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A Newly Discovered *Bacteroides* Conjugative Transposon, CTnGERM1, Contains Genes Also Found in Gram-Positive Bacteria

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Results of a recent study of antibiotic resistance genes in human colonic *Bacteroides* strains suggested that gene transfer events between members of this genus are fairly common. The identification of *Bacteroides* isolates that carried an erythromycin resistance gene, *ermG*, whose DNA sequence was 99% identical to that of an *ermG* gene found previously only in gram-positive bacteria raised the further possibility that conjugal elements were moving into *Bacteroides* species from other genera. Six of seven *ermG*-containing *Bacteroides* strains tested were able to transfer *ermG* by conjugation. One of these strains was chosen for further investigation. Results of pulsed-field gel electrophoresis experiments showed that the conjugal element carrying *ermG* in this strain is an integrated element about 75 kb in size. Thus, the element appears to be a conjugative transposon (CTn) and was designated CTnGERM1. CTnGERM1 proved to be unrelated to the predominant type of CTn found in *Bacteroides* isolates—CTns of the CTnERL/CTnDOT family—which sometimes carry another type of *erm* gene, *ermF*. A 19-kbp segment of DNA from CTnGERM1 was cloned and sequenced. A 10-kbp portion of this segment hybridized not only to DNA from all the *ermG*-containing strains but also to DNA from strains that did not carry *ermG*. Thus, CTnGERM1 seems to be part of a family of CTns, some of which have acquired *ermG*. The percentage of G+C content of the *ermG* region was significantly lower than that of the chromosome of *Bacteroides* species—an indication that CTnGERM1 may have entered *Bacteroides* strains from some other bacterial genus. A survey of strains isolated before 1970 and after 1990 suggests that the CTnGERM1 type of CTn entered *Bacteroides* species relatively recently. One of the genes located upstream of e*rmG* encoded a protein that had 85% amino acid sequence identity with a macrolide efflux pump, MefA, from *Streptococcus pyogenes*. Our having found >90% sequence identity of two upstream genes, including *mefA*, and the remnants of two transposon-carried genes downstream of *ermG* with genes found previously only in gram-positive bacteria raises the possibility that gram-positive bacteria could have been the origin of CTnGERM1.

The human intestinal tract is thought to be a site in which horizontal transfer of genes might occur fairly often. The concentration of bacteria in the colon is high, and factors such as the availability of abundant nutrients and surfaces on which biofilms could form would seem to be conducive to gene transfer (8, 11). It has been difficult to test this hypothesis, however, because of limited information about gene transfer elements in the predominant genera of colon bacteria. The numerically predominant bacteria in the colon are all obligate anaerobes, including *Bacteroides* species and a mixture of gram-positive genera (8). Virtually nothing is known about gene transfer elements of the gram-positive anaerobes, but some of the *Bacteroides* gene transfer elements have been characterized (13). Accordingly, attempts to assess the extent to which horizontal gene transfer occurs in the colon have focused on *Bacteroides* species.

Many *Bacteroides* strains carry plasmids, but a family of self-transmissible integrated elements called conjugative transposons (CTns) seems to be particularly widespread and may be the primary drivers of horizontal gene transfer in this genus (13, 18). These CTns are exemplified by CTnDOT and CTnERL, two CTns that are virtually identical except for a 13-kbp region in CTnDOT that contains an erythromycin resistance gene, *ermF* (23). Both CTnERL and CTnDOT carry the tetracycline resistance gene *tetQ*, and transfer of both of these CTns is stimulated 1,000- to 10,000-fold by exposure of donors to tetracycline (12, 13, 24). More than 80% of *Bacteroides* strains isolated since 1980 harbor one or more of these CTns, compared to 22 to 30% of strains isolated prior to 1970 (18). Clearly, these CTns have been in *Bacteroides* strains for some time and are continuing to spread actively within the genus. These data do not, however, address the question of the extent to which horizontal gene transfer might be introducing new DNA elements into *Bacteroides* species.

A recent survey of 289 *Bacteroides* isolates showed that, as expected, *ermF* was the predominant erythromycin (MLS_B) resistance gene in *Bacteroides* species (18, 23). Nonetheless, nine isolates were identified that contained *ermG*—a class of *erm* gene that has been found previously only in a gram-positive bacterium, *Bacillus sphaericus* (7). The DNA sequences of the *ermG* genes found in the *Bacteroides* isolates were over 99% identical to the DNA sequence of the *Bacillus sphaericus* gene (3). Finding virtually identical antibiotic resistance genes in two such distantly related genera suggested that *ermG* is being transmitted horizontally, possibly from gram-positive bacteria into *Bacteroides* species. Moreover, since the *ermG* was found

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant phenotypes[a]	Description and source or reference
Bacterial strains		
E. coli		
DH5α MCR	RecA$^-$ McrA$^-$ McrB$^-$ Mrr$^-$ HsdM$^-$ HsdR$^-$	Gibco BRL
S17-1	RecA$^-$ Tp^r TraRP4	Contains the transfer functions of RP4 integrated in the chromosome (20)
B. thetaiotaomicron BT5482 strains		
BT4001	Rif^r	Rif^r derivative of BT5482A
BT4100	Thy$^-$ Tp^r	Spontaneous thymidine-requiring mutant of BT5482A
BT4007	Rif^r Em^r Tc^r	BT4001 with CTnDOT in the chromosome
BT4004	Rif^r Tc^r	BT4001 with CTnERL in the chromosome
BT4009	Rif^r Em^r	BT4001 with CTn*7853* in the chromosome
BT4001ΩWH504	Rif^r Em^r	BT4001 with a self-transmissible element from WH504 that confcrs low-level Em^r (~3 μg/ml) but with no known *erm* gene detected (this study)
Plasmids		
pLYL001	Tc^r (Ap^r)	*Bacteroides* suicide vector that contains the $oriT_{RK2}$ and *tetQ* cloned into pUC19; can be mobilized by IncPα plasmid RP1 or the RP4 transfer functions in *E. coli* S17-1 (10)
pYP01	Tc^r (Ap^r)	The 442-bp internal *ermG* PCR was blunted and cloned into the *Hinc*II site of pLYL001 (this study)
pGRW6	Tc^r (Ap^r)	5-kb fragment downstream of *ermG* was cloned into pLYL001 by plasmid rescue of the *Pst*I-digested chromosome DNA (this study)
pGRW8	Tc^r (Ap^r)	10-kb fragment upstream of *ermG* was cloned into pLYL001 by plasmid rescue of the *Bam*HI-digested chromosome DNA (this study)
pGW47	(Kn^r)	A 3.0-kb *Aat*II/*Nar*I fragment containing the *Bacteroides* pBI143 *rep* and *mob* regions was purified from pFD160R (21), blunted, and then cloned into the *Ssp*I site on the p15A derivative pK184 (6) to form a low-copy-number *E. coli-Bacteroides* shuttle vector (G. Whittle)
pYP66	(Kn^r)	4.9-kb *Pst*I-*Eco*RV fragment of CTnGERM1 containing *mefA* was cloned into the *Pst*I-*Sma*I site of pGW47 to look for expression of *mefA*-encoded erythromycin resistance in *Bacteroides* hosts (this study)

[a] Phenotypes in parentheses are expressed only in *E. coli*, and phenotypes outside parentheses are expressed in *Bacteroides* strains. Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Gen, gentamicin; Kn, kanamycin; Rif, rifampin; Spec, spectinomycin; Tc, tetracycline; Thy$^-$, thymidine auxotroph; Tp, trimethoprim.

primarily in *Bacteroides* strains isolated after 1990, it was clear that *ermG* had come into *Bacteroides* species fairly recently—presumably from some other genus (3).

Broad host range transfers usually occur by conjugation, a process thought to help DNA escape restriction modification systems in the recipients, but whether *ermG* was on a conjugative element was not known. We report here that the *ermG* gene is carried on an integrated conjugative element, a CTn, which is different from CTnERL and CTnDOT and may have entered *Bacteroides* species much more recently than did the CTnERL- and CTnDOT-type elements. Our findings provide additional evidence which suggests that the exchange of DNA in the human colon may involve genera other than *Bacteroides.*

MATERIALS AND METHODS

Bacterial strains, growth conditions, and DNA manipulations. Strains and plasmids used in this study are described in Table 1, and the *ermG*-containing strains are described in Table 2. The strain designated WH was isolated from sewage by Caroline Plugge, a student in the microbial diversity summer course at the Marine Biological Laboratory (Woods Hole, Mass.). All other strains were

TABLE 2. Characteristics of nine *Bacteroides ermG*-containing strains

Strain	Species	Genotype[a]	Phenotype[b]	Group[c]	Transfer frequencies[d]		Retransfer frequencies[d]		Upstream of *ermG*[e] homology
					ermG	*tetQ*	*ermG*	*tetQ*	
Bov7991	*B. ovatus*	QGDNB	TE	I	0/0	$10^{-6}/10^{-4}$	NR	NT	−
BT7853	*B. thetaiotaomicron*	QG	TE	II	$10^{-5}/10^{-5}$	$10^{-5}/10^{-5}$	$10^{-5}/10^{-5}$	$10^{-5}/10^{-5}$	+
DH4083	*B. fragilis*	QGDN	TE	II	NT	NT	NT	NT	−
DH4072	*B. thetaiotaomicron*	QG	TEA	II	$10^{-6}/10^{-5}$	$10^{-6}/10^{-5}$	10^{-6}	10^{-6}	+
DH3716	*B. ovatus*	QGDN	TEA	III	$10^{-5}/10^{-5}$	0/0	10^{-4}	NR	+
DH3717	*B. distasonis*	QGDN	TEA	III	$10^{-5}/10^{-6}$	0/0	NT	NR	+
DH4140	*B. thetaiotaomicron*	QGDN	TEA	III	NT	NT	NT	NT	+
WH713	*Bacteroides* sp.	QGDN	TE	III	$10^{-7}/10^{-7}$	$10^{-8}/10^{-7}$	NT	NT	+
BF6436-5	*B. fragilis*	QGDN	TE	IV	$10^{-6}/10^{-6}$	0/0	10^{-7}	NR	+

[a] Genotype determined by Southern blot or dot blot hybridization to specific probes (18): Q, *tetQ* gene; G, *ermG* gene; D, CTnDOT joined ends; N, NBU1 primase and *mob* gene area; B, *ermB* gene.

[b] Phenotype: T, tetracycline resistance (>1 μg/ml); E, erythromycin resistance (>3 μg/ml); A, ampicillin resistance (>50 μg/ml).

[c] Group classification is based on the sequence identity of the PCR products of internal regions of the *ermG* genes relative to that of the *ermG* of *Bacillus sphaericus* (18).

[d] Frequencies are given as transconjugants per recipient at the end of the mating: the transfer frequency of *ermG* or *tetQ* without Tc induction/with Tc (1 μg/ml) induction. Definitions of notations used are as follows: 0/0, $<10^{-9}$; NR, not relevant (gene not present in donor); NT, not tested.

[e] The + indicates that both the original strain and the BT4001 transconjugants hybridized to the probes made from 10-kb regions upstream of *ermG* of CTnGERM1 from DH3716; the − indicates the lack of same.

clinical isolates obtained from various sources in the United States: DH indicates strains obtained from David Hecht, Loyola VA Hospital, Maywood, Ill.; BF6436-5 was obtained from the Yale Medical Center, and the other strains were obtained from Sydney Finegold, Wadsworth Anaerobe Laboratory, Los Angeles, Calif. (18). Species identifications were made by phenotypic testing in the laboratory of origin or by partial sequencing of the 16S rRNA gene in our laboratory or both. The species identity of one of the strains, WH713, has not been determined.

The methods used for cultivation of *Bacteroides* strains, DNA isolation, and cloning have been described previously (14, 16). The antibiotic concentrations used were as follows: ampicillin, 50 to 100 μg/ml; erythromycin (Em), 3 to 10 μg/ml; gentamicin (Gen), 200 μg/ml; tetracycline (Tc), 3 μg/ml; rifampin (Rif), 10 μg/ml; and trimethoprim (Tp), 100 μg/ml. The concentration of thymidine (Thy) was 100 μg/ml.

Mating experiments. To test whether an *ermG* gene was carried on a transmissible element, mating experiments were performed as described previously for the CTnERL- and DOT-type CTns (16). Thy-requiring spontaneous mutants of the *ermG*-containing *Bacteroides* strains served as donors, and the *B. thetaiotaomicron* 5482A Rif-resistant strain BT4001 served as the recipient (17). The transconjugants were selected on medium containing Gen, Em, and Rif—and no Thy. Since *tetQ* was coresident in all of the donors, the transconjugants were tested for cotransfer of the tetracycline resistance. To confirm that *ermG* was on a self-transmissible element, the resulting Em^r BT4001 transconjugants were used as donors in filter matings to BT4100 (Thy^-,Tp^r, and Rif^s) recipients. The secondary transconjugants were selected on medium containing Gen, Em, Thy, and Tp and patched onto Rif plates to identify any Thy mutations of the donors. The *ermG* element from strain DH3716 was chosen for further study because it transferred only *ermG* and not *tetQ*—a trait which suggested that it might be a new type of *Bacteroides* CTn. Four of the six strains transferred *ermG* constitutively (i.e., they required no growth in an antibiotic for induction), and the transfer was independent of the *tetQ* in the donor.

PFGE. To determine whether the DH3716-derived element was an integrated element, and if so, whether it integrated site specifically, pulsed-field gel electrophoresis (PFGE) analysis was performed. In six independent conjugation experiments, Thy^- strain DH3716 was mated with a BT4001 recipient. Two transconjugants from each of the six matings (total, 12 colonies) were inoculated into 10 ml of Trypticase-yeast extract-glucose. Cells were pelleted by centrifugation and lysed in situ in an agarose plug (14). The plugs were treated with lysozyme to lyse the cells, and proteinase K was added to inactivate nucleases before loading the plug onto a 1% agarose gel.

*Not*I digestion (40 U per plug) produced 12 well-spaced bands. The PFGE was performed according to the manufacturer's instructions (CHEF-DR III; Bio-Rad). *Not*I-digested DNA from the 12 independent primary transconjugants, *Not*I-digested DNA from strain BT4001, and *Saccharomyces cerevisiae* DNA standards were loaded onto the pulsed-field gel. The gel was run at 6 V/cm for 24 h with a switching angle of 120° and a switching time of 50 to 130 s.

Cloning segments of the *ermG* element by using plasmid rescue. Initial attempts to clone segments of the *ermG* element by constructing a cosmid library of *Bacteroides* chromosomal DNA and probing it with an *ermG* probe were unsuccessful. As an alternative strategy, we turned to a plasmid rescue approach. A 442-bp PCR product internal to *ermG* was cloned into the *Sma*I site of a *Bacteroides* suicide vector pLYL001 (10) in order to produce pYP01. pYP01 was then transformed into *E. coli* (S17-1), which contains the transfer regions of the IncPα plasmid RP4 (17). Filter mating between S17-1(pYP01) and a BT4001 transconjugant containing the CTnGERM1 element was performed. pYP01 integrates into *ermG* in the BT4001ΩCTnGERM1 recipient by homologous recombination. The transconjugants were selected on medium containing Gen and Tc. The chromosomal DNA of a Gen^r Tc^r transconjugant was digested with *Bam*HI, and a 16-kb fragment containing the vector and DNA adjacent to the *ermG* was isolated from low-melting-point agarose gel. DNA from this fraction was treated with T4 DNA ligase, which was used to transform *Escherichia coli* MCR. Transformants containing the plasmid and adjacent DNA sequences were isolated by selection for ampicillin resistance. A 16-kb plasmid, pGRW8, was isolated and was shown to contain pLYL001 (6 kb) and 10 kb of chromosomal DNA from the *ermG* element, which was later used as a probe. To clone more DNA from the element, the distal end of this 10-kb fragment was subcloned into pLYL001 and the process was repeated. A total of 19 kb from the regions adjacent to the *ermG* gene were cloned and sequenced.

DNA sequence of a 19-kb region adjacent to *ermG*. By using an Applied Biosystems model 373A version 2.0.1A dye terminator automated sequencer, workers at the University of Illinois Biotechnology Genetic Engineering Facility performed DNA sequencing. The primers were synthesized by workers at the University of Illinois Biotechnology Genetic Engineering Facility or at Operon Technologies, Inc. (Alameda, Calif.).

Southern hybridization analysis. Southern blot analysis was used to identify the band containing the *ermG* element in the PFGE gels and to determine the restriction fragment length polymorphisms of the various *ermG* transmissible elements integrated in strain BT4001 relative to CTnGERM1 probes. In both cases, the chromosomal DNAs from the agarose gels were transferred to nylon membrane filters and Southern hybridization was performed according to the previously described protocol (14). Probes were made from cloned DNA segments of CTnGERM1 and were labeled with fluorescein-dUTP by using random primers as specified in the NEN Life Sciences Renaissance Kit protocol. The Southern blots were developed by using a chemiluminescent substrate according to instructions provided by the manufacturer.

Nucleotide sequence accession number. The sequence of the 19-kb *ermG* region has been submitted to the EMBL nucleotide sequence database; the accession number is AJ557257.

RESULTS

Characteristics of nine *ermG*-containing *Bacteroides* strains. Previously, the nine *ermG*-containing *Bacteroides* strains identified in our survey of *Bacteroides* isolates had been divided into groups (I to IV) based on small sequence differences (1 to 4 bp within a 442-bp region) between the *ermG* alleles (18). The *ermG* genes were found in strains of different *Bacteroides* species (Table 2), thus indicating that they might be located on transmissible elements. At least one strain from each group was chosen to test for conjugal transfer of the *ermG* gene in a mating experiment in which the original isolate was the donor and a *Bacteroides thetaiotaomicron* strain, BT4001, was the recipient. Characteristics of the *ermG* strains and the results of the filter mating experiments are shown in Table 2.

The group II strains, BT7853 and DH4072, cotransferred *tetQ* and *ermG* to strain BT4001, suggesting that the *tetQ* and the *ermG* genes in these strains were carried on the same mobile element. However, the group III strains, WH713, DH3716, and DH3717, and a group IV strain, BF6436-5, transferred the *ermG* gene independently of the coresident *tetQ* gene, suggesting that in these strains the mobile elements carried only the *ermG* gene and might thus be a novel element different from CTnERL and CTnDOT. One of these strains, DH3716, was chosen for further study. As shown in Table 2, the *ermG* element in DH3716 was able to retransfer in a separate mating from a BT4001 transconjugant to a BT4100 recipient and thus appears to be self-transmissible and therefore not mobilized by a CTnDOT-like element. The CTnDOT-type elements have been shown previously to mobilize a variety of plasmids and integrated elements (13). Another indication that the *ermG* element from DH3716 was not mobilized by a CTnDOT-type element was that the transfer of the *ermG* gene from DH3716 to BT4001 was not enhanced by treating the donor DH3716 with Tc. That the transfer of CTnDOT itself and of mobilization of plasmids and integrated elements such as NBU1 and NBU2 by CTnDOT is seen only after exposure of donors to Tc is typical, so this was a difference between the *ermG* element and CTnDOT (12). Also, the transfer frequency of the *ermG* element was not enhanced by exposure of donors to Em. Transfer frequencies were about 10^{-5} transconjugants per recipient under all conditions tested.

The *ermG* element from DH3716 is about 75 kb in size and integrates into the chromosome site selectively. Both the donor DH3716 and the recipient BT4001 contained a 35-kb plasmid, p5482. This plasmid does not encode Em resistance, since BT4001 (which carries p5482) does not grow in the presence of

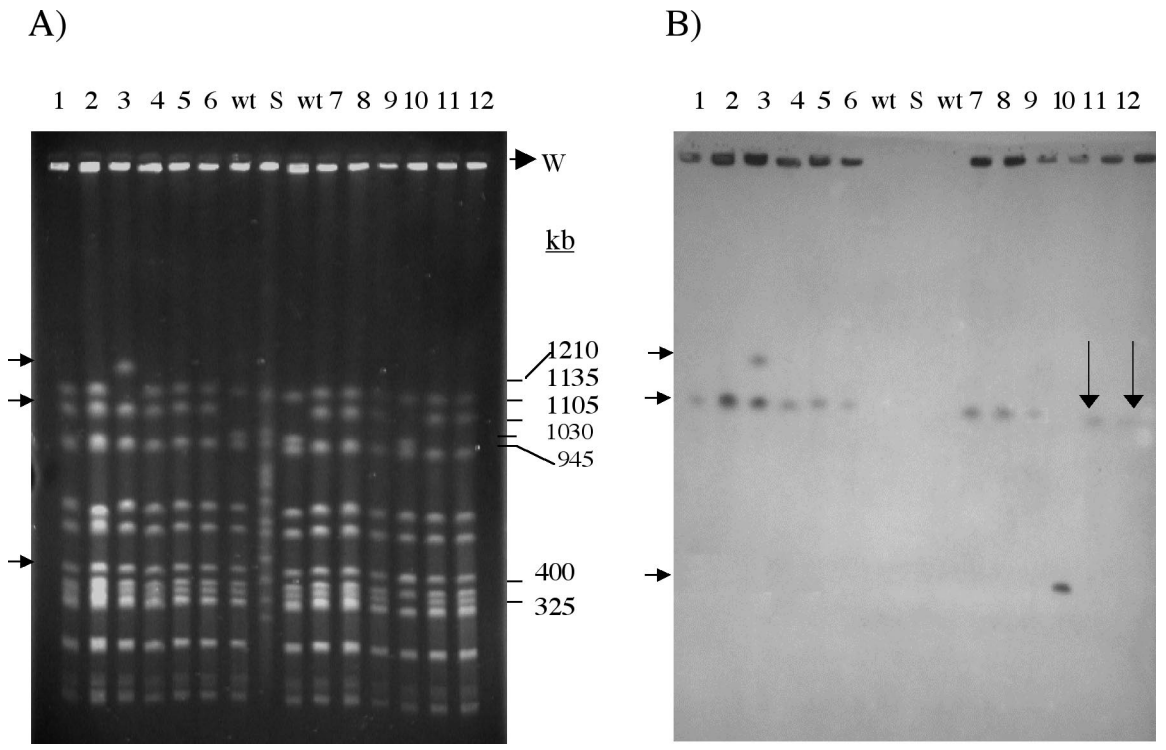

FIG. 1. Results of PFGE and Southern blotting to determine the size and insertion sites of CTnGERM1. (A) Ethidium bromide-stained pulsed-field gel of BT4001 Em^r transconjugants. The DNA from 12 *ermG* transconjugants, two from each of six matings between BT4001 and DH3716, was digested with *Not*I and run on a pulsed-field gel (lanes 1 to 12). S indicates *S. cerevisiae* DNA size standards. The sizes of the bands are given in kilobase pairs at the right side of the gel. Compared to the band sizes seen for BT4001, the transconjugants 1 to 9, 11, and 12 all have a band that shifted from 1,030 to 1,105 kb (arrow on left). Transconjugant 3 has an additional band that shifted from 1,135 to 1,210 kb (arrow on left). Transconjugant 10 has a band that shifted from 325 to 400 kb (arrow on left). In each case, the larger bands are 75 kb larger than the missing smaller bands, thus indicating that the integrated element is about 75 kb in size. (B) The Southern blot of the pulsed-field gel. The blot was probed with the 2.8-kb *Sac*I-SacI *ermG*-containing fragment (probe F in Fig. 2). In all cases, the *ermG*-hybridizing bands were the ones that shifted in molecular weight. The two arrows indicate the positions of two bands that were stronger after longer exposure of the Southern blot. The three arrows on the left indicate the same band positions as the arrows in panel A.

1 μg of Em per ml; therefore, the plasmid in these strains did not carry *ermG*. Furthermore, the *ermG* probe did not hybridize to the plasmid DNA preparations from either the donor or the BT4001 transconjugants, thus indicating that the *ermG* element was not a plasmid but instead might be integrated into the chromosome. To test the hypothesis that the *ermG* element was an integrated element, PFGE was performed to compare *Not*I digestion patterns of DNA from 12 independent transconjugants—obtained in matings between DH3716 and BT4001—to the *Not*I digestion pattern of the recipient BT4001 strain. Band shifts were observed in all transconjugants (Fig. 1A). In 10 of 12 transconjugants (83%), a band shifted in size from 1,030 to 1,105 kb. The increase in the size of the shifted band indicated that the integrating element was about 75 kb in size. DNA from the transconjugant in lane 3 exhibited two band shifts. Although one of the two shifts was the same as the other 10, the second shift involved a different band—suggesting that the *ermG* element integrated into a secondary site. Both of the shifts correspond to an increase in size of about 75 kb. Lane 10 shows the third band shift pattern. In this case, too, there was an increase of about 75 kb. Southern hybridization analysis demonstrated that all of the shifted *Not*I bands seen in Fig. 1A hybridized with the *ermG* probe and thus clearly contained the *ermG* element (Fig. 1B). This *ermG* element is an integrating, transmissible genetic element and thus appears to be a CTn. Since it carries the *ermG* gene, it was named CTnGERM1.

Sequence analysis of CTnGERM1 DNA in the *ermG* gene region. To assess the possible origin of CTnGERM1 and to ascertain whether the other *ermG*-containing strains carried similar CTns, we cloned 19 kb of DNA from the region containing *ermG* by using the plasmid rescue strategy described in Materials and Methods and obtained the complete DNA sequence of this region. The BLAST search results are summarized in Fig. 2 and Table 3. Thirteen putative ORFs were identified. According to the BLAST search results, *tnpIS* encodes a protein that has 90% identity with the transposase of *B. fragilis* IS613 (accession number BAA95632). There is 92% identity at the nucleotide level, suggesting that this is a *Bacteroides* insertion element transposase. Since there were 20 amino acids (aa) missing from the N-terminal end of the TnpIS protein, it was not clear if it was functional and possibly contributing to the transfer of the element. This gene was disrupted by single-crossover disruption using an internal fragment of the gene (C in Fig. 2). Southern blots were performed to confirm the result (data not shown). The transfer frequency of this mutant was the same as that of wild-type CTnGERM1 (i.e., $\sim 10^{-5}$ transconjugants per recipient). Thus,

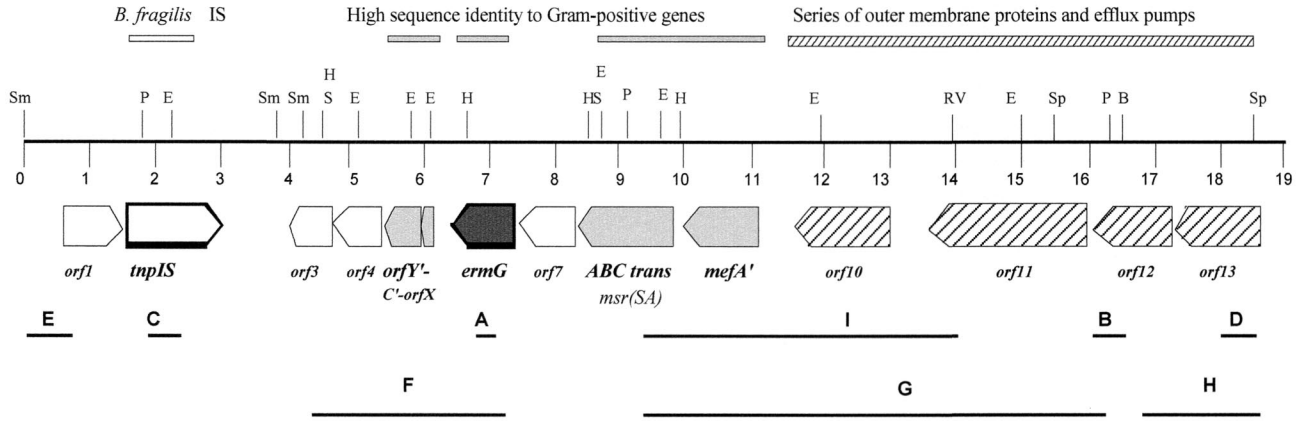

FIG. 2. Schematic diagram of the 19-kb region isolated from CTnGERM1. Restriction enzymes shown include *Sma*I (Sm), *Pst*I (P), *Eco*RI (E), *Sac*I (S), *Bam*HI (B), *Sph*I (Sp), *Hin*dIII (H), and *Eco*RV (RV). Size in kb is indicated by numbers from 0 to 19. Fragments A, B, C, D, and E were used either as inserts cloned into pLYL001 for plasmid rescue or as probes for hybridization to the chromosome DNA. Fragment F was used as the probe for detecting the *ermG*-containing bands in Fig. 1B. Fragments G and H were used as probes for detecting the distribution of the upstream *ermG* multidrug resistance region in *Bacteroides* strains. Fragment I, a 4.9-kb *Pst*I-*Eco*RV fragment including the *mefA*, was cloned into pGW47 to determine whether this fragment contained a gene that was responsible for low-level erythromycin resistance in *Bacteroides* hosts. Description of the functions and sequence identities are indicated by the bars above the map. Genes in light gray are homologs to known gram-positive genes, the striped arrows indicate the genes that are homologs to efflux and membrane proteins involved in antibiotic resistance mechanisms, and the putative *Bacteroides* IS transposase is indicated.

this putative transposase is not essential for the transfer of CTnGERM1. There are no copies of this insertion element transposase gene in the *B. thetaiotaomicron* 5482A genome that were detectable by Southern blot hybridization.

Strain BT4001 containing CTnGERM1 could grow in medium containing erythromycin concentrations as high as 200 μg/ml. A disruption of the *ermG* gene caused by using fragment A in Fig. 2 was made to determine whether this gene was solely responsible for the Em^r phenotype. The mutant with the disrupted *ermG* gene still exhibited a low level of erythromycin resistance (MIC of 5 μg/ml, compared to <0.2 μg/ml for strain BT4001). This result indicates that although most of the resistance to Em is due to *ermG*, CTnGERM1 may carry a second Em^r gene that has not yet been identified. This is presumably an *erm*-type gene because it confers resistance to lincosamide as well as erythromycin. If it is an *erm* gene, we have not yet identified it in our probe analyses as belonging to any of the known *erm* gene classes (18).

Six contiguous open reading frames (ORFs) of the 13 ORFs (bp 8,137 to the end) encoded putative proteins that had amino acid similarity to multidrug resistance efflux proteins (Table 3 and Fig. 2). The ABC*trans* and *mefA* homologs had over 90% sequence identity with the genes found in *Streptococcus pyogenes* and *S. pneumoniae*. The protein translations of the ORFs indicated that there were some nonsense mutations that truncated the products or deleted the N-terminal ends in some cases (see Table 3). The fact that all of these ORFs were transcribed in the same direction raised the possibility that this collection of genes might be an integron, but the typical conserved sequence (GTTRRRY), usually found between cassettes of classical integrons (5), was not found between these ORFs. There was also no evidence of repeated sequences flanking the genes that had high nucleotide sequence identity with gram-positive genes (Table 3 and Fig. 2), although most of them are found on transposons and plasmids in *Staphylococcus*, *Enterococcus* and *Streptococcus* hosts (1, 4, 15).

The *mefA* on CTnGERM encoded a protein that had 86% amino acid sequence identity and 94% nucleotide sequence identity with a macrolide efflux protein (MefA, accession number AAC44785) of *Streptococcus pyogenes*. To determine wheth-

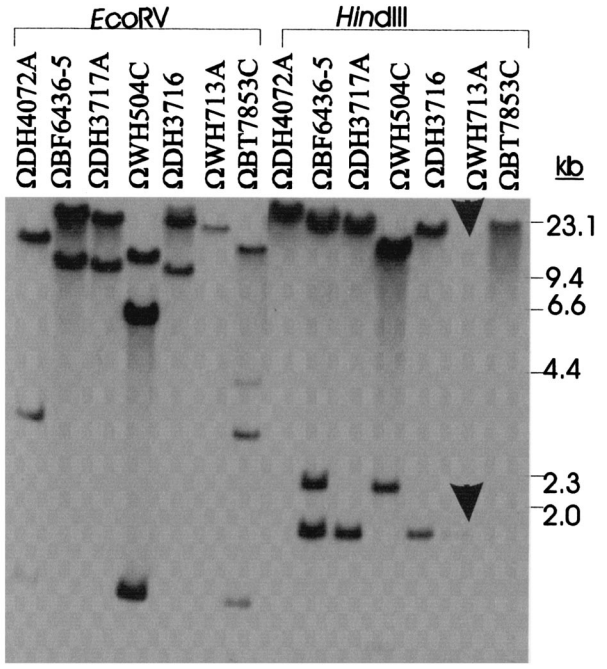

FIG. 3. Southern blot analysis of BT4001 transconjugants from different donors. The Southern blot analysis was done on *Eco*RV- and *Hin*dIII-digested chromosomal DNA from six *ermG*-containing BT4001 transconjugants and an Em^r transconjugant from WH504, BT4001ΩWH504. The blot was probed with fragment G, which contains sequences upstream of the *ermG* on CTnGERM1 (Fig. 2). The two arrows indicate the positions of two bands, which were more visible after longer exposure time of the Southern blot.

TABLE 3. Sequence analysis of 19-kb region of CTnGERM1

Location bp–bp 5′-3′	ORF or designation	No. of aa	% aa identity (no. of aa involved/ total no. of aa)	Results and descriptions of BLAST searches (accession no.)
1009–1368	1	119	32 (16/50)	*Rhizobium melioti* NodM (606 aa) homology in C-terminal end (X58632)
1556–2785	*tnpIS*	409	90 (369/409)	*Bacteroides fragilis* transposase (428 aa) of IS*613* and closely related IS elements in imipenem-resistant strains (BAA95632.1). This protein is missing first 20 aa. There is 92% identity at the nucleotide level. Contains transposase DDE domain of the IS*4* group. There are other related *Bacteroides* insertion elements.
4006–4240	3	121	35 (33/93)	*B. fragilis* Orf9 (481 aa) of unknown function located on mobilizable transposon Tn*4555* (AAD43601)
5259–4764	4	164	58 (94/162)	*Desulfitobacterium hafniense* (181 aa) hypothetical protein (00098450.1); *Clostridium acetobutylicum* HD superfamily hydrolase (163 aa, AE007856). The translated *orf4* protein has the HD and HDc motifs for metal-dependent phosphohydrolases.
5260–6290	*orfY′* and *C′-orfX*	230	85 (204/243)	*Staphylococcus intermedius* OrfY (244 aa) of unknown function. Associated with *ermB* and other antibiotic resistance genes (AF299292). The nucleotide identity is ~92%. There is a frameshift in the sequence. The 220 nucleotides of the 3′ end of the upstream *orfX* (unknown function) are also present. These genes are also found on the *Enterococcus faecium* plasmid pUW786 (17,510 bp) that contains *ermB* and vancomycin resistance genes (AF516335). OrfX and OrfY are carried on Tn*5404*/ Tn*5405* originally isolated in *Staphylococcus* (4).
6631–7365	*ermG*	244	99 (236/239)	*Bacillus sphaericus* ErmG (244 aa) on a plasmid (M15332). >99% identical at the nucleotide level. Also found on *Bacteroides* CTn*7853* (L42817).
7366–7860	7	125	33 (50/161)	*Bacillus firmus* glutamine-rich protein (AAB05373.1)
9420–8137	ABC transporter or *mel*	428	97 (414/427)	*Streptococcus pneumoniae* Mel (487 aa), a homolog of the product of the macrolide-streptogramin B efflux resistance gene *msr*(SA) (AAK69028). Has 96% nucleotide identity. Called an ABC transporter or *orf5* on Tn*1207.1* (15). Located downstream of *mefA*. Protein missing N-terminal end due to nonsense mutations.
10854–9922	*mefA*	310	86 (248/287)	*Streptococcus pyogenes* MefA (405 aa) macrolide-cfflux protein (AAC44785). 94% nucleotide identity. There are two stop codons in the end corresponding to the N terminus. Also found on Tn*1207.1* in an *S. pneumoniae* clinical strain (15) situated upstream of ABC transporter or *mel*. This MefA protein is missing the N-terminal end due to nonsense mutations.
12895–11537	*orf10*	452	38 (179/472)	*Chlorobium tepidum* FusA/NodT (475 aa) family of multidrug-resistant proteins (AAM72516.1). Contains To1C and OEP and outer membrane and efflux motifs.
15937–13490	*orf11*	815	46 (378/821)	*Chlorobium tepidum* AcrB/AcrD (1,063 aa) family of multidrug-resistant proteins (AAM72517.1). Has the motif of the AcrB/AcrD/AcrF family of proteins associated with antibiotic resistance due to efflux pumps.
17127–16101	*orf12*	373	34 (128/367)	*Chlorobium tepidum* AcrA/AcrE (396 aa) family of multidrug-resistant proteins (AAM72518.1). Has a motif contained by AcrA, HlyD, and EmrA families of proteins involved with membrane fusion, secretion, and multidrug resistance efflux.
18373–17171	*orf13*	400	N terminus, 32 (59/ 182); C terminus, 33 (55/164)	N-terminal end *B. subtilis* TagO (358 aa) tcichoic acid linkage unit synthesis (B69721). C-terminal of end acetyltransferase (GNAT) family YnaD (170 aa) *B. subtilis* (AAB41084.1). Contains motifs for two families of proteins: Rfe, UDP-N-acetylmuramyl pentapeptide phosphotransferase, and RimL, acetyltransferases.

er this *mefA* was responsible for the low level of Em^r seen in the absence of *ermG*, a 4.9-kb *Pst*I-*Eco*RV fragment (fragment I, Fig. 2) that contained *mefA* and *orf10* and which had homology to an efflux protein (Table 3) was cloned into a shuttle vector pGW47 to produce pYP66. S17-1(pYP66) was used as a donor to transfer pYP66 to BT4001. If the CTn-MefA was responsible for the residual Em^r that was not attributable to *ermG*, transconjugants resistant to 3 to 5 μg of Em per ml should have been isolated. No Em^r transconjugants were isolated ($<10^{-8}$ per recipient). Thus, neither of the genes in this region contributes to the Em^r seen in the *ermG* disruption strain.

More than one copy of CTnGERM1 can coexist in the same strain. In transconjugant 3 (txg3 in lanes 3 of Fig. 1), two *ermG* elements had integrated in the same host. Southern blot analysis was performed to test whether these two elements were copies of CTnGERM1 or different elements. Restriction enzymes *Eco*RV and *Hin*dIII were used to digest txg3 and other transconjugants, which contain only one copy of CTnGERM1. Probes were made from the 19-kb fragment cloned from CTnGERM1. The digestion patterns of the two elements in txg3 were exactly the same as those seen in the transconjugants containing only one copy of CTnGERM1 (data not shown). Thus, there are two copies of CTnGERM1 integrated into the chromosome of txg3.

The *ermG* genes in other *Bacteroides* strains are associated with an element related to CTnGERM1. In our initial analysis of the four strains that were capable of transferring *ermG* without cotransferring *tetQ* (i.e., DH3716, DH3717, WH713, and BF6436-5), we had transferred all of the *ermG* elements into strain BT4001. To determine whether these four transmissible elements were all related to CTnGERM1, chromosomal DNA from the four *ermG*-containing transconjugants was digested with two different restriction enzymes, *Eco*RV and *Hin*dIII. Southern blot analysis was performed with fragment G (Fig. 3) used as the probe. Probe G (Fig. 2) was used to eliminate *ermG* sequences, which had already been shown to be present in all *ermG* strains. In all cases, the upstream region probe hybridized to DNA in the transconjugants, thus indicating that all four elements were related to CTnGERM1. This was true even of the two elements that cotransferred *tetQ* and *ermG* from BT7853 and DH4072. Although the different

Upstream *ermG* alignment

```
                -26       -16         1                                  +#
CTn7853         gagATGCAATAAA----TTAAGGAGAGGTTATAATGAACAAAGTAAATATAAAAGATAGT 27
CTnGERM1        TtAATGaAATAAA----TTAAGGAGGGGTTATAATGAACAAAGTAAATATAAAAGATAGT 27
Bacillus        TgAATtCAtTAtAACCTTTAAGGAGAGGTTATAATGAACAAAGTAAATATAAAAGATAGT 27
                   **  * ** *    ******** **********************************
```

Downstream *ermG* region alignment

```
                                                              stop
CTn7853         GAACAATTTGTATCGCTATTTAATAGTTATAAAATATTTAACGGCTAAAAACAATAGGCC 727
CTnGERM1        GAACAATTTGTATCGCTATTTAATAGTTATAAAATATTTAACGGCTAAAAACAATAGGCC 727
Bacillus        GAACAATTTGTATCGCTATTTAATAGTTATAAAATATTTAACGGCTAAAA-CAATAGGCC 726
                ************************************************** *********

CTn7853         ACATGCAACTGTAAATGTTTAGTTATAGGTAGGGTAGCATAAGTTAAAATGCTATTCTGC 787
CTnGERM1        ACATGCAACTGTAAATGTTTAGTTATAGGTAGGGTAGCATtAGTTAAAATGCTATTCTGC 787
Bacillus        ACATGCAACTGTAAATGTTTAGTTATAGGTAGGGTAGCATAAGTTAAAATGCTATTCTGC 786
                **************************************** *******************

CTn7853         CTTTTAAAAGTATGGTATACATTCgtagGcGtccaATaacAaggtgTatCGcATctatcT 811/847
CTnGERM1        CTTTTAAAAGTATGGTATACATTCCTGAGAGA--GATTGGATATGTTCCCGTATACCGAT 845
Bacillus        CTTTTAAAAGTATGGTATACATTCCAGAGAGA--GATTGGATATGTTCCCGTATACCGAT 845
                ************************    * *    **   *     *  ** **     *

CTnGERM1        TAGTGCCAAAAGCGAAGTGGATATGTTTCCGAGAACGTACTCAAATGACATTCATTCTGT 905
Bacillus        TAGTGCCAAAAGCGAAGTGGATATGTTTCCGAGAACGTACTCAAATGACATTCATTCTGT 905

CTnGERM1        CCTCTCcAGCCAcGTgGAaaGTtTtagtTTGtctGAcgAAac 947
Bacillus        CCTCTCAAGCCAtGTtGAgtGTgTggcaTTGctgGAaaAAtg 947
```

FIG. 4. Nucleotide sequence alignment of *ermG* genes from CTn*7853*, *B. sphaericus*, and CTnGERM1. Each hyphen (-) represents a gap inserted because of dissimilarities in the alignment. ∗, 100% nucleotide identity of the three sequences. Lowercase letters were used when one of the sequences differed. The *ermG* start codon ATG and stop codon TAA are shown in boldface. The extended sequence identity between CTnGERM and the *B. sphaericus ermG* is continued at the bottom. The nucleotide position numbers are relative to the A of the ATG start codon: + for downstream positions and − for upstream positions. Alignment was performed with the ClustalW program. GenBank accession numbers are as follows: CTn*7853*, L42817; *ermG* region from *B sphaericus*, M15332; and CTnGERM1, AJ557257.

transconjugants contained DNA that was clearly related to DNA from CTnGERM1 from DH3716, the restriction patterns differed in the case of at least one restriction enzyme—except for the *ermG* element in DH3717. This indicates that even though the CTnGERM elements seem to have entered *Bacteroides* species only recently, either they are mutating rapidly or different variants have moved into *Bacteroides* species in multiple transfer events.

The *ermG* gene may be capable of moving as a cassette. In previous studies, a CTn that cotransferred *tetQ* and *ermG* had been identified (9). This CTn was designated CTn*7853* and is the *ermG* element that transfers out of BT7853 (ΩBT7853 in Fig. 3). A small amount of DNA around the *ermG* gene in CTn*7853* had been cloned and sequenced (3). The sequence identity between the *ermG* region of CTnGERM1 and that of CTn*7853* ends at 26 bp upstream of *ermG* (bp −26) and 96 bp downstream of *ermG* (bp +811; Fig. 4). Clearly, if CTn*7853* is in fact a member of the CTnGERM1 family, as the results in Fig. 4 suggest, *ermG* is inserted in different locations in the element. A comparison between the *ermG* regions of CTnGERM1 and *B. sphaericus* revealed that the sequence identity between the *ermG* region on CTnGERM1 and that of *B. sphaericus* ended at 16 bp upstream of the gene (bp −16). In this case, however, the sequence identity downstream of the *ermG* gene continues an additional 100 bp (>bp +910). Thus, if *ermG* is on a cassette, the cassette has different endpoints from those seen in the comparison of the CTnGERM1 *ermG* gene and the *ermG* from CTn*7853*.

If *ermG* had integrated into a preexisting CTn as a cassette, we would expect to find examples of strains that carried an element related to CTnGERM1 but did not carry *ermG.* To determine whether such elements were found in *Bacteroides* species, DNA from our collection of 289 *Bacteroides* strains was hybridized on dot blots with probes representing the *ermG* upstream region, the 7.2-kb *Pst*I-*Pst*I fragment and the 2.5-kb *Bam*HI-*Sph*I fragment (fragments G and H; Fig. 2). Of the 289 strains, DNA from 36 strains (12%) hybridized to both probes. Positive strains included seven of the nine *ermG*-containing strains and three strains (i.e., WH504, WH505, and WH506) that were able to transfer low-level Em^r but contained no known *erm* genes (Fig. 3). The remaining 26 cross-hybridizing strains were not Em^r. The hybridization to the upstream probes G and H may be due to the presence of an *ermG*-like element or the presence of one or more genes acquired independently.

Location of the 19-kb *ermG* region in CTnGERM1. According to the PFGE results, CTnGERM1 integrated into different positions in the chromosomal DNA of txg9 and txg10. Thus, a different digestion pattern should be seen if the restriction enzyme cuts outside the element DNA. DNA from both

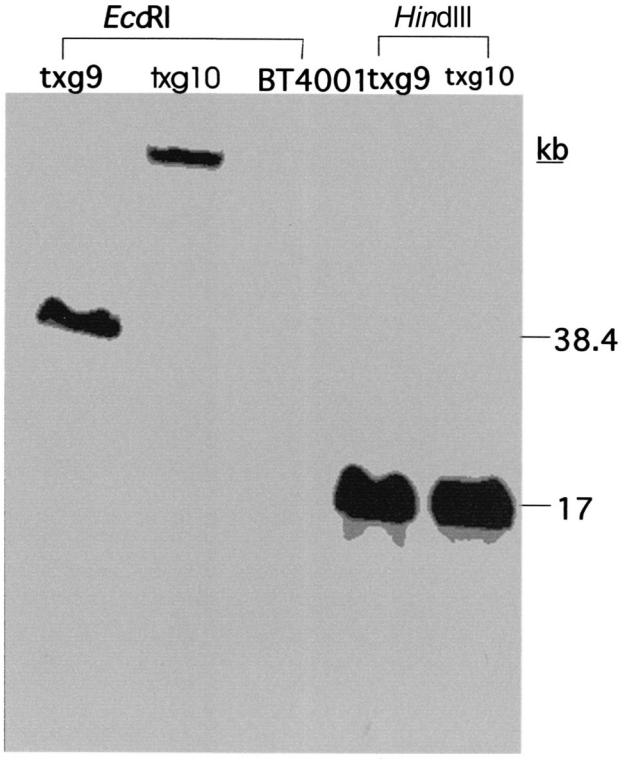

FIG. 5. Southern blot of the *Eco*RI- and *Hin*dIII-digested chromosomal DNA of BT4001ΩCTnGERM1 txg9 and txg10 shown in Fig. 1. The digested DNA samples were run on a field inversion electrophoresis gel, and the Southern blot of the gel was probed with labeled fragment B (Fig. 2). *Eco*RI-digested DNA from txg9, txg10, and BT4001 is shown in lanes 1, 2, and 3, respectively. *Hin*dIII-digested txg9 and txg10 are in lanes 4 and 5. The locations and sizes (in kb) of the relevant high-molecular-weight markers (BRL) are indicated on the right.

transconjugants was digested with *Eco*RI and *Hin*dIII and resolved in 1% agarose field inversion electrophoresis gel and then probed with fragment B (Fig. 5). The probe hybridized to the same 17-kb *Hin*dIII in both cases, suggesting that another *Hin*dIII site is 17 kb away from the *Hin*dIII at position kb 10 in Fig. 2 and that this *Hin*dIII site is inside CTnGERM1.

The *Eco*RI-digested chromosomal DNA of txg9 and txg10 showed different patterns. The hybridizing band of txg9 is 35 kb, but that of txg10 is >50 kb. Thus, one of the *Eco*RI sites is outside CTnGERM1, and the right end of CTnGERM1 is less than 35 kb away from the *Eco*RI site at position kb 15 in Fig. 2. Thus, the right end of CTnGERM1 is more than 10 kb away (based on *Hin*dIII digestion results) and less than 31 kb away from the end of the 19-kb cloned fragment. Since the whole element is about 75 kb in size, the left end must be 25 to 46 kb away from the left end of the cloned region (Fig. 2). These results are consistent with the hypothesis that the cloned 19-kbp *ermG*-containing DNA segment is near the middle of CTnGERM1.

DISCUSSION

Our results demonstrate that the transmissible element found in DH3716, CTnGERM1, is a 75-kbp element that is normally integrated into the *Bacteroides* chromosome. CTnGERM1 integrated into the same *Not*I band in most of the independently isolated transconjugants, thus indicating that integration may be relatively site specific. At this point, however, we cannot rule out the possibility that there is more than one site in the same large *Not*I band. By taking advantage of the fact that the CTn integrated into a different site in one of the transconjugants (i.e., tgx10), we were able to show that the cloned 19-kbp region is probably located in the middle of the 75-kbp element. Attempts to clone the ends of the CTn were unsuccessful. In our experience with CTnERL and CTnDOT, we found that regions near the ends were very difficult to clone and that deletions were common in clones that contained this DNA. Obtaining DNA sequence information from the ends of CTnGERM1 and related elements is of interest because one of the few features that seem, so far, to link the known CTns and the integrated elements they excise and mobilize is that their integrase genes are members of the phage lambda integrase family (2, 19, 22). Relatively few CTns have been characterized at this level, however, so it is not clear that this feature will prove to be a defining characteristic of integrating transmissible elements.

Even though only a 19-kbp segment of CTnGERM1 was obtained, this cloned DNA enabled us to show that all six of the elements that transferred *ermG* are closely related to each other and that there are CTnGERM1-type elements in *Bacteroides* strains that do not carry *ermG*. In fact, these elements that did not contain *ermG* were more common than the *ermG*-containing versions of the element. The *ermG* gene appears to be capable of integrating as a cassette. The fact that some of these elements also carry *tetQ* raises the question of whether *tetQ* is also integrating as a cassette into preexisting elements.

The fact that the CTnGERM1-type elements exhibited restriction length polymorphisms in the region detected by the probe used in Fig. 3 and that CTnGERM1-type elements that lacked *ermG* are more common than the forms that carry *ermG* points to the likelihood that CTnGERM1-type elements have entered members of the genus *Bacteroides* more than once. In this connection, it is worth noting that the strains carrying DNA that hybridizes with the CTnGERM1 probe were isolated from people in different parts of the United States. This result suggests that once these elements entered *Bacteroides* strains, some of them may have acquired *tetQ*, since *tetQ* appears to have been in *Bacteroides* strains much longer than the CTnGERM1 elements or *ermG*, based on our survey of *Bacteroides* strains. Only 3% of the 88 pre-1970 strains had DNA that hybridized to the *ermG* upstream probe, compared to 22 to 30% of these strains that already carried *tetQ* and a CTnDOT type element.

The origin of CTnGERM1 and related elements remains a mystery. The CTnGERM1 elements could have come from gram-positive bacteria. The *ermG* gene has previously been found only in a plasmid of *B. sphaericus* (7), and sequence identity of CTnGERM1 and the *B. sphaericus ermG* gene extends downstream of the gene. In *B. sphaericus*, the *ermG* gene is located on a plasmid, so the gene—rather than the entire CTn—seems to be what is moving between genera. Another CTnGERM1 gene had high nucleotide and amino acid sequence identity (>90%) with *mefA*, a macrolide efflux gene found in *S. pyogenes* and *S. pneumonia*. It is also upstream of a second gene, an ABC transporter or *msr*(SA) homolog, found

in *Streptococcus pneumoniae* on transposable element Tn*1207.1* (15). The nucleotide identity of the CTnGERM ORFs with these two genes is also ~90%, but other sequences associated with the Tn-like element have not been detected. It is interesting that another homolog of MefA (MefE2) encoded by a gene found on a *Bacteroides* mobilizable transposon, NBU2 (22), is much less similar to the MefA on CTnGERM1: MefE2 has only 33% amino acid identity with the MefAs encoded by genes found in *S. pyogenes* and CTnGERM1. Thus, the high sequence identity between *mefA* on CTnGERM1 and the *mefA* in S. *pyogenes* is not due to conservation of the *mefA* sequence. The sequences of two more gram-positive genes downstream of *ermG*, *orfX′ and orfY′*, are found on *Staphylococcus* transposons Tn*5404* and Tn*5405* (1, 4), which are often located on plasmids and other transmissible elements in the gram-positive bacteria, and these sequences may be remnants of Tn insertions into the CTnGERM elements. Tn*5404*- and Tn*5405*-related elements have been found to be associated with *ermB* in canine staphylococcal isolates (1).

If gram-positive bacteria were the source of CTnGERM1, the source is probably not *B. sphaericus* itself. The percentage of G+C content of the *ermG* gene is 26.8%, whereas that of the *Bacteroides* chromosome DNA is about 42% and that of *B. sphaericus* is 47%. This observation indicates that *ermG* is a newcomer in both species. Also, in CTnGERM1, the percentage of G+C content of the ORFs adjacent to *ermG* are 34 and 36.2%, respectively, and many of them, as previously described, have >90% nucleotide identity with known gram-positive genes (Table 3). Low percent G+C values are typical of the gram-positive cocci, but the sequence we have now does not implicate any of the known integrated conjugal elements from these bacteria. It is interesting that despite differences in the percentage of G+C content, if CTnGERM originated in the gram-positive bacteria, enough genes on the incoming element were expressed well enough for the CTn to be fully transmissible in a foreign host. Not all the genes on CTnGERM1 are functional, however, as is indicated by the fact that the putative macrolide efflux gene did not confer resistance to erythromycin on a *Bacteroides* host. The lack of expression is at least partly due to mutations and deletions. Whatever the origin of CTnGERM1, it is clear that in contrast to the CTnDOT-type elements that are transferring widely within the genus *Bacteroides*, conjugal elements can also move into *Bacteroides* strains, possibly from another genus.

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