities of the protein standards (86,000 to 14,000 daltons) were calculated and the molecular weight of the ß-lactamase was estimated from a plot of the relative mobility versus the log molecular weight.

Location of Enzymatic Activity

Cells were grown in 400 ml BHIS to 7-9 x 10^8 cells/ml. A portion (100 ml) was removed to prepare a crude ß-lactamase preparation (whole cell) as described above. The remaining 300 ml were centrifuged at 10,000 x g for 15 min at 4°C. A sample of the supernatant was saved for analysis of extracellular proteins. The cells were then fractionated by an osmotic shock technique slightly modified from Huang and Forsberg (1987). Cells were washed twice in 50 mM phosphate buffer, pH 7 containing 0.8% NaCl, then suspended in 0.5 M sucrose with 30 mM Tris, pH 7.2 at a volume of 50 ml per gram of cells, and shaken for 10 min at 22°C. The cells were centrifuged at 20,000 x g for 20 min at 22°C and then quickly suspended in ice cold distilled water at a volume of 30 ml per gram of cells. The cell suspension was shaken gently for 10 min at 4°C, then centrifuged for 20 min at 4°C. The supernatant containing the periplasmic fraction was removed and saved. The pellet was suspended in 5 ml 20 mM phosphate buffer, pH 7 and the cells were ruptured in a French pressure cell as described above. Cellular debris were removed by centrifugation at 35,000 X g for 1 hr at 4°C. The supernatant was removed and centrifuged at 200,000 X g for 2 hr at 4°C to obtain the cytoplasmic fraction in the supernatant and the membrane fraction in the sediment. The membranes were suspended in 5 ml 20 mM phosphate buffer, pH 7. The crude whole cell extract, extracellular fluid and cellular

fractions were assayed for ß-lactamase activity, protein concentration, and marker enzymes.

Marker Enzyme Assays

Acid phosphatase, the marker for the periplasmic fraction (Anderson and Salyers, 1989), was assayed by the Sigma Chemical Co. procedure No. 104 provided with the diagnostic kit. The cytoplasmic fraction was identified by the presence of ß-galactosidase (Neu and Heppel, 1965). Activity was measured colorimetrically using the substrate orthonitrophenylgalactoside (ONPG). Reaction mixtures of 1 ml containing assay buffer (100 mM sodium phosphate, pH 7, 1 mM $MgSO_4$, and 100 mM 2-mercaptoethanol), 2.5 mM ONPG and enzyme were incubated at 30°C for 10 min. The reaction was stopped by the addition of 0.5 ml of 1 M sodium carbonate and the absorbance at 420 nm recorded. The units of activity (nmoles produce formed per min) were calculated using the nitrophenyl molar extinction coefficient, 4860 (Scheif and Wensink, 1981). Succinate dehydrogenase was used as a marker for themembrane fraction (Kotarski and Salyers, 1984). Enzyme activity was measured by following NADH oxidation in the presence of fumarate. One ml reactions containing 50 mM sodium phosphate buffer, pH 7, 0.2 µmoles NADH, cell extract (100-500 µg protein), and 2.5 µmoles disodium fumarate were incubated at 30°C and the change in absorbance recorded at

340 nm over 5 min. One unit of activity is expressed as µmoles NADH oxidized per min as determined by the molar extinction coeffient for NADH, 6220 (Ernster and Lee, 1967).

RESULTS

Transfer of Cefoxitin Resistance

CLA341 was able to transfer Cf^r and Tc^r to *B. fragilis* 638 and to *B. uniformis* V528 in filter matings as shown in Table 2. Transconjugants were selected on plates containing Cf and Rf or Tc and Rf. Co-transfer of Cf^r and Tc^r was observed in 50% of the progeny tested. Tetracycline resistance transfer and transfer of Cf^r were enhanced in both strains when CLA341 was incubated with Tc before mating. CLA341 was able to transfer Cf^r almost 100 times more frequently to 638 than to V528 (expts. #1,4). Although CLA341 is resistant to Cc, transfer of this resistance was never observed. Pre-exposure of CLA341 to 5 µg/ml Cc had no effect on Cf^r transfer (expts. #2,5). Selected progeny from the primary mating were tested for the ability to re-transfer Cf^r. Isolate IB245, one of the transconjugants that had inherited only Cf^r and not Tc^r, was used as the donor and Cc^r *B. fragilis* IB220 was used as the recipient in a secondary transfer experiment. Selection plates in these matings contained Cf and Cc. IB245 was unable to retransfer Cf^r at detectable frequencies (expt. #7) but could be made capable of mobilizing Cf^r by introducing a conjugal transfer element. IB245.1 was made by transferring a Tc^r transfer element from V479-1 to IB245 and selecting for Tc^r and Cc sensitive transconjugants. The Tc resistance determinant

Table 2. Transfer of antibiotic resistance by *Bacteroides* strains.

Expt. No.	Donor	Recipient	Treatment	Marker transfer frequency[a] Cf	Tc
1	341	V528	Tc	2.9×10^{-8} (±3.4)	6.2×10^{-4} (±.61)
2	341	V528	Cc	$<10^{-8}$	$<10^{-8}$
3	341	V528	none	$<10^{-8}$	$<10^{-8}$
4	341	638	Tc	3.6×10^{-6} (±1.5)	3.3×10^{-4} (±3.1)
5	341	638	Cc	$<10^{-8}$	$<10^{-8}$
6	341	638	none	$<10^{-8}$	4.7×10^{-7} (±2.7)
7	IB245	IB220	none	$<10^{-8}$	ND[b]
8	IB245.1	IB220	Tc	1.2×10^{-5} (±1.1)	6.6×10^{-6} (±5.4)
9	IB246	IB220	Tc	3.3×10^{-4} (±2.9)	5.5×10^{-4} (±3.6)
10	IB246	IB220	none	7.4×10^{-7} (±5.5)	$<10^{-8}$
11	341	638	DNase	6.0×10^{-6}	ND
12	341	638	Sterile donor filtrate	$<10^{-8}$	ND
13	341	638	Donor/recipient separated by filter	$<10^{-8}$	ND
14	341	638	Liquid broth mating	$<10^{-8}$	ND

[a]Transfer frequency is the number of transconjugants divided by the number of input donor cells. Values are the average of at least three experiments. Numbers in parentheses are standard deviations.
[b]ND-not determined.

in V479-1 is not carried on any detectable extrachromosomal element. It is inducibly expressed and is transferred by conjugation. Cefoxitin resistance could be transferred from IB245.1 to IB220 when the donor was pretreated with Tc (expt. #8). Transconjugants that were Cf^r and Tc^r possessed an inducible Tc^r which was identical to that as the parent. When one of the Cf^r/Tc^r *B. fragilis* transconjugants, IB246, was used in secondary matings it was able to transfer Cf^r or Tc^r together or separately to IB220 (expt. #9). Cf^r transfer could be detected when IB246 was not pretreated with Tc (expt. #10) but frequencies were nearly 1000-fold lower.

Transfer of Cf^r required cell-to-cell contact on filters. Cefoxitin resistance transfer did not occur with sterile filtrates of the donor, nor did transfer occur in liquid broth matings, nor when donor and recipient were separated by a membrane filter. The inclusion of DNase did not inhibit transfer (expts. 11-14). These findings suggest the transfer of Cf^r occurred by conjugation.

The MIC data and the ß-lactamase specific activities for the transconjugants and parents are shown in Table 3. MIC values for strains IB246 and IB247 against cefoxitin, cefotaxime, pipercillin and ampicillin were similar to that of CLA341 though slightly lower, and significantly higher that of the parents 638 and V528. All of the strains were sensitive to imipenem though the Cf^r strains were slightly

less sensitive. The ß-lactamase specific activities of IB 246 and IB247 were higher than their cefoxitin sensitive parents.

Table 3. Susceptibility of Cf resistant and sensitive strains to various ß-lactam drugs and specific activities of their ß-lactamases.

Organism	Cf	Ct	MIC Pp	Ap	Ip	Specific Activity
CLA341	256	32	>256	>256	1.0	2.1
638	8	2	4	16	0.25	0.0094
V528	2	2	2	2	0.25	0.0238
IB246	128	32	64	128	1.0	4.3
IB247	128	16	128	256	0.5	1.8
638/pJST61.cfx	128	64	128	256	1.0	2.0

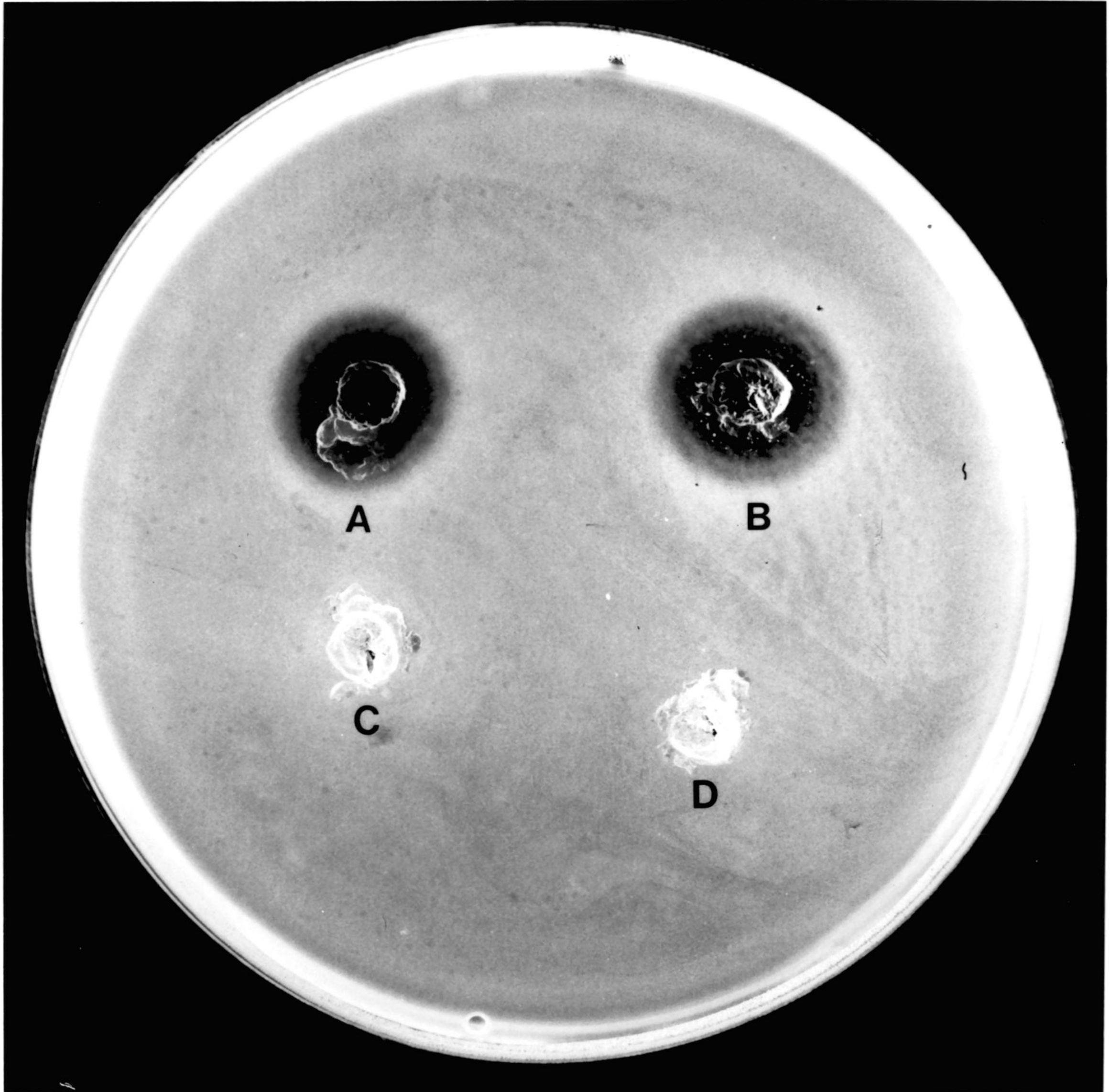
Abbreviations not explained in the text: Ct, cefotaxime; Pp, piperacillin; Ip, imipenem.

Cefoxitin Inactivation

Using the bioassay, the mechanism of Cf^r was examined to determine if the resistance was due to enzymatic inactivation of the drug or to some other mechanism such as an alteration in the binding of Cf or an alteration in cell permeability to Cf. The results are shown in Plate 1. Crude extracts from both CLA341 and the transconjugant showed the ability to inactivate Cf. The 638 cell extract produced a zone of inhibition of 13 mm and the control with Cf alone produced a zone of inhibition of 16 mm. CLA341 and IB246 extracts had no detectable zones of inhibition.

Plate 1. Bioassay for the hydrolysis of cefoxitin.

A: cefoxitin only. B: ß-lactamase extract from 638. C: ß-lactamase extract from IB246. D: ß-lactamase extract from CLA341. Positive hydrolysis is confirmed by the diminution in diameter of the zone of inhibition, as shown by the growth of the indicator strain near the well. The amount of protein per well is approximately 100 µg.

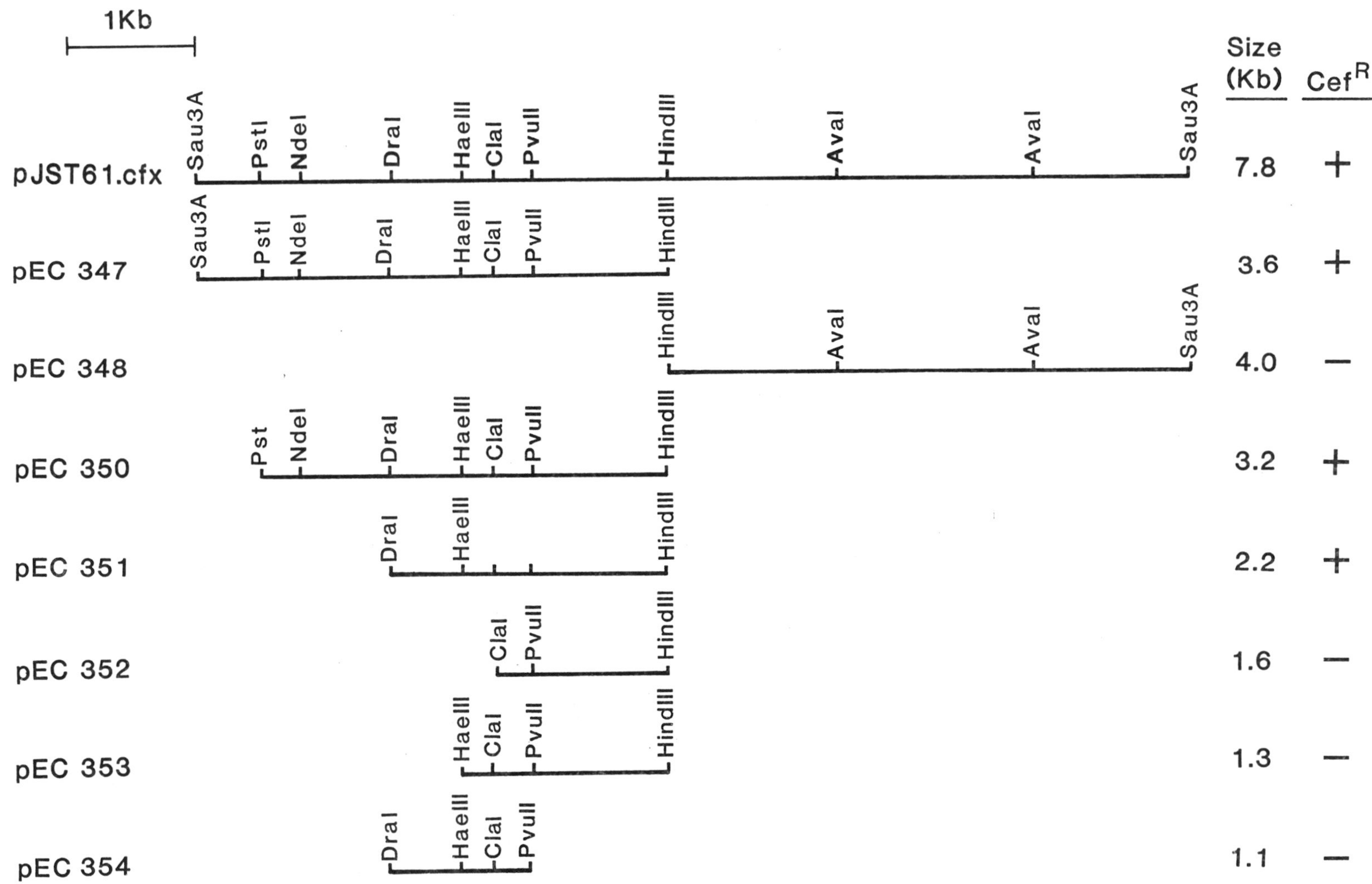

A
B
C
D

Cloning of CLA341 ß-lactamase

The ß-lactamase structural gene was isolated from a plasmid pool containing *Sau*3AI fragments of CLA341 chromosomal DNA ligated to the unique *Bgl*II site of shuttle vector pJST61. This library was transformed into *E. coli* HB 101 and then mated with 638. One Cf^r transconjugant was observed and analysis of the plasmid DNA from this isolate revealed a single 7.8 kb *Sau*3AI fragment at the pJST61 *Bgl*II site. The isolate containing the recombinant plasmid, designated pJST61.cfx, was analyzed for susceptibility to various ß-lactam drugs and the specific activity of the ß-lactamase determined (Table 3). To construct a physical map of the clone, pJST61.cfx was digested with various restriction endonucleases and analyzed by electrophoresis on 0.6% to 1.0% agarose gels depending on the fragment size. The location of the restriction enzyme sites was established by comparing a series of single and double digest with the restriction pattern of the vector. The resulting map is shown in Figure 1. To localize the ß-lactamase gene (*cfx*A) within the cloned fragment, pJST61.cfx was digested with *Sau*3AI and the 7.8 kb insert was purified from gel. The insert was then digested with *Bam*HI and *Hind*III to produce two fragments of 4.0 and 3.6 kb in size. These fragments were ligated to the *Bam*HI and *Hind*III sites of pBR322 to obtain the unique *Eco*RI and *Sph*I sites for further subcloning. The 4.0 and 3.6 kb fragments were ligated to the *Eco*RI and *Sph*I sites of

shuttle vector pFD288. Detection of pFD288 recombinants was based on the disruption of the ß-galactosidase alpha peptide on the plasmid. The resulting plasmids containing the 3.6 and 4.0 kb fragments designated pFD347 and pFD348 (Figure 1), were transferred to *B. fragilis* 628 to test for Cf^r. *B. fragilis* transconjugants carrying pFD347 displayed Cf^r levels similar to those of pJST61.cfx, while transconjugants with pFD348 were Cf sensitive. Further deletions were made between restriction sites in the shuttle vector and the insert to obtain the smallest subclone that expressed Cf^r in *B. fragilis*, a 2.2 kb *Dra*I/*Hind*III fragment, pFD351. Plasmid pFD353 which lacks the leftmost 500 base pairs (bp) from the *Dra*I to the *Hae*III sites and pFD354 which lacks 900 bp rightward from the *Pvu*II to the *Hind*III sites did not express Cf^r in *B. fragilis*. The shuttle vector pFD288 contains a spectinomycin resistance determinant for selection in *E. coli* instead of the *bla* ampicillin resistance gene present in pJST61. This allowed us to examine whether the Cf^r determinant from CLA341 could be expressed in *E. coli*. No expression of Cf^r was seen in *E. coli* DH5-alpha or HB 101 when cells were plated on media containing ampicillin.

Figure 1. Restriction endonuclease map of the cloned 7.8 kb fragment from CLA341 and plasmids carrying sub-fragments. Their sizes and the ability to confer Cf^r to *Bacteroides* are indicated.


1Kb
Size (Kb)
Cef^R
pJST61.cfx
Sau3A
PstI
NdeI
DraI
HaeIII
ClaI
PvuII
HindIII
AvaI
AvaI
Sau3A
7.8
+
pEC 347
3.6
+
pEC 348
4.0
—
pEC 350
3.2
+
pEC 351
2.2
+
pEC 352
1.6
—
pEC 353
1.3
—
pEC 354
1.1
—

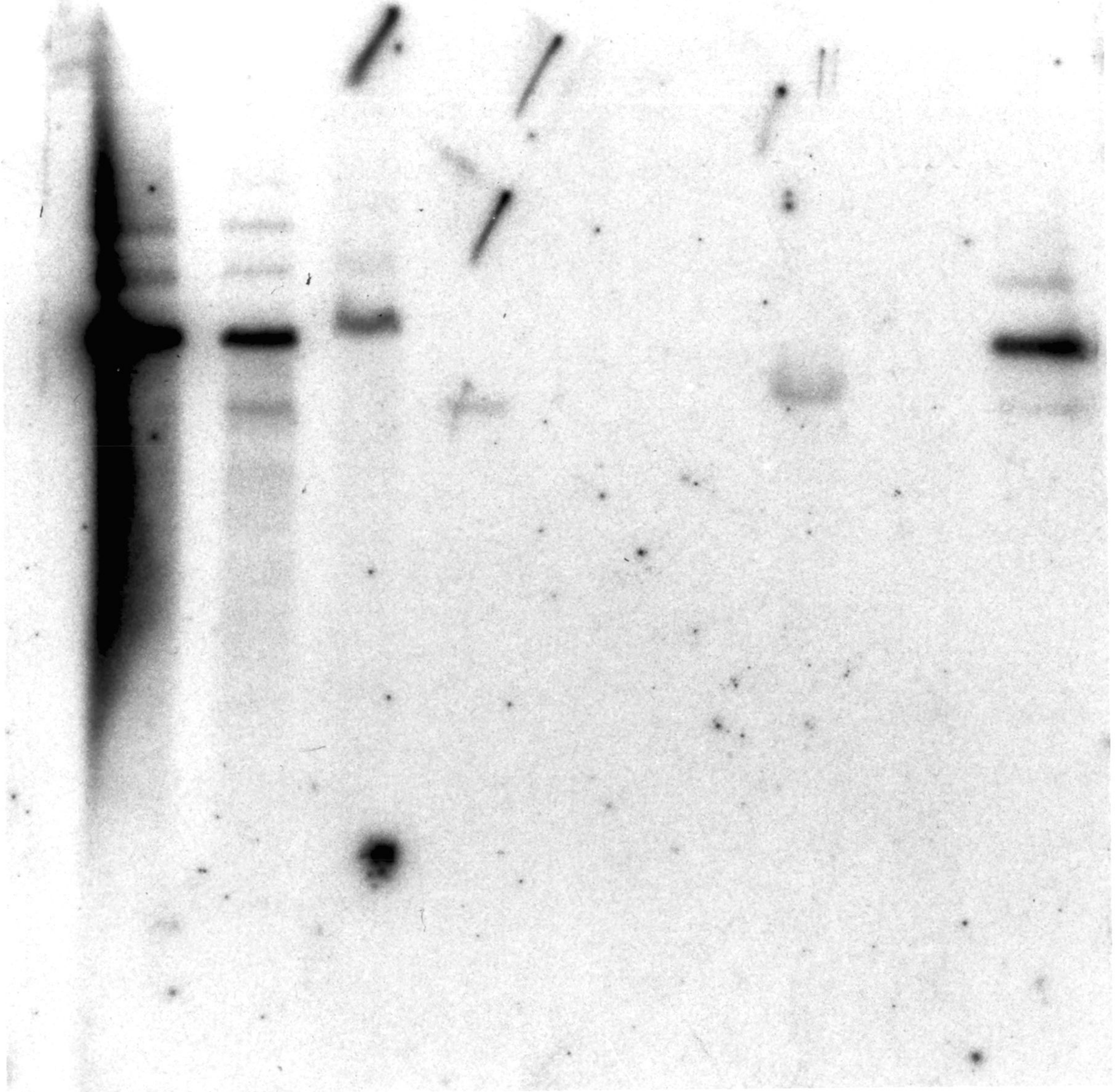
Southern Analysis

CLA341 contains at least four closed circular plasmid bands of approximately 2.8, 4.0, 7.2, and 58 kb when analyzed on agarose gel. In mating experiments, the 2.8 kb plasmid was always transferred to both Cf^r and Cf^r/Tc^r progeny. The 4.0 kb plasmid was transferred about 50% of the time. To test the hypothesis that the Cf^r determinant was not part of the extrachromosomal elements and to confirm that we had cloned the transferable Cf^r gene of CLA341, Southern analysis was performed. Plasmid pJST61, chromosomal DNA from IB245, IB246, IB247, 638, V528, HB101, and CLA341 and plasmid DNA from CLA341 were digested with *Sau*3AI and probed with the 2.2 kb subcloned fragment from pFD351 encoding Cf^r. The results in Plate 2 show that the probe hybridized strongly to the chromosomal DNA from CLA341 and the Cf^r transconjugants (lanes A, B, C, and I). The faint bands in these lanes probably result from partially digested DNA. There were no bands of homology detected in HB101 or CLA341 plasmid DNA. Weakly hybridizing bands were observed in 638 and in the vector pJST61 that may represent some homology to sequences on adjoining the Cf^r gene. These results clearly show that the transferable Cf^r gene is encoded on the chromosome. Furthermore, attempts to transform 638 to Cf^r with total plasmid DNA from CLA341 by eletroporation or the PEG methods were unsuccessful.

Southern analysis was also performed on Cf^r and Cf^r/Tc^r

Plate 2. Origin of *cfx*A.

Autoradiogram obtained after Southern transfer of 0.6% agarose gel of DNA restricted with *Sau*3AI followed by hybridization with ^{32}P-labelled 2.2 kb probe.
Lanes: A, IB245 chromosomal DNA; B, IB246 chromosomal DNA; C, IB247 chromosomal DNA; D, 638 chromosomal DNA; E, V528 chromosomal DNA; F, HB 101 chromosomal DNA; G, pJST61; H, CLA341 plasmid DNA; I, CLA341 chromosomal DNA.


A B C D E F G H I

transconjugants to examine the insertion of *cfx*A in the recipient chromosome. Chromosomal DNA from transconjugants from four independent matings was digested with *Pst*I and probed with a 0.6 kb *Pvu*II/*Hae*III fragment located within the *cfx*A structural gene. The DNA sequence of the 2.2 kb Cf^r subclone has been determined (Genebank accession number M72418) so the location of *Pvu*II/*Hae*III fragment is now known. This probe hybridized to several different sites on the chromosome of the transconjugants but not to the recipient (Plate 3). In most instances, *cfx*A inserted in one preferred site in the chromosome of the 638 Cf^r transconjugants suggesting a primary insertion site. However, more than half of the transconjugants analyzed had two or more alternate sites that hybridized to the probe. These multiple bands indicate that following conjugative transfer *cfx*A inserted into different sites on the recipient chromosome. Due to the transmissible nature of *cfx*A, we examined a set of Cf^r *Bacteroides* isolated from clinical specimens for homology to *cfx*A. Chromosomal DNA from eight clinical *Bacteroides* strains resistant to at least 60 µg/ml Cf was purified and restricted with *Eco*RI and analyzed by Southern hybridization. The DNA was probed with the 0.6 kb *Pvu*II/*Hae*III fragment (Plate 4). Homology to the probe was observed in the DNA of 7 of the 8 strains.

Plate 3. Insertion of *cfx*A in the recipient chromosome.

Autoradiogram obtained after Southern transfer of 0.6% agarose gel of 638 Cf^r transconjugants chromosomal DNA restricted with *Pst*I following hybridization with ^{32}P labelled 600 bp probe. Transconjugants in lanes A, B, C, D, E, and F were selected on 20 µg/ml Cf. Transconjugants in lanes G, H, and I were selected on 100 µg/ml Ap. Lane J: transconjugant selected on 60 µg/ml Cf. Lane K: transconjugant selected on 90 µg/ml Cf. Lane L; CLA341. Lane M; 638. Transconjugants in lanes A, C, E, G, H, J, and K were Cf^r. Transconjugants in lanes B, D, F, and I were Cf^r and Tc^r.

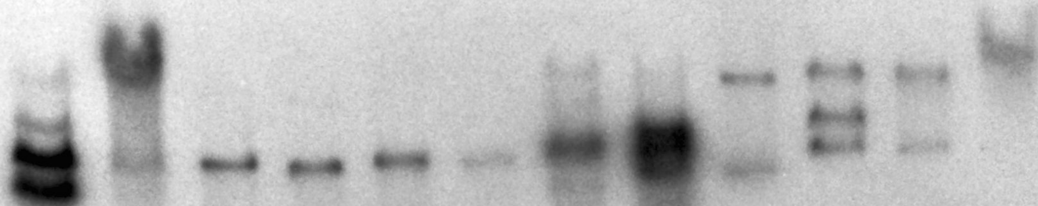
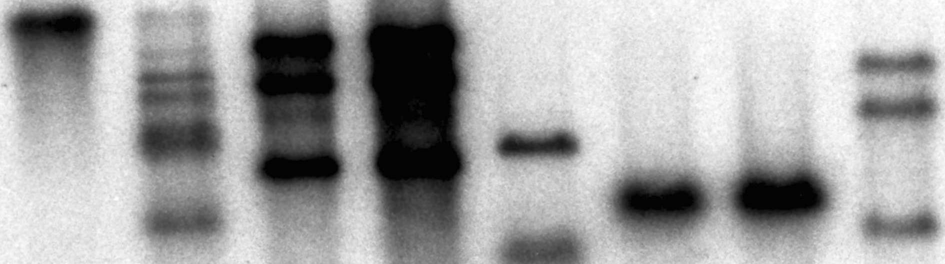

A B C D E F G H I J K L M

Plate 4. Homology of *cfx*A to other Cf^r *Bacteroides*.

Autoradiogram of 0.6% agarose gel of Cf^r *Bacteroides* strains restricted with *Eco*RI following hybridization with ^{32}P labelled 600 bp probe. Lanes: A, *B. vulgatus* CLA341; B, *B. fragilis* CLA262; C, *B. fragilis* CLA 276; D, *B. fragilis* CLA346; E, *B. uniformis* 7088; F, *B. fragilis* 7505; G, *B. ovatus* 7606; H, *B. fragilis* IB 113; I, *B. vulgatus* 7062.


A B C D E F G H I

Characterization of Enzyme Activity

The rates of hydrolysis of selected cephalosporins and penicillins are shown in Table 4. The substrate profiles of CLA341 and the Cf[r] transconjugant, IB246, were similar. The rates of hydrolysis for penicillin and ampicillin were slightly higher for the cloned gene protien. The Cf[r] enzyme from CLA341 hydrolyzed the first and second generation cephalosporins (cephaloridine, cephalothin, and cefuroxime) better than the penicillins. The third generation cephalosporins (cefotaxime and cefoperazone) were more resistant to hydrolysis. The enzyme was unable to hydrolyze cefoxitin or the carbampenem, imipenem, at appreciable rates (<0.1%).

Table 4. Substrate profiles of CLA341, IB246, and 638/pJST61.cfx.

	Relative hydrolysis rate[a]		
Antibiotic[b]	CLA341	IB246	638/pJST61.cfx
Cephaloridine	100	100	100
Cephalothin	67.5	91.7	80.0
Cefuroxime	19.2	26.0	10.9
Penicillin	10.7	13.5	36.5
Ampicillin	7.2	5.6	31.0
Cefotaxime	0.96	1.27	3.3
Cefoperazone	0.49	0.51	0.76

[a]Percentage of rate relative to cephaloridine (100%).

[b]The concentration for all antibiotics was 0.1 mM.

The K_m and relative V_{max} values for hydrolysis of two cephalosporins and penicillins presented in Table 5 show

that the Cf^r enzyme is predominantly cephalosporinase in character. Cephaloridine and cephalothin were hydrolyzed faster than benzylpenicillin or ampicillin in terms of V_{max} rates. The enzyme had less affinity for ampicillin than benzylpenicillin as shown by the respective K_m values. The ß-lactamase was sensitive to inhibition by sulbactam, clavulanic acid, PCMB, and iodine. The enzyme was not affected by 1,10 phenanthroline, EDTA, or DDT.

Table 5. Kinetics of hydrolysis of ß-lactam antibiotics by CLA341 cell extract.

Antibiotic	Relative V_{max}[a]	K_m[b]
Cephaloridine	100	113.2
Cephalothin	51.6	30.4
Penicillin	13.7	30.9
Ampicillin	13.4	107.5

[a]Rates of hydrolysis are relative to an arbitrary value of 100% for cephaloridine.

[b]K_m is expressed as µM.

Location of Enzyme Within the Cell

CLA341 was fractionated and the ß-lactamase activity determined for each fraction (Table 6). The small amount of ß-lactamase activity detected in the extracellular fraction was probably due to the release of the enzyme during cell lysis since the cytoplasmic enzyme ß-galactosidase was also present in this fraction. When the osmotic shock procedure was used to release enzymes in the periplasmic space,

less than 20% of the ß-lactamase was released. Over 80% of the periplasmic enzyme, acid phosphatase, was released from the periplasmic fraction indicating that we had adequately secured the periplasmic contents from the cell. The ß-lactamase activity fractionated most similarly to the membrane marker enzyme succinate dehydrogenase therefore Cf^r ß-lactamases can be localized to the membrane portion of the cell. Similar results were obtained for the transconjugant IB246.

Table 6. Cellular location of enzyme activity in CLA341.

	Percentage of total enzymatic activity[a]			
Cell Fraction	ß-lac	ß-gal	AcPhos	SDH
Extracellular	6.0	10.3	ND[b]	ND
Periplasmic	17.0	19.6	83.6	18.6
Cytoplasmic	19.0	47.6	13.6	15.7
Membrane	58.0	22.5	2.8	65.6

[a]Abbreviations: ß-lac, ß-lactamase; ß-gal, ß-galactosidase; AcPhos, acid phosphatase; SDH, succinate dehydrogenase.

[b]ND, None detected.

Physical Characteristics

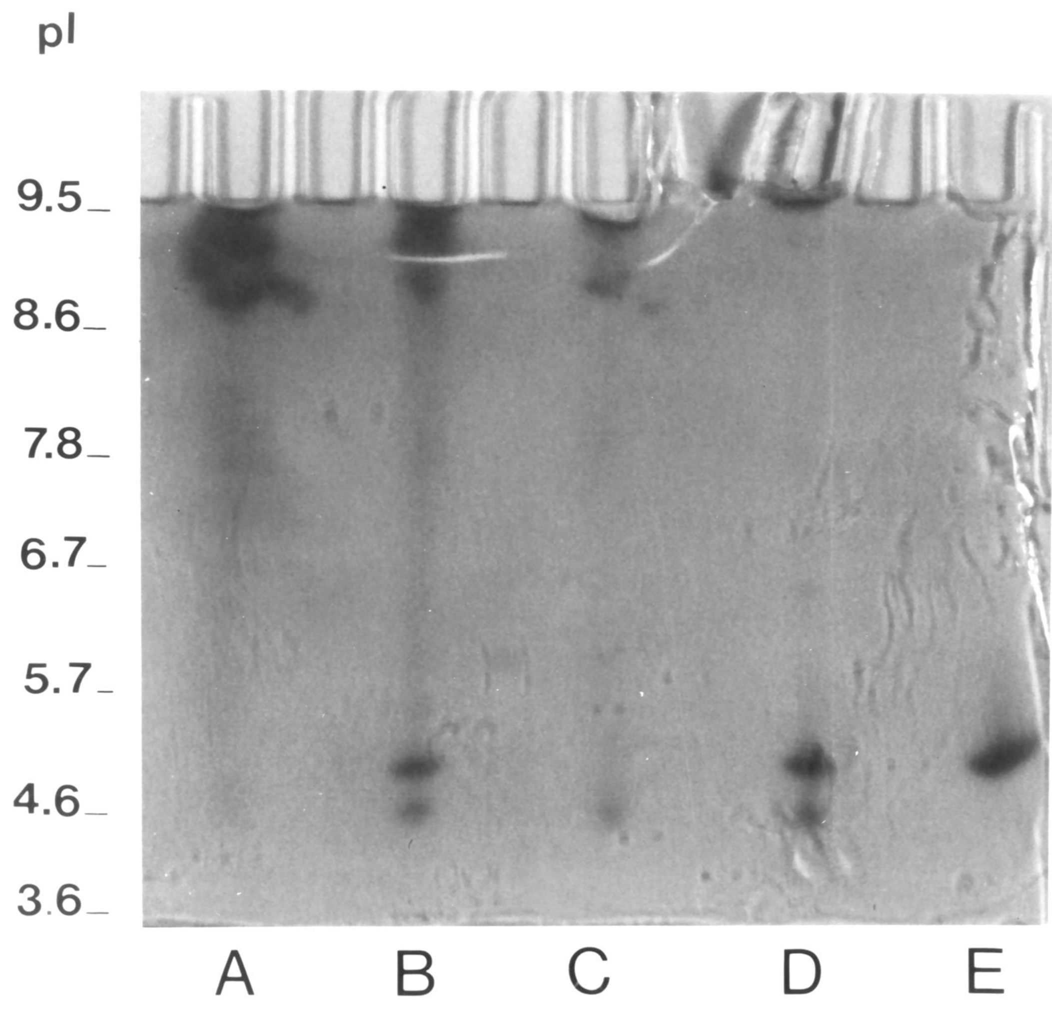
Attempts to electrofocus crude cell extracts of CLA341 ß-lactamase were unsuccessful although the ß-lactamase from crude cell extracts of 638 and V528 electrofocused satisfactorily. Electrofocusing of CLA341 crude cell extracts re-

sulted in large amounts of activity remaining at the loading site and a streak of activity from the loading site to approximately pH 5 (Plate 5, lane A). The streak was also observed in the cloned ß-lactamase and in the Cf^r transconjugants along with two bands with pIs of 5.2 and 4.7 (lane B). This streak of activity suggested incomplete release of the enzyme from the cellular components and is consistent with a membrane location of the enzyme. Dufresne et al. (1988) reported that Triton X-100 facilitates enzyme release so that the bands could be made visible upon electrofocusing. Incubation of CLA341 crude cell extracts with 1% or 2% Triton X-100 and the inclusion of 1% Triton X-100 in the gel did not eliminate the streak of activity. We were able to focus the ß-lactamase successfully in a sample from the periplasmic fraction of CLA341 concentrated by ultrafiltration (YM 100 Diaflo ultrafilter, Amicron Div., W. R. Grace & Co., Beverly, MA). The periplasmic fraction of CLA341 displayed a band of activity at pH 4.7 (lane C). The periplasmic fraction of IB246 had two ß-lactamase bands with pIs of 5.2 and 4.7, the 5.2 band corresponding to the ß-lactamase of the parent, 638 (lanes D and E).

The crude cell extracts of CLA341, 638/pFD351, IB246, IB247, V528, and 638 were run on SDS-polyacrylamide gels to compare the molecular weights of the ß-lactamases (Plate 6). The whole cell extract of CLA341 had a broad ß-lactamase band which upon closer analysis revealed two ß-lactamases of

Plate 5. Isoelectric focusing gel of ß-lactamase band patterns.

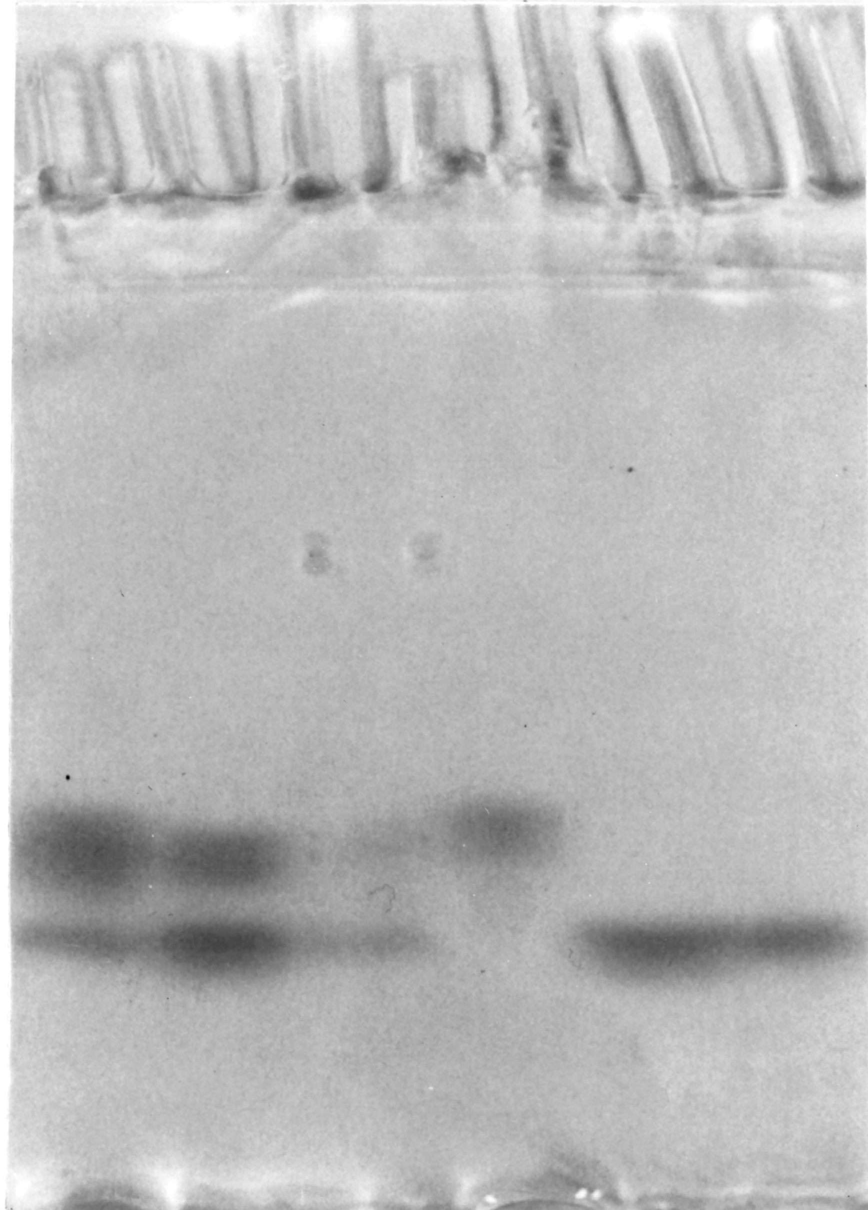
Lanes: A, CLA341 whole cell extract; B, IB246 whole cell extract; C, CLA341 periplasmic extract; D, IB246 periplasmic extract; E, 638 periplasmic extract.


pI
9.5
8.6
7.8
6.7
5.7
4.6
3.6
A
B
C
D
E

approximately 40,000 and 37,000 daltons (Plate 7, lane A). Since two different forms of the CLA341 ß-lactamase were observed in the isoelectric focusing studies, we decided to look at the ß-lactamase from the periplasm on SDS gels. Molecular weight determinations of ß-lactamases present in periplasmic fractions showed that the 37,000 dalton ß-lactamase was more predominant (Plate 7, lane B). The crude cell extacts of IB246, IB247, and the cloned ß-lactamase in 638/pFD351 showed the 40,000 and 37,000 dalton bands presumably from CLA341 and the 29,000 dalton band present in the parent strains 638 and V528 (Plate 6). The results from the isoelectric focusing and the SDS-PAGE clearly demonstrate that the Cf^r transconjugants had acquired a new ß-lactamase with identical characteristics as the Cf inactivating enzyme of CLA341.

Plate 6. SDS-PAGE gel showing the band patterns of ß-lactamases in whole cell extracts.

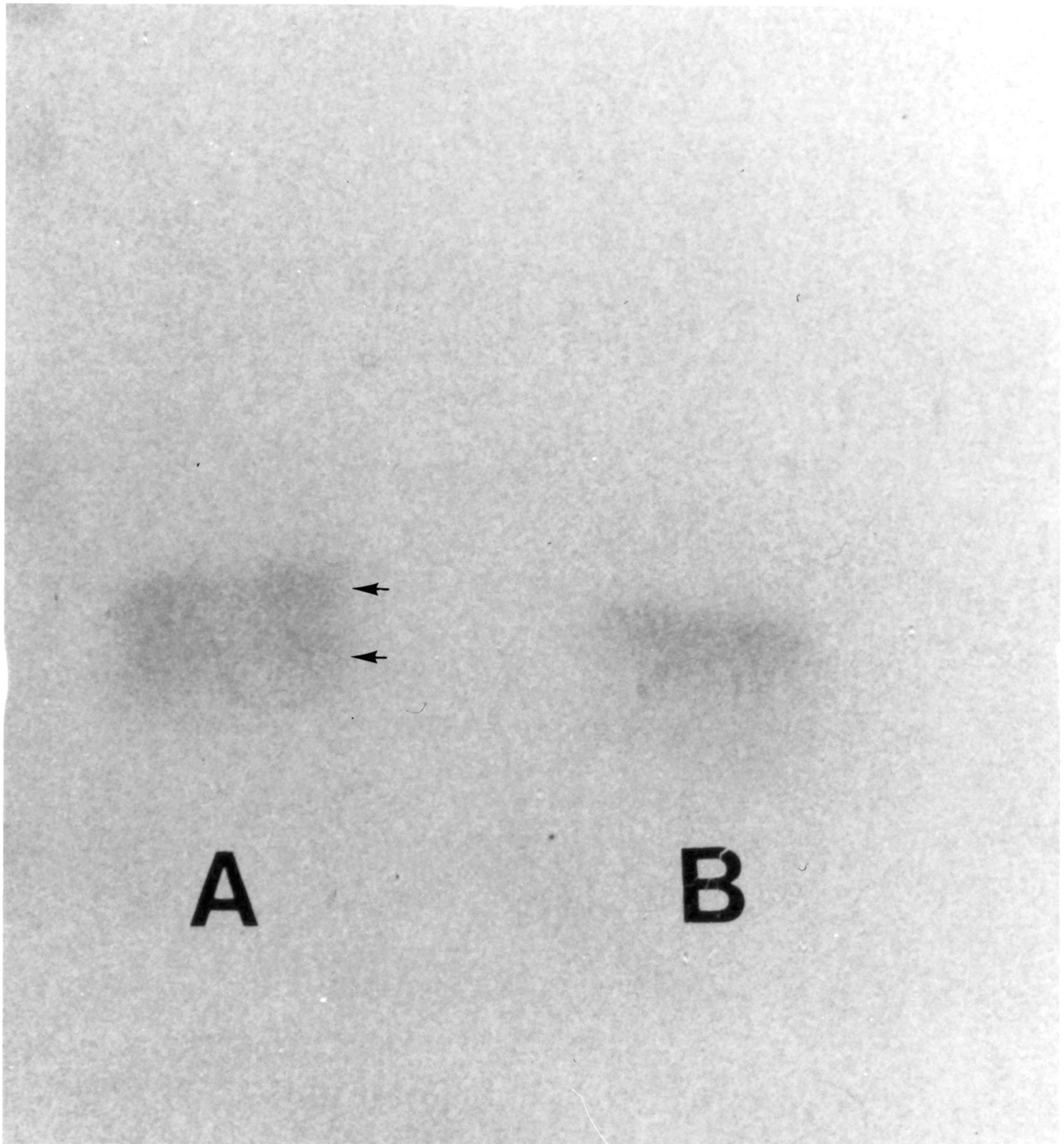
Lanes: A, 638/pFD351; B, IB246; C, IB247; D, CLA 341; E, 638; F, V528.



M W
x
1000
205
97
66
45
29
A
B
C
D
E
F

Plate 7. SDS-PAGE gel of CLA341 ß-lactamase.

Lane A: ß-lactamase obtained from the CLA341 whole cell extract. Lane B: ß-lactamase obtained from the periplasmic fraction. Arrows in lane A denote the positions of the two ß-lactamase bands.


A
B

DISCUSSION

ß-lactamase production is considered to be the main mechanism of resistance of anaerobes to many of the expanded and broad spectrum cephalosporins (Wexler et al., 1991). Generally, all the members of the *B. fragilis* group produce chromosomally encoded ß-lactamases that inactivate cephalosporins but not cefoxitin (Olsson et al., 1976). However, CLA341 possesses a ß-lactamase that is capable of inactivating Cf. Evidence supporting this conclusion was obtained in the bioassay (Plate 1) which showed that CLA341 and a Cf^r transconjugant have an enzymatic activity capable of inactivating Cf. The results from the isoelectric focusing and SDS-PAGE clearly demonstrate that the Cf^r transconjugants had acquired a new ß-lactamase with identical characteristics as the Cf inactivating enzyme of CLA341.

The ß-lactamase of CLA341 is closely associated with the membrane (Table 6) unlike the Cf sensitive *Bacteroides* ß-lactamases which are located in the periplasmic space (Britz and Wilkerson, 1978). ß-lactamases of the gram negative rods are usually highly soluble (Medeiros, 1984) and the observed membrane association of the *cfx*A ß-lactamase was unexpected. Membrane associated ß-lactamases have been reported in only two other gram negative bacteria, *Pseudomonas pseudomallei* (Livermore et al., 1987) and *Capnocytophaga* spp. (Foweraker et al., 1990). The *P. pseudomallei* and *Capnacytophaga* ß-

lactamases are also cephalosporinases but the CLA341 ß-lactamase differs from these in its pI, substrate profile, and in the inability to electrofocus after treatment with Triton X-100.

Although the majority of CLA341 ß-lactamase was associated with the membrane, some activity was located in the periplasmic space (Table 6). We observed that periplasmic extracts from CLA341 yielded a ß-lactamase with a pI of 4.7 and a molecular weight of 37,000. Crude cell extract displayed two forms of the ß-lactamase; one unelectrofocusable with a molecular weight of 40,000 and a second identical to that obtained in the periplamic extract. We hypothesized from these observations that the larger of the two forms is the immature pre-ß-lactamase attached to a hydrophobic signal peptide. This unprocessed form is cleaved as the enzyme is transported through the cytoplasm to the periplasm. One explaination for the inability to electrofocus ß-lactamase activity from crude cell extracts is that much of the enzyme was unprocessed and remained associated with the membrane. This membrane complex may remain at the loading site and would not migrate through the electric field. There has been a report of ß-lactamases of the *B. melaninogenicus* group (*B. bivus, B. asaccharolyticus, B. melaninogenicus*, and *B. oralis*) that could not be focused by conventional methods (Timewell et al., 1981a). These ß-lactamases were found to be in complexes with molecular weights greater than or equal

to 40 x 10^6 which were not electrofocusable. The enzymes could be electrofocused in periplasmic material released by osmotic shock and had molecular weights of 30,000 to 40,000.

Two forms of ß-lactamase have been reported in *Bacillus licheniformis*; one that was membrane bound and the other secreted into the culture medium. It was demonstrated that the excreted ß-lactamase originated in part by the enzymatic cleavage of the membrane form at its amino terminus (Simmons et al., 1978). It was proposed that the amino terminal region contained a sequence which functioned to transport the enzyme across the plasma membrane. It is possible that a similar situation exists between the cytoplasmic and periplamic ß-lactamases in CLA341. In this regard, Sherrill and McCarthy (1984) have demonstrated that the cytoplasmic form of a *Bacteroides* ß-lactamase was modified upon entry into the periplasmic space.

The physical properties of the CLA341 enzyme were similar to ß-lactamases of the *B. fragilis* group. The molecular weight of the periplasmic enzyme, 37,000, is within the range of other intestinal *Bacteroides* ß-lactamases (Britz and Wilkerson, 1978). In this study, the molecular weight of the CLA341 ß-lactamase was found to be greater than the molecule weight of the Cf sensitive ß-lactamases of 683 and V582 (29,000). The ß-lactamase of CLA341 had a more acidic pI than that of the cefoxitin sensitive strains 638 and V528. Similar results were reported by Eley and

Greenwood (1986) who found isoelectric points of 4.5-4.6 for high ß-lactamase producing strains that could inactivate Cf and isoelectric points of 5.0-5.3 for Cf sensitive strains. Cuchural et al. (1986b) found the pI of a transferable Cf^r ß-lactamase to be 8.1 for a *B. fragilis* strain. This difference in pI indicates that the CLA341 enzyme and the *B. fragilis* enzyme are probably two distinct ß-lactamases. There is a possibility, as in the case of the ß-lactamases TEM-1 and TEM-2, that one amino acid substitution could have resulted in a change in pI with no major change in kinetic properties (reviewed by Bush, 1989).

Inhibition by clavulanic acid, sulbactam, PCMB and iodine are other characteristics that the CLA341 ß-lactamase shares with the typical *B. fragilis* group enzymes (Eley and Greenwood, 1986; Olsson et al., 1976). PCMB inhibition could indicate the of the presence of one or more essential cysteines. Inhibition by iodine suggests the participation of a serine, tyrosine, or cysteine residue. The active site and structure of ß-lactamases show that many of these enzymes are serine amidases (Reading and Slocombe, 1986). The enzyme was not inhibited by EDTA or 1,10 phenanthroline which may suggest that a metal cofactor is not required for activity. The MIC data shows the CLA341 enzyme is more resistant to inhibition by imipenem than 638 and V528 but does not appear to able to hydrolyze it. Therefore, *cfx*A gene product does not appear to be the same as the enzymes pro-

duced by the *ccr*A and *cfi*A genes. These enzymes are capable of degrading both cefoxitin and imipenem, require Zn^{2+} as a cofactor for activity, are unaffected by clavulanic acid and sulbactam, and are not associated with the membrane (Rasmussen et al., 1990; Thompson and Malamy, 1990). The CLA341 enzyme hydrolyzes cefoxitin very slowly compared to other cephalosporins and the penicillins (Table 5). The rate of hydrolysis of penicillin and ampicillin was somewhat higher for the cloned *cfx*A protein than for the enzymes from CLA341 and IB246. However the cloned *cfx*A gene protein was still clearly a cephalosporinase.

The dissemination of antibiotic resistance genes has important medical consequences, and the Cf^r phenotype was transmissible to other *Bacteroides* strains. This transfer event required pretreatment of the donor cells with Tc and occurred at a frequency of 10^{-8} in the *B. uniformis* and 10^{-6} in the *B. fragilis* strains tested (Table 2). Transfer of Cf^r was not inhibited in the presence of DNase, eliminating transformation as the mode of transfer. Nor was transfer mediated by cell free donor filtrates providing evidence against generalized transduction. The transfer of the Cf^r element required direct contact between cells suggesting transfer by conjugation (Table 2). Raschtchian et al. (1982) reported that Cf^r transfer from a Cf^r/Tc^r *B. thetaiotaomicron* was not affected by pretreatment with Tc. Cuchural et al. (1986b) found a Cf^r/Tc^r *B. fragilis* that could transfer Cf^r

at low frequencies (10^{-7}) without Tc pretreatment. In contrast to these previous reports, the Cf^r element in CLA341 was not able to transfer at detectable frequencies except when induced with Tc. Similar results were seen in secondary matings and strains that did not also contain the Tc^r element failed to transfer at all. The ability to transfer Cf^r was restored in strains that contained the Cf^r determinant and the conjugal Tc^r element from V479-1 (Table 2).

An exception to this observation was the transconjugant IB246 which could transfer Cf^r at low levels without Tc induction to another *B. fragilis* strain (Table 2, expt. #10). Transfer of Cf^r in this mating could have occurred as a result of the Tc^r element having a low level transfer ability and transfer was observed because the donor and recipient were isogenic pairs. Still another possibility is that the Cf resistance determinant is being mobilized by *B. fragilis* 638. Smith and Spiegel (1987) showed that *B. fragilis* 638 can mobilize plasmids in the absence of Tc induction so it is possible that *B. fragilis* 638 can likewise mobilize Cf^r at low frequencies. Taken together, these results suggest that Cf^r does not have the ability to transfer on its own, but requires the aid of a conjugal transfer element. The genetic information for the transfer of Cf is separate from the Cf^r determinant.

The involvement of plasmids in the transfer of *cfx*A was difficult to rule out because the 2.8 kb plasmid was al-

ways transferred to Cf^r progeny. It is likely that the 2.8 kb plasmid and 4.0 kb plasmid (which appears to be at lower frequencies in transconjugants) are being mobilized by the conjugal tetracycline resistance element. Small cryptic plasmids have previously been shown to co-transfer with *Bacteroides* conjugal elements. At least seven different conjugal resistance elements recognize a mobilization region of the 4.2 kb plasmid pB8-51 which is found in several intestinal *Bacteroides* spp. (Valentine et al., 1988). Another cryptic plasmid, pVA503, co-transfers with a Cc^r/Tc^r element. Progeny devoid of pVA503 or its sequences were able to transfer Cc^r and Tc^r showing that the plasmid was not involved in the transfer process or expression of resistance (Mays et al.,1982). Plasmidless Cf^r progeny from matings were not isolated but Cf^r/Tc sensitive transconjugants were obtained that did contain the 2.8 kb plasmid. Although these strains did have the plasmid, they did not transfer Cf resistance. In addition, data from our Southern analysis showed that the plasmids shared no homologous sequences with the *cfx*A gene and therefore are not involved in the expression of Cf^r. Further, if *cfx*A were on a plasmid, the hybridization patterns in all the transconjugants would have been identical and would have resembled the profile of the donor. This was not the case seen in the transconjugants DNA profile (Plate 3). The fact that hybridization was observed in several different sites in the recipient chromosome suggest that

*cfx*A is on a transposon or some element that is involved in genetic rearrangements. The non-plasmid drug resistance transfer in CLA341 could involve mechanisms similar to the gram positive conjugative transposons described in *Streptococcus agalactiae* (Inamine and Burdett, 1985) and *Streptococcus pneumoniae* (Vijayakumar et al., 1986). These elements confer the multiple drug resistances chloramphenicol-tetracycline and chloramphenicol-tetracycline-MLS (macrolide, lincosamide, streptogramin B), respectively, and can integrate at either random or preferred sites. Analysis of these and other streptococcal transposons indicate that they have substantial homology to each other especially in the tetracycline resistance determinants which suggest that various drug resistance determinants insert within or delete from a basic tet transposable unit (Guild et al., 1982).

The transmissibility of *cfx*A between intestinal *Bacteroides* species may have important clinical significance since these organisms have been shown to transfer antibiotic resistance determinants *in vivo* (Butler et al., 1984). Our Southern analysis of other ß-lactam resistant *Bacteroides* showed that 7 of 8 contained sequences that shared homology to *cfx*A. The fact that we saw homologous bands in such a variety of Cf^r strains suggests that distribution of *cfx*A may be widespread (Plate 4).

The CLA341 ß-lactamase is unique among the ß-lactamases of the *B. fragilis* group in that it is membrane bound

and that ithas the ability to hydrolyze cefoxitin. This research marks the first time that a Cf^r ß-lactamase has been cloned from the intestinal *Bacteroides*. The ß-lactamase of CLA341 may be one of a new group of ß-lactamases associated with the membrane of gram negative bacteria. From an evolutionary standpoint it would be interesting to determine if this enzyme is a variation of a gram negative species or if it were acquired from a gram positive bacterium. Further study should focus on the mechanism of Tc resistance induction in the mobilization of Cf resistance.

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