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Survey of the Extrachromosomal Gene Pool of *Clostridium difficile*

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Pseudomembranous colitis, a severe diarrheal disease, has been linked to the administration of antibiotics and to two toxins produced by *Clostridium difficile*. Eighty-two strains of *C. difficile* isolated from humans and hamsters were assayed for the presence of plasmid DNA. Agarose gel electrophoresis of Sarkosyl-lysed cells indicated that 18% of the strains contained from one to four plasmids. The plasmid DNA in these strains ranged in molecular weight from 2.7×10^6 to 60×10^6. Strains with and without plasmids were examined for the cytopathogenic effect of the toxins on MRC-5 cells. No correlation was observed between plasmid content and cytopathogenic effect. The results of in vitro antibiotic susceptibility testing with plasmid-containing strains revealed that 33% of the strains tested exhibited growth with four or more of the antimicrobial agents used.

Clostridium difficile recently has been linked to antibiotic-associated enterocolitis and pseudomembranous colitis (1, 2, 24), which are severe and sometimes fatal diarrheal syndromes (32). This toxin-producing, strictly anaerobic bacterium was first described in 1935 (14), but has been recognized as a clinically significant pathogen only within the last 4 or 5 years. Evidence exists that the pathogenic effect of this organism is mediated by two toxins. These toxins have been partially characterized biochemically (25, 28a, 29), and studies indicate that one of them is the mediator of the cytopathogenic effect (10, 21, 22a). Previous investigations on the susceptibility of *C. difficile* to various antibiotics demonstrated that multiply drug-resistant strains may exist (4, 12, 26) and that transfer of tetracycline resistance can occur by a conjugation-like event (18). Smith et al. also published a study on the transfer of tetracycline resistance and proposed that the tetracycline resistance determinants may be located in the chromosome of *C. difficile* (27).

Studies on the genetic determinant(s) responsible for the expression of *C. difficile* toxins have not been reported. However, plasmid- and virus-mediated production of toxin and other extracellular enzymes have been demonstrated previously in other clostridial species such as *Clostridium perfringens* (6, 15), *Clostridium tetani* (20), *Clostridium botulinum* (8), and *Clostridium novyi* (8). Because of the previously reported linkage of toxin genes to extrachromosomal DNA and the well-established occurrence of antibiotic resistance genes on plasmids, we chose to examine the plasmid DNA population in *C. difficile*. This report presents the results obtained from screening 82 strains of *C. difficile* for extrachromosomal DNA. These results are correlated to the toxigenic profiles and antimicrobial susceptibilities of the strains.

MATERIALS AND METHODS

Bacterial strains. All strains of *C. difficile* were obtained from the Centers for Disease Control, Atlanta, Ga. Four strains were isolated from hamsters with antibiotic-associated diarrhea, and the remaining strains were isolated from humans with several gastrointestinal syndromes, including two forms of foodborne disease. All isolates were identified by standard procedures (5, 16).

Plasmid screening. Cells from a 3-ml overnight culture were inoculated into 50 ml of prereduced Schaedler broth (Difco Laboratories, Detroit, Mich.) and grown at 37°C in an anaerobic chamber (Model 1024; Forma Scientific, Marietta, Ohio) to an optical density at 620 nm of 0.3. The cells were then harvested by centrifugation ($600 \times g$ for 10 min at 4°C) and suspended in 0.5 ml of freshly prepared lysozyme mixture (10 mg of lysozyme per ml, 25% sucrose, 0.01 M Tris, pH 8). After a 20-min incubation at 37°C, 0.2 ml of EDTA (0.25 M in 0.025 M Tris, pH 12.6) was added, and the cells were incubated at room temperature for 5 min. Next, 3 ml of lysing solution (0.5 M Tris, 3% sodium dodecyl sulfate, pH 12.6) was added, and the suspension was agitated briefly and heated at 65°C for 30 min (19). Two volumes of unbuffered phenol:chloroform solution (1:1, vol/vol) were added, and the sediment was emulsified gently and centrifuged ($5{,}000 \times g$, 30 min, 4°C). The aqueous phase was removed and extracted with 2 volumes of isoamyl alcohol:chloroform (1:24, vol/vol) and centrifuged as

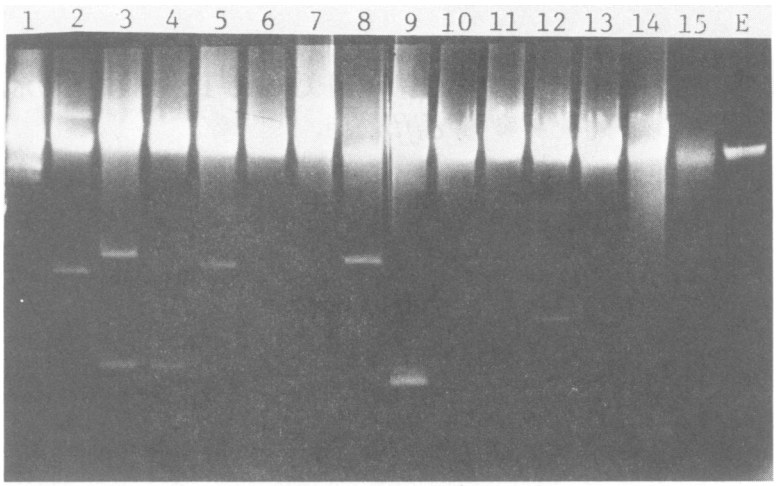

FIG. 1. Agarose gel electrophoresis of plasmid-containing strains of *C. difficile*. Molecular weights were determined from known weights of reference *E. coli* V517 (lane E), kindly supplied by F. Macrina (23).

described above; again the aqueous layer was removed, and the precipitate at the interface was avoided. The DNA in the aqueous phase was then concentrated with polyethylene glycol 6000 Carbowax (Fisher Scientific Co., Fair Lawn, N.J.) as described by Humphreys et al. (17) and suspended in 0.3 to 0.5 ml of buffer (50 mM Tris, 20 mM EDTA, pH 8).

Polyethylene glycol-precipitated DNA was subjected to electrophoresis in 0.8 or 0.45% agarose (BioRad Laboratories, Richmond, Calif.) that had been melted in TA buffer (40 mM Tris, 25 mM sodium acetate, 2 mM sodium EDTA, 0.45% glacial acetic acid). The DNA samples (40 μl) were mixed with 10 μl of bromophenol blue tracking dye (0.25% bromophenol blue, 25% glycerol, 5% sodium dodecyl sulfate) before being electrophoresed. Electrophoresis was conducted in TA buffer at 40 mV overnight. After the tracking dye had migrated 15 cm, the gel was stained in ethidium bromide (0.5 μg/ml) for 1 h at room temperature. Gels were placed on a short-wavelength UV light (model C-61; Ultraviolet Products, Inc., San Gabriel, Calif.) and photographed with type 55 Polaroid film through a Kodak 23A filter.

Cytotoxicity testing. *C. difficile* strains were grown for 24 h in chopped meat glucose (Carr Scarborough, Decatur, Ga.) and then filtered with a 0.45-μm filter (Millipore Corp., Bedford, Mass.). Filtrates (50 μl) were added to 180 μl of complete Eagle minimal essential medium containing MRC-5 embryonic human lung fibroblasts that had been grown as monolayers in flat-bottom microtiter plates. Cultures in which 50% of the cells exhibited the characteristic actinomorphic change within 24 to 48 h were considered positive for *C. difficile* toxin (7, 10).

Antibiotic susceptibility testing. Micro-Media Systems Inc. (Potomac, Md.) anaerobe minimal inhibitory concentration (MIC) test panels were used to test strains of *C. difficile* for resistance to carbenicillin, cefoxitin, chloramphenicol, clindamycin, penicillin, and tetracycline.

Resistance to colistin (16 μg/ml), erythromycin (4 μg/ml), gentamicin (16 μg/ml), rifampin (2 μg/ml), and vancomycin (8 μg/ml) was tested at single concentrations. All steps, including prereduction and inoculation of the anaerobe test panels, were performed as recommended by the manufacturer. After being inoculated, the panels were incubated for 48 h at 35°C in an anaerobic glove box (model 1024; Forma). Growth was recorded if turbidity was seen. Two strains of *C. difficile* (A-279 and A-280) with known antibiotic resistance were used as control organisms for the MIC tests.

RESULTS

Plasmid screen. Of the 82 strains of *C. difficile* tested, 15 strains had one to four plasmids, ranging in molecular weight from 2.7×10^6 to 60×10^6. An agarose gel with the DNA profiles of the plasmid-positive *C. difficile* strains is shown in Fig. 1. With the exception of strain 2, all *C. difficile* strains shown were human isolates.

The plasmids had similar molecular weights; strains 3 through 5 contained plasmids with molecular weights of 4.1×10^6 to 4.3×10^6, strains 6 and 7 contained plasmids with molecular weights of 4.7×10^6 to 4.9×10^6, and strains 10 through 15 contained plasmids with a common molecular weight of 2.7×10^6 to 2.9×10^6 (Table 1).

Cytopathogenic effect. All 82 strains of *C. difficile* were examined for their cytotoxigenic effect on MRC-5 embryonic human lung fibroblasts. Altogether, 4 of the 15 plasmid-containing strains and 10 of the 67 strains without plasmids were negative for cytopathogenic effect (Table 1).

In vitro susceptibility. Results of in vitro antibiotic susceptibility tests for the plasmid-containing strains revealed that 33% of the strains

TABLE 1. Cytopathogenic effects and molecular weights of plasmids in 15 strains of *C. difficile*

Strain no.	Cytopathogenic effect[a]	Plasmid mol wt ($\times 10^{-6}$) ± SE[b]
1	−	44.3 ± 2.8; 60 ± 5.3
2	+	12.3 ± 0.6
3	+	4.1 ± 0.2; 11.3 ± 1.2
4	−	4.1 ± 0.3
5	+	4.3 ± 0.3; 10.4 ± 1.0
6	−	4.9 ± 0.3
7	+	4.7 ± 0.2
8	−	3.9 ± 0.4; 13.2 ± 2.3
9	+	3.8 ± 0.2; 5.8 ± 0.4; 12.5 ± 0.9; 26.4 ± 1.4
10	+	2.9 ± 0.3
11	+	2.8 ± 0.2; 7.8 ± 0.4
12	+	2.7 ± 0.1
13	+	2.8 ± 0.1
14	+	2.8 ± 0.2
15	+	2.7 ± 0.2; 3.7 ± 0.2

[a] +, Characteristic actinomorphic effect on at least 50% of MRC-5 tissue culture cells; −, no cytopathogenic effect.

[b] Mean ± standard error of four different plasmid isolation procedures.

exhibited growth in four or more of the antimicrobial agents used (Table 2). MIC tests indicated that 27% of the strains tested were resistant to two or more antibiotics. Growth of *C. difficile* in antibiotics that were tested at only one concentration varied except with vancomycin, which did not allow growth of any of the strains. MICs for non-plasmid-containing strains are not presented.

DISCUSSION

More than 18% of the strains of *C. difficile* tested contained detectable plasmid DNA. Although there was no observed correlation between the cytopathogenic effect (i.e., toxin production) and the presence or absence of extrachromosomal DNA in these strains, this does not exclude the possibility that extrachromosomal DNA may carry genes for the production of toxin or manifestation of the pathological syndrome. The *ent* gene in *Escherichia coli* is one example of a system in which only 17% of 96 toxin-positive strains were shown to contain a plasmid that coded for the production of toxin (13). Cytopathogenicity was also demonstrated in the six *C. difficile* strains that contained the small-molecular-weight plasmids (2.7×10^6 to 2.9×10^6), allowing the possibility of a relationship between toxin production and the presence of plasmids.

It has been reported that toxin-producing strains of *C. difficile* have been isolated from healthy adults (22) and children (11, 22) who did not display disease symptoms. Therefore, it is possible that the manifestation of *C. difficile* infection may involve other factors in addition to toxin production. The pathological symptoms caused by toxigenic *E. coli* are an example of development of diarrheal disease being dependent not only on toxin production but also on a plasmid-encoded colonization factor which promotes attachment of the organism to the intestinal wall (28). Similar or different mechanisms may occur in *C. difficile* infections, which may be disclosed by further genetic studies.

TABLE 2. In vitro antibiotic susceptibility results for plasmid-containing strains of *C. difficile*

Strain no.	MIC (μg/ml)[a] of following antibiotic (range tested [μg/ml])						Growth in noninhibitory antibiotic (μg/ml of medium)				
	Carbenicillin (8–512)	Cefoxitin (1–64)	Chloramphenicol (0.5–32)	Clindamycin (0.25–16)	Penicillin (0.06–4)	Tetracycline (0.25–16)	Colistin (16)	Erythromycin (4)	Gentamicin (16)	Rifampin (2)	Vancomycin (8)
1	≤8	≤1	≤0.5	≤0.25	≤0.06	≤0.25	+	−	−	−	−
2	≤8	>64*	2	≤0.25	0.25	≤0.25	+	−	+	−	−
3	≤8	>64*	2	≤0.25	0.25	≤0.25	+	−	+	−	−
4	≤8	>64*	2	1	1	4	+	+	+	−	−
5	≤8	>64*	2	>16*	0.25	≤0.25	+	+	−	−	−
6	16	>64*	4	1	1	>16*	+	−	+	−	−
7	32	>64*	32*	≤0.25	>4*	>16*	+	−	+	+	−
8	≤8	>64*	2	≤0.25	0.5	≤0.25	+	−	−	−	−
9	≤8	>64*	2	≤0.25	0.5	≤0.25	+	−	+	−	−
10	≤8	>64*	2	≤0.25	0.5	≤0.25	+	−	+	−	−
11	16	>64*	16	16*	1	>16*	+	+	−	−	−
12	≤8	>64*	≥0.5	≤0.25	≤0.06	≤0.25	−	−	−	−	−
13	≤8	>64*	2	≤0.25	0.5	≤0.25	+	−	+	−	−
14	≤8	>64*	2	≤0.25	0.5	≤0.25	+	−	+	−	−
15	≤8	>1	1	≤0.25	≤0.06	≤0.25	+	−	+	−	−

[a] *, Resistant.

Susceptibility profiles of *C. difficile* have been reported in earlier studies (4, 7, 12, 26), and the overall findings of this investigation concur with those reported previously. Other investigators have shown that pseudomembranous colitis can occur after the administration of a broad spectrum of antibiotics (21) such as ampicillin (7), lincomycin (3), clindamycin (30), tetracycline (31), and the cephalosporins (9); however, the relationship of pseudomembranous colitis to antibiotic therapy remains an enigma (7).

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LITERATURE CITED

1. **Bartlett, J. G., N. S. Taylor, T. Chang, and J. Dzink.** 1980. Clinical and laboratory observations in *Clostridium difficile* colitis. Am. J. Clin. Nutr. **33**:2521–2526.
2. **Bartlett, J. S., T. W. Chang, N. S. Taylor, and A. B. Onderdonk.** 1979. Colitis induced by *Clostridium difficile*. Rev. Infect. Dis. **1**:370–378.
3. **Binner, E. J., and W. H. Tellman.** 1970. Pseudomembranous colitis as a sequel to oral lincomycin therapy. Am. J. Gastroenterol. **54**:55–58.
4. **Burdon, D. W., J. D. Brown, and D. J. Young.** 1979. Antibiotic susceptibility of *Clostridium difficile*. Antimicrob. Agents Chemother. **5**:307–310.
5. **Dowell, V. R., Jr., and T. M. Hawkins.** 1974. Laboratory methods on anaerobic bacteriology. CDC Laboratory Manual. Center for Disease Control, Atlanta, Ga.
6. **Duncan, C. L., E. A. Rokar, and C. M. Christerson.** 1978. Multiple plasmids in different toxigenic types of *Clostridium perfringens*: possible control of beta-toxin production, p. 246–248. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
7. **Dzink, J., and J. G. Bartlett.** 1980. In vitro susceptibility of *Clostridium difficile* isolated from patients with antibiotic-associated diarrhea or colitis. Antimicrob. Agents Chemother. **4**:695–698.
8. **Eklund, M. W., F. T. Poysky, J. A. Meyers, and G. A. Pelroy.** 1974. Interspecies conversion of *Clostridium botulinum* type C to *Clostridium novyi* type A by bacteriophage. Science **186**:456–458.
9. **Fee, X. J., N. E. Ament, and L. C. Holmes.** 1977. Pseudomembranous colitis associated with cephalazolin therapy. Am. J. Surg. **133**:247–248.
10. **Florin, L., and M. Theslan.** 1981. Intoxication of cultured human lung fibroblasts with *Clostridium difficile* toxin. Infect. Immun. **33**:66–74.
11. **Holst, E. L., and P. Helin.** 1982. Recovery of *Clostridium difficile* from children. Scand. J. Infect. Dis. **13**:41–45.
12. **George, W. L., V. L. Sutter, and S. M. Finegold.** 1978. Toxigenicity and antimicrobial susceptibility of *Clostridium difficile*, a cause of antimicrobial agent-associated colitis. Curr. Top. Microbiol. **1**:55–58.
13. **Gyles, C., S. Magdalene, and S. Falkow.** 1974. The enterotoxin plasmids of *Escherichia coli*. J. Infect. Dis. **130**:40–49.
14. **Hall, I. C., and E. O'Toole.** 1935. Intestinal flora in newborn infants, with a description of a new pathogenic anaerobe, *Bacillus difficilis*. Am. J. Dis. Child. **44**:390–402.
15. **Hans, P. B., and M. Solbery.** 1981. Isolation of a plasmid responsible for caseinase activity in *Clostridium perfringens* ATCC 326B. J. Bacteriol. **147**:262–266.
16. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, Va.
17. **Humphreys, O., G. A. Willshaw, and E. S. Anderson.** 1975. Simple method for the preparation of large quantities of pure plasmid DNA. Biochim. Biophys. Acta **383**:457–463.
18. **Ionesco, H.** 1978. Transfer de la resistance a la tetracycline chez *Clostridium difficile*. Ann. Microbiol. (Paris) **131**:141–174.
19. **Kado, C. I., and S. T. Lieu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1364–1373.
20. **Laird, W. J., W. Aaronson, R. R. Siler, W. H. Habig, and M. C. Hardegree.** 1980. Plasmid-associated toxigenicity in *Clostridium tetani*. J. Infect. Dis. **142**:623.
21. **Larson, H. E., and A. B. Prince.** 1977. Pseudomembranous colitis: presence of clostridial toxin. Lancet **i**:1312–1314.
22. **Larson, H. E., A. B. Prince, P. Honour, and S. P. Barriello.** 1978. *Clostridium difficile* and the etiology of pseudomembranous colitis. Lancet **i**:1063–1066.

22a. **Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins.** 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1147–1150.

23. **Macrina, F. L., D. J. Kopecho, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1**:417–420.
24. **Medline, A., D. H. Skin, and N. M. Medline.** 1976. Pseudomembranous colitis associated with antibiotics. Hum. Pathol. **7**:693–703.
25. **Ralfe, R. D., and S. M. Finegold.** 1979. Purification and characterization of *Clostridium difficile* toxin. Infect. Immun. **25**:191–201.
26. **Shuttleworth, R., M. Taylor, and D. M. Jones.** 1980. Antimicrobial susceptibilities of *Clostridium difficile*. J. Clin. Pathol. **33**:1002–1005.
27. **Smith, C. J., S. M. Mardowitz, and F. L. Macrina.** 1981. Transferable tetracycline resistance in *Clostridium difficile*. Antimicrob. Agents Chemother. **19**:997–1003.
28. **Sock, R. B.** 1980. Enterotoxigenic *Escherichia coli*: identification and characterization. J. Infect. Dis. **142**:279–286.

28a. **Sullivan, N. M., S. Pellett, and T. D. Wilkins.** 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1032–1040.

29. **Taylor, N. S., and J. G. Bartlett.** 1979. Partial purification and characterization of a cytotoxin from *Clostridium difficile*. Rev. Infect. Dis. **1**:379–385.
30. **Tedesco, F. J.** 1976. Clindamycin-associated colitis. Am. J. Dig. Dis. **21**:26–31.
31. **Toshmiwal, R., R. Fekety, and J. I. Silva, Jr.** 1979. Etiology of tetracycline-associated pseudomembranous colitis in hamsters. Antimicrob. Agents Chemother. **16**:167–170.
32. **Totten, M. A., J. A. Gregg, P. Fremont-Smith, and M. Legg.** 1978. Clinical and pathological spectrum of antibiotic-associated colitis. Am. J. Gastroenterol. **69**:311–319.