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Transferable Resistance to Clindamycin, Erythromycin, and Tetracycline in *Clostridium difficile*

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Resistance to clindamycin, erythromycin, and streptogramins in *Clostridium difficile* could be transferred to a susceptible strain by mixed culture at low frequencies (1×10^{-8} to 4×10^{-8} per donor cell). Transfer of tetracycline resistance occurred at frequencies of 3×10^{-7} to 5×10^{-7}. No plasmid DNA involved in these transfers could be detected.

Clostridium difficile has been implicated as the primary cause of pseudomembranous colitis and, at least in part, of diarrhea associated with antimicrobial or anticancer chemotherapy (2, 7, 12, 18). Resistance of *C. difficile* to antimicrobial agents may play a role in the development of such infections (5). Wide variation has been described in the susceptibility of *C. difficile* to clindamycin, chloramphenicol, erythromycin, and tetracycline (8, 9, 24, 30). In addition, large differences were found in the susceptibility to rifamycin in strains isolated in the Zurich area (J. Wüst, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B123, p. 38). In the study presented here, attempts were directed at (i) the transfer of chloramphenicol, clindamycin, erythromycin, rifamycin, and tetracycline resistance from resistant to susceptible *C. difficile* strains; (ii) a search for plasmid DNA related to antimicrobial resistance; and (iii) an elimination of resistance determinants.

Clinical isolates of *C. difficile* were cultured from fecal specimens with CCFA medium (11). Strains CD18, CD37, and CD38 were obtained from C. J. Smith, Medical College of Virginia, Richmond (26). The isolates were identified according to Holdeman et al. (15). For the experimental work, prereduced anaerobically sterilized brain heart infusion broth (15) and brucella agar (28) were used as basic media. Cultures were incubated in an anaerobic chamber (28). Minimal inhibitory concentrations were determined by the proposed National Committee for Clinical Laboratory Standards agar dilution method (28). Transfer of antibiotic resistance markers was based on filter mating techniques described previously (3, 29, 31). Mixed culture on filters was performed by mixing midlogarithm-phase broth cultures (ca. 10^8 bacteria per ml) of the donor and the recipient at ratios from 1:2 to 1:10. In some experiments, donor and recipient cultures were mixed, incubated for several hours in broth, and centrifuged, and samples were plated on the selective media. Attempts to increase transfer frequency were made by embedding the filters in agar during incubation (27) or by adding low amounts of agar to the donor-recipient mixtures (13). Controls in all experiments consisted of donor or recipient strains alone treated in the same manner. Curing of antibiotic resistance was attempted in brain heart infusion broth cultures as described by Carlton and Brown (4), with novobiocin, ethidium bromide, storage for up to 1 year, or incubation at 42°C. The curing agents were used at concentrations which allowed slight to moderate growth in overnight cultures. In some experiments, the curing agent was allowed to act at a higher concentration (23) or at an elevated temperature. Screening of strains for their plasmid content was performed as described by Smith et al. (26) and Guerry et al. (14). Attempts to isolate large plasmids were made by the method of Crosa and Falkow (6). Enzymatic activities were determined by using API ZYM strips (API System S.A., La Balme les Grottes, France) (19).

The results shown in Tables 1 and 2 are evidence that clindamycin, erythromycin, and streptogramin resistance could always be transferred together from donor 630 to recipient CD37 by using mixed culture on filters, which provided close contact between donor and recipient cells. Transfer occurred only in one donor-recipient pair of many combinations tested, and transfer frequencies were very low at the limits of detection despite attempts to optimize transfer conditions, including pretreatment of the donor with clindamycin or erythromycin (25). In some experiments performed exactly in the same way as successful transfers, no transconjugants could be found. No transfer could be detected when filters were incubated for only 1 and 4 h instead of 18 h before plating the mixture

TABLE 1. Transfer of resistance to clindamycin, erythromycin, and tetracycline by mixed culture

Donor	Recipient	Selective medium[a]	Transconjugants per input donor	Phenotype of transconjugants
630	CD37	Rifamycin + Clindamycin	3×10^{-8}[b]	$Clin^r$ Ery^r Rif^r Tet^s
		Rifamycin + Tetracycline	5×10^{-7}	$Clin^s$ Ery^s Rif^r Tet^r
		Rifamycin + Erythromycin	$<1 \times 10^{-8}$	
662	CD37	Rifamycin + Clindamycin	$<1 \times 10^{-8}$	
		Rifamycin + Tetracycline	3×10^{-7}	$Clin^s$ Ery^s Rif^r Tet^r
		Rifamycin + Erythromycin	$<1 \times 10^{-8}$	

[a] The selective media contained the antibiotics at concentration of 10 μg/ml.
[b] Averaged results of five independently performed experiments in which transconjugants could be observed. Additional experiments with the same donor-recipient combination under the same conditions failed to yield clindamycin-erythromycin resistant transconjugants. In the successful transfer experiments, donor and recipient were at a ratio of 1:2, and the filters were incubated for 18 h anaerobically before being plated on the selective media.

on the selective plates, when filters were embedded in agar (27), or when agar was added to the broth mixtures (13). All transconjugants had the phenotypes that were expected from the transfer of the resistance markers from donor to recipient (Table 2). Resistance to clindamycin and erythromycin was constitutively expressed in both donor and transconjugants by the techniques described by Ionesco (17) and Macrina et al. (20). Because of the unreliability of transfer at low frequencies, experiments concerning the transfer mechanism, e.g., susceptibility of the transfer to DNase (26), yielded no conclusive results. The disturbing unreliability of transfer cannot be explained for the time being; killing of the recipient by the donor or vice versa was ruled out by quantitative cell counts in donor, recipient, and donor-recipient cultures.

Transfer of tetracycline resistance could be achieved with donors 630 and 662. In addition, the experiments described by Smith et al. (26) with their donor CD38 and recipients CD18 and CD37 could be easily repeated. Tetracycline resistance was constitutively expressed in both donors and transconjugants. Chloramphenicol and rifamycin resistance could not be transferred to recipient strains made resistant to fusidic acid in vitro.

Although a total of 38,000 colonies were screened, resistance to chloramphenicol, clindamycin, erythromycin, rifamycin, and tetracycline could not be eliminated by various procedures known to affect plasmid replication.

The results presented here document the occurrence of transferable determinants of resistance against clindamycin-erythromycin-streptogramin and tetracycline in *C. difficile* by filter procedures. These results confirm the finding by Ionesco (17) and Smith et al. (26) that tetracycline resistance in *C. difficile* can be transferred. However, these authors were not successful in transferring resistance against clindamycin and erythromycin. Although the resistant donor 630 contained two plasmids, no involvement of these plasmids in the resistance transfers could be detected. Donor 662 was devoid of plasmid

TABLE 2. Properties of donor, recipient, and transconjugant strains

Strain	MIC (μg/ml)[a]						Plasmid DNA detected (Mdal)	API ZYM reactions	
	CLIN	ERY	PTNI	PTNII	RIF	TET		C_4^- esterase	Leucin-amino-peptidase
Donor									
630	256	≥2,048	256	32	≤0.1	64	5, 22	−	+
662	256	≥2,048	256	64	≤0.1	64	ND[b]	−	+
Recipient									
CD37	≤0.1	0.25	4	4	128	≤0.1	ND	+	−
Transconjugants									
630 × CD37 ($Clin^r$)	128–256	≥2,048	128–256	32–64	128–256	0.25–0.5	ND	+	−
630 × CD37 (Tet^r)	0.25–0.5	0.5–1.0	4	4	128–256	32–64	ND	+	−
662 × CD37 (Tet^r)	≤0.1	0.5–1.0	4	4	64–256	16–128	ND	+	−

[a] MIC, Minimal inhibitory concentration; CLIN, clindamycin; ERY, erythromycin; PTNI and PTNII, pristinamycin I and II, respectively; RIF, rifamycin; TET, tetracycline.
[b] ND, Not detected.

DNA but could nevertheless donate tetracycline resistance. The genetic elements responsible for transfer of clindamycin-erythromycin and tetracycline resistance in *C. difficile* do not appear to be connected with readily detectable plasmid DNA. The mechanism of transfer seems to be a conjugation-like phenomenon, since a close contact between donor and recipient cells such as that provided by mixed culture on filters is necessary. An attractive hypothesis is that the determinants of clindamycin-erythromycin and tetracycline resistance are part of chromosomally located transferable genetic elements as described recently in streptococci and *Bacteroides* spp. (10, 16, 20–22).

The clinical implications of the fact that resistance to clindamycin-erythromycin and tetracycline can be transferable are open to discussion, as resistance to antimicrobial agents is not the only mechanism by which disease due to *C. difficile* can be caused (1, 8). In addition, transfer of resistance determinants in *C. difficile* is, at least in vitro, a rare event.

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