Carbon Source Utilization Tests as an Aid to the Classification of Non-fermenting Gram-negative Bacteria

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SUMMARY

Some factors which affect the results of carbon source utilization (CSU) tests were investigated with 187 strains of Gram-negative bacteria, chiefly non-fermentative. There was little difference between the results at 37 and 30 °C. Repetition of tests showed that 5% of the results were non-reproducible. The variability was most pronounced with acetate, maleate and sucrose amongst the substrates and *Loefflerella mallei* amongst the bacteria. Prior induction of the bacterial enzymes before testing did not reduce variability nor show any other advantage. Analysis of the results by numerical taxonomic methods revealed differences in the groups of bacteria derived from the results of CSU tests compared with those derived from conventional biochemical test results. CSU tests readily provide a large number of taxonomic features with the advantage of a simple standardized testing procedure.

INTRODUCTION

Carbon source utilization (CSU) tests have been used for the classification and identification of bacteria for many years in the form of simple tests such as citrate utilization (Koser, 1923; Simmons, 1926). A wide range of carbon sources was first used for classification by Den Dooren de Jong (1926), whose work on pseudomonads was developed by Stanier, Palleroni & Doudoroff (1966). CSU tests have recently been used in the classification of *Acinetobacter* (Baumann, Doudoroff & Stanier, 1968*a*; Pintér & Bende, 1968), *Moraxella* and *Neisseria* (Baumann, Doudoroff & Stanier, 1968*b*), mycobacteria (Tsukamura, 1966), *Nocardia* (Gordon & Mihm, 1957), various non-fermenting bacteria (Gilardi, 1968*a*; *b*), and soil bacteria (Skyring & Quadling, 1969).

The patterns of CSU test results obtained with some non-fermentative Gram-negative bacteria have been described by Snell (1972). Difficulties in the use of these tests prompted an investigation of some of the factors which affect the results. The classification derived by numerical taxonomy on the results of CSU tests was compared with that derived from the results of conventional tests. The value of CSU tests in classification was assessed.

METHODS

Strains. The 187 strains used, mostly of non-fermenting bacteria, have been described by Snell (1972). They were representatives of the genera *Acinetobacter*, *Alcaligenes*, *Bordetella*, *Chromobacterium*, *Flavobacterium*, *Moraxella*, *Neisseria* and *Pseudomonas*.

Conventional tests. These 43 tests include all tests other than CSU tests and comprized: hydrolysis of aesculin, urea and Tween 80; production of β -galactosidase (ONPG test),

hydrogen sulphide, catalase, oxidase, indole and pigment; acid from 1% (w/v) ethanol, glucose, maltose, mannitol and xylose in ammonium salt medium; 1% (w/v) arabinose, glucose, glycerol, maltose and xylose in peptone water medium; and 10% (w/v) lactose agar slopes; growth in Christensen's and Simmons's citrate, KCN broth, and nutrient broth at 5, 22, 37 and 44 °C; gelatin liquefaction; gluconate oxidation; malonate utilization; Møller's decarboxylases (arginine, lysine, ornithine); motility at 22 and 37 °C; reduction of nitrate, nitrite and selenite; oxidation/fermentation test; phenylalanine deamination (PPA test); Thornley's arginine desimidase; tyrosine decomposition and Park's modified Hugh and Lefson test. References to the methods used are given in Snell (1972).

CSU tests. The medium of Owens & Keddie (1968) was used and consisted of a basal mineral medium and two stock vitamin supplement solutions. Although none of the strains showed absolute requirements for the vitamins, they improved the growth of some strains of *Alcaligenes*, *Loefflerella mallei* and *Chromobacterium violaceum*. The fluid medium was solidified with 1% (w/v) agar (Ionagar, Oxoid) and, after addition of the carbon source, distributed in 9 cm Petri dishes in volumes of 33 ml. The carbon source substrates were kept in stock solutions of 10% (w/v) at pH 7.0 and sterilized by filtration through sintered glass. For use they were incorporated in the basal medium at 0.2% (w/v). Asparagine, creatinine, methionine, oxalate, serine, tryptophan and valine were insoluble at 10% and were prepared as suspensions in sterile distilled water and heated at 60 °C for 60 min once only, a treatment which proved adequate to prevent contamination of the medium.

Inoculation of CSU tests. Carbon source media were inoculated with an even light stroke of a wire loop charged with bacteria from a solid medium. Up to 20 strains were inoculated in a Petri dish of 9 cm diam. which left room between inocula to avoid cross-feeding during growth. Application of dense saline suspensions using a dropping pipette or mechanical inoculator was also a satisfactory method for inoculating CSU tests.

Each series of plates for inoculation contained two control plates; one of nutrient agar to demonstrate viability of the inoculum, and a second of the basal mineral medium without added carbon source to indicate any growth due to carry over of nutrient with the inoculum.

Nitrogen sources. Some organisms failed to grow with only the ammonium salts. Horse serum, beef extract, yeast extract, peptone, casein hydrolysate or nutrient broth were not suitable since concentrations which provided adequate nitrogen also provided enough carbon to enable growth without an added carbon source. Also tested separately were methionine 20 mg % (w/v) and an amino acid mixture (Proom, 1955) of DL-glutamic acid, 0.14 mg/ml; L-proline, 0.011 mg/ml; DL-leucine, 0.057 mg/ml; DL-cysteine, 0.002 mg/ml; and DL-methionine, 0.006 mg/ml. The amino acids were prepared as stock solutions in sterile distilled water, heated at 60 °C for 1 h and added to the basal medium to give the stated concentrations.

Conditions of incubation. Cultures were routinely incubated at 37 °C except those of *Pseudomonas fluorescens* and *Chromobacterium lividum* which were incubated at 22 °C. Plates were incubated for 5 days in large tin boxes with a wad of moist cotton-wool in each box to ensure excess humidity. For a trial period, 94 strains were tested for their ability to utilize 10 different carbon sources on duplicate plates incubated at 30 and at 37 °C. Growth at these two temperatures was compared and results were recorded as: growth developing at one temperature and not at the other; growth more luxuriant at either temperature; or no difference in growth at either temperature. All results which showed differences between the two temperatures were repeated.

Induction of enzymes. Overnight cultures of 129 of the nutritionally non-fastidious strains were grown on nutrient agar, then subcultured on to nutrient agar plates each containing

0.2 % (w/v) of one of the 43 carbon sources. After further incubation overnight, the growth on these plates was inoculated on to the basal mineral medium with the given carbon source. The tests were repeated when the results showed differences between the induced and non-induced methods. When the results were still different on the first repeat, they were carried out yet again.

Repetition of carbon source tests. Initially strains were tested up to five times on the same carbon source. Later they were retested only if they had shown differences between the induced and non-induced tests.

Coding and scoring of results, and computer analysis. An S.D.S. 9300 computer at the Medical Research Council Computer Unit, Pentonville Road, London, was used. The classification was carried out with the program 'MINICLASP', a modification by Mr R. Jagoe of 'CLASP' (Rothamsted Experimental Station, Harpenden, Hertfordshire). Negative matches were included and sorting was by single linkage.

The method of coding the results of the conventional tests is described by Snell (1972). A simplified dendrogram in Fig. 1 shows the results of the computer classification of all the strains using conventional test results. The test results of the carbon sources used in the classification were coded as two state qualitative characters, positive (+) or negative (-). Weakly positive results were considered as positive as it was considered easier to distinguish absence of growth from growth, than degrees of poor growth from good growth. As urea, catechol, creatinine and oxalate gave uniformly negative results with all the 187 strains both with and without induction, these substrates were not used in the classification and are not included in the evaluation of results. Acetate, maleate and sucrose gave such poor reproducibility on repeated tests that results from these substrates were not used in the classification.

For comparison, three methods of scoring were used for variable results when these were found for a strain with a given substrate: (i) score 'positive' if a positive result had ever been found; (ii) use the result obtained at the first time of testing; and (iii) score as 'no comparison'. Three separate classifications of the strains were carried out by computer analysis on the CSU test results using a different one of these three methods in each case to score the variable results. The resulting dendrograms are shown in simplified form in Fig. 2, 3 and 4. Simplification of these dendrograms was needed in order to compare them visually as a large number of strains was involved. They were simplified both by joining branches meeting above a chosen similarity level (94 %) and by the omission of branches representing single strains. Some information was lost but the relations between the main groups were retained.

RESULTS

Nitrogen requirements. Amino acids were needed for the growth of all strains of *Moraxella*, *Neisseria*, *Flavobacterium*, *Bordetella parapertussis*, *Pseudomonas maltophilia*, two of the 36 strains of *Acinetobacter* and three of the ten *Comamonas*/*Pseudomonas* strains.

Methionine, known to be an absolute growth requirement for *Pseudomonas maltophilia* (Iizuka & Komagata, 1964; Stanier *et al.* 1966; Gilardi, 1969) supported growth of this organism at 20 mg % (w/v) on the mineral medium with a suitable carbon source. It also enabled one *Acinetobacter* strain and the three strains of the *Comamonas*/*Pseudomonas* group to grow with a suitable carbon source.

The amino acid mixture of Proom (1955) enabled growth to take place of all strains of *Bordetella parapertussis* and *Flavobacterium* and the remaining *Acinetobacter* strain, with a suitable carbon source. *Moraxella* and *Neisseria* strains grew poorly or not at all even when the medium was supplemented with the amino acid mixture.

Fig. 1. Simplified dendrogram derived from conventional test results.

Fig. 2. Simplified dendrogram derived from carbon source results with variable results scored as first result observed.

Fig. 3. Simplified dendrogram derived from variable results scored as positive.

Fig. 4. Simplified dendrogram derived from variable results scored as no comparison.

Effect of temperature on CSU tests. Taylor (1945) in a comparison of the effects of temperature on the biochemical activities of bacteria found, in general, that more positive results were obtained at 30 than 37°C. In the present study, 94 strains were compared at the two temperatures with 10 carbon sources and 940 test pair differences were possible but only 31 (3·3%) were found. These differences in activity at the two temperatures largely reflected the optimum growth temperature of the bacteria concerned. Only six of the differences were due to the growth of strains at one temperature and not at the other, the remaining 25 differences were only in the degree of growth. Of the 94 strains, 14 showed more activity at 37°C and eight strains more at 30°C.

Table 1. *The distribution of variable results with carbon sources*

Carbon source	Strains variable	Carbon source	Strains variable
Benzylamine	0 (0)*	Mannitol	7 (4)
DL-Tryptophan	2 (1)	L-Valine	7 (4)
Glycollate	1 (1)	DL-Aspartate	9 (5)
Pelargonate	2 (1)	Sarcosine	10 (5)
Nicotinate	2 (1)	D-Pantothenate	10 (5)
DL-Methionine	1 (1)	Propionate	12 (6)
Hippurate	3 (2)	Succinate	13 (7)
D-Xylose	3 (2)	L-Proline	13 (7)
Betaine	3 (2)	Fumarate	14 (7)
meso-Inositol	4 (2)	Adipate	14 (7)
DL-Glutamate	5 (3)	Malonate	14 (7)
L-Asparagine	5 (3)	Citrate	17 (9)
L-Arginine	6 (3)	D-Glucose	17 (9)
L-Hydroxyproline	6 (3)	n-Valerate	16 (9)
Glyoxylate	6 (3)	Pyruvate	18 (10)
Hydroxybenzoate	5 (3)	1-Ketoglutarate	18 (10)
Lactate	7 (4)	Maleate	21 (11)
L-Histidine	8 (4)	Acetate	20 (11)
DL-Ornithine	8 (4)	Sucrose	31 (17)
DL-Serine	7 (4)		

* The percentage of variable results shown in parenthesis is calculated as the number of strains showing variable results expressed as a percentage of the total number of strains.

Induction of enzymes and repetition of tests. The total variability, expressed as the percentage of variable results for the total number of tests, was 7.1 % for the 5031 induced tests and 5.7 % for the same number of non-induced tests, and induction, therefore, does not reduce the variability. The total number of positive results, i.e. all positive results from both primary and repeat tests, was approximately the same for both methods, 2315 for induced tests and 2378 for the non-induced. The only consistent difference was that induction was necessary to obtain growth of four strains of *Bordetella bronchiseptica* on valerate which was assumed to be due to the selection of mutants capable of utilizing valerate (Wegener, Reeves, Rabin & Ajl, 1968).

The rate of growth was about the same in the induced and non-induced tests, and there was little difference in the total number of strains yielding weak growth.

Reproducibility in CSU tests. In the case of non-induced tests, strains which grew on a carbon source on one occasion but not at another time were classed as variable for that particular carbon source. The numbers of these strains and their percentage of the total number of strains are arranged by substrates in Table 1.

The overall variability in these 7293 tests was 5 %. Amongst the substrates sucrose (17 %), acetate (11 %), and maleate (11 %) gave the highest rate of variability and deletion of these three tests reduced the variability to 4.2 %. The variability amongst the groups of bacteria is shown in Table 2 and variability is most marked (18 %) with strains of *Loefflerella mallei*.

Computer analysis. Dendrograms (Fig. 2, 3 and 4) each based on one of the three methods of scoring results for the CSU tests show that scoring the first result observed (Fig. 2) and scoring the results as positive if a positive result had ever been obtained (Fig. 3) produced similar groups. Treating the variables as 'no comparison' (Fig. 4) resulted in a different grouping, chiefly characterized by lack of discrimination and merging of groups.

Table 2. The distribution of carbon source variability in the groups of bacteria

	No. of strains	No. of variable results	% of variable results*
<i>Acinetobacter anitratus</i>	13	9	2
<i>Acinetobacter</i> (intermediate)	7	9	3
<i>Acinetobacter lwoffii</i>	16	34	5
<i>Alcaligenes odorans</i> group	4	15	10
<i>Bordetella bronchiseptica</i>	14	26	5
<i>B. parapertussis</i>	3	11	10
<i>Chromobacterium lividum</i>	8	14	5
<i>C. violaceum</i>	14	28	5
<i>Flavobacterium</i>	10	19	5
<i>Loefflerella mallei</i>	8	57	18
<i>Moraxella</i>	14	11	2
<i>Neisseria</i>	14	35	7
<i>Pseudomonas aeruginosa</i>	13	21	4
<i>P. alcaligenes</i>	5	14	7
<i>P. fluorescens</i>	4	4	3
<i>P. maltophilia</i>	3	0	0
<i>P. cepacia</i>	1	1	3
<i>P. pseudoalcaligenes</i>	5	6	3
<i>P. pseudomallei</i>	11	12	3
<i>P. putida</i>	5	0	0

* The percentage of variable results is calculated as the number of variable results expressed as a percentage of the product of the number of strains in a group of bacteria and the 39 carbon source substrates tested.

For comparison with the classification derived from the conventional tests (Fig. 1) the classification produced by scoring of the first result observed (Fig. 2) can be chosen. In general, the named groups formed by the results of conventional tests are more homogeneous than those derived from the CSU tests and the relations between some of the groups are altered.

Pseudomonas aeruginosa, *P. pseudomallei* and *P. fluorescens* seem to merge in the CSU dendrogram, and are further separated from each other in the conventional test dendrogram. *Pseudomonas putida* is more widely separated from all other groups compared to its position in the dendrogram derived from conventional tests. In contrast, *Chromobacterium violaceum* is more isolated in the conventional test classification. *Loefflerella mallei* appears as a heterogeneous species in both dendrograms but more markedly so in that derived from CSU tests in which the high variability of test results may have played a part. A group of *Acinetobacter* strains separate from *A. anitratus* and *A. lwoffii* is formed by the conventional tests, but merges with some *A. lwoffii* strains in the CSU dendrogram. However, *Alcaligenes odorans* strains are more widely separated from *Bordetella bronchiseptica* using CSU tests.

Evaluation of tests. The test differences between the 20 groups studied are shown for the CSU tests in Table 3 and for conventional tests in Table 4. The order and numbering of the groups of bacteria correspond with that of Snell (1972). Comparison of these two tables shows that more test differences are found between the groups in the case of conventional tests than with CSU tests, and that groups separated by only a few conventional tests are also not usually well separated by carbon source tests.

Table 3. *The number of carbon source test differences between groups of bacteria*
The groups of bacteria are arranged both vertically and horizontally. The number of test differences between any two groups of bacteria is found at the intersection of the row of one group with the column of the other group.

	Group																			
<i>Loefflerella mallei</i>	1	0																		
<i>Chromobacterium violaceum</i>	2	1	0																	
<i>Pseudomonas maltophilia</i>	3	2	3	0																
<i>Bordetella bronchiseptica</i>	4a	1	2	6	0															
<i>Alcaligenes odorans</i>	4b	5	6	7	4	0														
<i>Pseudomonas alcaligenes</i>	4c	0	0	1	0	6	0													
<i>P. pseudoalcaligenes</i>	5	0	0	3	2	5	0	0												
<i>Bordetella parapertussis</i>	6	3	5	8	12	2	4	0												
<i>Neisseria</i>	7a	1	1	1	1	5	0	0	0											
<i>Moraxella</i>	7b	3	3	4	6	8	2	2	0	0										
<i>Acinetobacter Iwoffii</i>	7c	1	2	2	1	4	0	0	0	1	0									
<i>Acinetobacter (intermediate)</i>	8	1	2	4	3	6	0	0	5	0	0	0								
<i>Acinetobacter anitratus</i>	9	4	5	5	6	3	3	4	12	7	10	3	5	0						
<i>Flavobacterium</i>	10	3	4	5	7	13	1	3	4	0	3	1	5	11	0					
<i>Pseudomonas aeruginosa</i>	11	8	7	7	8	6	6	6	17	8	13	6	8	2	15	0				
<i>P. pseudomallei</i>	12	8	8	11	11	6	9	10	22	11	17	8	13	3	18	0				
<i>P. fluorescens</i>	13	6	5	9	7	9	6	5	14	6	10	6	7	6	11	4				
<i>P. cepacia</i>	14	14	15	18	17	11	14	16	29	18	25	15	19	5	24	2				
<i>P. putida</i>	15	6	5	8	10	7	7	5	12	7	8	6	7	4	12	4				
<i>Chromobacterium lividum</i>	16	1	2	6	4	6	3	4	8	4	7	4	6	4	5	4				
Group	1	2	3	4a	4b	4c	5	6	7a	7b	7c	8	9	10	11	12	13	14	15	16

Table 4. The number of conventional test differences between groups of bacteria

The groups of bacteria are arranged both vertically and horizontally. The number of test differences between any two groups of bacteria is found at the intersection of the row of one group with the column of the other group.

	Group															
<i>Loefflerella mallei</i>	1	0														
<i>Chromobacterium violaceum</i>	2	4	0													
<i>Pseudomonas maltophilia</i>	3	12	9	0												
<i>Bordetella bronchiseptica</i>	4a	11	8	8	0											
<i>Alcaligenes odorans</i>	4b	13	7	8	3	0										
<i>Pseudomonas alcaligenes</i>	4c	7	4	8	2	3	0									
<i>P. pseudoalcaligenes</i>	5	8	5	6	2	1	1	0								
<i>Bordetella parapertussis</i>	6	8	9	13	6	8	4	4	0							
<i>Neisseria</i>	7a	4	4	10	7	8	2	3	3	0						
<i>Moraxella</i>	7b	6	7	8	5	6	2	3	1	0	0					
<i>Acinetobacter lwoffii</i>	7c	6	6	11	7	5	5	5	2	2	2	0				
<i>Acinetobacter</i> (intermediate)	8	6	8	17	15	13	11	13	10	7	9	6	0			
<i>A. anitratus</i>	9	8	8	15	13	12	11	12	11	8	9	5	2	0		
<i>Flavobacterium</i>	10	7	6	11	14	10	11	7	10	6	5	5	6	8	0	
<i>Pseudomonas aeruginosa</i>	11	8	3	14	10	8	7	6	14	11	11	8	10	8	7	0
<i>P. pseudomallei</i>	12	7	3	12	13	12	10	10	19	15	15	12	9	9	3	0
<i>P. fluorescens</i>	13	5	6	13	10	11	7	7	13	12	12	9	7	9	8	4
<i>P. cepacia</i>	14	12	10	16	17	17	15	13	21	17	17	14	13	13	11	10
<i>P. putida</i>	15	3	4	13	9	9	4	6	12	8	9	8	7	7	9	4
<i>Chromobacterium lividum</i>	16	9	8	12	12	13	9	9	12	10	11	10	11	11	6	9
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DISCUSSION

Before the introduction of a new series of tests for taxonomic use, the technical aspects of the tests need investigation as well as their value in discrimination between the taxa concerned. The discrimination obtained can be assessed by comparing the classifications formed by the new tests and by conventional tests. In this study with the group of bacteria investigated, a technical disadvantage of CSU tests is that some bacteria fail to grow on the nutritionally demanding mineral medium when a suitable carbon source was added. Even with added amino acids some strains of *Moraxella* and *Neisseria* failed to grow. Baumann *et al.* (1968*b*) attempted to use CSU tests for *Neisseria* and *Moraxella* by the addition of various defined and non-defined supplements to the carbon source medium. The validity of comparing the patterns of results obtained from bacteria with widely different growth requirements may be questioned, though Sneath (1968) has suggested statistical methods of comparing fast and slow growing strains.

Variability in CSU tests. Rhodes (1959) found some variability on repeating CSU tests and in the present study a variation of 5% was found, reduced to 4.2% by removal of the three most variable substrates. Although the overall value of a test is reduced by inherent variability, it still may provide uniform distinction between particular groups of bacteria in which the character is constant.

The high rate of variability shown by individual strains of *Loefflerella mallei* suggests that degrees of variability might be characteristic of different bacteria and could be scored as a character. Fascinating problems in both classification and identification by numerical methods would be raised.

Scoring of variability. The three methods of scoring were justified as follows. (i) Score as positive if a positive result was ever obtained. The necessary enzymes for utilization of a given substrate are assumed to be present even though not always demonstrable. It is, however, possible that the occasional positive result could arise from infrequent mutants, which would be more likely if the test was repeated a large number of times. (ii) Use the first result obtained. This corresponds to diagnostic usage when the test will usually be carried out only once. It also statistically represents the results since the probability of the first obtained result being the most frequent is the frequency itself. For example, if a test was performed four times and was positive in three of these, the probability of the first test being positive would be 0.75. (The most frequent result could have been used instead of the first result and in the case above would have led to scoring a positive result. However, when the test results were equally divided between positive and negative, e.g. positive two times out of four results, then neither a positive or negative result can be chosen. The use of the first result obviates this difficulty since a result is automatically chosen.) (iii) 'No comparison.' The success of this method depends on the percentage of variable results as too high a number might lead to comparisons based on small numbers of tests. In practice with a variability of 4.2% classification was less satisfactory than with methods 1 and 2 (Fig. 2 to 4).

Variable results could have been scored quantitatively, allotting different values according to the number of times a given test was positive in the series. However, as the average number of times tests were repeated in this study was four, significant probabilities could not be allotted and we had not the facilities to carry out the extensive further repeated tests which would be required to derive such probabilities.

Evaluation of carbon source tests. The lower number of test differences between the groups shown by the CSU tests compared to the conventional tests (Tables 3, 4) suggests that CSU tests should be used as a second line of investigation. The degree of standardization required

and the frequency of variable results make CSU tests by present techniques not entirely suitable for the routine diagnostic laboratory. Occasionally they do, however, provide differentiation between groups not differentiated by conventional tests and have proved useful in specialist identification in the National Collection of Type Cultures.

The differences in the classifications produced by the CSU and conventional tests may be due to the small number of tests used, none the less tests should be drawn from as many different types of property as possible for numerical classification.

The increasing use of CSU tests for classification and to a lesser extent in identification prompted the present study. CSU tests have many advantages. Once a testing procedure is established many strains can be tested in a large number of substrates, and the reading of test results is simple and does not require reagents. Use of a chemically defined medium ensures a high degree of standardization, and the tests are suited to mechanization and automation. Carbon source utilization patterns may provide information on the biochemical pathways present and hence comparison of these pathways in different bacteria may perhaps prove more rational than that of unrelated biochemical tests.

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