IDENTIFICATION AND TYPING OF BACTERIA

Media and tests to simplify the recognition and identification of members of the *Proteeae*

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Several important and diverse human pathogens are found in the tribe *Proteeae*. By identifying and concentrating on key biochemical reactions, it has been possible to devise six simple media that permit the identification of all the important members of the tribe with ease, speed and accuracy. This was confirmed by optional additional confirmatory media and tests.

Introduction

The tribe *Proteeae* comprises three genera, *Proteus*, *Morganella* and *Providencia*, and 10 species. Its members have diverse characteristics. Some, like *P. myxofaciens* – which was isolated from larvae of the gypsy moth (*Porthetria dispar*) [1] and *Prov. heimbachae* [2] which has been isolated from penguin faeces – have as yet never been associated with infections in man. On the other hand, *P. mirabilis* is one of the most frequently encountered organisms in the diagnostic medical microbiology laboratory.

Members of the tribe are associated with a wide variety of infections. After *Escherichia coli*, *P. mirabilis* is probably the commonest cause of urinary tract infection, particularly in elderly patients of both sexes [3]. Unlike *E. coli*, it has a predilection for the upper urinary tract, where it can cause stone formation and pyelonephritis. *Prov. stuartii* is also a major cause of urinary tract infection particularly in long-term catheterised elderly patients [4, 5]. However, *Prov. alcalifaciens* is rarely associated with urinary tract infections but is now a recognised cause of diarrhoea [6, 7]. *M. morganii*, which can be isolated from diarrhoeal stools in the absence of other known bacterial enteric pathogens, has often been suspected to cause diarrhoea but this has yet to be proved. Excepting the two organisms that are not associated with human infection, all other members of the tribe are also encountered frequently in infections of wounds, blood and sputum.

Members of the *Proteeae* also differ widely in their

antibiotic susceptibility. For example, *Prov. stuartii* isolates are some of the most antibiotic resistant of the Enterobacteriaceae, whereas problems of antibiotic resistance are unusual in *Prov. alcalifaciens* and *Proteus* spp. The antibiotic susceptibility of *M. morganii* strains is very different from that of *Proteus* and *Providencia* spp.

Therefore, accurate and rapid identification of members of the *Proteeae* is essential. This should not be difficult, although it frequently does not happen. *Prov. stuartii* is frequently misidentified, particularly when some commercial test kits are used, possibly because it may reveal traits of the plasmid-borne genes isolates often carry. Moreover, the description 'indole-positive *Proteus*' which is often seen in the literature may be a misnomer for any lactose negative, indole and urease forming member of the Enterobacteriaceae and as such could be *M. morganii*, *Prov. rettgeri*, *Prov. stuartii* or *P. vulgaris*. The latter organism is the only true 'indole-positive *Proteus*'.

The purpose of this study was to evaluate the key biochemical reactions useful in the identification of members of the *Proteeae* and combine them in an appropriate way so that an accurate, speedy and unambiguous identification of each species within the tribe could be made with the minimal number of tests and volume of media.

Materials and methods

Bacterial strains

A collection of 198 strains was examined. This comprised *P. mirabilis* (24 strains), *P. vulgaris* (24), *P. penneri* (15), *M. morganii* subsp. *morganii* (26), *M.*

morganii subsp. *sibonii* (25), *Prov. rettgeri* (17), *Prov. stuartii* (14), *Prov. alcalifaciens* (24), *Prov. rustigianii* (17) and *Prov. heimbachae* (12). The strains were selected at random from a large collection of isolates from around the world. They had been identified by extensive biochemical tests and all gave reactions characteristic for the species. The collection included NCTC and ATCC reference strains and, excepting isolates of *Prov. heimbachae* (kindly donated by Dr H. E. Müller) from penguin faeces, all were clinical isolates from man.

Media and tests

Phenylalanine deaminase (PAD) medium contained Tryptone water (Oxoid CM 87) 1.5 g, L-phenylalanine 1 g and agar 1.3 g in 100 ml of distilled water. After sterilisation at 121°C for 15 min, it was dispensed in either 200- μ l amounts into microwell plates or 2-ml amounts into sterile tubes which were sloped.

Urea-indole medium was prepared by supplementing, when cool, tryptone water (1.5 g in 100 ml of distilled water) which had been sterilised at 121°C for 15 min, with filtered sterile urea (40% w/v in water) to 2% w/v and with a 1 in 200 dilution of phenolphthalein 1% in isopropanol. The medium was dispensed aseptically in 1-ml volumes into sterile tubes.

Ornithine decarboxylase medium contained tryptone water 0.5 g, L-ornithine.HCl 1 g and 2.5 ml of bromocresol purple dye 0.08% in 100 ml of distilled water and was sterilised at 121°C for 15 min. When cool, the medium was supplemented aseptically with sterile glucose 10% w/v in water to 0.1% w/v and dispensed aseptically in either 200- μ l amounts into microwell plates or 2.5-ml amounts into screw-capped bottles.

Peptone water sugars were made by supplementing, aseptically, when cold, Peptone water (Oxoid CM 9) (1.5 g and 2.5 ml of bromocresol purple 0.08% in 100 ml of distilled water) which had been sterilised at 121°C for 15 min, with a sugar or sugars, each to a final concentration of 1% w/v, from a sterile (steamed for 1 h) stock solution of each sugar, 10% w/v in water. They were dispensed in either 200- μ l amounts into microwell plates or 1-ml amounts into sterile tubes.

All media were inoculated with one drop (c. 25 μ l) of either a nutrient broth culture of the strain incubated overnight at 37°C, or a suspension of a colony in saline, and incubated at 37°C. For tests in microwell plates, after inoculation of the media, drops of sterile liquid paraffin were added to wells containing ornithine decarboxylase medium and the plates were sealed with tape. This was punctured over wells containing PAD medium. Tests were read after incubation for 16–24 h. Development of a dark green

colour a few seconds after the addition of a drop of aqueous ferric chloride 10% w/v to the PAD medium indicated phenylalanine deaminase activity and the formation of phenylpyruvic acid. The formation of a pink colour in the urea-indole medium indicated urease formation. The subsequent development of a pink colour at the surface of this medium after the addition of Ehrlich's or Kovac's reagent indicated indole formation. Growth and the development of alkalinity (blue colouration) in the ornithine decarboxylase medium indicated formation of this enzyme. Fermentation of sugars resulted in a pH change to acid which was indicated by a colour change from blue to yellow.

Results and discussion

The results indicate that accurate identification of members of the *Proteeae* can be made satisfactorily with only a few tests if selection is made of key reactions unique to the tribe or a given species within it.

Excepting the recently defined rare organisms *Tatumella ptyseos* [8] and *Rahnella aquatilis* [9], the ability to oxidatively deaminate certain amino acids, usually phenylalanine or tryptophan, to the corresponding keto acid and ammonia, is a feature among the Enterobacteriaceae found only in strains belonging to the *Proteeae*. Upon the addition of aqueous ferric chloride (10%) the deaminated product of phenylalanine becomes dark green and later fades, whereas that of tryptophan remains red/brown. Members of the *Proteeae* are also readily recognised by their formation of a similar coloured product, but this time of a diffusible melanin-like pigment, when grown under aerobic conditions on media containing iron and certain L aromatic amino acids including tryptophan [10, 11]. This reaction is unique to *Proteeae* within the Enterobacteriaceae, but the same or a similar reaction and the formation of a product of a similar colour is also given by some other bacteria including species of *Pseudomonas* [12], *Aeromonas* and *Acinetobacter* [13] and *Legionella* [14].

Because of the importance of the test, and the possible confusion of the brown colour with that of the medium, the phenylalanine deaminase test with its distinctive coloured reaction product was used here in preference to that of tryptophan deaminase and red-brown pigment production.

All strains tested formed phenylalanine deaminase. However, with one isolate of *Prov. heimbachae* the PAD reaction was weak after incubation for 24 h. It is important that the test is done under aerobic conditions. Although PAD-negative isolates of *Proteeae* may exist, they are rare. In general, therefore, any isolate which

than they form it and hence appear to be indole negative. In order to reduce these variables to a minimum, the indole test was read after incubation of urea-indole medium containing tryptone water for 24 h as recommended [15].

In general, species in the *Proteeae* that form urease produce it in large amounts and, in unbuffered media, the pH rises to values exceeding pH 8.3. To detect this yet eliminate detection of weak urease production by other organisms, the pH indicator selected was phenolphthalein which is colourless below pH 8.3 but pink at a more alkaline pH. Experimentation showed that supplementing tryptone water with urea and phenolphthalein to produce the urea-indole medium did not affect the ability of any isolate to form either indole or urease or both. Both reactions could be read without confusion. After incubation and reading of the urease reaction, the addition of a few drops of Erlich's or Kovac's reagent (which is acidic) to the surface of the medium, caused a layer of pink phenolphthalein to become colourless and be separated from a deeper red layer which formed in the reagent at the surface of indole positive isolates. Among the *Proteeae*, *Proteus* spp., *M. morganii*, *Prov. rettgeri* and some (up to 30%) isolates of *Prov. stuartii* are the only ones that produce urease. The test for indole formation and urease production was always performed in tubes because a weak indole reaction above a positive urease reaction was difficult to read in wells outwith the perimeter of microwell plates.

The results of tests for urease and indole formation when combined with the results of previous tests permitted the unambiguous identification of *P. mirabilis*, *P. vulgaris*, *P. penneri*, *M. morganii*, *Prov. rettgeri* and *Prov. heimbachae* (Table 1).

Trehalose is fermented by many strains of all species of *Proteus* and, therefore, is not a very useful discriminating test for this genus. However, only c. 10% of isolates of *M. morganii* ferment trehalose. These are now called *M. morganii* subsp. *sibonii* and are distinct from *M. morganii* subsp. *morganii* isolates

that do not ferment this sugar [16]. Among *Providencia* strains, trehalose fermentation is restricted solely to virtually all isolates of *Prov. stuartii* of all biogroups. The results of this test in combination with the previous tests now permitted identification of the subspecies of *M. morganii* and *Prov. stuartii* (Table 1).

Maltose fermentation is an important reaction in that, in the tribe, it is positive only with *Proteus* spp. other than *P. mirabilis*, and by *Prov. heimbachae* in which it is a delayed reaction. On the other hand, adonitol fermentation is restricted in the tribe to nearly all isolates of most *Providencia* spp. except *Prov. rustigianii* and some isolates of *Prov. stuartii*. By combining both sugars into one test, added confirmation was given to the identity of isolates of the different species of *Proteus* and, in combination with all the previous tests, identification of isolates of *Prov. alcalifaciens* (adonitol positive) and *Prov. rustigianii* (adonitol negative) could now be made (Table 1).

The media and tests described are those which will give accurate identification of isolates of species within the *Proteeae* from the least number of tests. They were selected from many other tests and developed, initially with a small number of representative strains of all the species in the tribe except *P. myxofaciens*, in order to give results which could be read after incubation for 16–24 h. For example, sugars were used in media at a 1% concentration because it was found that some fermentative isolates did not show a positive reaction within 24 h when a lower concentration of the sugar was used. *P. myxofaciens* was not included in the study because it is not associated with man and there is only one isolate. When development of media and tests was complete, the 198 isolates to be examined were coded to hide their identity before test media were inoculated.

The results and identity of isolates are presented in Table 2. The results of reactions in tubes were identical to those in microwells. From Table 2 it can be seen that all except two of the 198 isolates were

Table 2. Results of accuracy of tests in identifying organisms in the tribe *Proteeae*

Species	Number of isolates tested	Number (%) of isolates whose reactions were consistent with the identity of the organism tested
<i>P. mirabilis</i>	24	24 (100)
<i>P. vulgaris</i>	24	24 (100)
<i>P. penneri</i>	15	15 (100)*
<i>M. morganii</i> subsp. <i>morganii</i>	26	26 (100)
<i>M. morganii</i> subsp. <i>sibonii</i>	25	25 (100)
<i>Prov. rettgeri</i>	17	17 (100)
<i>Prov. stuartii</i>	14	14 (100)
<i>Prov. alcalifaciens</i>	24	23 (96)*
<i>Prov. rustigianii</i>	17	17 (100)
<i>Prov. heimbachae</i>	12	11 (92)*

*For explanation see text.

Table 3. The confirmatory reactions of organisms in the *Proteeae*

Species	Mannose	Maltose	Ornithine	Mannitol	Trehalose	Galactose	Adonitol	Inositol	Rhamnose
<i>P. mirabilis</i>	-	-	+	-	+	+	-	-	-
<i>P. vulgaris</i>	-	+	-	-	v	+	-	-	-
<i>P. penneri</i>	-	+	-	-	v	+	-	-	-
<i>M. morganii</i>	+	-	+	-	-	+	-	-	-
subsp. <i>morganii</i>	+	-	+	-	-	+	-	-	-
subsp. <i>sibonii</i>	+	-	+	-	-	+	-	-	-
<i>Prov. rettgeri</i>	+	-	+	+	-	+	+	+	(+)
<i>Prov. stuartii</i>	+	-	-	-	+	+	+	+	-
<i>Prov. alcalifaciens</i>	+	-	-	-	-	+	+	-	-
<i>Prov. rustigianii</i>	+	-	-	-	-	+	+	-	-
<i>Prov. heimbachae</i>	+	-	-	-	-	+	+	v	+

+, formation of enzyme or fermentation of sugar; -, enzyme not formed or sugar not fermented; (), reaction of most isolates; v, different isolates give different results; the shaded areas denote the most important reactions in establishing identity.

identified correctly. One isolate of *Prov. alcalifaciens* gave a negative indole reaction and thereby was wrongly identified as *Prov. heimbachae*. Further testing of the isolate with Kovac's reagent, a more sensitive detector of indole than Ehrlich's reagent, confirmed it to be a weak indole-producing isolate of *Prov. alcalifaciens*. One isolate of *Prov. heimbachae* that gave a delayed PAD positive reaction would have been discarded as not belonging to the tribe. Six isolates among those correctly identified as *P. penneri* through being isolates of *Proteus* that were indole negative and ornithine decarboxylase negative, were unusual in giving delayed results (some for several days) for maltose fermentation although they fermented sucrose within 24 h. However, their identity would have still caused confusion even if a more extensive range of tests had been performed. *P. myxofaciens* would have been identified as *P. penneri* on the basis of the tests but could have been distinguished in that *P. myxofaciens* is the only *Proteus* spp. unable to ferment xylose.

Although additional confirmatory tests were not and should not be required, they were prepared and tested (Table 3). The following information and tests have been found to be helpful in identifying occasional unusual isolates. With very rare exceptions, true swarming on appropriate media is restricted to some isolates of *Proteus* spp. only. *P. mirabilis* is the only member of the tribe unable to ferment both mannose and maltose. Both sugars can be combined as a single test. *P. vulgaris* and *P. penneri* never form ornithine decarboxylase but acidify maltose. They differ in their ability to form indole. *P. penneri* isolates are uniformly salicin and aesculin negative, which is not the case for some *P. vulgaris* isolates. *M. morganii* isolates are readily recognised by their ability to ferment mannose and form ornithine decarboxylase. *Prov. rettgeri* is the only mannitol-fermenting member of the tribe. *Prov. stuartii* is the only trehalose-fermenting species of *Providencia* and, together with *Prov. rustigianii*, they are the only species of *Providencia* unable to ferment adonitol. *Prov. alcalifaciens* is the only *Providencia* spp. unable to ferment galactose. *Prov. rustigianii* isolates are distinctive by their inability to ferment adonitol, inositol or trehalose. *Prov. heimbachae* isolates are distinguished by the ability, shared in the tribe only with some isolates of *Prov. rettgeri*, to ferment rhamnose, and by the ability, unique in the tribe, of growing in the presence of KCN.

When the isolates, whose identity had been deduced from the results of the tests in Table 1 or as explained above, were examined by the confirmatory tests in Table 3, with the exception of the late maltose-fermenting isolates of *P. penneri*, all gave reactions as detailed in Table 3. Thus the accuracy of the methods, media and selected tests for identifying members in the *Proteeae* was confirmed.

This work shows that through knowledge of the key biochemical reactions of members of the *Proteeae*, it has been possible to devise a small number of media and tests that will enable the identification of all the important yet diverse members of the tribe to be made with speed, ease and accuracy.

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