Isolation and Characterization of Multiply Antibiotic-Resistant *Clostridium perfringens* Strains from Porcine Feces

JULIAN I. ROOD,[†]* EILEEN A. MAHER,^{††} EILEEN B. SOMERS, ELENA CAMPOS,^{††} AND CHARLES L. DUNCAN^{†††}

Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 7 November 1977

Multiply antibiotic-resistant strains of *Clostridium perfringens* were isolated from porcine feces. Strains that were resistant to tetracycline, erythromycin, clindamycin, and lincomycin were isolated, but no penicillin- or chloramphenicol-resistant strains were obtained. Typical minimal inhibitory concentrations for resistant strains were 16 to 64 μ g of tetracycline per ml, 64 to >128 μ g of erythromycin per ml, $\geq$ 128 μ g of lincomycin per ml, and 16 to 128 μ g of clindamycin per ml. Resistance to erythromycin was always associated with resistance to lincomycin and clindamycin. Minimal inhibitory concentrations were determined for 258 strains from six farms that used antibiotics in their feeds and 240 strains from five farms that did not use antibiotics. The results show that 77.9 and 22.7% of the strains from the former farms were resistant to tetracycline and erythromycin-clindamycin-lincomycin, respectively. The comparable data from the latter farms were 25.0 and 0.8%, respectively. Agarose gel electrophoresis failed to reveal a plasmid band that was common to the resistant strains but absent in the susceptible strains. Attempts to transfer tetracycline, erythromycin, and clindamycin resistance from one strain, CW459, were not successful. Antibiotic-susceptible mutants were not isolated from this strain, despite the use of a variety of curing agents.

Plasmids have been shown to code for resistance to a variety of antibiotics (1, 9). In many cases this resistance is transferable either to different strains of the same species or to different bacterial species (9, 36). Such R-plasmids may carry the genes for resistance to a number of chemically unrelated antibiotics. As a result, they constitute a problem with pathogenic bacteria, since treatment with a single antibiotic may result in the development of resistance to a number of different antibiotics (5, 6, 23).

A number of studies have implicated the use of antibiotics in animal feeds in the development of antibiotic resistance by the intestinal microorganisms of animals consuming such feeds (10, 16, 18, 30). Concern was raised that the use of antibiotics in feeds would lead to the selection of antibiotic-resistant pathogenic bacteria that may be involved in human and animal infections (9, 34). Anderson and Lewis (2) documented

outbreaks of infection in humans due to a *Salmonella typhimurium* strain whose resistance was selected for by the use of antibiotics in animals, and Levy et al. (19) recently showed the transfer of an R-plasmid from chickens to humans. In another report, it was shown that selective pressure for resistant bacteria in chickens consuming a tetracycline (Tc) feed extended to human beings in contact with the chickens and the feed (18). All of the reports mentioned above were studies of antibiotic resistance in gram-negative bacteria such as *Escherichia coli* and *Salmonella* sp. Very little work has been done on gram-positive sporulating anaerobes such as *Clostridium perfringens*.

C. perfringens is a common inhabitant of the gastrointestinal tract in both humans and animals. It has been shown to cause gas gangrene and food poisoning in humans (8, 31) and a variety of enterotoxemic diseases in animals (4, 13, 14, 20). Smith (29) studied the effect of Tc- and penicillin (Pc)-containing feeds on the development of resistant strains of *C. perfringens* in pigs and chickens. He was able to isolate a number of Tc-resistant *C. perfringens* strains, but no Pc- or chloramphenicol (Cm)-resistant strains were isolated. The percentage of the *C.*

[†] Present address: Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra 2601, Australia.

^{††} Present address: Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706.

^{†††} Present address: Campbell Institute for Food Research, Campbell Place, Camden, NJ 08101.

perfringens population that was resistant to Tc was higher in Tc-fed animals than in animals not fed Tc.

In 1975, Sebald et al. (27, 28) reported the isolation of a Tc-, Cm-, erythromycin (Em)-, and clindamycin (Cc)-resistant *C. perfringens* strain from a human clinical source. Resistance appeared to be coded for by two plasmids, pIP401 and pIP402, that carried the Tc-Cm determinants and the Em-Cc determinants, respectively. The R-plasmid pIP401 was transferable to other *C. perfringens* strains, but pIP402 was not transferable. Studies in our laboratory (8a) revealed that many strains of *C. perfringens*, of all toxigenic types, carry plasmid deoxyribonucleic acid. All of these strains were susceptible to Pc, Tc, Em, Cc, and Cm.

The study reported here was commenced to see if multiply antibiotic-resistant strains of *C. perfringens* could be isolated and to see if resistance in such strains was plasmid determined. This paper reports the isolation of Tc- and Em-, Cc- and lincomycin (Ln)-resistant *C. perfringens* strains from porcine feces. It also details the plasmid profiles of several of these resistant strains. The results presented in this paper were previously reported, in part, in abstract form (J. I. Rood, E. A. Maher, E. B. Somers, E. Campos, and C. L. Duncan, *Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother.* 17th, New York, Abstr. no. 20, 1977).

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. Strain NCTC 8798 was from the National Culture Type Collection, England. All freshly isolated strains were given *Clostridium* Wisconsin numbers and were permanently stored in the lyophilized state at room temperature. Working stock cultures were stored either in glycerol storage medium or cooked meat medium (CMM, Difco Laboratories, Detroit, Mich.) at -20°C . Glycerol storage medium contained 60 ml of glycerol, 3.7 g of brain heart infusion (BHI) broth (Difco), 0.1 g of sodium thioglycolate, 0.55 ml of a 0.1% solution of rezasurin, and demineralized water to a volume of 100 ml. BHI agar (BHI broth with 1.5% agar), Trypticase peptone glucose broth (5.0% Trypticase, 0.5% proteose peptone, 0.4% glucose, and 0.1% sodium thioglycolate), and egg yolk-free tryptose sulfite cycloserine agar (SC agar) (11, 12) were made up as previously described. CMM broth and fluid thioglycolate (FTG) broth (Baltimore Biological Laboratories, Cockeysville, Md.) were both made up as directed by the manufacturer. All media were sterilized by autoclaving for 15 min at 121°C . Solid media were incubated at 37°C under an atmosphere of 80% (vol/vol) N_2 , 10% (vol/vol) H_2 , and 10% (vol/vol) CO_2 . Liquid media were also incubated at 37°C ; when growth was followed using a Klett-Summerson Photoelectric Colorimeter (Klett Manufacturing Co., N.Y.). Chloramphenicol (lot 300902) was from Calbiochem, San Diego, Calif.; D-cycloserine,

nalidixic acid, and penicillin G were from Sigma Chemical Co., St. Louis, Mo.; L-hydrochloride (lot 211BE), Cc-hydrochloride (lot 275BK), and Tc-hydrochloride (lot 4423C) were generous gifts of the Upjohn Co., Kalamazoo, Mich.; and Em base (lots 50-505-CD and 260-2759) was a generous gift of Abbott Laboratories, North Chicago, Ill.

Isolation of strains from porcine feces. Fresh porcine fecal specimens were collected at various intervals from eleven farms in southern Wisconsin. After transportation to the laboratory, each specimen was examined as follows. A 10-g fecal sample was added to 100 ml of sterile demineralized distilled water and homogenized for 30 s at low speed using a blender. A loopful of homogenate was then streaked onto SC agar and SC agar containing either Tc (10 $\mu\text{g}/\text{ml}$), Pc (10 U/ml), Cm (5 $\mu\text{g}/\text{ml}$), Ln (10 $\mu\text{g}/\text{ml}$), or Em (25 $\mu\text{g}/\text{ml}$). An enrichment procedure was carried out by subculturing 1 ml of homogenate into 10 ml of CMM broth, heat shocking it for 20 min at 75°C , and cooling and incubating it at 37°C . After 24 h, this enriched culture was plated onto SC agar and SC agar containing the various antibiotics. Following incubation for 24 to 48 h, plates derived from both the enriched and non-enriched cultures were examined for the presence of black colonies. Where possible, 10 black colonies were selected from SC agar, and 3 black colonies were selected from each of the SC agars with antibiotics. All colonies were purified by replating onto medium of the same composition as that from which they were isolated. After incubation at 37°C for 24 h, the purified strains were subcultured into CMM broth, incubated for 24 to 48 h, and then stored at -20°C .

Identification and antibiotic susceptibility testing of isolates. *C. perfringens* strains were identified on the basis of their nonmotility, gelatin liquefaction, nitrate reduction, and lactose fermentation, using lactose-gelatin broth and nitrate-motility medium (3, 11). Several non-*C. perfringens* strains were identified by L. V. Holdeman, Virginia Polytechnic Institute, Blacksburg, Va. Simultaneous with the identification of *C. perfringens* strains, the susceptibility of each strain to eleven antibiotics was determined by disk susceptibility tests as follows. An overnight FTG broth culture of each strain was diluted with 0.1% (wt/vol) peptone to the turbidity of a 0.5 McFarland no. 1 standard using a Bausch and Lomb Spectronic 20 Colorimeter (turbidity of 0.08 to 0.10 at 620 nm). The diluted culture was swabbed onto a BHI agar plate (15 by 150 mm) and the sterile antibiotic disks were dispensed. The diameter of each zone of inhibition was recorded after incubation of the plates for 24 h at 37°C . All disks were from Baltimore Biological Laboratories and were stored at 4°C prior to use. The antibiotics tested and the amount on each disk were as follows: Tc (30 $\mu\text{g}/\text{ml}$), Em (15 $\mu\text{g}/\text{ml}$), kanamycin (30 $\mu\text{g}/\text{ml}$), rifampin (5 $\mu\text{g}/\text{ml}$), aureomycin (30 $\mu\text{g}/\text{ml}$), Cm (30 $\mu\text{g}/\text{ml}$), Ln (2 $\mu\text{g}/\text{ml}$), Cc (2 $\mu\text{g}/\text{ml}$), doxycycline (30 $\mu\text{g}/\text{ml}$), Pc (10 U/ml), and streptomycin (10 $\mu\text{g}/\text{ml}$).

Determination of MICs. Minimal inhibitory concentrations (MICs) were determined by the agar dilution method (35) using BHI agar. Working stock cultures of each strain were subcultured into FTG broth and incubated overnight at 37°C . The resultant cultures were plated onto BHI agar and reincubated.

For each strain, after 48 h of incubation at 37°C, four colonies were subcultured into 6 ml of FTG broth, incubated for 5 to 6 h at 37°C, diluted to a 0.5 McFarland no. 1 standard as before, and further diluted 1:4 with FTG broth. Approximately 1- to 2- μ l samples were applied, using a Steer's Replicator (Melrose Machine Shop, Woodlyn, Pa.) to duplicate BHI agar plates containing various concentrations of antibiotic. The antibiotics tested were Tc, Em, Cc, Ln, Cm, and Pc. Each plate was prepared on the day of use and contained from 0.25 to 128 μ g of one of these antibiotics per ml. MICs were read, after 24 h at 37°C, as the lowest concentration of antibiotic to inhibit growth of the organism. A faint haze of growth, or just a few colonies, were regarded as negative. All MICs reported are the average of at least two separate duplicate determinations. Strains NCTC 8798 and CW459 were used each day as susceptible or resistant controls, respectively.

Growth of cells and preparation of lysates. Strains were grown at 37°C to the end of log phase in 100 ml of Trypticase peptone glucose broth containing 5.0% Trypticase (Baltimore Biological Laboratories), 0.5% proteose peptone (Difco), 0.4% glucose, and 0.1% sodium thioglycolate. Cells were harvested by centrifugation and washed once with tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.5 M, pH 8.0), containing 0.005 M Na₂-ethylenediaminetetraacetic acid and 0.05 M NaCl (TES buffer). Cells were either lysed immediately or frozen at -20°C until required. For lysis, cells were suspended in a mixture of 2.0 ml of 25% sucrose in TES and 0.4 ml of lysozyme (10 mg/ml in TES) and incubated at 37°C for 15 to 30 min. After addition of 0.8 ml of Na₂-ethylenediaminetetraacetic acid (0.25 M, pH 8.0), mixing, and incubation for a further 20 to 30 min at 37°C, 3.6 ml of 2.0% sodium lauryl sarcosinate-97 (Chemical Additives Co., Farmingville, N.Y.) in TES was added. Following incubation at 37°C for 10 min, the deoxyribonucleic acid in the lysate was sheared by either aspiration from a 5.0-ml pipette 10 to 15 times or by 10 passages through an 18-gauge needle. Cellular debris was removed by centrifugation at 31,300 $\times$ g for 20 min.

Isolation of plasmid DNA. The lysates prepared as above were adjusted to a volume of 8 ml by the addition of TES. Ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide), 0.4 ml of a 10-mg/ml solution in TES, was added with subsequent protection of the lysate from light. Cesium chloride was added to the lysate to achieve a final refractive index of about 1.3960 to 1.3970. Dye-buoyant density gradient centrifugation was carried out by centrifugation for 40 to 48 h at 40,000 rpm using a Beckman (Beckman Instruments, Palo Alto, Calif.) type 50 fixed angle rotor in a Beckman L5-65 or L5-50 preparative ultracentrifuge. Plasmid bands were observed under long-wave ultraviolet illumination and were extracted from the gradient with an 18-gauge needle. Ethidium bromide was removed by extraction (four times) with CsCl-saturated isopropanol. CsCl was removed by dialysis against TES at either a 1 $\times$ or 0.25 $\times$ concentration. The preparations were either used as is or concentrated by pervaporation to approximately 0.5 ml and stored at 4°C until required.

Agarose gel electrophoresis. Tris(hydroxy-

methyl)aminomethane-borate buffer [1.08% tris- (hydroxymethyl)aminomethane base, 0.093% Na₂-ethylenediaminetetraacetic acid, 0.55% boric acid, pH 8.0] containing 0.5 μ g of ethidium bromide per ml was used as the electrophoresis buffer. Cylindrical glass tubes (0.6 by 15 cm) were filled to a height of 13.5 cm with 0.6% agarose (Seakem ME, Marine Colloids Inc., Rockland, Me.) in electrode buffer. Sucrose-dye solution (30% sucrose, 0.005% bromophenol blue in TES), 25 μ l, was added to 50 μ l of plasmid DNA (diluted if necessary). The sample was applied to the top of the gel, overlaid with electrode buffer, and electrophoresed at a constant current of 1 mA/gel until the dye band was approximately 1 cm from the end of the gel (4 to 5 h). Gels were removed from the tubes, placed onto a shortwave ultraviolet lightbox (Ultraviolet Products Inc., San Gabriel, Calif.), and photographed with a Polaroid MP-3 camera with both red and ultraviolet filters and using Polaroid type 57 film.

Attempted curing of antibiotic resistance. A single colony of strain CW480 was subcultured into 6 ml of FTG broth and incubated for 8 h at 37°C. Portions (0.1 ml) of a 1:1,000 dilution of the resultant culture were subcultured into 10 ml of FTG broth containing the required curing agent. After incubation for 24 h at 37°C (in the dark if acridines were used), the cultures were diluted to give approximately 50 to 200 colonies per plate and were spread onto BHI agar. These plates were incubated overnight at 37°C and replicated (17), first onto BHI agar containing Tc (10 μ g/ml), Em (15 μ g/ml), and Cc (15 μ g/ml), and then onto BHI agar without antibiotics. The plates were examined after 24 h at 37°C for colonies that grew on BHI agar but did not grow on BHI agar with antibiotics.

RESULTS

Isolation of multiply resistant *C. perfringens* strains. Initial studies were centered on six farms in southern Wisconsin. All of these farms were currently using swine feed that contained various combinations of antibiotics. The antibiotics in use were Pc (0.11 to 4 lb/ton [ca. 0.05 to 1.8 kg/tonne] of feed), sulfamethazine (0.22 lb/ton [ca. 0.1 kg/tonne] of feed), aureomycin (0.04 to 7 lb/ton [ca. 0.02 to 3.2 kg/tonne] of feed), Ln (0.09 to 4.4 lb/ton [ca. 0.04 to 2 kg/tonne] of feed), streptomycin (0.05 to 4 lb/ton [ca. 0.02 to 1.8 kg/tonne] of feed), oxytetracycline (0.04 lb/ton [ca. 0.02 kg/tonne] of feed), and tylosin (0.09 to 0.18 lb/ton [ca. 0.04 to 0.08 kg/tonne] of feed). Due to the diversity of the antibiotics and the wide range of concentrations used, it was not possible to group the specimens according to the antibiotic or antibiotics present in the feeds.

A total of 100 specimens were collected from these initial six farms. *C. perfringens* strains were isolated from 35 specimens by either direct or enriched plating on SC agar. A further 20 specimens yielded *C. perfringens* isolates when

plated onto SC agar containing antibiotics. *C. perfringens* could not be detected in the remaining 45 specimens even after enrichment. Disk susceptibility testing revealed that most *C. perfringens* isolates were resistant to kanamycin and streptomycin and susceptible to rifampin. These antibiotics were not studied further. Many strains that were resistant to Tc or Tc, Em, Cc, and Ln were isolated. MICs of a typical selection of these strains are shown in Table 1. Strain NCTC 8798 is included as a susceptible control, as is strain CW473. For the purposes of discussion, a resistant strain is arbitrarily defined as one for which the MIC of a particular antibiotic is ≥ 16 $\mu\text{g/ml}$. The isolation procedure also yielded antibiotic-resistant non-*C. perfringens* strains. The MICs for three of these strains are also shown in Table 1. No other studies were carried out on the non-*C. perfringens* isolates.

In *C. perfringens* Tc resistance was often, but not always, associated with Em, Cc, and Ln resistance. No Pc- or Cm-resistant *C. perfringens* strains were isolated. Resistance to Em, Cc, and Ln was always associated, although, as seen in Table 1, the MICs were not in any fixed ratio. This correlation was not the case for non-*C. perfringens* strains, for example, CW486. All further discussion in this paper refers only to *C. perfringens* isolates.

Comparison of MICs of strains from different groups of farms. Following the ready isolation of these resistant *C. perfringens* strains from farms using antibiotics in their feeds (AB farms), it was decided to examine specimens from farms that did not use antibiotics (NAB farms). The five farms that were selected for study had not used antibiotics in their swine feeds or therapeutically for at least two years prior to this trial. A total of 29 specimens were obtained from these farms. All specimens yielded *C. perfringens* isolates from SC agar. To compare the relative percentage of resistant isolates in strains from the two groups of farms,

MICs were determined against all six antibiotics studied (Tc, Em, Cc, Ln, Cm, and Pc). Every *C. perfringens* isolate from non-antibiotic-containing SC agar was examined in this manner. In all, MICs were determined for 258 strains from AB farms and 240 strains from NAB farms. The results show that for Tc, Em, Cc, and Ln, a higher percentage of resistant *C. perfringens* strains was found from the AB farms (Fig. 1). For Tc, 201 strains (77.9%) of the *C. perfringens* isolates from AB farms were resistant. These strains came from 31 different specimens from all 6 farms. In comparison, only 60 (25.0%) of the isolates from NAB farms were resistant. These 60 strains came from 12 different specimens from the 5 farms. Six of the latter specimens were from farm J. For Em, Cc, and Ln resistance, 58 (22.7%) of the *C. perfringens* strains from AB farms were resistant. These strains came from 12 different specimens on 4 farms. In comparison, only 2 strains (0.8%) from NAB farms were resistant. Both of these isolates came from the same specimen on farm J. Of the 7 farms from which specimens were obtained that did not yield Em-, Cc-, and Ln-resistant *C. perfringens* strains on SC agar, only specimens from 3 farms still failed to have these resistant strains after selection on SC agar with Em, Ln, or Tc.

As shown in Fig. 1E, no Cm-resistant strains were isolated, and the cumulative inhibition curves for this antibiotic were almost identical. Although all the isolates were regarded as Pc susceptible (Fig. 1F), the strains from AB farms were less susceptible than those from NAB farms. Direct selection on SC agar with Pc or Cm failed to isolate any strains resistant to Pc or Cm.

Examination of plasmid profiles of resistant strains. We decided to examine the plasmid profiles of a number of these strains, first, to see if there was a plasmid band or bands common to the resistant strains, and second, to facilitate the choice of a resistant strain for use

TABLE 1. Typical MICs for strains from porcine feces

Species	Strain	MIC ($\mu\text{g/ml}$)					
		Tc	Em	Cc	Ln	Cm	Pc
<i>C. perfringens</i>	CW459	32	64	64	128	4	1
	CW471	32	>128	16	128	2	≤ 0.25
	CW502	32	128	128	>128	2	1
	CW500	32	1	2	4	2	≤ 0.25
	CW473	4	0.5	≤ 0.25	≤ 0.25	2	≤ 0.25
	NCTC 8798	≤ 0.25	0.5	≤ 0.25	≤ 0.25	1	≤ 0.25
<i>C. butyricum</i>	CW505	16	2	≤ 0.25	4	≤ 1	≤ 0.25
<i>C. sporogenes</i>	CW486	8	64	2	32	0.5	≤ 0.25
<i>C. paraputrificum</i>	CW498	16	128	16	64	1	0.5

FIG. 1. Cumulative inhibition curves of *C. perfringens* strains from AB farms (—) and NAB farms (---). The total number of strains examined was 258 and 240, respectively. All strains were isolated on SC agar without antibiotics.

in curing experiments. Agarose gel electrophoresis was carried out on partially purified plasmid preparations as described in the text. Initially seven strains, all from farm A, were examined. Five of these strains (CW459, CW468, CW476, CW477, and CW470) were Tc, Em, Cc, and Ln resistant, one strain (CW461) was resistant to Tc only, and the remaining strain CW473 was susceptible to all four antibiotics. The results (Fig. 2) show that all of the resistant strains had one or two high-molecular-weight bands that were not present in strain CW473. The number of bands varied from strain to strain, as expected. It was not possible to correlate any one plasmid band with any particular resistance phenotype. These observations were followed by examining the agarose gel profiles of matched pairs of strains from each of farms B to F. Each

pair of strains was isolated from the same specimen on the farm indicated (Fig. 3). Either susceptible or resistant or, in one case (B), Tc-resistant and Tc-, Em-, and Cc-resistant strains were paired. The results show that the susceptible strains in general had more plasmid bands than the resistant strain of the pair. Strains CW500 and CW502 had the same apparent plasmid profile, as shown by co-electrophoresis, despite the fact that they differ in their resistance patterns. Again it was not possible to correlate any one plasmid band with a particular resistance phenotype.

Due to the relatively simple nature of its plasmid profile (only one band observed on agarose gels [Fig. 2]), strain CW459 was chosen for further study. Attempts were made to cure strain CW480 (a spontaneous mutant of strain

CW459 that was resistant to nalidixic acid [50 $\mu\text{g/ml}$] and spectinomycin [500 $\mu\text{g/ml}$] for its resistance to Tc, Em, or Cc. The curing agents used and the number of colonies screened are shown in Table 2. Although a total of 27,253 colonies were screened, no mutants that were sensitive to any of these three antibiotics were obtained. Attempts by mixed-plate mating (28) to transfer Tc, Em, or Cc resistance from strain CW459 to a susceptible recipient strain were equally unsuccessful. The efficiency of transfer was less than 5×10^{-8} transipients per donor cell after 20 h of incubation on BHI agar at 37°C. The recipient strain used here had previously been shown to act as a recipient under these conditions (J. I. Rood, V. N. Scott, and C. L. Duncan, manuscript in preparation).

DISCUSSION

A number of workers from various laboratories have studied the antibiotic susceptibility patterns of *C. perfringens* isolates from clinical specimens or human feces. In general, the strains examined were all susceptible to Pc and Cm (15, 21, 22, 25, 26, 33). Staneck and Washington (32) reported that of 24 *C. perfringens* strains examined 96% were susceptible to Pc at 0.4 $\mu\text{g/ml}$, but that one strain had an MIC of 12.5 $\mu\text{g/ml}$. Strains resistant to Cc and Ln have been observed, but not in great numbers. Wilkins and Thiel (37) looked at ten isolates and found one strain with a Cc MIC of $>12.5 \mu\text{g/ml}$. Sapico et al. (25) reported one strain with an Ln MIC of 25 $\mu\text{g per ml}$, the remaining 42 strains examined

FIG. 2. Agarose gel electrophoresis profiles of plasmids from strains isolated from farm A. Electrophoresis was at 1 mA/gel in 0.6% (wt/vol) agarose gels. The anode is at the bottom. The C. number of each strain is indicated beneath each gel. C indicates the chromosome band.

being susceptible to 12.5 μ g of Ln per ml. Other studies on Cc (21, 22, 26, 32, 33, 38) or Ln (21, 22, 38) as well as Em (15, 21, 22, 25, 33) failed to detect strains with MICs of 16 μ g/ml or higher. However, the strain studied by Sebald et al. (27) had MICs of >1,000, 90, 40, and 50 μ g of Em, Cc, Tc, and Cm per ml, respectively.

Several workers have reported Tc-resistant *C. perfringens* isolates. Schwartzman et al. (26) examined 57 strains and found that five strains were resistant to 8 μ g of Tc per ml. Mathias et

al. (22) found that 8 (of 30) strains examined had MICs of 12.5 μ g of Tc per ml or higher, and Sutter and Finegold (33) found that 3 (of 9) strains had MICs of $\geq$ 16 μ g of Tc per ml. Six percent of the 34 strains examined by Martin et al. (21) were resistant to more than 25 μ g of Tc per ml and three of the strains reported by Johnstone and Cockcroft (15) had MICs of $\geq$ 30 μ g/ml.

Dornbusch et al. (7) examined 252 *C. perfringens* isolates from clinical sources in Sweden.

FIG. 3. Agarose gel electrophoresis profiles of plasmids from strains isolated from farms B to F. Electrophoresis was at 1 mA/gel in 0.6% (wt/vol) agarose gels. The anode is at the bottom. The letters at the top of the gels refer to the farm from which the strain was isolated. The C. number of each strain is indicated at the top of each gel, and the resistance phenotype is at the bottom of the gel. All Em- and Cc-resistant strains were also resistant to Ln.

TABLE 2. Attempted curing of antibiotic resistance from strain CW480^a

Curing agent	Concn (µg/ml)	No. of colonies screened
Control		5,105
46°C		4,310
Ethidium bromide	2	2,100
Acriflavine	0.5	2,025
Proflavine	5	2,105
Quinacrine	20	4,265
Acridine orange	20	3,960
9-Aminoacridine	5	1,980
Sodium dodecyl sulfate	20	1,403

^a Approximately 10⁴ cells were subcultured into FTG broth containing the indicated concentration of curing agent. After incubation for 24 h at 37°C, the cultures were diluted, plated onto BHI agar, incubated, and then replicated onto BHI agar containing antibiotics. No susceptible mutants were obtained.

Their results appear to be somewhat different from those of the above-mentioned workers, as the incidence of resistant strains was higher. Eight strains had MICs of ≥ 16 µg of Pc per ml, 3 strains had MICs of ≥ 16 µg of Em per ml, 6 strains had MICs of ≥ 16 µg of Cc per ml, 40 strains had MICs of ≥ 16 µg of Ln per ml, and 78 strains had MICs of ≥ 20 µg of Tc per ml. All 252 strains were susceptible to 4 µg of Cm per ml. The reason for this higher level of resistance is not immediately apparent. It may reflect differences in the media or assay procedures or differences in the type and source of the isolates obtained.

Among the porcine strains examined in this paper, especially those from AB farms, there was a higher incidence of resistance to Em, Cc, Ln, and Tc than reported from the human isolates previously discussed. This was not so with Pc and Cm. Resistance to Em, Cc, and Ln was generally of a higher level; in many cases MICs were ≥ 64 µg/ml and were always associated. Dornbusch et al. (7) did not find any such correlation, but other workers (27) were able to associate Em and Cc resistance with a single R-plasmid in one strain of *C. perfringens*.

In our study, the percentage of *C. perfringens* isolates that were resistant to either Tc or Em, Cc, and Ln was significantly higher from AB farms than from NAB farms. One possible explanation for these results is that the use of antibiotics in the feed results in a direct selection of multiply antibiotic-resistant *C. perfringens* strains. Double-blind experiments where different groups of pigs were given identical feeds (except for the antibiotics studied) and housed under identical conditions would be required to verify this hypothesis. Smith (29) studied the

effects of Pc and Tc on the isolation of *C. perfringens* strains from pigs and chickens. He observed that Tc-resistant strains formed a higher proportion of the *C. perfringens* population of the feces of Tc-fed pigs than they did from non-Tc-fed pigs. No Pc- or Cm-resistant isolates were obtained. Resistance to the macrolide antibiotics was not studied, and MICs were only determined for Tc-resistant strains, not for the whole *C. perfringens* population. Our observations suggest that the recovery of *C. perfringens* isolates from antibiotic-fed pigs was much poorer than from non-antibiotic-fed animals (35 of 100 specimens from AB farms yielded *C. perfringens* isolates from SC agar, compared with 29 of 29 specimens from NAB farms), and that resistant strains could, in some cases, be isolated from NAB specimens by direct plating on SC agar with antibiotics; this agrees with the results of Smith (29). He noted that Tc-resistant strains were present in similar proportions in specimens from both sources, the important difference being that in the feces of Tc-fed animals, they formed a much greater proportion of the total *C. perfringens* population. It was postulated that the constant presence of Tc in the intestine of Tc-fed animals suppressed the levels of susceptible *C. perfringens* strains, leaving the resistant strains, if present, as the main component of the *C. perfringens* population.

Strain CW459 (Tc, Em, Cc and Ln resistant) could not be cured of its resistance by any of the commonly used curing agents, nor did it transfer its resistance in mixed-plate mating experiments. This does not necessarily rule out the possibility that resistance in this strain is plasmid determined. It is possible that an R-plasmid present in strain CW459 was of a nontransferable type and that conditions other than those used in these experiments could result in the elimination of the plasmid, with concomitant loss of one or all of the resistance determinants.

Previous workers (28) demonstrated that Tc resistance in *C. perfringens* may be determined by a transferable R-plasmid. Experiments in this laboratory (J. I. Rood, V. N. Scott, and C. L. Duncan, manuscript in preparation) have shown that *C. perfringens* strain CW92, isolated from a clinical source, also carries a transferable Tc resistance plasmid. Although attempts to transfer an R-plasmid from strain CW459 were unsuccessful, preliminary experiments (J. I. Rood, and E. A. Maher, unpublished results) indicate that some of the Tc-resistant porcine strains isolated in the present study may have the ability to transfer their resistance in a similar manner. Further studies on these strains are currently in progress.

Pc is generally accepted as the antibiotic of

choice in the treatment of *C. perfringens* infections (15, 24, 39). Em (15, 22, 24) and Cc (22, 26) have both been suggested as alternatives in cases where Pc is contraindicated. The discovery in our experiments of an apparent animal reservoir of Em-, Cc-, and Ln-resistant strains serves as a warning that use of these antibiotics in the treatment of *C. perfringens* infections may not be effective if the causative strain has similar resistance patterns to those reported here. As already discussed, there has been one report of a human clinical isolate with similar resistance profiles (27). *C. perfringens* also causes a variety of enterotoxemic diseases in farm animals (4, 13, 14, 20). The effect of multiply antibiotic-resistant strains on the course and severity of these diseases in animals consuming antibiotics in their feed remains to be determined, as does their economic effect, if any, to the meat industry.

ACKNOWLEDGMENTS

We acknowledge the most capable technical assistance of Dave Ripple and Pat Way. The former is especially thanked for his invaluable assistance in procuring specimens from farms that were not utilizing antibiotics in their swine feeds. We would also like to thank L. V. Holdeman for the identification of several of the non-*C. perfringens* isolates.

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by contributions to the Food Research Institute by member industries. C. L. Duncan was the recipient of Public Health Service Research Career Development Award AI-70721-03 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

- Anderson, E. S. 1968. The ecology of transferable drug resistance in the enterobacteria. *Ann. Rev. Microbiol.* **22**:131-180.
- Anderson, E. S., and M.-J. Lewis. 1965. Drug resistance and its transfer in *Salmonella typhimurium*. *Nature (London)* **206**:579-583.
- Anonymous. 1976. *Clostridium perfringens* Official First Action. *J. Assoc. Off. Anal. Chem.* **59**:490-492.
- Bergeland, M. E. 1975. Clostridial infections, p. 525-540. In H. W. Dunne and A. D. Leman (ed.), *Diseases of swine*, 4th ed. Iowa State University Press, Ames, Iowa.
- Datta, N. 1971. R factors in *Escherichia coli*. *Ann. N. Y. Acad. Sci.* **182**:59-64.
- Datta, N., M. C. Faiers, D. S. Reeves, W. Brumfitt, F. Ørskov, and I. Ørskov. 1971. R factors in *Escherichia coli* in faeces after oral chemotherapy in general practice. *Lancet* **i**:312-315.
- Dornbusch, K., C. E. Nord, and A. Dahlbäck. 1975. Antibiotic susceptibility of *Clostridium* species isolated from human infections. *Scand. J. Infect. Dis.* **7**:127-134.
- Duncan, C. L. 1970. *Clostridium perfringens* food poisoning. *J. Milk Food Technol.* **33**:35-41.
- Duncan, C. L., C. M. Christenson, and J. I. Rood. 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.
- Falkow, S. 1975. Infectious multiple drug resistance. Pion Ltd., London.
- Guinée, P. A. M. 1971. Bacterial drug resistance in animals. *Ann. N. Y. Acad. Sci.* **182**:40-51.
- Hauschild, A. H. W., R. J. Gilbert, S. M. Harmon, M. F. O'Keefe, and R. Vahlefeld. 1977. ICMSD methods studies. VIII. Comparative study for the enumeration of *Clostridium perfringens* in foods. *Can. J. Microbiol.* **23**:884-892.
- Hauschild, A. H. W., and R. Hilsheimer. 1974. Evaluation and modifications of media for enumeration of *Clostridium perfringens*. *Appl. Microbiol.* **27**:78-82.
- Hogle, R. M. 1975. Clinical and laboratory diagnosis of enterotoxaemia of cattle. *Vet. Med. Small Anim. Clin.* **70**:983-984.
- Jensen, R. 1974. *Diseases of sheep*. Lea and Febiger, Philadelphia.
- Johnstone, F. R. C., and W. H. Cockcroft. 1968. *Clostridium welchii* resistance to tetracycline. *Lancet* **i**:660-661.
- Katz, S. E., C. A. Fassbender, P. S. Dinnerstein, and J. L. Dowling, Jr. 1974. Effects of feeding penicillin to chickens. *J. Assoc. Off. Anal. Chem.* **57**:522-526.
- Lederberg, J., and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. *J. Bacteriol.* **63**:399-406.
- Levy, S. B., G. B. Fitzgerald, and A. B. Maccone. 1976. Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. *N. Engl. J. Med.* **295**:583-588.
- Levy, S. B., G. B. Fitzgerald, and A. B. Maccone. 1976. Spread of antibiotic-resistance plasmids from chicken to chicken and chicken to man. *Nature (London)* **240**:40-42.
- Long, J. R. 1973. Necrotic enteritis in broiler chickens. I. A review of the literature and the prevalence of the disease in Ontario. *Can. J. Comp. Med.* **37**:302-308.
- Martin, W. J., M. Gardner, and J. A. Washington II. 1972. In vitro antimicrobial susceptibility of anaerobic bacteria isolated from clinical specimens. *Antimicrob. Agents Chemother.* **1**:148-158.
- Mathias, R., M. Gurwith, A. Ronald, and S. Hoban. 1974. Susceptibility of clinical isolates of *Clostridia perfringens* to 13 antibiotics. *Can. J. Public Health* **65**:64.
- Møller, J. K., A. L. Bak, A. Stenderup, H. Zachariae, and H. Afzelius. 1977. Changing patterns of plasmid-mediated drug resistance during tetracycline therapy. *Antimicrob. Agents Chemother.* **11**:388-391.
- Parker, M. T. 1969. Postoperative clostridial infections in Britain. *Br. Med. J.* **3**:671-676.
- Sapico, F. L., Y. Y. Kwok, V. L. Sutter, and S. M. Finegold. 1972. Standardized antimicrobial disc susceptibility testing of anaerobic bacteria: in vitro susceptibility of *Clostridium perfringens* to nine antibiotics. *Antimicrob. Agents Chemother.* **2**:320-325.
- Schwartzman, J. D., L. B. Reller, and W. L. Wang. 1977. Susceptibility of *Clostridium perfringens* isolated from human infections to twenty antibiotics. *Antimicrob. Agents Chemother.* **11**:695-697.
- Sebald, M., D. Bouanchaud, and G. Bieth. 1975. Nature plasmidique de la résistance à plusieurs antibiotiques chez *C. perfringens* type A souche 659. *C. R. Acad. Sci. Paris Ser. D* **280**:2401-2404.
- Sebald, M., and G. Bréfort. 1975. Transfert du plasmide tétracycline-chloramphenicol chez *Clostridium perfringens*. *C. R. Acad. Sci. Paris Ser. D* **281**:317-319.
- Smith, H. W. 1959. The effect of the continuous administration of diets containing tetracyclines and penicillin on the number of drug-resistant and drug-sensitive *Clostridium welchii* in the faeces of pigs and chickens. *J. Pathol. Bacteriol.* **77**:79-92.
- Smith, H. W., and W. E. Crabb. 1957. The effect of the continuous administration of diets containing low levels of tetracyclines on the incidence of drug resistant *Bac-*

- terium coli* in the faeces of pigs and chickens: the sensitivity of the *Bacterium coli* to other chemotherapeutic agents. *Vet. Rec.* **69**:24-30.
31. **Smith, L. D. S., and L. V. Holdeman.** 1968. The pathogenic anaerobic bacteria, p. 201-255. Charles C Thomas, Publisher, Springfield, Ill.
 32. **Staneck, J. L., and J. A. Washington II.** 1974. Antimicrobial susceptibilities of anaerobic bacteria: recent clinical isolates. *Antimicrob. Agents Chemother.* **6**:311-315.
 33. **Sutter, V. L., and S. M. Finegold.** 1976. Susceptibility of anaerobic bacteria to 23 antimicrobial agents. *Antimicrob. Agents Chemother.* **10**:736-752.
 34. **Swann, M., K. L. Blaxter, H. I. Field, J. W. Howie, I. A. M. Lucas, E. L. M. Millar, J. C. Murdoch, J. H. Parsons, and E. A. White.** 1969. Report of the joint committee on the use of antibiotics in animal husbandry and veterinary medicine. Her Majesty's Stationary Office, London.
 35. **Washington, J. A., II, and A. L. Barry.** 1974. Dilution test procedures, p. 410-417. In E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), *Manual of clinical microbiology*, 2nd ed. American Society for Microbiology, Washington, D.C.
 36. **Watanabe, T.** 1963. Infective heredity of multiple drug resistance in bacteria. *Bacteriol. Rev.* **27**:87-115.
 37. **Wilkins, T. D., and T. Thiel.** 1973. Resistance of some species of *Clostridium* to clindamycin. *Antimicrob. Agents Chemother.* **3**:136-137.
 38. **Williams, K., J. M. Brandish, and A. T. Willis.** 1972. Some observations on clindamycin. *Med. Lab. Technol.* **29**:233-242.
 39. **Willis, A. T.** 1969. *Clostridia of wound infections*, p. 41-156. Butterworths, London.