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Transferable Tetracycline Resistance in *Clostridium difficile*

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The transfer of tetracycline resistance among strains of *Clostridium difficile* is described. Transfer occurred by a conjugation-like event that was insensitive to deoxyribonuclease, could not be mediated by donor culture filtrates or chloroform-treated donor cultures, and required cell-to-cell contact. Tetracycline-resistant progeny recovered from matings displayed a resistance phenotype identical to that of the donor in level of resistance, constitutive expression, and transmissibility. Although the original tetracycline-resistant donor contained 5×10^6- and 22×10^6-dalton plasmids, standard physical analyses of antibiotic-resistant transconjugants revealed no plasmid deoxyribonucleic acid molecules in common with the donor strain. Furthermore, tetracycline-susceptible derivatives of the original donor always possessed a plasmid complement identical to that of the resistant parental strain as determined by restriction endonuclease digestion analysis. The results indicate that the tetracycline resistance determinant(s) was not encoded by readily detectable plasmid deoxyribonucleic acid and may be chromosomally located.

Recently a number of studies have implicated *Clostridium difficile* as the etiological agent in most cases of antibiotic-associated diarrhea and pseudomembranous colitis (2, 4, 17). *C. difficile* is a normal member of the indigenous, anaerobic bacterial flora in the bowel of adult humans (7, 9) and infants (11), but it is usually numerically insignificant and represents less than 0.1% of the cultivable flora found in healthy subjects. After various antibiotic therapies, *C. difficile* may proliferate at the expense of the repressed normal bowel flora and produce a toxin which is believed to be involved in the pathogenesis of pseudomembranous colitis (1).

Antibiotic-resistant strains of *C. difficile* have been described previously (6, 9), and recently, Ionesco (14) reported the transfer of low-level tetracycline resistance among such strains. This resistance determinant, encoding resistance to 15 to 20 μg of tetracycline per ml, may have been transferred by a conjugation-like mechanism. Despite these observations and the potential role that antibiotic-resistant strains may play in pseudomembranous colitis, there is a paucity of information concerning the genetic basis of antibiotic resistance in these organisms. Antibiotic resistance plasmids have been documented in other obligate anaerobic bacteria commonly found in the human intestinal tract. In *Bacteroides fragilis*, transferable resistance to the macrolide, lincosamide, and streptogramin B group drugs has been reported to be plasmid mediated (23, 26, 28). Transferable tetracycline resistance in *Clostridium perfringens* is also plasmid linked (3, 25). We describe here a transferable antibiotic resistance determinant in *C. difficile*, encoding resistance to ~50 μg of tetracycline per ml, which does not appear to be plasmid mediated.

(A preliminary account of this work has appeared previously [C. J. Smith, F. L. Macrina, and S. M. Markowitz, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 362, 1980].)

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study and their relevant characteristics are described in Table 1. All strains (clinically isolated in the United States) were obtained from T. D. Wilkins at the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, and were maintained anaerobically in chopped meat medium (13). Brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 5 g of yeast extract (Difco) per liter and 1 g of L-cysteine per liter (BHIS) was used for all routine culturing. The BHIS medium was prepared by the anaerobic methods previously described (13), and broth cultures were incubated in stoppered tubes with an atmosphere of 90% N_2 and 10% CO_2. For solid media 15 g of agar (Difco) per liter and 0.09 g of $FeSO_4 \cdot 7H_2O$ per liter were added to the BHIS broth. Culture plates were incubated anaerobically in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) under an atmosphere of 80% N_2,

TABLE 1. *C. difficile* strains

Strain designation	Phenotype[a]	Plasmid size (Mdal)
CD18[b]	Cc^s Tc^s Rf^r	None detected
CD37[b]	Cc^s Tc^s Rf^r	None detected
CD84	Cc^r Tc^s Rf^s	4.2 and 8.3[c]
CD34	Cc^r Tc^r Rf^s	5.1 and 22.1[c]

[a] Minimum inhibitory concentrations for the antibiotic resistance markers: clindamycin (Cc^r), 50 μg/ml; Tetracycline (Tc^r), 60 μg/ml; rifampin (Rf^r), >50 μg/ml; Cc^s, <1 μg/ml; Tc^s, <1 μg/ml; Rf^s, <1 μg/ml.

[b] Strains were selected as multistep, spontaneously occurring rifampin-resistant mutants.

[c] Plasmid size represents the average of four independent experiments as determined by agarose gel electrophoresis of plasmid DNA purified by cesium chloride-ethidium bromide centrifugation (19, 21).

10% CO_2, and 10% H_2. Filter-sterilized antibiotics were added to autoclaved selective media at the following concentrations: tetracycline, 15 μg/ml; rifampicin, 20 μg/ml; clindamycin, 10 μg/ml; and penicillin G, 100 μg/ml.

Antibiotic susceptibility testing was performed by the agar dilution method (20), except for tetracycline resistance, which was determined in broth cultures as described previously (13), because the $FeSO_4$ present in the solid medium interfered with the results. The donor strain used in our studies, CD34, grew on agar plates containing 65 to 75 μg of tetracycline per ml. In broth cultures, growth of CD34 was inhibited by 60 μg of tetracycline per ml. The susceptible strains, CD18, CD37, and CD84, would not grow on agar plates containing 5 μg of tetracycline per ml and in broth cultures were inhibited by less than 1 μg of tetracycline per ml.

Filter mating and curing procedures. Filter matings were performed as previously described (28), but with the following minor modifications. Mid-log-phase cultures of the donor (1 ml, ~10^8 cells per ml) and recipient (2 ml, ~10^8 cells per ml) were mixed and sedimented (3,000 × *g*, 5 min, 23°C) in stoppered tubes under an N_2 atmosphere. These were suspended in 0.1 ml of BHIS broth and transferred with a Pasteur pipette to sterile nitrocellulose filters (0.45-μm pore size, type HA; Millipore Corp., Bedford, Mass.). Filters were incubated anaerobically for 24 h on the surface of BHIS agar plates. After incubation, cells were removed from the filters by blending in a Vortex mixer with 1 ml of BHIS broth in stoppered tubes with an N_2 atmosphere. The resulting cell suspensions then were plated on the appropriate selective medium and incubated anaerobically for 48 h.

Curing of antibiotic resistance was performed by the method of Rood et al. (24). In some cases curing followed by penicillin enrichment (22) was used to isolate antibiotic-susceptible variants.

Plasmid DNA preparation and analysis. For the routine isolation of plasmid deoxyribonucleic acid (DNA), cells obtained from 300-ml cultures were lysed by the method of Rood et al. (24) and then cleared of chromosomal DNA by precipitation with 1 M NaCl as described by Guerry et al. (10). The resulting cleared lysate was then subjected to cesium chloride-ethidium bromide ultracentrifugation, and the plasmid-containing fraction was isolated as previously described (28). Plasmid DNA was analyzed by agarose slab gel electrophoresis with the appropriate-sized reference plasmid molecules (19, 21).

Screening of strains for their plasmid content was performed by preparing cleared lysates from 5-ml cultures by a scaled-down version of the technique described above. The resulting cleared lysate (ca. 0.3 ml) then was sequentially extracted with equal volumes of phenol and then chloroform-isoamyl alcohol (24:1). The aqueous layer was retained, and the DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 8.6) and 2 volumes of ethanol. This mixture was held at −70°C for 2 h and then centrifuged at 12,100 × *g* at −20°C for 20 min. The ethanol was decanted, and the precipitated DNA was redissolved in 60 μl of water. These plasmid-enriched preparations (20 to 40 μl) were analyzed directly by agarose gel electrophoresis.

A variety of other techniques also were used for the isolation of plasmid DNA, and these methods were modified only to the extent necessary to achieve lysis of the *C. difficile* strains. The different techniques included those described by Clewell and Helinski (5), Hansen and Olsen (12), and LeBlanc and Lee (16). Restriction endonuclease digestions were performed according to the instructions of the supplier (Bethesda Research Laboratories, Rockville, Md.) (18).

RESULTS

Tetracycline resistance transfer. Evidence for the transfer of tetracycline resistance in *C. difficile* was obtained by using a filter mating system (28). The data in Table 2 (experiments 1 and 3) indicate that tetracycline resistance was transferred from strain CD34 to two different suitably marked recipient strains at frequencies between 2×10^{-7} and 8×10^{-7} transconjugants per input donor cell. The transconjugants all displayed a phenotype expected from the unidirectional transfer of tetracycline resistance from the putative donor strain to the recipient. No clindamycin resistance transfer was observed within the limits of detection of this system (ca. 3×10^{-8} transconjugants per ml; Table 2, experiments 2 and 4). Control experiments in which either the donor or recipient strains alone were placed on filters, incubated overnight, and then plated on selective media resulted in no growth, except for the infrequent occurrence of spontaneous rifampin-resistant mutants of CD34.

Transconjugants obtained from the primary matings were able to transfer tetracycline resistance to the clindamycin-resistant recipient strain CD84. As shown in Table 2, transfer occurred at slightly higher frequencies (experiments 5 and 6) relative to the primary crosses with CD34 as donor. It is important to note that

TABLE 2. *Transfer of tetracycline resistance by filter matings*[a]

Experiment	Donor	Recipient	Selective medium[b]	Transconjugants		
				Per ml	Per input donor	Phenotype
1	CD34	CD18	Tc, Rf	252 ± 58	7.8×10^{-7}	$Tc^r\ Rf^r\ Cc^s$
2	CD34	CD18	Cc, Rf	0	$<3 \times 10^{-8}$	
3	CD34	CD37	Tc, Rf	157 ± 25	4.2×10^{-7}	$Tc^r\ Rf^r\ Cc^s$
4	CD34	CD37	Cc, Rf	0	$<3 \times 10^{-8}$	
5	CD101[c]	CD84	Tc, Cc	1,270 ± 232	4.5×10^{-6}	$Tc^r\ Rf^s\ Cc^r$
6	CD104[c]	CD84	Tc, Cc	1,360 ± 275	3.1×10^{-6}	$Tc^r\ Rf^s\ Cc^r$
7	CD84	CD18	Cc, Rf	0	$<3 \times 10^{-8}$	
8	CD108[d]	CD18	Tc, Rf	122 ± 29	2.1×10^{-7}	$Tc^r\ Rf^r\ Cc^s$

[a] Each experiment represents the averaged results of at least three independently performed matings.
[b] The selective media contained the indicated antibiotics at the following concentrations: tetracycline (Tc), 15 μg/ml; rifampin (Rf), 20 μg/ml; clindamycin (Cc), 10 μg/ml.
[c] Strains CD101 and CD104 are independently obtained transconjugants from experiment 1.
[d] Strain CD108 is a transconjugant obtained from experiment 5.

CD84 was unable to transfer clindamycin resistance to the recipient strain CD18 (experiment 7). Tetracycline-resistant transconjugants recovered from these crosses also were fertile and able to transfer their resistance to the original recipient strain CD18. An example of such transfer is illustrated in Table 2, experiment 8.

The transfer of tetracycline resistance to various recipient strains resulted in progeny cells with a tetracycline resistance phenotype identical to that of the parental strain. In all cases the level of resistance displayed by transconjugants of CD34 as tested in broth cultures was >45 and <60 μg/ml. This resistance also was constitutively expressed in both the donor strain and transconjugants. Specifically, cultures of CD34 and a variety of independently isolated transconjugants were grown overnight in the presence or absence of tetracycline (1 μg/ml), diluted, and then challenged with an inhibitory concentration of tetracycline (20 μg/ml, final concentration). Analysis of growth curves obtained in such experiments showed that both induced and noninduced cultures of all strains grew at similar rates (data not shown). Furthermore, none of the strains displayed a lag phase in their growth cycle after addition of the antibiotic.

Characterization of transfer. To better describe the nature of the transfer event, we performed a number of mating controls by using the primary mating system of CD34 × CD18. The data in Table 3 indicate that the transfer process was insensitive to deoxyribonuclease when it was added to either the mating mixture, the nonselective mating agar, or both. Furthermore, donor culture cell-free filtrates or chloroform-treated donor cells failed to mediate transfer of tetracycline resistance (Table 3, treatments 5 and 6). Direct cell-to-cell contact of the donor strain and the recipient was required for

TABLE 3. *Properties of the tetracycline resistance transfer event*

Treatment[a]	Transconjugants	
	Per ml	Per input donor
1. None	125	6.2×10^{-7}
2. 100 μg/ml DNase in 5 mM $MgSO_4$ buffer added to mating mixture	350	1.7×10^{-6}
3. 5 mM $MgSO_4$ buffer added to mating mixture	210	1.0×10^{-6}
4. 100 μg of deoxyribonuclease per ml in 5 mM $MgSO_4$ buffer added to the nonselective mating agar	200	1.0×10^{-6}
5. Cell-free filtrates of donor added to recipient[b]	None detected	$<3 \times 10^{-8}$
6. Chloroform-treated donor cells added to recipient	None detected	$<3 \times 10^{-8}$
7. Donor and recipient separated by membrane filter[c]	None detected	$<3 \times 10^{-8}$
8. Liquid broth matings[d]	None detected	$<3 \times 10^{-8}$

[a] Results presented are from one experiment but similiar observations were made during additional trials. The standard mating of CD34 × CD18 was used for all experiments, and matings were performed as described in the text.
[b] Cell-free filtrates of the donor CD34 were prepared by filtration of log-phase cells through 0.45-μm-pore size filters (Millipore type HA).
[c] The donor strain CD34 and the recipient CD18 were collected separately on 0.45-μm-pore size membrane filters and then placed one on top of the other cell side up on the nonselective mating plate. These filter "sandwiches" were then treated as normal matings.
[d] Liquid broth matings were carried out by adding log-phase cells of both donor and recipient to BHIS broth and incubating overnight. The resulting culture was sedimented by centrifugation at 3,000 × *g* for 5 min in stoppered tubes under an N_2 atmosphere. The cell pellet was resuspended in one-tenth the original volume of BHIS medium and plated on selective medium.

transfer. No tetracycline-resistant progeny were observed after matings performed in broth cultures or when the donor and recipient were separated by a 0.45-μm-pore size filter (Table 3, experiments 7 and 8).

Molecular characterization of transfer. The presence of plasmids in the donor strain CD34 and the transferable nature of the tetracycline resistance suggested the presence of a conjugative R plasmid. However, no plasmid species present in the original donor strain was observed in any of the transconjugants recovered in matings. Figure 1 shows typical results obtained after horizontal agarose gel electrophoresis of cleared lysates. In lane A the two cryptic plasmids (5.1×10^6 and 22.1×10^6 daltons [5.1 and 22.1 Mdal]) of CD34 are seen. Lane C contains the plasmidless recipient CD18, and lanes D through J show the complete absence of detectable plasmid DNA in any of the primary transconjugants. Similar results were obtained with cleared lysates of clones obtained from secondary crosses. The CD84 strain in lane K contains two cryptic plasmids (4.2 and 8.3 Mdal), and these are the only plasmids observed in any of the transconjugants (lanes L through O) obtained from crosses where this strain was used as a recipient.

The results obtained from these "screening" experiments were supported by analysis of cleared lysates after cesium chloride-ethidium bromide ultracentrifugation. Although the plasmids observed in strains CD34 and CD84 were easily purified, no plasmid bands were observed in CD18, CD37, or any of the primary transconjugants examined. Likewise, only the resident cryptic plasmids present in CD84 were isolated from transconjugant clones recovered in secondary crosses with CD84 as recipient. A variety of other methods were also used to prepare lysates for analysis by cesium chloride-ethidium bromide ultracentrifugation. In our laboratory the Hansen and Olsen technique (12) was successfully used for the isolation of 110- to 120-Mdal plasmids from *Pseudomonas putida* (Durham and Welch, manuscript in preparation), but no large plasmids were seen in any of the *C. difficile* strains when this technique was used. The method of LeBlanc and Lee (16) has been found useful for the isolation of plasmid DNA from strains where plasmids were not previously observed. However, no new plasmid species were observed in our *C. difficile* strains when this technique was used for lysate preparation. Similar results were observed with the method of Clewell and Helinski (5).

Loss of tetracycline resistance. The isolation of antibiotic-susceptible variants of resistant bacterial strains has in the past proven to be useful for determination of the cellular location

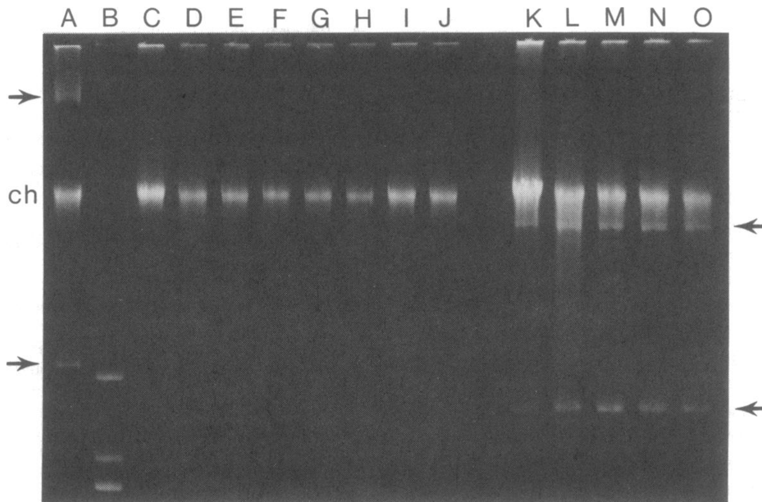

FIG. 1. *Agarose gel electrophoresis of C. difficile lysates. Cleared lysates were electrophoresed on a horizontal 0.8% agarose slab gel (28 by 18 by 1 cm) for 8 h at 70 V. Lane A, CD34, the tetracycline-resistant donor strain containing two plasmids, 5.1 and 22.1 Mdal (noted by arrows to the left of the photograph); bands corresponding to contaminating chromosomal fragments (ch) are also seen; lane B, reference plasmid molecules (19) with sizes from top to bottom of 35.8, 4.8, 3.7, and 3.4 Mdal, respectively; lane C, plasmidless recipient strain CD18; lanes D through J, progeny of CD18 × CD34 matings (Table 2, experiment 1); lane K, CD84 containing two cryptic plasmids, 4.2 and 8.3 Mdal (noted by arrows to the right of the photograph); lanes L and M, progeny of CD104 × CD84 mating (Table 1, experiment 6); lanes N and O, progeny of CD101 × CD84 mating (Table 2, experiment 5).*

of these antibiotic resistance determinants. Tetracycline-susceptible derivatives of the donor strain, CD34, were obtained at low frequencies (~0.3%), and treatment of cultures with various curing agents or heat (42°C) did not significantly alter the frequency at which susceptible clones were observed (data not shown). Six independently isolated tetracycline-susceptible derivatives were examined for their plasmid content, and analysis revealed in all strains the presence of both plasmids originally found in the parental strain CD34. Purified plasmid DNA from one such derivative, CD116, was subjected to restriction endonuclease digestion with either *Hin*dIII (Bethesda Research Laboratories) or *Hpa*II (Bethesda Research Laboratories). Results obtained from agarose gel electrophoresis of these digests indicated that the plasmid species in CD116 were identical to the 5.1- and 22.1-Mdal species present in CD34, within the limits of this restriction endonuclease fingerprint analysis (data not shown).

A number of experiments were performed to determine whether the tetracycline-susceptible derivatives suffered an irreversible loss of resistance. Six independently isolated clones were tested for their ability to revert to tetracycline resistance by spreading ca. 8×10^8 cells on plates containing 5, 10, or 20 μg of tetracycline per ml. In no case were resistant colonies observed. Similar results were obtained when cells were plated on tetracycline-containing plates by an agar overlay method. The addition of mutagens (ethidium bromide or ethyl methane sulfonate) contained in paper disks placed in the center of tetracycline-containing agar plates also failed to produce reversion of the susceptible strains. Finally, all six susceptible strains were mated in combination with each other by the filter mating protocol described above. After plating on selective medium (15 μg of tetracycline per ml) no tetracycline-resistant clones were observed. However, each of these susceptible strains was able to act as a recipient for tetracycline resistance in crosses with CD101 as the donor (data not shown).

DISCUSSION

The results presented in this paper have documented the occurrence of a transferable tetracycline resistance determinant in the intestinal pathogen *C. difficile*. The acquisition of this resistance by susceptible strains uniformly resulted in a tetracycline resistance phenotype identical to that of the donor strain in level of resistance, constitutive expression, and transmissibility (Table 2). These results are similar to those recently reported for a tetracycline resistance determinant in a French isolate of *C. difficile* (14), except that we observed the transfer of a higher level of resistance. In addition, we have shown that the nature of the transfer event is consistent with a conjugation-like mechanism (Table 3). However, the results do not rigorously rule out the possibility that a difficult-to-detect, specialized transducing phage was involved with the transfer.

Although the original tetracycline-resistant donor strain CD34 contained two plasmid species, we have been unable to demonstrate the involvement of either in the resistance transfer phenomenon. Transconjugant clones obtained from matings between the plasmidless recipient CD18 and the donor CD34 did not contain any detectable plasmid DNA (Table 2 and Fig. 1). The inability to detect plasmids in these transconjugant strains did not appear to be an event unique to the CD18 background. Clones obtained in the secondary crosses with a plasmid-containing recipient CD84 (Table 2) always displayed the cryptic plasmids originally present in this recipient (Fig. 1), but no other plasmid species were seen. It seems likely that if an additional plasmid were present in these transconjugants it would have been observed. The finding that all of our tetracycline-susceptible derivatives of CD34 contained both the 5.1- and 22.1-Mdal plasmids present in the resistant parental strain, is also consistent with the view that these plasmids play no role in tetracycline resistance.

The molecular nature of the genetic element(s) responsible for tetracycline resistance transfer in *C. difficile* is open to interpretation, although it appears that readily detectable plasmid DNA plays no role. In this regard it is possible that a very large or difficult-to-isolate plasmid which has escaped detection is involved with the resistance transfer process. The isolation of nonreverting tetracycline-susceptible variants of CD34 is certainly consistent with the loss of an undetectable plasmid species. However, in view of the ease with which plasmid DNA was isolated from the donor strain CD34 and the recipient CD84, and the different methods (5, 10, 12, 16, 24) used for the preparation of lysates, this explanation seems unlikely.

An attractive alternative hypothesis is that the tetracycline resistance determinant(s) is part of a chromosomally located transferable transposon. Transferable tetracycline and chloramphenicol resistance determinants in some strains of *Streptococcus pneumoniae* are found on the chromosome (27). Recently, Franke and Clewell (8) reported the conjugal transfer of a chromosomally located tetracycline resistance transpo-

son (*Tn*916) in *Streptococcus faecalis*. The transfer data and physical analyses of *C. difficile* shown in the present study are consistent with such a mechanism. In addition, transposon-mediated deletion formation is known to occur in a variety of bacterial systems (15). Perhaps the irreversible loss of tetracycline resistance in CD34 is the result of a deletion formation associated with a transposon-like element located on the chromosome. To more precisely identify the location of the tetracycline resistance determinant(s) in CD34 we are in the process of cloning this determinant by using recombinant DNA methodologies.

The transfer of antibiotic resistance between strains of *C. difficile* may have future clinical implications. Although the presence of antibiotic-resistant strains of *C. difficile* is not always necessary for development of pseudomembranous colitis, the acquisition of resistance by toxigenic strains could predispose patients under antibiotic therapy to pseudomembranous colitis by favoring the multiplication of such organisms. Our observations and those of Ionesco (14) indicate that resistance to clindamycin, an antibiotic often associated with pseudomembranous colitis, was not transferable. However, in view of the transferable nature of tetracycline resistance, some *C. difficile* strains may possess the potential to transfer resistance to this and other clinically important antibiotics.

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