Decarboxylase and other Reactions of some Gram-negative Rods

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SUMMARY

Tests for decarboxylation of amino acids, oxidation of gluconate, growth in potassium cyanide broth, utilization of malonate and deamination of phenylalanine were applied to 269 strains of aerobic Gram-negative rods, other than members of the Enterobacteriaceae. The results indicated that some of these tests might be of value in the identification of organisms outside this family. The decarboxylase and KCN tests permitted separation of *Pasteurella septica* from *P. pestis* and *P. pseudotuberculosis*. Phenylalanine deaminase was detected in only one of the strains tested. Possible mis-classification of *Pseudomonas maltophilia* and of the glanders bacillus was suggested.

INTRODUCTION

Haynes (1951) used the oxidation of gluconate to ketogluconate as a means of characterizing *Pseudomonas aeruginosa*; a test based on this reaction has proved valuable within the Enterobacteriaceae (see Cowan, 1955; Shaw & Clarke, 1955). Tests for amino acid decarboxylase activity, utilization of malonate, deamination of phenylalanine and ability to grow in the presence of potassium cyanide are of value in characterizing members of this family, and it has been suggested (Sneath & Cowan, 1958) that genera such as *Pasteurella* and *Vibrio* should be included in surveys of the Enterobacteriaceae. These tests have now been applied to a selection of aerobic Gram-negative rods (other than enterobacteria); the results are reported here.

METHODS

Strains. A total of 269 strains maintained in the National Collection of Type Cultures was used.

Biochemical tests used were as follows.

Amino acid decarboxylase activity. By Møller's (1955) method, with incubation of the cultures for 4 days. Falkow's (1958) method was not used since, at least among the Enterobacteriaceae, the results do not always agree with Møller's method; Ewing (1960) regarded the latter as the standard reference method for use in taxonomy.

Gluconate oxidation. By the method of Shaw & Clarke (1955); cultures were tested for the presence of reducing substances after incubation for 2 and 7 days.

Growth in the presence of KCN. By the method described by Rogers & Taylor (1961), a modification of that of Møller (1954); incubation was for 2 days at 37° or 3 days at 30° or 22°.

Malonate utilization and phenylalanine deamination. By the combined medium of Shaw & Clarke (1955); cultures were examined after incubation for 1 and 7 days.

For the detection of phenylalanine deaminase in the *Proteus* and *Providencia* groups, Ewing, Davis & Reavis (1957) found this combined medium to be less satisfactory than phenylalanine agar.

Cultures were incubated at 37° with the following exceptions: *Chromobacterium amethystinum*, *Pasteurella pestis*, *Pseudomonas chlororaphis*, *P. diminuta*, *P. fluorescens*, *P. graveolens*, *P. mucidolens*, *P. syncyanea*, *Vibrio metchnikovii*, *V. percolans* and *V. proteus* at 30°; *Chromobacterium lividum* at 22°.

RESULTS

The results are shown in Table 1. Because of their exacting nutritional requirements, strains of the genus *Haemophilus* were not included in this work. Huet (1959), however, reported the presence of lysine decarboxylase in some strains of *H. influenzae*. The two strains of *Bordetella pertussis* tested were in phase IV (Leslie & Gardner, 1981) and grew well on simple media. Media were not supplemented to ensure the growth of the more fastidious organisms. In a few instances a negative result may have been due to failure of the organism to grow under the conditions of the test; e.g., the three strains of *Moraxella lacunata* failed to grow in the basal medium used for the KCN test.

Decarboxylases

Attempt to distinguish the reactions whereby arginine is metabolized (Møller, 1955) were not made. A positive reaction is here referred to, for convenience, as indicating the presence of arginase; for our purpose it was immaterial whether an arginase, a decarboxylase or a dihydrolase system was involved. Arginase activity was pronounced in *Aeromonas* spp., *Chromobacterium violaceum*, *Loefflerella* spp. and most strains of *Pseudomonas*. Strains of *Brucella* spp. (except *B. ovis*) showed arginase activity after incubation for 6–10 days but are here recorded as negative.

Ornithine decarboxylase activity was found in most strains of *Pasteurella septica* and in some strains of *P. haemolytica* var. *haemolytica* but not in *P. haemolytica* var. *ureae*. Lysine decarboxylase activity was detected in *Pseudomonas maltophilia* and in the genus *Vibrio* where it was usually associated with ornithine decarboxylase; this is in agreement with the results of Ewing, Davis & Edwards (1960) and Hugh & Ryschenkow (1961a). With strains of *Alcaligenes* difficulty was encountered in reading the reactions, due to alkalization of the controls; this effect was not found with other organisms which did not produce acid from glucose (e.g. *Bordetella bronchiseptica*). Ewing *et al.* (1960), who studied only 5 strains of *A. faecalis*, detected weak arginase activity in one of them.

Gluconate oxidation

The results of this test are liable to different interpretations by different workers. Shaw & Clarke (1955) stated 'a positive test showed a yellowish-brown precipitate of cuprous oxide and there was a characteristic smell of decaying cabbage'. Haynes (1951) after heating and rapid cooling of his reaction mixtures set them aside overnight and then compared the amount of precipitate with standards prepared from calcium ketogluconate. A worker familiar with the qualitative examination of urine for 'sugar' would record a green precipitate (indicating about 0.5% glucose) as +, and a yellowish one (about 1%) as ++. In the present work a

Table 1. The reactions of some Gram-negative bacilli

Organism	No. of strains tested	Number of positive reactions								
		Decarboxylases			Gluconate oxidation		Growth in KON	Malonate utilization		Phenylalanine deaminase
		Arginine	Lysine	Ornithine	At 2 days	At 7 days		At 1 day	At 7 days	
<i>Achromobacter anitratus</i>	11	.	.	.	1w	1	9	.	2	.
<i>A. equuli</i>	7	.	.	.	.	.	1	.	.	.
<i>Actinobacillus lignieresii</i>	6	.	.	.	.	.	.	.	.	.
<i>Aeromonas</i> spp.	7	7	.	.	7	5	7	.	1w	.
<i>Alcaligenes denitrificans</i>	1	.	.	.	.	.	1	.	.	.
<i>A. faecalis</i>	5	1w	.	.	.	1	4	.	1w	1
<i>A. viscosus</i>	1	.	.	.	.	.	1	.	.	.
<i>Bordetella bronchiseptica</i>	11	.	.	.	.	.	11	.	.	.
<i>B. paraptussis</i>	3	.	.	.	.	.	.	.	.	.
<i>B. pertussis</i> (phase IV)	2	.	.	.	.	.	.	.	.	.
<i>Brucella abortus</i>	13	.	.	.	.	.	.	.	.	.
<i>B. melitensis</i>	7	.	.	.	.	.	.	.	.	.
<i>B. neotomae</i>	4	.	.	.	.	.	.	.	.	.
<i>B. ovis</i>	1	.	.	.	.	.	.	.	.	.
<i>B. suis</i>	7	.	.	.	.	4	.	.	.	.
<i>Chromobacterium amethystinum</i>	1	.	.	.	.	.	.	.	.	.
<i>C. lividum</i>	3	1	.	.	.	.	2v	.	.	.
<i>C. typhi-flavum</i>	1	.	.	.	.	1	1	.	1	.
<i>C. violaceum</i>	14	14	.	.	.	.	14	.	.	.
<i>Flavobacterium meningosepticum</i>	1	.	.	.	.	.	.	.	.	.
<i>Loefflerella mallei</i>	9	9	.	.	.	.	5	.	.	.
<i>L. pseudomallei</i>	9	9	.	.	.	.	9	.	1	.
<i>Lophomonas alcaligenes</i>	1	.	.	.	.	.	.	.	1	.
<i>Moraxella bovis</i>	3	.	.	.	.	.	.	.	.	.
<i>M. lacunata</i>	3	.	.	.	.	.	.	.	.	.
<i>M. liquefaciens</i>	1	.	.	.	.	.	.	.	.	.
<i>M. woelfii</i>	3	.	.	.	.	.	.	.	.	.
<i>Pasteurella haemolytica</i>	4	.	.	2	.	.	3v	.	.	.
<i>P. haemolytica</i> var. <i>ureae</i>	4	.	.	.	.	.	.	.	.	.
<i>P. pestis</i>	10	.	.	.	.	.	.	.	.	.
<i>P. pseudotuberculosis</i>	20	.	.	.	.	.	.	.	.	.
<i>P. septica</i>	26	.	.	23	.	.	24v	.	.	.
<i>Pseudomonas aeruginosa</i>	14	14	.	.	14	14	14	3	14	.
<i>P. chlororaphis</i>	1	1	.	.	1	1	.	.	1	.
<i>P. diminuta</i>	1	.	.	.	.	.	.	.	.	.
<i>P. fluorescens</i>	4	4	.	.	4	4	2	.	3	.
<i>P. graveolens</i>	1	1	.	.	.	.	.	.	.	.
<i>P. maltophilia</i>	3	.	3	.	.	.	3	.	3	.
<i>P. mucidolens</i>	1	.	.	.	.	.	1	.	.	.
<i>P. ovalis</i>	2	2	.	.	1	1	.	1	2	.
<i>P. polycolor</i>	1	1	.	.	1	1	1	.	1	.
<i>P. synyanea</i>	1	1	.	.	.	.	.	.	.	.
<i>P. spp.</i> (achromogenio)	5	5	.	.	.	.	3v	1	4	.
<i>Vibrio alcaligenes</i>	3	.	.	.	.	.	.	.	.	.
<i>V. cholerae asiaticae</i>	14	.	14	14	.	.	4	.	.	.
<i>V. el Tor</i>	9	.	9	9	3	2	7	.	.	.
<i>V. metchnikovii</i>	1	.	1	.	.	1	.	.	.	.
<i>V. percolans</i>	1	1w	.	.	.	.	.	.	.	.
<i>V. proteus</i>	2	.	2	.	.	.	.	.	.	.
<i>Vibrio</i> spp.	6	.	6	6	4	2	5	.	.	.

v = variable results. w = weak reaction. . = negative.

strain of *Klebsiella aerogenes* was used as a positive control organism; hence the results of positive tests are strong reactions, indicated by a yellow to dark brown precipitate after heating. Traces of green precipitate may have been due to excess phosphate and were ignored. Some tests which were recorded as negative showed, after standing overnight at room temperature, a small precipitate of cuprous oxide; this was most evident with *Loefflerella pseudomallei* but was also noted with the strain of *Lophomonas alcaligenes* and with some strains of *Achromobacter anitratus*.

Strong gluconate-oxidizing ability was shown by *Aeromonas* and most *Pseudomonas* strains. Strains of *P. aeruginosa* gave similar results when tested after 2 and 7 days, but two of the aeromonads were positive at 2 and negative at 7 days;

presumably the ketogluconate had undergone further metabolism. (Since opinions on the taxonomy of the genus *Aeromonas* are conflicting: see Eddy, 1960; Ewing, Hugh & Johnson, 1961, attempts to distinguish species within this genus have not been made). Occasional strains of *Achromobacter anitratus*, *Alcaligenes* and *Vibrio* spp. showed gluconate oxidation, as did the single available strain of *Chromobacterium typhi-flavum*. This latter organism has not yet been allotted to a suitable taxon; other strains received under this designation have all been re-characterized as *Enterobacter cloacae*. It is not a chromobacterium as defined by Sneath (1960), who reported the gluconate reaction of *C. lividum* as often positive and that of *C. violaceum* as doubtful or negative. Neither of these *Chromobacterium* species showed gluconate oxidation in our study, nor did *C. amethystinum* which Sneath (1960) regarded as a gelatinous psychrophilic chromobacterium; our strain will now grow at 37°. Three of 4 American strains and 1 of 3 Danish strains of *Brucella suis* appeared to be gluconate-positive, after incubation for 7 days. All strains which appeared to oxidize gluconate were tested in control medium without gluconate to show the absence of reducing substances from any capsular or slime material produced. False positives were not observed.

Growth in the presence of KCN

With the exception of *Moraxella lacunata*, *Brucella ovis* and a few other strains of *Brucella* spp., all the organisms tested grew in the basal medium without added cyanide. Strains of *Pasteurella septica* generally grew in the presence of KCN but some gave variable results in different tests. Mollaret (1961) reported the variability of *P. septica* in this test. Hugh & Ryschenkow (1961*a*) noted subspecies differences with the KCN test in their study of 152 strains of *Vibrio comma* (*V. cholerae asiaticae*). Our results indicated the El Tor variety to be less sensitive to the presence of cyanide. Four of five strains of *Alcaligenes faecalis* grew in KCN broth; Doxiadis, Pavlatou & Chrysosostomidou (1960) reported 33 strains of 'Bacillus faecalis alcaligenes' to be KCN-negative. The KCN-sensitive strain (NCTC 1347) was considered by Galarneault & Leifson (1956) to be one of *Lophomonas alcaligenes*. Fey (1959) reported that 8 of 9 strains of *A. faecalis*, 3 strains of 'B. viscosum equi' and 9 strains of *Bordetella bronchiseptica* did not grow in KCN broth: in our hands, all strains of *B. bronchiseptica* and 1 of 7 strains of *Achromobacter equuli* (*Actinobacillus equuli* in *Bergey's Manual*, 1957) grew in the presence of KCN.

Utilization of malonate

Use of malonate after incubation for 24 hr. was shown only by some strains of *Pseudomonas*. After incubation for 7 days, strong positive reactions were given by many *Pseudomonas* spp., two strains of *Achromobacter anitratus* and the single strains of *Chromobacterium typhi-flavum* and *Lophomonas alcaligenes*; a few strains of other organisms gave weak reactions.

Many strains of *Achromobacter anitratus* caused some alkalization of the medium but by comparison with a known positive (*Klebsiella aerogenes*) no difficulty was encountered in reading the results. Pigment production at the surface of the medium by pigmented pseudomonads caused some difficulty in determining the use of malonate; this was overcome by using an indicator-free medium and determining the alkalinity after incubation. The colour of *Chromobacterium violaceum* tended

to interfere and this was overcome in the same way. Our results confirmed Sneath (1960) who reported that chromobacteria rarely used malonate.

Mollaret (1961) reported 3 strains of *Pasteurella pseudotuberculosis* of serotype IV as malonate-positive in 48 hr. at 28° or 4–7 days at 18°; 301 strains of other serotypes were malonate-negative as were *P. pestis* and *P. septica*. These incubation temperatures were not used by us for *P. pseudotuberculosis* and use of malonate was not found in this genus.

Phenylalanine deaminase

The presence of phenylalanine deaminase is no longer considered to be a specific character of the genus *Proteus* although its occurrence in other genera is uncommon. Shaw & Clarke (1955) noted that weak reactions were obtained occasionally with strains of *Klebsiella aerogenes*, *Escherichia coli* and some 'paracolons'. The occurrence of weak reactions for phenylpyruvic acid in a minority of strains of *Aeromonas* spp. was noted by Eddy (1960), Ewing *et al.* (1961) and Lysenko (1961).

The single strain of *Alcaligenes faecalis* (NCTC 415) which gave a positive reaction after incubation for 24 hr. was that designated as 'type' by Winslow, Kligler & Rothberg (1919); phenylpyruvic acid was not detected after incubation for 48 hr. Phenylalanine deaminase activity was not found in any other genus tested in our work, after 1 or 7 days of incubation. The absence of phenylalanine deaminase activity has been reported for *Chromobacterium* (Sneath, 1960), *Pasteurella* (Henriksen & Jyssum, 1961; Mollaret, 1961), *Pseudomonas* (Lysenko, 1961) and *Vibrio cholerae asiaticae* (Hugh & Ryschenkow, 1961*a*).

DISCUSSION

The *Achromobacter-Pseudomonas* group, at present poorly defined, is under investigation by many workers. The strains of *Achromobacter anitratus* studied by us were oxidase-negative non-motile Gram-negative rods or coccobacilli which oxidized glucose but did not reduce nitrate. Buttiaux (1961) separated 'B. anitratum' from *Achromobacter*; by using the arginine hydrolysis test of Sherris, Shoesmith, Parker & Breckon (1959) he found that 7 of 14 strains of 'B. anitratum' hydrolysed arginine but none of 11 strains of *Achromobacter*. Thornley (1960) devised a test for the anaerobic breakdown of arginine which permitted separation of *Achromobacter* and *Pseudomonas* strains. In our study, arginase activity was characteristic of the genus *Pseudomonas*, the exceptions being *P. diminuta*, *P. maltophilia* and *P. mucidolens*. Thornley (1960) noted that *P. diminuta* did not break down arginine in her test and suggested that the organism should be regarded as belonging to some other genus than *Pseudomonas*; Rhodes (1958) thought that *P. diminuta* might be more closely related to *Acetobacter* or *Vibrio*. The absence of lysine decarboxylase from the pseudomonads makes it necessary to consider whether *P. maltophilia* (Hugh & Ryschenkow, 1961*b*) should be included in this genus; Thibault (1961) would exclude it for this reason. The achromogenic pseudomonads tested here were not a homogeneous group in their reactions although all showed arginase activity, but in one strain it was weak and delayed. Buttiaux (1961) found arginine to be metabolized by only 11 of 16 non-pigmented strains of *Pseudomonas*.

Disagreement exists about the taxonomy of the genus *Pseudomonas*. Gaby (1955) considered *P. chlororaphis* and *P. putida* to be indistinguishable from *P. aeruginosa*

on the basis of biochemical and growth characters, whereas *P. fluorescens*, *P. fragi*, *P. graveolens*, *P. mildenbergii*, *P. mucidolens*, *P. ovalis* and *P. putrefaciens* differed primarily from *Ps. aeruginosa* in their optimum growth temperature; *P. mildenbergii* was further found to be indistinguishable from *P. ovalis* and *P. fluorescens* from *P. putrefaciens*. Sneath (1960) considered *P. syncyanea* to be closely related to *P. aeruginosa*, whereas Haynes (1961) reported that a strain (ATCC 9979, NCTC 9943) received as *P. syncyanea* corresponded to the erroneous description of *P. mildenbergii* in *Bergey's Manual* (1957) and was really *P. convexa*. The identity of the phytopathogen *P. polycolor* with *P. aeruginosa* was shown by Elrod & Braun (1942) and Hoff & Drake (1960). Rhodes (1959) considered that further subdivisions of soil- and water-inhabiting fluorescent pseudomonads were not justified beyond *P. fluorescens*. She was of the opinion that *P. convexa*, *P. mildenbergii* and 7 other species agreed well with her definition of *P. fluorescens* and that 9 other species, including *P. ovalis* and *P. syncyanea*, showed only minor differences from it. Lysenko (1961) considered that Rhodes' definition of *P. fluorescens* was too broad and that insufficient account had been taken of other species in the genus. Our results, on the basis of only four tests, cannot be expected to clarify the position, but the single strain of *P. polycolor* reacted in an identical manner with that of strains of *P. aeruginosa*, and some similarities between *P. chlororaphis*, *P. fluorescens* and *P. ovalis* were found.

The finding of ornithine decarboxylase activity in *Pasteurella septica* and the ability of this organism to grow in the presence of KCN are two further criteria for distinguishing it from *Pasteurella pestis* and *P. pseudotuberculosis*. From an analysis of characters with the aid of an electronic computer, Talbot & Sneath (1960) suggested that *Pasteurella septica* was distantly related to these two species, which were closely related to each other.

Leifson (1960) regarded *Vibrio percolans* as a *Lophomonas*; in our study it showed weak arginase activity unlike the type strain (NCTC 9991) of *Lophomonas alcaligenes* (Galarneault & Leifson, 1956). We have studied only the one strain of *L. alcaligenes* although Galarneault & Leifson regarded our strain NCTC 1847 of *Alcaligenes faecalis* as a strain of *L. alcaligenes*. We did not follow these authors in regarding *Vibrio alcaligenes* as synonymous with *L. alcaligenes*.

The presence of arginase activity in *Loefflerella pseudomallei* is in accordance with current opinions on its classification—as *Pseudomonas pseudomallei* in *Bergey's Manual* (1957). *Loefflerella mallei* also showed arginase activity; this organism is listed in *Bergey's Manual* as *Actinobacillus mallei*. Cowan & Steel (1961) noted that *L. mallei* oxidized glucose whereas *A. lignieresii* fermented it, a fundamental difference in their opinion. The reactions given by organisms now listed in *Bergey's Manual* (1957) as species of *Actinobacillus* (e.g. *A. equuli*, *A. lignieresii* and *A. mallei*) serve to indicate that this genus is taking over the function once fulfilled by 'Bacterium' as a convenient place for poorly studied Gram-negative rods.

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Note added in proof

With the exception of *Pseudomonas polycolor*, we did not study any *Pseudomonas* strains from plant sources. Dr B. P. Eddy of the Low Temperature Research Station, Cambridge, has informed us that some of the plant pathogenic pseudomonads he investigated were arginine dihydrolase negative; Dr A. M. Paton of the University of Aberdeen, in a personal communication, has stated that a large proportion of the pseudomonads found as saprophytes or pathogens on plants do not produce 2-ketogluconate from gluconate.

Davis & Park (1962) have reported that the generic name *Lophomonas* is invalid and they have proposed the new generic name *Comamonas* to replace it. The type strain *C. percolans* is maintained as NCTC 1937 and in our paper is called *Vibrio percolans*.

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