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Biochemical Classification of *Proteus* and Providence Cultures

By CONSTANCE SHAW AND PATRICIA H. CLARKE*

National Collection of Type Cultures, Colindale Avenue, London, N.W. 9

SUMMARY: Biochemical reactions of 153 Providence (29911 type) and 63 *Proteus* cultures were examined. Providence cultures are included in the genus *Proteus*, as their cultural characters are similar; this relationship is strengthened by the production of L-amino acid oxidase and glutamic acid decarboxylase by both. *Proteus inconstans* Ornstein nov.comb. is the name proposed for Providence cultures.

The family Enterobacteriaceae includes many groups which have a position intermediate between the established genera; of these the paracolons 29911 type (Stuart, Wheeler, Rustigian & Zimmerman, 1943) or Providence group (Brooke, 1951; Kauffmann, 1951) were found by biochemical tests to be related to the genus *Proteus* (Ewing, Tanner & Dennard, 1954). We have investigated the distribution of several biochemical characters among the Enterobacteriaceae, in particular the ability to metabolize potassium gluconate, sodium malonate, and also to attack amino acids by oxidative deamination or decarboxylation. The results we have obtained confirm the close relation of the Providence cultures to the species of the genus *Proteus*.

METHODS

Strains. The cultures of *Proteus* spp. and other genera examined were those maintained at the National Collection of Type Cultures. The strains examined included: *Bacillus inconstans* (Ornstein, 1921), an intestinal organism described and deposited by Braun in 1926; the three original strains of the Wakefield bacillus (Berger, 1945); B 81 and B 105 of Sachs's mannitol-negative dysentery organisms (Sachs, 1943); Stuart's paracolons 29911 type. Later, we obtained 120 Providence cultures from Dr W. H. Ewing (these are listed as Providence (2) in Table 2), and freshly isolated strains from faeces collected by different laboratories of the Public Health Laboratory Service during investigations of diarrhoea.

Biochemical tests

Gluconate test. The medium was a modification of that used by Haynes (1951) for pseudomonads. It consisted of: Evans's peptone, 1.5 g.; Yeastrel, 1.0 g.; K_2HPO_4 , 1.0 g.; potassium gluconate, 40.0 g.; distilled water, 1000 ml.; adjusted to pH 7.0; sterilized 10 lb./sq.in. for 10 min. After incubation of the culture for 48 hr. at 37°, 1.0 ml. of Benedict's qualitative reagent for reducing sugars was added and the tubes placed in boiling water for 10 min.

* Present address: Department of Biochemistry, University College, London, W.C. 1.

A positive test showed a yellowish brown precipitate of cuprous oxide and there was a characteristic smell of decaying cabbage.

Phenylalanine and malonate tests. Leifson's (1933) sodium malonate and Henriksen's (1950) phenylalanine tests were combined by adding phenylalanine to Leifson's medium: the combined medium was supplemented with 0.1 % Yeastrel to increase growth. The medium was as follows: $(\text{NH}_4)_2\text{SO}_4$, 2.0 g.; K_2HPO_4 , 0.6 g.; KH_2PO_4 , 0.4 g.; NaCl, 2.0 g.; sodium malonate, 3.0 g.; DL-phenylalanine, 2.0 g.; Yeastrel, 1.0 g.; 0.5 % (w/v) ethanolic bromthymol blue, 5 ml.; distilled water, 1000 ml.; sterilized 10 lb./sq.in. for 10 min. Cultures were incubated overnight at 37° and organisms using malonate produced an alkaline reaction. When a few drops of 0.1 N-HCl and a few drops of 0.5 M-ferric chloride were added to the tubes, a deep green colour indicated the production of phenylpyruvic acid from phenylalanine (Henriksen & Closs, 1938).

Indole and H₂S production were shown, respectively, by oxalic acid papers (Holman & Gonzales, 1923) and lead acetate papers inserted above cultures in Lemco broth + 0.01 % cysteine incubated at 37° for up to 7 days (Clarke, 1953).

Gelatin liquefaction was observed in nutrient gelatin stab cultures incubated for 30 days at 22°.

Hydrolysis of urea. Stuart, van Stratum & Rustigian's (1945) highly buffered medium and also Christensen's (1946) weakly buffered nutrient urea agar were used. The tubes incubated at 37° were read daily for 7 days.

Methyl-red test (MR). Cultures were grown in glucose phosphate peptone (Evans) medium and incubated for 5 days at 30°.

Voges-Proskauer test (VP). Three media, each containing Evans's peptone, were used. Glucose phosphate peptone medium; glucose peptone medium with NaCl but without phosphate (Smith, Gordon & Clark, 1946); and glucose peptone medium without salt and phosphate (Abd-el-Malek & Gibson, 1948). Cultures were incubated at 30° for 5 days and tested for the presence of acetoin by Batty-Smith's (1941) modification of Barritt's (1936) α -naphthol method.

Use of citrate as sole source of carbon. All tests were made by needle inoculation from a liquid culture. Koser's citrate medium (1923) was incubated for 7 days at 30°. All tubes showing turbidity were regarded as positive when confirmed by needle reinoculation into citrate.

Fermentation tests. Medium consisted of peptone water + bromcresol purple + 1 % (w/v) of 'sugar' and final readings made after incubation of cultures for 14 days at 37°. The 'sugars', tested were glucose, arabinose, xylose, rhamnose, lactose, sucrose, maltose, trehalose, glycerol, mannitol, dulcitol, sorbitol, inositol, adonitol and salicin.

Microtests

In most cases tests were carried out simultaneously on growing cultures and as microtests with washed suspensions (Clarke & Cowan, 1952). Heavy suspensions were made from cultures grown overnight on Lemco agar at 37°. The microtests were carried out as follows:

Phenylalanine. Two drops suspension, 2 drops 0.03 M-phenylalanine, 2 drops 0.025 M-phosphate buffer, pH 6.8. Incubated in a water-bath and after 1 hr. at 37° tested by adding 2 drops 0.5 M-FeCl₃. Other amino acids were also used as substrates.

Malonate. Two drops suspension, 2 drops 0.03 M-sodium malonate, 2 drops 0.025 M-phosphate buffer, pH 6.0 + phenol red as indicator. Results were read up to 24 hr. at 37° and positive tests showed an alkaline reaction.

Decarboxylases. Two drops suspension, 2 drops 0.03 M solution amino acid adjusted to pH 5.0, 2 drops 0.0125 M-phthalate buffer, pH 5.0 + bromocresol purple. At this pH value CO₂ is liberated so that the reaction mixture will show an indicator colour change when decarboxylation has taken place. Readings were taken at 2, 4 and 24 hr. at 37°. Toluene was added in later tests. Each set of tests included a suspension control without amino acid; when this suspension was alkaline buffer was added to all tubes until a yellow colour was given by the control.

RESULTS

Table 1 summarizes the results of the tests made on the *Proteus* spp. and the Providence strains. Included among the Providence strains are Sachs's B 81 and B 105 and the Wakefield bacillus, which Stuart, Wheeler & McGann (1946) showed to be members of the Providence group. NCTC 2481, *Bacillus inconstans* Ornstein, in our routine examination was found to have the biochemical characters of the Providence group. Dr W. H. Ewing confirmed this and has also shown that serologically the strain belongs to Providence 0 group 9.

Motile Providence strains, like *Proteus* spp., have a tendency to spread on agar. Therefore very dry plates were used to obtain single colonies. The cells were found to be peritrichously flagellate, though the flagella were few in number as compared with typical *Proteus* spp.

All strains of *Proteus* spp. and all but two of the Providence group produced phenylpyruvic acid by oxidative deamination of phenylalanine. The two negative cultures were positive by the micromethod, but had lower than average activity. There was no clear-cut division between any of the *Proteus* spp. and the Providence group when histidine was used as substrate (Singer, 1953). Stumpf & Green (1944) found that freshly prepared suspensions of *P. vulgaris* could oxidatively deaminate nearly all the naturally occurring amino acids, and manometric studies on the Providence cultures show that there is considerable variation in the relative rates of oxidation of phenylalanine and histidine by different strains. The phenylalanine microtest was particularly sensitive; after 15 min. incubation *Proteus* spp. and Providence cultures produced a deep green colour on the addition of ferric chloride. Other species of enterobacteria were negative (Table 2), but after 24 hr. incubation weak reactions were occasionally obtained with strains of *Klebsiella aerogenes*, *Escherichia coli* and some paracolons, so that the enzyme is not completely absent from these species.

Gluconate oxidation, originally used by Haynes to distinguish the pseudo-

monads, is a useful test for klebsiellas. Providence and *Proteus rettgeri* strains do not oxidize gluconate, but a few strains of the other *Proteus* spp. give positive results in this test. The malonate test is invariably negative with *Proteus* and Providence cultures.

Table 1. *Cultural characters of Proteus and Providence strains*

	<i>P. mirabilis</i>		<i>P. morganii</i>		<i>P. rettgeri</i>		<i>P. vulgaris</i>		Providence strains	
	+	-	+	-	+	-	+	-	+	-
Biochemical tests										
Motility	22	1	22	2	8	1	2	5	144	9
Gluconate test	13	10	1	23	0	9	0	7	0	153
Malonate test	0	23	0	24	0	9	0	7	0	153
Phenylalanine deamination	23	0	24	0	9	0	7	0	151	2
Indole	0	23	24	0	8	1	7	0	151	2
H ₂ S produced	22	1	24	0	9	0	7	0	153	0
MR	8	15	8	16	7	2	5	2	101	52
VP	17	6	0	24	0	9	1	6	1	152
Gelatin liquefied	18	5	0	24	0	9	4	3	0	153
Urea hydrolysed	23	0	24	0	9	0	7	0	11	142
Citrate utilized	6	17	1	23	8	1	4	3	96	57
Acid from										
Glucose	23	0	24	0	9	0	7	0	153	0
Arabinose	1	22	0	24	3	6	0	7	5	148
Xylose	23	0	5	19	0	9	6	1	17	136
Rhamnose	0	23	0	24	7	2	0	7	5	148
Lactose	0	23	0	24	0	9	0	7	2	151
Sucrose	19	4	1	23	9	0	7	0	146	7
Maltose	1	22	0	24	0	9	7	0	9	144
Trehalose	23	0	3	21	0	9	5	2	32	121
Glycerol	22	1	20	4	8	1	7	0	80	73
Mannitol	0	23	0	24	9	0	0	7	17	136
Dulcitol	0	23	0	24	0	9	0	7	3	150
Sorbitol	0	23	0	24	0	9	0	7	12	141
Inositol	0	23	0	24	8	1	0	7	32	121
Adonitol	0	23	0	24	9	0	0	7	110	43
Salicin	4	19	0	24	5	4	5	2	9	144

Table 2. *Microtests for Proteus and Providence strains*

	No. tested	Amino acid decarboxylase			Deaminase
		Arginine	Lysine	Histidine	Phenylalanine
		No. positive			
<i>P. vulgaris</i>	6	0	0	0	6
<i>P. mirabilis</i>	22	0	0	0	22
<i>P. morganii</i>	23	0	0	0	23
<i>P. rettgeri</i>	9	0	0	0	9
Providence (1)	11	0	0	0	11
(2)	47*	1†	0	2†	47
Other enterobacteria	75	65	42	10	0

* Toluene added to the buffer in these tests (see text).
 † Weak reactions.

The decarboxylase tests showed that unlike most of the other enterobacteria tested, *Proteus* spp. and Providence cultures did not decarboxylate arginine or lysine (Table 2). To later tests toluene was added to the phthalate buffer as suggested by Møller (1954*a*) and under these conditions glutamic acid decarboxylase was detected in 45 of 47 Providence cultures, most of the *Proteus* cultures, and 7 of 8 *Escherichia coli* cultures (Table 3). Møller (1954*c*) described the decarboxylase reactions of a large number of enterobacteria which he has examined by a similar method. He increased the sensitivity of this test, and a simplified test with growing cultures (Møller, 1955), by using an acid medium to obtain the maximum amount of enzyme. We have found that some decarboxylase activity can be detected in *E. coli* cultures grown on Lemco agar at a neutral pH reaction and that the glutamic decarboxylase activity of *Proteus rettgeri* measured manometrically, is affected less than that of *E. coli* by the original pH reaction of the growth medium.

Table 3. *Glutamic acid decarboxylase shown by toluene-treated suspensions*

	No. tested	No. positive
<i>P. vulgaris</i>	5	4
<i>P. mirabilis</i>	9	8
<i>P. morganii</i>	7	1
<i>P. rettgeri</i>	6	6
Providence	47	45
<i>E. coli</i>	8	7

A washed suspension of *Clostridium welchii* used for the estimation of glutamic acid was tested by the micromethod and gave positive results for glutamic acid decarboxylase in 5–10 min. This mixture was then examined by paper chromatography, using 80 % phenol as solvent, and γ -aminobutyric acid could be detected. A suspension of *Proteus rettgeri* (NCTC 7477) gave a positive glutamic acid decarboxylase reaction in 30 min. and also a similar chromatogram.

DISCUSSION

Other workers have pointed out the relation between species of the genus *Proteus* and the Providence group (Kauffmann, 1951; Møller, 1953, 1954*a–c*; Ewing *et al.* 1954; Singer & Bar-Chay, 1954; Buttiaux, Osteux, Fresnoy & Moriametz, 1954). In general, our results are in agreement, but we have added more evidence in the tendency to swarm and in the production of H_2S by Providence strains, as well as the amino acid reactions. The addition of a known sulphur source to our medium accounts for the greater sensitivity of our test for H_2S (Clarke, 1953). Only a few Providence strains split urea, but as Singer & Bar-Chay (1954) showed, the urease-positive strains do not differ in any other respect from the urease-negative strains.

King (1953) described leucine and valine decarboxylases of *Proteus vulgaris* which were not present in other species of Enterobacteriaceae. We obtained positive results for almost all *Proteus* and Providence cultures by our micro-test method. As this enzyme has a neutral pH optimum, and leucine is a good substrate for the deaminase, the leucine decarboxylation was probably

obscured by its deamination. A washed suspension of *P. rettgeri* (NCTC 7477) was tested manometrically (pH 6·8, 0·025 M-phosphate buffer, gas phase N₂, with acid tip at the end of the reaction period). The suspension failed to decarboxylate leucine, but a small amount of CO₂ was produced from endogenous substrate by the cells alone. Under these conditions *P.morganii* (NCTC 3667) was shown to decarboxylate leucine, but *P. mirabilis* (NCTC 5887), which had given a doubtful reaction in Proom & Woiwod's amine test, also failed to decarboxylate leucine.

Proteus rettgeri was separated from other *Proteus* spp. by Proom & Woiwod (1951) because it gave negative results in a test for amines which were produced from valine and leucine in their medium, and did not require nicotinic acid for growth. Kauffmann (1953) went a step further and created separate genera for *P. rettgeri* (Rettgerella), *P. morganii* (Morganella) and Providence strains (Providencia), leaving *Proteus* with only two species, *P. vulgaris* and *P. mirabilis*. The similarities between *Proteus* spp. and the Providence group suggest a natural relationship, and we do not agree with Kauffmann (1953) that separate genera are required for these different species, an opinion which he revised in 1954. We think that our work adds to the evidence linking the Providence group to the genus *Proteus*. The first description of a Providence culture appears to be that of *Bacillus inconstans* by Ornstein (1921), which was amplified by Braun & Löwenstein (1923-4). Consequently the correct specific epithet will be *inconstans*, and the species should be designated as *Proteus inconstans* (Ornstein) nov. comb.

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