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ErmB Determinants and Tn*916*-Like Elements in Clinical Isolates of *Clostridium difficile*

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Erythromycin and tetracycline resistance was analyzed in 37 *Clostridium difficile* clinical isolates. Strains of different clonal origins showed different erythromycin and tetracycline resistance determinants and different genetic arrangements of the elements. In strains of recent isolation, the presence of Tn*916*-like elements, never found before in *C. difficile* clinical isolates, has been demonstrated.

In *Clostridium difficile*, macrolide, lincosamide, and streptogramin B (MLS_B) resistance is usually due to an *erm*(B) gene carried by the Tn*5398*, a mobile element that shows heterogeneous genetic organization (8, 21). *C. difficile* strains can be grouped in different phenotypic classes on the basis of erythromycin and clindamycin resistance patterns, and these classes seem to be related to the presence of different alleles of the *erm*(B) gene (21, 22). Tetracycline resistance is predominantly due to a *tet*(M) gene carried by the conjugative transposon Tn*5397* (13). This element differs from Tn*916* since it contains a group II intron and has different integration/excision modules (16, 17, 24). The Tn*916* conjugative transposon has never been found in *C. difficile*, except in one environmental isolate (25).

The purpose of this study was to characterize the erythromycin and tetracycline resistance elements harbored by 37 selected clinical isolates of *C. difficile*, resistant to erythromycin and/or tetracycline and *erm*(B) and/or *tet*(M) positive.

The MICs were determined by E-test (AB Biodisk), and the breakpoints were ≥4 mg/liter for erythromycin and clindamycin and ≥8 mg/liter for tetracycline (14). *tet*(M) and *erm*(B) genes were detected by PCR, by using primers TETMd-TETMr to amplify 1.0 kb of *tet*(M) and primers E5-E6 to amplify 0.6 kb of *erm*(B) (22). The isolates belonged to four PCR ribotypes identified in our country. Two of them, PCR ribotypes A and D, grouped strains mainly isolated before 1990, whereas PCR ribotypes L and R grouped strains mainly isolated in the years 2000 and 2001 (22). As shown in Table 1, all PCR ribotype A strains harbored both *erm*(B) and *tet*(M), whereas the copresence of both genes was observed only in two strains belonging to PCR ribotype R and one to PCR ribotype D. Five strains, one PCR ribotype D and four PCR ribotype R, were resistant to erythromycin and resistant or inducibly resistant to clindamycin but *erm*(B) negative. By using the primers reported in Marilyn C. Roberts' website, http://faculty.washington.edu/marilynr/, these strains were also examined by PCR for *erm*(A), *erm*(C), *erm*(F), *erm*(Q), and *mef*(A) genes and resulted negative (data not shown). These results were confirmed by hybridization assays using a DIG High Prime DNA labeling and detection kit (Roche Applied Science, Penzberg, Germany) and, as probes, the amplified gene fragments from the following control strains and plasmids: *Staphylococcus aureus* RN4658 for *erm*(A), *C. difficile* 630 for *erm*(B), *S. aureus* RN2442 for *erm*(C), *Streptococcus pneumoniae* PN137 for *mef*(A), R751Ω 4 for *erm*(F), and JIR2879 for *erm*(Q). The results suggest that erythromycin resistance in these isolates could be due to an *erm* or *mef* class different from those examined or to a different MLS_B resistance mechanism and indicate the need to monitor the circulation of resistant strains, particularly in hospital environments.

TABLE 1. Characteristics of the 37 *C. difficile* isolates examined in the study

PCR ribotype	MICs/MIC range (mg/liter)[a]			Detection of:			*erm*(B)-negative strains resistant to erythromycin	Detection of:	
	ERY	CLI	TET	*erm*(B)	*tet*(M)	*erm*(B)/*tet*(M)		*tndX*	*int*
A (21)[b]	256 (4)/12–48 (17)	256 (21)	256 (2)/12–64 (19)	−	−	+ (21)		+ (21)	−
D (4)	256 (4)	256 (3)/3 (1)[c]	32 (1)/0.016–0.064 (3)	+ (3)	−	+ (1)	1	+ (1)	−
L (1)	1.0 (1)	4 (1)	1.0 (1)	−	+ (1)	−		−	+ (1)
R (11)	256 (8)/0.032–1.0 (3)	256 (4)/4–12 (3)/0.5–1.5 (4)[c]	12–16 (6)/0.023–4 (5)	+ (2)	+ (7)	+ (2)	4	−	+ (9)

[a] ERY, erythromycin; CLI, clindamycin; TET, tetracycline.
[b] The number of strains is shown in parentheses throughout.
[c] One strain of PCR ribotype D and one of PCR ribotype R were inducibly resistant to clindamycin. Resistance to clindamycin was induced by a pregrowth on blood agar plates containing 0.05 mg/liter of erythromycin.

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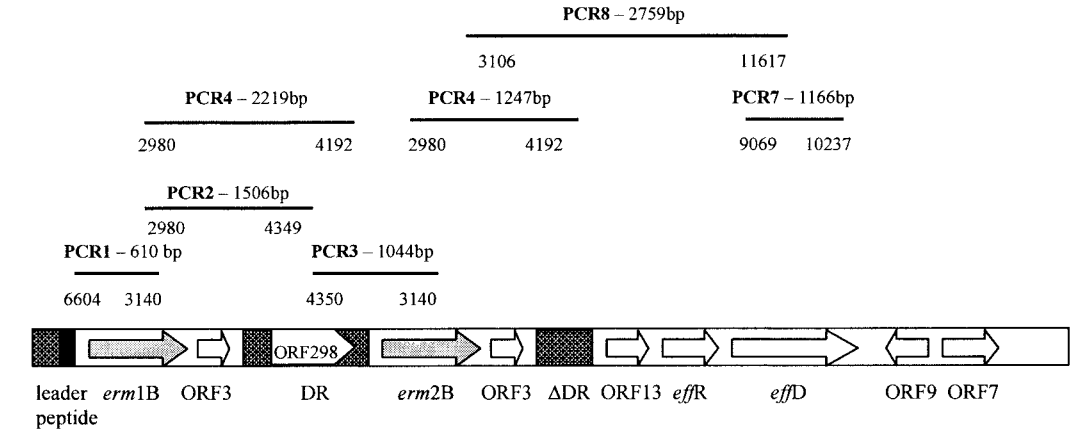
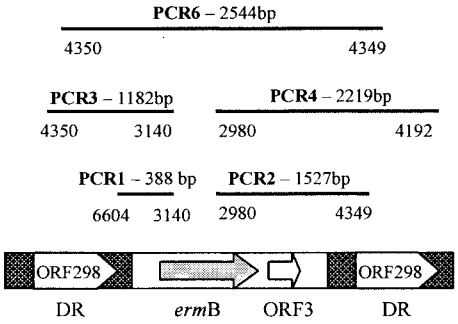
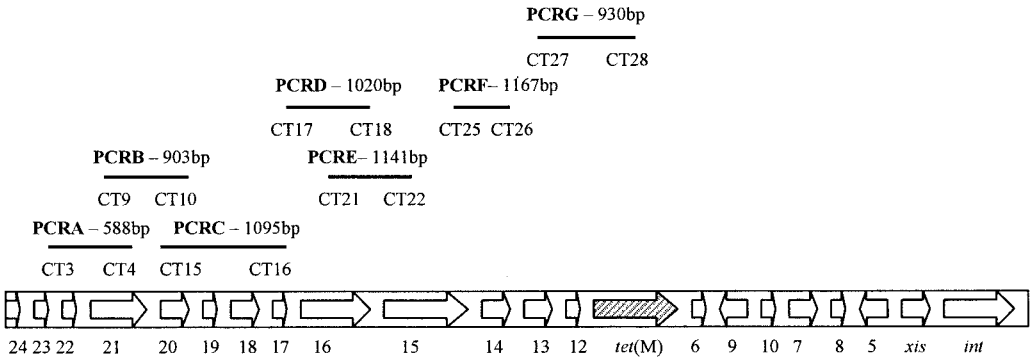


ErmB determinant of *C.difficile* 630



ErmB determinant of *C.perfringens* CP592



Tn*916* of *E. faecalis* DS16

FIG. 1. PCR analysis of the genetic organization of the ErmB determinants and the Tn*916*-like elements. The primers used to analyze the ErmB determinants and the Tn*916*-like elements were described by Farrow et al. (8) and Wang et al. (25), respectively. The expected amplified fragments are shown as bars in relation to the arrangements of the elements from *C. difficile* 630 (GenBank accession no. AF109075), *C. perfringens* CP592 (GenBank accession no. U18931), and *E. faecalis* DS16 (GenBank accession no. U09422). The length of the fragments are reported in bp, and the primers used for each amplification are indicated below the bars.

TABLE 2. Results of the molecular analysis of the ErmB determinants detected in the *C. difficile* clinical isolates examined in this study

PCR ribotype	ErmB determinant arrangements (no. of strains)	Results of PCR assays (length in bp)[a]							*C. difficile* strains with a similar element (reference)
		1	2	3	4	6	7	8	
A	*E1* (4)	+ (610)	+ (1,506)	+ (1,044)	+ (2,219/1,247)	−	+ (1,166)	+ (2,759)	630 (8)
	E2 (17)	+ (610)	−	−	+ (1,247)	−	−	−	C191 (21)
D	*E3* (1)	+ (388)	+ (1,527)	−	+ (2,219)	−	−	−	F17 (21)
	E4 (2)	+ (388)	+ (1,527)	−	−	−	−	−	662 (8)
R	*E4* (1)	+ (388)	+ (1,527)	−	−	−	−	−	662 (8)
	E5 (1)	−	−	−	+ (1,247)	−	−	−	This study
	E6 (1)	+ (388)	−	−	−	−	−	−	L289 (8)
	E7 (1)	+ (2,000)	−	−	−	−	−	−	This study

[a] Results of PCR assays for amplification of Erm determinant regions and approximate length of fragments in bp.

Erythromycin resistance determinants were characterized using seven of the eight amplifications described by Farrow et al. (8). The amplified regions in *C. difficile* 630 (7) and *Clostridium perfringens* CP592 (2) ErmB elements and the expected size of PCR fragments are shown in Fig. 1. *C. difficile* 630, F17, and C191 (21) were used as control strains. Seven different arrangements of the ErmB determinant were identified and named, for convenience, types *E1* to *E7* (Table 2). All strains were highly resistant to erythromycin and clindamycin (MIC ≥ 256 mg/liter), except isolates with a genetic arrangement *E2*, which showed lower resistance levels to erythromycin (MICs of 12 to 48 mg/liter) (data not shown). Genetic arrangements *E1*, *E2*, *E3*, *E4*, and *E6* were similar to those already identified (7, 8, 21), whereas two arrangements, *E5* and *E7*, found in PCR ribotype R strains, were new. Type *E5*, with a product of about 1,247 bp by PCR 4, probably had an incomplete direct repeat sequence located downstream of the *erm*(B) gene, whereas type *E7* showed only the *erm*(B) gene, with a PCR 1 product of about 2 kb. The number of *erm*(B) genes and their sequence were detected by hybridization assay, by using the *erm*(B) PCR product as probe and by PCR-restriction fragment length polymorphism, respectively (21, 22). Types *E1* and *E2* had an *erm*(B) similar to that of *C. difficile* 630, whereas types *E3* to *E7* had an *erm*(B) similar to that of *C. perfringens* CP592 (2, 22). As already reported (8, 21), all *E1* strains had two *erm*(B) copies with two hybridizing bands at 2.0 and 2.3 kb, whereas the other strains had one *erm*(B) copy with a band at 3.0 or 2.3 kb, when probed with the *erm*(B) gene (data not shown).

The *int* gene, a marker for the Tn*916*-like elements, was detected using the primer couple INTf-INTr (11), whereas the *tndX* gene, characterizing the Tn*5397*-like elements, was detected using primers tndx1, 5′ TACATTGTTAAAACAGC AAGC 3′, and tndx3, 5′ TATCAATGAGACACTGCTA 3′. *S. pneumoniae* PN20 (11) and *C. difficile* 630 (26) were used as controls for the *int* and *tnd*X genes, respectively. All PCR ribotype A and D strains, showing a *tet*(M) gene, were positive for *tndX*, whereas all PCR ribotype L and R strains carrying a *tet*(M) gene were *int* positive (Table 1). No strain had both the *tndX* and *int* genes, confirming previous results obtained in vitro by Wang et al. (24). All *C. difficile* strains with a Tn*5397*-like element were resistant to tetracycline, with MICs between 12 and 256 mg/liter, whereas *C. difficile* strains with a Tn*916*-like element could be resistant, inducibly resistant, or susceptible to tetracycline, with MICs between 0.023 and 16 mg/liter.

Tn*916*-like elements were characterized using seven of the primer couples reported by Wang et al. (25). The primers were designed on the Tn*916* element of *Enterococcus faecalis* DS16 (10). The amplified regions and the expected sizes of PCR fragments are shown in Fig. 1. PCR analysis of the Tn*916*-like elements showed four different genetic organizations that, for convenience, we named *Ta* to *Td* (Table 3). *C. difficile* isolates with MICs from 8 to 16 mg/liter, resistant or inducibly resistant to tetracycline, showed elements very similar to those found in *E. faecalis* and this arrangement, named *Ta*, was the prevalent. Type *Tb* was identified in one inducibly resistant strain showing the amplified fragments by PCR C (*orf* 20-19-18-17) and G [*orf* 13-12-*tet*(M) partial] of about 4 kb and 870 bp, respectively. The same size variation in the PCR G product was observed in one resistant strain (type *Tc*), whereas one tetracycline-susceptible isolate was characterized as type *Td* for the absence of amplification of the region containing *orf* 16-15 (PCR E).

The Tn*5397*-like elements were always found in *C. difficile* strains harboring an ErmB determinant, whereas Tn*916*-like elements were found either in *erm*(B)-positive strains or in 80% of *C. difficile* isolates resistant to erythromycin but *erm*(B) negative (Table 1). Further studies will be necessary to verify whether *erm*(B) and *tet*(M) are linked on the same element, as observed in other microorganisms (3, 5, 6, 15).

The Tn*916* transposon and related elements are widespread in many clinically relevant gram-positive bacteria (5, 15, 18,

TABLE 3. Results of the molecular analysis of the Tn*916*-like elements detected in the *C. difficile* clinical isolates examined in this study

Tn*916*-like element arrangement (no. of strains)	Results of PCR assays (length in bp)[a]						
	A	B	C	D	E	F	G
Tb (1)	+ (588)	+ (903)	+ (4,000)	+ (1,020)	+ (1,141)	+ (1,167)	+ (870)
Ta (7)	+ (588)	+ (903)	+ (1,095)	+ (1,020)	+ (1,141)	+ (1,167)	+ (930)
Tc (1)	+ (588)	+ (903)	+ (1,095)	+ (1,020)	+ (1,141)	+ (1,167)	+ (870)
Td (1)	+ (588)	+ (903)	+ (1,095)	+ (1,020)	−	+ (1,167)	+ (930)

[a] Results of PCR assays for amplification of Tn*916* regions and approximate length of fragments in bp.

19). Their ability to mobilize plasmids or other conjugative transposons could be relevant for acquisition of multiple antibiotic resistance and other virulence characteristics by *C. difficile* (1, 4, 9, 12, 20, 23). Further investigations will be carried out to better characterize the erythromycin and tetracycline resistance determinants detected in this study and their relevance in *C. difficile* epidemiology.

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