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Typing of *Pseudomonas cepacia* by Bacteriocin Susceptibility and Production

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The significance of *Pseudomonas cepacia* as an opportunistic pathogen in immunocompromised patients has become increasingly recognized. Particularly disturbing is its increased incidence, reported by several North American centers, in respiratory tract cultures from patients with cystic fibrosis. Epidemiological studies of *P. cepacia* have been hampered by a lack of typing methods. In this paper we report the development of a typing scheme based on bacteriocin production and susceptibility. For bacteriocin production, test isolates of *P. cepacia* were rapidly applied to the surfaces of agar plates with a multiple inoculator. After incubation of these test isolates for 5.5 h and their exposure to chloroform, indicator strains were applied in agar overlays without prior removal of the test strain growth. After 18 h of incubation, inhibition zones caused by bacteriocin activity were recognized. A similar procedure was used to examine the bacteriocin susceptibility of the test strain. The bacteriocin type of the test strain was defined based on its bacteriocin production as judged by zones of inhibition against a set of eight indicator strains and by susceptibility or resistance of the test strain to bacteriocin produced by six producer strains. Of 373 strains of *P. cepacia*, 95.2% were typed into a total of 44 type combinations. Bacteriocin typing provided a suitable procedure for epidemiological studies of colonization or infection by *P. cepacia*. The technique described in this paper was simple to perform, gave a result within 24 h, provided good strain discrimination, and was suitable for clinical, environmental, and phytopathogenic strains.

Pseudomonas cepacia is arguably the most nutritionally versatile species of the genus *Pseudomonas* and the species most resistant to antibiotics, antiseptics, and disinfectants (3, 12, 14). The organism is even able to utilize penicillin G as a sole carbon source (2). Isolation of *P. cepacia* from clinical specimens and hospital environments, particularly from wet surfaces, equipment, pharmaceutical solutions, and antiseptics, has became increasingly common, and the presence of *P. cepacia* in these areas has been shown to lead to colonization and infection (13, 15, 16). The virulence of *P. cepacia* is low, and most clinical isolations are not associated with symptoms, but in 1980, Randall (15) emphasized that the species ". . . could become a potentially lethal pathogen in a compromised patient." Subsequently, this observation was strikingly confirmed when several centers in North America noted an increasing percentage of *P. cepacia* in respiratory tract cultures from patients with cystic fibrosis; the incidence was highest either at postmortem examination or immediately before death (L. L. Mackenzie and P. H. Gilligan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C34, p. 317), and infection with *P. cepacia* had become a major clinical problem (9). Epidemiological investigations and judgments have been hampered by a lack of suitable typing or "fingerprinting" methods for *P. cepacia*. Gonzalez and Vidaver (5) divided 30 strains into two distinct groups based on differences in bacteriocin production and in maceration of onion slices; all six clinical isolates were negative for these characteristics. Recently, a serotyping system has been described (8) which allows differentiation of 282 isolates into seven somatic (O) and five flagellar (H) groups, with 98.5% of strains being typable by this method. To achieve greater discrimination, we developed an additional or alternative typing system based on susceptibility to and production of bacteriocins. The technique described in this paper was simple to perform and allowed differentiation of 373 isolates of *P. cepacia* into 44 cepaciacin types, with 95.2% of strains being typable by this method.

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MATERIALS AND METHODS

Bacterial strains. A total of 243 pseudomonads, consisting of 130 *P. cepacia* (including ATCC 25416, 25609, and 17759), 98 *P. fluorescens*/*P. putida* (including NCIB 1007 and 10525), 10 *P. maltophilia* (*Xanthomonas maltophilia*; including ATCC 13637), and 1 each of *P. mendocina* (NCIB 10541), *P. diminuta* (ATCC 11568), *P. pseudoalcaligenes* (NCIB 9946), *P. testosteroni* (NCIB 9393), and *P. stutzeri* (NCIB 9040), were used to select suitable producer and indicator strains which either produced bacteriocins active against *P. cepacia* or were susceptible to the bacteriocins produced by strains of *P. cepacia* (Table 1). The 373 strains of *P. cepacia* which were typed represented one strain from each patient, plant, or environmental specimen. These strains had also been isolated from different geographical locations and included strains generously provided by H. Monteil, Institut de Bacteriologie de la Faculte de Medicin de Strasborg, Strassburg, France; J. D. Klinger, Rainbow Babies Childrens Hospital, Cleveland, Ohio; A. King, St. Thomas Hospital, London; and T. Pitt, Public Health Laboratory, Colindale, London. The identity of the strains was confirmed with the arginine glucose medium of Stewart (17), the API 20NE system (Analytab Products, Plainview, N.Y.), and the Uni-N/F-Tek multiple medium circular plate (Flow Laboratories, Inc., McLean, Va.). *P. aeruginosa* 430 and *P. aeruginosa* indicator strains 5 and 6 were from the laboratory collection (6), and strains PAO3047 and PML15161 (11) were kindly supplied by M. Kageyama, Mitsubishi-Kasei Institute of Life Sciences, Tokyo.

TABLE 1. Producer and indicator strains for bacteriocin typing of *P. cepacia*

Strain type and no.	Strain name	Source (reference)
Producer (CP)		
1	*P. cepacia* JC2346	Govan and Harris
2	*P. cepacia* B12417	H. Monteil
3	*P. cepacia* B68061	H. Monteil
4	*P. fluorescens* NPF26	Govan and Harris
5	*P. cepacia* 18979	H. Monteil
6	*P. cepacia* 77-16	J. Klinger
Indicator (CS)		
1	*P. cepacia* B12290	H. Monteil
2	*P. cepacia* PC8604	H. Monteil
3	*P. fluorescens* NPF26	Govan and Harris
4	*P. cepacia* B68303	H. Monteil
5	*P. cepacia* B68061	H. Monteil
6	*P. cepacia* G2	Govan and Harris
7	*P. putida* PAW85	Bayley et al. (1)
8	*P. cepacia* D36	H. Monteil

Typing of *P. cepacia* by production of bacteriocin. The method used for typing *P. cepacia* by bacteriocin production was based on our technique for pyocin typing of *P. aeruginosa* (4). The strains of *P. cepacia* to be typed were streaked for single colonies onto nutrient agar (Columbia agar base; Oxoid Ltd., Basingstoke, London) and incubated at 30°C overnight. The single colonies that arose from each test strain were used to prepare a bacterial suspension of 10^8 to 10^9 organisms in 1 ml of sterile physiological saline (absorbance at 550 nm, 0.5).

A multipoint inoculator (model A400; Denley Instruments Ltd., Sussex, England), incorporating 21 stainless-steel pins (one being a marker pin; diameter of each pin, 2 mm; pin distance apart, 16 mm), was used to dispense 1-μl volumes of the bacterial suspensions onto a set of eight plates (diameter, 90 mm) each containing 10 ml of tryptone soy agar (Oxoid). In this way 20 test strains could be typed simultaneously against each indicator strain. After the spots dried, usually within a few minutes, the plates were incubated at 30°C for 5.5 h. Filter paper disks (5 cm; Whatman, Inc., England) were impregnated with chloroform, and the plates were placed over the disks for 15 min to allow the chloroform vapor to kill the bacteria. The plates were then exposed to air for an additional 15 min to eliminate residual chloroform vapor. Cultures of the indicator strains, grown without agitation in nutrient broth (Oxoid no. 2) for 4 h at 30°C to a population size of approximately 10^7 organisms per ml, were applied to the plates by adding 0.1 ml of each bacterial indicator culture to 2.5 ml of molten, semisolid agar (1% Proteose Peptone [Difco Laboratories, Detroit, Mich.] in 0.5% agar [Oxoid L11]) held at 45°C and poured as overlays (a separate indicator strain was applied to each plate). When the overlays had set, the plates were incubated for 18 h at 30°C, and the cepaciacin producer type (P1, 2, etc.) was determined by the patterns of inhibition produced on the eight indicator strains (Table 2, Fig. 1),.

Typing by susceptibility of the test strain to bacteriocin. The technique used to type strains by their susceptibility to bacteriocin was similar to that described above for typing by bacteriocin production except that the test strain was prepared as described for an indicator strain and that its susceptibility to the bacteriocins produced by six standard producer strains (CP1 through CP6) was determined. The cepaciacin susceptibility types (S1, 2, etc.) were determined by the susceptibility pattern of the test strain against the standard producer strains (Table 3).

Cepaciacin susceptibility to trypsin. Trypsin (Difco) was incorporated into molten tryptone soy agar held at 45°C at a concentration of 50 mg/ml. The influence of trypsin on the bacteriocin activity of the six producer strains (CP1 through CP6) was investigated. Controls included non-trypsin-containing medium and strains of *P. aeruginosa* producing both trypsin-resistant and trypsin-sensitive bacteriocins. *P. aeruginosa* 430 produces the phage-tail-like, trypsin-resistant pyocins F1 and R2, which are active against *P. aeruginosa* indicator strains 5 and 6, respectively (6, 11); *P. aeruginosa* PAO3047 produces the trypsin-sensitive pyocin S2, which is active against strain PML15161.

Production and induction of cepaciacin in liquid medium. The medium used was sodium glutamate broth (10). Of an overnight culture of the bacteriocinogenic strain, 1 ml was added to 20 ml of sterile broth and incubated in a 100-ml flask in an orbital incubator (model 1H460; Gallenkamp, London) at 140 rpm for 2 h at 30°C. Mitomycin C was added to a final concentration of 5 μg/ml, and incubation was continued for an additional 2 h. The culture was centrifuged at 1,430 × *g*

TABLE 2. Inhibition patterns of cepaciacin producer types of *P. cepacia*

Cepaciacin producer type (P)	Inhibition of indicator strain (CS) no[a]:							
	1	2	3	4	5	6	7	8
1	+	−	−	+	−	+	+	−
2	−	−	+	−	+	+	−	−
3	−	−	−	+	+	+	+	−
4	+	−	+	+	+	+	−	+
5	−	+	−	−	+	+	−	+
6	+	+	−	−	+	+	−	+
7	+	−	−	+	−	−	−	−
8	−	−	−	+	−	−	−	−
9	−	−	−	−	−	+	−	−
10	−	−	−	−	−	+	−	+
11	+	−	−	−	−	−	−	−
12	+	−	−	−	−	+	−	−
13	+	−	−	+	−	+	−	−
14	+	−	+	+	−	+	−	−

[a] +, Inhibition; −, no inhibition.

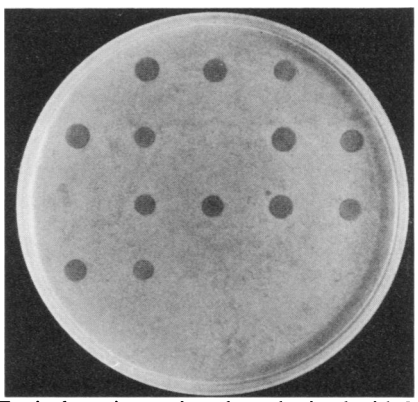

FIG. 1. Typical routine typing plate obtained with 20 test strains of *P. cepacia* tested for bacteriocin activity against indicator strain CS1; 13 strains show inhibition of CS1.

for 30 min to remove bacterial cells, and the supernatant was membrane filtered (0.2 μm; Millipore Corp., London).

Assay of cepaciacin activity. The procedure used for the assay of cepaciacin activity was that described by Govan (7) for an assay of pyocin activity with *P. cepacia* CS1 used as the bacteriocin-susceptible strain.

Electron microscopy. Mitomycin C-induced cepaciacin preparations were centrifuged for 90 min at 95,000 × *g*, and the pellets were gently suspended in 1 M ammonium acetate (pH 7.0). Equal volumes of each suspension and 2% potassium phosphotungstate (pH 7.0) were mixed on the surface of a clean glass slide, and a drop of the mixture was transferred to the surface of a collodion-coated grid. After 30 s, excess fluid was removed, and the preparation was allowed to dry. Preparations were viewed with a Hitachi electron microscope at 50 kV.

RESULTS

Development of a bacteriocin typing technique. Initial studies carried out on 243 pseudomonads, including 130 isolates of *P. cepacia*, indicated that the strains of *P. cepacia* could be differentiated based on their production of and susceptibility to bacteriocins. Examples were also observed of the susceptibility of *P. cepacia* to bacteriocins produced by other pseudomonad species and, in turn, of the production of bacteriocins by *P. cepacia* strains active against other species. Based on these observations, a preliminary set of producer and indicator strains was assembled. Using these strains, we investigated the use of different media, temperatures, and periods of incubation to obtain optimal conditions for the production and detection of bacteriocins suitable for typing *P. cepacia*; the selected technique is described above. We next sought to improve strain discrimination and reduce the number of untypable strains by testing an additional 100 previously untested strains of *P. cepacia* for their ability to act as producer or indicator strains. By a process of elimination and selection, we assembled a final set of eight indicator strains (CS1 to CS8) and six producer strains (CP1 to CP6). The origin and description of these strains is given in Table 1.

TABLE 3. Inhibition patterns of cepaciacin susceptibility types of *P. cepacia*

Cepaciacin susceptibility type (S)	Inhibition pattern produced on test strain by producer strain (CP) no.[a]:					
	1	2	3	4	5	6
1	+	+	+	−	−	+
2	+	+	−	+	−	−
3	+	−	+	−	+	+
4	+	+	−	−	+	+
5	−	−	−	+	−	−
6	+	+	+	−	+	−
7	+	−	+	+	+	−
8	+	+	−	−	−	−
9	−	+	−	+	−	−
10	−	+	−	−	+	−
11	+	+	−	+	+	−
12	+	+	−	−	−	+
13	+	−	−	−	+	+
14	+	−	+	+	+	+
15	−	−	+	+	+	−
16	+	+	+	−	+	+
17	+	+	−	−	+	−
18	+	−	+	−	−	−
19	−	−	−	+	+	+
20	+	−	+	+	−	−
21	−	−	−	−	+	−
22	+	−	−	−	−	+
23	+	+	−	+	+	+

[a] +, Inhibition; −, no inhibition.

TABLE 4. Distribution of cepaciacin types of 186 nonepidemic *P. cepacia* isolates from human, environmental, and phytopathogenic specimens[a]

Cepaciacin type[b]	No. of strains[c] from:			Total no. (%)
	France	U.S.	U.K.	
S3/P0	27	8	1	36 (19.5)
S2/P0	10	4	9	23 (12.4)
S16/P0		15	1	16 (8.5)
S7/P6	12			12 (6.5)
S1/P0	7	3		10 (5.4)
S13/P0	5	2		7 (3.8)
S14/P6	7			7 (3.8)
S4/P13	4			4 (2.2)
S5/P4	1	1	2	4 (2.2)
S0/P4	2		2	4 (2.2)
S8/P0	3			3 (1.6)
S9/P11	3			3 (1.6)
S11/P13	3			3 (1.6)
S17/P13	3			3 (1.6)
S2/P11			2	2 (1.1)
S7/P5	1			1 (1.1)
S18/P0		1	1	2 (1.1)
S20/P0			2	2 (1.1)
S22/P0		1	1	2 (1.1)
S0/P0	10	3	3	16 (8.5)
Other types[d]	13	8	4	25 (13.4)

[a] Only one strain from each specimen or from each epidemic outbreak is represented.

[b] For example, a strain designated as type S7/P6 gives a susceptibility pattern 7 (Table 3) and a production pattern 6 (Table 2).

[c] France, from the collection of Dr H. Monteil; U.S., from the collection of J. Klinger; U.K., from T. Pitt and A. King and from strains isolated in our laboratory.

[d] One each of types S2/P13, S2/P3, S3/P6, S3/P8, S4/P1, S4/P12, S5/P0, S6/P13, S6/P0, S6/P9, S10/P13, S11/P12, S12/P13, S13/P9, S13/P10, S13/P14, S16/P7, S17/P7, S19/P0, S19/P4, S21/P0, S23/P12, S23/P6, S0/P2.

Bacteriocin typing of *P. cepacia*. In the collection of 373 isolates of *P. cepacia* investigated, a total of 23 susceptibility types (S1, S2, etc.) and 14 producer types (P1, P2, etc.) were distinguished. By combining the characteristics of bacteriocin susceptibility and production, we divided 95.2% of the isolates into a total of 44 type combinations with 19 types represented by more than a single isolate. The results of typing demonstrated epidemics of cross-infection among clinical isolates obtained from certain hospitals. For example, 128 strains of *P. cepacia* were recovered from 128 patients in an intensive care unit over a period, and all strains were cepaciacin type S3/P0. Similarly, all 30 strains recovered from 30 patients in a different intensive care unit were cepaciacin type S7/P6. The distribution of 186 nonepidemic *P. cepacia* isolates from human, environmental, and phytopathogenic specimens is shown in Table 4. Clinical, environmental, and phytopathogenic strains could be typed based on bacteriocin susceptibility and production.

Reproducibility of typing patterns. All 373 strains of *P. cepacia* were retyped after 6 months of storage at −70°C. In addition, 60 strains (maintained on minimal medium at 12 to 15°C) were typed on six occasions during this 6-month period. No differences were observed with strains maintained at low room temperature (12 to 15°C), and in the single retyping of the entire collection, only three strains showed a

pattern of production or susceptibility which differed from the original; in each instance this difference involved only a single reaction.

Nature of the bacteriocins of *P. cepacia*. When the cultural conditions of the typing technique were used, without exception every zone of inhibition observed in the typing of 373 strains of *P. cepacia* appeared as a narrow band extending only 1 to 2 mm from the outer edge of the producer growth, and each zone had a sharp edge (Fig. 1). Similar zones were observed with the R and F pyocins of *P. aeruginosa* 430, whereas the S pyocin of strain PAO3047 produced zones of 5 to 8 mm and had a diffuse edge. The exposure of the bacteriocins produced by the six *P. cepacia* producer strains, CP1 through CP6, to trypsin had no effect on their inhibitory activity; a similar result was observed in the case of the R and F pyocins of *P. aeruginosa* 430, whereas the inhibitory activity of the S pyocin of *P. aeruginosa* PAO3047 was completely lost in the presence of trypsin. When producer strains CP1 and CP2 were grown in sodium glutamate broth and their inhibitory activity was assayed against the indicator CS1, titers of activity were in the range of 8 to 16; in the presence of mitomycin C at 5 μg/ml, bacteriocin activity was induced to 256 titers. Titers of activity were taken as the highest dilutions of the bacteriocin preparation that gave clear zones of inhibition of the indicator strain. When bacteriocin-containing preparations were centrifuged at 95,000 × *g* for 90 min, bacteriocin activity was found to be sedimentable, with no residual activity remaining in the supernatant. Electron microscopy of the sedimented pellets revealed contractile, phage-tail-like bacteriocins resembling the R pyocins of *P. aeruginosa* (7). No evidence of plaques of inhibition due to phage activity was observed in the course of these assays.

DISCUSSION

In the early stages of this investigation, considerable difficulty was encountered in maintaining the viability of *P. cepacia* cultures during storage at 4°C. Although cultures remained viable as suspensions at −70°C in 10% skimmed milk (Oxoid), in contrast to our experience with *P. aeruginosa*, most *P. cepacia* died rapidly when maintained on nutrient agar slopes at 4°C. Since it is an advantage to maintain cultures for several days for experimental, typing, and transport purposes, it was necessary to find a suitable storage medium. We found that cultures of *P. cepacia* could be adequately maintained for at least 6 months at low room temperature (12 to 15°C) on synthetic minimal media; a suitable medium was the minimal medium of Vogel and Bonner (18).

The technique for bacteriocin typing of *P. cepacia* described in this report was simple to perform and required no special materials for its performance. Strain discrimination was good and an improvement on existing methods of typing. In vitro, both production of and susceptibility to bacteriocins were stable characteristics of *P. cepacia*. Other indices of reliability for a satisfactory typing technique are the constancy of type in replicate isolates obtained on different days from the same site in the same patient and the degree of uniformity of type in strains from the same epidemic focus. In the case of *P. cepacia*, evidence for such in vivo stability was difficult to acquire since the large collection of strains used to develop the technique had of necessity been collected over a period of years for a variety of purposes and since epidemiological data were not always available. However, in several patients colonized with *P. cepacia* over a period of 6 months, in vivo stability could be demonstrated. The second index of reliability was more strikingly demonstrated in the case of the Strassburg isolates, in which the high incidence of cepaciacin type S3/P0 and S7/P6 resulted from two epidemic episodes and, in each epidemic, the uniformity of the strains was confirmed by serotyping (8).

The good reproducibility of the typing results may be due to the typing procedure, which was developed from a similar technique which has been used successfully for the pyocin typing of *P. aeruginosa*. Cepaciacin typing, however, revealed several interesting comparisons regarding the bacteriocinogeny of these two pseudomonads. In the case of *P. aeruginosa*, production of bacteriocins is very common and found in over 95% of isolates (4). In contrast, production of bacteriocins by *P. cepacia* was less common and was observed in only 30% of isolates. Since the detection of bacteriocins requires the use of suitable indicator strains, it could be argued that the low bacteriocinogeny of *P. cepacia* isolates resulted from inadequate detection. This seems unlikely, however, since several unsuccessful attempts were made to detect bacteriocin activity in nonproducers by testing a large number of potential indicator strains and by induction with mitomycin C. Consequently, the typing of isolates based on bacteriocin production, as is the case with *P. aeruginosa*, was not found to be suitable for *P. cepacia*; therefore, to obtain maximum typability and discrimination, cepaciacin typing was based on both bacteriocin production and susceptibility. A second comparison of bacteriocinogeny between *P. aeruginosa* and *P. cepacia* concerns the nature of the bacteriocins per se. Under the conditions of the typing procedure, the type of inhibition zone observed with *P. cepacia* was without exception a narrow, sharp-edged zone, resistant to the presence of trypsin and similar to the zones produced by the trypsin-resistant, phage-tail-like R and F pyocins of *P. aeruginosa* (6). No evidence was found in *P. cepacia* of the wide, diffuse-edged zones resulting from low-molecular-weight S pyocins, which have been observed in over 70% of *P. aeruginosa* isolates (4). Electron microscopy of mitomycin C-induced preparations from each of the six producer strains of *P. cepacia* (CP1 through CP6) confirmed that the cepaciacins did indeed resemble the phage-tail-like, contractile R pyocins. Cepaciacin typing was suitable for clinical, environmental, and phytopathogenic isolates of *P. cepacia*. This observation appears to conflict with the report of Gonzalez and Vidaver (5), who found that only environmental and phytopathogenic isolates are bacteriocinogenic and then only in liquid media. The likely explanation for this discrepancy lies in the small number of clinical isolates investigated by these authors and the fact that the technique described in this report is probably more suited to the production and detection of cepaciacins.

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