Epidemiological Differentiation of *Serratia marcescens*: Typing by Bacteriocin Sensitivity

J. J. FARMER III

Department of Microbiology, The University of Alabama, University, Alabama 35486

Received for publication 24 September 1971

Strains of *Serratia marcescens* were compared and differentiated by a new method. Bacteriocin lysates were prepared from mitomycin-induced *S. marcescens* and added to lawns of test strains. From 100 bacteriocin producers, 12 were chosen with the aid of computer analysis as the most useful in differentiation. Uniform drops of the 12 standard bacteriocins were added simultaneously with a bacteriocin-bacteriophage dropper to each strain to be typed. All 93 strains of *S. marcescens* tested were typable and were differentiated into 79 different sensitivity patterns. One pattern had three strains, 12 patterns had two strains each, and 66 patterns had only one strain. The bacteriocins also inhibited *Shigella, Klebsiella, and Enterobacter*, but no other *Enterobacteriaceae*. Bacteriocin sensitivity was less stable as an epidemiological marker than bacteriocin production. Several colonial mutants had sensitivity patterns different from the wild types, but most mutants were identical. In three different instances when cross-infection had been shown by other methods, bacteriocin sensitivity also gave the correct epidemiological results. Until the significance and frequency of genetic variations are known, a more stable epidemiological technique should be used in conjunction with bacteriocin sensitivity.

Bacteriocins are bactericidal proteins which are synthesized by bacteria and are active against other strains of the same species or closely related species (10, 13). Strains from different sources can usually be differentiated by differences in their bacteriocin production or bacteriocin sensitivity patterns. For this reason, bacteriocin typing has become a useful technique for comparing strains in hospital epidemiology.

Bacteriocin production has been used to differentiate *Pseudomonas aeruginosa* (5, 7, 18), *Shigella sonnei* (1, 6), *Escherichia coli* (17), *Proteus* (2), and *Klebsiella* (15). Bacteriocin sensitivity has been used to differentiate *P. aeruginosa* (5, 11), *S. sonnei* (1), *E. coli* (17), *Klebsiella* (14), and *Neisseria meningitidis* (8). In the article immediately preceding, I showed that *Serratia marcescens* can be typed by bacteriocin production. However, it was not possible to determine which would be the better epidemiological marker—bacteriocin production or bacteriocin sensitivity. The purpose of this article is to explain the new method for typing *S. marcescens* by bacteriocin sensitivity and to show some typical results. Independently, Traub, Raymond, and Startman (16) recently devised a similar method.

**MATERIALS AND METHODS**

The report immediately preceding should be consulted for a more detailed description of bacterial strains and procedures.

**Bacteriocin production.** Mitomycin lysates which contained bacteriocins (bacteriocin lysates) were prepared as previously described, except that 0.3 ml of chloroform was added, and the contents of the tubes were vigorously mixed with an orbital mixer for 10 sec. After the chloroform had settled, 2 ml was removed from the top layer into a sterile tube. Traces of chloroform evaporated during the next 30 min. There was a slow decline in bacteriocin activity when the lysates were stored at 4°C, so all lysates were used only on the day they were prepared. However, preliminary experiments have indicated that gelatin or other proteins may stabilize the bacteriocins. The chloroform step was omitted in the host-range experiments, and the viable cells remaining after mitomycin induction were removed by centrifugation (twice for 10 min at 2,000 × g). This preserved bacteriocins of both "A" and "B" types (12).

**Standard set of 12 bacteriocin producers.** A set of 102 bacteriocin producers was first screened.
About half of these inhibited few of the other strains tested, and were eliminated. The remaining 50 producers were then tested against 63 randomly selected strains of S. marcescens. The array of host-range data was analyzed with the aid of a computer, and 12 bacteriocin producers which best differentiated the 63 strains were selected. These standard producers were designated P 1 to P 12 and were used to type all strains.

Typing by bacteriocin sensitivity. Each strain to be typed was grown on Brain Heart Infusion Agar (BHIA) for 16 hr at 37 C. A small amount of growth was removed and visually adjusted to the same turbidity as the turbidity standard. From this, 0.1 ml was removed and added to 3 ml of medium B which had been melted and cooled to 50 C. The contents of the tube were mixed, and the suspension was overlaid onto BHIA. The overlay was allowed to solidify, and the plate was dried with the top off for 3.5 hr at room temperature.

Uniform drops of the 12 bacteriocin lysates and a control were added simultaneously with an ACCU-DROP multidropper (3) or manually with tuberculin syringes. After the drops had dried, the plates were incubated at 37 C for 16 hr. Each of the 12 zones was compared with the control, and any inhibition greater than the control was defined as positive. This end point was more reproducible than any other. The 12 positive or negative reactions were converted into a four-digit number according to the notation previously described (4).

RESULTS

Differentiation by bacteriocin sensitivity. Figure 1 shows four plates after incubation. Clear zones are due to inhibition of the test strain by bacteriocins from the standard producers. Strains one, two, and three have different sensitivity patterns, but strain three is identical to strain four. Strains three and four were isolated from different patients, but the striking similarity in their sensitivity patterns implies that the strains are identical and suggests that either cross-infection or a single source of infection was responsible. Table 1 shows how the results are tabulated and changed into simplified notation.

Distribution of bacteriocin sensitivity patterns. Table 2 shows that 93 different strains were differentiated into 79 different bacteriocin sensitivity patterns. Pattern 1428 had three strains, 12 other patterns had two strains each, and each of the other 66 patterns had only one strain each. Each of the strains was sensitive to at least one bacteriocin, so all were “typable.”

Bacteriocin sensitivity of colonial mutants. Most colonial mutants had the same bacteriocin sensitivity pattern; the one exception which differed significantly in sensitivity from the wild type is shown in Table 3. Several other mutants differed slightly from the wild type, but these variations were not enough to change a positive to a negative or conversely.

Bacteriocin sensitivity of an epidemic strain isolated from different patients. Three different instances of cross-infection or single-source outbreak were documented by serological typing, bacteriocin production, and antibiotic susceptibility. These strains were then compared by bacteriocin sensitivity. Table 4 shows that the bacteriocin sensitivity patterns in each separate case were the same, which indicated that the correct results would have been obtained had bacteriocin sensitivity been the only epidemiological method used.

Bacteriocin sensitivity of other Enterobacteriaceae. Table 5 shows that other species of Enterobacteriaceae were sensitive to lysates prepared from the 12 standard strains of S. marcescens. The following strains were typable: 2 of the 4 Shigella species; 8 of 11 K. pneumoniae, 3 of 8 E. aerogenes, 1 of 3 E. cloacae, and the 1 strain tested each of K. oz- enae and E. liquefaciens. There was no inhibition of any of the other Enterobacteriaceae (listed in the previous paper) tested. The inhibition pattern of the test strain was related to treatment of the bacteriocin lysates. Some of the bacteriocins were destroyed by chloroform, but others were resistant.

DISCUSSION

Bacteriocin typing can be done in either of two ways. In typing by bacteriocin production, each test strain is grown under standard conditions during which bacteriocins are produced. The bacteriocins are then added to a set of standard indicator strains. The advantage of this method is that the only variable which must be controlled is the set of indicator strains. Lyophilization and storage at reduced temperature without transfer (5) have been used successfully to prevent possible changes in indicator strains. In typing by bacteriocin sensitivity, bacteriocins are produced from standard strains under controlled conditions and added to each strain to be typed. The advantage of this method is that many more strains can be typed at one time. The disadvantage of typing by bacteriocin sensitivity is that the bacteriocins must be prepared for each new experiment or they must be titrated against standard indicator strains to insure that the strength has not changed. The former approach appears to be much easier, but it may be possible to stabilize bacteriocins with
**FIG. 1.** Four plates after incubation. Clockwise from the top left, the plates are of test strains 1, 2, 3, and 4. Bacteriocins P 1, 2, and 3, were applied on the top row from left to right; P 4, 5, and 6 on the second row, etc.

**TABLE 1. Tabulation of the results from Figure 1**

| Strain | Reaction against bacteriocin | Notation |
|--------|-----------------------------|----------|
|        | P 1 2 3 4 5 6 7 8 9 10 11 12 |          |
| 1      | + + + + + + + + + + + + + + + | 1112     |
| 2      | + + + + + + + + + + + + + + + | 1284     |
| 3      | + + + + + + + + + + + + + + + | 3537     |
| 4      | + + + + + + + + + + + + + + + | 3537     |

* These results are not tabulated in Table 2.

* Symbols: + = inhibition of the strain by the bacteriocin; – = no inhibition.

proteins such as gelatin or bovine serum albumin (9), or to lyophilize them.

Bacteriocin production appears to be more stable as an epidemiological marker than bacteriocin sensitivity. This is explained by the genetic basis of the two. Bacteriocin production is usually controlled by episomes (10, 13), and bacteriocins are produced as long as the
TABLE 2. Bacteriocin sensitivity patterns of 93 strains of S. marcescens

| Pattern | No. of strains | Pattern | No. of strains | Pattern | No. of strains |
|---------|----------------|---------|----------------|---------|----------------|
| 1111    | 1              | 2858    | 1              | 6138    | 1              |
| 1114    | 1              | 2866    | 1              | 6148    | 1              |
| 1117    | 1              | 6258    | 1              |         |                |
| 1118    | 2              | 3142    | 2              | 6455    | 1              |
| 1124    | 1              | 3164    | 1              | 6516    | 1              |
| 1126    | 1              | 3342    | 1              | 6577    | 1              |
| 1127    | 1              | 3365    | 1              | 6656    | 1              |
| 1128    | 2              | 3422    | 1              | 6856    | 2              |
| 1141    | 2              | 3465    | 1              | 6858    | 1              |
| 1144    | 2              | 3466    | 1              |         |                |
| 1161    | 1              |         |                | 7838    | 1              |
| 1228    | 1              | 4158    | 1              | 7858    | 2              |
| 1341    | 1              | 4258    | 1              | 7888    | 2              |
| 1428    | 3              | 4428    | 1              |         |                |
| 1458    | 1              | 4628    | 1              | 8326    | 1              |
| 1642    | 1              | 4655    | 1              | 8327    | 1              |
| 1658    | 1              | 4838    | 1              | 8356    | 1              |
| 1755    | 1              | 4858    | 1              | 8526    | 1              |
|         |                |         |                | 8587    | 1              |
| 2118    | 1              | 5127    | 1              | 8628    | 1              |
| 2171    | 1              | 5142    | 2              | 8686    | 1              |
| 2176    | 1              | 5172    | 2              | 8855    | 1              |
| 2214    | 1              | 5316    | 1              | 8875    | 1              |
| 2217    | 1              | 5356    | 1              | 8885    | 2              |
| 2227    | 1              | 5366    | 1              | 8887    | 1              |
| 2428    | 1              | 5656    | 1              |         |                |
| 2626    | 1              | 5858    | 1              |         |                |
| 2656    | 2              | 5883    | 1              |         |                |
| 2657    | 1              | 5887    | 1              |         |                |
| 2758    | 1              |         |                |         |                |
| 2838    | 1              |         |                |         |                |

TABLE 3. Bacteriocin sensitivity patterns of different colony types of the same strain

| Colonial mutant | Pattern |
|-----------------|---------|
| 18-68-1*        | 1161    |
| 1A              | 1161    |
| 1B              | 1161    |
| 1C              | 1161    |
| 18-68-6         | 3554    |
| 6A              | 3554    |
| 18-68-7         | 3571    |
| 7A              | 3871    |
| 18-68-9         | 2658    |
| 9A              | 2658    |
| 9B              | 2658    |

* The parent colony type is the first of the group. The colonial mutants follow with an A, B, or C.

organism maintains the episome. Fortunately, episomes are usually lost only after drastic manipulation, such as acridine treatment, and spontaneous losses during epidemiological studies have not been reported. Bacteriocin sensitivity is based on an entirely different genetic property—cell wall and cell membrane structure (10, 13). Many ecological pressures such as antibiotic treatment, formation of anti-cell wall antibodies, or even serial transfer in the laboratory can select for mutants with altered cell walls or membranes. The structural changes in the mutants are also likely to affect bacteriocin receptor sites and result in an altered bacteriocin sensitivity pattern. It is difficult to predict the importance of such changes in practical epidemiology. However, Abbott and Shannon (1) studied colicin production and sensitivity of Shigella sonnei, but rejected sensitivity as an epidemiological marker because different sensitivity patterns were observed in strains that were considered identical from other epidemiological evidence. They found colicin production stable under both in vitro manipulations and actual epidemiological conditions. The present study has shown that different colonial types, which presumably reflect changes in cell wall structure, can have altered bacteriocin sensitivities. Most epidemiologists receive their strains from laboratory technicians who have carefully picked single colonies. Since colonial mutants are frequently produced by S. marcescens, this is an important consideration. However, in all three instances of cross-infection, bacteriocin sensitivity gave epidemiological results which agreed with other methods. These results are in agreement with Traub (16), who found that multiple isolates over a period of weeks from the same patient had identical bacteriocin sensitivity patterns. Bacteriocin sensitivity is well
suit for routine typing, but the significance of possible genetic variation must await further investigations.

Other species of *Enterobacteriaceae* were sensitive to bacteriocin lysates from *S. marcescens*. This is in agreement with Prinsloo (12), who found *Salmonella, Escherichia*, and *Enterobacter* sensitive. Other cross-reactions will probably be detected as more strains are tested. Since several strains of *Klebsiella* and *Enterobacter* were sensitive, it should be possible to devise a single typing system for the whole *Klebsiella-Enterobacter-Serratia* division. It is now feasible to test many strains for bacteriocin production and to choose only those with host-range patterns that extend into all three genera. The method described in this report would meet this requirement if several new bacteriocin producers were added to increase the percentages of *Klebsiella* and *Enterobacter* which are typable.

It will be interesting to compare the typing method described here with that of Traub. The 8% untypable strains observed by Traub may be sensitive to bacteriocins from P 1 to P 12. The best features of both methods should be combined, and a standardized method should be proposed. Standardization would enhance reproducibility and the usefulness of the method.

**ACKNOWLEDGMENTS**

I thank I. L. Roth, M. T. Kenny, R. J. Heckly, J. A. Currie, F. Denis, M. J. Ramirez, G. P. Bodey, J. N. Wilpert, C. H. Zierdt, V. R. Dowell, and Leo Hart for their kind gifts of cultures; L. S. McClung and J. A. Currie for helpful discussions and the latter for the serotyping data; L. G. Herman and J. J. Farmer, Jr., for encouragement; and L. H. Farmer for her assistance in most experiments.

**LITERATURE CITED**

1. Abbott, J. D., and R. Shannon. 1958. A method of typing *Shigella sonnei*, using colicine production as a marker. J. Clin. Pathol. 11:71-77.
2. Cradock-Watson, J. E. 1965. The production of bacteriocines by Proteus species. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. Orig. 196:385-388.
3. Farmer, J. J., III. 1970. Improved bacteriophage-bacteriocin applicator. Appl. Microbiol. 20:517-519.
4. Farmer, J. J. 1970. Mnemonic for reporting bacteriocin and bacteriophage types. Lancet 2:96.
5. Farmer, J. J., III, and L. G. Herman. 1969. Epidemiological fingerprinting of Pseudomonas aeruginosa by the production of and sensitivity to pyocin and bacteriophage. Appl. Microbiol. 18:760-765.
6. Gillies, R. R. 1964. Colicine production as an epidemiological marker of Shigella sonnei. J. Hyg. 62:1-9.
7. Gillies, R. R., and J. R. W. Govan. 1966. Typing of Pseudomonas pyocyanea by pyocine production. J. Pathol. Bacteriol. 91:339-345.
8. Kingsbury, D. T. 1966. Bacteriocin production by strains of Neisseria meningitidis. J. Bacteriol. 91:1696-1699.
9. Mitsui, E., and D. Mizuno. 1969. Stabilization of colicin E2 by bovine serum albumin. J. Bacteriol. 100:1136-1137.
10. Nomura, M. 1967. Colicins and other related bacteriocins. Annu. Rev. Microbiol. 21:257-284.
11. Osman, M. A. M. 1965. Pyocine typing of Pseudomonas aeruginosa. J. Clin. Pathol. 18:200-202.
12. Prinaloo, H. E. 1966. Bacteriocins and phages produced by Serratia marcescens. J. Gen. Microbiol. 45:205-212.
13. Reeves, P. 1965. The bacteriocins. Bacteriol. Rev. 29:24-45.
14. Slopek, S., and J. Marecz-Babczyzyn. 1967. A working scheme for typing Klebsiella bacilli by means of pneumocins. Arch. Immunol. Ther. Exp. 15:525-529.
15. Stouthamer, A. H., and G. A. Tieze. 1966. Bacteriocin production by members of the genus Klebsiella. Anto nie van Leeuwenhoek J. Microbiol. Serol. 32:171-182.
16. Traub, W. H., E. A. Raymond, and T. S. Starsman. 1971. Bacteriocin (marcescin) typing of clinical isolates of Serratia marcescens. Appl. Microbiol. 21:837-840.
17. Vosti, K. L. 1968. Production of and sensitivity to colicins among serologically classified strains of Escherichia coli. J. Bacteriol. 96:1947-1952.
18. Zabransky, R. J., and F. E. Day. 1969. Pyocine typing of clinical strains of Pseudomonas aeruginosa. Appl. Microbiol. 17:293-296.