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Molecular Cloning and Genetic Analysis of a Chloramphenicol Acetyltransferase Determinant from *Clostridium difficile*

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A gene bank from a clinical isolate of *Clostridium difficile* expressing high chloramphenicol acetyltransferase activity was constructed by cloning *Sau*3A-cleaved clostridial DNA fragments into the plasmid vector pUC13. Among 1,020 clones tested, 11 were resistant to chloramphenicol; 1 of these, with an insert size of 1.9 kilobases (pPPM9), was studied further. The clone pPPM9 was mapped using a variety of restriction enzymes, and a 0.27-kilobase *Eco*RV-*Taq*I restriction fragment was shown to be within the chloramphenicol resistance (Cm^r) gene by using transposon (Tn*1000*) mutagenesis. The 0.27-kilobase fragment and the 1.9-kilobase insert were radiolabeled and used as DNA probes in hybridization studies. Southern blot analysis with the gene probes against chromosomal DNA from Cm^r strains of *C. difficile* obtained from five distinct geographical locations revealed that at least two copies of the same chloramphenicol acetyltransferase gene were present for each strain. Hybridization of the gene probes against Cm^r strains of *Staphylococcus epidermidis, Staphylococcus aureus, Klebsiella edwardsii, Escherichia coli,* and to four other clostridial species revealed no homology even under conditions of low stringency.

Clostridium difficile is a gram-positive, spore-forming anaerobic bacterium which is the causative agent of pseudomembranous colitis, antibiotic-associated colitis, and antibiotic-associated diarrhea (3, 10, 20). Epidemiological studies have shown that *C. difficile* can be acquired in the hospital (16); multiresistant strains, including those expressing chloramphenicol resistance (Cm^r), have been responsible for cross-infection (25, 36). The administration of antibiotics appears to be important in the clinical manifestation of the disease (10, 20). Two toxins have been identified as being produced by *C. difficile*, namely, toxin A, an enterotoxin causing fluid accumulation in the rabbit ileal loop assay, and toxin B, a potent cytotoxin (19, 22). Various studies have shown a possible correlation among the organism's resistance to antibiotics and toxin-producing capabilities and the manifestation of disease (5, 29). However, apart from a few reports on the transfer of tetracycline (13, 18, 30) and erythromycin-clindamycin resistance (12), little is known about the molecular and genetic basis of antibiotic resistance for the organism.

The aim of our research is to study, at the molecular level, the role of Cm^r and other antibiotic resistance determinants in the pathogenicity of *C. difficile*. By cloning and characterizing a Cm^r gene, a suitable DNA probe can be constructed to study the dissemination and transfer of this determinant among *C. difficile* strains and other bacterial species. The cloning of a *C. difficile*-derived Cm^r gene should also prove useful as a genetic marker in the development of clostridial shuttle vector systems.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors. The clinical isolate of *C. difficile* (W1) used in this study as the source of *C. difficile* genomic DNA was obtained from a patient with pseudomembranous colitis at this hospital in 1983 (16). The relevant details of other bacterial strains used in this study are summarized in Table 1. The *Escherichia coli* K-12 derivative JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1*, Δ(*lacproAB*) *F' traD36 proAB lacI*^{q}*ZΔM15*] was the recipient for all transformation experiments (14) and was the host strain for the vector plasmid pUC13 (Ap^r *lac*$^+$) (37).

Culture media. Clostridial strains were grown in prereduced Nakamura broth (24) and incubated at 37°C overnight under anaerobic conditions. All other strains were grown in Luria-Bertoni broth (23) or on Luria-Bertoni agar (23).

Determination of MICs. MICs of chloramphenicol, clindamycin, erythromycin, rifampin, and tetracycline were determined by an agar dilution method. Overnight cultures in Nakamura broth were diluted 1:100 in prereduced Nakamura broth. A multipoint inoculator (Denley Scientific Instruments, Billingshurst, United Kingdom) was used to deposit an inoculum of approximately 10^3 organisms onto the surface of Wilkins-Chalgren agar plates (Oxoid, Ltd., Basingstoke, United Kingdom) containing 7% (vol/vol) defibrinated horse blood (Oxoid) and serial twofold dilutions of antibiotic. Plates were incubated for 24 h either aerobically (*E. coli*) or anaerobically (*C. difficile*), and the MICs were recorded as the lowest antibiotic concentration which prevented the formation of visible growth.

CAT assay. The chloramphenicol acetyltransferase (CAT) activities for Cm^r strains were determined from 1 ml of sonicated cell extracts by the method of Shaw and calculated by using a suitable colorimetric assay (27).

Inducibility studies. Cultures were grown to an optical density at 550 nm of 0.8 with and without a subinhibitory concentration (1 μg/ml) of chloramphenicol. The cultures (0.5 ml) were diluted in 20 ml of fresh medium containing 20 μg of chloramphenicol per ml. Cultures were incubated at 37°C; samples were taken at 30-min intervals, and their optical density at 550 nm was measured.

DNA preparation. *C. difficile* genomic DNA was prepared by a method developed in this laboratory (35). Plasmid DNAs from *E. coli* and *C. difficile* were prepared by the methods of Birnboim and Doly (4) and Roberts et al. (26), respectively.

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TABLE 1. Bacterial strains

Strain and reference no.	Source of strain	Chloramphenicol MIC (μg/ml)	CAT sp act (mmol/min per mg)	Hybridization of 0.27-kb CAT fragment to bacterial genomic DNA
C. difficile				
W1	Clinical isolate, this laboratory	48	5.21	+
E1	Clinical isolate, this laboratory	4[a]	0	−
Y1	Clinical isolate, this laboratory	4[a]	0	−
SGC0545	M. Delmee, Brussells, Belgium	24	5.01	+
3026	E. Kuiper, Amsterdam, Holland	24	4.68	+
NBL13	C. Nord, Stockholm, Sweden	24	4.88	+
C250	A. Pantosti, Rome, Italy	24	5.01	+
E. coli				
JM109(pUC13)	Host strain and vector plasmid	1[a]	0	−
JM109(pPPM9)	Host strain and 1.9-kb Cm^r insert	>250	13.21	+
JM109(pPPM10)	Host strain and inverted Cm^r insert	1[a]	0	−
NCTC 50055(pR726), CAT type I clone	PHLS, Colindale, United Kingdom	>250	NT[b]	−
NCTC 50128(p50), CAT type II clone	PHLS, Colindale, United Kingdom	>250	NT	−
NCTC 50022(pR387), CAT type III clone	PHLS, Colindale, United Kingdom	>250	NT	−
NCTC 50070(pR1033), nonenzymatic CAT clone	PHLS, Colindale, United Kingdom	>250	NT	−
1428	Clinical isolate, this laboratory	>250	NT	−
1520	Clinical isolate, this laboratory	>250	NT	−
SP29	Clinical isolate, this laboratory	>250	NT	−
5040	Clinical isolate, this laboratory	>250	8.20	−
S. aureus				
F142	Clinical isolate, this laboratory	64	6.21	−
1858	Clinical isolate, this laboratory	64	6.82	−
S. epidermidis				
BLC 157	Clinical isolate, this laboratory	16	NT	−
BLC 1633	Clinical isolate, this laboratory	16	3.92	−
K. edwardsii				
126	Clinical isolate, this laboratory	32	NT	−
F398	Clinical isolate, this laboratory	64	7.28	−
F452	Clinical isolate, this laboratory	16	NT	−
KL390	Clinical isolate, this laboratory	32	NT	−
Clostridium sordellii				
S321	Clinical isolate, this laboratory	32	6.87	−
R1200	Clinical isolate, this laboratory	16	3.21	−
R2710	M. Phillips, PHLS, Luton, United Kingdom	16	0	−
Clostridium innocuum				
R657	M. Phillips, PHLS, Luton, United Kingdom	16	1.29	−
R1001	M. Phillips, PHLS, Luton, United Kingdom	16	0	−
Clostridium tertium R673	M. Phillips, PHLS, Luton, United Kingdom	16	1.64	−
Clostridium fallax R2720	M. Phillips, PHLS, Luton, United Kingdom	16	2.82	−

[a] MICs of <4 μg/ml were considered to indicate a chloramphenicol-susceptible isolate.
[b] NT, Not tested.

Construction and screening of *C. difficile* genomic library. *C. difficile* genomic DNA was partially cleaved with the restriction endonuclease *Sau*3A, and fragments between 2 and 10 kilobases (kb) were pooled. The fragments were ligated into the *lacZ* gene of *Bam*HI-cleaved pUC13 DNA and transformed into competent *lac E. coli* JM109 cells by the method of Hanahan (14). Transformants were selected on agar plates containing ampicillin (50 μg/ml) and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma Chemical Co., Poole, United Kingdom). The colorless (*lac*) colonies, which contain *C. difficile* insert DNA, were then replica plated onto agar containing 24 μg of chloramphenicol per ml.

Restriction enzyme analysis and transposon mutagenesis. The clone with the smallest DNA insert size (pPPM9) was mapped with a variety of restriction endonucleases (*Acc*I, *Ava*I, *Bam*HI, *Bsc*I, *Dde*I, *Eco*RI, *Eco*RV, *Hinc*II, *Hind*III, *Mbo*I, *Pst*I, *Sma*I, *Sst*I, *Taq*I, *Xba*I, and *Xbo*I) as specified by the suppliers (NBL, Cramlington, United Kingdom). To further localize the CAT gene, transposon mutagenesis with Tn*1000* (gamma-delta region of the F factor of *E. coli* K-12) was performed to generate insertion mutations of pPPM9

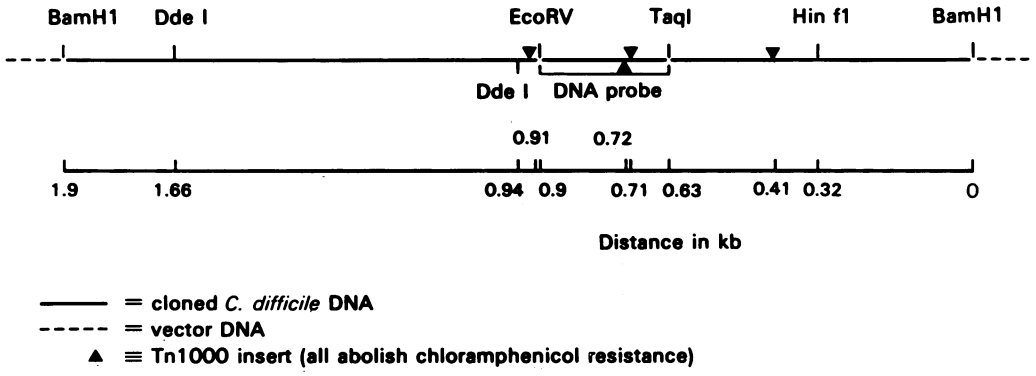

FIG. 1. Linearized restriction cleavage map of the 1.9-kb insert from plasmid pPPM9. The *Bam*HI site nearest the *lacZ* gene in the vector DNA is taken as the origin for kilobase distance measurements.

which were no longer Cm^r. These insertions were localized by digesting separately with *Hin*dIII and *Eco*RI essentially as described by Taylor et al. (32).

Preparation of radioactive DNA probes and hybridization procedures. DNA fragments were radiolabeled in vitro with [γ-^{32}P]dCTP (Amersham International, Amersham, United Kingdom) by the random sequence hexamer method of Feinberg and Vogelstein (9). Colony hybridization was performed on nitrocellulose filters (New England Nuclear Corp., Boston, Mass.) by the method of Grunstein and Hogness (11), with an extra denaturing and neutralization step to ensure complete lysis of all bacterial cells. Further DNA analysis on restriction endonuclease-digested genomic DNA from *C. difficile* chloramphenicol-susceptible (Cm^s) and Cm^r strains was performed by transferring the digested DNA to Gene Screen hybridization transfer membrane (New England Nuclear) by the method of Southern (31). Southern blot hybridizations were carried out under conditions of high stringency (50% formamide), whereas for colony hybridization conditions of lowered stringency (25% formamide) were employed as recommended by New England Nuclear. All blots were dried, exposed to Fuji-RX X-ray film, and stored at −70°C for 1 to 3 days.

RESULTS

***C. difficile* strains.** Five clinical isolates of *C. difficile* from 53 examined were found to be resistant to chloramphenicol; the MICs for the antibiotic are shown in Table 1. All *C. difficile* Cm^r strains had CAT activity (Table 1) and were found to be inducible with subinhibitory concentrations of chloramphenicol. One strain (W1) was chosen for construction of a gene bank, since it had the highest MIC (48 μg/ml) for chloramphenicol and was also resistant to a variety of other antibiotics (MICs: clindamycin, 200 μg/ml; erythromycin, 500 μg/ml; rifampin, 200 μg/ml; and tetracycline, 75 μg/ml). Furthermore, this strain was particularly pathogenic (in terms of severity of disease) and had high cytotoxin and enterotoxin titers (34).

Construction and screening of *C. difficile* genomic library. A total of 1,020 pUC13 clones with *C. difficile* insert DNA were screened for resistance to chloramphenicol; 11 positive clones were detected. One positive clone (pPPM9) which contained the smallest *C. difficile* DNA insert (1.9 kb) was chosen for further study.

The chloramphenicol MIC for the pPPM9 clone in *E. coli* JM109 was 250 μg/ml and was expressed constitutively in contrast to the original *C. difficile* strain (W1) with an MIC of 48 μg/ml, expressed by induction with subinhibitory concentrations of chloramphenicol.

Restriction mapping and transposon mutagenesis of pPPM9. A preliminary restriction cleavage map of pPPM9 is shown in Fig. 1. The restriction cleavage map of pPPM9 was used to confirm the identity of the clone pPPM10, in which the 1.9-kb DNA insert was cloned in the opposite orientation. No expression of CAT activity was observed for pPPM10. Mutants of pPPM9 were obtained from spontaneous Tn*1000* insertions, giving Cm^s strains. The positions of four Tn*1000* insertion mutations are shown in Fig. 1.

Hybridization studies. The 0.27-kb *Eco*RV-*Taq*I restriction fragment was isolated and radiolabeled to use as a DNA probe for *C. difficile*-derived Cm^r determinants. The radiolabeled 0.27-kb CAT gene probe was hybridized against *Hin*dIII digests of chromosomal DNA from five Cm^r *C. difficile* strains (Fig. 2). Lane f shows positive hybridization against two *Hin*dIII DNA fragments (approximate sizes, 16 and 19 kb) from the strain from which the gene library was

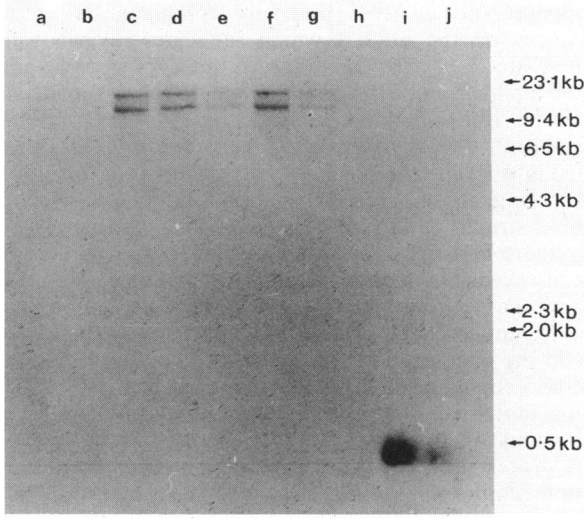

FIG. 2. Southern hybridization analysis. An *Eco*RV-*Taq*I restriction fragment from pPPM9 was radiolabeled with [^{32}P]dCTP and used to probe for chloramphenicol resistance. Lanes: a, Cm^s *C. difficile* strain (E1) digested with *Hin*dIII; b, pC221 Cm^r determinant from *S. aureus*; c through g, Cm^r *C. difficile* C250, 3026, NBL13, W1, and SGC0545, respectively, digested with *Hin*dIII; h, *C. difficile* W1 plasmid DNA; i, *C. difficile* W1 digested with *Eco*RV and *Taq*I; j, *Eco*RV-*Taq*I restriction fragment from pPPM9.

constructed (W1). Lanes c, d, e, and g showed positive hybridization against *Hin*dIII fragments of apparent similar size for Cm^r *C. difficile* strains from Italy, Holland, Sweden, and Belgium, respectively. Lanes a, b, and h revealed no hybridization to a Cm^s *C. difficile* strain (Y1), to a plasmid vector pC221 derived from a Cm^r determinant from *Staphylococcus aureus* (6), and to two plasmids (18.3 and 4.2 megadaltons) from *C. difficile* strain W1, respectively. Lane i showed positive hybridization to W1 genomic DNA digested with *Eco*RV and *Taq*I which was stronger than the hybridization to the 0.27-kb *Eco*RV-*Taq*I DNA fragment used as the CAT gene probe (lane j). Identical results were obtained when the whole 1.9-kb insert from pPPM9 was used as a DNA probe (data not shown).

Colony hybridization with the 0.27- and 1.9-kb gene probes against a variety of *Staphylococcus epidermidis*, *S. aureus*, *Klebsiella edwardsii*, *E. coli*, and *Clostridia* strains resistant to chloramphenicol showed no homology even under conditions of low stringency (Table 1).

DISCUSSION

We have cloned and expressed a CAT determinant from a gram-positive anaerobe, *C. difficile*, into an *E. coli* host strain. Our results indicate that the expression of the CAT gene in the pPPM9 clone can only occur in one direction, suggesting that the *C. difficile* promoter sequences may be absent or may not function in *E. coli*. DNA sequencing of pPPM9 is currently in progress to further analyze the *C. difficile*-derived CAT gene. The CAT gene from pPPM9 was constitutively expressed at a higher MIC (250 μg/ml compared with 48 μg/ml) in *E. coli*, in contrast to the inducible expression of the same gene in the original *C. difficile* host strain. This feature has been observed for other CAT genes cloned into *E. coli* from other gram-positive bacteria, namely, pC194 (17) and pC221 (6) derived from *S. aureus* and pUB110 (15) derived from *Bacillus pumilus*. Various lines of evidence suggest that the control of expression of the CAT gene from gram-positive organisms is at the level of translation and involves inversely complimentary repeat sequences on the mRNA which sequester the ribosome-binding site (7, 8, 15). Induction of the CAT gene requires the destabilization of the complimentary base sequences to make the ribosome-binding site available for translation initiation (2, 7). We presumed that once the CAT gene is cloned into *E. coli* the regulatory mechanism for the expression of the gene is not recognized or has not been cloned into the host strain.

Southern blot hybridization data clearly showed the presence of two distinct bands from the *Hin*dIII digestions of the genomic DNA from Cm^r *C. difficile* strains obtained from five different geographical locations. As there are no *Hin*dIII sites in the gene probes, this suggests that there may be at least two copies of the CAT gene in each of the *C. difficile* strains, that are resistant to the antibiotic. Also, the presence of an intense band for *Eco*RV-*Taq*I-digested *C. difficile* (W1) DNA suggests that the 0.27-kb gene probe has hybridized to multiple copies of the CAT gene. Furthermore, 11 Cm^r clones were originally obtained from the gene library which consisted of a single genome equivalent of *C. difficile* (W1) DNA, again suggesting that multiple copies of the CAT gene may be present in the genome of the (W1) *C. difficile* strain. No hybridization was observed between the CAT gene probe and two plasmids isolated from the W1 strain; in the absence of any detectable plasmids from the strains from Belgium, Holland, Sweden, and Italy, we assumed that the CAT determinants are located on the chromosome of *C. difficile*, although linkage studies are required for confirmation.

Several Cm^r genes have been biochemically and genetically characterized from widely diverse organisms (28). The most intensely studied have been CAT genes isolated from Cm^r strains of *E. coli* and *S. aureus*, because of their potential as genetic markers for in vitro cloning systems. The *C. difficile* Cm^r strains used in this study show significant CAT activity. However, the 0.27- and 1.9-kb gene probes showed no homology by colony hybridization to three distinguishable variants of the CAT gene (types I, II, and III [28]) and to a nonenzymic Cm^r *E. coli* strain (Table 1). Both the 0.27- and 1.9-kb gene probes also failed to hybridize to DNA from Cm^r *S. aureus*, *S. epidermidis*, *K. edwardsii*, and other clostridial species. This suggests that the *C. difficile*-derived CAT determinant and the DNA immediately flanking the CAT gene may be unique.

Recently, Hachler et al. (13) showed DNA homology between a transferable tetracycline resistance determinant of *C. difficile* and the conjugative transposon Tn*916*, originally isolated from *S. faecalis*. Furthermore, they successfully transferred an erythromycin-clindamycin resistance determinant from *C. difficile* to *S. aureus* in the absence of detectable plasmid DNA (12). Also, Abraham and Rood (1) reported two Cm^r genes from a *Clostridium perfringens* strain, originally located on a large plasmid, which were capable of undergoing site-specific excision and transposition when cloned into *E. coli*. We have not tested for DNA homology between the *C. perfringens* Cm^r transposon and the CAT determinant we have cloned. It is possible that the *C. difficile* CAT gene is located on a chromosomally borne transposon. However, since Southern blot data on the genomic DNA of the five *C. difficile* Cm^r strains revealed identical banding patterns, this would require that the transposable element integrates into the *C. difficile* chromosome at specific sites. Alternatively, the five *C. difficile* strains analyzed may, in fact, be the same strain, but this appears unlikely as the isolates were obtained from five different parts of the world.

The role of the CAT gene and other antibiotic resistance determinants in the pathogenicity of the *C. difficile* remains to be evaluated. However, it was notable that all five Cm^r *C. difficile* strains were multiresistant, highly toxigenic, and obtained from patients with pseudomembranous colitis. Toxin genes have been found on transposable elements from the heat-stable toxin of enterotoxic *E. coli* (21). We have previously reported the cloning of the *C. difficile* enterotoxin (toxin A) (33) and are currently investigating the possibility of multiple antibiotic resistance determinants and/or toxin genes existing on the same or different transposable elements in *C. difficile*.

Finally, the cloning and characterization of a CAT determinant from *C. difficile* should also be useful as a genetic marker in the construction of a shuttle vector cloning system for the exchange of genetic information between *E. coli* and the *Clostridium* genus. This will make it possible to reintroduce into *C. difficile* various cloned genetic elements, for example, toxin genes, and assess their role, if any, in the pathogenicity of the organism.

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