

Bacteriocin Susceptibility of *Clostridium perfringens*: a Provisional Typing Schema

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Ninety-four strains of *Clostridium perfringens* were examined for bacteriocin production. Bacteriocins produced by ten of these strains were selected for typing 274 cultures of *C. perfringens*. The bacteriocins were prepared by growing the producer strains in broth and precipitating the active principle from the supernatant fluids of centrifuged cultures with ammonium sulfate. All bacteriocins were titrated against a common indicator strain, adjusted to equivalent titers, and spotted onto blood agar plates seeded with the test organisms. Fifty different bacteriocin sensitivity patterns were observed. These patterns were organized into seven groups bearing some relationship, and the largest number of strains falling into any one pattern did not exceed 16% of the total strains tested. Ninety-nine percent of all isolates were typable. The new method should prove useful in studies where strains must be fingerprinted.

Bacteriocin typing has proved to be a valuable epidemiological tool in recent years. Although the bacteriocins of *Clostridium perfringens* have not been as extensively studied as other bacteriocins, their existence has been documented (6, 7). In 1971 we reported that four of 33 strains of *C. perfringens* produced bacteriocins (3), and more recently our survey has included 94 strains of the species.

Since *C. perfringens* represents not only a member of the normal bowel flora but also a pathogen capable of causing such diseases as gas gangrene, clostridial cellulitis, and food poisoning, an examination of a bacteriocin typing scheme for this species was considered of value. A proposed schema is presented in this paper.

MATERIALS AND METHODS

Bacterial strains and isolates. Many strains of *C. perfringens* were gratefully received from the collections of V. Fredette, Institute of Microbiology and Hygiene, Laval-des-Rapides, Que.; L. S. McClung, Indiana University, Bloomington, Ind.; A. Sasarman, Department of Microbiology and Immunology, University of Montreal, Montreal, Que.; A. Hauschild, Food Research Laboratories, Health Protection Branch, Health and Welfare Canada, Ottawa, Ont.; as well as from the Department of Microbiology and Immunology, McGill University, Montreal, Que.; the Department of Bacteriology, Pathology Institute, Halifax, Nova Scotia; and the American Type Culture Collection, Rockville, Md. An additional 180 isolates were cultured from stool specimens obtained locally. Cultures were either lyophilized or maintained in

cooked meat medium (Difco Laboratories, Detroit, Mich.) at 4 C. All incubations were at 37 C.

Detection of bacteriocin-producing strains. The supernatant fluids of 94 strains of *C. perfringens* were spotted in checkerboard fashion against the bacterial growth of these same strains to determine which strains produced inhibitors of growth. Briefly the methodology was as follows.

Indicator strains were grown for 3 h in brain heart infusion broth (Difco), after which 0.8 ml of each culture was respectively removed and added to 2.5 ml of 0.6% semi-solid agar (Difco) which had been melted and cooled to 45 C. The inoculated agar was poured over the surface of a brain heart infusion agar plate and allowed to solidify. Standard bacteriological loopfuls of the above supernatant fluids were placed on such overlays and allowed to dry before anaerobic incubation for 18 h. The appearance of a marked zone of inhibition in the lawn of bacterial growth at the site of inoculation was evidence of bacteriocin activity.

Bacteriocin production. Ten strains of *C. perfringens* were selected for their bacteriocin production. Overnight cooked meat cultures of each strain were subcultured by adding a 10% inoculum to freshly boiled and cooled brain heart infusion broth. This culture was grown for 2.5 h, after which 30 ml was transferred to 500 ml of broth. After 5 h, the culture was centrifuged at $6,000 \times g$ for 10 min to sediment the bacteria, and the bacteriocin contained in the supernatant fluid was precipitated by adding powdered ammonium sulfate to 40% saturation at 4 C. The precipitate was collected by centrifugation at $6,000 \times g$ for 10 min and resuspended in 5 ml of sterile brain heart infusion broth. The bacteriocin was sterilized by filtration or by the addition of a few drops of chloroform.

Bacteriocin assay and typing. Strain no. 2 (type

A1; see Table 4) was sensitive to all 10 bacteriocins and served as the indicator strain for all titrations. This indicator strain was grown for 18 h in cooked meat medium, and 1 ml was subcultured to 10 ml of broth. After 3 h of incubation, the culture was diluted 1:100 in brain heart infusion broth and swabbed onto the surface of a blood agar plate. Bacteriocins were serially diluted and assayed by placing a loopful of the serial dilutions onto the surface of the swabbed plate. The reciprocal of the highest dilution showing any inhibition of bacterial growth in this assay was defined as the "titer" expressed in arbitrary units of bacteriocin activity.

For bacteriocin typing, the bacteriocins were adjusted to a strength of 320 U when possible. Drops containing 16 μ liters of each bacteriocin were systematically placed on plates which were seeded with test strains of *C. perfringens* (as described for the indicator strain above) with a Pipetman P20 pipette (Mandel Scientific Co., Montreal, Que.). After the inocula had dried, the plates were incubated anaerobically for 18 h under an atmosphere of nitrogen gas. The plates were examined for zones of growth inhibition, and the sensitivity pattern for each strain was recorded. Very weak zones of inhibition with bacterial growth within the zone were defined as negative.

RESULTS

Selection of bacteriocin-producing strains.

The results of the cross-plating experiment indicated that a large percentage of the 94 strains produced an inhibitory factor active against at least one other strain of *C. perfringens*. Many of these inhibitory factors had a wide spectrum of activity, whereas others had a very limited host range. Table 1 shows the number of bacteriocins which inhibited various percentages of the test strains, whereas Table 2 reveals the number of strains which were inhibited by various percentages of the bacteriocins. Those specific bacteriocin-producing strains listed in Table 1 were first examined as potentially useful, since they inhibited a considerable number of the test organisms. Strain no. 96 used in the typing scheme, and not listed here, was a known producer of bacteriocin obtained from A. Sasarman. Thirteen of the strains tested in the screening experiment carried bacteriophages, but no phage lysed more than two strains. Distinct plaques were observable in each of these cases.

The strains which were selected for bacteriocin production and typing and their origins are listed in Table 3. The rationale for utilizing 320 U of bacteriocin as a standard concentration for typing was established by testing a series of dilutions of bacteriocin against several indicator strains. With titers of less than 320 U, the inhibition patterns of the test strains began to vary with negative or weak reactions replacing

TABLE 1. Number of strains producing inhibitors of one or more other strains of *C. perfringens*

Percent of strains inhibited (94 tested)	No. of bacteriocin-producing strains which inhibited that percent (94 tested) ^a
1-10	61
11-20	14
21-30	5 (32, 54, 78, 82, 83)
31-40	5 (43, 48, 51, 73, 75)
42	1 (4)
69	1 (28)
77	1 (55)
84	1 (63)

^a Numbers in parentheses are strain numbers.

TABLE 2. Number of strains inhibited by one or more other strains of *C. perfringens*

Percent of bacteriocin-producing strains which inhibit other strains	No. of strains inhibited by that percent ^a
1-10	49
11-20	25
21-30	8 (3, 17, 31, 38, 42, 65, 49, 75)
33	1 (36)
42	1 (60)

^a Numbers in parentheses are strain numbers.

TABLE 3. Bacteriocin-producing strains and their origin

Our culture no.	Source	Source designation	Toxin type	Strain designation	Origin
4	McClung	694	A	117-A	Chicken broth
28	VGH ^a	35230			Survey
43	HI ^b	05022			Vulva swab
48	HI	03352			Survey
55	Hauschild	PH-2	A		
63	Hauschild	CO-2	A		
73	ATCC ^c	10388			Lamb
75	ATCC	12915	A	Agg. T1 ^d	Steak
78	ATCC	12918	A	Agg. T4	Feces
96	Sasarman	61/65			

^a VGH, Victoria General Hospital, Halifax.

^b HI, Halifax Infirmary, Halifax.

^c ATCC, American Type Culture Collection.

^d Agg. T., Agglutinating type.

previously positive reactions. Although most titers obtainable by ammonium sulfate precipitation well exceeded 320 U, strains no. 73 and 75 consistently produced low titers, and such bacteriocins were used at the highest concentration possible (but not exceeding 320 U).

Bacteriocin titers were stable at 4 C over several weeks when preserved in broth. The ammonium sulfate step also precipitates alpha- and theta-toxins (and probably other biological products), but the presence of these contaminants was judged not to be detrimental to the typing system. The bacteriocin can be purified considerably by column chromatography and other separation techniques, but the end product is very unstable.

Establishment of a typing pattern. Table 4 shows the patterns of susceptibility of 274 strains of *C. perfringens* typed with 10 bacteriocins. Fifty patterns were obtained, and these were placed in seven alphabetical groupings for ease of recognition. The strains in the Group A typing pattern are susceptible to bacteriocins 4, 28, 43, 48, 55, 63, and 73 with variation in response to bacteriocins 75, 78, and 96. Strains in the Group B typing pattern were sensitive to bacteriocins from strains 4, 28, 43, and 48 with variation in response to the remaining bacteriocins. The other groups are largely defined by the sensitivity to bacteriocins 4, 28, and 43. Some bacteriocin types represent only one

strain of *C. perfringens*, whereas the typing pattern with the largest number of strains is C3, which contains 15.7% of the 274 organisms tested (Table 5). Only three strains were untypable.

Some of the isolates of *C. perfringens* came from repeated stool cultures on the same individual, so that the typing patterns could be compared on different occasions. Of ten such examples (Table 6) the typing pattern in three paired cases was identical, whereas it varied in the remaining seven cases. Also, the typing patterns of *C. perfringens* isolated from more than one member of the same family were compared in four instances (Table 7). The patterns were identical within each family pair in two cases but different within the other two.

The reproducibility of bacteriocin typing was examined by repeatedly typing four cultures of *C. perfringens* as follows. Each culture was streaked onto blood agar to obtain isolated colonies, from which four colonies (A, B, C, D) were randomly selected. A portion of each colony was inoculated into cooked meat medium for growth and subsequent bacteriocin

TABLE 4. Provisional bacteriocin typing schema for *C. perfringens*

Type	Sensitivity to bacteriocin from strain: ^a										Type	Sensitivity to bacteriocin from strain: ^a									
	4	28	43	48	55	63	73	75	78	96		4	28	43	48	55	63	73	75	78	96
A	1	+	+	+	+	+	+	+	+	+	4	+	-	-	-	-	-	-	-	-	
	2	+	+	+	+	+	+	+	-	+	5	+	-	-	-	-	-	+	+	-	
	3	+	+	+	+	+	+	-	-	+	6	+	-	-	-	-	+	-	+	-	
B	1	+	+	+	-	+	+	+	+	+	7	+	-	-	-	-	+	-	-	-	
	2	+	+	+	-	+	+	+	+	-	8	+	-	-	+	-	+	-	-	-	
	3	+	+	+	-	+	+	+	-	+	E	1	-	+	+	+	+	+	-	-	+
	4	+	+	+	-	+	+	+	-	+		2	-	+	+	+	+	+	-	+	+
	5	+	+	+	-	+	+	-	+	+		3	-	+	+	-	+	+	+	-	+
	6	+	+	+	-	+	+	-	+	-		4	-	+	+	-	+	+	+	-	+
	7	+	+	+	-	+	+	-	-	+		5	-	+	+	-	+	+	+	+	-
	8	+	+	+	-	+	-	-	-	+		6	-	+	+	-	+	+	-	+	+
C	1	+	+	-	+	+	+	+	+	+		7	-	+	+	-	+	+	-	+	
	2	+	+	-	+	+	+	-	+	+		8	-	+	+	-	-	-	+	+	
	3	+	+	-	-	+	+	+	+	+	9	-	+	-	-	+	+	-	+		
	4	+	+	-	-	+	+	+	-	+	10	-	+	-	-	+	+	-	-		
	5	+	+	-	-	+	+	+	-	+	11	-	+	-	-	+	+	-	-		
	6	+	+	-	-	+	+	+	-	+	12	-	+	-	-	+	+	-	+		
	7	+	+	-	-	+	+	+	-	+	13	-	+	-	-	+	-	+	+		
	8	+	+	-	-	+	+	-	+	+	14	-	+	-	-	+	+	+	+		
	9	+	+	-	-	+	+	-	-	+	15	-	+	-	+	+	-	+	+		
	10	+	+	-	-	+	+	-	+	-	F	1	-	-	+	-	-	+	-	-	
	11	+	+	-	-	+	+	-	+	+		2	-	-	+	-	-	+	+	+	
D	1	+	-	+	-	-	-	-	+	-	G	1	-	-	-	+	-	-	-	-	
	2	+	-	+	-	-	-	+	-	2		-	-	-	-	+	-	-	-		
	3	+	-	-	-	-	-	-	+	-											

^a Symbols: +, susceptible to bacteriocin; -, resistant to bacteriocin. Untypable strains were resistant to all bacteriocins.

TABLE 5. Distribution of 274 strains of *C. perfringens* into bacteriocin sensitivity patterns

Type	No. of strains in type	% of strains in type	No. of strains in alphabetical groups	% of strains in alphabetical groups	Type	No. of strains in type	% of strains in type	No. of strains in alphabetical groups	% of strains in alphabetical groups		
A 1	19	6.9	27	9.9	5	1	0.4	12	4.4		
2	4	1.5			6	1	0.4				
3	4	1.5			7	3	1.1				
B 1	21	7.7	56	20.4	8	1	0.4			26	9.5
2	2	0.7			E 1	5	1.8				
3	10	3.6			2	1	0.4				
4	7	2.6			3	2	0.7				
5	7	2.6			4	2	0.7				
6	1	0.4			5	1	0.4				
7	7	2.6			6	1	0.4				
8	1	0.4			7	1	0.4				
C 1	6	2.2	145	52.9	8	1	0.4	3	1.1		
2	2	0.7			9	3	1.1				
3	43	15.7			10	2	0.7				
4	1	0.4			11	1	0.4				
5	14	5.1			12	2	0.7				
6	6	2.2			13	1	0.4				
7	37	13.5			14	2	0.7				
8	28	10.2			15	1	0.4				
9	6	2.2			F 1	2	0.7				
10	1	0.4			2	1	0.4				
11	1	0.4			G 1	1	0.4				
D 1	1	0.4	Untypable		2	1	0.4	3	1.1		
2	2	0.7									
3	1	0.4									
4	2	0.7									

typing. A second portion of colony A was streaked onto blood agar to obtain isolated colonies from which colonies A₁, B₁, C₁, and D₁ were randomly selected. These colonies were typed as described above, and a portion of colony A₁ was streaked onto blood agar for isolated colonies. The procedure was repeated 10 times for each culture, each time typing four colonies and subculturing the A colony. Thus, 40 typings were done on each culture.

In this experiment there was essentially no change in the typing patterns of three of the four cultures, although there was an occasional loss of susceptibility to a particular bacteriocin which was regained on a subsequent passage. The fourth culture became consistently susceptible to a bacteriocin to which it was originally resistant.

DISCUSSION

The division of 274 strains of *C. perfringens* into 50 types based upon their susceptibility to bacteriocins indicates that this species is heterogeneous with regard to bacteriocin sensitivity.

We (4) previously indicated that these bacteriocins brought about the removal of the cell wall of susceptible strains of *C. perfringens* and, if this is the sole mode of action, then our present findings indicate a very heterogeneous cell wall composition as recognized by bacteriocins or, alternatively, a number of sites in the pathway of cell wall synthesis are capable of blockage by bacteriocin. It has been suggested by a serological study (1) that classical toxin type A strains of *C. perfringens* are a heterogeneous group in terms of antigenic structure, but this has not been implemented in typing. Some food poisoning varieties of these type A strains have been grouped serologically by Hobbs et al. (2) with some success.

Although most of the strains in our collection were type A or presumed type A (because of isolation from human sources [5]), we did possess a few known type C and D strains. Both types C and D were inhibited by bacteriocins produced by type A strains and bacteriocins produced by types C and D could inhibit type A strains, suggesting that there is no obvious

TABLE 6. Typing patterns of *C. perfringens* isolated from more than one stool specimen from the same individual

Individual	Isolate no.	Date of culture	Bacteriocin type
A	533	8-2-73	B6
	619	14-2-73	C2
B	325	24-1-73	B7
	326	24-1-73	B7
C	102	28-2-72	C3
	108	29-2-72	C3
D	123	11-4-72	D4
	126	12-4-72	C5
E	141	7-12-72	D7
	145	8-12-72	D1
F	101	28-2-72	C8
	104	28-2-72	B3
G	125	12-4-72	B1
	132	18-4-72	C1
H	154	14-3-72	B3
	168	15-3-72	B5
	194	20-6-72	B5
I	266	27-6-72	C7
	234	27-7-72	B1
	235	27-7-72	A1
J	152	14-3-72	B1
	156	14-3-72	B1

TABLE 7. Typing patterns of *C. perfringens* isolated from more than one member of the same family

Individual	Isolate no.	Typing pattern
Husband	195	B1
	196	B1
Brother	253	C3
	254	C7
Sister	269	C7
	270	C3
Father	165	C7
	166	C7

relationship between either the production of, or susceptibility to, bacteriocins and the classical typing scheme based upon toxin production.

The design of the typing system allows for the insertion of new types within the major groups. Although there is no clear relationship between typing patterns and known food poisoning

strains, many such strains were of the Group E patterns.

Henriksen (cited in ref. 1) suggested by serological technique that an individual's feces may contain more than one serological type of *C. perfringens* at any given time, and from our data it appears that more than one bacteriocin type of *C. perfringens* may be carried at one time. A much more detailed study is indicated in this area. Because of a potential change in susceptibility of isolates of *C. perfringens* to bacteriocins upon repeated subculturing, it is advisable to perform typing on fresh isolates of the organism.

This paper demonstrates the feasibility of typing *C. perfringens* by means of bacteriocin sensitivity. The methodology is simple and, with very few exceptions, the results are easily read.

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LITERATURE CITED

- Henderson, D. W. 1940. The somatic antigens of the *Cl. welchii* group of organisms. *J. Hyg.* 40:501-512.
- Hobbs, B. C., M. E. Smith, C. L. Oakley, G. H. Warrack, and J. C. Cruickshank. 1953. *Clostridium welchii* food poisoning. *J. Hyg.* 51:75-101.
- Mahony, D. E., and M. E. Butler. 1971. Bacteriocins of *Clostridium perfringens*. Isolation and preliminary studies. *Can. J. Microbiol.* 17:1-6.
- Mahony, D. E., M. E. Butler, and R. G. Lewis. 1971. Bacteriocins of *Clostridium perfringens*. 2. Studies on mode of action. *Can. J. Microbiol.* 17:1435-1442.
- Taylor, A. W., and W. S. Gordon. 1940. A survey of the types of *Cl. welchii* present in soil and in the intestinal contents of animals and man. *J. Pathol. Bacteriol.* 50:271-277.
- Tubylewicz, H. 1966. Experimental studies on bacteriocinogeneity in *Clostridium perfringens* type A. 1. Isolation of bacteriocins and their antibacterial spectrum. *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* 14:31-36.
- Uchiyama, K. 1966. Studies on bacteriocin-like substances produced by *Clostridium perfringens*. 1. Activity spectrum of the inhibitory substances produced by *Cl. perfringens*. *Med. J. Kagoshima Univ.* 18:131-144.