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RESEARCH PROJECT INITIATION

Date: July 29, 1974

Project Title: A Study of Pathogenic Pseudomonas Strains from Surface and Swimming Waters and From Clinical Materials

Project No: E-20-658

Principal Investigator Dr. Alfred W. Hoadley

Sponsor: Public Health Service

Agreement Period. From 7/23/74 Until 5/31/75

Type Agreement: Biomedical Sciences Support Grant (Internal)

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Reports Required: Final Report due 7/15/75

Sponsor Contact Person (s): Dr. John W. Crenshaw
Biomedical Sciences Support Grant Committee
School of Biology
Campus

Assigned to: Civil Engineering

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RA-3 (6-71)

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RESEARCH PROJECT TERMINATION

Date: October 1, 1975

Project Title: A Study of Pathogenic Pseudomonas Strains from Surface and Swimming Waters and From Clinical Materials

Project No: E-20-658

Principal Investigator: Dr. A. W. Hoadley

Sponsor: DHEW/PHS - Biomedical Sciences Support Grant

Effective Termination Date: 5-31-75

Clearance of Accounting Charges: N/A

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Assigned to School of Civil Engineering

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Other ~~Dr. John W. Crenshaw, Jr.~~
File G-32-603

RA-4 (5/70)

BILL HOADLEY

Final Report to:

The Committee on Biomedical Sciences Support Grant

Report No. E.-20-658

A Study of Pathogenic *Pseudomonas* Strains from Surface and Swimming Waters and from Clinical Materials

Submitted by:

Alfred W. Hoadley, Ph.D.
School of Civil Engineering
Georgia Institute of Technology

July, 1975

Pseudomonas aeruginosa is a highly versatile bacterial species which causes a wide range of diseases in plants, animals, and man. It is a pathogen of increasing concern to man because it is resistant to a wide variety of antibiotics and frequently invades patients receiving antibiotic therapy. It is a major problem in cancer wards, burn wards, and newborn nurseries of hospitals where patients have low resistance to disease.

While wound infections and carriers are often the sources of outbreaks, it has become clear that water supplies, vegetables, and flowers (which in turn may acquire the organisms from water and soil) entering hospitals are significant sources of the organisms. Thus, there is increasing concern among specialists in infectious diseases for the ecology of the species outside of the hospital environment.

A problem in the clinical laboratory and the sanitary microbiology laboratory evaluating the quality of surface and swimming waters has been the identification of apyocyanogenic strains of P. aeruginosa (which fail to produce the typical blue pigment, pyocyanin). As Gilardi (1968) has pointed out, "the identification of pigmented strains of P. aeruginosa presents no problem; however, apyocyanogenic strains are not uncommon, which presents a problem in their differentiation from other pseudomonads." Often, identification of apyocyanogenic P. aeruginosa is based upon growth at 42C and fluorescence or upon odor; or bacterial strains which are Gram negative, oxidase positive, exhibit oxidative glucose metabolism, and grow at 42C are designated P. aeruginosa. However, other characteristics which give consistent reactions with pyocyanogenic P. aeruginosa become highly variable among apyocyanogenic strains. As a result, in recent years many authors have proposed schemes for the identification of P. aeruginosa. Some have consisted of selected tests designed to distinguish the species economically in the clinical laboratory.

Gilardi in published reports (Gilardi 1968, 1971) and in periodic unpublished guides for the "Identification of Nonfermentative Gram Negative Bacteria" has provided tables listing the characteristics of strains and percentages of strains positive, and indicating tests of particular diagnostic value. Among tests which are more generally considered to be of diagnostic value and which are commonly employed to identify P. aeruginosa are denitrification, oxidation of gluconate to 2-ketogluconate, and liquefaction of gelatin. Among pyocyanogenic strains of P. aeruginosa each of these characteristics is typically positive. Furthermore, pyocyanogenic strains typically hemolyse blood and utilize mannitol. Indeed, P. aeruginosa appears to be a very tight species with respect to all of its properties. On the other hand, among 63 apyocyanogenic strains investigated by Gilardi ("Identification of Nonfermentative Gram Negative Bacteria," revised December, 1974), these characteristics were found to be variable:

Characteristic	Percent of Strains Positive
Hemolysis of blood	44
Gelatinase	59
Nitrogen gas production	60
Gluconate oxidation	64
Acid from mannitol	67

Thus, about 60% of apyocyanogenic strains exhibited the expected results, and apyocyanogenic strains of P. aeruginosa are generally considered to exhibit a high degree of variability. According to Hugh and Gilardi (1974), the following universal features of pyocyanogenic strains of P. aeruginosa are unreliable for the identification of apyocyanogenic strains:

nitritase
gelatinase
caseinase
lipase
production of 2-ketogluconate
triphenyltetrazolium chloride tolerance
utilization of adipate
suberate
acetamide

Hugh and Gilardi suggested, however, that the comparison of cellular fatty acids and nucleic acid reactions of intact cells represent potentially useful tools for the identification of P. aeruginosa.

Other workers have accepted more fully the significance of characteristics viewed by Gilardi as variable. Thus Haynes (1951) studied 57 bacterial strains which grew at 41C. Among these strains, 43 produced pyocyanin and 9 were identified as apyocyanogenic strains of P. aeruginosa based upon their growth at 41C, oxidation of gluconate, and slime production in gluconate broth. Five apyocyanogenic strains failing to oxidize gluconate and to produce slime in gluconate broth were considered not to meet criteria for inclusion in the species. More recently, Sutter (1968) suggested that 4 fluorescent Pseudomonas strains able to grow at 41C but failing to denitrify or to hydrolyze gelatin probably were not P. aeruginosa. Among 65 strains, including 14 apyocyanogenic strains, considered by Sutter to be P. aeruginosa, only single strains failed to denitrify, grow at 41C, and hydrolyze gelatin. Phillips (1969) recommended a key for the identification of P. aeruginosa, which, while it did not include the ability to denitrify, eliminated from the species strains failing to oxidize gluconate.

Most recently, Hoadley and Ajello (1972) examined 29 strains of apyocyanogenic fluorescent pseudomonads capable of growth at 41C, but apparently differing in several respects from P. aeruginosa. Nearly all strains failed to denitrify and to utilize mannitol, and gluconate, and many failed to utilize acetamide. Some strains were identical to Pseudomonas mendocina in the stutzeri group, save that they were fluorescent. DNA from these strains also exhibited a high degree of homology with P. mendocina strains (Palleroni, personal communication). The predominant biotype frequently produced a brown pigment and exhibited moderate homology with P. aeruginosa. Such strains constituted over 90% of the populations of fluorescent pseudomonads able to grow at 41C which were demonstrated in surface waters by Hoadley and Ajello (1972).

It was the objective of the present study to obtain apyocyanogenic Pseudomonas strains capable of growth at 42C from clinical materials and from surface waters, and to characterize these strains by means of selected biochemical tests, serological typing, and antibiotic susceptibility testing. From the information obtained, it was anticipated that the similarity of strains to P. aeruginosa and the occurrence of strains differing from P. aeruginosa in clinical material could be determined.

Furthermore, it was the objective to determine serotypes of P. aeruginosa strains to determine whether certain serotypes survived better in water and thus predominated in water.

MATERIALS AND METHODS

Cultures examined. Approximately 413 cultures from surface waters, swimming pools, and clinical sources were screened to determine whether they were indeed fluorescent pseudomonads capable of growth at 41C. The cultures consisted of isolates from surface and swimming waters considered to differ from P. aeruginosa, and strains obtained from workers at other laboratories, most of which were received as apyocyanogenic P. aeruginosa. Screening consisted of preliminary screening for production of fluorescent pigment and pyocyanin, and growth at 41C. Strains producing pyocyanin were accepted as P. aeruginosa. Strains failing to produce pyocyanin, but producing fluorescent pigment and growing at 41C, were examined to confirm oxidase production and oxidative glucose metabolism. Many of the cultures obtained as apyocyanogenic P. aeruginosa or unidentified Pseudomonas strains able to grow at 41C (UFP strains) were examined also for their ability to hemolyze human blood, gelatin liquefaction, casein hydrolysis, denitrification, gluconate oxidation, arginine dihydrolase production, and egg yolk reaction. All strains were typed employing antisera for P. aeruginosa. Some strains received from other laboratories as apyocyanogenic strains produced pyocyanin in our hands; many exhibited reactions characteristic of P. fluorescens or P. putida and were not investigated further. From the cultures received, 13 pyocyanogenic P. aeruginosa strains, 47 apyocyanogenic P. aeruginosa strains, and 129 UFP strains were selected for study of substrate utilization, antibiotic susceptibilities, and pyocine typing. Selected strains were analysed also for cellular fatty acid composition.

In addition to the above strains, 12 pyocyanogenic and 13 apyocyanogenic strains of P. aeruginosa, and 14 UFP strains from swimming pools; and 7 pyocyanogenic and 4 apyocyanogenic strains of P. aeruginosa, and 2 UFP strains from ears

Table 1. Sources of Strains Examined

Source	No. of Strains	Origin
D. E. Knight and A. W. Hoadley - Georgia Institute of Technology, Atlanta, Georgia	29	Isolated from surface waters in Georgia
G. Ajello - Georgia Institute of Technology, Atlanta, Georgia	42	Isolated from surface waters in Brazil
A. W. Hoadley, G. Ajello, and N. Masterson, Georgia Institute of Technology, Atlanta, Georgia, and the University of Florida, Gainesville, Florida	39	Isolated from swimming pool waters in Florida
D. Mooney and A. W. Hoadley - Georgia Institute of Technology, Atlanta, Georgia	69	Isolated from hospital wastes and receiving stream in Georgia
Dr. M. H. Brodsky, Ontario Ministry of Health, Toronto, Ontario	24	Isolated from swimming pools in Canada
Dr. J. Shulman, Grady Memorial Hospital, Atlanta, Georgia	46	Isolated from clinical materials
Dr. Rudolph Hugh, George Washington University Medical School	7	Isolated from clinical materials and other sources
Dr. R. Weaver, Center for Disease Control, Atlanta, Georgia	13	Isolated from clinical materials
Dr. G. L. Gilardi, Hospital for Joint Diseases and Medical Center, New York, N. Y.	20	Isolated from clinical materials
Dr. E. Yourassowsky, Hospital Universitaire Brugmann, Brussels, Belgium	12	Isolated from clinical materials
A. W. Hoadley, Georgia Institute of Technology, Atlanta, Georgia	97	Isolated from infected outer ears
A. W. Hoadley, G. Ajello, N. Masterson, Georgia Institute of Technology, Atlanta, Georgia, and the University of Florida, Gainesville, Florida	14	Isolated from infected and healthy outer ears and rectal swabs
Dr. S. D. Kominos, Mercy Hospital, Pittsburgh, Pennsylvania	1	Isolated from clinical materials

and rectal swabs from swimmers and non-swimmers were examined. All strains were examined by immunotyping and pyocin typing. Apyocyanogenic P. aeruginosa and UFP strains were examined for gelatin liquefaction, denitrification, ability to utilize 7 substrates, and susceptibility to 7 antibiotics.

Strains of known P. aeruginosa from surface waters and wastes were typed by immunotyping and were tested for their resistance to carbenicillin. Strains selected for immunotyping included 288 strains from non-polluted lake waters and 214 strains from a stream receiving domestic and hospital sewage. Strains examined for resistance to carbenicillin included 35 strains isolated from a stream above a hospital waste discharge, 109 strains isolated from the hospital wastes, and 186 strains isolated from the stream below the hospital waste discharge.

Characterization of isolates. Pigment production, while it was investigated employing a variety of media, was in all strains demonstrated on commercially available media. Fluorescent pigment was detected on King's B medium (Flo Agar, BBL) or Pseudomonas F Agar (Difco) slants incubated at 30C for 72 hr. Slants were observed for fluorescence daily under a Wood's lamp. Pyocyanin production was tested on slants of King's A medium (Tech Agar, BBL) or Pseudomonas P Agar (Difco) incubated at 30C for up to 7 days. Blue pigment was detected visually in slants, or if not readily detectable, was extracted with chloroform and then partitioned into several drops of 1 N HCl where it exhibited a red color.

Growth at 41C was tested on slants of Haynes medium (Haynes, 1951) warmed to 41C prior to inoculation, and inoculated slants were incubated in a water bath for 24 and 48 hr. Indophenol (cytochrome) oxidase was determined by the method of Gaby and Hadley (1957). Glucose metabolism was tested on the oxidative-fermentative medium of Hugh and Leifson (1953). Incubation was overnight at 30C. Liquefaction of gelatin was determined on plates of nutrient agar containing 0.4% gelatin. Plates were incubated at 30C. After 48 hr incubation, plates were

flooded with acid mercuric chloride. Liquefaction was indicated by a zone of clearing surrounding streaks of cells. Casein hydrolysis was determined on the medium of Brown and Scott Foster (1970) incubated at 30C for 48 hr. Denitrification was determined as described by Stanier et al. (1966), or by the fermentation tube method (Manual of Clinical Microbiological Methods, 1974). Oxidation of gluconate to 2-ketogluconate was determined by the methods of Gaby and Free (1958) and Arai et al. (1970). The egg yolk reaction was determined as described in the Manual of Clinical Microbiological Methods (1974).

Arginine dihydrolase activity was detected in oxidative-fermentative basal medium (lacking glucose) containing 1% arginine. Tubes of medium containing arginine and control tubes lacking arginine were covered with mineral oil to a depth of about 1 cm and incubated at 30C for 24 hr. A blue color in tubes containing arginine, but not in control tubes, indicated a positive test.

Utilization of sebacate, saccharate, mannitol, glycollate, gluconate, geraniol, and acetamide were determined by the methods of Stanier et al. (1966).

Flagella stains were prepared according to the method of Leifson (1960).

Immunotyping was performed using either the F antisera of Parke-Davis & Co. or the 16 antisera of Difco. The F antisera of Parke-Davis & Co. were provided by Dr. H. B. Devlin and typing was done according to accompanying instructions. The 16 antisera of Difco were provided by Mr. Duke Bunner and typing was done according to accompanying instructions.

Pyocine typing was performed employing the 18 indicator strains of Jones et al. (1974).

Susceptibility to 11 antibiotics was determined by the method of Bauer et al. (1966). Antibiotics included tetracycline (30 µg), chloramphenicol (30 µg), streptomycin (10 µg), kanamycin (30 µg), neomycin (30 µg), carbenicillin (100 µg), cephalothin (30 µg), ampicillin (10 µg), colistin (10 µg),

and polymyxin B (300 units).

Cellular fatty acid compositions of selected strains were determined by Dr. C. W. Moss and Mrs. Sally Dees of the Center for Disease Control as described by Moss et al. (1972).

RESULTS AND DISCUSSION

Characteristics of strains. Of 123 apyocyanogenic fluorescent Pseudomonas strains contributed by workers at other institutions, most were readily identified as P. aeruginosa. Many produced the blue pigment pyocyanin which is characteristic of the species, even though they were sent as strains incapable of producing the pigment. Most of the strains sent by one worker were readily identified as P. putida and failed to exhibit the characteristics attributed to them by the sender. Six truly apyocyanogenic strains from clinical material appeared to exhibit the characteristics being sought in this study, i.e. characteristics differing from those of P. aeruginosa in several respects described below. One clinical worker late in the study reported having seen many cultures which he identified as P. aeruginosa, but which exhibited exceptional antibiotic susceptibility patterns which resembled unidentified fluorescent Pseudomonas (UFP) strains. Cultures are now being sent as they are isolated. One hundred and twenty three UFP strains originating from surface waters and from hospital wastes were examined.

A summary of characteristics of strains examined is presented in Table 2. Strains selected for study included 13 pyocyanogenic and 47 apyocyanogenic strains of P. aeruginosa from environmental and clinical sources as well as culture collections, as well as 129 UFP strains. On the basis of the tests employed, it was possible to divide the UFP strains into 8 groups.

All strains examined were oxidase positive, exhibited oxidative glucose metabolism and produced fluorescent pigment and arginine dihydrolase. All strains examined thus far possess polar flagellation. All but 2 strains exhibited negative egg yolk reactions, i.e. were lecithinase negative. These characteristics are typical of the fluorescent group of pseudomonads, and, with the exception of the 2 strains exhibiting positive egg yolk reactions, are consistent with P. aeruginosa.

Table 2. Characteristics of *P. aeruginosa* and UFP strains examined

	P. aeruginosa		UFP Strains							
teristic	Pyocyanogenic	Apyocyanogenic	I	II	III	IIIa	IIIb	IV	V	Va
lla	1	1	1	1	>1	>1	>1	>1	?	?
ats										
nazine	+ 100%+ (13/13)*	- 0%+ (0/47)	- 0%+ (0/50)	- 0%+ (0/3)	- 0%+ (0/41)	- 0%+ (0/2)	- 0%+ (0/2)	- 0%+ (0/12)	- 0%+ (0/18)	- 0%+ (0/1)
otenoid	+ 0%+ (0/13)	- 10.6%+ (5/47)	V 38%+ (19/50)	- 0%+ (0/3)	V 17.1%+ (7/41)	- 0%+ (0/2)	- 0%+ (0/2)	- 0%+ (0/12)	- 0%+ (0/18)	- 0%+ (0/1)
ysis	+ 100%+ (13/13)	+ 100%+ (47/47)	V 82%+ (41/50)	- 0%+ (0/3)	V 78%+ (32/41)	+ 100%+ (2/2)	V 50%+ (1/2)	V 16.7%+ (2/12)	V 77.8%+ (14/18)	- 0%+ (0/1)
in liquefaction	+ 100%+ (13/13)	+ 89.4%+ (42/47)	+ 100%+ (50/50)	- 0%+ (0/3)	+ 100%+ (41/41)	+ 100%+ (2/2)	+ 100%+ (2/2)	- 0%+ (0/12)	+ 100%+ (18/18)	+ 100%+ (1/1)
n hydrolysis	+ 92.4%+ (12/13)	+ 91.5%+ (43/47)	+ 87.8%+ (43/49)	- 0%+ (0/3)	+ 100%+ (41/41)	+ 100%+ (2/2)	- 0%+ (0/2)	V 25%+ (3/12)	+ 89%+ (16/18)	+ 100%+ (1/1)
rification	+ 100%+ (13/13)	+ 89.4%+ (42/47)	- 0%+ (0/50)	- 0%+ (0/3)	- 0%+ (0/41)	- 0%+ (0/2)	V 50%+ (1/2)	V 25%+ (3/12)	- 0%+ (0/18)	- 0%+ (0/1)
nate oxidation	+ 100%+ (13/13)	+ 95.9%+ (45/47)	- 0%+ (0/50)	V 33.3%+ (1/3)	- 0%+ (0/41)	+ 100%+ (2/2)	+ 100%+ (2/2)	V 75%+ (9/12)	- 0%+ (0/18)	- 0%+ (0/1)
ine dihydrolase	+ 100%+ (13/13)	+ 100%+ (47/47)	+ 98%+ (49/50)	+ 100%+ (3/3)	+ 100%+ (41/41)	+ 100%+ (2/2)	+ 100%+ (2/2)	+ 100%+ (12/12)	+ 100%+ (18/18)	+ 100%+ (1/1)
olk reaction	- 0%+ (0/13)	- 0%+ (0/47)	- 0%+ (0/50)	- 0%+ (0/3)	- 0%+ (0/41)	- 100%+ (2/2)	- 0%+ (0/2)	- 0%+ (0/12)	- 0%+ (0/18)	- 0%+ (0/1)
lzation of:										
bacate	+	+ 100%+ (3/3)	+ 100%+ (19/19)	V 33%+ (1/3)					+ 92%+ (13/14)	+ 100%+ (1/1)
charate	-	- 0%+ (0/3)	- 0%+ (0/19)	+ 100%+ (3/3)					- 0%+ (0/14)	+ 100%+ (1/1)
nnitol	+	+ 100%+ (3/3)	- 0%+ (0/19)	V 33%+ (1/3)					- 0%+ (0/14)	+ 100%+ (1/1)
ycollate	-	V 66.7%+ (2/3)	- 0%+ (0/19)	+ 100%+ (3/3)					- 0%+ (0/14)	+ 100%+ (1/1)
uconate	+	V 66.7%+ (2/3)	- 5.3%+ (1/19)	V 67%+ (2/3)					- 7.1%+ (1/14)	+ 100%+ (1/1)
raniol	+	+ 100%+ (3/3)	+ 94.7%+ (18/19)	+ 100%+ (3/3)					+ 100%+ (14/14)	+ 100%+ (1/1)
etamide	+	+ 100%+ (3/3)	- 10.5%+ (2/19)	V 33%+ (1/3)					+ 100%+ (14/14)	+ 100%+ (1/1)
stance to:										
ntamicin	S 0%+ (0/13)	S 0%+ (0/46)	S 0%+ (0/50)	S 0%+ (0/3)	S 0%+ (0/40)	S 0%+ (0/2)	S 0%+ (0/2)	S 0%+ (0/9)	S 0%+ (0/18)	S 0%+ (0/1)
rbenicillin	V 46.2%+ (6/13)	V 42.3%+ (19/45)	R 89.4%+ (42/47)	V 66.7%+ (2/3)	R 92.5%+ (37/40)	R 100%+ (2/2)	R 100%+ (2/2)	R 88.9%+ (8/9)	R 88.9%+ (16/18)	R 100%+ (1/1)
loramphenicol	R 100%+ (13/13)	R 95.6%+ (43/45)	R 91.9%+ (45/49)	R 100%+ (3/3)	R 87.5%+ (35/40)	R 100%+ (2/2)	R 100%+ (2/2)	R 100%+ (9/9)	R 94.5%+ (17/18)	R 100%+ (1/1)
reptomycin	V 77%+ (10/13)	V 76.1%+ (35/46)	S 2%+ (1/50)	V 33.3%+ (1/3)	S 2.5%+ (1/40)	V 50%+ (1/2)	S 0%+ (0/2)	V 55.6%+ (5/9)	S 0%+ (0/18)	S 0%+ (0/1)
tracycline	R 92.4%+ (12/13)	R 100%+ (45/45)	V 66%+ (33/50)	S 0%+ (0/3)	R 85%+ (34/40)	S 0%+ (0/2)	V 50%+ (1/2)	V 66.7%+ (6/9)	R 100%+ (18/18)	R 100%+ (1/1)
omycin	S 7.7%+ (1/13)	V 47.9%+ (22/46)	V 70%+ (35/50)	S 0%+ (0/3)	V 77.5%+ (31/40)	S 0%+ (0/2)	S 0%+ (0/2)	S 0%+ (0/9)	V 27.8%+ (5/18)	R 100%+ (1/1)
anamycin	R 92.4%+ (12/13)	R 88.9%+ (40/45)	S 2%+ (1/50)	S 0%+ (0/3)	S 0%+ (0/40)	S 0%+ (0/2)	V 50%+ (1/2)	S 0%+ (0/9)	S 0%+ (0/16)	S 0%+ (0/1)
ephalothin	R 100%+ (12/12)	R 100%+ (38/38)	R 100%+ (21/21)	R 100%+ (3/3)	R 100%+ (5/5)	R 100%+ (2/2)	R 100%+ (1/1)	R 100%+ (9/9)		
mpicillin	R 100%+ (12/12)	R 97.4%+ (36/37)	R 100%+ (21/21)	R 100%+ (3/3)	R 100%+ (5/5)	R 100%+ (2/2)	R 100%+ (1/1)	R 100%+ (9/9)		
olistin	S 7.7%+ (1/12)	S 0%+ (0/37)	S 0%+ (0/21)	S 0%+ (0/3)	S 0%+ (0/5)	S 0%+ (0/2)	S 0%+ (0/1)	S 0%+ (0/9)		
olymyxin B	S 7.7%+ (1/12)	S 0%+ (0/37)	S 0%+ (0/21)	S 0%+ (0/3)	S 0%+ (0/5)	S 0%+ (0/2)	S 0%+ (0/1)	S 0%+ (0/9)		
nable by:										
Pyocine typing	+ (1/1)	+ (3/3)							- (0/14)	
Serological typing	+ (11/13)	+ (39/47)	- (0/50)	- (0/3)	- (0/41)	- (0/2)	- (0/2)	- (0/12)	- (0/18)	- (0/1)

positive (more than 85% positive)
negative (less than 15% positive)
variable (15% to 85% positive)
numbers in parentheses indicate number of positive or resistant strains over total strains examined

Furthermore, all strains grew at 41C, in this respect resembling P. aeruginosa.

Patterns of flagellation varied among strains (Table 3). Among strains of P. aeruginosa, single polar flagella were observed most commonly, as expected. According to Lautrop and Jessen (1964) and Jessen (1965), if less than 10% of cells are multitrichous, a Pseudomonas strain may be considered to possess single polar flagella. If more than 25% of cells are multitrichous, the strain should be considered to possess polar multitrichous flagellation. However, if between 10 and 25% of cells possess polar flagellar tufts, the flagellar pattern is intermediate, and the strain cannot be assigned to either well defined group. For convenience in Table 2, all strains exhibiting flagellar indices (percent multitrichous) in excess of 10 were considered to possess more than 1 polar flagellum. One of the 13 pyocyanogenic strains of P. aeruginosa was of an intermediate type (12% multitrichous) and 9 of 47 apyocyanogenic strains were of an intermediate type or were lophotrichous (12, 12, 14, 14, 16, 24, 28, 28, and 36%, respectively). While the pyocyanogenic strain and several of the apyocyanogenic strains differed little from the ideal monotrichous flagellar indices, the number of apyocyanogenic strains exhibiting multitrichous polar flagellation was higher than normally would be anticipated.

Among the 129 UFP strains, intermediate and multitrichous strains were more common (Table 3). It should be pointed out that among the 2 major groups of UFP strains (Groups I and III) based upon data obtained thus far, the only difference is to be found in the patterns of flagellation. Lateral flagella of the type described by Palleroni et al. (1970) in the stutzeri group were not observed. Polar tufts found among Group III strains frequently included a single flagellum of normal appearance and straight flagella having an irregular and abnormal appearance. The latter structures did not resemble the lateral flagella of Beneckea strains described by Bauman, Bauman and Mandel (1971). It may be of significance

Table 3. Identity and flagellar indices of UFP Strains

Group	Strain	Flagellar Index	Group	Strain	Flagellar Index	Group	Strain	Flagellar Index
I	201	3	I	DM2	0	III		
	202	2	cont'd	DM49	8.5	cont'd	DM48	32
	203	6		DM52	9		DM50	50
	204	4		DM53	1		DM51	23
	205	2		DM55	0		DM54	10
	207	2	II	217	0	IIIa	247	10
	209	8		219	0		414	58
	211	0		231	0	IIIb	208	13
	212	0	III	DM1	40		415	66
	213	0		DM9	42	IV	230	76
	214	0		DM11	53		233	26
	215	0		DM12	30		234	46
	216	0		DM13	50		235	70
	218	0		DM15	70		236	58
	220	0		DM17	15		237	36
	221	6		DM18	64		244	42
	222	0		DM19	17		245	64
	223	0		DM20	60		246	88
	224	0		DM21	15		410	74
	225	0		DM22	19		416	76
	226	0		DM23	13		417	50
	227	0		DM24	28	V	206	
	228	0		DM25	68		305	
	229	0		DM26	29		306	
	239	0		DM27	29		319	
	301	4		DM28	52		323	
	302	8		DM29	46		324	
	303	1		DM30	40		325	
	304	0		DM31	21		327	
	307	0		DM32	28		328	26
	308	3		DM33	14		329	
	309	0		DM34	58		330	
	310	1		DM35	40		331	
	311	0		DM36	40		332	
	312	4		DM37	40		333	
	313	1		DM38	17		334	
	314	1		DM39	59		335	
	315	1		DM40	40		336	
	316	6		DM41	56		337	
	317	4		DM42	68	Va	326	
	318	2		DM43	42			
	320	0		DM44	62			
	321	0		DM45	50			
	322	0		DM46	35			
	423	0		DM47	56			

that all Group III strains were isolated from hospital sewage and thus presumably originated from the hospital environment.

UFP strains could be recognized by their lack of hemolytic activity, ability to hydrolyze gelatin and casein, or their lack of ability to denitrify or oxidize gluconate. Whereas some pyocyanogenic and apyocyanogenic P. aeruginosa strains differed from the ideal biotype with respect to a single characteristic, all UFP strains differed with respect to at least 2 characters. Most commonly, UFP strains failed to denitrify, to oxidize gluconate, or both. Many strains differed with respect to other characteristics as well. In addition, whereas both pyocyanogenic and apyocyanogenic P. aeruginosa strains exhibited strong hemolytic activity, the activity of most UFP strains was very weak, often detectable only after scraping cells from the surface of blood agar plates. Based primarily upon these characteristics and flagellar patterns, UFP strains were assigned to 8 groups.

The 2 predominant UFP groups, Groups I and III, apparently differing from each other only with respect to their flagellation, differed from P. aeruginosa in the above characteristics only with respect to their generally low hemolytic activity and their failure both to denitrify and to oxidize gluconate. It is of interest that many Group I and III UFP strains (38 and 17%, respectively) produced brown carotenoid pigments, a property exhibited among the aerobic pseudomonads only by P. mendocina in the stutzeri group. It must be pointed out that GC contents of 3 Group I UFP strains determined by Dr. Manley Mandel at the M.D. Anderson Hospital and Tumor Institute indicated a similarity to P. aeruginosa rather than to P. mendocina. DNA hybridizations conducted by Dr. N. J. Palleroni of the University of California in Berkeley indicated a low homology between 2 UFP Group I strains and P. mendocina.

Tests for the utilization of 7 substrates were limited. None of the 13

pyocyanogenic strains of P. aeruginosa included in this study were tested. Expected reactions are included in Table 2, however, and are based upon results obtained in our laboratory on other strains and results reported by Stanier et al. (1966). Among the 3 apyocyanogenic strains examined, there was exhibited considerable variability. This probably is of little significance in view of the small number of strains examined. Only the ability of 2 apyocyanogenic P. aeruginosa strains to utilize glycollate was unusual.

On the other hand, significant differences existed between UFP strains and P. aeruginosa. Among the 19 Group I UFP strains, none utilized mannitol and only 1 and 2 strains utilized gluconate and acetamide, respectively. While the lack of ability to utilize gluconate might be anticipated in view of the failure of these strains to oxidize gluconate, these differences, and particularly the failure to utilize acetamide, suggest real differences between UFP strains and P. aeruginosa. The ability to utilize geraniol, however, is limited to P. aeruginosa and P. mendocina.

Among the 3 Group II UFP strains, utilization of the 7 substrates was variable, and, like the reactions of the 3 apyocyanogenic P. aeruginosa strains, these results probably are of limited taxonomic value. However, the patterns of reactions do, in some ways, resemble those of P. mendocina, a non-fluorescent pseudomonad in the stutzeri group. In particular, only single strains utilized sebacate, mannitol, and acetamide, none of which are utilized by P. mendocina. Furthermore, all strains utilized saccharate, glycollate, and geraniol, and 2 of the 3 strains utilized gluconate, all of which are utilized typically by P. mendocina. It should be pointed out that a fourth strain isolated from clinical material has been received from Dr. Spyros Kominos at Mercy Hospital in Pittsburgh. This strain was fluorescent, oxidase positive, exhibited oxidative glucose metabolism, and grew at 42C, but resembled Group II strains in failing to hemolyze blood, liquefy

gelatin, hydrolyze casein, denitrify, oxidize gluconate, or exhibit lecithinase activity on egg yolk agar, while exhibiting arginine dihydrolase activity. Ordinarily, this strain would be identified as P. aeruginosa on the basis of fluorescence, oxidase reaction, oxidative glucose metabolism, and growth at 42C. However, this strain was susceptible to kanamycin and resistant to carbenicillin, which differs from the normal pattern of susceptibilities exhibited by P. aeruginosa (see discussion below).

It is significant that strain 217 in UFP Group 2 isolated by us from surface waters had been studied by Dr. N. J. Palleroni, who, applying the battery of tests employed by Stanier et al. (1966), had found the strain to be identical to P. mendocina save that it was fluorescent and that it failed to produce carotenoid pigments. GC determinations performed by Dr. Manley Mandel confirmed the similarity of strain 217 to P. mendocina. DNA hybridizations conducted by Dr. Palleroni demonstrated 65% competition at both 70 and 80C between strain 217 and P. mendocina, indicating a considerable homology between the UFP strain and that species.

Among other UFP groups, only 15 strains of Groups V and Va were examined for utilization of the 7 substrates. With the exception of 1 of these strains, none has been examined for flagellation. Among strains of Group V, none utilized mannitol or gluconate, and, with the exception of the utilization of acetamide, these strains resembled Group I strains. The single strain of Group Va was unusual in its ability to utilize each of the 7 substrates, thus differing from P. aeruginosa in its ability to utilize saccharate and glycollate.

With only rare exceptions, strains of P. aeruginosa and UFP strains were susceptible to gentamicin, colistin, and polymyxin B and were resistant to cephalothin and ampicillin. Most strains examined were resistant to chloramphenicol, although among both Group I and III UFP strains susceptibility was not rare, and susceptible strains occurred among apyocyanogenic P. aeruginosa and Group V UFP strains.

Neomycin susceptibility was highly variable. While resistance was demonstrated in only 1 of 13 pyocyanogenic P. aeruginosa strains, it occurred in approximately 48% of apyocyanogenic P. aeruginosa strains and between 70 and 80% of Group I and III UFP strains. Thus, susceptibility to neomycin, while it is characteristic of pyocyanogenic strains, is of little value in distinguishing apyocyanogenic P. aeruginosa from UFP strains. Furthermore, it might be anticipated that neomycin would be of doubtful value in the treatment of infections caused by apyocyanogenic fluorescent pseudomonads.

In contrast to the failure of susceptibility to the above antibiotics to provide useful diagnostic information, susceptibility to carbenicillin, streptomycin, and kanamycin do appear to provide a basis for distinguishing UFP strains from pyocyanogenic and apyocyanogenic P. aeruginosa. The clearest distinction is between resistance of P. aeruginosa strains and susceptibility of UFP strains to kanamycin. Approximately 90% of both pyocyanogenic and apyocyanogenic UFP strains were resistant to kanamycin, whereas only 2 of 123 UFP strains examined were resistant to this drug. This observation is similar to that of Blazevic et al. (1973) who recommended susceptibility to kanamycin to distinguish between P. aeruginosa on the one hand and P. fluorescens and P. putida on the other.

Susceptibility to carbenicillin also may serve well to distinguish P. aeruginosa from other fluorescent pseudomonads, as suggested by Blazevic et al. (1973). While less than half of the P. aeruginosa strains examined were resistant to carbenicillin, UFP strains characteristically exhibited resistance to this drug. This is particularly the case when it is recognized that all resistant P. aeruginosa strains were of clinical origin. No environmental P. aeruginosa strains included in this study were resistant to carbenicillin. This does not mean that carbenicillin resistant strains may not be isolated from the environment. Green et al. (1974) reported isolation of a carbenicillin resistant strain from soil in a culti-

vated field in California. In addition, hospital sewage may serve as a reservoir of carbenicillin resistant strains reaching surface waters (see discussion below). Thus, as suggested by Blazevic et al. (1973), resistance to carbenicillin, together with susceptibility to kanamycin, provides good evidence that a strain differs significantly from P. aeruginosa.

Of less certainty is susceptibility to streptomycin. Between 76 and 77% of P. aeruginosa strains examined were resistant to streptomycin. On the other hand, resistant UFP strains, except in Groups IIIa and IV (11 of 125 strains), were rare. Thus, resistance to carbenicillin, together with susceptibility to strepomycin and/or kanamycin should provide strong evidence of a UFP strain differing in many other respects from P. aeruginosa.

Only very limited studies of pyocin production were undertaken employing strains reported in Table 2. Of a total of 4 P. aeruginosa strains examined, all were typable employing the 18 P. aeruginosa indicator strains of Jones et al. (1974b). In studies of 35 strains of P. aeruginosa examined by us but not reported in Table 2, 32 were typable employing the above indicator strains, and Jones et al. (1974) were able to type all of 100 strains which they examined. Of 14 UFP strains reported in Table 2, none could be shown to produce pyocins. In previous studies, it was demonstrated that none of 9 UFP Group I strains produced pyocins active against the 8 indicator strains of Gillies and Govan (1966). These results suggest a divergence from P. aeruginosa, but more extensive studies of both production of and sensitivity to pyocins will be undertaken to clarify this point.

Results of serological typing of strains employing the 16 antisera of Difco similarly suggested a divergence of UFP strains from P. aeruginosa. Whereas over 80% of both pyocyanogenic and apyocyanogenic strains of P. aeruginosa were typable, none of the 129 UFP strains were typable.

Further evidence suggesting that UFP strains may differ significantly from

P. aeruginosa was obtained from the cellular fatty acid compositions of selected P. aeruginosa and UFP strains. Cellular fatty acid compositions of 7 apyocyanogenic P. aeruginosa strains (including 4 strains included in Table 2) and 23 UFP strains were determined by Dr. C. W. Moss and Mrs. Sally Dees of the Center for Disease Control by gas-liquid chromatography (as decribed by Moss et al. [1972]). The cellular fatty acid compositions of all 7 apyocyanogenic P. aeruginosa strains resembled those of the fluorescent group. Only 2 UFP strains (in Group IV) resembled the fluorescent group. The remaining 21 UFP strains, while exhibiting some variability in their cellular fatty acid compositions, resembled the alcaligenes and stutzeri groups.

Substantial evidence has been cited suggesting the existence of fluorescent Pseudomonas strains which, while they resemble P. aeruginosa in certain important respects, including ability to grow at 42C, possession of single polar flagella and utilization of geraniol, they differ in many respects including ability to denitrify, ability to oxidize gluconate, failure to utilize acetamide, resistance to carbenicillin, susceptibility to streptomycin and kanamycin, and failure to agglutinate in the presence of P. aeruginosa antisera. In surface and swimming pool waters UFP strains are of interest because they can cause false positive reactions in most probable number tests for P. aeruginosa. The clinical significance of UFP strains cannot be judged from the present study. However, 8 UFP strains from clinical sources were included in the study, 4 of unknown origin, 3 from infected outer ears, and 1 from a healthy outer ear. One additional culture isolated from clinical material and resembling UFP Group II strains was received too late for inclusion in the study. Identical UFP strains(included in the present study)were isolated by Hoadley et al. (1975) from swimming pool waters and from the healthy auditory canal of 1 swimmer and the infected outer ear of a second.

Frequencies of P. aeruginosa immunotypes in polluted and non-polluted surface waters. The frequencies of immunotypes among P. aeruginosa strains isolated from surface waters resembled in some respects frequencies reported from hospitalized patients. Frequencies among strains from unpolluted or remotely polluted surface waters and from stream waters receiving hospital wastes are presented in Table 4. The incidences of type 1 strains in both polluted and unpolluted waters were similar to those reported among 742 strains from hospitalized patients by Moody et al. (1972). On the other hand, while less than 1% of strains examined by Moody were untypable, approximately 20% of all strains from surface waters were untypable by the 7 antisera of Parke-Davis and Co. Also, types 3, 5, and 6 were encountered less frequently among isolates from surface waters than among isolates (10.5%, 6.6%, and 19.9%, respectively). Type 4 was encountered more frequently, approximately 9% of isolates from surface waters as compared to 4.6% of isolates from clinical sources being of that type. Lanyi et al. (1966) and Nemedi and Lanyi (1971) recognized a similar striking similarity of P. aeruginosa serotypes in water samples from numerous sources, sewage, and human feces employing 13 antisera of Lanyi (1966).

Carbenicillin resistance of P. aeruginosa strains from waters and wastes. Pseudomonas aeruginosa in hospital wastes discharged to streams may be of special interest because they can bear resistance to certain antibiotics. In recent years, P. aeruginosa strains resistant to carbenicillin and to gentamicin have appeared in hospitals. While strains isolated from hospital wastes appears not to be resistant to gentamicin, carbenicillin resistant strains were demonstrated in both hospital wastes and their receiving stream below the waste outfall (Table 5). Strains from the stream above the outfall were susceptible to carbenicillin.

Table 4. Frequencies of P. aeruginosa immunotypes among isolates from surface waters

(Data of Ajello, Mooney, and Hoadley, unpublished)

Parke-Davis immunotype	Lake waters (288 strains)	Polluted stream (214 strains)
1	37.5*	28.1
2	5.9	13.1
3	2.8	4.2
4	8.7	9.3
5	1.7	0.7
6	2.1	1.4
7	6.2	10.3
3,7	5.6	10.3
Other strong cross reactions	4.9	1.4
Rough	1.4	3.7
Untypable	23.2	17.3

*Percent of strains

Table 5. Occurrence of P. aeruginosa isolates resistant to carbenicillin* in hospital wastes and a receiving stream

(Data of Hoadley and Ajello, unpublished)

Source of isolates	No. tested	No. resistant	% resistant
Steam above outfall	35	0	0
Hospital Wastes	109	20	12.0
Stream below outfall	186	24	18.4

*Resistance determined by the method of Bauer et al. (2) with 100 µg discs

PUBLICATIONS

Two published papers were supported in part by this grant, and it is anticipated that a third publication and a presented paper will be possible in the fall of 1975. The following papers were supported in part by this grant:

Hoadley, A. W., G. Ajello, and N. Masterson. 1975. Preliminary studies of fluorescent pseudomonads capable of growth at 41C in swimming pool waters. Appl. Microbiol. 29:529-531. Characterization of cultures, including minimal physiological and biochemical tests, testing of resistance to 7 antibiotics, pyocin typing, and immunotyping of P. aeruginosa and UFP strains from swimming pools and swimmers.

Hoadley, A. W. 1975. Pseudomonas aeruginosa in surface waters. In V. M. Young (ed.) The ecology of Pseudomonas aeruginosa and its role in the colonization of hospitalized patients. C. H. Thomas, Springfield (In press). Serotyping of P. aeruginosa strains from polluted and non-polluted surface waters. Carbenicillin resistance of P. aeruginosa strains in hospital wastes and receiving waters.

After testing of strains has been completed, a presentation and a paper will be prepared to present the findings of this study relating to the characteristics and possible significance of UFP strains:

Proposed presentation: Annual Meeting of Southeast Branch, American Society for Microbiology.

Proposed publication: Journal of Clinical Microbiology.

PERSONNEL SUPPORTED

This contributed to the support one half-time associate for a period of 10 months:

Gloria W. Ajello, M.S.
Research Associate
School of Civil Engineering

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