APPLIED MICROBIOLOGY, Oct. 1968, p. 1532-1538
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Vol. 16, No. 10
Printed in U.S.A

Identification of *Pseudomonas* Species Isolated from Hospital Environment and Human Sources

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Received for publication 15 July 1968

Methods and procedures are described for the identification of aerobic pseudomonads isolated from clinical specimens. Fluorescence is used as a means of primary differentiation. Final identification is based on the observation of characteristics such as flagellation, storage of intracellular fat, growth at 4 C and at 41 C, denitrification, gelatin hydrolysis, arginine dihydrolase activity, and oxidase activity. The value of this schema to the diagnostic laboratory is discussed.

The differentiation of pseudomonads from other bacteria and the differentiation of *Pseudomonas aeruginosa* from other pseudomonads found in clinical material has been the subject of several investigations (2, 3, 5, 10, 12, 19). The pathogenic pseudomonads, *P. aeruginosa* and *P. pseudomallei*, may be distinguished from other species that are thought to be nonpathogenic by their production of heat-resistant alkaline phosphatase (9). Identification of other species of pseudomonads is usually not attempted in the routine diagnostic laboratory. That some of these these species are found in clinical specimens, and may be of significance, is indicated by the sources of strains that were identified and reported by von Graevenitz (17, 18), Hugh and Ryschenkow (4), King (*unpublished data*), and Stanier et al. (14). Recent systematic comparative studies (11, 14) of aerobic pseudomonads appear to clarify the taxonomic positions of many members of the genus *Pseudomonas*. These workers have suggested some general characteristics that are of diagnostic value for differentiating species of *Pseudomonas*. Because of increasing interest in infections caused by nonfermentative, gram-negative bacteria, it was considered that the use of some of the characteristics suggested would enable the diagnostic laboratory to identify many of the aerobic pseudomonads encountered among this group of bacteria.

The purposes of this study were to (i) select characteristics most useful in identifying pseudomonads encountered in a diagnostic laboratory, (ii) adapt these to commercially available or easily prepared media and reagents, and (iii) develop for the identification of pseudomonads a simple dichotomous schema that could be utilized in a routine diagnostic laboratory. The methods and procedures are not designed to replace those recommended for more exacting taxonomic studies.

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MATERIALS AND METHODS

Bacteria. Twenty-two known cultures were obtained from the American Type Culture Collection (ATTC) and from other investigators (Table 1). Except for the one strain of *Bacillus megaterium*, species designations are those of Stainer et al. (14). Unknown cultures either were isolated by the author during surveys for human carriers and environmental sources of *P. aeruginosa*, or they were isolated from clinical specimens in hospital diagnostic laboratories.

Media and tests. Production of fluorescent pigment was determined on Pseudomonas Agar F (Difco) by incubating inoculated slants overnight at 37 C and then examining them for fluorescence by means of ultraviolet light (3,660 pm). Negative cultures were left at room temperature and reexamined daily for up to 1 week before they were considered finally negative. Strains that were not fluorescent, but that were thought to be of the fluorescent species, were later retested on Pseudomonas F Agar and Flo Agar (Baltimore Biological Laboratories, Baltimore, Md.).

Production of pyocyanine and pyorubrin pigments was determined on Pseudomonas Agar P (Difco) by incubating inoculated slants overnight at 37 C and then at room temperature for up to 1 week. When diffusible pigment was observed, the color was recorded and chloroform was then added to extract pyocyanine. Red diffusible pigment was considered to be pyorubrin; chloroform-extractable (blue) pigment was considered to be pyocyanine. Strains that did not produce pyocyanine or pyorubrin were retested later on Pseudomonas Agar P and Tech Agar (BBL).

Motility was observed microscopically by the

TABLE 1. *Sources of strains used for testing materials and methods*

Species designation	Received as	Donor
Bacillus megaterium	*Bacillus megaterium*	E. Ishiguro, as Meg 47-5
Pseudomonas aeruginosa	*P. aeruginosa*	D. C. Birdsell, as Hoff & Drake, no. 44
P. aeruginosa	*P. aeruginosa*	D. C. Birdsell, as Hoff & Drake, no. 249
P. aeruginosa	*P. aeruginosa*	C. Wilson, as Holloway H-1
P. aeruginosa	*P. aeruginosa* (melanogenic)	P. Liu, as no. 117
P. aeruginosa	*P. aeruginosa* (melanogenic)	P. Liu, as no. 1184
P. alcaligenes	*P. alcaligenes*	ATCC, as no. 14909
P. fluorescens	*P. chlororaphis*	W. C. Haynes, as B560
P. fluorescens	*P. aureofaciens*	W. C. Haynes, as B1543P
P. fluorescens	*P. aureofaciens*	W. C. Haynes, as B1576
P. fluorescens	*P. reptilivora*	W. C. Haynes, as B963
P. fluorescens	*P. reptilivora*	W. C. Haynes, as B964
P. pseudomallei	*P. pseudomallei*	M. Smith
P. pseudomallei	*P. pseudomallei*	M. Smith
P. putida	*P. putida*	W. C. Haynes, as B13
P. putida	*P. putida*	W. C. Haynes, as B805
P. maltophilia	*P. maltophilia*	ATCC, as no. 13637
P. maltophilia	*P. maltophilia*	A. von Graevenitz, as no. 53
P. maltophilia	*P. maltophilia*	A. von Graevenitz, as no. 64
P. stutzeri	*P. stutzeri*	ATCC, as no. 17588
P. stutzeri	*P. stutzeri*	C. B. van Niel, as no. MD41
P. stutzeri	*P. stutzeri*	A. von Graevenitz, as no. 20
P. stutzeri	*P. stutzeri*	A. von Graevenitz, as no. 24

hanging-drop method. Suspensions to be used to test for motility, flagella, fat, and Gram stains were prepared from the overnight growth on Pseudomonas Agar F. In the application of Liefson's method (6) for flagella staining, commercially available precleaned slides were used. Intracellular fat granules were stained by the method of Burdon (1); a smear of an overnight slant culture of *B. megaterium* was used as a positive control. The oxidase test was performed by Kovac's method (5).

Ability to grow anaerobically in glucose was determined in OF Medium [BBL or Difco; modified by the addition of 0.3% (w/v) Ionagar (Colab Laboratories Inc., Chicago Heights, Ill.], tubed to a depth of approximately 10 cm. Filter-sterilized glucose was added to the melted base to a concentration of 1% (w/v). The medium was cooled to approximately 48 C, then inoculated sparingly by means of a straight wire to the bottom of the tube. Cultures were incubated at 37 C for up to 3 days, and then examined for growth in the lower half of the tube. The deep tube of relatively solid medium made the use of a petrolatum or agar seal unnecessary.

Growth at 4 C and 41 C was determined by inoculating slants of Brain Heart Infusion Agar (Difco) and then incubating them in water baths. Growth at 4 C was recorded after 10 days; growth at 41 C was recorded after 24 and 48 hr.

Denitrification (the ability to utilize nitrate as an electron acceptor and grow anaerobically, usually with the production of gas) was observed by inoculating melted and cooled Nitrate Agar (tubed to a depth of about 10 cm to provide anaerobic conditions in the lower portions of the tube). A small inoculum was introduced to the bottom of the tube by means of a straight wire. Cultures were incubated at 37 C for 48 hr. If no anaerobic growth or gas was apparent at this time, a second tube was inoculated from the first, and incubated for 5 days. Growth in the lower half of the tube (or growth and gas) was recorded as positive.

The presence of arginine dihydrolase was detected by heavily inoculating a deep tube of melted and cooled OF Medium to which 1% (w/v) arginine and 0.3% (w/v) Ionagar had been added. A tube of the basal medium to which no arginine had been added was inoculated as a control. After incubation at 37 C for 24 to 48 hr, the test was recorded as positive if the tube containing arginine was blue throughout and the control either showed no change in color or was blue only at the surface.

Gelatin and starch hydrolysis were determined by the plate methods described elsewhere (13).

Identification procedure. All cultures received were first streaked on a nonselective nutrient agar and a single, well-isolated colony subcultured to Kligler Iron Agar (KIA). Only those strains that failed to produce acid in the butt of the KIA tube were retained for further study. Pseudomonas Agar F and Pseudomonas Agar P were inoculated from the growth on KIA. After overnight incubation, pigment production was observed; then motility tests, oxidase tests, Gram, fat, and flagella stains were done. Cultures selected for complete identification were inoculated to the remaining seven test media.

RESULTS

The characteristics of 21 known strains of pseudomonads are indicated in Table 2. Of 228 unknown cultures examined, 60 were nonmotile and did not possess flagella; 5 were motile and peritrichous. These were therefore not *Pseudomonas* and were not considered further. On the basis of fluorescent and pyocyanine pigment production, 63 were considered to be *P. aeruginosa* and were not characterized further. The remaining 100 cultures were tested for all the characteristics described above. The species of 93 of these were readily identified, whereas seven could not be identified by these characteristics (Table 3).

From these results and from the data of Stanier et al. (14), a simple, dichotomous schema, based on the observation of from 2 to 10 characteristics, was developed (Fig. 1). Other characteristics (such as number of flagella, denitrification, growth at 4 C, and gelatin hydrolysis) may be utilized to confirm the identity of the fluorescent species (Table 4). Confirmatory tests are necessary for the identification of *P. aeruginosa* strains that do not produce pyocyanine or pyorubrin. The four unidentified fluorescent species would have been identified as *P. aeruginosa* on the basis of fluorescence and growth at 41 C. However, none of them denitrified and none hydrolyzed gelatin; therefore, they probably are not *P. aeruginosa*.

TABLE 2. *Characteristics of known strains of Pseudomonas*[a]

Characteristic	No. of positive strains						
	P. aeruginosa	*P. alcaligenes*	*P. fluorescens*	*P. maltophilia*	*P. pseudomallei*	*P. putida*	*P. stutzeri*
Polar flagella	5	1	6	3	2	2	4
Predominantly 1	5	1	0	1	0	0	4
Predominantly >1	0	0	6	2	2	2	0
Intracellular fat	0	0	0	0	2	0	0
Fluorescence	5	0	6	0	0	2	0
Pyocyanine or pyorubrin	3	0	0	0	0	0	0
Denitrification	5	0	0	0	2	0	4
Growth at 4 C	0	0	5	0	0	1	0
Growth at 41 C	4	0	0	0	2	0	3
Gelatin hydrolysis	4	1	6	3	2	0	0
Starch hydrolysis	0	0	0	0	0	0	4
Oxidase	5	1	6	0	2	2	4
Arginine dihydrolase	5	1	5	0	2	2	0

[a] The no. of strains tested were as follows: *P. aeruginosa*, 5; *P. alcaligenes*, 1; *P. fluorescens*, 6; *P. maltophilia*, 3; *P. pseudomallei*, 2; *P. putida*, 2; and *P. stutzeri*, 4.

TABLE 3. *Characteristics of unknown strains of Pseudomonas*

Characteristic	No. of positive strains identified as					
	P. aeruginosa	*P. fluorescens*	*P. maltophilia*	*P. putida*	Fluorescent species	Nonfluorescent species
Polar flagella	65	2	15	11	4	3
Predominantly 1	65	0	1	1	3	0
Predominantly >1	0	2	14	10	1	3
Intracellular fat	0	0	0	0	0	0
Fluorescence	65	2	0	11	4	0
Pyocyanine or pyorubrin	51	0	0	0	0	0
Denitrification	64	0	0	0	0	0
Growth at 4 C	0	2	0	4	1	0
Growth at 41 C	64	0	0	0	4	0
Gelatin hydrolysis	64	2	15	0	0	1
Starch hydrolysis	0	0	0	0	0	0
Oxidase	65	2	0	11	4	3
Arginine dihydrolase	65	2	0	11	4	0

[a] The no. of strains tested were as follows: *P. aeruginosa*, 65; *P. fluorescens*, 2; *P. maltophilia*, 15; *P. putida*, 11; fluorescent species, 4; nonfluorescent species, 3.

Characteristics useful in confirming the identity of nonfluorescent pseudomonads are listed in Table 5. Although no strains of *P. multivorans* or of the acidovorans group were encountered in this study, they are included in Table 5 and in Fig. 1 to indicate characteristics that should be of value in identifying them. Sources of strains studied by Stanier et al. (14) indicate that these may be found in clinical specimens. *P. pseudoalcaligenes* is not included because of its variability in several of the characteristics that were used in this study. For example, Stanier et al. (14) describe *P. pseudoalcaligenes* as being intracellular fat, variable; gelatin hydrolysis, variable; and arginine dihydrolase, variable.

Because pigment formation and motility are key characteristics in this schema, it is important that tests to demonstrate these be as reliable as possible, or that they be repeated if results are questionable. The presence of fluorescent pigment should be determined by examining cultures under ultraviolet light. Several of the strains in the study produced little, if any, pigment visible under ordinary light, and would have been considered negative had they not been examined with ultraviolet light. Fluorescent pigment production was found to be variable in a few strains. Of six strains retested because they were thought to belong to one of the fluorescent species, two were fluorescent on both Pseudomonas Agar F and Flo Agar; one was fluorescent only on Pseudomonas Agar F, and three were fluorescent only on Flo Agar.

Pyocyanine pigment production also was variable in a few strains. Of 24 strains retested, five produced pyocyanine on both Pseudomonas Agar P and Tech Agar and three produced it only on Tech Agar, whereas the remaining 16 did not

TABLE 4. *Confirmatory characteristics for identification of fluorescent pseudomonads*

Characteristic	*P. aeruginosa*	*P. fluorescens*	*P. putida*
Polar flagella	1	>1	>1
Denitrification	+	V[a]	0
Growth at 4 C	0	+	V
Gelatin hydrolysis	+	+	0

[a] V = variable.

TABLE 5. *Confirmatory characteristics for identification of nonfluorescent pseudomonads*

Characteristic	Acidovorans group[a]	*P. alcaligenes*	*P. maltophilia*	*P. multivorans*[a]	*P. pseudomallei*	*P. stutzeri*
Polar flagella	>	11	>1	>1	>1	1
Intracellular fat	+	0	0	+	+	0
Gelatin hydrolysis	0	0	+	+	+	0
Starch hydrolysis	0	0	0	0	0	+
Oxidase	+	+	0	+	+	+

[a] Based on data of Stanier et al. (14). None was encountered among the strains studied.

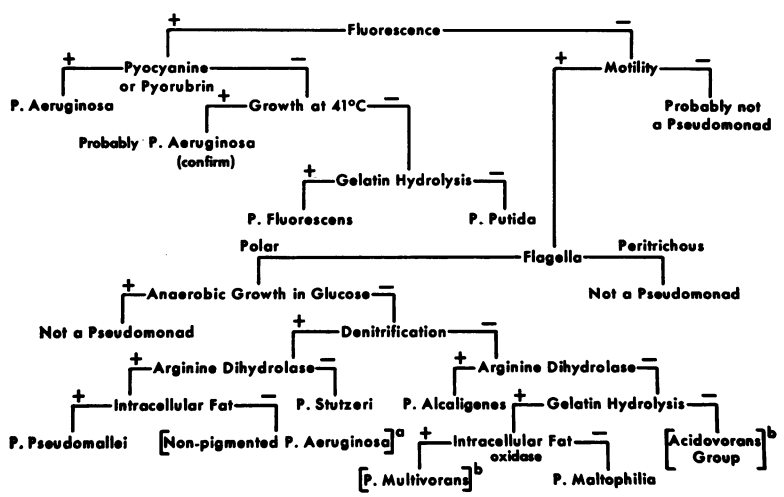

FIG. 1. *Schema for identification of pseudomonads. (a) Identity based on theoretical considerations; no such strains encountered in this study. (b) Identity based on data reported by Stanier et al. (14).*

TABLE 6. *Source and significance of pseudomonads other than P. aeruginosa*

Culture identified	Source	Significance
P. fluorescens	Blood culture	Septicemia; 27 days postoperative, abdominal abscess following bowel resection; organism recovered in pure culture from three successive blood cultures
P. fluorescens	Saliva	Carrier survey-mixed flora
P. maltophilia	Blood culture	No clinical or postmortem evidence of sepsis
P. maltophilia	Ear drainage	Records not available
P. maltophilia	Urine	Part of mixed flora (*E. coli*) urinary tract infection
P. maltophilia	Urine	Few colonies in pure culture; urinary tract infection
P. maltophilia	Urine	Epididymitis
P. maltophilia	Urine	20,000/ml with 50,000/ml of Mima-Herellea and 15,000/ml of *E. coli*
P. maltophilia	Tracheal secretions (tracheotomy tube)	Pneumonitis; serratia and *E. coli* in same specimen
P. maltophilia	Sputum	Mixed flora (*Klebsiella-Aerobacter*); atelectasis, right lower lobe
P. maltophilia	Sputum	Mixed flora; no indication in records of respiratory tract infection
P. maltophilia	Sputum	Mixed flora; no indication in records of respiratory tract infection
P. maltophilia	Throat	Mixed flora (*Klebsiella-Aerobacter*); pneumonia
P. maltophilia	Drainage—eye	Discharge from eye of blind patient; treatment with polymixin-neomycin-bacitracin ointment cleared exudate
P. maltophilia	Cervix	Records not available
P. maltophilia	Dog bowel tissue	Experimental surgery-contaminant in tissue perfusion experiment; dog had preoperative bowel preparation with neomycin
P. putida	Bile	Drainage; 2 days postoperative, following common bile duct exploration; no clinical evidence of infection; *P. aeruginosa* in same specimen
P. putida	Urine	More than 10,000,000/ml in pure culture from indwelling catheter; patient afebrile
P. putida	Urine	5,000,000/ml; chronic urinary tract infection; 10,000,000/ml *E. coli* also found in same specimen
P. putida	Sputum	Mixed flora following pneumococcal pneumonia and treatment with erythromycin; after tetracycline therapy, "*Achromobacter*" not recovered; significance questionable
P. putida	Feces	Carrier survey; *P. aeruginosa* and mixed enteric flora also present
P. putida (isolates from six individuals)	Saliva	Carrier survey-mixed flora
Fluorescent species (isolates from two individuals)	Saliva	Carrier survey-mixed flora
Fluorescent species	Open tibial fracture	Part of mixed flora (*P. aeruginosa*, *Aerobacter*, *Staphylococcus*); instillation of polymixin and neomycin cleared *Pseudomonas* and *Aerobacter* from wound
Fluorescent species	Whirlpool bath	Environmental survey on burn ward
Nonfluorescent species	Ureterostomy drainage	Part of mixed flora (*Aerobacter*, enterococci); infection following surgery; Hodgkin's disease
Nonfluorescent species	Vesicular infection of gangrenous leg	Dry gangrene following snake bite; infection cleared after sulfamylon therapy
Nonfluorescent species	Urine	Part of mixed flora (*Serratia* and *E. coli*); persistent bacilluria following kidney transplant

produce pyocyanine on either medium. Five of these strains produced brown to black-brown pigment on both media, and pyocyanine was extracted from four of them by the use of chloroform.

Because nonfermenting bacteria usually are strict aerobes, they will not grow much below the surface in the various semisolid media that are recommended for motility tests. For this reason, hanging-drop preparations, as suggested by Leifson (7), are necessary to determine motility.

The source and clinical significance of each of the pseudomonads studied, other than *P. aeruginosa*, are indicated in Table 6. A few of the strains appeared to be of clinical importance. *P. fluorescens* was isolated from three successive blood cultures from a patient with septicemia; *P. maltophilia* was isolated from two patients with urinary tract infection, from the urine of one patient with epididymitis, and from purulent exudate from the eye of a blind patient. The significance of isolations of *P. putida* and *P. maltophilia* from various other specimens, as part of a mixed flora, is questionable. Isolation of strains of *P. fluorescens* and *P. putida* from saliva or feces of healthy individuals indicates that these are part of the normal flora of a few individuals. Nonfluorescent pseudomonads were not found in saliva or feces of healthy individuals (15, 16) because methods for detection were selective for fluorescent pseudomonads capable of growing on Cetrimide Agar.

DISCUSSION

The system for classification of aerobic pseudomonads recently proposed by Stanier et al. (14) offers to clarify the positions of several species of the genus *Pseudomonas*. That this work is of importance to a diagnostic medical microbiologist is evidenced by the fact that many of the strains studied were from humans. Although their taxonomic studies were based largely on nutritional needs, certain morphological and physiological characteristics were also used as distinguishing features to differentiate species.

To what extent such a proposed system would be useful in a diagnostic medical microbiology laboratory depends on the simplicity of tests and the methodology required to identify various members of the genus. To determine the wide array of substances usable as carbon sources by the many isolates of nonfermentative, gram-negative bacteria that confront the diagnostic microbiologist would be an overwhelming task. It should be within the capability of many diagnostic laboratories to use some of the other characteristics in an identification schema.

The schema developed in this study was based on a few general morphological and physiological characteristics. It was found useful in identifying species of aerobic pseudomonads that made up a large proportion of the nonfermentative, gram-negative bacteria contributed to this study. As with any dichotomous key that is based on a few characteristics, there are obvious limitations. Lack of motility, for example, would exclude nonmotile variants of motile species, and it would exclude identification of *P. mallei* by this system. Occasional strains aberrant in other features would not fit into the schema, or they might fit only into a nondefinitive group category.

Despite its limitations, the schema leads to more accurate definition of strains than is achieved in many diagnostic laboratories by conventional methods. For example, of the 18 nonfluorescent strains of *Pseudomonas* contributed to this study, none had been recognized by the contributors as *Pseudomonas*. They had been reported as *Alcaligenes*, *Flavobacterium*, or *Mima-Herellea*. Examination of motility and flagellation would have been of great assistance to the laboratory in providing a more correct diagnosis. Although for many years Leifson (7, 8) has urged the use of flagella staining to distinguish *Pseudomonas*, *Alcaligenes*, and other genera, diagnostic microbiologists are reluctant to incorporate this method into their routine or to use it even as a special test. Leifson's method (6) was found to be a relatively simple technique by which any reasonably adept technician should obtain good results.

The relative frequency of occurrence of various members of the genus *Pseudomonas* in clinical specimens cannot be determined from reported data. Contributions of strains was voluntary, and somewhat selective.

ACKNOWLEDGMENTS

I am deeply grateful to the many contributors of strains of known species of *Pseudomonas* and to the contributors of clinical isolates. My special thanks are due to my colleague, Valerie Hurst, for her encouragement and advice, and for the use of her laboratory facilities for this study.

This study was supported by Public Health Service research grant CC-00092 from the National Communicable Disease Center, Atlanta, Ga.

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