Fluorescent *Pseudomonads* Capable of Growth at 41°C but Distinct from *Pseudomonas aeruginosa*

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One hundred and twenty-seven apyocyanogenic fluorescent *Pseudomonas* strains capable of growth at 41°C, but differing from *Pseudomonas aeruginosa*, were typed serologically and tested for pyocin production, antibiotic susceptibility, selected biochemical reactions, and utilization of selected substrates. Results were compared with those from 40 apyocyanogenic and 14 pyocyanin-producing strains of *P. aeruginosa*. Unidentified fluorescent *Pseudomonas* (UFP) strains generally were not agglutinated by *P. aeruginosa* antisera and showed little or no pyocin activity. In contrast to *P. aeruginosa* strains, UFP strains usually failed to oxidize D-gluconate or reduce nitrate to nitrogen gas. They could not use D-gluconate or D-mannitol as sole carbon source and were susceptible to kanamycin. The cellular fatty acid compositions of major UFP groups resembled those of the *alcaligenes-stutzeri* groups.

Apyocyanogenic fluorescent *Pseudomonas* strains capable of growth at 41°C but differing in several respects from *P. aeruginosa* have been isolated over a period of several years from environmental sources and clinical materials. In two previous papers (13, 14), we reported that most probable numbers of fluorescent pseudomonads isolated at 39°C could exceed by as much as 25 times the most probable numbers of pyocyanogenic *P. aeruginosa* in surface and swimming pool waters. It seemed important in evaluating the quality of surface and swimming waters to be able to differentiate between apyocyanogenic *P. aeruginosa* and other fluorescent organisms that resemble it.

In clinical laboratories also, the differentiation of apyocyanogenic *P. aeruginosa* from other pseudomonads may present a problem. Bacterial strains that are gram negative and oxidase positive, metabolize glucose oxidatively, and grow at 41°C usually are designated as *P. aeruginosa*. Sometimes identification is based only on fluorescence and growth at 41°C. Other characteristics that give consistent reactions with pyocyanin-producing *P. aeruginosa* strains are variable among apyocyanogenic strains. Among tests that generally are considered to be of value for the identification of *P. aeruginosa* are denitrification, oxidation of D-gluconate to 2-keto-D-gluconate, liquefaction of gelatin, hemolysis of erythrocytes, and utilization of mannitol. However, Gilardi (11) found these characteristics to be variable among apyocyanogenic strains, and Hugh and Gilardi (15) stated that denitrification, hydrolysis of

gelatin and casein, oxidation of gluconate to 2-ketogluconate, and utilization of acetamide are unreliable tests for the identification of apyocyanogenic strains. Furthermore, Gilardi (10) reported that apyocyanogenic strains of *P. aeruginosa* were more often susceptible to tetracycline, streptomycin, and kanamycin than were pyocyanogenic strains. Similarly, von Graevenitz and Redys (30) reported that nearly half of 14 apyocyanogenic strains were sensitive to kanamycin and neomycin.

Other investigations have followed more rigid criteria for the identification of *P. aeruginosa*. Haynes (12) did not include in the species five of nine apyocyanogenic strains studied by him because they failed to oxidize gluconate or produce slime in gluconate broth. Sutter (28) suggested that four fluorescent *Pseudomonas* strains able to grow at 41°C but unable to denitrify or to hydrolyze gelatin probably were not *P. aeruginosa*. Phillips (26) recommended a key for the identification of *P. aeruginosa* which, although it did not include the ability to denitrify, omitted from the species strains failing to oxidize gluconate. Sixty percent of 43 apyocyanogenic fluorescent *Pseudomonas* strains able to grow at 41°C as reported previously by us failed to denitrify, whereas 88% did not utilize gluconate and mannitol. Seventy-four percent used acetamide poorly or not at all (13).

It was the objective of the present study to obtain apyocyanogenic strains capable of growth at 41°C from clinical materials and from surface waters and to characterize these strains by means of selected biochemical and chemical

tests, serological and pyocin typing, and tests of susceptibility to antibiotics. From the resulting data, it was our aim to determine the source of the reported variability among apyocyanogenic strains of *P. aeruginosa* and to establish whether inclusion of unidentified fluorescent pseudomonads resembling, but distinct from, *P. aeruginosa* could explain this variability.

MATERIALS AND METHODS

Fourteen pyocyanin-producing strains of *P. aeruginosa*, 40 apyocyanogenic strains of *P. aeruginosa*, and 127 unidentified fluorescent *Pseudomonas* (UFP) strains were obtained from clinical and environmental sources (Table 1) in Europe and North and South America and were characterized by means of selected tests. UFP strains were assigned to one of four groups on the basis of physiological properties and flagellation (Table 2). Unless otherwise indicated, all incubations were at 30°C.

Pigment production. Slants of King's B medium (Flo agar, BBL) and pseudomonas F agar (Difco) incubated for 3 to 5 days were examined under a Wood's lamp to detect fluorescent pigment. Pyocyanin production was tested on slants of King's A medium (Tech agar, BBL) and pseudomonas P agar (Difco) incubated for up to 7 days. Blue pigment was detected visually in slants or, if not readily detectable, was extracted with chloroform. A color change to red after acidification with several drops of 1 N HCl confirmed the presence of pyocyanin.

Growth at 41°C. Slants of Haynes's medium (12) were warmed to 41°C before inoculation. Inoculated slants were incubated in a water bath at 41°C, and the presence of growth within 48 h was recorded.

Oxidase. The method of Gaby and Hadley (9) and Pathotec paper strips (Warner-Lambert Co.) were used to test for the presence of oxidase.

Glucose metabolism. The medium of Hugh and Leifson (16) containing 1.0% D-glucose was used to confirm the oxidative metabolism of D-glucose. Tubes were read daily for periods up to 7 days.

Hydrolysis of gelatin. A procedure based on Smith's modification of Frazier's technique as cited in the *Manual of Microbiological Methods* (6) was used to test for the hydrolysis of gelatin. Plates of nutrient agar containing 4% gelatin were divided

into quadrants, and each quadrant was inoculated with a small patch of inoculum from the test strain. After 48 h of incubation, plates were flooded with approximately 5 ml of acid-mercuric chloride. Hydrolysis was indicated by a zone of clearing surrounding the patch of growth. This test is more sensitive for detecting hydrolysis of gelatin by fluorescent pseudomonads than are tubes of nutrient gelatin commonly used for identification of *Enterobacteriaceae*.

Hemolysis. All strains were streaked on plates of Trypticase soy agar (BBL) to which had been added 5% defibrinated human blood. Plates were incubated for 48 h. A clear zone surrounding the streaks or individual colonies indicated hemolytic activity.

Casein hydrolysis. Plates containing 15 to 20 ml of the medium of Brown and Foster (5) were inoculated in the same way as the gelatin plates. After 48 h of incubation, plates were checked for a clear zone surrounding the growth.

Denitrification. The nitrate broth fermentation tube method described by Hugh and Gilardi (15) was used to detect strains able to produce nitrogen gas. Strains failing to produce gas were retested by the method of Stanier et al. (27). Growth from tubes of semisolid nitrate medium was transferred to deep tubes of the same medium, which were subsequently sealed with an agar overlay and incubated for up to 5 days. Although time consuming, the method appears to be more reliable for detection of denitrification than the fermentation tube method. Four strains of apyocyanogenic *P. aeruginosa* yielding negative results (failing to produce gas) in fermentation tubes were found to be positive (produce gas) with the method of Stanier et al.

Oxidation of D-gluconate. The tests of Arai et al. (1) and Gaby and Free (8), using gluconate substrate tablets (Key Scientific Products Co.) and one-half of a Clinitest reagent tablet (Ames Co.), were used to determine the presence of 2-ketogluconate. In the latter procedure, inoculated tubes of substrate were incubated for 18 to 20 h. All tests negative at 18 to 20 h were repeated with 48 h of incubation.

Egg yolk reaction. Plates of Trypticase soy agar (BBL) to which was added a 10% commercial (Difco) egg yolk emulsion (29) were divided into quadrants and inoculated in the same manner as the gelatin plates. A white precipitate surrounding the growth

TABLE 1. Sources of strains studied

Source	<i>P. aeruginosa</i>		UFP strains			
	Pyocyanogenic	Apyocyanogenic	Group I	Group II	Group III	Group IV
Environmental						
Water ^a		2	53	4		7
Hospital wastes and receiving stream	3	6	10		46	
Clinical						
Infected and normal outer ears	2	5	4			
Other ^b	9	27				3

^a Swimming pools and unpolluted surface waters.

^b Cultures of known clinical origin and cultures received from clinical laboratories without information on sources.

TABLE 2. Characteristics of *P. aeruginosa* and UFP groups

Characteristic	<i>P. aeruginosa</i>		UFP strains			
	Pyocyano- genic (14) ^a	Apyocyano- genic (40)	Group I (67)	Group II (4)	Group III (46)	Group IV (10)
No. of flagella	1	1	1	1	>1	>1
Pigments						
Phenazine	+ ^b (100) ^c	— (0)	— (0)	— (0)	— (0)	— (0)
Carotenoid	— (0)	— (13)	V (25)	— (0)	V (22)	— (0)
Hemolysis	+ (100)	+ (100)	V (81)	— (0)	V (83)	— (0)
Gelatin hydrolysis	+ (100)	+ (88)	+ (100)	— (0)	+ (100)	— (0)
Casein hydrolysis	+ (93)	+ (90)	+ (87)	— (0)	+ (96)	V (30)
Denitrification	+ (100)	+ (100)	— (0)	V (25)	— (2)	V (40)
Gluconate oxidation	+ (100)	+ (95)	— (0)	V (25)	— (2)	V (70)
Utilization of:						
Sebacate	+ (100)	+ (98)	+ (100)	V (50)	+ (100)	V (20)
Saccharate	— (0)	— (0)	— (0)	V (50)	— (0)	— (10)
Mannitol	+ (100)	+ (93)	— (0)	V (25)	— (0)	V (50)
Glycolate	— (7)	— (0)	— (2)	+ (100)	— (0)	V (40)
Gluconate	+ (100)	+ (93)	— (2)	V (50)	— (0)	+ (100)
Geraniol	+ (93)	V (83)	+ (99)	+ (100)	+ (100)	V (80)
Acetamide	+ (100)	+ (100)	— (2)	— (0)	— (0)	— (0)
Resistance to:						
Gentamicin	S (0)	S (0)	S (0)	S (0)	S (0)	S (0)
Carbenicillin	V (36)	V (41)	R (89)	V (75)	R (96)	V (80)
Chloramphenicol	R (100)	R (95)	R (94)	R (100)	V (84)	R (90)
Streptomycin	V (79)	V (73)	S (2)	V (25)	S (2)	V (50)
Tetracycline	R (100)	R (100)	V (75)	S (0)	V (82)	V (60)
Neomycin	V (21)	V (45)	V (31)	S (0)	V (20)	S (0)
Kanamycin	R (93)	R (88)	S (2)	S (0)	S (0)	S (0)
Cephalothin	R (100)	R (100)	R (100)	R (100)	R (100)	R (100)
Ampicillin	R (100)	R (100)	R (100)	R (100)	R (100)	R (100)
Colistin	S (0)	S (3)	S (0)	S (0)	S (0)	S (0)
Polymyxin B	S (0)	S (0)	S (0)	S (0)	S (0)	S (0)

^a Number in parentheses is number of strain examined.^b Symbols: +, Positive (>85% positive); —, negative (<15% positive); R, resistant (>85% resistant); S, sensitive (<15% resistant); V, variable (15 to 85% positive or resistant).^c Percent positive or resistant.

indicated lecithinase production and a positive reaction.

Arginine dihydrolase activity. Deep tubes of OF basal medium (Difco) containing 0.3% Ionagar and 1% arginine and control tubes lacking arginine were inoculated by stabbing. Sterile mineral oil to a depth of 1 cm was added to all tubes, which were then incubated for 1 to 5 days. A blue color in tubes containing arginine but not in the control tubes constituted a positive test.

Nutritional tests. Utilization of acetamide, geraniol, D-gluconate, glycolate, D-mannitol, saccharate, and sebacic acid was determined as described by Stanier et al. (27). A positive test was indicated by the presence of growth within 7 days.

Tests for the utilization of acetamide by UFP strains on the medium of Stanier et al. often did not give clear results. To evaluate the potential usefulness of acetamide utilization to distinguish UFP strains from apyocyanogenic *P. aeruginosa* in the clinical laboratory, all strains were tested on the acetamide broth of Favero et al. (7) and on slants of

acetamide broth containing 15 g of agar/liter. A positive test was indicated by the formation of a strong alkaline reaction (red color) in the medium. The use of slants incubated at 37°C rather than broth improved the sensitivity of the test. Of six apyocyanogenic strains of *P. aeruginosa* failing to yield a positive reaction in broth incubated at 37°C for 24 h, five yielded positive reactions on slants, and reactions produced by the 35 other strains examined were stronger on slants than in broth. One strain failed to give a positive reaction within 7 days, although it did grow on Stanier's medium containing acetamide. No UFP strains produced positive reactions on acetamide slants within 24 h, although two strains gave weakly positive reactions after 6 and 7 days.

Flagella stains. Light suspensions of organisms from washed nutrient broth cultures incubated for 24 h were used to prepare slides that were stained by the method of Leifson (20). Flagella indexes (FIs) were determined as described by Lautrop and Jessen (19).

Immunotyping. Slide agglutination tests were performed by using 7 *P. aeruginosa* antisera supplied by Parke-Davis & Co. and 16 antisera provided by Difco. All UFP strains and nontypable *P. aeruginosa* strains were tested with the Difco antisera.

Pyocin typing. Production of pyocins was tested with the 18 indicator strains of Jones et al. (18).

Antibiotic susceptibility. Susceptibility to 11 antibiotics was determined by the technique of Bauer et al. (3). Antibiotic disks used were: tetracycline, 30 μ g; chloramphenicol, 30 μ g; streptomycin, 10 μ g; kanamycin, 30 μ g; neomycin, 20 μ g; carbenicillin, 100 μ g; cephalothin, 30 μ g; ampicillin, 10 μ g; colistin, 10 μ g; polymyxin B, 300 U; and gentamicin, 10 μ g (BBL or Pfizer). Incubation was at 35°C.

Fatty acid composition of whole cells. Selected strains were grown on nutrient agar slants and submitted to C. W. Moss and Sally Dees of the Center for Disease Control, Atlanta, Ga., for cellular fatty acid analysis as described by Moss et al. (23).

DNA base ratios and hybridization. Four UFP I strains and a single UFP II strain were submitted to N. J. Palleroni, who determined deoxyribonucleic acid (DNA) base compositions by buoyant density gradient centrifugation as described by Mandel (21). DNA-DNA hybridization between two UFP group I strains, one UFP group II strain, and *P. mendocina* CH20 (reference strain) at 70 and 80°C was performed as described by Ballard et al. (2). Base compositions were expressed as moles percent guanine plus cytosine (G+C). The results of hybridization experiments were expressed as percent DNA competition, that is, as relative competition of heterologous DNA (from the test strain) and homologous DNA (from the reference strain) with the binding of radioactive homologous DNA to reference DNA.

RESULTS AND DISCUSSION

All strains selected for study were polarly flagellated and oxidase positive, oxidized D-glucose, and produced fluorescent pigment and arginine dihydrolase. The egg yolk test for lecithinase was negative for all strains but two, which grew poorly at 41°C. Each strain was placed into one of three groups: pyocyanin-producing *P. aeruginosa*, apyocyanogenic *P. aeruginosa* and unidentified fluorescent *Pseudomonas*. Pyocyanogenic *P. aeruginosa* was readily identified on Tech agar or pseudomonas P agar. A few strains produced pyocyanin only after repeated subculture. Apyocyanogenic *P. aeruginosa* was separated from the UFP strains on the basis of serotypability, all nontypable strains being designated initially as UFP. Ultimately, two of the nontypable strains were reassigned to the apyocyanogenic *P. aeruginosa* group. All but three serotypable apyocyanogenic strains and nine UFP strains grew well at 41°C in 24 h, and all grew well at 37°C.

Lautrop and Jessen (19) considered strains having FIs (FI = percentage of multitrichous

organisms) of 0 to 10 as polar monotrichous and those with FIs greater than 25 as lophotrichous, whereas those exhibiting FIs between 10 and 25 were regarded as intermediate and of uncertain flagellar classification. As expected, all pyocyanogenic *P. aeruginosa* strains were polar monotrichous but one, which was intermediate (FI = 12). Among the 40 apyocyanogenic *P. aeruginosa*, 4 were intermediate and 3 were lophotrichous; all remaining strains exhibited polar monotrichous flagellation. Most UFP strains possessing intermediate FIs were grouped with multitrichous strains.

Although 71 UFP strains bore single polar flagella, 44 were multitrichous and 12 were intermediate. Two major groups of UFP strains differed only in their patterns of flagellation, group I (composed of 67 strains) being polar monotrichous and group III (46 strains) bearing atypical polar tufts. Group III organisms frequently possessed several straight flagella-like appendages in addition to a single flagellum of normal appearance (Fig. 1d,e,h,i). The flagella did not stain with ordinary stains. UFP groups II and IV included heterogeneous strains, differing substantially from groups I and III. Group II strains possessed single polar flagella, and group IV strains possessed multiple polar flagella.

Whereas most strains of *P. aeruginosa* were hemolytic and hydrolyzed casein and gelatin, UFP strains varied with respect to these characteristics (Table 2). Hemolysis, when it did occur, tended to be very weak among UFP strains. Ninety-three percent of all *P. aeruginosa* strains examined produced N₂ gas in nitrate broth, whereas 95% of the UFP strains failed to do so. With the exception of two apyocyanogenic strains, all strains of *P. aeruginosa* oxidized D-gluconate, whereas only 6% of UFP strains did so.

Results of tests for the utilization of seven substrates were consistent with the observation of Stanier et al. (27) that *P. aeruginosa* strains could use acetamide, geraniol, D-gluconate, D-mannitol, and sebacic acid but not glycolate or saccharate. In contrast, although most UFP strains used geraniol and sebacic acid, they failed to utilize gluconate or mannitol. Twenty UFP strains reported previously (13, 14) to produce moderate or scanty growth on plates of Stanier's medium containing acetamide failed to grow on the medium when retested during the present study. Four UFP strains previously reported to grow on that medium and four additional strains produced trace or poor growth during the present study. Only one strain producing poor growth was recorded as positive in Table 2. None of the UFP strains gave positive

FIG. 1. Flagella stains of representative *P. aeruginosa* and group III UFP strains. (a) Strain 106 (pyrocyanogenic *P. aeruginosa*); (b) strain 139 (apycyanogenic *P. aeruginosa*); (c,d) strain DM-15 (group III UFP); (e,f) strain DM-25 (group III UFP); (g,h,i) strain DM-26 (group III UFP). Bar represents 2 μ m.

reactions in the acetamide broth or on slants of Favero's medium (7). Utilization of geraniol by all but three UFP strains suggests a close relation to *P. aeruginosa* or *P. mendocina*, the only species known to utilize this substrate.

Most *P. aeruginosa* and UFP strains were susceptible to gentamicin, neomycin, colistin, and polymyxin B and were resistant to tetracycline, cephalothin, ampicillin, and chloramphenicol (Table 2). The majority of UFP strains differed from the majority of *P. aeruginosa* strains in their resistance to carbenicillin and in their susceptibility to streptomycin and kanamycin. Ninety percent of UFP strains were resistant to carbenicillin. Most carbenicillin-resistant *P. aeruginosa* strains were of clinical origin. Ninety-three percent of UFP strains were susceptible to streptomycin in contrast to 26% of *P. aeruginosa* strains. *P. aeruginosa* and UFP strains were most clearly distinguished on the basis of kanamycin resistance, which occurred in 89% of *P. aeruginosa* strains but in less than 1% of UFP strains. Susceptibility to kanamycin and resistance to carbenicillin were characteristics recommended by Blazevic et al. (4) to distinguish *P. putida* and *P. fluorescens* from *P. aeruginosa*.

Differences between UFP strains and *P. aeruginosa* also occurred with respect to the

production of pyocins. With few exceptions, *P. aeruginosa* strains were typable and the zones of inhibition of indicator strains were sharp and clear. The majority of UFP strains were not typable, and when inhibition did occur, the zones usually were fuzzy, indicating weak pyocin activity. Jones et al. (17) found that sensitivity to pyocins produced by *P. aeruginosa* was common among the other species of the fluorescent group, but their ability to produce pyocin-like substances was not investigated.

Further evidence suggesting that UFP strains may differ from *P. aeruginosa* was obtained by C. W. Moss and Sally Dees from the cellular fatty acid composition of selected *P. aeruginosa* and UFP strains as determined by gas-liquid chromatography. The single pyrocyanogenic and six apycyanogenic strains examined possessed each of three hydroxy fatty acids, resembling the fluorescent group (Table 3). Of 27 UFP strains tested, 4 resembled the fluorescent group; the remaining 23 UFP strains resembled the *alcaligenes-stutzeri* groups, lacking the 2-OH 12:0 acid but possessing the 3-OH 10:0 and 3-OH 12:0 acids. *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. stutzeri* lack the 2-OH 12:0 acid, but produce the 3-OH 10:0 and 3-OH 12:0 acids (22, 23).

The results of determinations of DNA base

compositions and homology studies are presented in Table 4 and indicate that the G+C content of the UFP II strain (64.3%) resembled that of *P. mendocina* (21). Furthermore, the moderately high degree of competition with *P. mendocina* CH20 at both 70 and 80°C suggests a relatively high degree of homology with *P. mendocina*. This was suggested also by nutritional screening by N. J. Palleroni. The G+C contents of the four UFP I strains resembled those of *P. aeruginosa*. On the other hand, competition between two UFP I strains and *P. mendocina*, although low, suggested a substantial degree of homology. On the basis of both matching coefficients and DNA homology with *P. pseudoalcaligenes*, Palleroni et al. (25) concluded that *P. mendocina* could be regarded as a link between the stutzeri group and the alcaligenes group.

Evidence has been cited suggesting the existence of fluorescent *Pseudomonas* strains

which, while they resemble *P. aeruginosa* in their ability to grow at 41°C, in their possession of single polar flagella, and in their utilization of geraniol, also consistently differ in certain respects. Strains examined failed to denitrify or to oxidize gluconate; mannitol and usually acetamide were not utilized. They were resistant to carbenicillin, susceptible to streptomycin and kanamycin, and failed to agglutinate in *P. aeruginosa* antiserum or exhibit pyocin activity. The apparent variability among apyocyanogenic strains of *P. aeruginosa* reported by other workers (10, 11, 15) may have been caused by inclusion of UFP strains among the cultures tested. In the clinical laboratory, UFP strains probably can be recognized by their failure both to denitrify and oxidize gluconate (groups I and III) or by their failure to hydrolyze both gelatin and casein (groups II and IV). No strain of *P. aeruginosa* examined during this investigation yielded negative results in more than one of the four tests, whereas all UFP strains yielded negative results in at least two of the tests. Susceptibility to kanamycin also may indicate a UFP strain. Of potential value to distinguish *P. aeruginosa* from UFP strains is the production of an alkaline reaction on slants of the acetamide medium of Favero et al. (7) containing 15 g of agar/liter by strains of *P. aeruginosa* and the failure of UFP strains to do so.

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TABLE 3. Cellular hydroxy fatty acids of selected *Pseudomonas aeruginosa* and UFP strains^a

Group	No. of strains	Fatty acid ^b		
		3-OH 10:0	2-OH 12:0	3-OH 12:0
<i>P. aeruginosa</i>				
Pyocyanogenic	1	+	+	+
Apyocyanogenic	6	+	+	+
UFP I	16	+	—	+
UFP II	1	+	+	+
	3	+	—	+
UFP III	3	+	—	+
UFP IV	3	+	+	+
	1	+	—	+

^a Analyses performed by S. Dees and C. W. Moss, Center for Disease Control.

^b Numbers to left of colon refer to number of carbon atoms; numbers to right refer to number of double bonds; 2-OH and 3-OH refer to the position of the hydroxyl group.

^c Symbols: +, Present; —, absent.

TABLE 4. DNA base composition and homologies among UFP strains^a

Group	Strain	G+C mol%	% DNA competition ^b	
			70°C	80°C
UFP I	211	67.3		
	212	67.9	49	25
	213	67.9		
	218	67.3	44	28
UFP II	217	64.3	65	64

^a Analyses performed by N. Palleroni.

^b DNA competition between UFP strains and *P. mendocina* CH20.

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