

# Bacteriocine typing of *Proteus*

I. J. AL-JUMAILI

From the Department of Microbiology, Royal Victoria Infirmary, Newcastle upon Tyne

**SYNOPSIS** A method of typing isolates of *Proteus mirabilis* and *Proteus vulgaris* is described based on the sensitivity of the organisms to bacteriocine. Twelve standard proteocine producing strains were selected from a large number of isolates tested, and from these liquid proteocine preparations were prepared. The sensitivities of 1805 isolates to these 12 preparations were then determined and none was found to be untypable. From the results a bacteriocine typing system has been developed.

Bacteriocine typing has been shown to be successful in the characterization of specific strains of several genera of Gram-negative bacilli. Such typing enabled Abbott and Shannon (1958), Abbott and Graham (1961), and Gillies (1964) to study outbreaks of dysentery caused by *Shigella sonnei*. Likewise a detailed study of the bacteriocines (pyocines) produced by *Pseudomonas aeruginosa* led to typing schemes based on pyocine production (Wahba, 1963; Darrell and Wahba, 1964; Gillies and Govan, 1966). Linton (1960) described a method of colicine typing of strains of *Escherichia coli*. However, the production of bacteriocines (proteocines) by members of the genus *Proteus* has been little studied. An initial investigation into the typing of strains of *Proteus* by this method was unsatisfactory as only three proteocine types were discovered (Cradock-Watson, 1965).

Nevertheless it has now been established that, by using liquid preparations of proteocines, a much more satisfactory typing scheme may be developed based on proteocine sensitivity.

## Method and Materials

### MEDIA

(a) PP3 broth—Difco Proteose-peptone No. 3, 20 g, and sodium chloride, 5 g, are dissolved in 1.0 litre distilled water, adjusted to pH 7.4, distributed in 5 ml amounts in test-tubes or in 50 ml amounts in 250 ml conical flasks, and autoclaved at 121°C for 15 minutes.

(b) Bile-salt-brain-heart infusion Agar—(BBHIA). Brain-heart infusion powder (oxid) 36 g, Difco Bile-Salt No. 3, 1.5 g, and oxid Agar No. 3, 10 g, were dissolved in 1.0 litre distilled water, adjusted to pH 7.4, and the medium was sterilized by autoclav-

ing. Before use, 12 ml amounts of melted medium were distributed into sterile 9 cm diameter plastic Petri dishes, and the plates were dried in the incubator at 37°C for 2 hours.

### SOURCE OF STRAINS

Five hundred and sixty-eight *Proteus* strains were obtained from clinical specimens from hospital patients during a period of nine months. The majority of organisms were isolated from urine and smaller numbers of pus, nasal, and wound swabs. Duplicate isolates from the same patients were avoided.

### BIOCHEMICAL IDENTIFICATION

The conversion of phenylalanine to phenylpyruvic acid and the hydrolysis of urea on Christensen's medium were used as criteria to place non-lactose-fermenting Gram-negative bacilli in the genus *Proteus*. *Proteus mirabilis* and *Proteus vulgaris* were identified on the basis of indole and H<sub>2</sub>S production, decarboxylation of ornithine, and fermentation of maltose. It was found that 59% were *P. mirabilis* and 41% *P. vulgaris*.

### PRODUCTION OF PROTEOCINE

For production of proteocines, isolates are inoculated into 5 ml amounts of PP3 broth and incubated at 25°C. After 18 hours this culture is added to 50 ml of pre-heated (25°C) PP3 broth and incubated with orbital shaking at 25°C. After one hour mitomycin C (55 µg) is added to each culture to give a final concentration of 1 µg mitomycin C per ml and the incubation is continued for a further 24 hours. The broth cultures are centrifuged at 3000 rev/min for 20 minutes, and the supernatant fluid is transferred to fresh sterile containers. Chloroform, 0.5 ml, is added to each flask, the contents of which are then thoroughly agitated for 5 minutes. The cultures are then

centrifuged again at 3000 rev/min for 20 minutes, and the chloroform-free sterilized proteocine preparation forms the supernatant which is finally removed to sterile screw-capped bottles and stored at 4°C until required. The proportion of bacteriocinogenic isolates is shown in table I.

Species	No. of Isolates Tested	No. of Isolates Producing Proteocines	% Bacteriocinogenic Organisms
<i>P. mirabilis</i>	333	290	87
<i>P. vulgaris</i>	235	145	60

Table I Production of proteocine by *Proteus* species

#### SENSITIVITY TESTING

Isolates to be screened for proteocine sensitivities are grown in 5 ml PP3 broth in a water bath at 37°C for 7 hours. Two millilitres of a 10<sup>-2</sup> suspension of the culture are flooded over the surface of a dried BBHIA plate to produce a uniform lawn of bacteria, excess broth being removed with a sterile Pasteur pipette. After drying, standard drops of proteocine preparations are transferred to the surface of the seeded agar plate using the LIDWELL phage applicator (Biddulph & Co). The plates are finally dried and incubated at 37°C for 18 hours. Proteocine activity is indicated by clear punched-out zones of inhibition within the confluent growth of the bacterial lawn (complete inhibition) or by inhibition with discrete colonies within the zones (partial inhibition).

#### Results

##### SELECTION OF STRAINS FOR PRODUCTION OF PROTEOCINE PREPARATIONS FOR ROUTINE SCREENING

Proteocine preparations of 333 isolates of *P. mirabilis* and 235 isolates of *P. vulgaris* were tested individually against the same 568 strains used as indicators. Treating the proteocine preparations as operational taxonomic units and the suppression of growth on any particular lawn as a positive character for binary coding, the results of this survey were subjected to cluster analysis according to the method of Ward (1963) and using computer programs available in the Clustan IA package (Wishart, 1969) on an IBM 360/67 computer. It was found that the preparations from proteocine producing strains fell for the most part into one or other of 12 major groups or clusters, the inhibition patterns produced by members of each group being more like those produced by other members of the group than any produced by members of other groups.

Thus, in order to ensure as wide a spectrum of activity as possible, a representative isolate was selected from each of these 12 major groups and proteocine containing preparations were prepared from these. An indicator strain was selected for each of the 12 proteocine preparations. These strains were particularly susceptible to the proteocine being tested. The 12 proteocine preparations were then decimally diluted in saline so that dilutions of up to 10<sup>-4</sup> were obtained. Each dilution was spotted onto the same lawn of its particular organism, and the titre of each preparation was given as the reciprocal of the highest dilution showing complete inhibition of the indicator strain. Thus the neat preparations were standardized so that each had an in use titre of 10<sup>4</sup> with respect to its own indicator strain. These were used in the subsequent routine tests.

None of the 12 standard proteocine preparations has any effect on 100 isolates of *E. coli*.

##### DEVELOPMENT OF TYPING SYSTEM

The 12 standard proteocines were then used to type 1000 isolates of *Proteus* obtained during the routine examination of clinical specimens. These included isolates obtained repeatedly from the same sites of infection. To record the results the signs (+) and (-) were used. A (+) designated proteocine activity shown by either complete or partial inhibition, and a (-) indicated no inhibition. For example, 35 isolates gave the same result, with (+) reactions to preparations 4 and 5 only, which formed sensitivity pattern No. 1. All isolates were sensitive to at least one of the proteocine preparations and a total of 48 proteocine-sensitivity patterns were demonstrated (table II). These were arbitrarily numbered and each isolate was subsequently assigned to one of the numbered sensitivity patterns. Thus proteocine patterns 1 to 48 were designated and any individual isolate was described as belonging to a particular sensitivity pattern.

It is possible to simplify the description of the proteocine inhibition pattern by the introduction of a simple code. The 12 standard proteocine producers are divided into four lots of three, and each triplet is numbered (421, 421, etc.) (table III). A reaction in the first column of any triplet is given the numerical value 4, the second 2, and the third 1. No reaction is given zero value. Thus, by summing the numerical values of each member of each triplet, a number is obtained which describes uniquely the pattern of each triplet. For instance, in table III, strain A is coded 5 (ie, 4 + 1) 2, 2, 2. Strain B is 0, 1, 0, 4 and strain C is 2, 6 (ie, 4 + 2), 0, 2.

##### REPRODUCIBILITY OF THE SYSTEM

In order that any typing system can be of epidemio-

Sensitivity Pattern No.	BJ												Code	No. of Isolates	Percentage
	1	2	3	4	5	6	7	8	9	10	11	12			
1	-	-	-	+	+	-	-	-	-	-	-	-	0600	35	3.5
2	-	-	-	+	+	+	-	-	-	-	+	-	0702	46	4.6
3	+	-	-	-	-	-	-	-	-	+	-	+	4012	27	2.7
4	+	-	-	-	-	-	-	-	-	+	-	+	4011	21	2.1
5	-	+	-	-	+	+	-	-	-	-	+	-	2602	16	1.6
6	-	-	-	-	-	+	-	-	-	+	-	+	0111	16	1.6
7	-	+	+	-	-	-	-	-	-	-	+	-	3004	34	3.4
8	-	+	+	-	-	+	-	-	-	-	-	-	3100	15	1.5
9	+	-	-	-	-	-	+	-	-	-	-	-	4100	23	2.3
10	+	-	+	-	-	-	-	-	-	+	-	+	5012	16	1.6
11	-	+	-	-	-	-	+	-	-	-	+	-	2102	23	2.3
12	-	-	-	-	-	+	-	-	-	-	-	-	0100	25	2.5
13	-	-	-	+	+	-	-	-	-	-	-	-	0700	37	3.7
14	+	-	-	-	-	+	+	-	-	+	-	-	4112	42	4.2
15	+	+	+	-	-	-	-	-	-	-	-	-	7000	20	2.0
16	+	-	-	-	-	+	-	-	-	+	-	-	4110	18	1.8
17	-	-	-	+	-	-	-	+	-	-	-	-	0440	51	5.1
18	-	-	-	+	-	+	-	-	-	+	-	-	0504	16	1.6
19	-	+	-	-	-	+	-	-	-	+	+	-	2106	16	1.6
20	-	+	+	-	-	-	-	-	-	+	-	-	3002	18	1.8
21	+	-	-	+	+	-	-	-	-	-	-	-	4600	19	1.9
22	+	-	+	-	-	-	-	-	-	-	-	-	5000	16	1.6
23	-	-	-	-	-	+	-	-	+	-	-	+	0121	15	1.5
24	-	-	-	-	-	-	+	-	+	+	-	-	0134	13	1.3
25	-	-	-	-	-	-	-	-	-	+	-	-	0010	12	1.2
26	-	-	-	-	-	-	-	-	-	+	-	+	0011	15	1.5
27	-	-	-	-	-	-	-	-	-	+	-	-	0004	18	1.8
28	-	-	-	-	-	-	-	-	-	-	-	+	0001	19	1.9
29	-	-	-	-	-	-	-	+	-	-	-	-	0040	18	1.8
30	-	-	-	-	-	-	+	-	-	-	+	-	0102	15	1.5
31	-	-	-	-	-	-	+	-	+	-	-	-	0120	19	1.9
32	-	-	-	-	-	-	+	-	-	-	-	+	0101	20	2.0
33	-	-	-	-	+	+	-	-	-	-	+	-	0602	31	3.1
34	-	-	-	-	+	+	-	-	-	-	+	-	0502	17	1.7
35	-	-	-	-	+	-	-	+	-	-	+	-	0442	18	1.8
36	-	-	-	+	-	-	-	-	-	-	-	-	1000	13	1.3
37	-	+	+	+	-	-	-	-	-	-	+	-	3402	15	1.5
38	-	+	-	-	-	-	-	-	-	-	+	-	2002	16	1.6
39	-	+	-	-	-	-	+	-	-	-	-	-	2100	20	2.0
40	+	-	-	-	-	-	+	-	-	-	-	-	5100	17	1.7
41	-	+	+	+	-	-	+	-	-	-	+	-	3102	15	1.5
42	-	+	+	+	-	-	-	-	+	-	-	-	3020	17	1.7
43	-	+	-	+	+	+	-	-	-	-	+	-	2702	17	1.7
44	-	+	-	-	-	+	-	-	-	+	-	-	2104	15	1.5
45	-	-	-	+	-	-	+	+	-	-	-	-	0542	21	2.1
46	-	-	+	+	-	-	-	-	-	-	-	-	1400	15	1.5
47	-	+	+	-	-	-	+	-	-	+	-	-	3104	24	2.4
48	-	-	-	-	-	+	-	-	-	+	-	-	0110	15	1.5

Table II Patterns of inhibition produced by the 12 different proteocine preparations on 1000 isolates of *Proteus*

BJ	4			2			1			4			2			1			
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	
A	+	-	+	-	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-
B	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
C	-	+	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-

Table III Coding system for the sensitivity patterns

logical value it must be shown to be reproducible and consistent.

In a bacteriocine typing system four major features must remain stable and constant. These are:

- the bacteriocine production of the producing strains,
- the sensitivities of the indicator strains,
- the inoculum of the indicator organisms, and
- the specificity of the system.

#### (a) Bacteriocine Production

Three sets of the standardized preparations produced at different times were used to type 1000 isolates of *Proteus*. All such preparations were found to give rise to identical inhibition patterns on all three occasions, and thus it was concluded that proteocine production was a reproducible feature of a given bacteriocinogenic strain. In addition, storage of the

preparations at 4°C for several months was found not to reduce bacteriocin activity.

#### (b) Bacteriocine Sensitivity

It must be demonstrated that the same organism displays an identical inhibition pattern when tested repeatedly against the same set of bacteriocine preparations and that organisms isolated from the same known source on several occasions also exhibit the same inhibition pattern on testing against the same set of bacteriocines. It was found that, in practice, no change in sensitivity patterns was detectable when the same isolate, stored on a nutrient agar slope, was re-tested over a period of months, nor was any change in sensitivity patterns demonstrated with organisms isolated on as many as 10 separate occasions from the same site of the same infected patient. No patient was found to excrete more than one strain of *Proteus* simultaneously.

#### (c) Standardization of the Indicator Inoculum

As with antibiotic sensitivity testing, differences in the density of bacteria on the surface of the plate can cause apparent variations in the sensitivity of an organism to a bacteriocine preparation. The ability of the dilution method to give a standardized bacterial lawn was therefore checked by the method of Miles and Misra. It was found that the dilution used contained approximately  $10^6$  organisms per ml which gave almost confluent growth of the indicator organism.

#### (d) Specificity of the System

The 48 different sensitivity patterns obtained showed a fairly uniform distribution of the 1000 isolates tested. The highest number of isolates in one pattern is 51, giving a percentage of 5.1 of the total. The lowest number of organisms falling into one sensitivity pattern is 12, which is 1.2% of the total (table II). The specificity of the system is shown by this uniformity in the distribution of the 1000 strains.

In addition to the 1000 strains dealt with here, 204 isolates of epidemiological importance were also typed using this system and compared with other typing methods. The results appear in this issue (Al-Jumaili, 1975).

#### Discussion

The increase in *Proteus* infections in hospitals has made it desirable to develop a satisfactory method to

identify strains which may be responsible for cross-infection. Although serological methods were tried and met with a certain amount of success (Perch, 1948), it has now been shown that bacteriocine typing methods can be used successfully as an epidemiological tool for use with *Proteus*. Previous attempts to bacteriocine or bacteriophage-type these organisms have been unsatisfactory (Cradock-Watson, 1965). As bacteriocine typing provides a technically simple and reliable epidemiological method, it seems likely that systems developed along the lines described in this paper will eventually result in a simple and quick method of typing *Proteus*. Areas of further investigation include the possibility of improving the bacteriocine yield by varying media, cultural conditions, etc. (George, 1975), the improved standardization of proteocine preparations by study of individual pure proteocines, and the extension of such systems to cover *Proteus rettgeri*, *Proteus morgani*, and possibly even *Providencia* isolates.

#### References

- Abbott, J. D. and Graham, J. M. (1961). Colicine typing of *Shigella sonnei*. *Mth. Bull. Minist. Hlth Lab. Serv.*, **20**, 51-58.
- Abbott, J. D. and Shannon, R. (1958). A method for typing *Shigella sonnei* using colicine production as a marker. *J. clin. Path.*, **11**, 71-77.
- Al-Jumaili, I. J. (1975). An evaluation of two methods of bacteriocine typing of organisms of the genus *Proteus*. *J. clin. Path.*, **28**, 788-792.
- Cradock-Watson, J. E. (1965). The production of bacteriocine by *Proteus* species. *Zbl. Bakt. I Abt. (orig.)*, **196**, 385-388.
- Darrell, J. H. and Wahba, A. H. (1964). Pyocine-typing of hospital strains of *Pseudomonas pyocyanea*. *J. clin. Path.*, **17**, 236-242.
- George, R. H. (1975). Comparison of different media for bacteriocine typing of *Proteus mirabilis*. *J. clin. Path.*, **28**, 25-28.
- Gillies, R. R. (1964). Colicine production as an epidemiological marker of *Shigella sonnei*. *J. Hyg. (Lond.)*, **62**, 1-8.
- Gillies, R. R. and Govan, J. R. W. (1966). Typing of *Pseudomonas pyocyanea* by pyocine production. *J. Path. Bact.*, **91**, 339-345.
- Linton, K. B. (1960). The colicine typing of coliform bacilli in the study of cross-infection in urology. *J. clin. Path.*, **13**, 168-172.
- Perch, B. (1948). On the serology of the *Proteus* group. *Acta path. microbiol. scand.*, **25**, 703-714.
- Wahba, A. H. (1963). The production and inactivation of pyocines. *J. Hyg. (Lond.)*, **61**, 431.
- Ward, J. H. (1963). Hierarchical grouping to optimize an objective function. *J. Amer. stat. Ass.*, **58**, 236.
- Wishart, D. (1969). Clustan 1A. The Computing Laboratory, University of St. Andrews.