Characterization and Identification of Gram-Negative, Nonfermentative Bacteria

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The morphological and physiological characteristics of 593 strains of nonfermentative, gram-negative bacteria are described. A battery of 46 tests was used to identify and differentiate strains representing 8 genera and 31 species of named and group-designated bacteria. Seven selected amides and organic salts were closely examined to determine their usefulness, individually or as a battery, in characterizing and identifying the organisms. Of these, allantoin and acetamide showed the most promise in differentiating the more commonly occurring organisms from biochemically similar species. Susceptibility patterns to 12 antimicrobics also proved useful in differentiation, especially among atypical strains.

In the past several years, increased attention has been given to the recognition and identification of nonenteric, gram-negative bacteria. Of these, the nonfermentative, gram-negative rods and cocci have gained prominence because of the increased frequency of recovery of these organisms from clinical materials as well as the heightened awareness of their possible role as pathogens rather than as simply contaminants. Also, the nomenclature and taxonomy of these bacteria have been clarified with considerable success. The media and test conditions used in the identification and differentiation of nonfermentative bacteria (NFB) are diverse, and the choice of each presents a continuous challenge to many clinical laboratories. Currently, there are three practical and widely used schemata for identification. These include those of Weaver et al. (19), Hugh and Gilardi (6), and Pickett and Pedersen (15, 16), wherein the approaches to the problems of identification and the criteria used for identification differ to some extent. Just recently, an additional scheme has been introduced into the literature (13).

Assimilation tests of organic compounds using basal mineral media (2, 4), and basal salt solutions with the incorporation of an indicator to determine alkalization of selected compounds (15, 16), have been used for characterization of the NFB. In many instances, characterization based on such biochemical and nutritional parameters as suggested in these studies and others (17) is still beyond the capabilities of most clinical laboratories. Although our laboratory follows the criteria set forth by the Center for Disease Control (CDC) for identification of

NFB, tests that showed promise in clinical applications were adopted from the findings of other investigators (15, 16). The purposes of this report are as follows: (i) to describe the methods used in this laboratory to identify oxidative and nonoxidative NFB, (ii) to describe the morphological and physiological characteristics of the organisms encountered, and (iii) to describe results of tests with selected amides and organic salts that have proven to be useful, as a battery and individually, in assisting in differentiation.

MATERIALS AND METHODS

Organisms. The organisms described in this report were strains of nonfermenting rods and cocci received by the Microbiology Section, Brooke Army Medical Center, Fort Sam Houston, Tex., over a 3.5-year period. Cultures were referred for identification or confirmation and were received as "gram-negative bacilli" or "*Pseudomonas* species." The criteria used for identification were principally those described by Weaver et al. (19). Supplemental tests (2, 4, 15, 16) resulted in 46 characteristics used for identification. Flagella stains were performed on most organisms by using the Leifson technique (7), and most organisms were examined by electron microscopy to confirm flagellar arrangement.

Morphological studies. Cultures received were streaked on 5% defibrinated sheep blood, eosin-methylene blue, and MacConkey (MAC) agars for isolation as well as for studies of colonial morphology, pigmentation, and hemolysis. Growth characteristics were observed on MAC agar (Difco), salmonella-shigella agar (SS agar; Difco), and triple sugar iron agar (TSI; Difco). Trypticase soy agar slants (TSA; BBL) and Trypticase soy broths (TSB; BBL) were used for preparation of the Gram stain, slide motility, flagella stain, and oxidase tests. All media

were incubated at 35°C for 18 to 24 h and held at room temperature for an additional 2 or 3 days for further observations.

Biochemical studies. The following media or tests were inoculated with a drop or stab from an 18-h TSB culture and incubated at 35°C: Casitone broth for indole (Difco), Simmons citrate (Difco), GI motility medium (Difco), phenylalanine agar (Difco), Christensen urea agar (BBL), nitrate broth with inverted tube (BBL), Seller's agar (Difco), litmus milk, malonate broth (Difco), cetrimide agar (0.09%), esculin slants, 10% glucose and lactose slants with phenol red, Moeller decarboxylase medium (Difco), rapid decarboxylase medium (12), deoxyribonucleic acid test agar (Difco), spirit blue agar for lipase (Difco), starch agar for amylase (TSA with 0.2% soluble starch), egg yolk agar for lecithinase (TSA with 10% enrichment; Difco), potassium gluconate broth for gluconate oxidation (Difco), and *o*-nitrophenyl- β -D-galactopyranoside (ONPG) disks for the β -galactosidase test (Difco). Nutrient gelatin (Difco) and *Pseudomonas* agars P and F (Difco) for pyocyanine and fluorescein production were incubated at both 25 and 35°C.

The following tests or observations were made at 24 h: phenylalanine deaminase (FeCl₂), gluconate oxidation (Clinitest tablets; Ames Co.), deoxyribonuclease (DNase) (1 N HCl), starch hydrolysis (Lugol's iodine), ONPG, and oxidase tests (N disks, Kovacs' test). Tests for nitrite production and indole (Ehrlich reagent after xylene extraction) were performed after 2 and 7 days. All other tests were observed daily for 4 days and again at 7 days before discarding.

Oxidation of carbohydrates. Kings semisolid oxidative-fermentative (O-F) medium was used throughout the study. The basal medium contained: Casitone (Difco), 2 g; phenol red, 2.0 ml of a 1.5% aqueous solution; agar, 3.0 g; reagent grade water, 900 ml. The pH was adjusted to 7.3, and 4.5 ml was dispensed into screw-capped tubes (13 by 100 mm). After autoclaving, a Seitz-filtered 10% solution (0.5 ml) of each sugar was added to appropriate tubes at the time of use to give a final concentration of 1%. Media were inoculated by stabbing four times with a loopful of overnight broth culture. The open tests were incubated at 35°C and observed daily for 4 days and at 7 days. Acidification was judged by a perceptible indicator change from red to yellow.

Alkalinization of amides and salts. The test for alkalinization of the various substrates was similar to that described for acetamide (11). The basal medium was that used for Simmons citrate agar and is available commercially (Difco). The substrates were as follows (grams per liter): acetamide, 10; allantoin, butyramide, formamide, nicotinamide, propionate, and tartrate, 5. The completed media (pH adjusted to 6.8) were dispensed in 6-ml amounts into screw-cap tubes (16 by 125 mm), autoclaved, and allowed to cool in the slanted position. The slants were inoculated with a drop of inoculum and examined daily for 4 days and again at 7 days. A pronounced blue coloration of the medium was recorded as a positive test, indicating alkalinization of the substrate. Uninoculated controls were included pe-

riodically to monitor substrate stability. Each batch of newly prepared medium was quality control checked, using *Pseudomonas aeruginosa* (ATCC 27853), *P. acidovorans* (ATCC 17438), and stock strain of *P. alcaligenes*.

Antimicrobial susceptibility tests. Susceptibility tests were performed using the Bauer-Kirby standardized single disk method (1). Zone sizes were measured to the nearest millimeter and the results were recorded.

RESULTS AND DISCUSSION

The physiological characteristics of oxidative, yellow-pigmented organisms and nonoxidative organisms are presented in Tables 1 to 4. Table 5 shows the compiled results of alkalization of selected amides and organic salts. Table 6 shows the antibiograms of the NFB.

Fluorescent oxidizers. The *P. aeruginosa* cultures submitted to this laboratory for identification presumably were originally recovered as nonpigmented isolates. Pigment was not evident on the original plated media, but, after several passages, 25 cultures (27%) produced pyocyanine and 5 cultures (5%) produced pyomelanine. In Table 1, results of tests on P medium are recorded at 35°C and those on F medium and gelatin are recorded at 25°C. Comparative studies of the 91 cultures of *P. aeruginosa* showed that pyocyanine production was more favorable at 35°C (80%) than at 25°C (75%), although fluorescein production was equivocal at the two temperatures. In sharp contrast, fluorescein production by *P. putida* and *P. fluorescens* was decidedly lacking at 35°C when compared with that at 25°C. In addition, reactions were more rapid and intense at 25°C, which obviated the need for culture examination under ultraviolet light. Indeed, 38 strains (73%) of *P. putida* and 12 strains (67%) of *P. fluorescens* produced fluorescein at 25°C, whereas only 17 strains (33%) and 6 strains (33%), respectively, produced fluorescein at 35°C. No isolates of the two species produced fluorescein at 35°C and not at 25°C. This study and others (2, 9) have shown that the group features of pyocyanine and fluorescein are not constant characteristics among the fluorescent pseudomonads. Nevertheless, the selection of proper incubation temperatures greatly assisted in the enhancement of pigment formation (9).

Liquefaction of gelatin was also influenced by incubation temperature. Significantly, 7 of the 17 gelatin-positive strains of *P. fluorescens* were negative in 7 days at 35°C but positive in 2 days at 25°C. Although most other organisms capable of gelatinase production were active at both temperatures (i.e., *P. aeruginosa* and *P.*

Amides/salts	(87)	(52)	(18)	(5)	(15)	(3)	(19)	(25)	(4)
Acetamide ^c	79 (6)	98	6	0 (2)	40	0	0	0	0
Allantoin ^c	55 (24)	91	4	0 (1)	0	13	0	0	0
Butyramide	1 (11)	14	23	0	0	0	0	0	1 (1)
Citrate	86 (1)	100	18	5	100	87	3	0	4
Formamide	33 (47)	92	50	3	60	2	13	6 (2)	32
Malonate ^c	81 (5)	99	69	12 (2)	100	1	33	6	24
Nicotinamide	0 (6)	6	0	0	0	0	0	0	8
Propionate	71 (13)	97	63	6 (3)	40	20	1 (2)	4 (8)	0
Tartrate	0 (1)	1	25	11	0	0	0	0	48
									0
									3 (1)
									100

^a Symbols: +, number positive within 2 days; (+), number positive within 3 to 7 days; %, percent positive after 7 days.

^b All strains were indole, H₂S, lysine, and ornithine decarboxylase negative.

^c Useful test for species differentiation.

^d Results reported for rapid method.

maltophilia), liquefaction was generally faster at 25°C by 1 to 5 days.

The *P. aeruginosa* isolates demonstrated considerable activity against the amides and organic salts. Alkalinization of acetamide, allantoin, formamide, and propionate was consistent, and, with the exception of formamide, activity was rapid and intense against these substrates. There was little difference between the pyocyanogenic and apyocyanogenic strains in the extent or rate of activity. *P. putida* was most active against butyramide, formamide, propionate, and tartrate, whereas *P. fluorescens* was active principally against formamide and propionate. The inability of the two fluorescent organisms to alkalinize allantoin and acetamide was diagnostically significant. The variability of the isolates of *P. putida* to alkalinize butyramide and tartrate is a departure from previous reports (13, 15) that ascribed almost 100% activity against these substrates. The discrepancy in our findings and that reported by these workers may reflect not only differences in methodology, but also differences in population and size of population of organisms studied. Interestingly, in one report all six strains of *P. putida* were allantoin negative (15), whereas in a recent report all five strains tested were allantoin positive (13).

Nonfluorescent oxidizers. *P. pseudomallei* isolates were characterized by production of nitrates and gas, peptonization of litmus milk, and production of gelatinase, lipase, and lecitinase. All cultures showed three or more flagella and were active against carbohydrates, but failed to grow on SS agar. Pigmentation and a wrinkled appearance were variable characteristics. Members of the *P. stutzeri* complex were monopolar organisms that produced nitrites and gas and occasionally deaminated phenylalanine. Pigment production was variable and urease production was often delayed. The *P. stutzeri* (Vb-1) isolates hydrolyzed starch, occasionally developed wrinkled colonies, and oxidized maltose and mannitol. Cultures of the CDC group Vb-3 (*P. stutzeri*-like) closely resembled *P. stutzeri*, except for the positive arginine test using the Moeller medium and lesser ability to produce nitrites and gas. Many of the isolates of *P. stutzeri* and Vb-3 failed to denitrify in both nitrate broth and in Seller's agar. Denitrification is generally considered a universal property of *P. stutzeri* (4, 6, 14). This property has been demonstrated (17) after several passages through semisynthetic nitrate medium, but the technique has not been totally successful in other hands (15). The results of the present study and others (13, 15)

TABLE 2. Biochemical characteristics of *Acinetobacter* and *Achromobacter* species^a

Test or medium ^b	<i>A. calcoaceticus</i> (53 strains)		<i>A. xylosoxidans</i> (15 strains)		CDC VD-1 (5 strains)		<i>Achromobacter</i> sp. 1 (8 strains)		<i>Achromobacter</i> sp. 2 (3 strains)	
	+(+)	%	+(+)	%	+(+)	%	+(+)	%	+(+)	%
Motility ^c										
Slide	0	0	15	100	5	100	6	75	3	100
Tube	0	0	13	87	5	100	6	75	3	100
Flagellar arrangement			P ^a		P		P		P	
Oxidase ^c	0	0	15	100	5	100	8	100	3	100
Pyocyanine (35°C)	0	0	0	0	0	0	0	0	0	0
Fluorescein (25°C) ^c	0	0	0	0	0	0	0	0	0	0
Gluconate oxidation	0	0	0	0	0	0	0	0	0	0
Phenylalanine deaminase	0	0	0	0	3	60	0	0	2	67
Urease ^c	3 (8)	21	0	0	5	100	5 (3)	100	1 (1)	67
Nitrite production	0	0	12	80	2 (1)	60	3 (1)	50	1	33
Denitrification ^c	0	0	13	87	5	100	1	13	3	100
Litmus milk peptonized	2	4	0	0	0	0	6 (1)	88	0	0
Gelatinase (25°C)	0	0	0	0	0	0	0	0	0	0
DNase	0	0	0	0	0	0	0	0	0	0
Lipase	38 (2)	75	1	7	0	0	1 (4)	63	0	0
Lecithinase	1 (6)	13	0	0	0	0	0	0	0	0
Starch hydrolysis	0	0	0	0	0	0	0	0	0	0
Esculin hydrolysis ^c	0	0	0	0	4 (1)	100	8	100	0	0
Growth on cetrimide ^c	3	6	15	100	0	0	1 (1)	25	0	0
Growth on MAC agar	52	98	15	100	5	100	8	100	3	100
Growth on SS agar ^c	3	6	15	100	5	100	1	13	3	100
Hemolysis	0	0	0	0	0	0	0	0	0	0
Pigment	0	0	0	0	0	0	0	0	0	0
Wrinkled colonies	0	0	0	0	0	0	0	0	0	0
Arginine dihydrolase ^{c, d}	2 (5)	13	0	0	5	100	0 (5)	63	0	0
OF										
Glucose ^c	53	100	1 (3)	27	0 (2)	40	5 (3)	100	3	100
Lactose	33 (20)	100	0	0	0	0	0 (5)	63	0	0
Sucrose	0	0	0	0	0	0	4 (4)	100	0	0
Maltose	2 (5)	13	0	0	0	0	5 (3)	100	0	0
Mannitol	0	0	0	0	0	0	6 (1)	88	0	0
Xylose ^c	53	100	15	100	1 (4)	100	8	100	3	100
Fructose ^c	0 (2)	4	1 (3)	27	1 (4)	100	8	100	2 (1)	100
10% Glucose	53	100	0	0	0	0	2 (4)	75	3	100
10% Lactose	53	100	0	0	0	0	0 (2)	25	0	0
ONPG	0	0	0	0	0	0	8	100	0	0
Amides/salts	(51)		(15)		(5)		(8)		(3)	
Acetamide ^c	1 (2)	6	4 (8)	80	0	0	0	0	0	0
Allantoin ^c	0 (2)	4	10 (2)	80	5	100	7 (1)	100	2 (1)	100
Butyramide	2 (1)	6	11 (4)	100	0	0	0 (3)	38	0	0
Citrate	50	98	15	100	1 (3)	80	3 (5)	100	0	0
Formamide	2 (7)	18	15	100	1 (1)	40	8	100	0 (1)	33
Malonate ^c	41 (2)	84	0 (3)	20	0	0	0	0	0	0
Nicotinamide	0	0	0 (12)	80	0	0	0	0	0	0
Propionate	17 (17)	67	0 (2)	13	0	0	0 (4)	50	0	0
Tartrate	14 (3)	33	0	0	0 (1)	20	0	0	0	0

^a Symbols are the same as in Table 1, footnote a. P, Peritrichous flagellar arrangement.^b All strains were indole, H₂S, lysine, and ornithine decarboxylase negative.^c Useful test for species differentiation.^d Results reported for rapid method.

indicate that this characteristic is not stable for all strains of *P. stutzeri*. In addition, arginine activity is generally considered to be a negative characteristic of *P. stutzeri* (4, 6), and only a few reports have found otherwise (8) or refer to arginine-positive, *P. stutzeri*-like organisms (19). Recently, dihydrolase reactions of NFB, using a rapid test medium, were described (12). Tests for arginine dihydrolase in the rapid medium suggested that this medium was more

sensitive than the conventional Moeller medium. As a result, notable discrepancies were seen in tests with *P. stutzeri* isolates. Whereas arginine activity was not evident in Moeller medium, 67% of the isolates tested demonstrated arginine activity in the rapid medium. Thus, most of the organisms listed as *P. stutzeri* in Table 1 should more rightly be placed in the Vb-3 group (*P. stutzeri*-like). The arginine dihydrolase test is the only criterion separating

these two organisms, and since antibiograms are also similar (Table 6), consideration should be given to designating these organisms as biotypes. The infrequently recovered *P. mendocina* were arginine positive, but failed to produce lipase, hydrolyze starch, show wrinkling on agar, or oxidize maltose and mannitol. Except for the negative mannitol reaction, *P. mendocina* (4, 6, 14) appears to correspond to CDC Vb-2 (19). *P. pseudoalcaligenes* was monopolar, but variable in phenylalanine and arginine activity, ability to grow on SS agar, and utilization of citrate. Oxidation of glucose was delayed although activity against fructose was usually prompt. As seen in Table 1, except for the limited activity against formamide and propionate by these groups of organisms, overall activity against the selected amides and salts was lacking. Only four organisms conforming to the CDC designation V-A, type 1, were encountered in this 3.5-year period. These were distinctive by rapid urease production, nitrite production with gas formation, and failure to grow on cetrimide and SS agars. Oxidation of sugars, with the exception xylose, was delayed up to 6 days. Three strains were nonmotile in semisolid medium and when examined by wet mount, although all organisms possessed monopolar flagella as confirmed by electron microscopy. Activity against formamide and tartrate was rapid in most instances.

The oxidase-negative *Acinetobacter calcoaceticus* (*A. anitratus*) were nonmotile coccobacilli with a tendency to occasionally appear as diplobacilli and rods. Most cultures alkalinized citrate and malonate within 24 h although alkalization of the selected amides and salts was variable (Table 2).

Peritrichous oxidizers. The most predominant of the peritrichous oxidizers was *Achromobacter xylosoxidans* (Table 2). Biochemical characteristics were similar to the pseudomonads in that they were motile and oxidase positive, reduced nitrates to nitrites and gas, grew on SS agar, and were tolerant to cetrimide. The organisms were consistent in the oxidation of xylose, but showed variable ability to attack glucose and fructose. Tatum et al. (18) found that all strains of *A. xylosoxidans* oxidized glucose although reactions were delayed. The strains in this report were received from diverse sources and can be judged as atypical strains. Activity against the amides was diagnostic. In addition to *P. aeruginosa*, *A. xylosoxidans* was the only oxidative organism tested that alkalinized both allantoin and acetamide. It was also the only oxidative organism that regularly alkalinized both butyramide and nic-

otinamide in 7 days.

Sixteen isolates of achromobacteria encountered were unusual both morphologically and biochemically. The flagellar arrangements consisted of monopolar as well as lateral and occasional peritrichous flagella. On the basis of the abundant lateral flagella, these organisms were considered peritrichous. Eight isolates were designated *Achromobacter* sp. 1. These organisms appeared as gram-negative coryneform rods, many of which were banded as seen with *A. xylosoxidans*. Two strains were nonmotile although flagella were seen by stain. The organisms grew on MAC agar, were oxidase positive, produced nitrites, urease, and lipase, peptonized litmus milk, and hydrolyzed esculin. All strains were ONPG positive. One strain produced gas and grew on SS agar. Tests for arginine dihydrolase and oxidation of 10% carbohydrates required at least 3 days for reactions to be evident. Similar to *A. xylosoxidans*, these organisms alkalinized allantoin and formamide, but failed to alkalinize acetamide and nicotinamide. The biochemical characteristics of two additional infrequently encountered achromobacter species are included in Table 2. Five organisms were consistent with the CDC designation VD-1. Three other organisms were designated *Achromobacter* sp. 2. Characteristics of both groups included rapid urea hydrolysis, phenylalanine deamination, nitrite production, denitrification, and growth on SS agar. Group VD-1 hydrolyzed esculin although oxidation of sugars was delayed 3 or more days. The strains of *Achromobacter* sp. 2, resembled *A. xylosoxidans*, except for their failure to utilize citrate or tolerate cetrimide. Significantly, these were the only organisms that singly, but consistently, alkalinized allantoin.

Yellow-pigmented oxidizers. Table 3 presents the characteristics of the yellow-pigmented, oxidative bacteria. Nineteen strains (24%) of *P. maltophilia* presented a light yellow pigment on TSA slants. There were no significant biochemical differences between the pigmented and nonpigmented biotypes of *P. maltophilia*. The oxidase-positive strains (28%) gave weak, but definitely positive, reactions on N disks. Gelatin and esculin were rapidly hydrolyzed, although peptonization of litmus milk and decarboxylation of lysine (Moeller) required 3 or more days in many instances. Lysine decarboxylation, using the rapid method, was diagnostically useful (12). Deoxyribonucleic acid was hydrolyzed by most organisms, whereas a positive ONPG test was seen with less than half of the strains. Eight of the eleven organisms that oxidized glucose required 3 or

TABLE 3. Biochemical characteristics of yellow-pigmented, oxidative, nonfermenting bacteria^a

Test or medium ^b	<i>P. maltophilia</i> (80 strains)			<i>P. cepacia</i> (7 strains)			<i>F. meningosepticum</i> (8 strains)			<i>Flaobacterium</i> IIb (22 strains)			CDC IIK-1(12 strains)			CDC IIK-2 (7 strains)			CDC VE-1 (5 strains)			CDC VE-2 (14 strains)		
	+	(+)	%	+	(+)	%	+	(+)	%	+	(+)	%	+	(+)	%	+	(+)	%	+	(+)	%	+	(+)	%
Motility ^c	79	99	6	86	0	0	0	0	0	0	0	0	3	25	0	0	0	5	100	10	71			
Slide Tube	68 (1)	86	4	>2	57	0	0	0	0	0	0	0	0	0	0	0	0	5	100	8	57	1		
Flagellar arrangement	22	28	5	71	8	100	22	100	11	92	7	100	0	0	0	0	0	>2	0	0	0			
Oxidase ^c	0	0	0	0	8	100	12	55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Indole ^c	0	0	4	56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	14		
Glucanate	5	6	0	0	0	0	2	9	1	8	2	29	0	0	0	0	0	0	0	5	36			
Phenylalanine	8 (39)	59	4 (2)	86	0	0	0	0	0	0	0	7	100	1 (3)	80	1 (3)	29	0	0	1 (3)	80	1 (3)	29	
Urease ^c	26 (2)	35	2	29	0	0	6 (1)	32	0	0	0	0	0	0	0	1	20	0	0	0	0	0	0	
Nitrite production	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Denitrification	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Litmus milk peptonized ^c	63 (15)	98	5 (2)	100	8	100	22	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Gelatinase (25°C) ^c	80	100	3 (4)	100	6 (1)	88	21 (1)	100	1	8	0	0	0	0	0	2 (2)	80	0	0	0	0	0	0	
DNase ^c	70	88	0	8	100	8	36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Lipase	50 (16)	83	6	86	5	63	9	41	9	75	1 (3)	57	2	40	7	50	0	0	0	0	0	0	0	
Lecithinase	0 (1)	1	7	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Starch hydrolysis	0	0	0	0	0	5	63	11	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Esculin hydrolysis ^c	70 (10)	100	5 (2)	100	8	100	21	95	8 (4)	100	7	100	4	80	0	0	0	0	0	0	0	0	0	
Growth on cetrimide	33 (6)	49	6	86	0	0	0 (3)	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Growth on MAC agar ^c	80	100	7	100	0	0	25	0 (2)	9	0	0	2	29	5	100	14	100	5	100	14	100			
Growth on SS agar	23 (1)	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Hemolysis	9	11	1	14	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0	1	7		
Pigment ^c	19	24	3	43	3	38	22	100	12	100	4	57	5	100	14	100	5	100	14	100				
Wrinkled colonies ^c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Lysine ^{c, d}	80	100	7	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Arginine ^{c, d}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Ornithine ^{c, d}	0	0	3	43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
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Glucose ^c	3 (8)	14	7	100	8	100	16 (6)	100	6 (6)	100	7	100	5	100	14	100	5	100	14	100				
Lactose ^c	0	0	6 (1)	100	0	38	1	5	4 (4)	67	7	100	0	0	0	0	0	0	0	0	0	0	0	
Sucrose	0	0	5 (2)	100	0	0	3 (2)	23	7 (4)	92	6 (1)	100	0	0	0	0	0	0	0	0	2 (1)	21		
Maltose ^c	68 (11)	99	5 (1)	86	6 (1)	88	12 (10)	100	7 (5)	100	7	100	4	80	13 (1)	100	4	80	13 (1)	100				
Mannitol ^c	0	0	2 (3)	71	7	88	1 (1)	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Xylose ^c	0 (2)	3	4 (2)	86	0	0	5 (2)	32	9 (3)	100	7	100	5	100	14	100	5	100	14	100				
Fructose	11 (23)	43	2 (4)	86	8	100	8 (10)	82	7 (1)	67	7	100	5	100	11 (3)	100	5	100	11 (3)	100				
10% Glucose	0	0	7	100	0	0	13	36	1 (3)	33	6 (1)	100	5	100	12 (2)	100	5	100	12 (2)	100				
10% Lactose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
ONPG	32	40	7	100	8	100	9	41	7	58	7	100	2	40	0	0	0	0	0	0	0	0	0	

Amides/salts	(68)	(5)	(5)	(15)	(8)	(5)	(4)	(10)
Acetamide ^c	1 (7)	12	5	100	0 (1)	20	0	20
Allantoin ^c	0	0	0	0	0 (1)	0	0	0
Butyramide	1 (6)	10	2 (1)	60	0	0	0	0
Citrate ^c	45 (17)	91	5	100	0 (2)	25	0 (1)	0
Formamide	41 (15)	82	4 (1)	100	0	0	100	100
Malonate ^c	34 (28)	91	5	100	1 (1)	40	4	50
Nicotinamide	0 (5)	7	1	20	0	0	100	100
Propionate	23 (33)	82	4	80	0 (1)	13	0	10
Tartrate ^c	1 (8)	13	5	100	0 (2)	25	0	80

^a Symbols are the same as in Table 1, footnote a.

^b All strains were H₂S, P, and F negative.

^c Useful test for species differentiation.

^d Decarboxylase and dihydrolase reported for rapid method.

more days to do so. Formamide and propionate were alkalized in 7 days. In contrast, *P. cepacia* was biochemically versatile, oxidizing most carbohydrates. In addition, acetamide, formamide, propionate, and tartrate were alkalized by most strains. All strains decarboxylated lysine and three (43%) decarboxylated ornithine. Three of the seven strains presented a sulfur-yellow pigment, which mimicked a positive test on F medium.

Isolates of *Flavobacterium meningosepticum* were nonmotile, pale yellow organisms that grew poorly on MAC agar. All organisms failed to oxidize sucrose, xylose, and 10% lactose. All peptonized litmus milk, hydrolyzed esculin, and produced β -galactosidase. Most liquefied gelatin and showed DNase, lipase, and amylase activity. In contrast, isolates of *Flavobacterium* II-b were yellow-orange on blood and Trypticase soy agars. Additionally, they were less consistent in tests for indole, DNase, lipase, amylase, and β -galactosidase. The results of indole tests are not consonant with those of Tatum et al. (18), who found all 25 cultures of II-b to produce indole after 48 h. Since the organisms in this report were received as reference specimens, it is presumed that the indole-negative characteristics had presented difficulty in the original identification attempts. Allantoin was alkalized by most isolates of II-b in 7 days. This is in agreement with a recent report in which all of the 4 strains of *Flavobacterium* II-b were allantoin positive (13), but a departure from an earlier report in which all of 16 strains were allantoin negative (15).

Cultures of the CDC group IIK were predominantly nonmotile and oxidase positive. The yellow-pigmented IIK-1 cultures failed to split urea or grow on MAC agar. Most strains alkalized formamide. Isolates of IIK-2 were urease positive, grew on MAC agar, acidified 10% glucose and lactose slants, and alkalized allantoin. Pigmentation of these strains developed only after overnight incubation at room temperature.

Cultures of the CDC groups VE-1 and VE-2 were motile, oxidase-negative organisms with a tendency to form wrinkled colonies after 48 h of incubation. All organisms grew well on MAC agar, and all presented yellow colonies on both blood and MAC medium. Strains of group VE-1 were multitrichous and esculin, arginine, and ONPG positive and grew on SS agar. Formamide and, occasionally, propionate were alkalized. Group VE-2 consisted of monopolar organisms that failed to hydrolyze esculin, dihydrolyze arginine, grow on SS agar, or produce β -

TABLE 4. Biochemical characteristics of nonoxidative bacteria^a

Test or medium ^b	<i>P. acidovorans</i> (16 strains)		<i>P. testosteroni</i> (11 strains)		<i>P. alcaligenes</i> (8 strains)		<i>P. diminuta</i> (9 strains)		<i>A. odorans</i> (11 strains)		CDC IV-C (6 strains)		<i>B. bronchiseptica</i> (4 strains)		<i>A. luoffii</i> (45 strains)		<i>M. osloensis</i> (11 strains)	
	+	(+)	+	(+)	+	(+)	+	(+)	+	(+)	+	(+)	+	(+)	+	(+)	+	(+)
Motility ^c	16	100	11	100	8	100	9	100	11	100	5	83	4	100	0	0	0	0
Slide	10	63	9	82	8	100	9	100	10	91	4	67	4	100	0	0	0	0
Tube	>2		>2		1		1		P		P		P					
Flagellar arrangements	16	100	11	100	8	100	9	100	11	100	6	100	4	100	0	0	11	100
Oxidase ^c	0	0	1	9	1	13	2	22	0	0	0	0	0	0	14	31	0	0
Phenylalanine	0	0	1	18	0	(1)	13	0	0	0	5	(1)	100	4	100	2	(1)	0
Urease ^c	16	100	8	73	8	100	0	0	0	0	0	0	4	100	0	0	0	0
Nitrite production ^c	0	0	0	0	0	0	0	0	0	9	(2)	100	0	0	0	0	0	0
Denitrification ^c	1	(2)	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gelatinase (25°C)	0	0	0	0	0	0	2	(3)	56	0	0	0	0	0	0	0	0	0
DNase ^c	3	19	0	27	1	13	1	11	0	0	0	(1)	17	1	25	40	(1)	91
Lipase	2	(1)	19	0	7	88	0	0	10	91	1	17	0	0	1	2	1	36
Growth on cetrimide	16	100	11	100	8	100	9	100	11	100	6	100	4	100	42	93	2	(1)
Growth on MAC agar	8	50	0	0	3	38	0	0	5	45	0	0	4	100	2	(1)	7	0
Growth on SS agar ^c	0	0	0	0	0	0	5	56	0	0	0	0	1	25	3	7	1	9
Pigment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arginine dihydrolase ^{c, d}	0	0	0	0	6	(1)	88	0	0	0	0	0	0	0	0	0	0	0
Amides/salts	(16)		(10)		(8)		(9)		(11)		(3)		(4)		(45)		(11)	
Acetamide ^c	16	100	0	0	0	0	0	0	10	(1)	100	0	0	0	0	(7)	16	0
Allantoin ^c	7	(9)	100	3	30	0	0	0	0	0	3	100	1	25	0	(1)	2	0
Butyramide ^c	15	(1)	100	0	0	0	0	0	9	(2)	100	0	(4)	100	0	(2)	4	0
Citrate	15	94	4	40	2	25	0	0	11	100	2	(1)	100	4	100	17	(6)	51
Formamide ^c	16	100	9	(1)	100	0	0	0	11	100	3	100	3	(1)	100	2	4	0
Malonate	3	(10)	81	0	(1)	10	0	0	10	(1)	100	1	(1)	25	6	(2)	18	0
Nicotinamide	3	(3)	38	0	0	0	0	0	9	(2)	100	1	33	0	0	(1)	2	0
Propionate	0	(2)	13	0	50	0	0	0	7	(4)	100	0	2	100	7	(14)	47	1
Tartrate ^c	16	100	1	10	0	0	0	0	0	0	1	(1)	67	0	1	(1)	4	0

^a Symbols are the same as in Table 1, footnote a.^b All strains were negative for the following tests: indole, H₂S, pyocyanine, fluorescein, gluconate oxidation, peptonization of litmus milk, lecithinase, starch hydrolysis, esculin hydrolysis, hemolysis, lysine and ornithine decarboxylase, oxidation of 1% carbohydrates and 10% glucose and lactose, ONPG.^c Useful test for species differentiation.^d Results reported for rapid method.

TABLE 5. Alkalinization of selected amides and organic salts by nonfermenting bacteria^a

Strain	No. tested	Acetamide		Allantoin		Butyramide		Formamide		Nicotinamide		Propionate		Tartrate	
		+	%	+	%	+	%	+	%	+	%	+	%	+	%
<i>P. aeruginosa</i>	87	79	(6)	98		55	(24)	91		1	(11)	33	(47)	92	
<i>P. putida</i>	52	2	(1)	6		0	(2)	4		3	(9)	15	(11)	50	
<i>P. fluorescens</i>	18	0	(1)	6		0	(1)	6		0		7	(4)	61	
<i>P. pseudomallei</i>	5	0	(2)	40		0		0		0		3		60	
<i>P. stutzeri</i>	15	1	(1)	13		0		0		0		2		13	
<i>P. stutzeri</i> -like, Vb-3	19	0	(2)	11		0		0		0		8	(1)	47	
<i>P. pseudoalcaligenes</i>	25	0		0		0		0		0		6		24	
CDC VA-1	4	0		0		0		0		0		0		75	
<i>A. xylooxidans</i>	15	4	(8)	80		10	(2)	80		11	(4)	15		100	
CDC VD-1	5	0		0		5		100		0		0		40	
<i>Achromobacter</i> sp. 1	8	0		0		7	(1)	100		0		8		100	
<i>Achromobacter</i> sp. 2	3	0		0		2	(1)	100		0		0		33	
<i>P. maltophilia</i>	68	1	(7)	12		0		0		1	(6)	41	(15)	82	
<i>P. cepacia</i>	5	5		100		0		0		2	(1)	60		100	
CDC group VE-1	4	0		0		0		0		0		4		100	
CDC group VE-2	10	1	(1)	20		0		0		0		5		50	
<i>Flavobacteria</i>	20	0	(1)	5		6	(5)	55		0		1	(5)	30	
CDC group IIK-1	8	0		0		0		0		0		25		75	
CDC group IIK-2	5	0		0		5		100		0		0		20	
<i>P. acidovorans</i>	16	16		100		7	(9)	100		15	(1)	16		100	
<i>P. testosteroni</i>	10	0		0		3		30		0		9	(1)	100	
<i>A. odorans</i>	11	10	(1)	100		0		0		9	(2)	100		100	
<i>A. calcoaceticus</i>	51	1	(2)	6		0		4		2	(1)	11		100	
<i>A. lwoffi</i>	45	0	(7)	16		0	(1)	2		2		2	(7)	18	
<i>B. bronchiseptica</i> ^b	7	0		0		4		57		0	(7)	6	(1)	100	

^a Symbols are the same as in Table 1, footnote a.
^b *B. bronchiseptica*, four isolates; CDC IV-C, three isolates.

TABLE 6. Antimicrobial susceptibility of oxidative and nonoxidative bacteria

Strain	No. tested	% Susceptible ^a											
		Ampicil- lin	Carbeni- cillin	Cephalo- thin	Chloram- phenicol	Kanam- cin	Colistin	Gentami- cin	Tetracy- cline	Strepto- mycin	Sulfisoxa- zole	Nalidixic Acid	Nitro- furantoin
<i>P. aeruginosa</i>	85	5	82	0	22	24	100	88	19	28	42	15	0
<i>P. putida</i>	52	0	0	0	21	86	100	88	61	69	34	0	0
<i>P. fluorescens</i>	17	0	0	0	36	100	100	100	76	77	71	29	0
<i>P. pseudomallei</i>	6	83	83	0	100	100	0	0	100	0	83	100	0
<i>P. stutzeri</i> ^b	28	89	96	4	54	100	100	100	100	96	75	86	0
<i>A. xylosoxidans</i>	15	73	100	20	93	13	87	0	0	0	80	27	0
<i>Achromobacter</i> sp. 1	8	75	100	50	50	88	88	100	100	13	38	88	88
CDC VD-1	5	0	0	0	0	20	80	100	100	20	100	80	0
<i>P. maltophilia</i>	73	1	19	1	77	15	70	37	32	24	77	92	3
<i>P. cepacia</i>	7	0	14	0	100	57	0	57	0	14	86	100	0
<i>F. meningosepticum</i>	8	0	0	0	63	0	0	13	25	13	13	100	0
<i>Flavobacterium</i> II-b	21	5	19	5	81	5	0	67	29	43	34	100	24
CDC IIK ^c	15	67	93	33	93	33	20	67	100	7	73	60	47
CDC VE ^d	14	93	93	14	93	100	100	100	100	100	86	50	14
<i>P. acidovorans</i>	15	0	46	0	100	33	13	0	100	0	93	100	93
<i>P. testosteroni</i>	10	50	100	70	100	100	100	80	100	60	90	100	70
<i>P. alcaligenes</i>	7	14	100	0	58	100	100	86	100	29	100	100	0
<i>P. pseudocaligenes</i>	23	61	100	0	69	91	100	100	100	65	91	91	0
<i>P. diminuta</i>	9	11	100	56	100	89	22	89	100	22	22	0	0
<i>A. odorans</i>	10	90	100	100	70	50	100	100	100	10	70	100	90
<i>B. bronchiseptica</i> ^e	8	38	100	88	100	75	88	100	100	13	100	75	13
<i>A. luoffi</i>	39	93	98	49	93	100	100	100	85	82	92	95	27
<i>A. calcoaceticus</i>	47	32	98	4	22	96	100	98	89	77	91	98	4
<i>M. osloensis</i>	11	100	100	100	100	100	100	100	100	82	82	100	82

^a Susceptibilities are not intended as a guide for therapy, but are used as supplemental information for identification.^b *P. stutzeri*, 13 strains; *P. stutzeri*-like (CDC Vb-3), 15 strains.^c CDC IIK-1, 10 strains; CDC IIK-2, 5 strains.^d CDC VE-1, 5 strains; CDC VE-2, 9 strains.^e *B. bronchiseptica*, 4 strains; CDC IV-C, 4 strains.

galactosidase. Some isolates deaminated phenylalanine and most alkalized tartrate.

Nonoxidative bacteria. Table 4 shows the characteristics of the nonoxidative bacteria. Isolates of the *P. acidovorans*-*P. testosteroni* group were motile organisms possessing three or more polar flagella, with tufts frequently appearing at both poles. Activity against the amides and salts was significantly different between these two organisms, whereas the alkalization of allantoin, acetamide, butyramide, and tartrate by *P. acidovorans* was diagnostic. The alkalization of formamide by *P. testosteroni* was useful in differentiating it from *P. alcaligenes* and *P. diminuta*, neither of which alkalized the substrates. *P. alcaligenes* was monopolar, with an ability to grow on cetrimide and to occasionally dihydrolyze arginine. *P. diminuta* was monopolar. Flagella appeared tight and of uniform wavelength resembling the often described corkscrew appearance. Gelatin liquefaction, DNase production, and brown pigment were variable characteristics. *Alcaligenes odorans* isolates were short rods, often coccobacillary, with flagellar arrangements varying from degenerate to conspicuous peritrichous flagella. These organisms produced gas in Sella's medium, grew on cetrimide agar, and often produced a pronounced odor. Gas was not produced in nitrate broth. Alkalization of substrates was consistent, except for allantoin and tartrate. Strains of the rapid urease-positive *Bordetella bronchiseptica* and CDC IV-C species were biochemically similar, except that the former reduced nitrate and grew on SS agar and the latter failed to do so. Similar to the achromobacteria, isolates of group IV-C actively alkalized allantoin. The cellular morphology of *Acinetobacter lwoffii* varied considerably. Most cultures showed coccobacilli and diplococci although diplobacilli and short rods were often present. Many cultures deaminated phenylalanine and most produced lipase. In contrast to *A. lwoffii*, *Moraxella osloensis* isolates were oxidase positive, failed to deaminate phenylalanine, and did not grow on MAC and citrate agars. Many of the moraxella strains were oxidase negative with N disks, but positive with Kovacs' reagent. The activity of these two species against the selected substrates was limited. The alkalization of the selected substrates by the nonoxidative organisms are in general agreement with the findings of other workers using different basal media (13, 16). A notable discrepancy was the failure of our strains of *B. bronchiseptica* to alkalize allantoin, nicotinamide, and tartrate. Pickett and Pedersen (16) showed

that all 13 strains of *B. bronchiseptica* alkalized allantoin and nicotinamide, but failed to alkalize formamide. Their findings are more consistent with our strains of CDC IV-C.

Alkalization of selected substrates. Growth on the media containing selected amides and organic salts appeared as scant or abundant, with little correlation to the ability of the organisms to alkalize the various substrates. Pickett and Pedersen (15) found that weakly buffered suspensions of their media, in the absence of exogenous substrates, became alkaline and that determination of pH changes was difficult to interpret. The basal and completed agar media described in this report were sufficiently stable to discern alkalization after 1 to 7 days of incubation. Some isolates presented extremely weak reactions, but were regarded as negative tests since reactions did not increase in intensity even after extended incubation. Hence, only a pronounced blue coloration was recorded as a positive test.

Table 5 summarizes the results of alkalization of the amides and salts by various organisms. The results show the activity of each organism against the individual substrates as well as the time that was necessary to obtain results. The species of organisms predominantly allantoin positive were rapid in their activity. Alkalization of acetamide, butyramide, and tartrate was also quite rapid. Activity against formamide and propionate varied considerably, suggesting a requirement for extended incubation. Allantoin was useful in differentiating *P. aeruginosa* from other fluorescent pseudomonads, the achromobacteria from other oxidative organisms, group IIR-2 from yellow-pigmented organisms, and *P. acidovorans* from other nonoxidative organisms. Acetamide also was useful in differentiating *P. aeruginosa*, *A. xylosoxidans*, *P. cepacia*, *P. acidovorans*, and *A. odorans* from related organisms. Allantoin and acetamide combined as a battery clearly separated biochemically similar organisms, especially those that were encountered most frequently. Alkalization of formamide and propionate was quite universal and of limited use as individual tests. Nicotinamide activity was limited to *A. xylosoxidans* and *A. odorans*. Although the number of isolates was small in some instances, alkalization of tartrate was consistent among strains of group VA-1, *P. cepacia*, and *P. acidovorans*, as well as among most strains of group VE-2.

Antimicrobial susceptibility. Antibiograms of the NFB are presented in Table 6, with the intermediate category of susceptibility included as susceptible. Susceptibilities are not intended

as a guide for therapy especially since, with the exception of *P. aeruginosa*, the Bauer-Kirby method has not been standardized for these organisms. Nevertheless, antibiograms are readily available to the clinical microbiologists and can offer supplemental information for identification of the NFB (3). The inverse relationship in susceptibility to carbenicillin and kanamycin was marked among the fluorescent pseudomonads. Whereas *P. aeruginosa* was susceptible to carbenicillin and resistant to kanamycin, the reverse was seen for *P. putida* and *P. fluorescens*. *P. putida* and *P. fluorescens* were also susceptible to tetracycline and streptomycin to a greater degree than was *P. aeruginosa*. In contrast to *P. pseudomallei*, isolates of the *P. stutzeri* group were sensitive to a wide range of agents, except for cephalothin and nitrofurantoin. A striking difference in susceptibility was seen with the various species of achromobacteria. Whereas *A. xylosoxidans* strains were susceptible to ampicillin, carbenicillin, and chloramphenicol and resistant to gentamicin and tetracycline, the exact opposite was seen for group VD-1. The *Achromobacter* sp. 1 were similar to *A. xylosoxidans* in susceptibility to ampicillin, carbenicillin, and chloramphenicol and identical to group VD-1 in susceptibility to gentamicin and tetracycline. The susceptibility of these organisms to nitrofurantoin was especially marked. For the most part, the yellow-pigmented organisms were resistant to the antimicrobics. Most strains of *P. maltophilia* were susceptible to chloramphenicol, colistin, sulfisoxazole, and nalidixic acid. *P. cepacia* and the flavobacteria demonstrated susceptibility only to chloramphenicol and nalidixic acid. The II-K and V-E groups differed from the other yellow-pigmented organisms in that they were susceptible to a wide range of agents, especially ampicillin and carbenicillin. There was a noticeable difference between *P. acidovorans* and *P. testosteroni*, with *P. testosteroni* being the more susceptible to ampicillin, cephalothin, gentamicin, and streptomycin. The lack of resistance of these organisms to nitrofurantoin was also marked. *P. alcaligenes* and *P. pseudoalcaligenes* were similar in susceptibility, except against ampicillin and streptomycin. Susceptibility of *P. diminuta* differed from the other nonoxidative organisms with respect to colimycin and nalidixic acid. *A. odorans* and *B. bronchiseptica* were susceptible to a wide range of antibiotics, except for streptomycin. *A. lwoffii* and *A. calcoaceticus* presented the same antibiograms, except for ampicillin and chloramphenicol.

The methods presented in this report, especially with the addition of amides and organic salts, were designed to simplify the characteri-

zation of oxidative and nonoxidative, nonfermenting bacteria.

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