A Study of Features used in the Diagnosis of *Pseudomonas aeruginosa*

By RITA R. COLWELL

*Department of Biology, Georgetown University,
Washington, D.C. 20007, U.S.A.*

SUMMARY

Thirty-three strains of *Pseudomonas aeruginosa*, previously described as a species group by a quantitative study, were subjected to an extended schedule of tests used in bacterial classification and identification. Adansonian analysis by electronic computer confirmed previous results, indicating that the 33 strains should be treated as a species group. The 172 features coded for each strain were analysed by computer, yielding a 'probability of occurrence' of each feature within the species, *P. aeruginosa*. This output was then used to select features appropriate for diagnostic purposes. From the analyses it was also possible to measure the sensitivity of several methods for determining the presence or absence of characteristics such as pigment production and oxidation of gluconate to ketogluconate. The general applicability of the use of the computer to cooperative pooling of data by bacteriologists is discussed.

INTRODUCTION

The incidence of *Pseudomonas aeruginosa* in clinical infections and in various natural environments, coupled with the difficulty of diagnosis of this micro-organism, has led to an increased interest in its identification and classification (Ringén & Drake, 1952; Gould & McLeod, 1960; Rogers, 1960; Pulverer & Korth, 1962; Curtin, Petersdorf & Bennett, 1961). A large number of papers have been published concerning *P. aeruginosa*, particularly on methods for the selective isolation of the organism, methods for enhancement of pigment production and new tests for classification (Seleen & Stark, 1948; Bühlmann, Vischer & Bruhin, 1961*a, b*). Several tests have been proposed as specific for *P. aeruginosa* (Haynes, 1951; Gaby & Free, 1953, 1958; Kovacs, 1956). A comparative study of the tests suggested for identification of *P. aeruginosa* should be useful for those workers who isolate this micro-organism frequently in routine studies. Heretofore, the difficulty in carrying out a study of this type lay in the handling and correlation of the large amount of necessary data. For such problems, electronic computers are a valuable bacteriological tool. Diagnostic characters can be extracted from the total taxonomic data for given groups of micro-organisms and the relative merits of the characteristics considered.

Several years ago, the application of electronic computers to bacterial taxonomy was studied by an analysis of data for a number of pseudomonads, both culture collection strains and fresh isolates (Colwell & Liston, 1961*a, b*; Rhodes, 1961). 'Taxonomic clusters' within the genus *Pseudomonas* were identified. Subsequently, using 33 strains from the American Type Culture Collection, a species cluster was defined for *P. aeruginosa*, and a quantitative basis for species definition was

established (Liston, Wiebe & Colwell, 1962, 1963). As the next logical step, it appeared appropriate to increase the battery of tests as widely as possible, thereby extending the description of *P. aeruginosa* and providing a means of determining the reliability of the various tests proposed for identification and classification of the organism. Furthermore, the data so obtained could be mathematically sifted by the computer to extract significant diagnostic features. The present paper describes a study of the characteristics used in diagnosis of *P. aeruginosa* and of the application of the electronic computer as an aid in classifying this species.

METHODS

The organisms studied were 33 strains of *Pseudomonas aeruginosa* received from the American Type Culture Collection and treated previously in a quantitative study by electronic computer as a species group (Liston *et al.* 1963); see Table 1.

Methods used were those described by Colwell & Liston (1961*a*) or the Society of American Bacteriologists (1957), except as indicated below. The standard inoculum for all tests was a single drop (1/20 ml.) from a sterile disposable pipette (Fisher Scientific Company) of a 24–48 hr broth culture. The stock culture medium (YE) was a modification of that used by Rhodes (1959): Difco yeast extract, 0.3 g.; Difco Bacto-Proteose Peptone, 1.0 g.; NaCl 0.5 g.; agar 1.5 g. (agar omitted from liquid stock media); distilled water 1 l.; adjusted to pH 7.2–7.4 with NaOH. Tests were carried out at room temperature (25°) except where otherwise indicated.

Effect of environmental conditions on growth

Temperature range of growth. The ability of strains to grow on YE agar slopes and in YE broth at 0°, 5°, 10°, 15°, 25°, 30°, 35° and 40° was screened by incubation of the inoculated media in incubator rooms temperature-controlled to $\pm 0.5^\circ$, and the upper and lower limits of growth determined by incubation in a water bath at the desired temperature. As suggested by Haynes & Rhodes (1962), a strain was considered incapable of growth at a given temperature unless it could survive at least three serial transfers at that temperature. In addition, where possible, the medium was pre-incubated at the test temperature for 6–12 hr before inoculation. The incubation period extended from 5 days to 4 weeks, depending on the temperature of incubation.

Salinity range of growth. The strains were inoculated into YE broth tubes, with final NaCl concentrations of 0, 0.5, 3.0, 5.0 and 10.0%. The tubes were incubated for 14 days after inoculation.

pH range of growth. YE broth, adjusted to pH 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0 and 9.0, with HCl or NaOH as required, was inoculated and incubated for 14 days, growth being observed at 1, 2, 7 and 14 days.

Sensitivity to antibiotics and a 'vibriostatic compound'. The strains were tested for sensitivity to penicillin (high concentration), dihydrostreptomycin (10 μ g.), chloramphenicol (30 μ g.), chlortetracycline (30 μ g.), oxytetrocycine (30 μ g.), polymyxin B (300 units), tetracycline (30 μ g.; Bauer, Roberts & Kirby, 1959–60) and the vibriostatic compound (2,4-diamino-6,7-diisopropylpteridine) described by Shewan, Hodgkiss & Liston (1954). YE agar plates were used, with sensitivity discs (Baltimore Biological Laboratory, Inc.) for the antibiotics listed.

Physiological tests

Glucose utilization. The method of Hugh & Leifson (1953) was used to determine whether glucose was used oxidatively or fermentatively. The miniature tube method (Colwell & Quadling, 1962) was also used. Growth in glucose with and without added iodoacetate (Lysenko, 1961) was tested.

Production of acid from carbohydrate. Cultures were incubated for 4 weeks in a bromocresol-purple broth basal medium (Difco Laboratories) with 1.0% filter-sterilized test carbohydrate added. Glucose, lactose, maltose, sucrose, galactose, mannitol, mannose, melibiose, melezitose, dextrin, glycerol and sorbitol were used. In addition, the Hugh & Leifson (1953) basal medium without agar and with Durham tubes was used, with added filter-sterilized glucose, lactose or maltose as test carbohydrates. Each culture was examined at 1, 7, 14 and 28 days for acid and gas production in all tests.

Hydrolysis of aesculin. The agar plate technique of Sneath (1956) was used.

Production of 2-ketogluconic acid. Cultures were incubated for 7 days in Paton's broth medium (Paton, 1959); the presence of reducing sugars was determined by adding Benedict's reagent (Clinitest tablet, Ames Co. of Canada, Ltd., Toronto). The method of Haynes (1951) was also used and the production of slime and 'oyster' formation noted. The rapid test for gluconate oxidation (Gaby & Free, 1958) was done with gluconate substrate tablets (Key Scientific Products Inc., Los Angeles, U.S.A.) but the incubation time was increased to 72 hr at room temperature on a rotary shaker.

Oxidation of calcium lactate through acetate to carbonate and production and accumulation of dihydroxyacetone in media containing glycerol and the production of acetic acid from ethanol were tested by the methods given by Shimwell, Carr & Rhodes (1960).

Production of pigment. Several media, designed for, or described as enhancing, pigment production by *Pseudomonas aeruginosa* were tested. All the media used were scored for production of a green water-soluble pigment. Media designed for production of pyocyanine (Burton, Eagles & Campbell, 1947; Burton, Campbell & Eagles, 1948; King, Ward & Raney, 1954), pyorubin (Meader, Robinson & Leonard, 1925; King, Ward & Raney, 1954), and fluorescein (King, Campbell & Eagles, 1948; King, Ward & Raney 1954; Paton, 1959), were prepared as described by these authors, inoculated and incubated up to 7 days, being routinely examined for the production of visible pigment or pigment fluorescence under ultraviolet radiation in the case of fluorescein. Pigment production was tested for on Sabouraud maltose agar (Martineau & Forget, 1958). Pyocyanine and pyorubin were identified by the presumptive tests outlined by Wetmore & Gochenour (1956). Finally, sporadic production of oxychlororaphin and chlororaphin crystals was noted but, as observed by Haynes & Rhodes (1962), no single medium served as indicator for consistent production of these compounds.

Ammonia production was tested for by Nessler's reagent after incubation for 7 days in YE broth.

Hydrogen sulphide formation from sodium thiosulphate and from cystine was tested by the miniature tube method of Colwell & Quadling (1962). Also used, for comparative purposes, were the Difco lead acetate agar slopes.

Trimethylamine oxide reduction. Cultures were incubated for 14 days in Wood & Baird's medium (1943) and the presence of trimethylamine was then tested for according to the method given by the authors.

Hydrolysis of Tween 20, 40, 60 and 80. The use of the water-soluble Tweens for easy detection of lipolytic activity was described by Sierra (1957). His technique, using 1.0% Tween 20, 40, 60 or 80 in calcium chloride peptone agar, was followed. Cultures were kept for 14 days, being examined routinely for the appearance of conspicuous precipitates around the colonies.

Catalase, cytochrome oxidase and oxidase formation. Catalase was tested for by adding a drop of ('10 vol.') hydrogen peroxide to a smear of a 24–48 hr YE agar colony on a glass slide; for cytochrome oxidase and oxidase formation the methods of Gaby & Free (1958) and Kovacs (1956), respectively, were used.

De-amidation of acetamide was tested for by the method outlined by Bühlmann, Vischer & Bruhin (1961*b*).

Lecithinase was tested for by spotting a drop of a 24–48 hr YE broth culture on to an egg-yolk agar plate consisting of Difco Nutrient Agar containing 10% (v/v) egg-yolk suspension. The egg-yolk suspension was prepared as follows: an egg yolk was washed in 0.2% NaCl, placed in a Blendor jar with 200 ml. 0.2% NaCl and the resulting suspension centrifuged at 13,000 rev./min. for 5 min. in a high-speed refrigerated centrifuge. The supernatant fluid was sterilized by Seitz filtration and added to cooled sterile Difco Nutrient Agar before pouring plates.

Arginine dihydrolase was tested for by the method of Thornley (1960).

Melanin production from tyrosine and from phenylalanine was observed by the method of Brisou & Menantaud (1957).

Production of phenylpyruvic acid from phenylalanine was tested for by the method of Ewing, Davis & Reavis (1957).

Nutrition

The standard inoculation for all the tests was carried out by spotting agar plates containing basal salts medium (NaCl 5.0 g.; MgSO₄.7H₂O, 0.2 g.; K₂HPO₄, 1.0 g.; distilled water 1 l.; adjusted pH 6.8) with added organic acids and ammonium-N or amino acids. A control plate with only the basal salts medium was tested at the same time. Vitamin-free Casamino acids, proline, DL-alanine, β -alanine, arginine, lysine, phenylalanine (California Biochemical Corp.) and sodium formate were tested for ability to support growth.

Computer analysis

The results of each test were recorded and coded, yielding 172 coded characteristics per strain. These data were transferred to IBM punch cards and were processed by electronic computer. Similarity values were obtained and sorted as described previously (Colwell & Liston, 1961*a*). The data for the 33 strains were then processed by electronic computer so that the frequency of occurrence of each feature in the group was computed by using the programmed formula $v = n/N$, where n = the number of strains showing the characteristics and N = the total number of strains in the cluster. The output, sorted by decreasing frequency, was then compiled, grouping characteristics into morphological, cultural, etc., feature clusters.

The IBM 1620 40,000K electronic computer was used in these studies and the programs have been documented (Colwell, in *Taxometrics* no. 3).

RESULTS AND DISCUSSION

Quantitative analysis of the species group

Previous study indicated that all strains listed in Table 1, with the exception of 257, 7701 and 9027, can be considered members of the species *Pseudomonas aeruginosa* (Liston *et al.* 1963). Seventy-eight coded features, representing 134 character states, were used to describe and quantify the species. The $\bar{\Delta}S$ for the 30 strains comprising the group was 77%. Other parameters were: $\bar{N}_s = 31$; $\bar{N}_a = 9$; $\bar{N}_0 = 38$ and $\bar{M} = 88\%$, where N_s = number of shared features, N_a = number of features present in one strain and not the other, and M = matching coefficient, or similarity index in which the number of concordances between negative responses as well as positive responses is taken into account (Silvestri, Turri, Hill & Gilardi, 1962). In the study described in this paper, the number of coded features was increased to 172 for each strain tested and the calculations repeated, giving: $\bar{\Delta}S = 82\%$; $\bar{N}_s = 84$;

Table 1. *American Type Culture Collection numbers of Pseudomonas aeruginosa strains used in this study**

97	10145	14209
257	10197	14210
260	10752	14211
262	13388	14212
7700	14149	14213
7701	14203	14214
8689	14204	14215
8707	14205	14216
8709	14206	14217
9027	14207	14218
8721	14208	14219

* Additional information for each strain is given by Liston, Wiebe & Colwell (1963).

Table 2. *Similarity of each strain to the calculated median organism*

Organism (ATCC no.)	S value with median	Organism (ATCC no.)	S value with median
14214	98		
14216	92	7701	89
14208	92	14213	88
14149	92	8707	88
8689	91	97	87
14208	91	14219	87
14209	91	14210	87
14215	91	10145	86
7700	91	10752	86
14218	91	9027	86
14204	90	14212	86
14217	90	9721	85
13388	90	14207	85
260	89	257	84
14205	89	8709	84
14206	89	10197	82
14211	89	262	70

$\bar{N}_a = 19$; $\bar{N}_0 = 69$ and $\bar{M} = 89\%$. The values obtained were comparable to those from the previous published calculations except that now higher similarity indices resulted for strain/strain comparisons and the intra-group calculations.

Listed in Table 2 are the similarity indices for each strain compared with the hypothetical Median Organism which was derived according to the method of Liston *et al.* (1968). Using their '75 % rule', strain 262 would not be included in the species group. The question of whether to include the strains 9027, 7701, 257 and 262 in the *Pseudomonas aeruginosa* species was considered in the earlier published study and the conclusion drawn was that 9027, 7701 and 257 should be excluded. From the data listed in Table 2, only strain 262 would now be considered of doubtful membership. However, the exclusion of strain 262 does not effect a very large change in the values listed above. Recalculated, these were: $\bar{\Delta S} = 83\%$; $\bar{N} = 85$; $\bar{N}_a = 18$; $\bar{N}_0 = 69$ and $\bar{M} = 90\%$.

Analysis of features

Using the *Pseudomonas aeruginosa* strains as a quantitatively defined species group, a description of the species can be derived which may be assumed to approximate to the population phenotype. A listing of the features in terms of frequency of occurrence should provide a best estimate of the probability of occurrence of the various characters within the species. In other similar samplings these data may then be useful in the identification and classification process. The compiled output of features and frequency of occurrence, condensed according to operational fields, viz. morphology, physiology, etc., is given in Tables 3-7.

Cultural characteristics of the strains are given in Table 3. All strains were Gram-negative rods with polar flagella, occurring as singles or pairs. Slender, round or tapered organisms of short to medium length were observed, with no curved, oval-shaped organisms or chains scored. The characteristics of growth in YE broth were variable, with moderate even turbidity most frequent, granular turbidity rare, and pellicle formation occurring with a frequency of 0.48.

The majority of the cultures formed grey, flat, entire, translucent colonies, about 2-5 mm. in diameter on YE agar, but there were opaque, off-white colonies occurring at low frequency. Variation in the colonial morphology of *Pseudomonas aeruginosa* has long been known (Hadley, 1937) and has been studied by several investigators (Gaby, 1946; Gaby & Free, 1953; Williamson, 1956). The observations made in the present work confirm the results of other investigators that colonies on agar may be exclusively of one type or a mixture of both types. Attempts to correlate pathogenicity with the colonial variants have been of relatively limited value. However, serological studies of the species *P. aeruginosa* have depended largely on selection of stable smooth colonies for propagation of antigens (Verder & Evans, 1961).

The effect of temperature, pH value and salt concentration on growth are among the coded features included in Table 3. In this study, all cultures grew at temperatures ranging from 25° to 41°; none was able to grow at temperatures from 0° to 5°. Thus, the full temperature range of growth was 5.5-45°. However, it should be stated that the majority of strains grew at temperatures ranging from 8° to 42°. On the basis of the data given, the test for growth at 42° suggested by Haynes (1951)

for the diagnosis of *Pseudomonas aeruginosa* is not absolute but is a feature which occurs with sufficiently high frequency to justify its use in a diagnostic procedure.

Growth was observed in YE broth media in the range pH 4.0–9.0, most strains growing in the YE broth initially in the range pH 5.0–9.0. Sodium chloride concentrations, ranging from none added to 5% sodium chloride were tolerated by all but one strain.

Table 3. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: cultural characteristics*

YE broth					
Rods	1	Motile by polar flagella	0.94	Ovals	0
Singles	1	Tapered	0.88	Stout (0.6–1.0 μ)	0
Pairs	1	Short (0.2–0.6 μ)	0.76	Chains	0
Gram-negative	1	Round end	0.58	Gram-variable	0
		Slender (0.2–0.6 μ)	0.42		
		Medium length (0.6–1.2 μ)	0.30		
		Curved rods	0.06		
		'Filaments'	0.03		
		Even turbidity	0.85	Heavy turbidity	0
		Moderate turbidity	0.70		
		Pellicle	0.48		
		Slight turbidity	0.30		
		Granular turbidity	0.12		
		Ring	0.12		
YE agar					
		Grey	0.85	White	0
		Entire	0.82	Convex	0
		Medium (2–5 mm) colony	0.85		
		Translucent	0.73		
		Opaque	0.30		
		Small (< 2 mm) colony	0.18		
		Off-white	0.15		
Growth at 25°–41°	1	42°	0.97	0°–5°	0
		44°	0.73		
		15°	0.97		
		10°	0.97		
		8°	0.91		
		7°	0.79		
		6°	0.07		
Growth at pH 5.5–9.0	1	pH 5.0	0.97		
		pH 4.5	0.30		
		pH 4.0	0.06		
Growth in 0.5% NaCl	1	0% NaCl	0.97	10% NaCl	0
		3.0%–5.0% NaCl	0.97		

Several media were devised in an attempt to determine a nutritional pattern for the species. Single amino acids in a buffered salts agar basal medium were tested for ability to support growth. Results of studies with amino acids are given in Table 4. All strains grew in vitamin-free Casamino acids, buffered salts agar basal medium and in the basal medium to which the amino acids, proline, β -alanine, DL-alanine, phenylalanine, lysine and arginine, separately, had been added. Less than half the strains were able to utilize lysine as sole nutrient source, although all

strains produced abundant growth in the lysine + peptone broth medium of Falkow (1958). Growth on phenylalanine agar was rare (0.08) and sparse when it did occur. The test for phenylalanine deaminase (Ewing *et al.* 1957) was negative for all strains.

Table 4. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: nutritional characteristics and antibiotic sensitivities*

Growth in					
Koser's citrate medium	1	0.3 % malonate	0.97	0.1 % sodium formate	0
Simmon's citrate medium	1	0.1 % DL-alanine + salts	0.97		
Vitamin-free Casamino acids	1	0.1 % β -alanine + salts	0.97		
		0.1 % arginine + salts	0.97		
Lysine broth (Falkow, 1958)	1	1.0 % calcium lactate (oxidation of calcium lactate through acetate to carbonate)	0.94		
0.1 % proline + salts*	1				
NH ₄ H ₂ PO ₄ + salts	1	0.1 % lysine + salts	0.45		
		0.1 % phenylalanine + salts	0.08		
Sensitive to					
		Oxytetracycline 30 μ g.†	0.97	Erythromycin 15 μ g.	0
		Polymyxin B 300 units	0.91	Penicillin high concn.	0
		Tetracycline 30 μ g.	0.85	Pteridin compound	0
		Kanamycin 30 μ g.	0.78		
		Dihydrostreptomycin 10 μ g.	0.73		
		Chlortetracycline 30 μ g.	0.45		
		Chloramphenicol 30 μ g.	0.15		
		Novobiocin 30 μ g.	0.06		

* g/l.: NaCl, 5.0; MgSO₄·7H₂O, 0.2; K₂HPO₄, 1.0; pH 6.8.

† Sensitivity discs, Baltimore Biological Laboratory, Inc.

Other compounds were tested for ability to support growth and several antibiotics were tested for growth inhibition (Table 4). All strains were citrate-positive in both Koser's and Simmons's media. This, of course, suggested that ammonium-N was a suitable source of nitrogen for growth, and was verified by the observation that all strains were able to grow on a basal medium to which NH₄H₂PO₄ had been added.

Growth in a 0.3 % malonate broth (Ewing, 1962) and oxidation of calcium lactate to carbonate (Shimwell *et al.* 1960) were features which occurred at very high frequencies within the group.

Of the antibiotics which were used (Table 4), all strains tested were resistant to penicillin, erythromycin and the pteridine compound. A majority of the strains tested were resistant to novobiocin and chloramphenicol. The antibiotics, oxytetracycline, polymyxin B, tetracycline, kanamycin, and dihydrostreptomycin, were inhibitory for > 70 % of the strains. Sensitivity to chlortetracycline occurred at a frequency of 0.45. From figures given in Table 4, an estimate of antibiotic sensitivities for strains of *Pseudomonas aeruginosa* can be determined.

Reactions in various carbohydrate media were examined (Table 5). Attack on carbohydrates was oxidative when both the methods of Hugh & Leifson (1953) and Colwell & Quadling (1962) were followed. There was no fermentation by any of the 33 strains. Acid without gas was produced from glucose, and iodoacetate did not inhibit this acid production. Of the carbohydrates tested, only glucose (0.97) and

galactose (0.40) were utilized by many of the strains. Acid production from mannose, melezitose, glycerol and sucrose were features of infrequent occurrence. Positive methyl-red and Voges-Proskauer reactions and hydrolysis of aesculin were also features of rare occurrence in the group. Dihydroxyacetone was not accumulated from glycerol by any of the strains, nor was acetic acid produced from ethanol. Starch was not hydrolysed.

Table 5. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: carbohydrate reactions*

Growth in glucose+ iodo- 1 acetate medium	Glucose oxidized in presence of iodoacetate	0.97	Glucose fermented (Hugh & Leifson)	0
	Glucose oxidized (Hugh & Leifson)	0.97	Glucose fermented (miniature tube method)	0
	Glucose oxidized (miniature tube method)	0.97	Gas from carbohydrates	0
	Gluconate → ketogluconate (Haynes)	0.97	Acid from lactose	0
	Gluconate → ketogluconate (Gaby & Free)	0.92	Acid from maltose	0
	Gluconate → ketogluconate (Paton)	0.94	Acid from mannitol	0
	Slime production in gluconate medium (Haynes)	0.88	Acid from melibiose	0
	Acid from galactose	0.40	Acid from sorbitol	0
	Acid from mannose	0.17	Acid from dextrin	0
	Acid from melezitose	0.03	Starch hydrolysed	0
	Acid from glycerol	0.03	Glycerol → dihydroxyacetone	0
	Acid from sucrose	0.03	Ethanol → acetic acid	0
	Methyl-red positive	0.03	Agar digested	0
	Voges-Proskauer positive	0.03		
	Aesculin hydrolysed	0.03		

Three methods for detection of gluconate oxidation were used and the results are given in Table 5. Most of the strains were able to produce ketogluconate from gluconate substrate. The technique of Gaby & Free (1958) was quick and simple to carry out, but the chance of not detecting a positive reaction was greater than with the method proposed by Haynes (1951).

Additional data obtained from other biochemical tests are given in Table 6. All strains produced ammonia in peptone water, reduced nitrates and nitrites, and showed catalase, cytochrome oxidase, oxidase, acetamide deamidase, lipase and lecithinase. The presence of urease and arginine dihydrolase were features which occurred at significantly high frequencies. Acid peptonization of litmus milk, hydrolysis of casein and production of hydrogen sulphide from cystine were characters of frequent occurrence. Gelatin liquefaction was somewhat less constant a feature (0.61). Characteristics such as melanin production from tyrosine and indole production from tryptophan were features of low occurrence, indicating a sporadic incidence in the group. A trimethylamine oxide reductase was not detected.

Certain features such as production of hydrogen sulphide depend greatly on the medium and method used. The lead acetate agar technique cannot be considered satisfactory for detection of sulphide production from proteins or their split products, at least for pseudomonads. Other investigators have commented on the inadequacies of the lead acetate agar method (Sherwood, Johnson & Radotincky, 1926; Rhodes,

1959). As seen from Table 6, the miniature tube method of Colwell & Quadling (1962) is more suitable for the detection of hydrogen sulphide. Hydrogen sulphide production from sodium thiosulphate, as examined by the miniature tube method, was not a characteristic of the 83 strains of *Pseudomonas aeruginosa* examined.

Table 6. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: additional biochemical characteristics*

Ammonia production in peptone water	1	Cytochrome oxidase present (Gaby & Free, 1953)	0.97	Trimethylamine oxide reduced to trimethylamine	0
Nitrate reduced to nitrite	1	Urease present	0.97	Litmus milk reduced	0
Nitrite reduced	1	Arginine dihydrolase present	0.97	Litmus milk acid → alkaline	0
Catalase present	1	Lipase (Tween 20)	0.97	Litmus milk alkaline → acid	0
Oxidase present (Kovacs, 1956)	1	Litmus milk peptonized	0.94	Phenylalanine → phenylpyruvic acid	0
Acetamide deamidated	1	Litmus milk acid	0.91	Phenylalanine → melanins	0
Lipase present (Tween 40, 60, 80)	1	Casein hydrolysed	0.85		
Lecithinase present	1	H ₂ S produced from cystine	0.82		
		Gelatin liquefied	0.61		
		H ₂ S produced on lead acetate agar	0.21		
		Litmus milk surface peptonized only	0.21		
		Tyrosine → melanins	0.18		
		Tryptophan → indole	0.17		
		Litmus milk alkaline	0.06		
		H ₂ S produced from sodium thiosulphate	0.06		

Pigmentation, a 'key' feature in some classificatory schema, was examined rather carefully. Five different media, cited as specific for pigment production by *Pseudomonas*, were compared. The results are given in Table 7. In YE broth and on YE agar, both containing Difco Proteose Peptone, and on King, Campbell & Eagles (1954) agar medium, a green diffusible pigment was observed for all strains tested. The Sabouraud maltose agar, suggested by Martineau & Forget (1958) and Davis, Sellers, Orbach & Weddington (1960), was not as efficient for determining pigment production as either YE medium or the King *et al.* medium. However, not all pigments which were produced by these strains were pyocyanine; a green pigment which was chloroform-soluble and pH-sensitive, for the purposes of this study, was considered to be pyocyanine. Such a pigment was detected in 97% of the strains tested, but the frequency with which it was observed varied with the medium used (0.79–0.97). Similarly, the pigment fluorescein occurred with equally variable frequency among the strains (Table 7). The King *et al.* media designated for enhanced production of the pigments pyocyanine, pyorubin and fluorescein, gave less equivocal and somewhat more reliable results than the other media used.

Pigment production by *Pseudomonas aeruginosa* is considered an important diagnostic feature when it is present in a culture, but non-pigmented strains occur and create a difficult problem for the diagnostician who relies on 'key' features (Gaby & Free, 1958; Verder & Evans, 1961; Bühlman *et al.* 1961*b*). The variability of pigment production among strains of *P. aeruginosa* has long been known (Baerthlein, 1918; Charrin & Phisalix, 1892; Eisenberg, 1914). Requirements for pyocyanine, pyorubin and fluorescein production by *P. aeruginosa* have been studied

by a number of workers since Gessard's early publications on the 'pyocyanogenic bacillus' (1890, 1891, 1892, 1918). The type of peptone used (Georgia & Poe, 1932) and the inorganic salts present in the medium (Burton *et al.* 1948) have been shown to influence the amount and kind of pigment which is produced. This single feature, or any other feature whether 'computed' or designated arbitrarily, should not be relied upon as sole criterion for diagnosis.

Table 7. *Feature and frequency of occurrence within the Pseudomonas aeruginosa species cluster: pigmentation characteristics*

Green, diffusible, water-soluble pigment produced in			
YE agar	1	Sabouraud maltose agar	0.88
King <i>et al.</i> agar	1	Burton <i>et al.</i> medium	0.82
Production of specific pigments			
		Pyocyanine—King <i>et al.</i> agar	0.97
		Pyocyanine—Burton <i>et al.</i> medium	0.79
		Pyorubin—King <i>et al.</i> agar	0.79
		Fluorescein—King <i>et al.</i> agar	0.94
		Fluorescein—YE broth	0.90
		Fluorescein—Paton's medium	0.88
		Fluorescein—Burton <i>et al.</i> medium	0.70
		Oxychlororaphine	0.12

Since no single character can be selected as 'non-mutable', hence an essential prerequisite for membership in a given taxon (a monothetically as opposed to a polythetically constructed taxon), how can a diagnosis be made with high degree of confidence and minimum investment of labour and material? Obviously, the problem is one of equilibration of input and output information until an optimum for both is reached. Certainly it is safe to say that maximum information, that is, all possible information, about a given strain would result in an identification and classification of maximum confidence estimate, which is absolute certainty. Since it is not always possible or desirable to determine even 172 coded features for every new isolate, the question then is, how many features and what combination of these are needed for identification and classification? Proceeding from the data given, the description of *Pseudomonas aeruginosa* would be as follows: a Gram-negative, rod-shaped, polarly-flagellated organism, occurring as single organisms and in pairs, with no oval-shaped organisms or chains; which formed grey, flat, entire translucent colonies about 2–5 mm in diameter on YE agar medium; which grew at temperatures of 25–41° (but not at 0–5°) and at pH 5.0–9.0; tolerated NaCl concentrations up to 5%; was able to grow in an inorganic-N sodium citrate medium, or on a basal salts medium to which 0.1% proline or vitamin-free Casamino acids was added, and in a glucose medium with added iodoacetate; did not ferment glucose or produce acid from lactose, maltose, mannitol, melibiose, sorbitol, dextrin; did not hydrolyse starch, digest agar, produce ethanol from acetic acid or accumulate dihydroxyacetone from glycerol; which produced ammonia in peptone water; reduced nitrate and nitrite; produced catalase, oxidase, acetamide deamidase, lipase and lecithinase but not trimethylamine oxide reductase and phenylalanine deaminase; finally, which produced a green water-soluble pigment on YE agar. Yet no single character of those listed is either indispensable in defining the organism or is a sufficient single criterion.

In fact, then, the simultaneous possession of a group of characters of high frequency of occurrence among strains of a given species is that which is definite (Liston *et al.* 1963). Moller (1962) proceeded by a similar line of reasoning, and introduced the concept of the attribution decision, a decision determined by the probability of belonging of each element to one or more taxa with respect to each classification criterion. However, it may be more useful to deal with the taxonomic data available for bacterial species in terms of frequency of occurrence within the cluster of organisms as the estimate of reliability of classification, the 'probability of belonging' to a taxon increasing with increasing occurrence of characters of high frequency for that taxon.

Features found to have a very high probability of occurrence taken together with features of very low probability of occurrence provide the convenient diagnostic keys for recognition of additional isolates belonging to the species. It is possible, of course, to minimize the number of necessary attributes since, the more attributes which appear unique to the species, fewer are then required to define it. Conversely, those features of low frequency in the taxon will decrease the 'probability of belonging' for a test organism. Although these attributes may be rare for the taxon they should not be used to exclude strains from the group, except, of course, when many rare characters occur together. Furthermore, from the computed relationships to the hypothetical median organism, such as for the strains studied, it is possible to select a working neotype (Colwell, 1964).

The storing, sorting and sifting of taxonomic data by machine can be extremely useful for diagnosis as well as construction of taxa, that is, for selection of diagnostic features, comparison of methods, identification and choosing neotype strains. The total amount of data currently available for any single microbial group would be handled easily by computers presently installed in university, government and industrial laboratories. A co-operative study and subsequent data analysis may offer objective evaluation of diagnostic procedures currently followed in most laboratories.

This investigation was supported by a grant (G-19405) from the U.S.A. National Science Foundation. Appreciation is expressed to Dr N. E. Gibbons for assistance in obtaining laboratory and computer facilities during tenure as Guest Scientist at the National Research Council of Canada where part of the research was carried out, and to Dr W. A. Clark of the American Type Culture Collection for gift of the *Pseudomonas aeruginosa* strains.

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