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Novel Antibiotic Resistance Transfer in *Bacteroides*

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Resistance to tetracycline and lincosamide antibiotics was transferred en bloc from a strain of *Bacteroides fragilis* (V503) to a plasmidless strain of *Bacteroides uniformis* (V528) during in vitro filter matings. Resistance transfer was detected at frequencies of 10^{-5} to 10^{-6} drug-resistant progeny per input donor cell and was dependent on cell-to-cell contact of donors and recipients. Transfer was insensitive to DNase and was not mediated by chloroform- or filter-sterilized donor broth cultures. A determinant for resistance to cefoxitin in V503 was not transferred in this system. V503 contained a 3.7×10^6-dalton plasmid (pVA503). Drug-resistant progeny of V503 × V528 matings usually contained pVA503, but up to 20% of the total progeny of such crosses were plasmid free. Filter blot DNA hybridization studies (Southern method) confirmed that pVA503 was not integrated into the host chromosome of the plasmidless progeny. Drug-resistant progeny from V503 × V528 matings (with or without pVA503) conjugally transferred clindamycin resistance and tetracycline resistance to a suitable recipient strain. None of the drug resistance determinants of V503 were affected by treatment with standard plasmid curing regimens, and methods designed to detect very large plasmid molecules failed to suggest the involvement of extrachromosomal DNA in this resistance transfer system. The well-characterized *Bacteroides* R plasmid, pBF4 (conferring clindamycin resistance), was found to share hybridizing sequences with bulk cellular V503 DNA when examined by filter blot hybridization. Similarly sized sequences were found in drug-resistant progeny recovered from matings. Neither of the two pBF4 derivatives carrying deletions that abolished clindamycin resistance hybridized with V503 DNA.

In the last 3 years there have been several reports describing self-transferable resistance (R) plasmids isolated from anaerobic bacteria. Brefort et al. (2) described a 36×10^6-dalton (36-Mdal) plasmid (pIP401) isolated from a strain of *Clostridium perfringens* that was associated with the expression of resistance to tetracycline (Tc^r) and chloramphenicol (Cm^r). pIP401 was transferable among *C. perfringens* strains via a conjugation-like process. Tally et al. (22) reported transferable resistance to clindamycin (Cc^r)-erythromycin (Em^r) after intraspecies filter matings between strains of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*, and recently they have implicated the involvement of a 10-Mdal plasmid (pBFTM10) in Cc^r and conjugal transfer (21). Privitera et al. (14, 15) have described transferable Cc^r in *Bacteroides* mediated by a 28-Mdal plasmid. Transferable resistance to tetracycline in *B. fragilis* has also been described by Privitera et al. (14, 15), but the plasmid linkage of this marker is not clear at present. Workers in our laboratory (25) have described a 27-Mdal plasmid species (pBF4) isolated from a strain of *B. fragilis* which is self-transferable in interspecies matings and mediates the expression and transfer of resistance to clindamycin and erythromycin. A restriction enzyme site map of pBF4 has been prepared, and two pairs of inverted repeat sequences have been located on this plasmid (27).

In this communication, we describe a *B. fragilis* donor strain (V503) that transfers resistance to clindamycin and tetracycline without the involvement of detectable plasmid DNA. The successive en bloc transfer of these resistance determinants to additional recipients suggests a modular self-transferable genetic sequence which may be transposon-like in nature. Evidence that the Cc^r determinant of *B. fragilis* V503 shares homology with the analogous determinant of pBF4 also is presented.

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TABLE 1. Principal bacterial strains

Species	Strain	Selected phenotypic traits[a]	Plasmid DNA size (Mdal)	Source or reference
B. fragilis	V503	Tc^r Cc^r Em^r Ln^r Rf^s Cf^r ind^- cat^+ ara^-	3.7	T.D.W.[b]
B. uniformis	V528	Tc^s Cc^s Em^s Ln^s Rf^r Cf^s ind^+ cat^- rha^- ara^+	None present	(25)
B. ovatus	V211	Tc^s Cc^s Em^s Ln^s Rf^s Cf^s ind^+ cat^- rha^+ ara^+	None present	(25)

[a] Tc^r, Growth on medium containing 20 μg of tetracycline per ml; Tc^s, no growth on medium containing 5 μg of tetracycline per ml; Cc^r, growth on medium containing 200 μg of clindamycin per ml; Em^r, and Ln^r, growth on medium containing 200 μg of erythromycin and lincomycin, respectively, per ml; Em^s, Cc^s and Ln^s, no growth on medium containing 5 μg of erythromycin, clindamycin, and lincomycin, respectively, per ml; Rf^r, growth on medium containing 40 μg per ml of rifampicin per ml; Rf^s, no growth on medium containing 5 μg of rifampicin per ml; Cf^r, growth on medium containing 25 μg of cefoxitin per ml; Cf^s, no growth on medium containing 5 μg of cefoxitin per ml. Indole production (ind^+ or ind^-) and catalase production (cat^+ and cat^-) were determined as described by Holdeman et al. (7); arabinose fermentation (ara^+ or ara^-) was determined by the addition of colorimetric pH indicator; rha^+, ability to grow on rhamnose as the sole source of carbon; rha^-, inability to use rhamnose as the carbon source.

[b] Obtained as a clinical isolate (in October 1977) at the University of Illinois Hospital, Chicago Medical Center, and characterized at the Virginia Polytechnic Institute (VPI) Anaerobe Laboratory, Blacksburg (VPI no. 12256). Obtained from T. D. Watkins at VPI.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are described in Table 1. Stock cultures were maintained in chopped meat medium (7). Cells were cultivated in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) which was supplemented with tryptone (final concentration, 1%), cysteine hydrochloride (1%), hemin (5 μg/ml), and vitamin K (menadione) (1 μg/ml). The broth was prereduced anaerobically and sterilized as described in the *Anaerobe Laboratory Manual* (7). Solid medium was prepared by adding agar (1.5%) to the supplemented BHI broth. The defined medium of Varel and Bryant (24) supplemented with 0.1% Caseamino Acids, 0.05% yeast extract, and 0.05% tryptone was used in some instances as selective medium. Rhamnose (0.3%) was used as the sole source of carbon in this medium.

Broth cultures and test tube matings (see below) were inoculated under a stream of oxygen-free gas (nitrogen-carbon dioxide, 9:1). Agar plates were incubated anaerobically either in jars using the Gas-Pak system (BBL Microbiology Systems, Cockeysville, Md.) or in vented, gas evacuation-replacement jars (7). All incubations were at 37°C. Antibiotic sensitivity assays were performed by using the agar dilution method described previously (11).

Mating procedures. Filter matings were performed with cells that were grown in supplemented BHI broth to the midexponential growth phase (5×10^8 cells per ml). Donor and recipient cultures (0.5 and 1.0 ml, respectively) were placed together in sterile (1.5-ml) Eppendorf polypropylene centrifuge tubes (Agonics, Brooklyn, N.Y.). The cells were pelleted and then suspended in approximately 0.1 ml of broth. These mating mixtures were transferred by pipette onto sterile membrane filters (type HA, 25-mm diameter, 0.45-μm pore size; Millipore Corp., Bedford, Mass.). The filters were placed on supplemented BHI agar plates, and the plates were incubated under anaerobic conditions for 16 h unless otherwise noted. The filters then were transferred aseptically to sterile 40-ml polypropylene centrifuge tubes. Sterile prereduced buffered salts solution (1 ml) (7) was added to each tube. The cells were washed from the membrane filters by vigorous agitation on a Vortex mixer for 1 min at high speed. Appropriate dilutions of the cell suspension were plated on supplemented BHI agar plates containing appropriate concentrations of antibiotics. The levels of donor and recipient cells on the filter after mating were monitored to ensure that both members of the mating mixture had grown on the filter disk (25). Drug-resistant progeny were always reisolated two or three times on selective media. Reisolated colonies then were examined for unselected phenotypic traits.

Test tube matings were performed by aseptically adding donor and recipient cells (in the proportion described above for filter matings) to a rubber-stoppered tube containing 2 ml of prereduced and anaerobically sterilized BHI-supplemented agar. The cells were pelleted on the surface of the agar butt by briefly subjecting the tube to centrifugation in a clinical centrifuge. The supernatant was aseptically decanted, the tube was flushed with N_2–CO_2 (9:1), and the mating cell mixture was allowed to incubate at 37°C for the desired period of time. After the mating period, the cells were washed from the surface of the agar butt with 1 ml of sterile prereduced buffered salts solution and transferred to a sterile (1.5-ml) Eppendorf polypropylene centrifuge tube and disrupted by agitation on a Vortex mixer for 1 min at high speed. The cell suspension was then plated as described above.

Plasmid DNA isolation and analysis. Plasmid DNA content was determined by subjecting early stationary growth phase cultures to the lysis and cleared-chromosome technique of Guerry et al. (5). These lysates were extracted with an equal volume of phenol previously equilibrated with TE buffer (50 mM Tris, and 5 mM disodium EDTA, pH 8) followed by extraction with an equal volume of chloroform-isopentanol (24:1). The aqueous phase was concentrated by the

addition of 5 M NaCl (to a final concentration of 0.15 M) and 2 volumes of cold 95% ethanol. This preparation was held at −20°C overnight (16 h) or at −70°C for 1 h. It was then subjected to centrifugation at 12,000 × *g* at −20°C for 20 min. The ethanol solution was decanted, and the precipitated DNA was redissolved in 60 μl of TE buffer. Appropriate amounts (10 to 50 μl) of each preparation were placed on agarose gels and subjected to electrophoresis for size determinations (9). Plasmid DNA from *Bacteroides* or *Escherichia coli* used for endonuclease restriction digestion, molecular cloning, and hybridization studies was isolated by a modification of the same procedure. The culture volume was increased to 800 ml, and the plasmid DNA was further purified during ultracentrifugation in cesium chloride-ethidium bromide gradients (25). Bulk cellular DNA for filter blot hybridization studies was isolated by the procedure of Marmur (12). Contour length measurements of purified plasmid DNA were performed as described previously (10). Endonuclease restriction enzymes were obtained from Bethesda Research Laboratories (Rockville, Md.), and digestions were performed according to the manufacturer's instructions.

Molecular cloning on the *Eco*RI-D fragment of pBF4 into pBR325 (1) was accomplished by using standard recombinant DNA methodology as previously published from this laboratory (8).

Filter blot hybridizations. Bulk cellular DNA was transferred to nitrocellulose filters by the method of Southern (20). Radiolabeling of plasmid DNA was performed by the *in vitro* nick translation method with ^{32}P in the alpha position of deoxycytidine triphosphate (16). The materials and protocol were supplied by New England Nuclear Corp. (Beverly, Mass.). The ^{32}P-labeled probe DNA (specific radioactivity, 10^7 cpm/μg) was denatured in 90 mM Tris buffer with 1 M NaOH at pH 12.1 to 12.3 for 10 min. The probe DNA was adjusted to pH 8.0 by the addition of 1 M sodium acetate (pH 4.0). Hybridization of the ^{32}P-labeled probe DNA with the DNA transferred to the nitrocellulose and subsequent autoradiography were performed as described by Thayer (23).

Ethidium bromide or coumermycin treatment. Cultures of V503 were grown to the early exponential phase of growth in defined broth medium (24). Approximately 10^5 cells were transferred to each of a series of tubes containing 10 ml of the minimal broth and increasing concentrations of ethidium bromide (Sigma Chemical Co., St. Louis, Mo.) or coumermycin (Bristol-Meyers, Syracuse, N.Y.). After overnight incubation, the broth culture containing the highest concentration of drug in which growth was not inhibited was spread on nonselective BHI agar medium. At least 500 colonies then were scored for Cc^r and Tc^r by direct replication to drug-containing media.

RESULTS

Plasmid content of *B. fragilis* V503. The high level of Cc^r (>200 μg/ml) and Tc^r (20 μg/ml) displayed by *B. fragilis* V503 prompted us to examine it for plasmid DNA. Agarose gel electrophoretic analysis of cleared lysate preparations of V503 revealed a single plasmid species (pVA503). The size of pVA503 was 3.55 ± 0.23 Mdal based on comparative migration in agarose gels (9) (nine determinations with four different preparations of pVA503). A size estimate of 3.85 ± 0.19 Mdal was obtained from contour length measurements of 16 molecules of pVA503 photographed in the electron microscope. pSC101 (6.02 Mdal) was used as a size reference in these determinations (10).

Electrophoretic analysis of cleared, concentrated cell lysates often revealed a plasmid DNA component that migrated between the 3.7-Mdal covalently closed circular form of pVA503 and the host chromosome. This plasmid species migrated to a position similar to that of a 7.5-Mdal covalently closed circular plasmid or a 3.7-Mdal open circular species. Endonuclease restriction digests of purified pVA503 demonstrated this species to be open circular plasmid DNA. Specifically, *Hae*III was found to cleave pVA503 into two fragments: 2.9 and 0.8 Mdal. Limited digestion with this enzyme allowed us to follow the formation of circular and then linear pVA503 forms. Under standard conditions of electrophoresis (0.7% agarose), open circular pVA503 migrated in the 7.5-Mdal range (covalently closed circular), whereas linear pVA503 migrated in the 6.8-Mdal range (covalently closed circular) (data not shown).

To ensure that a large R plasmid in V503 had not gone undetected, cell lysates were subjected to the plasmid isolation procedure of Hansen and Olsen (6). This method can be used reliably to demonstrate extraordinarily large plasmids. Such analyses still revealed pVA503 to be the only detectable plasmid species in *B. fragilis* V503. Control experiments with the Hansen and Olsen method were performed in which plasmids as large as 220 Mdal could be successfully isolated from *Pseudomonas putida* strains (28).

Drug resistance transfer. *B. fragilis* V503 was examined for its ability to transfer Cc^r and Tc^r despite the absence of large candidate conjugative plasmids in this strain. Using the *Bacteroides uniformis* V528 recipient, progeny were selected for resistance to clindamycin and tetracycline alone or in combination (Table 2). Transfer of the Cc^r and Tc^r markers was readily detected in such matings (Table 2, matings 1 through 3), but the cefoxitin marker present in V503 was never observed to be transferred in this system (Table 2, mating 4). Progeny always were verified by scoring the unselected chromosomal traits of arabinose fermentation and the production of catalase and indol. Additionally, we found that *B. fragilis* V503 and *Bacteroides uniformis* V528 could be readily differentiated on the basis of the fragment pattern of their chromosomal DNAs after digestion with *Hind*III restriction endonuclease (data not shown). The phenotypic traits and the *Hind*III restriction

TABLE 2. Drug resistance transfer frequencies

No.	Mating	Donor	Recipient	Selected on[a]:	Frequency of transfer[b]	Representative progeny
1	Primary	*B. fragilis* V503	*B. uniformis* V528	Rf Tc Cc	1.0×10^{-6}	V619, V622
2	Primary	*B. fragilis* V503	*B. uniformis* V528	Rf Cc	7.6×10^{-6}	—
3	Primary	*B. fragilis* V503	*B. uniformis* V528	Rf Tc	1.2×10^{-6}	—
4	Primary	*B. fragilis* V503	*B. uniformis* V528	Rf Cf	$<1.0 \times 10^{-8}$	—
5	Secondary	*B. uniformis* V619	*B. ovatus* V211	Rham Tc Cc	1.1×10^{-6}	—
6	Secondary	*B. uniformis* V619	*B. ovatus* V211	Rham Cc	1.2×10^{-6}	—
7	Secondary	*B. uniformis* V619	*B. ovatus* V211	Rham Tc	3.7×10^{-6}	—
8	Secondary	*B. uniformis* V622	*B. ovatus* V211	Rham Tc Cc	2.0×10^{-6}	—
9	Secondary	*B. uniformis* V622	*B. ovatus* V211	Rham Cc	1.8×10^{-6}	—
10	Secondary	*B. uniformis* V622	*B. ovatus* V211	Rham Tc	5.5×10^{-6}	—

[a] Selective medium (BHI supplemented as described in the text) for matings 1 through 4 contained rifampicin (Rf), 10 μg/ml; clindamycin (Cc), 5 μg/ml; tetracycline (Tc), 5 μg/ml; and cefoxitin (Cf), 5 μg/ml. Progeny were identified as *B. uniformis* by indole production, lack of catalase production, fermentation of arabinose, and *Hin*dIII endonuclease restriction digest profile of bulk cellular DNA. Tetracycline was used at a concentration of 2.5 μg/ml in the secondary matings. Rhamnose (Rham) was used in defined medium at a concentration of 0.3%.

[b] Frequency of transfer = number of resistant progeny/viable input donor cell.

fragment patterns of the progeny obtained in these crosses always conformed to those of the recipient strain (V528). Attempts to obtain progeny containing only the Cc^r and Tc^r marker alone were unsuccessful. Progeny selected as Cc^r Rf^r always inherited Tc^r (>200 progeny colonies scored per experiment), and those selected as Tc^r Rf^r were always Cc^r (>200 progeny colonies scored per experiment).

The progeny from eight independently performed matings also were examined for plasmid DNA content (data not shown). Approximately 80% of the progeny (10 to 25 clones examined per experiment) examined contained the pVA503 plasmid, regardless of whether they were selected on medium containing both tetracycline and clindamycin or clindamycin alone.

Two independently obtained progeny from primary mating no. 1 (Table 2) were examined for conjugal proficiency. One strain, *B. uniformis* V619 (Table 2), contained the pVA503 plasmid, whereas *B. uniformis* V622 was devoid of detectable extrachromosomal DNA. *B. ovatus* V211 was used as a recipient in these matings, and the results are seen in Table 2 (matings 5 through 10). Both V619 and V622 were able to transfer their resistance en bloc; the Cc^r and Tc^r markers were never seen to segregate from one another in these matings (see above discussion).

Using previously published methods (25) we examined the V503 × V528 mating system with respect to mode of genetic exchange. The results of these experiments revealed that drug resistance was not transferred when the donor cultures were filter sterilized, treated with chloroform, or physically separated from the recipient strain by a membrane filter. The observed transfer was insensitive to DNase I. Further, when broth cultures of V503 and V528 were anaerobically mixed and suitably incubated, no genetic exchange of drug resistance was observed. Preliminary test tube matings were performed in which V503 and V528 were allowed to incubate on the surface of a 2-ml plug of supplemented BHI agar in a tube containing an anaerobic atmosphere. This enabled us to control the duration of mating under strict anaerobic conditions in contrast to filter matings incubated in Gas-Pak jars which were exposed to a decreasing concentration of oxygen until anaerobiosis was achieved (1 to 2 h). The minimum time required for the transfer of drug resistance between V503 and V528 in test tube matings was 45 min. Tc^r and Cc^r appeared to transfer as a unit. No progeny were found after 45 min of mating that were Tc^r and Cc^r alone (based on the screening of 100 progeny per experiment in two experiments).

Characterization of drug resistance phenotypes. The donor V503 and primary resistant progeny (V619, V622, and others tested from the secondary matings) expressed the same levels of resistance to erythromycin, lincomycin, clindamycin, and tetracycline (Table 1). Resistance to tetracycline in V503 was expressed inducibly. Cells cultivated in the presence of a sub-inhibitory concentration of tetracycline (0.1 μg/ml) did not demonstrate a lag in the growth rate during the early exponential phase after challenge with an inhibitory concentration (5 μg/ml) of tetracycline (F. L. Macrina, T. D. Mays, C. J. Smith, and R. A. Welch, J. Antimicrob. Chemother., in press). Cells cultivated in the absence of the drug showed a significant lag in growth when challenged with tetracycline. The same growth pattern was observed for V619 and V622 (Ma-

crina et al., in press). The inducible expression of Tc^r was supported by agar dilution susceptibility assays (data not shown). V503, V619, and V622 (Table 2) cultivated in a sub-inhibitory (0.1-μg/ml) concentration of tetracycline displayed 75% of the colony-forming activity on 20 μg of tetracycline per ml as compared with growth on drug-free medium. Colony-forming activity of noninduced cells was <5% on 20 μg of tetracycline per ml as compared with growth on drug-free medium. Clindamycin resistance was expressed constitutively in strain V503 and in V619 and V622 based on similarly performed growth curve experiments and viability determinations (data not shown).

B. fragilis V503 was grown in low concentrations (1 μg/ml) of tetracycline and tested for conjugal transfer of its Cc^r and Tc^r. No inductive effect on conjugal proficiency was seen, however, with transfer remaining at levels comparable to that seen in Table 2.

The Cc^r and Tc^r markers in V503 were stable in cells grown at 37 or 42°C. In addition these markers were stable in cells grown in the presence of the plasmid curing agents coumermycin or ethidium bromide. A minimum of 500 colonies were screened per curing experiment without detection of drug-sensitive clones.

Molecular analysis of V503, V528, V619, and V622. The presence of pVA503 as an autonomous plasmid species did not correlate with the expression of the drug resistance phenotypes in the primary resistant progeny or their ability to act as donors in subsequent matings (Table 2). To investigate whether pVA503 was present as an integrated segment of the host chromosome in the plasmidless progeny, filter blot hybridizations were carried out with a radiolabelled pVA503 probe (Fig. 1). From these hybridization studies, no sequences of pVA503 were found that hybridized to V528 (lane B) or a primary plasmidless progeny isolate (V622, lane D). pVA503 did hybridize to the 3.7-Mdal plasmid present in V503 (lane A) and in a plasmid-containing progeny (V619, lane C). It should be noted that the blotted DNA in lanes A through D represented material prepared by the method of Marmur (12) and digested with *Hind*III. pVA503 does not have any *Hind*III cleavage sites (unpublished data) and thus appears as covalently closed circular, open circular, and linear components in lanes A and C. These data enabled us to conclude that pVA503 is not present in an integrated state in a resistant progeny cell.

As a probe of the molecular basis of Cc^r in V503 we used the well-characterized *B. fragilis* R plasmid pBF4 (27). This 27-Mdal conjugative plasmid confers constitutively expressed Cc^r. Two deletion-bearing derivatives of pBF4 that are Cc^s were also used. A restriction endonuclease cleavage site map of pBF4 and the location of the two above-mentioned deletions (Δ1 and Δ2) are shown in Fig. 2A. Based on overlap of the Δ1 and Δ2 deletions we selected the *Eco*RI-D fragment (kilobase coordinates ~1 to 4 on map) as an additional molecular probe in these studies, and, accordingly, constructed a pBR325:*Eco*RI-D chimeric plasmid using recombinant DNA methodologies in an EK 1 host system. *Eco*RI-cleaved pBF4 Δ2 (lane A), pBR325:*Eco*RI-D (lane B), and pBF4 (lane C) analyzed by

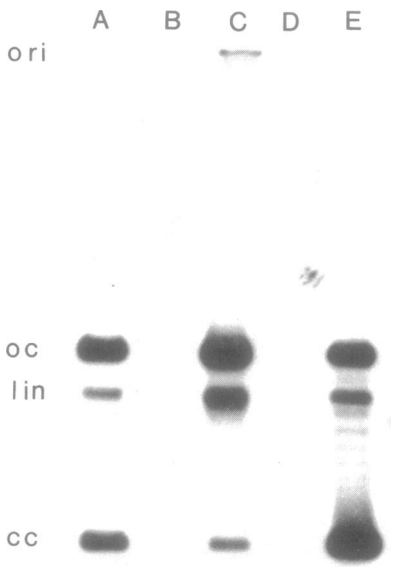

FIG. 1. Autoradiograph of filter-blotted DNA hybridized with ^{32}P-radiolabeled pVA503. Bulk cellular DNA was isolated by the method of Marmur (12). Purified pVA503 was prepared as described in the text. *Hind*III digestion of the bulk cellular DNA preparations (lanes A through D) was performed, and fragments were separated on electrophoretic agarose gels (0.7%) as described in the text. The DNA was transferred to nitrocellulose paper by the method of Southern (20). pVA503 was radiolabeled with ^{32}P by the nick translation method (16). Radiolabeled pVA503 was allowed to hybridize with the filter-blotted DNA, and autoradiographs were prepared as described by Thayer (23). The identity of the DNA preparations that appear above are as follows: (A) *B. fragilis* (donor) V503; (B) *B. uniformis* (recipient) V528; (C) *B. uniformis* V619; (D) *B. uniformis* V622; (E) uncleaved pVA503 purified from CsCl-ethidium bromide ultracentrifugation (approximately 1.5 μg). The origin (ori) of migration in the agarose gel appears at the top of the autoradiograph. The open circular (oc), linear (lin), and covalently closed circular (cc) forms of pVA503 appear in the lanes containing V503 (A), V619 (C), and purified pVA503 (E). The intermediate bands in lane E probably represent topoisomers present in this CsCl-ethidium bromide preparation of pVA503.

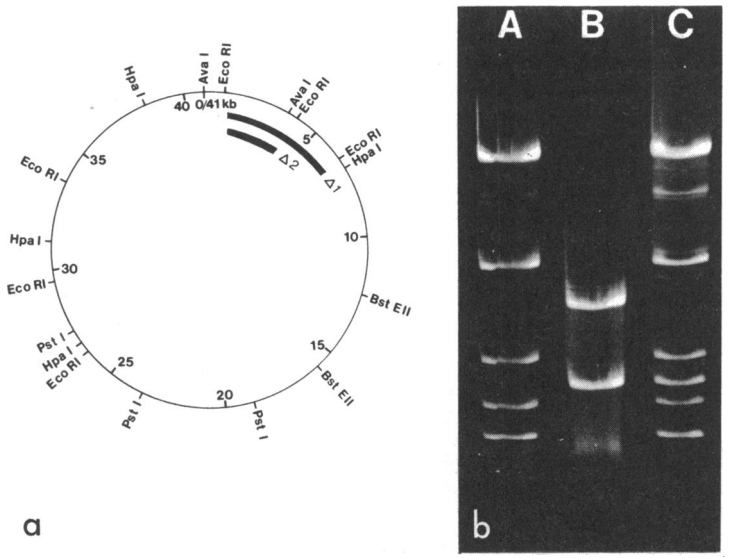

FIG. 2. pBF4 map and analyses of pBR325:*Eco*RI-D chimera. a, Restriction endonuclease cleavage site map of pBF4; the areas covered by the deletions carried by pBF4Δ1 and pBF4Δ2 are noted by the thick lines on the inside of the circle. b, Agarose gel analyses of the pBR325:*Eco*RI-D chimera. Lane A, *Eco*RI-cleaved pBF4Δ2; fragment sizes, top to bottom, are: 19.5, 8.1, 4.3, 3.2, and 2.6 kilobases. Lane B: *Eco*RI-cleaved pBR325:*Eco*RI-D recombinant plasmid; the uppermost component is linear pBR325, the lowermost component is the 3.7-kilobase *Eco*RI-D fragment of pBF4. Lane C, *Eco*RI-cleaved pBF4 DNA; components between the 19.5 and 8.1-kilobase bands and the single faint band below the 8.1-kilobase band are partially digested fragments.

agarose gel electrophoresis are seen in Fig. 2B.

Our purpose in these studies was twofold. First we wanted to determine if the Cc^r determinant of V503 (isolated in the United States) shared homology with the Cc^r determinant carried by pBF4 (isolated from a French *B. fragilis* clinical strain). Second, we wanted to test the hypothesis that the Cc^r Tc^r determinants were chromosomally located. The results of these experiments are presented in the composite autoradiogram in Fig. 3. The blotted DNA in all four panels of this figure are the same; all are *Hind*III-cleaved, Marmur-prepared (12) DNAs from each of the following strains: A, *B. fragilis* V479-1 (contains pBF4); B, V528 (recipient); C, *B. fragilis* V503 (donor); D and E, V619 and V622, respectively (independently obtained progeny; Table 2 and Fig. 1). Panel a represents the radiographic pattern seen when pBF4 was used as a molecular probe. All of the seven *Hind*III components of pBF4 present in the V479-1 DNA were readily observed (lane A), whereas no pBF4 hybridizing sequences to the recipient V528 strain were detected (lane B). Two pBF4 hybridizing components were seen in the V503 strain (lane C), however, and these same components were also present in the two independent progeny strains V619 (lane D) and V622 (lane E). The pBR322:*Eco*RI-D probe hybridized to the V479-1 DNA at a position corresponding to the *Hind*III-D component of pBF4 (lane A, panel b; *Hind*III-D of pBF4 is a doublet band [unpublished data]). No hybridizing activity to V528 DNA was observed (lane B, panel b), but the same two pBR325:*Eco*RI-D hybridizing components were seen in V503, V619, and V622 (panel b, lanes C, D, and E, respectively). These hybridizing components were found to be identical to those seen when pBF4 was used as probe DNA (Fig. 3, panel a) Panels c and d of Fig. 3 show the results obtained by using pBF4Δ2 (panel c) and pBF4Δ1 (panel d) as molecular probes in this system. Hybridization to the pBF4-containing V479-1 strain (lane A in both cases) was evident, with the lessened intensity of the *Hind*III-D component (panel c) and *Hind*-III-A and -D components (panel d) owing to the location of the deletions carried by the probe (27). No hybridizing activity of either of the deletion-bearing pBF4 derivatives to the V528 recipient (lane B), V503 donor (lane C), or the resistant progeny (lanes D and E) was observed, however. It should be noted that lanes B through E of panels c and d contained DNA in fivefold excess over that contained in lane A.

DISCUSSION

The existence of conjugative R plasmids in anaerobic bacteria (2, 14, 15, 21, 22, 25–27) is underscored by the overwhelming predominance of these microorganisms in the human indigenous microflora (13). Indeed, intestinal *B.*

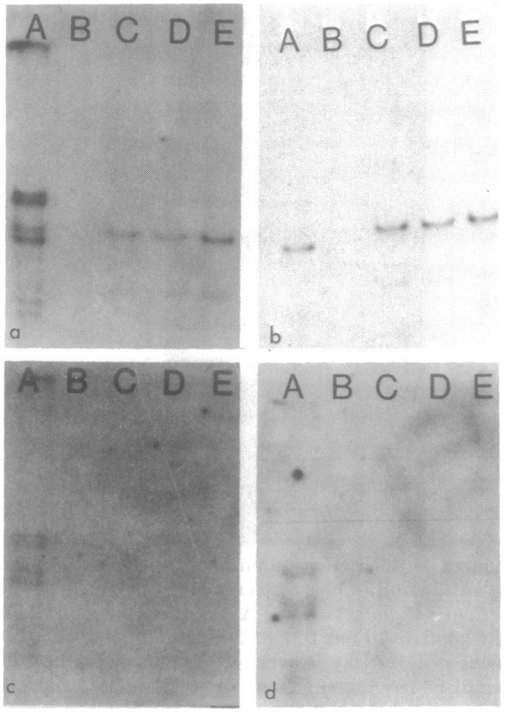

FIG. 3. Molecular probe analyses of *Bacteroides* donors, recipients, and progeny. Conditions for DNA isolation, preparation, and ^{32}P-labeling of probe DNA and Southern blot methodology (20) were as described for Fig. 1. The *Hin*dIII-cleaved blotted DNAs in all four panels were as follows: A, *B. fragilis* V479-1 (contains pBF4); B, *B. uniformis* V528 (recipient); C, *B. fragilis* V503 (donor); D, *B. uniformis* V619 (resistant progeny from V503 × V528 mating); E, *B. uniformis* V622 (resistant progeny from V503 × V528 mating). ^{32}P probe DNA was as follows: panel a, pBF4; panel b, pBF325:*Eco*RI-D chimera; panel c, pBF4Δ2; panel d, pBF4Δ1. In panel a about 1 μg of DNA was applied to each lane; about 0.5 μg of DNA per lane was used in panel b. In panels c and d about 0.5 μg of DNA was applied to lane A, whereas 2.5 μg of DNA was applied to each of lanes B through E. The sizes of the *Hin*dIII components of pBF4 seen in lane A (top to bottom) were: 12.1, 9.9, 5.7, 4.8 (doublet), 2.6, 2.3, and 1.8 kilobases.

fragilis strains are the most commonly isolated anaerobes in soft tissue infections (4). Clindamycin is the drug of choice for use against *B. fragilis*, a species which usually possesses inherent resistance to β-lactam and aminoglycoside antibiotics. The high level of clindamycin resistance in *B. fragilis* V503 was unusual. The fact that the Cc^r and Tc^r of V503 were transferable in the absence of detectable plasmid DNA is a novel finding in *Bacteroides*. Recipients that inherit the Cc^r and the Tc^r determinants (Table 2) do so in a process that fits the criteria of conjugation-like genetic exchange. It should be noted, however, that our data do not rigorously exclude specialized transduction by some difficult-to-detect (perhaps defective) prophage as a means of transfer.

The Cc^r and Tc^r of V503 determinants did not segregate from one another at detectable frequencies (Table 2) indicating their genetic linkage. Although *B. fragilis* V503 was resistant to cefoxitin (presumably via production of a cephalosporinase), this resistance was never observed to be transferable (Table 2, mating 4). The secondary matings (Table 2) clearly suggested that the genetic information for the transfer process was inherited with the resistance determinants. Cc^r Tc^r progeny isolated from primary crosses always were able to act as conjugal donors of that resistance.

The issue of plasmid involvement in the transfer system described here cannot be fully resolved at present. Our data do allow us to conclude that the 3.7-Mdal pVA503 plasmid is not involved in the transfer process or the expression of resistance. Progeny from V503 × V528 matings were able to transfer Cc^r and Tc^r despite the absence of pVA503 as monitored by standard plasmid DNA detection methods (Table 2). Indeed, such plasmidless strains (e.g., V622) have been shown to be devoid of any detectable pVA503 sequences when examined by filter blot hybridization (Fig. 1). This rules out the possibility of pVA503 integration into the V528 genome after transfer. Our reliable use of the Hansen and Olsen (6) technique argues against the presence of a large conjugative plasmid in V503. In control experiments, we could successfully demonstrate plasmids in the 200-Mdal range from *Pseudomonas* (28). However, it is possible that a plasmid (or plasmids) that is difficult to isolate by conventional methods is responsible for the conjugative drug resistance we have observed. On the other hand, the failure to obtain drug-susceptible segregants of *B. fragilis* V503 after treatment with plasmid curing agents such as coumermycin or ethidium bromide supports the notion that the Cc^r and Tc^r determinants are not extrachromosomally located. Roberts and Smith (17) recently have reported that seemingly plasmidless drug-resistant strains of *Haemophilus influenzae* can transfer their resistances by conjugation and that R plasmids can be subsequently recovered in resistant progeny. This strain-dependent isolation of plasmid DNA does not seem likely in our *Bacteroides* system. We have failed to detect plasmids in three different genetic backgrounds (V528, V211, and V531 [Macrina et al., in press]) after transfer from the V503 donor. Further, it should be noted that derivatives of both V528 and V531 have been used as hosts to reliably isolate conju-

gative R plasmid (pBF4) DNA (25).

The experiments designed to probe the genetic basis of Cc^r in V503 (Fig. 3) make an important point. There was clear homology between the pBF4 plasmid and DNA sequences present V503 (Fig. 3, panel a). Interpretation of this sequence homology was facilitated by using the *Eco*RI-D fragment of pBF4 (as cloned into pBR325) as a molecular probe (Fig. 3, panel b). The *Eco*RI-D fragment of pBF4 corresponded to the portion of pBF4 that, when deleted, resulted in irreversible loss of Cc^r. Hence, the similar hybridization patterns seen when either pBF4 (Fig. 3, panel a) or pBR325:*Eco*RI-D was used a probe strongly suggested that the hybridizing sequences of V503 represented at least a portion of the Cc^r determinant in this strain. Compelling evidence in support of this notion was provided by the blots obtained by using pBF4Δ2 (Fig. 3, panel c) and pBF4Δ1 (Fig. 3, panel d) as molecular probes. Neither of the deletion-bearing probes hybridized to V503, V619, or V622 DNA. Hence, we have concluded that the sequence homology between pBF4 and *B. fragilis* V503 resides in their Cc^r determinants. Unfortunately, these experiments failed to shed any light on the genetic location of the Cc^r determinant in V503 and its drug-resistant progeny. If the Cc^r Tc^r conjugal proficiency linkage unit were chromosomally located in V503, then one could predict reasonably that its location in independent progeny cells would vary based on random chromosomal integration sites. This should have resulted in hybridization patterns among the progeny that differed from that seen with V503. This obviously was not the case with *Hin*dIII-cleaved DNAs (Fig. 3). The use of other enzymes (e.g., *Bam*HI, *Eco*RI, *Ava*I, and *Bst*EII) also failed to render hybridization patterns of progeny DNA that differed from this donor. Indeed, even limit-digested DNAs (with *Eco*RI) from V503 and V619 and V622 gave the same array of partially cleaved fragments hybridizing to the pBR325:*Eco*RI-D probe (data not shown). Taken together, these data suggest that the Cc^r and Tc^r determinants do reside on a difficult-to-detect plasmid. Two other possibilities exist, however. First, it is possible that the resistance transfer segment is chromosomally located, but is relatively large. All restriction enzymes tried to date cleave within this sequence; thus, junction sequences have never been probed. The second possibility is that there is a chromosomal hot spot for integration of the resistance transfer sequence. We are continuing our studies of this system to resolve these possibilities.

Conjugal transfer of single or multiple resistance determinants without plasmid involvement has been reported to occur in *Streptococcus pneumoniae* (18). Shoemaker et al. (18) have described the DNase-insensitive transfer of chloramphenicol and tetracycline resistance genes (en bloc) between strains of pneumococci. This process is similar to what we have observed in that progeny always become donors of the resistance genes in question, and mobilization of other chromosomal markers (e.g., erythromycin resistance) does not appear to occur. Recently, Franke and Clewell (3) described the conjugation-like transfer of a chromosomally located 10-Mdal Tc^r transposon (Tn*916*) between strains of *S. faecalis*. This transfer occurred in the absence of apparent plasmid DNA and was specific for the Tc^r determinant. Other known chromosomal genes were not mobilized by Tn*916*, and Tc^r progeny always simultaneously acquired donor ability for the Tc^r marker.

The clinical and evolutionary significance of the apparent plasmidless Cc^r and Tc^r transfer in *B. fragilis* remains to be evaluated. However, precedent for such unusual transfer of resistance in anaerobic bacteria is beginning to accumulate. We have described plasmidless Tc^r transfer in *Clostridium difficile* (19), and Privitera et al. have made similar observations with *B. fragilis* (submitted for publication). Finally, Tally et al. (21) recently have described a transfer system in *Bacteroides* involving non-plasmid-associated en bloc transfer of Cc^r-Tc^r which appears to be similar to the one described here.

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