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Evidence for Extensive Resistance Gene Transfer among *Bacteroides* spp. and among *Bacteroides* and Other Genera in the Human Colon

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Transfer of antibiotic resistance genes by conjugation is thought to play an important role in the spread of resistance. Yet virtually no information is available about the extent to which such horizontal transfers occur in natural settings. In this paper, we show that conjugal gene transfer has made a major contribution to increased antibiotic resistance in *Bacteroides* species, a numerically predominant group of human colonic bacteria. Over the past 3 decades, carriage of the tetracycline resistance gene, *tetQ*, has increased from about 30% to more than 80% of strains. Alleles of *tetQ* in different *Bacteroides* species, with one exception, were 96 to 100% identical at the DNA sequence level, as expected if horizontal gene transfer was responsible for their spread. Southern blot analyses showed further that transfer of *tetQ* was mediated by a conjugative transposon (CTn) of the CTnDOT type. Carriage of two erythromycin resistance genes, *ermF* and *ermG*, rose from <2 to 23% and accounted for about 70% of the total erythromycin resistances observed. Carriage of *tetQ* and the *erm* genes was the same in isolates taken from healthy people with no recent history of antibiotic use as in isolates obtained from patients with *Bacteroides* infections. This finding indicates that resistance transfer is occurring in the community and not just in clinical environments. The high percentage of strains that are carrying these resistance genes in people who are not taking antibiotics is consistent with the hypothesis that once acquired, these resistance genes are stably maintained in the absence of antibiotic selection. Six recently isolated strains carried *ermB* genes. Two were identical to *erm*(B)-P from *Clostridium perfringens*, and the other four had only one to three mismatches. The nine strains with *ermG* genes had DNA sequences that were more than 99% identical to the *ermG* of *Bacillus sphaericus*. Evidently, there is a genetic conduit open between gram-positive bacteria, including bacteria that only pass through the human colon, and the gram-negative *Bacteroides* species. Our results support the hypothesis that extensive gene transfer occurs among bacteria in the human colon, both within the genus *Bacteroides* and among *Bacteroides* species and gram-positive bacteria.

Concern over the safety implications of antibiotic-resistant bacteria in foods has centered around the question of how likely such bacteria are to transfer resistance genes to human intestinal bacteria during their passage through the intestinal tract and what might happen to the transferred genes once they enter colonic bacteria. This question is part of a larger question about the amount of horizontal gene transfer that actually occurs in nature. Few attempts have been made to determine how much gene transfer occurs among bacteria in the colon or in other environments. Some studies have been done to assess the extent of horizontal gene transfer among microorganisms in soil and water (9, 13, 16), the intestines of laboratory mice (26), and experimental abscesses (4). These studies found evidence that horizontal gene transfer events do occur in these settings at frequencies similar to or higher than those observed in the laboratory.

In an earlier paper, Nikolich et al. examined a small number of tetracycline-resistant *Bacteroides* and *Prevotella* species from the human colon and the colons of farm animals (27). The results of that study suggested that horizontal gene transfer had occurred between members of these two genera. Transfer was also demonstrated between the black-pigmented oral *Prevotella* and *Bacteroides* species in the laboratory (11). Results of recent studies of vancomycin-resistant enterococci isolated from the intestines of animals and humans also support the hypothesis that horizontal gene transfer events, occur in the intestinal tract (15, 48; L. B. Jensen, A. M. Hammerum, R. L. Poulsen, and H. Westh, Letter, Antimicrob. Agents Chemother. **43:**724–725, 1999). In this paper, we report the results of the first systematic investigation of horizontal gene transfer events involving a major population of human colonic bacteria, *Bacteroides* species. Although this study focuses on transfer of antibiotic resistance genes, the conclusions could presumably be applied to the transfer of other genes that perform accessory functions.

The human colon is an environment that should be very conducive to horizontal gene transfer events. Nutrients are abundant, the concentration of bacteria is high (10^{12} per g [wet weight]), and there are many surfaces such as plant particles to which bacteria can adhere. Colonic bacteria have been shown to carry a variety of plasmids and integrated elements that can be transferred by conjugation. Yet, under optimized laboratory conditions, transfer of these elements occurs at a relatively low frequency, 10^{-5} to 10^{-7} per recipient or lower. This raised the question of how effectively such conjugal elements could spread in the colonic environment. Moreover, if a transfer event took place, how likely would the recipient be to maintain the newly acquired element?

A way to assess the degree of horizontal transfer among different strains of bacteria in a natural setting is to determine whether identical or virtually identical copies of the same gene

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are found in different species. This approach does not provide transfer rates in vivo, but it can answer questions about the extent to which horizontal transfer has occurred and what types of elements are most often responsible for such transfers. For this study, we chose to focus on *Bacteroides* species. *Bacteroides* species comprise a major part of the human colonic microbiota, accounting for about 25% of all colonic isolates (36, 52). *Bacteroides* species, especially *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*, are opportunistic pathogens, which can cause life-threatening infections if they escape from the colon as a result of abdominal trauma or surgery. Over the past several decades, *Bacteroides* clinical isolates have become increasingly resistant to antibiotics. Resistance to tetracycline has become so common that strains are often not tested for susceptibility to this antibiotic. In recent years, resistance to drugs used to treat *Bacteroides* infections, such as clindamycin, has also been increasing. Conjugal elements such as plasmids and conjugative transposons (CTn's) have been found in *Bacteroides* clinical isolates (34). Thus, horizontal gene transfer could conceivably have played a role in the rising incidence of resistance in this bacterial group. In this study, we assess the role of CTn's and plasmids in the transfer of antibiotic resistance genes.

MATERIALS AND METHODS

***Bacteroides* strains.** The 88 VPI strains are from the Anaerobe Laboratory at the Virginia Polytechnical Institute in Blacksburg. Some of these strains had been isolated from healthy volunteers (community isolates); others had been isolated from infected patients (clinical isolates). All of them were isolated before 1980, many before 1960 (17). The clinical isolates from 1980 to the present were obtained from various medical centers and hospitals: the majority were from the Wadsworth Anaerobe Laboratory (WAL) in Los Angeles, Calif. (n = 34) and Loyola Strich School of Medicine, in Maywood, Ill. (n = 65; designations begin with DH). The 1996-to-1997 community isolates were from rectal swabs taken from volunteers attending the Microbial Diversity Course at Woods Hole, Mass., in 1996 and 1997 (n = 86; designations begin with WH). The isolation of *Bacteroides* strains from these volunteers took advantage of the aerotolerance of *Bacteroides* species and their resistance to high levels of gentamicin (200 μg/ml). Samples were diluted and streaked directly onto supplemented BHI medium (7) containing 200 μg of gentamicin/ml under aerobic conditions and were then incubated anaerobically in BBL GasPak jars for 48 h. Fifty or more isolates were first checked by colony hybridization using *tetQ* and CTnDOT probes (Table 1), and four or five isolates from each source were selected at random, stocked, and saved for further analysis. The further verification of the identity of the isolates included hybridization to *Bacteroides*-specific probes (19), Gram staining, and some selective 16S ribosomal DNA (rDNA) sequencing using a universal prokaryotic forward primer and a *Bacteroides* group-specific reverse primer (30). This mode of isolation could have had a slight bias in favor of the species that are somewhat more aerotolerant and less fastidious than other species, such as *B. thetaiotaomicron* and *B. fragilis*. This bias could not have been too strong, however, because we isolated no *B. fragilis* using this procedure, and whereas many of the isolates were *B. thetaiotaomicron*, several were also *Bacteroides uniformis* and *Bacteroides eggerthii*. These ratios reflected the ratios of *Bacteroides* species in the colon, where the concentration of *B. fragilis* is more than 10-fold lower than that of *B. thetaiotaomicron* or *B. uniformis*.

The species of the VPI community and clinical isolates was first identified by biochemical analysis and later confirmed by DNA-DNA or rRNA hybridization studies (17, 18). The species of the isolates from the Loyola and Wadsworth Veterans Affairs (VA) hospitals and the other clinical sources were determined by biochemical analyses carried out at the institutions from which they were obtained. We checked some of these strains by partial sequencing of their 16S rRNA genes. In the case of the clinical isolates, there was probably a slight bias in favor of resistant over susceptible strains because patients infected with resistant strains are more likely to experience treatment failure and are thus more likely to have specimens of the infecting bacterium sent to the clinical laboratory. The two sets of strains from the Wadsworth VA hospital were obtained because of their resistance to chloramphenicol or tetracycline and therefore are not representative of all clinical isolates. Data for these are reported separately.

Antibiotic resistance phenotypes. Fresh overnight cultures were streaked onto supplemented BHI (7) agar plates containing the antibiotic. The strains were considered resistant if they grew on plates containing erythromycin (3 μg/ml), clindamycin (3 μg/ml), or tetracycline (1 μg/ml). *B. thetaiotaomicron* 5482 and *B. fragilis* 638 did not grow at these concentrations. No attempt was made to determine the MICs for each strain because the purpose of these phenotypic tests was to correlate the resistance phenotype with the presence of the resistance genes responsible and not to determine the precise level of resistance. Many of the clinical strains came with MIC results, and the resistances we observed correlated with the information provided.

DNA hybridizations. DNA dot blots using total DNA prepared from 2-ml overnight cultures of each strain (33, 50) were probed using fluorescein-dUTP-labeled DNA fragments or purified PCR products. Labeling was done as described in the Renaissance kit protocols (NEN Life Sciences). The blots were developed using a chemiluminescent substrate. Southern blotting was done on the DNA of 60 strains that hybridized to *tetQ* to determine how related they were to the known *Bacteroides* CTn's, especially CTnDOT. The DNA from each strain was digested with *Eco*RI and *Eco*RV, and the blots were probed with a series of fluorescein-labeled probes. The first probe used was a 7,700-bp *Eco*RI fragment of CTnDOT which contained *tetQ, rteA, rteB*, and *rteC* (46). Some of the blots were probed sequentially with an *rteC* probe, an *rteB* probe, and then a probe containing *tetQ* and *rteA* in order to identify restriction fragments containing each sequence. This was necessary because of the restriction site polymorphisms observed for some of the strains. See Table 1 for descriptions of primers for PCR-generated products and the sources of the isolated fragments used to make the probes.

PCR amplification of 16S rRNA sequences and *tetQ* genes. The PCR primers used to amplify 16S rDNA and the internal fragments for *tetQ, ermG*, and *ermB* genes are described in Table 1. The amplification was done using *Taq* I polymerase in 100-μl reaction mixtures containing 10 to 100 ng of DNA and 200 ng of each primer in a solution containing 1× Gibco-BRL PCR buffer, 1.5 mM $MgCl_2$, and 0.2 mM deoxyribonucleoside triphosphate mixture. The amplification cycles were as follows: 95°C for 5 min; 30 cycles of 95°C for 1 min, 50 to 52°C for 1 min, and 72°C for 2 min; and a final elongation step of 72°C for 5 min. The PCR products were extracted directly from the reaction mix using a Wizard PCR Clean up kit (Promega). The PCR products were sequenced using the PCR primers by the University of Illinois Biotechnology Center.

RESULTS

Hybridization analyses of community and clinical isolates. For an initial assessment of the extent to which horizontal gene transfer has occurred between colonic *Bacteroides* strains over the past 3 decades, we compared tetracycline and erythromycin resistance profiles and the carriage of possible resistance genes of two sets of isolates. One set (88 isolates) had been collected before 1980, many of the strains before 1960. The second set (211 isolates) had been collected after 1980, most during the 1990s. Both sets contained strains isolated from patients with *Bacteroides* infections (clinical isolates) and strains isolated from the intestines of healthy humans (community isolates). By comparing clinical and community isolates, it was possible to determine whether patterns seen in clinical isolates mirrored those seen in community isolates. If the spread of resistance was occurring in the community isolates, later causing clinical infections, the patterns should be similar. If resistance was arising in hospitals, the incidence of phenotype resistance and carriage of resistance genes should be higher in the clinical isolates than in the community isolates.

Results of the initial survey are shown in Table 2. As expected from studies of clinical isolates, the incidence of phenotypic resistance to tetracycline and erythromycin had risen dramatically since the pre-1970 period. All of the tetracycline-resistant strains tested contained a single tetracycline resistance gene, *tetQ*. *tetQ* encodes a ribosome protection type of

TABLE 1. Sources of the DNA fragments used to probe the *Bacteroides* isolates

Target gene(s)	Source of DNA for the probe[a]
Antibiotic resistance genes	
tet(Q)	The *tetQ* gene of CTnDOT cloned onto pNFD13-2 (28). A 1.55-kbp *Eco*RI-*Pvu*II internal fragment was used as a probe. Primers Q for (GGC TTCT ACG ACA TCT ATT A) and Q rev (CAT CAA CAT TTA TCT CTC TG) were used to PCR amplify a 758-bp internal fragment (21).
tet(C)	*Hin*dIII- *Nru*I fragment of pBR328 (*tetC*)
tet(L)	pMV158 (entire) (*tetL*) (20)
tet(M)	*Bam*HI-*Kpn*I *tetM* (Tn*916*) fragment from pFD310 (45)
tetB(P)	*Pst*I-*Eco*RI internal fragment of *tetB*(P) from pJIR667 (43)
ermB	A 639-bp internal fragment of pTV1-OK (Tn *917*) was used for sequencing and as a probe. Primers were B1 (GAA AAG GTA CTC AAC CAA ATA) and B2 (AGT AAC GGT ACT TAA ATT GTT TAC). The accession no. is X58285) (47).
ermF	A 0.85-kbp *Eco*RI fragment containing *ermFS* (accession no. M37699) of Tn*4551* cloned in pFD214 (44)
ermG	PCR primers G for1 (ACA TTT CCT AGC CAC AAT C) and G rev1 (CGC TAT GTT TAA CAA GC) were used to obtain a 442-bp internal product (bp 465 to bp 907) (accession no. L42817) (8).
ermA, *ermC*, *ermQ*	PCR products or fragments of cloned genes. The primers and targets for *ermA* and *ermC* are described by Sutcliffe et al. (47). A 380-bp internal fragment of *ermQ* was isolated from pJIR745 (2).
Element probes	
CTnDOT joined ends	1.1-kbp PCR product containing the CTnDOT joined ends; includes *att* site and part of the integrase gene (5)
NBU1–NBU2 shared region	*prmN1 oriT mobN1* region of NBU1 (41)
IS*4351*	1.2-kbp *Eco*RI-*Hin*dIII fragment (accession no. M17124) of Tn*4400* cloned onto pEG920 (31, 42)
tetQ-rteA-rteB and *rteC* region of CTnDOT	7.7-kbp *Eco*RI fragment of CTnDOT isolated from a cosmid clone of CTnDOT (40, 46)
rteC alone	1.1-kbp subclone from CTnDOT region on pLYL52 (23) (accession no. L02419)
rteB alone	500-bp *Eco*RV-*Bsa*I fragment of *rteB* from CTnDOT (accession no. M81439)

[a] Fragments of *tet*(C), *tet*(L), *tet*(M), and *tetB*(P) were isolated from plasmids and used as probes. See reference 22 for accession numbers.

tetracycline resistance (28). No hybridization was detected when probes representing other tetracycline resistance genes, *tetC, tetL, tetM*, or *tetB*(P), were used. It is clear from Table 2 that the incidence of strains carrying *tetQ* has risen steadily from the pre-1970 period to the 1990s. *tetQ* was also found in strains obtained from a sewage treatment plant, indicating that carriage of the gene was maintained in this environment.

Carriage of erythromycin resistance genes (*erm* genes) has also increased. Seventy-two percent of the erythromycin-resistant strains (51 of 71) contained either *ermF* or *ermG*. *ermF*

TABLE 2. Distribution of *tetQ* and of MLS- and CTnDOT-specific genes found in community and clinical *Bacteroides* sp. isolates

Isolate group[c] (*n*)	% of isolates with resistance or hybridizing to the indicated probe									
	Tc^r	CTnDOT element probes[a]				Em^r	MLS and IS probes[b]			
		tetQ	CTn ends	*rteB*	*rteC*		*ermF*	*ermG*	*ermB*	IS*4351*[d]
Community										
VPI, pre-1970 (32)	**28**	28	34	47	31	**0**	0	0	0	0
VPI, 1970–1980 (33)	**42**	42	48	48	42	**0**	0	0	0	0
WH, 1996–1997 (86)	**80**	83	81	83	76	**29**	15	0	2	5
Sewage, 1997 (16)	**62**	62	88	81	75	**38**	12	6	6	0
Clinical										
VPI, pre-1970 (23)	**22**	22	30	43	26	**9**	9	0	0	0
Loyola VA (65)	**83**	83	85	83	80	**31**	17	8	2	3
WAL Cm (20)	**ND**[e]	60	60	90	60	**5**	5	0	0	0
WAL Tc (14)	**100**	100	86	93	79	**64**	50	14	14	0
Other (10)	**80**	80	90	90	80	**70**	60	10	0	20

[a] Genes found on a family of *Bacteroides* 50- to 70-kbp CTn's called the CTnDOT family (38). The sources of the DNAs used for probes are described in Table 1.
[b] MLS genes or *erm* genes found by hybridization. Active genes confer resistance to all three groups of antibiotics. Several strains hybridized to two different *erm* probes.
[c] Strains are described in Materials and Methods. Cm^r, all strains in this group were chloramphenicol resistant. Only two other strains, one community isolate and one clinical isolate, were found to be chloramphenicol resistant. Tc^r, all the strains in this group were tetracycline resistant.
[d] IS*4351* has usually been found associated with *Bacteroides* regular compound transposons that also contain *ermF*. All of the IS*4351*-containing strains also hybridized to the *ermF* probe.
[e] ND, not done.

TABLE 3. Summary of the *Bacteroides tetQ* sequence data

Group (no. of strains)	% Identity (no. of strains) to the following *tetQ* allele, source, and RFLP pattern[a]:		
	tetQ-1, CTnDOT, A	*tetQ*-2, BF1126, C or D	*tet*-3, BF-2, B
VPI, 1960–1970s (13)[b]	100 (1) >96 (12)	100 (1)	100 (3) >98 (6)
Woods Hole community isolates (10)	>96 (10)	99–100 (2)	99–100 (6)
Clinical isolates, 1980–present[c] (10)	>96 (3)	99–100 (2)	100 (5)

[a] The accession numbers for the three *Bacteroides tetQ* alleles are as follows: for *tetQ*-1, X58717; for *tetQ*-2, Z21523; and for *tetQ*-3, Y08615. They are all >96% identical to each other, and they all were isolated from *Bacteroides* strains containing a CTn element. The RFLP pattern for the *tetQ rteA rteB rteC* region is shown in Fig. 1 and 2. The highest correlation (there were exceptions) of the *tetQ* allele identity with the Southern blot RFLP pattern observed with the *tetQ, rteA, rteB*, and *rteC* probes is also indicated.

[b] Ten different species are represented by these strains.

[c] The known CTnDOT element sequences (e.g., CTnDOT, CTnERL, CTn*12256*) described by Nikolich et al. (27) are not included. CTnV479 is still the only *tetQ* sequence with <96% identity to any of the three *tetQ* alleles shown.

and *ermG* are members of the macrolide-lincosamide-streptogramin B (MLS) family of resistance genes (32, 51). These genes also confer resistance to clindamycin, a drug that has been used to treat *Bacteroides* infections in the past. The fact that so many resistant strains harbored these two *erm* genes suggests that they too are being transferred horizontally. Although *ermF* has been associated primarily with *Bacteroides* and related genera (10, 12), *ermG* was first found in a gram-positive soil bacterium, *Bacillus sphaericus* (25). None of the strains in this survey hybridized to the *ermA*, *ermC*, or *ermQ* probes (Table 1); however, six of the strains surveyed contained DNA that cross-hybridized with the *ermB* probe. *ermB*, like *ermG*, has been found mainly in the gram-positive bacteria and is prevalent in clinical isolates of *Clostridium, Streptococcus*, and *Enterococcus* spp. (32). All of the *Bacteroides* strains carrying *ermB* or *ermG* were recent isolates, whereas some *ermF*-containing strains were found among the pre-1970 clinical isolates. Thus, it appears that *ermB* and *ermG* have entered the *Bacteroides* species more recently than *ermF*.

The strains included in the survey represented 10 different *Bacteroides* species in both the clinical and community isolates. These species are only distantly related to each other and share DNA-DNA hybridization values ranging from 5 to 45% (17). Finding the same resistance genes detectable by Southern hybridization, which indicates >80% nucleotide identity in such distantly related strains, suggested that horizontal gene transfer, and not dissemination of one or a few resistant strains, was responsible for the high incidence of carriage of *tetQ, ermB, ermF*, and *ermG*.

Sequence analysis of *tetQ* genes from different strains. If recent horizontal gene transfer was responsible for the widespread carriage of *tetQ* and the *erm* genes, the genes in different strains should be virtually identical at the DNA sequence level. Of the *erm* genes, we were particularly interested in the *erm*B and *erm*G genes because of their known gram-positive origin. The *Bacteroides ermF* genes found on transposons and CTn's are >99% identical, and the original source of this gene is not known (10, 12). *tetQ* PCR products from 33 isolates representing 10 species (13 pre-1970 strains and 20 post-1980 strains) were sequenced and compared. The results are summarized in Table 3. The sequences were compared to the *tetQ* sequences deposited in GenBank. The sequences of the 33 strains had 96 to 100% identity to the *tetQ*-3 gene found on the conjugative transposon CTnDOT (Table 3) (accession no. X58717). Genes from 20 of the 33 strains were 98 to 100% identical to *tetQ*-1 from *B. fragilis* strain BF2 (accession no. Y08615), including 9 of the 13 pre-1970 strains. The *tetQ* on CTnV479 is still the most divergent, with only 89 to 90% identity to the other *tetQ* genes in either *Bacteroides* or *Prevotella* strains (27). The high sequence identity of the *tetQ* alleles supports the hypothesis that *tetQ* has been spread by horizontal gene transfer. This result also rules out convergent evolution, the independent evolution of two versions of the same gene. The amino acid sequences of such genes can be very similar if there is strong selection for a particular sequence. Even two proteins with the same amino acid sequence, however, can be encoded by genes whose sequences differ by as much as 20% due to 3rd-base wobble.

Sequence analysis of the six *ermB*-containing isolates revealed that two contained genes that were identical to the *erm*(B)-P gene found previously in *Clostridium perfringens* and *Streptococcus pneumoniae* (3, 32), and the remaining four differed only by 1 to 3 nucleotides (Table 4). A similarly high level of sequence identity was seen when the 442-bp internal sequences of nine *ermG* genes were compared. These had 1 to 5 nucleotide differences within this region compared to the *ermG*

TABLE 4. *Bacteroides ermB* sequence comparisons to *C. perfringens erm*(B)-P

Strain	Source	Change at nucleotide position[a]:		
		A433	C450	C522
Bov7991[b]	Clinical, WAL			
WH202[c]	Community, Woods Hole, 1997			
WH207	Community, Woods Hole, 1997		G	
BF8371	Clinical, WAL		G	
DH3760	Clinical, Loyola	C	G	
WH714	Community (sewage), Woods Hole, 1997	C	G	T

[a] The 639-bp PCR-amplified *ermB* product from each of the *Bacteroides* strains was sequenced and compared to the *ermB* [*erm*(B)-P] of *C. perfringens* (accession no. X58285). Only the differences are indicated.

[b] A *Bacteroides ovatus* strain that contains both *ermB* and *ermG* sequences.

[c] A 1997 Woods Hole isolate that is phenotypically sensitive to erythromycin.

TABLE 5. Differences between the *Bacteroides ermG* sequences and *Bacillus sphaericus ermG*

Strain	Source	Group[a]	Change at nucleotide position[b]:						
			C509	G582	**G602**	T678	G715	T810	C853
Bov7991	WAL	I			**T**				
BT7853	WAL	II			**T**		A	C	
DH4083	Loyola	II			**T**		A	C	
DH4072	Loyola	II			**T**		A	C	
DH3716	Loyola	III		A	**T**	C			T
DH3717	Loyola	III		A	**T**	C			T
DH4140	Loyola	III		A	**T**	C			T
WH713	Sewage, 1997	III		A	**T**	C			T
BF6436-5	Clinical, Yale	IV	T	A	**T**	C			T

[a] The strains are grouped according to sequence identities. BT7853, group II, contains CTn*7853*. The other two strains in group II also hybridized to the CTn*7853*-specific probe.

[b] The sequences of the 442-bp *Bacteroides ermG* PCR products were compared to the sequence of *ermG* from *Bacillus sphaericus* (accession no. M15332). The G602-to-T602 difference shared by all of the *Bacteroides* sequences is indicated by boldface.

in *Bacillus sphaericus* (25) and could be grouped into four groups by sequence (Table 5). The *ermG* genes from two of the strains were identical to the *ermG* found on CTn*7853* and fell into group II, with 3 nucleotide differences. The finding that there was more sequence diversity among alleles of *tetQ* than among alleles of *ermG* and *ermB* is consistent with our results in Table 2, which indicate that *tetQ* has been in *Bacteroides* species longer than either *ermG* or *ermB*.

Type of gene transfer elements associated with the horizontal gene transfer. Previous studies, which were limited to a small number of clinical isolates, had shown that *tetQ* in *Bacteroides* spp. was carried on two different types of CTn's, one represented by CTnDOT and one represented by CTn*7853* (29, 39). *ermF* was found previously on several CTn's of the CTnDOT group and on three transmissible plasmids (24). *ermG*, however, has been found only on CTn*7853* (8). Virtually all of the strains that harbored *tetQ* also harbored DNA that hybridized with a probe from the ends of CTnDOT (5). Whereas more than 80% of CTnDOT has now been sequenced, making it easier to design probes that identify elements of this family of CTn's, only a small amount of sequence outside the *tetQ* region of CTn*7853* is available. Using a 1-kbp fragment located 6 kbp upstream of *tetQ* as a probe for CTn*7853* type CTn's, we found only two additional strains that cross-hybridized to the probe; they both contained *ermG* and were the other two strains in Group II (Table 5), with an *ermG* sequence identical to that of CTn*7853* in *B. thetaiotaomicron* 7853. Thus, it appears that CTn*7853* type CTn's are not widespread in *Bacteroides* and that the CTnDOT type elements are the predominant type of CTn. There were strains that hybridized to the CTnDOT end probe that did not contain *tetQ*, for example, the VPI strain *B. uniformis* 0061. *B. uniformis* 0061 has been shown previously to carry a cryptic CTn, CTnXBU4422, which is related to CTnDOT (42).

Bacteroides strains commonly have one or more plasmids, and some of these plasmids are either self-transmissible or mobilizable (24, 35, 37). *tetQ* has not been found on plasmids in *Bacteroides* spp., but it has been found on plasmids, e.g., pRR14, from *Prevotella* strains (27). *ermF*-carrying *Bacteroides* plasmids have been described, and in all of these plasmids the *ermF* gene was linked to an insertion sequence, IS*4351*, which provided a promoter for the resistance gene (24). IS*4351* is rare in *Bacteroides*; it was found in only 8 of 299 strains tested and was detected only in association with *ermF* sequences from the strains isolated after 1980 in this study (Table 2). IS*4351* has not been found on any CTn's (36). Only 1/5 of the strains that contained *ermF* also contained IS*4351*. In the other 4/5, *ermF* may well be carried on CTn's such as CTnDOT.

Finding *tetQ* in a strain containing DNA that also hybridizes to the CTnDOT probe does not necessarily prove that *tetQ* is carried on a CTnDOT type element. Downstream of *tetQ* on CTnDOT are three regulatory genes, *rteA, rteB*, and *rteC* (Fig. 1). The CTnDOT family of CTn's represents the only elements so far found to contain this entire region (37). CTn*7853* and *tetQ* from some *Prevotella* strains have a small fragment of *rteA* adjacent to *tetQ* but none of the other genes (29). If *tetQ* is carried on a CTnDOT type element, the *rteA rteB rteC* region should be intact and adjacent to *tetQ*. Results of the survey shown in Table 1 indicated that strains carrying the CTnDOT end sequences also carried DNA that hybridized with *rteB* and *rteC* (which are missing on CTn*7853*). To confirm that there was genetic linkage between *tetQ* and the *rte* genes in such strains, 60 strains were analyzed by Southern blotting. The majority of the strains tested (79%) had restriction patterns indicating that the *tetQ rteA rteB rteC* region was present, although there were some restriction fragment length polymorphisms (RFLP) (Fig. 1 and 2). The CTnDOT pattern A or A′ (*Eco*RV site missing or not cutting) with the two small fragments containing *rteC* sequences was not the predominant one. Instead, elements with pattern B, with the *rteC* sequences all contained on a 2.6-kbp fragment, as shown in Fig. 1 and 2, predominated in all but one of the groups of strains tested (Fig. 3). This pattern correlated fairly well with the strains whose *tetQ* sequence had 98 to 100% identity to *tetQ*-3 from *B. fragilis* BF-2 (accession no. Y08615; G. Reysset, unpublished data). Several of the strains tested contained more than one element (four instead of two end junction fragments) and had a mixed pattern such as A*. For example, *B. fragilis* ERL, a clinical isolate, had two CTnDOT type elements: one (CTnERL) had pattern A and the other (CTnERL2) had the A′ pattern (42).

An interesting trend was seen in CTn's contained in more recently isolated strains (Fig. 3). Whereas virtually all the older isolates carried CTn's with the A–A′ or B restriction pattern, the pattern in many of the newer isolates indicated that parts of the *rteA rteB rteC* region had been lost or rearranged, as seen in the patterns of C1, C2, and C3 in the Southern blot shown

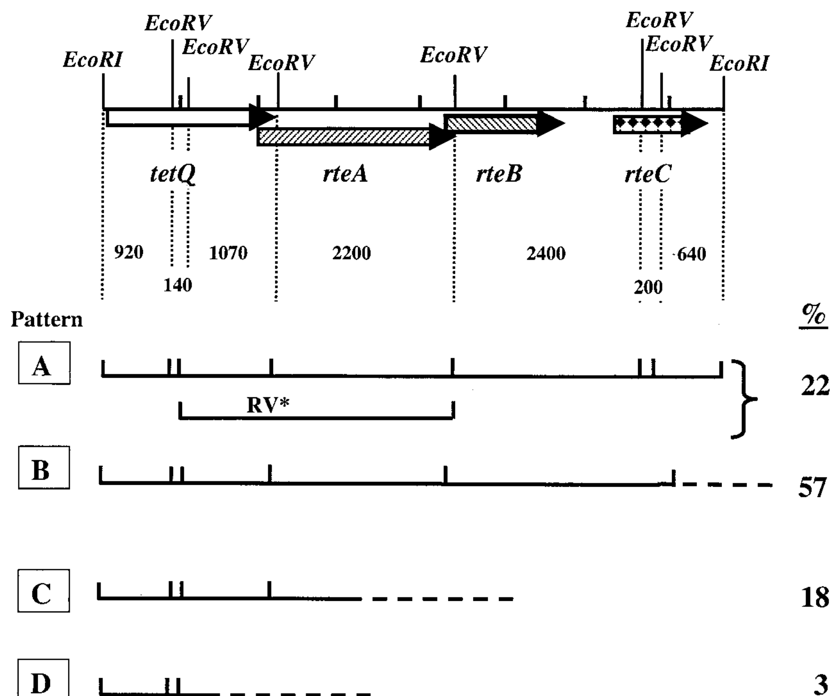

FIG. 1. Diagrammatic representation of the restriction patterns seen on Southern blots of DNA from different strains, which was hybridized with a probe that detects the *tetQ rteA rteB rteC* region of the CTnDOT type CTn's. The fragment sizes and the locations of the genes within the 7.7-kbp *Eco*RI fragment used as the probe are shown at the top. Pattern A has the restriction fragment profile of CTnDOT and closely related elements. Some of the CTnDOT family of elements (CTnERL2) lack an *Eco*RV site and have the fragment labeled RV*. This pattern is referred to as A′. Occasionally this *Eco*RV site does not cut completely, and a mixed pattern is observed (indicated as A* in the Southern blot in Fig. 2). Strains exhibiting pattern B are missing the two small *rteC* fragments ($C_{0.2}$ and $C_{0.64}$ in Fig. 2), and all of the *rteC* homology is located in the 2.6-kbp fragment labeled $BC_{2.6}$ in Fig. 2. Strains that exhibit the C pattern are heterogeneous. A few lack *rteC* completely (CTn*7853*; C3 in Fig. 2), and others just have a very different pattern, but all of the genes on the probe are present (CTnV479; C2 in Fig. 2). Pattern D is rare; these strains have *tetQ* and usually neither *rteB* nor *rteC* sequences. The percentage of the 60 strains exhibiting each of the patterns is shown at the right.

in Fig. 2 and summarized in Fig. 3. C3 is the rare pattern observed for CTn*7853* that lacks both *rteB* and *rteC*, whereas patterns C1 and C2 contained both *rteB* and *rteC* sequences (Fig. 3). C1 and C2 type isolates also hybridized to the CTnDOT end probe, whereas C3 isolates did not. Since the *rte* genes are essential for transfer of CTnDOT type elements, this may indicate that these C pattern strains that hybridized to the CTnDOT ends contain CTn's that are no longer transmissible.

DISCUSSION

Taken together, our results show that the substantial increase in tetracycline resistance among *Bacteroides* isolates that has occurred over the past few decades was due to the horizontal spread of a single gene, *tetQ*, on CTn's of the CTnDOT type. *ermF* and *ermG* also appear to be spreading by horizontal gene transfer, possibly also on CTn's such as CTnDOT and CTn*7853*. It is likely that these gene transfer events took place in the human colon, because *Bacteroides* species are found primarily in the human colon and are present, if at all, in low numbers in the intestines of other animals or in the environment. The only environmental site outside the human colon to harbor significant numbers of *Bacteroides* strains would be a sewage treatment plant. Transfer of resistant *Bacteroides* strains from sewage treatment plants to humans, however, would be unlikely to have produced the widespread colonization seen in our studies. Also, as is evident from the 16 sewage plant isolates tested (Table 2), carriage seems to be lower in strains obtained from this setting than in strains isolated from the human colon.

A feature of the CTnDOT type elements allows us to speculate about what may have caused this extensive spread of resistance genes. Most of the CTnDOT type elements exhibit regulated transfer (38). That is, transfer occurs only if the donors are first stimulated with low levels of the antibiotic tetracycline. After tetracycline induction, transfer frequencies rise 1,000- to 10,000-fold. No other class of antibiotics has this effect. Thus, tetracycline use in the community probably played a role not only in selecting for maintenance of *tetQ* but also in causing it to be transferred in the first place.

Our results show clearly that once a resistance gene enters *Bacteroides* species, it can be spread widely among these species if it becomes part of a CTn or some other transmissible element. Our survey also provided evidence that *Bacteroides* species may share DNA with members of genera outside the *Bacteroides-Prevotella* group. *ermF* was first found in *Bacteroides* species (12) but clearly originated in a low-G+C organism (33%). *tetQ*, which appears to have originated in *Bacteroides* spp., has 40% G+C, which is the average observed for this genus (17). The origin of *ermG* (27% G+C) is also unknown, but this gene has been found in *Bacillus sphaericus*, a gram-positive soil bacterium, as well as in *B. thetaiotaomicron*

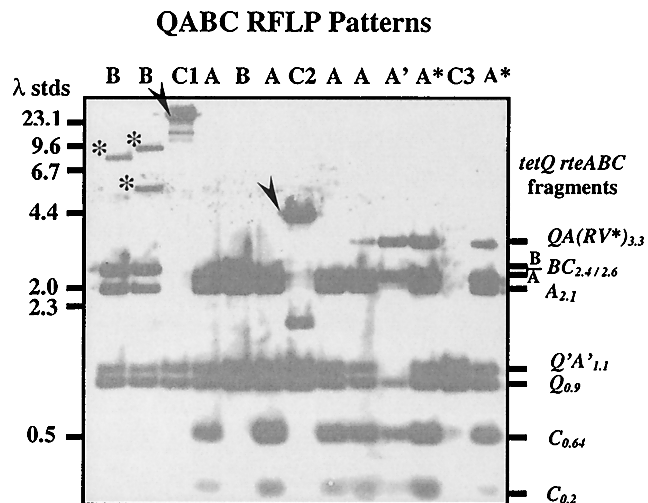

FIG. 2. Southern blot of the *Eco*RI- and *Eco*RV-digested cellular DNA from *tetQ*-containing *Bacteroides* isolates. The blot was first probed with an *rteC* probe and then reprobed with the *tetQ-rteA-rteB-rteC*-containing probe. The blot is overexposed so that the small *rteC*-containing bands ($C_{0.2}$ and $C_{0.64}$) can be observed for pattern A. These sequences appear in the $BC_{2.6}$ band of pattern B. The sizes of the *Hin*dIII lambda DNA size standards (stds) are given on the left. The sizes and contents of the major bands hybridizing to the probes are shown on the right. A schematic of the region being probed and the expected sizes is shown in Fig. 1. The patterns for each lane are labeled according to the scheme described in Fig. 1. The *rteC*-containing fragments for patterns C1 and C2 are indicated by arrows. These fragments also contain *rteB*, and C2 is for CTnV479. The B patterns in lanes 1 and 2 also contain extra hybridizing bands (indicated by asterisks) that hybridize to *rteB* but not *rteC or tetQ* probes. The A* patterns may be due to a strain containing both an A and an A′ CTn as is seen for *B. fragilis* ERL or the pattern may be due to partial digestion of the *Eco*RV site between *tetQ* and *rteA* (Fig. 1), as is sometimes observed for CTn*12256* (data not shown).

7853 (8). Our survey also turned up six *Bacteroides* strains that had acquired an *ermB* gene virtually identical to an *ermB* from *C. perfringens, Streptococcus pneumoniae*, and *Enterococcus faecalis*. This finding suggests that even bacteria that do not normally reside in the colon (*S. pneumoniae*) or that reside there in low numbers (*C. perfringens* and *E. faecalis*) can donate DNA to members of numerically predominant groups of colonic bacteria. Our results do not prove that DNA was transferred directly from these species to *Bacteroides* species, but the presence of *ermG* and *ermB* in multiple isolates from different geographical locations indicates that some genetic connection, however indirect, is open between the gram-positive bacteria where the genes appear to have originated and the gram-negative *Bacteroides* species.

Given that some gram-positive resistance genes appear to have moved into *Bacteroides*, it is surprising that *tetM*, a resistance gene that is distantly related to *tetQ* and confers the same type of resistance, was not found in any of these *Bacteroides* isolates. The *tetM* gene is carried on several gram-positive CTn's that appear to have a broad host range, at least under laboratory conditions. The failure to find *tetM* could indicate that there might be barriers to transfer of some types of elements in vivo or that there is a lack of selective pressure for tetracycline resistance determinants other than *tetQ*, since 80%

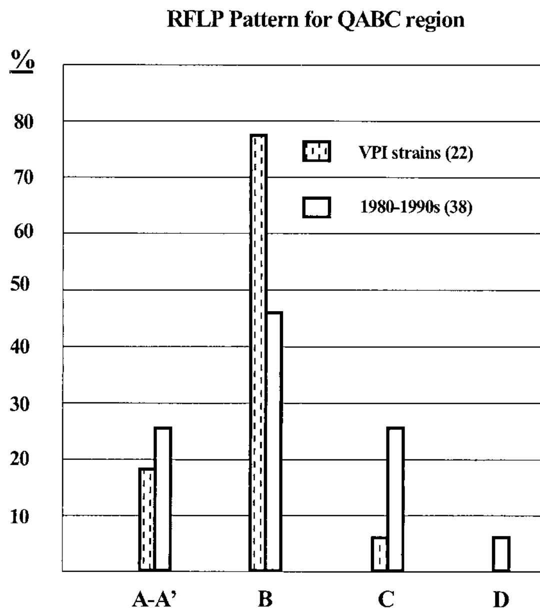

FIG. 3. Differences in restriction patterns (depicted in Fig. 1 and 2) between older isolates and modern isolates. Both groups of strains include both community and clinical isolates. The 22 VPI strains were isolated before 1970. The RFLP patterns are shown in Fig. 1, and Southern blot patterns A, B, and C are shown in Fig. 2.

of the *Bacteroides* strains are already tetracycline resistant due to *tetQ*.

Once in *Bacteroides* species, conjugal transfer mediated primarily by CTn's can spread these genes widely among different *Bacteroides* species. This study is the first to demonstrate what scientists have long suspected, that the human colon is a site that is highly conducive to horizontal gene transfer and to stable maintenance of transferred resistance genes. It is also the first study to associate an increase in antibiotic resistance with carriage of a particular type of conjugal element, in this case CTn's of the CTnDOT class. CTn's have also been found in gram-positive bacteria (1, 6) and in the *Escherichia coli* phylogenetic group of gram-negative bacteria (14, 49). The results of our study suggest that CTn's are making a major contribution to the transmission of antibiotic resistance genes.

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