JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1989, p. 677–680
0095-1137/89/040677-04$02.00/0

Vol. 27, No. 4

Production of Bacteriocin-Like Antagonism by Clinical Isolates of *Yersinia enterocolitica*

MARY T. CAFFERKEY,* KAY McCLEAN, AND MARY E. DRUMM

Department of Clinical Microbiology at Central Pathology Laboratory, St. James' Hospital, and Department of Microbiology, The Moyne Institute, Trinity College, Dublin, Ireland

Received 22 June 1988/Accepted 12 September 1988

Fourteen clinical isolates of *Yersinia enterocolitica* serotype O:3 and four well-documented virulent strains of serotypes O:3, O:8, and O:9 were biotyped and examined for plasmid-associated autoagglutination and calcium dependency and for epithelial cell adherence. These strains were tested for the production of bacteriocin-like antagonism by using tryptone soya blood agar at room temperature and at 37°C. By using the cross-streaking method, three clinical isolates produced inhibitory substances at room temperature. These substances were active against a variety of clinical isolates and their plasmid-cured derivatives at both room temperature and 37°C. The inhibition was easier to read after incubation of the cross-streaked plate at 37°C. The inhibition patterns indicate that two of the three producer strains appear to recognize potentially virulent O:3 strains, with or without the virulence plasmid.

Yersinia enterocolitica has been extensively studied in recent years (2, 3, 10, 24). Improved methods of isolation and identification have led to a greater appreciation of the clinical importance of the organism (10). Moreover, there has been a worldwide increase in reporting of this organism, but whether this is due to increasing incidence, an increased awareness of the clinical diseases produced, or improved diagnostic capability is uncertain (10, 23).

Laboratory methods to differentiate strains of *Y. enterocolitica* have been limited to biotyping, serotyping, comparison of antibiograms, and bacteriophage susceptibility testing. In the United States, most strains involved with human infection have been of serotypes O:3, O:8, and O:5, whereas in Canada O:3 is most common and in Europe O:3 and O:9 are most common (10, 24). Sensitive methods for differentiating strains within these serotypes have not been available, although the phage typing system has been useful in separating Canadian and European O:3 serotypes (24).

Bacteriocins are defined as antibacterial substances produced by various species of bacteria which are active usually against closely related organisms (7). Bacteriocins have found widespread application in epidemiological studies as specific markers of bacteria. In the genus *Yersinia*, bacteriocin-like activity has been reported for *Y. pestis* (1), *Y. pseudotuberculosis* (5), and *Y. intermedia* (4). The biology of yersiniae is markedly influenced by temperature. Virulent strains of *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* possess a related plasmid species which is essential for virulence and calcium-dependent growth at 37°C (19). The activity of pesticin 2 is closely dependent upon calcium ions (20). In addition, several biological attributes of *Y. enterocolitica* are enhanced by lower-temperature incubation, including enterotoxin production (17, 22) and fimbria synthesis (15).

Here we report the results of screening 18 strains of *Y. enterocolitica* as potential producers and as potential indicators of bacteriocin-like substances. Growth at two different temperatures, room temperature (ca. 22°C) and 37°C, was assessed.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. Eighteen isolates of *Y. enterocolitica* were examined for the production of bacteriocin-like antagonism. Fourteen of these were our own clinical isolates obtained over a 15-month period from patients with gastrointestinal symptoms, and cultures for *Salmonella*, *Shigella*, and *Campylobacter* spp. were negative. Details of the strains and clinical details are shown in Table 1.

Three well-characterized strains of established virulence were kindly supplied by other investigators. An additional strain of serotype O:3 was obtained from the Public Health Laboratory Service, Leicester, United Kingdom. Details of these strains and their sources are included in Table 1.

Screening for recognized *Yersinia* virulence features. All strains were screened for the presence of plasmids by the method of Portnoy and Falkow (18). Calcium dependency was tested by growth on magnesium oxalate agar at 37°C, and spontaneous mutants that were calcium independent were isolated as large colonies on magnesium oxalate agar (12). All strains and their calcium-independent mutants (when applicable) were tested for autoagglutination in RPMI 1640 medium as previously described (14). Adherence to human epithelial cell lines HEp-2 and HeLa was measured by a modification of the method of Brunius and Bolin (6), whereby each bacterial strain and its plasmid-cured variant, when applicable, were grown in tryptone soya broth at 37°C for 24 h and harvested and washed with phosphate-buffered saline to a suspension of 10^8 bacteria per ml; the epithelial cell monolayer and bacterial suspension were incubated at 37°C for 90 min. The remainder of the procedure was as described by Scott and associates (21).

Media. Tryptone soya agar (Oxoid Ltd.) was used to maintain the strains before testing. Tryptone soya broth (Oxoid) was used to prepare overnight broth cultures of the indicator strains from the respective tryptone soya agar cultures. Tryptone soya blood agar containing 5% (vol/vol) defibrinated horse blood (Biological Laboratories, Ballina Ltd.) was used as the test medium. The organisms were also maintained on tryptone soya blood agar at 4°C and were subcultured every 10 days.

Demonstration of bacteriocin-like antagonism. Deferred antagonism—the cross-streaking method. Each strain was

TABLE 1. Source, biotype, and serotype of strains tested

Strain no.	Source	Biotype	Serotype
6	Feces	4	O:3
9	Hospital water storage tank	1	O:3
15	Feces	1	O:3
17	Feces	1	O:3
19	Feces	1	O:3
20	Feces	2	O:3
22	Appendix	3	O:3
28	Feces	4	O:3
32	Colonic biopsy	3	O:3
33[a]	Colonic biopsy	3	O:3
34[a]	Colonic biopsy	3	O:3
38	Feces	1	O:3
45	Feces	3	O:3
51	Feces	4	O:3
Ye134 (Institut Pasteur)	N. S. Mair	4	O:3
8081	M. Skurnik	1	O:8
Ruokola/71	M. Skurnik	2	O:9
6471/76	M. Skurnik	4	O:3

[a] Separate samples from the same patient.

TABLE 2. Virulence-associated characteristics of strains

Strain no.	Plasmid-associated characteristic[a]			
	40–48-megadalton plasmid	AA[b]	Ca^+ dependency	Epithelial cell adherence
6	+	+	+	+
9	–	–	–	–
15	–	–	–	–
17	–	–	–	–
19	–	–	–	–
20	–	–	–	+
22	+	–	+	+
28	+	+	+	+
32	+	+	+	+
33	–	–	–	+
34	+	+	+	+
38	–	–	–	–
45	–	–	–	+
51	+	+	+	+
Ye134	+	+	+	+
Ruokola/71	+	+	+	+
6471/76	+	+	+	+
8081	+	+	+	+

[a] +, Positive; –, negative.
[b] AA, Autoagglutination.

grown at room temperature and subsequently tested by the cross-steaking method at room temperature and at 37°C. The cross-streaking method was performed as described by Gillies and Govan (8) and Govan and Gillies (9) with two modifications. The strain to be tested was inoculated as a 1.5-cm-wide streak (instead of 1 cm) diametrically across duplicate tryptone soya blood agar plates. The plates were incubated overnight at either room temperature or 37°C. A wider streak of the original inoculum was used because the inhibitory zones produced were larger and clearer. After overnight incubation, the inoculum was removed with a glass slide, and remaining viable growth was killed by exposure to UV light for 30 min. The indicator strains were streaked singly at right angles to the original inoculum by using a wire loop (8 strains per plate). The plates were incubated at room temperature or 37°C overnight, and inhibition was recorded where the indicator strains crossed the original inoculum. This procedure was followed until all of the strains had been tested against each other.

RESULTS

Table 2 gives details of the results of testing of the strains for virulence-associated characteristics.

Production of bacteriocin-like activity. Three *Y. enterocolitica* isolates, numbers 15, 20, and 38 (Table 1), produced bacteriocin-like antagonism by the cross-streaking methods. The inhibitory substance was produced only by incubation at room temperature, regardless of whether the strains had been maintained at room temperature or 37°C prior to testing. However, the inhibition was demonstrable after incubation at both room temperature and 37°C, and the cross-streaked plates incubated at 37°C were easier to read, with clearly defined zones of inhibition (Fig. 1).

The inhibitory substance was bactericidal, since the inhibition zones remained free of indicator colonies during incubation at room temperature for 2 weeks.

None of the producer strains was susceptible to its own active principle or to that of either of the other two producer strains.

Producer strains and inhibition patterns. Two of the producer strains, isolates 15 and 38, were of biotype 1, while the third producer, isolate 20, was of biotype 2. Isolate 20 adhered to the human epithelial cells tested, while isolates 15 and 38 did not. The three producer strains were virulence plasmid negative. Of the producer strains, 20 and 38 had identical spectra of activity against clinical isolates of biotypes 3 and 4 and their plasmid-cured derivatives (Table 3). In addition, two plasmid-free isolates which exhibited epithelial cell adherence were also inhibited by these producer strains. Strain 20 differed from strain 38 in that it also

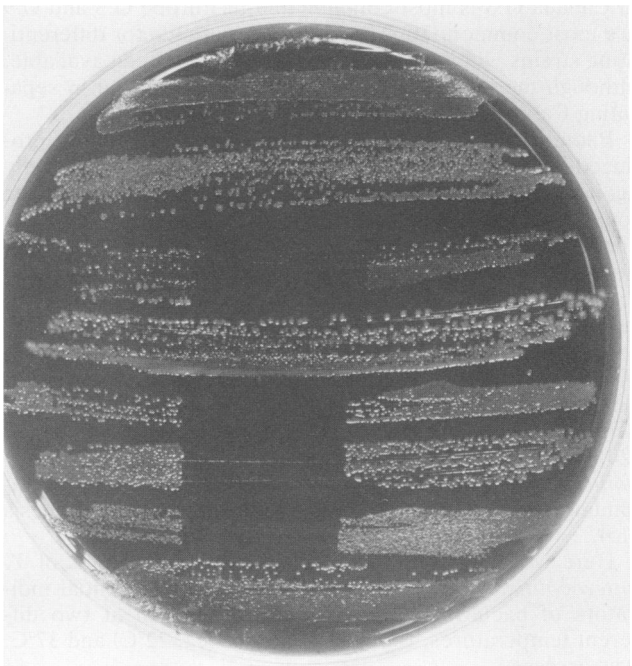

FIG. 1. Demonstration of bacteriocin-like antagonism by the cross-streaking method. Indicator strain 20 was incubated at room temperature overnight, and the cross-streaked plate was incubated at 37°C. Test strains, from top, are 9, 15, 17, 20, 22, 28, 32, and 38.

TABLE 3. Inhibition spectra of the three producer strains

Indicator strain[a]	Result for producer strain[b]:		
	15	20	38
6	−	+	+
6c	−	+	+
9	−	−	−
15	−	−	−
17	−	+	−
19	−	−	−
20	−	−	−
22	−	+	+
22c	−	+	+
28	−	+	+
28c	−	+	+
32	−	+	+
32c	−	+	+
33	−	+	+
34	−	+	+
34c	−	+	+
38	−	−	−
45	−	+	+
51	−	+	+
51c	−	+	+
Ye134	−	+	+
Ye134c	−	+	+
6471/76	−	+	+
6471/76c	−	+	+
Ruokola 71	−	+	+
Ruokola 71c	−	+	+
8081	+	−	−
8081c	+	−	−

[a] c, Virulence plasmid-cured strain.
[b] +, The indicator strain did not grow across the region of the producer inoculum; −, the indicator strain was not inhibited by the producer strain.

inhibited isolate 17, which was biotype 1 plasmid-free and did not adhere to HeLa or HEp-2 cells. Isolate 15 displayed inhibition of only one strain, 8081, and of its virulence plasmid-cured derivative.

DISCUSSION

In this study, 3 of 18 isolates of *Y. enterocolitica* produced an inhibitory substance during incubation at room temperature. The temperature dependence finding in our study is similar to that reported for *Y. intermedia* by Bottone and associates (4). They found that the bacteriocin-like antagonism produced by *Y. intermedia* exhibited temperature-dependent activity against *Y. fredricksenii*, *Y. intermedia*, *Y. kristensenii*, and *Y. enterocolitica* but not against other members of the family *Enterobacteriaceae*. Activity was observed only when the producer strain was grown at 25°C and not at 37°C, and was demonstrated by the lawn-spotting technique.

Antagonistic activity has been previously reported to be produced by *Y. enterocolitica*, but the bactericidal effect observed was attributed to the production of bacteriophage tails with bactericidal activity for a range of *Y. enterocolitica* strains (11). Nicolle and associates (16) have reported that a large proportion (>70%) of the *Y. enterocolitica* strains included in their study were lysogenic. The relationship, if any, between the antagonistic activity reported here and lysogenic bacteriophages or defective bacteriophages remains to be demonstrated.

Inhibitory bacterial products include a wide range of substances: "classical" low-molecular-weight antibiotics, lytic agents, enzymes, bacteriocins, and defective bacteriophages. Metabolic products such as ammonia, lactic acid, and hydrogen peroxide are capable of bacteriocin-like antagonism on solid media. In this study, none of the physicochemical or genetic criteria used in the definition of a bacteriocin were examined (16). However, the antagonistic substance was bactericidal, since the inhibition zone remained clear of indicator colonies during incubation at room temperature for 2 weeks. None of the three producer strains was susceptible to its own active principle or to that of either of the other two producer strains. The nature of the active substance remains to be elucidated.

The assumption that the production of bacteriocin-like antagonism can be related to a calcium-dependent function at 37°C could not be deduced from our experiments. No inhibitory activity was detected when the producer strains were incubated at 37°C on tryptone soya blood agar, a calcium-rich medium.

In conclusion, three clinical isolates of *Y. enterocolitica* have been shown to produce bacteriocin-like antagonistic substances active against strains of *Y. enterocolitica* O:3 and single strains of serotypes O:8 and O:9. The site of activity was not related to virulence plasmid-coded structures, since plasmid-cured strains were also inhibited. Three producers of bacteriocin-like activity were described. The antagonistic spectrum of two of the producer strains is almost identical to similar inhibition spectra of serotype O:3 isolates and the single O:9 isolate. These two producers appear to recognize potentially virulent O:3 strains, with or without the virulence plasmid present. In contrast, the third producer strain inhibited only the single O:8 strain tested.

LITERATURE CITED

1. **Ben-Gurion, R., and I. Hertman.** 1958. Bacteriocin-like material produced by *Pasteurella pestis*. J. Gen. Microbiol. **19**:289–297.
2. **Berche, P. A., and P. B. Carter.** 1982. Calcium requirement and virulence of *Yersinia enterocolitica*. J. Med. Microbiol. **15**: 277–284.
3. **Bottone, E. J.** 1977. *Yersinia enterocolitica*: a panoramic view of a charismatic micro-organism. Crit. Rev. Microbiol. **5**:211–241.
4. **Bottone, E. J., K. K. Sandhu, and M. A. Pisano.** 1979. *Yersinia intermedia*: temperature-dependent bacteriocin production. J. Clin. Microbiol. **10**:433–436.
5. **Brubaker, R. R., and M. J. Surgalla.** 1961. Pesticins. I. Pesticin-bacterium interrelationships, and environmental factors influencing activity. J. Bacteriol. **82**:940–949.
6. **Brunius, G., and I. Bolin.** 1983. Interaction between *Yersinia pseudotuberculosis* and the HeLa cell surface. J. Med. Microbiol. **16**:245–261.
7. **Fredericq, P.** 1957. Colicins. Annu. Rev. Microbiol. **11**:7–22.
8. **Gillies, R. R., and J. R. W. Govan.** 1966. Typing of *Pseudomonas pyocyanea* by pyocine production. J. Pathol. Bacteriol. **91**:339–345.
9. **Govan, J. R. W., and R. R. Gillies.** 1969. Further studies in the pyocine typing of *Pseudomonas pyocyanea*. J. Med. Microbiol. **2**:17–25.
10. **Gutman, L. T.** 1986. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, p. 65–84. *In* S. L. Gorbach (ed.), Infectious diarrhea. Blackwell Scientific Publications, Oxford.
11. **Hamon, Y., P. Nicolle, J.-F. Vieu, and H. Mollaret.** 1966. Recherche de la bacteriocinogenie parmi les souches de *Yersinia enterocolitica*. Ann. Inst. Pasteur **111**:368–372.
12. **Higuchi, K., and J. L. Smith.** 1961. Studies on the nutrition and physiology of *Pasteurella pestis*. VI. A differential plating medium for the estimation of the mutation rate to avirulence. J. Bacteriol. **81**:605–608.
13. **Ito, J., M. Kageyama, and F. Egami.** 1970. Isolation and characterisation of pyocins from several strains of *Pseudomonas aeruginosa*. J. Gen. Appl. Microbiol. **16**:205–214.
14. **Laird, W. J., and D. C. Cavanaugh.** 1980. Correlation of autoagglutination and virulence of yersiniae. J. Clin. Microbiol.

11:430–432.

15. **MacLagan, R. M., and D. C. Old.** 1980. Haemagglutinins and fimbriae in different serotypes and biotypes of *Yersinia enterocolitica*. J. Appl. Bacteriol. **49**:353–360.
16. **Nicolle, P., H. Mollaret, Y. Hamon, and J.-F. Vieu.** 1967. Etude lysogenique, bacteriocinogenique et lysotypique de l'espece *Yersinia enterocolitica*. Ann. Inst. Pasteur **112**:86–92.
17. **Pai, C. H., and V. Mors.** 1987. Production of enterotoxin by *Yersinia enterocolitica*. Infect. Immun. **19**:908–911.
18. **Portnoy, D. A., and S. Falkow.** 1981. Virulence-associated plasmids from *Yersinia enterocolitica* and *Yersinia pestis*. J. Bacteriol. **148**:877–883.
19. **Portnoy, D. A., H. Wolf-Watz, I. Bolin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43**:108–114.
20. **Reeves, P.** 1972. The bacteriocins, p. 101–103. Chapman & Hall, Ltd., London.
21. **Scott, T. G., C. J. Smyth, and C. T. Keane.** 1987. *In vitro* adhesiveness and biotype of *Gardnerella vaginalis* in relation to the occurrence of clue cells in vaginal discharges. Genitourin. Med. **63**:47–53.
22. **Serafim, M. B., A. R. Monteiro, and A. F. P. DeCastro.** 1983. Factors affecting detection of *Yersinia enterocolitica* heat-stable enterotoxin by the infant mouse test. J. Clin. Microbiol. **17**: 799–803.
23. **Vantrappen, G., H. O. Agg, K. Geboes, E. Ponette, and H. O. Agg.** 1982. *Yersinia* enteritis. Med. Clin. North Am. **66**:639–653.
24. **Winblad, S.** 1980. Immune response to *Yersinia* and *Pasteurella*, p. 474–478. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.