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Horizontal Transfer of Erythromycin Resistance from *Clostridium difficile* to *Butyrivibrio fibrisolvens*

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This study demonstrates for the first time the in vitro transfer of the erythromycin resistance gene *erm*(B) between two obligate anaerobes, the human spore-forming pathogen *Clostridium difficile* and the rumen commensal *Butyrivibrio fibrisolvens*, suggesting that this event might occur also in the natural environment.

Antibiotic resistance genes commonly reside on transmissible plasmids or on other mobile genetic elements which allow the horizontal transfer of these genes between strains, species, and even genera of the normal microflora as well as between pathogens of human and animal origin (6, 18, 24). It is known that antibiotics used in human and veterinary medicine may increase the selective pressure on bacterial populations, which could result in an increase of resistant bacteria (4, 12). The human and animal gastrointestinal tract is a good environment for horizontal gene transfer events (22).

rRNA methylation is the most frequent mechanism of resistance to macrolide-lincosamide-streptogramin B antibiotics in gram-positive bacteria (21). The most widely distributed *erm* gene class is *erm*(B), which is present in a large range of hosts, including *Clostridium difficile* (20, 21). *C. difficile* is a spore-forming gram-positive bacillus, an opportunistic pathogen that is responsible for many cases of antibiotic-associated diarrhea in humans and animals, and it has been recognized as one of the major causes of nosocomial diarrheic diseases (3, 26). *C. difficile* strains are frequently resistant to macrolides, and this resistance seems to be associated with strains more prone to cause epidemics (13). *C. difficile* ErmB resistance determinants can show different genetic arrangements and alleles (8, 27, 28).

Butyrivibrio fibrisolvens is one of the most abundant bacteria isolated from the rumen and has also been identified in the human gastrointestinal tract (29). It is a small, motile, curved rod with tapered ends that analysis of both cell wall structure and 16S rRNA gene sequences indicate it is gram positive (5), although it is currently classified as gram negative. Previous studies demonstrated that some tetracycline resistance determinants could be transferred, under laboratory conditions, from different rumen and human microorganisms to *B. fibrisolvens* (11, 15).

In this study, we examined the possibility of the *erm*(B) gene transfer between *C. difficile* clinical isolates 630, C191, F17, and CD51 harboring different ErmB determinants and *B. fibrisolvens* strains.

Relevant characteristics of the bacterial strains used in this study are listed in Table 1. The media used in mating experiments were the M2GSC (16) and the RGM (10) broths. Conjugation assays were performed as previously described (23), with the following specific modifications. Donor and recipient bacteria were grown to mid-exponential phase (optimal optical densities at 600 nm [OD_{600}] of 0.3 and 0.4 for *C. difficile* and *B. fibrisolvens*, respectively), mixed in a final ratio of 1:1, spread on a sterile nitrocellulose 0.45-μm-pore-size filter on a blood agar (BA) plate (supplemented with 0.1% hemin and 0.1% vitamin K), and incubated for 18 h in an anaerobic cabinet at 35°C. All media were supplemented with 10 μg/ml of tetracycline, 20 μg/ml of erythromycin, and 50 μg/ml of rifampin, as appropriate for each strain. Cycloserine-cefoxitin-fructose-agar (CCFA) (Oxoid, Limited Basingstoke, Hampshire, England), containing 5% egg yolk, was used to discriminate between *B. fibrisolvens* and *C. difficile* strains, when necessary.

Transfer mating results between *C. difficile* and *B. fibrisolvens* are shown in Table 2. A successful transfer of erythromycin resistance to the recipient *B. fibrisolvens* 2221^R (Rif^r) was obtained using *C. difficile* CD51 (Em^r) as the donor, whereas all attempts to transfer the erythromycin resistance from strains *C. difficile* 630, C191, and F17 were unsuccessful, even if erythromycin resistance transfer from *C. difficile* 630 to another two gram-positive bacteria has been previously demonstrated (9, 17). Erythromycin-resistant transconjugants from the mating between *C. difficile* CD51 and *B. fibrisolvens* 2221^R were obtained in each experiment at average frequencies of 4.7×10^{-8} per donor and 4.6×10^{-7} per recipient, respectively. Onward transfer of erythromycin resistance determinants from *B. fibrisolvens* RE1 (Rif^r, Em^r) to *B. fibrisolvens* 1.230 (Tc^r) was also obtained, with average transfer frequencies of erythromycin resistance of 6.4×10^{-4} per donor and 3.6×10^{-6} per recipient, respectively. In these mating experiments, we also observed the tetracycline resistance transfer from the recipient strain *B. fibrisolvens* 1.230 to the donor *B. fibrisolvens* RE1. The transconjugants that had acquired the erythromycin or the tetracycline resistance had a different colony morphology and were also identifiable by API rapid ID32A enzymatic test strips (Biomérieux, Marcy l'Etoile, France) for the production of arginine, glycine, and histidine arylamidase by *B. fibrisolvens* 2221^R and by 16S rRNA sequencing (2). The onward transfer of an *erm*(B) determinant among *B. fibrisolvens* strains indicates the possibility for the spread of erythromycin resistance among *B. fibrisolvens* popu-

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TABLE 1. Bacterial strains used in this study

Bacterial strain	Resistance phenotype[a]	Source or reference
C. difficile		
CD51	Em^r, Tc^s, Rif^s	This study
630	Em^r, Tc^r, Rif^s	30
C191	Em^r, Tc^r, Rif^s	27
F17	Em^r, Tc^r, Rif^s	27
CD37	Em^s, Tc^s, Rif^r	25
B. fibrisolvens		
2221^R	Em^s, Tc^s, Rif^r	1
1.230	Em^s, Tc^r, Rif^s	23
B. fibrisolvens transconjugants		
RE1	Em^r, Tc^s, Rif^r	This study (CD51 × 2221^R)
bTE1	Em^r, Tc^r, Rif^s	This study (RE1 × 1.230)

[a] Em^r, erythromycin resistant; Em^s, erythromycin sensitive; Tc^r, tetracycline resistant; Tc^s, tetracycline sensitive; Rif^r, rifampicin resistant; Rif^s, rifampicin sensitive.

TABLE 2. Frequency of erythromycin resistance transfer between *C. difficile* and *B. fibrisolvens* strains

Donor strain	Recipient strain	Avg transfer frequency		No. of replicates	Progeny saved for further study
		Per donor	Per recipient		
CD51	2221^R	4.8×10^{-7}	4.6×10^{-7}	3	RE1–RE9
RE1	1.230	6.4×10^{-4}	3.6×10^{-5}	3	bTE1–bTE9
CD51	1.230	4.2×10^{-6}	3.3×10^{-7}	3	TE1–TE9

lations in vivo and the potential role of these bacteria as a reservoir of antibiotic resistance genes.

Transfer of erythromycin resistance was also obtained between *C. difficile* CD51 and *B. fibrisolvens* 1.230, with average frequencies of 4.2×10^{-6} per donor and 3.6×10^{-7} per recipient, respectively. The transfer of the *C. difficile* CD51 *erm*(B) determinant to the two different *B. fibrisolvens* strains, 2221^R and 1.230, indicates that it is not influenced by the rapid autoaggregation shown by *B. fibrisolvens* 2221^R (19, 23).

All *B. fibrisolvens* transconjugants showed high levels of erythromycin resistance (MIC > 256 μg/ml), similar to the donor strain *C. difficile* CD51. The stability of this resistance was confirmed by replating selected colonies on supplemented BA plates containing or lacking erythromycin (20 μg/ml).

Erythromycin resistance transfer was confirmed in all transconjugants by PCR, using primers ermB1 (5′-CTCAAAACT TTTTAACGAGTG) and ermB2 (5′-CCTCCCGTTAAATAA TAGATA) to amplify a 711-bp fragment of *erm*(B). PCR conditions consisted of 30 cycles of 30 s at 94°C, 1 min at 50°C, and 1 min at 72°C.

The genomic DNA of *C. difficile* CD51 and of 27 selected *B. fibrisolvens* transconjugants (Table 2) were also examined by pulsed-field gel electrophoresis (PFGE) after digestion with SmaI and by hybridization assays. PFGE was performed using a CHEF-Mapper apparatus (Bio-Rad Laboratories) at 6 V/cm for 22 h at 14°C, with an included angle of 120° and linear ramping from 5 to 70 s for *C. difficile* and from 0.05 to 10 s for *B. fibrisolvens*. The PCR fragment obtained from the *erm*(B) amplification was used as probe. When the SmaI-digested *C. difficile* CD51 genome (Fig. 1A) was hybridized with the *erm*(B) probe, one hybridizing band at about 770.0 kb was observed (Fig. 1B). The nine *B. fibrisolvens* 2221^R transconjugants showed three different PFGE patterns: the first pattern differed from that of the recipient strain for two additional bands at 22 kb and 104 kb and the loss of one band at 89 kb, the second for two additional bands at 109 kb and 55 kb and the loss of one band at 129 kb, and the third for two additional bands at 19 kb and 104 kb and the loss of one band at 89 kb (Fig. 1C). Three transconjugants showed the first pattern and had an *erm*(B) hybridizing band at 22 kb, only one transconjugant showed the second pattern and had a hybridizing band at 109 kb, and five transconjugants showed the third pattern and had a band at 19 kb (Fig. 1D). The 18 *B. fibrisolvens* 1.230 transconjugants showed only one PFGE pattern with two additional bands at 133 kb and 19 kb and the loss of a band at 152 kb (Fig. 1C), regardless of the donor strain (CD51 or RE1). All showed an *erm*(B) hybridizing band at 19 kb (Fig. 1D). PFGE and hybridization results indicated the acquisition of about 35 kb of chromosomal DNA from the donor in all the examined transconjugants and suggest that the ErmB determinant has a preferred insertion site in the *B. fibrisolvens* chromosome. Since *B. fibrisolvens* 1.230 carried tetracycline resistance determinant *tet*(W) (1), the 18 selected transconjugants derived from this strain were also digested with ApaI, as previously described by Scott et al. (23), to examine this determinant. As already observed, *B. fibrisolvens* 1.230 showed a *tet*(W) hybridizing band at 134 kb, whereas the transconjugants showed a band at 120 kb (data not shown). Furthermore, all the transconjugants had an *erm*(B) hybridizing fragment at 44 kb (data not shown). These results suggest that the insertion site of the ErmB determinant in the *B. fibrisolvens* 1.230 chromosome is located in proximity of the *tet*(W) gene, so the insertion of this determinant could introduce a new ApaI restriction site changing the size of *tet*(W) hybridizing fragments in the *B. fibrisolvens* 1.230 transconjugants.

No transfer of erythromycin resistance was obtained when *B. fibrisolvens* transconjugants TE1 and RE1 (Table 2) were used as donors and *C. difficile* CD37 (Rif^r, Tc^s, Em^s) as recipient. The basis of this result is not clear, but the mechanism of the ErmB element transposition, its intermediate form, and the structure of the *B. fibrisolvens* donor strain and that of the *C. difficile* recipient strain cell wall could be implicated.

The *C. difficile* CD51 ErmB determinant showed a new genetic arrangement, named *E5* (28), that was also confirmed in all *B. fibrisolvens* transconjugants (data not shown). The best known element carrying an *erm*(B) determinant in *C. difficile* is Tn*5398*, which has been found in *C. difficile* 630 (Fig. 2) (8). PCR mapping based on the nucleotide sequence of this element indicated that *C. difficile* C191, F17, and CD51 ErmB determinants are not carried by Tn*5398*-like elements (28). The sequence analyses of the *C. difficile* 630, C191, and F17 ErmB determinants were performed in previous studies (7, 27), and their genetic context is shown in Fig. 2. To compare the ErmB determinants of the different *C. difficile* isolates used as donor strains, in this study we also completed the characterization of the *C. difficile* CD51 ErmB determinant. This isolate harbors one copy of the *erm*(B) gene, visualized as a

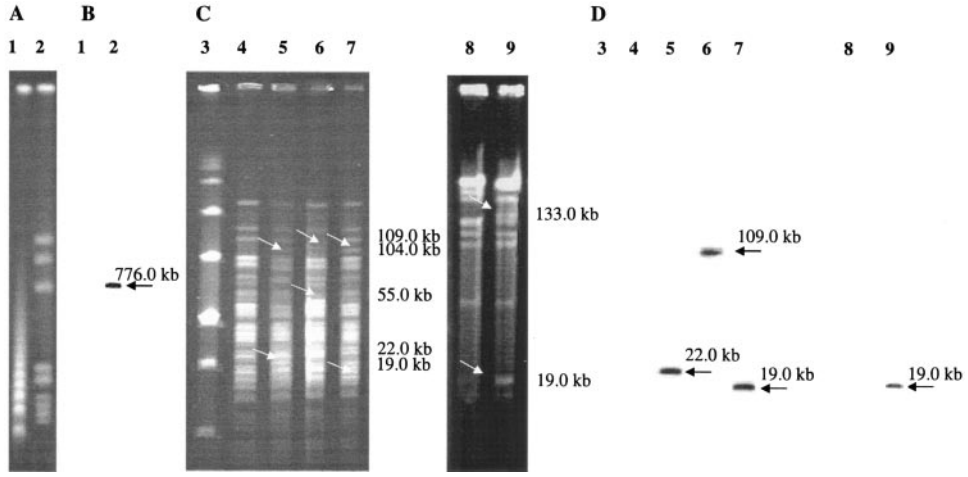

FIG. 1. (A and B) PFGE of *C. difficile* CD51 genomic DNA digested with SmaI and hybridization assay with the *erm*(B) probe, respectively. Lane 1, Lambda ladder PFG marker (New England Biolabs, Hitchin, Hertfordshire, United Kingdom); lane 2, *C. difficile* CD51. (C and D) PFGE of *B. fibrisolvens* transconjugant genomic DNAs digested with SmaI and hybridization assay with the *erm*(B) probe, respectively. Lane 3, low-range PFG marker (New England Biolabs, Hitchin, Hertfordshire, United Kingdom); lane 4, *B. fibrisolvens* 2221^R; lanes 5 to 7, *B. fibrisolvens* 2221^R transconjugants; lane 8, *B. fibrisolvens* 1.230; lane 9, *B. fibrisolvens* 1.230 transconjugant. The acquired and the hybridizing DNA fragments in *B. fibrisolvens* transconjugant chromosomes are indicated with arrows, and the relative sizes in kilobase pairs are reported at the side of the figure.

hybridizing band at 1.5 kb when the genomic DNA digested with Sau3A (7, 27) is hybridized with the *erm*(B) probe (data not shown). Using the primers designed by Farrow et al. (8), we were able to amplify and sequence a DNA fragment of 1,391 base pairs in length containing the *C. difficile* CD51 ErmB determinant (EMBL database accession number AJ968665). Sequence analysis demonstrated that this region is 100% identical to that of the ErmB determinant of *Arcanobacterium pyogenes* OX-7 (between base 1640 and base 3023; GenBank accession no. AY334073) (14). The *C. difficile* CD51 *erm*(B) allele, identical to that of *C. difficile* F17, is not preceded by a leader peptide sequence and is followed by *orf3* and by a partial direct repeat sequence due to a deletion of 947 bp (Fig. 2). Further experiments will be performed to complete a *C. difficile* CD51 erythromycin resistance element characterization and to investigate its mechanism of transfer.

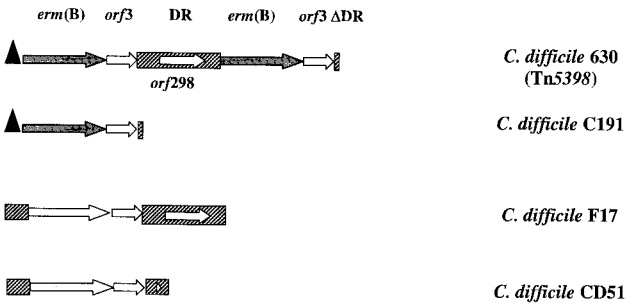

FIG. 2. Schematic representation of the ErmB determinant genetic arrangement in the different *C. difficile* isolates used in this study. The representation is based on the nucleotide sequence of the different ErmB determinants (*C. difficile* 630, GenBank accession number AF109075; *C. difficile* C191, GenBank accession number AJ294530; *C. difficile* F17, GenBank accession number AJ294529; *C. difficile* CD51, EMBL accession number AJ968665). The approximate extent and organization of the determinants are not necessarily to scale. Regions of nucleotide sequence similarity are indicated by the same shading. The arrows indicate the individual open reading frames and their respective direction of transcription. The two *erm*(B) variants are indicated by the different color of the arrows. The leader peptide sequence is represented by a black triangle. DR, direct repeat.

The data presented in this paper provide the first evidence of the erythromycin resistance transfer between the human pathogen *C. difficile* and the rumen commensal *B. fibrisolvens* by conjugation in vitro, supplying additional proof that the resistance gene horizontal transfer among gastrointestinal anaerobic microorganisms could involve bacteria belonging to different ecosystems and normally found in different hosts.

Nucleotide sequence accession number. The DNA sequence of the *C. difficile* CD51 ErmB determinant was submitted to the EMBL database under accession no. AJ968665.

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