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Bacteriocin Typing of Clinical Isolates of *Enterobacter cloacae*

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A total of 16 selected bacteriocins of *Enterobacter cloacae* were characterized presumptively. They proved to be noninfectious, sedimentable (105,000 × *g*), resistant against chloroform and trypsin, and nonfilterable. The host ranges were essentially species specific. Based on susceptibility to one or more of these 16 bacteriocins, 242 of 308 (78.6%) clinical *E. cloacae* isolates were typed and assigned to 52 provisional bacteriocin types. Several outbreaks of nosocomial cross-infection were discerned retrospectively. Thus, bacteriocin typing of *E. cloacae* isolates may prove useful for controlling hospital infection.

During the past 2 years we experienced a significant increase in the number of clinical isolates of *Enterobacter cloacae*. We wished to document outbreaks of nosocomial cross-infections. Therefore, a study was undertaken of the feasibility of bacteriocin typing based on the susceptibility of isolates to selected bacteriocins of these opportunistic pathogenic microorganisms (5). Previously, Hamon and Peron (4) found that 8 of 29 strains produced trypsin-resistant bacteriocins. Freitag and Friedrich (3) examined a total of 65 *E. cloacae* isolates of which 13 proved to be bacteriocinogenic. On the basis of susceptibility to bacteriocins, these authors were able to type 51 of 65 strains (78.5%) which were assigned to 23 bacteriocin types designated A through X (type Y contained 14 nontypable isolates). Decades ago, Sakazaki and Namioka (8) differentiated 79 serotypes among 170 isolates of *E. cloacae* and identified 53 O antigens and 57 H antigens. Mummery et al. (6) described a nosocomial outbreak of urinary tract infections due to atypical *E. cloacae* and found 58 isolates sharing a common O antigen. Very recently, Richard (7) utilized biotyping with 15 biochemical markers, and several episodes of nosocomial cross-infection were resolved with this technique. The data obtained in the present study indicated the potential usefulness of a simple bacteriocin typing procedure analogous to that developed for *Serratia marcescens* previously (11).

MATERIALS AND METHODS

Bacteria. A total of 308 *E. cloacae* isolates were examined; other *Enterobacter* species, including *Enterobacter sakazakii* (2), were excluded. The isolates were identified according to conventional criteria (1) by the API 20E miniature method. A total of 20 isolates each of *Klebsiella pneumoniae* and *S. marcescens* and several isolates of *E. sakazakii*, *Enterobacter agglomerans*, and *Enterobacter aerogenes* served for the determination of the host ranges of *E. cloacae* bacteriocins. The seven bacteriocinogenic (b^+) *E. cloacae* strains C1 through C5, C8, and C9 were received through the courtesy of V. Freitag, Hamburg, West Germany.

Of the 308 clinical *E. cloacae* isolates, 104 were obtained from respiratory tracts, 79 from urine, 50 from stool, 25 from wounds, 16 from ear, nose, and throat areas, 7 from blood, 7 from intravenous catheter tips, 5 from the gastric aspirate of newborns, 2 from cerebrospinal fluid, 1 from peritoneal fluid, 1 from an umbilical cord, and 11 from undetermined clinical sources.

Media. Brain heart infusion broth, tryptic soy agar, tryptic soy broth (TSB), and MacConkey agar with added crystal violet were purchased from Difco Laboratories, Detroit, Mich. The bacteria were maintained on tryptic soy agar slants at 4°C and in a mixture of one volume of brain heart infusion broth plus one volume of heat-inactivated (56°C, 30 min) bovine serum (Behringwerke AG, Frankfurt, West Germany) at −65°C.

Reagents. API 20E strips were purchased from API Bio Merieux GmbH, Nürtingen, West Germany. Mitomycin C (MC) (batch no. 49C-0411) was procured from Sigma Chemie GmbH, München, Taufkirchen, West Germany. Stock solutions of 20 μg mitomycin C per ml were sterilized with membrane filters (filter units, 0.2 μm; Nalge/Sybron Corp., Rochester, N.Y.) and stored in 5-ml portions at −15°C until further use. Trypsin (1:250) was purchased from Difco; stock solutions of 10 mg of trypsin per ml of phosphate-buffered saline (pH 7.4) were sterilized with membrane filters and stored at −65°C. Chloroform (analytical grade) was supplied by E. Merck AG, Darmstadt, West Germany.

Induction of bacteriocins. Candidate strains (259 isolates) of *E. cloacae* were subcultured to 5 ml of TSB and incubated at 31°C for 4 h, after which 1 ml of each culture was transferred to 8.5 ml of fresh prewarmed (31°C) TSB. These tubes were incubated at 31°C for 1

TABLE 1. Lethal activity of b$^+$ *E. cloacae* strains against relevant i strains

i Strain	Lethal activity of b$^+$ strain															
	9	59	70	80	91	99	132	167	184	C1	C2	C3	C4	C5	C8	C9
9i	−	+	−	+	+	+	−	−	+	−	−	−	−	+	−	−
23i	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−
27i	+	−	+	−	−	−	−	+	−	+	+	−	+	+	−	−
35i	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	+
127i	−	+	−	+	−	+	−	−	+	−	−	+	−	+	−	−
245i	+	−	+	−	+	−	+	+	+	+	+	−	+	+	−	−

h. Next, 0.5 ml of MC solution (20 μg/ml) was added (final MC concentration, 1 μg/ml). For control purposes, 9.5 ml of TSB received 0.5 ml of MC at a concentration of 20 μg/ml. The cultures were held at 31°C overnight. The next morning, 1 ml of chloroform was added, the tubes were shaken manually 20 times (or on a Vortex shaker for 10 s), and then the crude cell lysates were centrifuged at 3,500 × *g* at 4°C for 10 min. The supernatant fluids were harvested and aerated for 20 min at room temperature (10, 11). The bacteriocin cell lysates were stored at 4°C.

Bacteriocin typing. The technique was essentially identical to that introduced previously for *S. marcescens* (10, 11). Briefly, clinical isolates of *E. cloacae* were subcultured to 2.5 ml of TSB and were incubated at 31°C overnight. The next morning, the turbidity was adjusted to that of McFarland (barium sulfate) no. 0.5 standard in saline, after which the bacteria were diluted 10-fold further. Next, plates (diameter, 14 cm) of MacConkey agar with added crystal violet were swab streaked in three planes. The plates had been subdivided into 17 sectors (16 bacteriocins and 1 control sector with TSB-MC). With the aid of 15-cm Pasteur pipettes, 0.05-ml drop inocula of each bacteriocin and of the control solution were delivered to appropriate sectors and permitted to dry in (about 15 min at room temperature, open lids). The plates were incubated at 31°C overnight and were evaluated the next morning. Any zone of inhibition revealing nonconfluent bacterial growth was interpreted as positive; mere slight suppression of growth (fine background confluent growth or less luxuriant semimucoid growth) was scored as negative.

Preliminary characterization of bacteriocins. The representative *E. cloacae* bacteriocins 80 and C5 were tested for resistance against 5 mg of trypsin (1:250) per ml (1 ml of bacteriocin cell lysate plus 1 ml of trypsin at a concentration of 10 mg/ml; control, 1 ml of cell lysate plus 1 ml of phosphate-buffered saline, pH 7.4; exposure at 35°C for 1 h). After this, the cell lysates were titrated for residual lethal activity against control indicator (i) *E. cloacae* strain 127i. The titers obtained per 0.05 ml were multiplied by 20 (lethal units per ml). The b$^+$ *E. cloacae* strains 80 and C5 were induced with 0.5, 1, and 2 μg of MC per ml to determine the optimal concentration of MC. Furthermore, quintuplicate cultures of these two b$^+$ strains were induced with 1 μg of MC per ml. At 0, 1, 2, 3, and 4 h, appropriate cultures were lysed with chloroform and were assayed against *E. cloacae* strain 127i. Disposable 0.2-μm membrane filter units served to document the nonfilterability of bacteriocins 80 and C5. The noninfectivity of all 16 *E. cloacae* bacteriocins was ascertained through successive passages in relevant i strains (9 ml of log-phase i strain TSB culture plus 1 ml of cell lysate; incubation at 31°C overnight; addition of 1 ml of chloroform and processing of cell lysate; titration; subsequent combination of 1 ml of passaged cell lysate plus 9 ml of fresh i strain TSB culture, and so on). The 16 *E. cloacae* bacteriocins were subjected to ultracentrifugation at 105,000 × *g* at 4°C for 3 h (Kontron TGA 65 ultracentrifuge; fixed-angle rotor TFT 65.38; 37,000 rpm). The supernatant fluids were titrated for residual titers as compared with control cell lysates.

RESULTS

A total of 24 of 259 isolates (9.3%) of *E. cloacae* proved reproducibly (by two screen experiments) to be b$^+$ after induction with 1 μg of MC per ml. Lysogenized strains of *E. cloacae* were excluded from further study. Of the 24 candidate b$^+$ strains, 9 (strains 9, 59, 70, 80, 91, 99, 132, 167, and 184) were chosen as well as 7 b$^+$ strains (strains C1 through C5, C8, and C9) that had been received from V. Freitag, Hamburg. The 16 b$^+$ strains of *E. cloacae* differed in host range and consistently yielded bacteriocins with reproducible individual host ranges. Six *E. cloacae* isolates (strains 9i, 23i, 27i, 35i, 127i, and 245i) were selected as control i strains (Table 1).

All 16 *E. cloacae* bacteriocins proved to be noninfectious; their lethal activity was lost within two or three passages in appropriate i strains. Furthermore, all 16 chloroform-resistant bacteriocins were sedimented by centrifugation at 105,000 × *g* at 4°C for 3 h. The representative *E. cloacae* bacteriocins 80 and C5, which were assayed against strain 127i, resisted 5 mg of trypsin per ml, and their biological activity was lost after membrane filtration (0.2 μm). Bacteriocin activity was obtained with 0.5, 1, and 2 μg of MC per ml (bacteriocin 80 yielded 200, 400, and 800 lethal units per ml, respectively; bacteriocin C5 yielded 12,800, 12,800, and 25,600 lethal units per ml, respectively). Peak titers of bacteriocins 80 and C5 were obtained within 3 h after the addition of 1 μg of MC per ml. Therefore, all b$^+$ strains of *E. cloacae* subsequently were induced with 1 μg of MC per ml, and the

TABLE 2. Bacteriocin types of *E. cloacae*, based on susceptibility to 1 or more of 16 selected bacteriocins

Bacteriocin type	Susceptibility (+) or resistance (−) against bacteriocin															
	9	59	70	80	91	99	132	167	184	C1	C2	C3	C4	C5	C8	C9
1	+	−	+	−	−	−	+	+	−	+	+	−	+	+	−	−
2	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
3	−	+	−	+	+	+	−	−	+	−	−	−	−	+	−	−
4	−	−	−	+	−	+	−	−	−	−	+	−	−	+	−	−
5	−	+	−	+	−	+	−	−	+	−	−	−	−	+	−	−
6	+	−	+	−	−	−	−	−	+	+	+	−	−	−	−	−
7	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−
8	−	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−
9	−	+	−	+	−	−	−	−	+	−	−	+	−	+	−	−
10	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−
11	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	+
12	+	−	−	−	−	−	−	+	−	+	+	−	−	+	−	−
13	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
14	+	−	−	+	−	−	−	+	+	+	+	−	−	+	−	−
15	+	−	+	−	+	−	+	+	−	−	+	−	−	+	−	−
16	+	−	+	−	−	−	+	+	−	+	+	−	−	+	−	−
17	+	−	+	−	−	−	+	+	−	+	+	−	−	−	−	−
18	+	−	+	−	−	−	−	+	−	−	−	−	−	+	−	−
19	+	−	+	−	−	−	+	+	+	+	+	−	+	+	−	−
20	−	+	−	−	−	+	−	+	+	+	−	−	+	+	−	−
21	−	−	−	+	−	−	−	−	+	−	−	+	−	+	−	−
22	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−
23	−	−	−	+	−	+	−	−	−	−	−	−	−	+	−	−
24	−	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−
25	+	−	+	−	−	−	+	+	−	−	+	−	−	−	−	−
26	−	+	−	+	−	−	−	−	+	−	−	−	−	+	−	−
27	+	−	+	−	−	−	−	+	+	+	+	−	+	+	−	−
28	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+
29	−	−	−	+	+	+	−	−	−	−	−	−	−	+	−	−
30	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
31	+	−	+	+	−	−	−	+	−	−	+	−	−	+	−	−
32	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
33	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
34	−	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−
35	−	+	−	+	−	+	−	−	+	−	−	+	−	+	−	−
36	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
37	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−
38	+	−	+	−	−	−	+	+	−	−	+	−	−	+	−	−
39	−	−	−	+	−	+	−	−	+	−	−	−	−	+	−	−
40	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−
41	−	−	−	−	−	−	−	+	+	+	−	−	+	+	−	−
42	−	+	+	+	−	+	−	+	+	+	−	−	+	+	−	−
43	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−
44	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	−
45	−	+	−	−	−	+	−	−	+	+	−	−	+	+	+	+
46	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	−
47	+	−	+	+	−	−	+	+	−	−	+	−	−	+	−	−
48	+	−	+	−	+	+	+	+	+	+	+	−	+	+	−	−
49	+	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−
50	−	+	−	+	−	+	−	−	+	+	+	+	−	+	−	−
51	−	−	−	+	−	−	−	−	+	+	−	−	+	+	+	+
52	−	+	−	−	−	−	−	−	+	−	−	−	−	+	−	−

TABLE 3. Frequency of *E. cloacae* bacteriocin types[a]

Bacteriocin type	No. of isolates	Bacteriocin type	No. of isolates
1	13	31	1
2	24	32	1
3	1	33	3
4	3	34	38
5	11	35	1
6	1	36	1
7	29	37	3
8	1	38	1
9	1	39	13
10	11	40	2
11	3	41	2
12	1	42	1
13	13	43	1
14	1	44	1
15	1	45	1
16	2	46	3
17	1	47	1
18	1	48	1
19	11	49	8
20	2	50	2
21	2	51	2
22	2	52	1
23	11		
24	1		
25	1		
26	2		
27	1		
28	1		
29	1		
30	1		

[a] Totals: typable, 242 (78.6%); nontypable, 66 (21.4%).

cultures were held overnight at 31°C before further processing. The 16 *E. cloacae* bacteriocins were inactive against 20 representative isolates of *K. pneumoniae* and *S. marcescens*. Only one of five isolates of *E. sakazakii* proved susceptible; four isolates of *E. agglomerans* and two strains of *E. aerogenes* were refractory.

With respect to the feasibility of bacteriocin typing of *E. cloacae* strains, a total of 308 isolates from various clinical sources described above were typed twice based on susceptibility to 1 or more of 16 selected *E. cloacae* bacteriocins. Of these, 242 isolates (78.6%) could be typed and were assigned to 52 provisional bacteriocin types (Tables 2 and 3). The 10 most commonly encountered bacteriocin types, in descending order of frequency, were types 34, 7, 2, 1, 13, 39, 5, 10, 19, and 23 (Table 3). Several episodes of nosocomial cross-infection were uncovered retrospectively. For example, in an extramural neonatal intensive care unit, a strain of bacteriocin type 2 (12 isolates) accounted for infections in 11 infants; concurrently, a strain of type 7 (4 isolates) was recovered from 4 infants. An additional outbreak occurred in the intensive care unit of the Department of Anesthesiology. A strain of bacteriocin type 34 (19 isolates) was recovered from 14 patients (bronchial aspirates, wound and urine specimens). Urinary tract infection (two specimens) followed by postoperative wound infection (three isolates) in a patient owing to *E. cloacae* type 34 was noted during the course of 5 weeks. Dual *E. cloacae* infections were shown in a newborn infant, with one external drainage and two cerebrospinal fluid specimens of type 19 and one untypable urine specimen. On the other hand, a patient who suffered from long-standing *E. cloacae* urinary tract infection yielded a strain of bacteriocin type 3, followed by a strain of bacteriocin type 5, which was recovered twice during the subsequent 3-month period. Types 3 and 5 differed in one bacteriocin receptor only, bacteriocin 91 (Table 2).

DISCUSSION

This preliminary study indicated that bacteriocin typing of clinical isolates of *E. cloacae* is feasible. The data corroborated those of Freitag and Friedrich (3). Unfortunately, the percentage of nontypable *E. cloacae* isolates proved larger than that of *S. marcescens*. Nevertheless, in view of the antigenic diversity (O and H antigens) of this opportunistic pathogenic microorganism (8), we felt that a simple, inexpensive technique, based essentially on that developed years ago for *S. marcescens*, might help interested laboratories (especially reference laboratories) to investigate outbreaks of nosocomial cross-infections due to *E. cloacae*. It is not known at this stage whether potential phenotypic variations of one or more bacteriocin receptors are of epidemiological significance (9); this is also true, of course, to potential O-antigen and biotype variations. Most likely, the 16 selected *E. cloacae* bacteriocins represent products of defective lysogeny (10, 11).

With the increasing use of third- and fourth-generation cephalosporin antibiotics plus the concurrent spread of aminoglycoside and β-lactamase resistance plasmids (5) in large medical institutions, it is anticipated that strains of *E. cloacae* may surpass strains of *S. marcescens* in nosocomial significance. With regard to numerical incidence, *E. cloacae* already overtook *S. marcescens* at our institution. It remains to be determined whether bacteriocin typing of *E. cloacae* may prove beneficial for other clinical microbiology laboratories charged with responsibility in hospital infection control.

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