A STUDY OF THE SOMATIC ANTIGENS AND BIOCHEMICAL PROPERTIES OF SELECTED SPECIES OF THE GENUS <u>PSEUDOMONAS</u>

by

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INTRODUCTION

The genus Pseudomonas, as described by Breed, et al. (1948) consists for the most part of soil and water bacteria. Many plant pathogens are also included but few animal pathogens are presented. Bacteria within the genus Pseudomonas are characterized as being usually motile, with monotrichous or lophotrichous flagella. Many of the species are capable of producing a green, fluorescent, water-soluble pigment. Many possess an ability to ferment glucose, sometimes with gas. Nitrates are frequently reduced either to nitrites, ammonia or free nitrogen. Some species hydrolyze fats and attack hydrocarbons. Except for three species listed by Breed, et al. (1948) the organisms are Gram negative in staining properties. The type species representing this genus is Pseudomonas aeruginosa (Schroeter) Migula.

Pseudomonas aeruginosa is perhaps the best known organism within the genus probably due to the fact that this organism is one of the few animal pathogens included within the genus and because it is pathogenic to man.

Since Pseudomonas aeruginosa is the type species, this preliminary investigation was undertaken in order to determine the antigens exhibited by other species in the genus in common with the type species as well as the possibility of the exhibition of homologous or heterologous antigens by different strains of the same species.

At the same time it was felt that by the process of agglutination-adsorption techniques an identification scheme similar to that utilized in identifying members of the genus Salmonella could be developed for the genus Pseudomonas. Serological studies have been performed and reported previously by other workers. The results were in much disagreement as revealed in the literature review. Thus, it seems there is a definite need for new classification schemes, utilizing both biochemical and serological methods.

REVIEW OF LITERATURE

A review of the literature reveals much disagreement on the classification of the genus Pseudomonas and the differentiation of species within this group. However, the species which have gained most of the attention are Pseudomonas aeruginosa and Pseudomonas fluorescens, the former being associated with human infections and the latter being commonly found in soil and water. Fortunately, later studies of this genus include many of the 148 organisms placed in the genus by Breed, et al. (1948).

From clinical observations and animal experiments, Schneider (1926) noticed that Bacillus pyocyaneus (Pseudomonas aeruginosa)[1] and Bacillus fluorescens liquefaciens (Pseudomonas fluorescens) produced the same clinical pictures. The author concluded that no definite differentiation between the two organisms could be made on the basis of "ring abscess" formation. Ring abscesses were attributed to both or were absent.

Turfitt (1936), investigating the existing relationship of the pyocyaneus-fluorescens group, indicated that there was a remarkable resemblance, both morphologically and culturally. Along with Bacillus pyocyaneus (Pseudomonas aeruginosa), Bacillus fluorescens liquefaciens (Pseudomonas fluorescens) and Bacillus fluorescens non-liquefaciens (Pseudomonas eisenbergii), one hundred green, fluorescent organisms isolated from nature were studied.

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[1] The terminology for all bacteria is that used in Bergey's Manual of Determinative Bacteriology, 6th edition. Baltimore: Williams and Wilkins, 1948. When an author has used an older name it appears first, followed by the Bergey name in parentheses.

It was hypothesized earlier by Ruzicka (1899) that Bacillus fluorescens (Pseudomonas fluorescens) and Bacillus pyocyaneus (Pseudomonas aeruginosa) were not two distinct types, but merely extremes of the same type. To prove this theory he made the two organisms assume the same characteristics.

Results of experiments performed by Aoki (1926) were similar to those of Ruzicka (1899). The mode of investigation however, was different. Fifty strains of Bacillus pyocyaneus (Pseudomonas aeruginosa), isolated from human infections, were differentiated into 22 groups by cross-agglutinations. Classification of a given strain by agglutination did not always agree with classification by cultural methods. Regarding the idea that Bacillus putidum (Pseudomonas putida), Bacillus fluorescens (Pseudomonas fluorescens) and Bacillus pyocyaneus (Pseudomonas aeruginosa) are closely related, the author favored the idea that these three organisms were variations of one single species.

Kanzaki (1934), studying 102 strains, demonstrated 34 different types, of which six types contained the majority of those studied. He also verified Aoki's (1926) results, finding eighteen serological types within Aoki's twenty-two groups. In a later publication Kanzaki (1934) presented results showing that two immunological types of Bacillus pyocyaneus (Pseudomonas aeruginosa) presented specific and non-specific types, two others showed specific types and still two others showed non-specific types. Thus it was concluded that sera from specific forms contained receptors for specific forms and sera for non-specific forms contained equal receptors for the heterogenous types.

Pursuing the study further Kanzaki (1934) found that the specific receptors were heat stable and that the non-specific receptors were destroyed on boiling.

In contrast to the previous experiments, Trommsdorf (1916) investigating the possibility that Bacillus pyocyaneus, (Pseudomonas aeruginosa), Bacillus *fluorescens* (*Pseudomonas fluorescens*) and *Bacillus putida* (*Pseudomonas putida*) were variations of one organism, found that both the cultural and serological classification did not coincide. By agglutination, he was able to divide 25 of 27 *Bacillus pyocyanea* (*Pseudomonas aeruginosa*) strains into three groups. Cross-agglutination was obtained between the three species, but it was concluded on the basis of adsorption techniques that the three species were antigenically different.

On the other hand, Sandiford (1937) tested 50 strains of *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*) isolated from various sources. All cultures showed a negative indole reaction and all cultures formed acid from glucose. Those isolated from water were indistinguishable from organisms isolated from human infection by means of cultural characteristics which were employed to differentiate *Bacillus fluorescens* (*Pseudomonas fluorescens*) from *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*).

According to Sherwood, et al. (1926), efforts to produce high-titered sera with *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*) were not successful, the maximum being 1-5000. Cross agglutinations between strains gave irregular results.

Lilley and Bearup (1928) found that *Pseudomonas aeruginosa* isolated from tap water were practically identical serologically with the Lister Institute strain. The authors isolated *Pseudomonas aeruginosa* from the blood of four patients and agglutinins for these organisms were demonstrated in five cases including the four blood cultures. Symptoms described from the infection included fever, headache, back pains, diarrhea and constipation. Control sera used in identification of this infection consisted of sera collected from 100 diseases. Agglutination did not occur in dilutions higher than 1-20, except in *Pseudomonas* infections where the titer was higher.

Three main antigens were found distributed among strains of Pseudomonas pyocyanea (Pseudomonas aeruginosa) by Mayr-Harting (1948). The serological behavior of the strains studied depended on the presence and quantity of these antigens. It was not possible to distinguish between flagellar and somatic antigens, but those found were assumed to be somatic. A striking peculiarity was noticed in the study, in that a complete loss of agglutinability occurred on moderate heating, which left the antigenic activity unimpaired.

Seven serological groups were described by Christie (1948). These groups were distinguished by slide agglutination on the basis of somatic antigens with further subdivision on the basis of flagellar antigens. Examining 138 strains of Pseudomonas pyocyanea (Pseudomonas aeruginosa) the author also demonstrated that there were pathogenic and saprophytic groups which could be differentiated on the basis of ability to show hemolysis on sheep blood agar and to grow at 37° C.

Meader (1924), found in his study of serological and cultural reactions of Bacillus pyocyaneus (Pseudomonas aeruginosa) that all strains were identical except one culture isolated from rabbit urine which varied in its power to reduce nitrates and form gas from potassium nitrate. The other strains included in this study consisted of the following isolates: Three strains from water, presumably Bacillus fluorescens liquefaciens (Pseudomonas fluorescens); one from milk; three from rabbit urine; six from human urinary infections; one from a human wound; one from a human blood infection; one stock strain known to be 20 years old; and six unknown strains. Culturally these cultures had the following characteristics: None coagulated milk, but all digested it in 24 to 44 hours; no pigment was produced anaerobically. It was also observed that certain bacteria which produce a brilliant red water-soluble pigment culturally and morphologically resembled Bacillus pyocyaneus (Pseudomonas aeruginosa) and Bacillus fluorescens liquefaciens (Pseudomonas fluorescens). From these results the author concluded that a definite relationship did occur. He placed the organism producing the red water-soluble pigment midway between the human type Bacillus pyocyaneus (Pseudomonas aeruginosa) and the water type Bacillus fluorescens liquefaciens (Pseudomonas fluorescens).

Perhaps the most complete study of classification of the genus Pseudomonas was made by Munoz, et al. (1949). A representative group of strains of Pseudomonas was studied to determine whether there was any serological relationship among strains of this genus. The results indicated that a group of monotrichic strains that had the morphological and biochemical characteristics of Pseudomonas aeruginosa could be differentiated into two distinct serological groups, one of which was homogeneous and the other heterogeneous. A lophotrichic strain that had the same biochemical characteristics showed a strong flagellar relationship to the heterogeneous groups.

Pseudomonas caviae, Pseudomonas fragi, Pseudomonas graveolens, Pseudomonas mucidolens and Pseudomonas pavonacea species that were readily differentiated by their biochemical characteristics were also differentiated serologically. Cultures of Pseudomonas fluorescens, Pseudomonas mildenbergii, Pseudomonas ovalis and Pseudomonas putida that could not be satisfactorily differentiated by biochemical means were easily differentiated serologically.

Pseudomonas aeruginosa has also been studied in a role as a plant pathogen. This organism was recognized in previous studies as a human pathogen. Elrod and Braun (1942) found that isolates of Phytomonas polycolor (Pseudomonas polycolor) were indistinguishable from Pseudomonas aeruginosa on the basis of pyocyanin formation, growth at 37° C. and animal pathogenicity. Agglutination, complement fixation and agglutination-adsorption techniques proved the two plant organisms to be serologically identical with at least one animal isolate and to be closely allied to the others. Brief biochemical comparisons also indicated singleness of the group. These results failed to confirm the earlier work by Meader (1924) that by agglutination-adsorption the Pseudomonas aeruginosa group was serologically uniform.

Bacillus pyocyaneus (Pseudomonas aeruginosa) was also noticed to resemble the Whitmore Bacillus on passage through a guinea pig, by Blanc, et al. (1943). Culturally it did not form indole, failed to reduce nitrates and did not acidify arabinose medium. Serologically it had the same agglutinability as the Whitmore Bacillus.

Saint John-Brooks et al. (1925), also investigated phytopathogenic bacteria by cultural and serological methods. It was demonstrated that the N.A.B. strain of Bacillus marginale (Pseudomonas marginalis) differed from two other strains of the same species. Comparisons of the serological relationships of these strains with eleven strains of Bacillus pyocyaneus (Pseudomonas aeruginosa) isolated from various sources proved negative. Results of agglutination tests were variable using strains of Bacillus pyocyaneus (Pseudomonas aeruginosa) against high-titered serum for each organism.

Antigenic heterogeneity was also revealed by Pliszka (1939) studying Pseudomonas punctata. He isolated strains from infected carp which were not serologically identical. Polyvalent serum was recommended for treatment. Efforts to explain the agglutination reaction were made by Lasseur, et al. (1935), using Bacillus pyocyaneus var. pyocyanogenes (Pseudomonas aeruginosa) Bacillus chlororaphis (Pseudomonas chlororaphis) and Bacillus prodigiosus (Serratia marcescens). Harvested from nutrient agar the organisms were washed three times and resuspended in saline. After being shaken with glass beads the material was fractionally centrifuged four times. Deposits of each fraction were suspended in saline and subjected to homologous serum agglutination. Concerning Bacillus pyocyaneus (Pseudomonas aeruginosa) the speed of agglutination was much greater with the third fraction than the first. Discordant results were obtained with Bacillus chlororaphis (Pseudomonas chlororaphis). In all cases it was the longest and largest bacteria which centrifuged out most readily, the average size becoming smaller with each successive fraction. The authors consider that the greater agglutinability of cells which centrifuged more slowly was explained by the fact that these were smaller, younger and biochemically more active, therefore, adsorbing agglutinins more readily.

Continuing the study of Bacillus chlororaphis (Pseudomonas chlororaphis) Lasseur, et al. (1938), found that no cross-agglutination occurred among the corresponding colonial types Rb, Ra, and S.

Very similar experiments were performed on the corresponding types by Lasseur, et al. (1939), but in addition to studying cross-agglutinations among various phases of Bacillus chlororaphis (Pseudomonas chlororaphis) Castellani tests were performed. The activity of the serums in decreasing order were as follows: Ra type, S type and Rb type. The affinity of S agglutinin was greater than that of Ra agglutinin for Bacillus chlororaphis (Pseudomonas chlororaphis) S since the antigen could adsorb all the agglutinins of the Serum S and only part of those of the Serum Rb.

Results of a study of dissociation of Pseudomonas aeruginosa were reported by Gaby (1946). Colonies of Pseudomonas aeruginosa isolated from human infections varied considerably morphologically, but were shown to be derived from at least three basic colony types designated as A, B, and R.

The pattern of fermentative and proteolytic activities of the A, B, and R types were extremely variable and thought to be of little value as a means of classification. Agglutination reactions of the various cultures of Pseudomonas aeruginosa indicated that the somatic antigens were homologous, but agglutination-adsorption tests showed a definite heterologous relationship existing between the flagellar antigens of the three basic colony types.

Hosaya, et al. (1949), prepared an antigenic substance from the Tsuchijimi strain of Pseudomonas aeruginosa by precipitation with zinc chloride, dialysis electrodialysis and precipitation with acetone followed by drying. The material contained 11.5 percent nitrogen, 1.61 percent organic phosphorus, 2.1 percent ash, 5.8 percent sugar, and was considered to be nucleo-protein. A precipitin reaction with immune rabbit serum was obtained in the dilution of 1-250,000 and was not affected by heating to 100° C for one hour. One to two milligrams of this antigenic substance conferred an immunity to injections of live bacilli in guinea pigs which lasted for three weeks. Digestion with trypsin, pepsin or both had no affect on the antigenic properties.

MATERIALS AND METHODS

Cultures Employed

Eleven strains of the genus Pseudomonas were studied in these experiments, including an organism suspected of being a member of this genus and designated as "Species X".

Table 1 lists the organisms as well as their sources of isolation.

Table 1. Organisms and sources of isolation.

Identification Letter	Organism	Source
A.	Pseudomonas aeruginosa	N.R.R.L.* Strain B-23
B.	Pseudomonas aeruginosa	Infected pheasant
C.	Pseudomonas aeruginosa	Human ear infection
D.	Pseudomonas aeruginosa	Child's fecal specimen
E.	Pseudomonas aeruginosa	Unknown source
F.	Pseudomonas chlororaphis	N.R.R.L. B-560
G.	Pseudomonas fluorescens	N.R.R.L. B-10
H.	Pseudomonas indoloxidans	N.R.R.L. B-769
I.	Pseudomonas iodinium	N.R.R.L. B-141
J.	Pseudomonas reptilovorous	N.R.R.L. B-963
K.	Species X	Sewage

* Northern Regional Research Laboratory.

Cultures of these organisms were maintained on nutrient agar culture medium composed of the following ingredients:

Beef extract	3 g.
Proteose peptone	10 g.
Sodium chloride	5 g.
Agar	20 g.
Distilled water	1000 ml.

The medium was adjusted to pH 7.0. All media used throughout this study was sterilized by autoclaving at a steam pressure of 15 pounds per square inch (121° C). Periodically transfers were made onto fresh nutrient agar slants and incubated at 37° C until growth was apparent. Following incubation, the fresh slants were placed in a refrigerator and stored until used.

Biochemical Identification of Cultures

Identification of the species was confirmed using procedures outlined by Lord (1951) and comparison with keys in Breed, et al. (1948).

Carbohydrate fermentation was determined using the following medium:

Potassium bicarbonate	0.8 g.
Ammonium acid phosphate (monobasic)	1.0 g.
Potassium acid phosphate (monobasic)	1.7 g.
Proteose peptone	20 g.
Distilled water	1000 g.

Carbohydrates included in the study consisted of one percent solutions of glucose, maltose, sucrose and lactose. Brom thymol blue was the indicator used to determine the reaction during incubation.

A tube test was employed to determine gelatin liquefaction. Prior to inoculations the tubes containing approximately four milliliters of nutrient gelatin were chilled. When the gelatin was sufficiently firm a loopful of inoculum was stabbed into the medium and incubated at 20° C for a period of two weeks before final observations were made.

Potassium nitrate broth was utilized in studying the reduction of nitrates. The following constituents were included in the preparation of the medium:

Proteose peptone	5 g.
Beef extract	3 g.
Potassium nitrate	1 g.
Distilled water	1000 ml.

After dissolving the ingredients, the solution was adjusted to pH 7.0 and tubed in five milliliter amounts prior to sterilization.

In testing for nitrites two reagents were employed as designated by Lord (1951). The two reagents with their ingredients are listed below and were mixed in the order as listed.

No. 1

Distilled water	714 ml.
Glacial acetic acid	286 ml.
Sulfanilic acid	8 g.

No. 2

Distilled water	714 ml.
Glacial acetic acid	286 ml.
Dimethyl alpha naphthylamine	6 g.

To each tube containing the potassium nitrate culture medium, 0.5 ml. of each reagent was added. Production of a red color was a positive test for the reduction of nitrates to nitrites.

Production of indole was ascertained employing a medium described by Lord (1951) containing the following components:

Tryptone	10 g.
Beef extract	3 g.
Sodium chloride	5 g.
Distilled water	1000 ml.

This medium was also adjusted to neutrality before sterilization.

The Goré test employed for detecting the presence of indole used the following reagents:

Ehrlich's Reagent #1

95% ethyl alcohol	95 ml.
Para dimethyl amino benzaldehyde	1 g.
Concentrated hydrochloric acid	20 ml.

Ehrlich's Reagent #2

Potassium persulfate	5 g.
Distilled water	100 ml.

Goré's method as described by Lord (1951) proceeds as follows: The cotton stopper from the culture medium is removed, moistened on the bottom with Ehrlich's Reagent #1 and an equal volume of Ehrlich's Reagent #2. After replacing the stopper, it is pushed down into the tube within one inch of the medium. The tube is then placed in a boiling water bath for approximately ten minutes. Formation of a red color on the stopper indicates a positive test for indole.

The medium employed to study the production of hydrogen sulfide consisted of the following ingredients:

Proteose peptone	20 g.
Sodium phosphate (Dibasic)	2 g.
Agar	1 g.
Dextrose	1 g.
Distilled water	1000 ml.

The constituents were dissolved and the pH adjusted to 7.6, after which 10 ml. of a 1.5 percent solution of ferric ammonium citrate were added. About 5 ml. amounts were dispensed in tubes and sterilized by autoclaving. The medium is solidified while the tube is standing upright. Specimens of the culture were stabbed into the medium and incubated at 37° C for one week. The appearance of a black color throughout the medium was a positive test for hydrogen sulfide.

Reactions in litmus milk were also observed as a means of identification. This medium was prepared with the ingredients as follows:

Dry skim milk	100 g.
Powdered litmus	5 g.
Distilled water	1000 ml.

The constituents were dissolved, dispensed in 5.0 ml. amounts in tubes and sterilized by autoclaving.

Observations extended over a period of one week at which time the final reactions were noted and recorded.

Motility was determined by hanging drop preparations.

Pigment Production

Since the production of pyocyanin is typical of Pseudomonas aeruginosa, this character was also employed as a means of identification. The following medium proposed by Gessard (1890) was reported to be optimum for maximum pyocyanin production by Burton, Eagles and Campbell (1947). The medium has the following composition:

Glycerol	50 ml.
Proteose peptone	20 g.
Magnesium sulfate	20 g.
Potassium phosphate (dibasic)	0.4 g.
Ferrous sulfate	0.1 g.
Distilled water	1000 ml.

The medium was dispensed in 100 ml. amounts in 250 ml. Erlenmeyer flasks and autoclaved. According to Harris (1950) pyocyanin is formed in the late stages of growth, thus the flasks were allowed to incubate for one week before extraction.

Pyocyanin was extracted by the following method. An equal volume of chloroform was added to the medium and the resulting solution was placed in a separatory funnel. When the emulsion due to shaking broke, the chloroform fraction was withdrawn. To this fraction an equal volume of 0.01 Normal hydrochloric acid was added and again thoroughly mixed in a separatory funnel. The pink or red hydrochloric acid fraction was separated and the chloroform fraction was discarded. Presence of the characteristic blue color after neutralization with dilute alkali was presumed sufficient evidence of pyocyanin.

Antigen Preparations

Immunizing Antigen. The antigen prepared for immunizing purposes was one containing *Pseudomonas aeruginosa*, N.R.R.L. strain B-23. Actual preparation followed closely that outlined by Wadsworth (1939).

A saline suspension of organisms from the selected culture was streaked on a nutrient agar plate and incubated for 24 hours at 37° C. From that plate a typical smooth, isolated colony was selected and stained by the Gram's method in order to detect any obvious contamination. If the cellular morphology appeared uniform throughout the slide, nutrient agar slants were inoculated with organisms from the same colony. After 24 hours' incubation at 37° C, the organisms were removed by the addition of two or three milliliters of sterile physiological saline prepared with the following constituents:

Sodium chloride	0.85 g.
Distilled water	100 ml.

A suitable suspension of the organisms was obtained by carefully scraping the surface of the agar slant with a sterile loop. This suspension was transferred aseptically to a previously sterilized Blake bottle which contained

100 ml. nutrient agar which had been allowed to solidify on its side, thereby creating a larger surface for bacterial growth. By careful handling, the entire agar surface was exposed to the suspension of cells. Following inoculation, the bottles were incubated for 24 hours at 37° C.

The following day the surface growth was observed for any apparent contamination. A specimen was also removed and stained by Gram's method. If the appearance in either case was unsatisfactory the bottles were discarded and the process was repeated.

The procedure for harvesting the growth consisted of adding ten milliliters of physiological saline to which had been added 0.5 percent phenol. The cells were separated from the culture medium by carefully rotating the bottles. A sterile pipette was used in transferring the suspension of cells from the bottles to sterile, screwcap tubes. In these sterile tubes the cells were sedimented with a Servall angle head centrifuge and washed three times with the phenolized physiological saline.

After the final wash, a volume of 95 percent ethyl alcohol was added to give a final concentration of 30 percent absolute alcohol. An equation suggested by Wadsworth (1939) facilitated the calculations with each antigen. The equation proposed is as follows:

Volume of 95 percent alcohol = Volume of suspension X 0.54.[1]

The alcohol-treated suspension was allowed to stand overnight in order to inactivate flagellar antigens and streaked on a nutrient agar plate the following morning as a final test for sterility.

Antigen standardization was achieved employing the Coleman Model II Universal Spectrophotometer set at a wave length of 500 $m\mu$. A standard curve

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1 0.54 is a constant derived from the formula 95 Y = 33 1/3 (Volume suspension + Y), in which Y = the volume of 95 percent alcohol required.

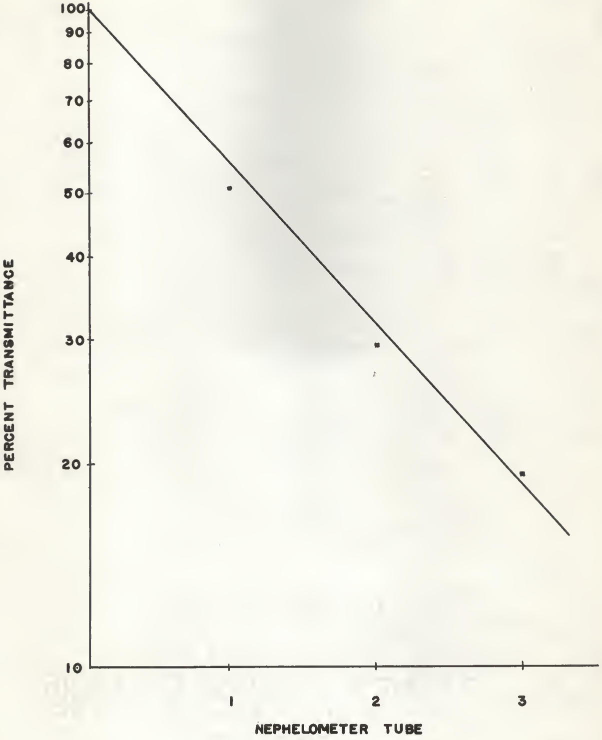

FIG. I: GRAPH OF READINGS ON NEPHELOMETER TUBE VS. PERCENT LIGHT TRANSMITTANCE was plotted utilizing the standard nephelometer tube series. (Fig. 1) For purposes of immunization the antigen was standardized at a concentration ten times that of nephelometer tube #3 and stored in rubber-capped, vaccine bottles.

Agglutinating Antigen. Agglutinating antigens were prepared for all organisms listed in Table 1. The procedure used in the preparation was similar to that utilized in preparing immunizing antigen except that the antigens were stored in sterile, screw-cap bottles to facilitate the removal of adequate amounts needed in the tests.

Antigens employed for agglutination tests were stored in three different concentrations. First, a portion of the original suspension was held. The second concentration was standardized to the turbidity of nephelometer tube #4 and employed in adsorption procedures. The third concentration separately stored was standardized to nephelometer tube #1 and was utilized in that concentration for agglutination test procedures.

Preparation of Immune Serum

A goat was selected as the animal to be immunized, the reason being that large amounts of blood were needed in order to extract enough serum for the study.

Subcutaneous injections were made on alternate days over a period of four weeks beginning with a small volume and increasing the dosage until reaching a maximum of five milliliters. After 31 days injections were stopped and the animal was allowed to rest for 10 days in order to obtain a maximum titer. The animal was then bled aseptically by way of the jugular vein, and the blood was defibrinated with glass beads in the bleeding flask. Table 2 shows the immunization chart according to days and dosage.

Table 2. Protocol for immunization of goat with Pseudomonas aeruginosa B-23 antigen.

Day	Amount injected (ml)	Remarks
0	0	1st bleeding (control serum)
1	1	
3	1	
5	1	
7	1	
9	1	
11	2	
13	2	
15	3	
17	3	
19	3	
21	3	
31	5	
41	0	2nd bleeding
45	3	
47	5	
49	5	
51	5	
53	5	
63	0	3rd bleeding
65	5	
67	5	
69	5	
71	5	
73	5	
75	5	
85	0	4th bleeding

Blood cells was separated from the immune serum by centrifugation using a Model 2 Universal International centrifuge at 2500 to 3000 R.P.M. for approximately 15 minutes. With a sterile pipette the serum was removed and placed in sterile, screw-cap bottles. In order to eliminate diluting of the serum by the addition of a preservative, the small bottles were placed in a freezer and quickly frozen at -10° C. This temperature was maintained until the serum was used.

Serum titers were determined by tube agglutination procedures according to the American Public Health Association (1950). Serial dilutions of antigen and serum were made and incubated for 48 hours at 37° C. Degrees of agglutination were observed in each tube after incubation. Table 2 provides the standards by which the results were tabulated.

Table 3. Agglutination standards.

Degree of Agglutination	Appearance	
	Supernatant	Precipitate
+ + + +	clear	unevenly clumped
+ + +	turbid	unevenly clumped
+ +	turbid	restricted clumping
+	turbid	clumping barely visible
-	turbid	button formed due to settling

Adsorption Procedures

Agglutination adsorption tests were performed according to the procedure outlined by Stafseth, et al. (1954). Equal amounts of serum and antigen (the concentration equal to Nephelometer Tube #4) were placed in large sterile tubes (16 x 150 mm) and incubated from four to five hours at 37° C. Following incubation, the tubes were placed at 5° - 8° C for a period of 12 hours. The mixture was then centrifuged in a Universal International Centrifuge at 3000 R.P.M. for a period of 20 to 30 minutes. Carefully avoiding disturbance of the sediment, the supernatant was removed aseptically and placed in screw-cap tubes for freezing. Prior to freezing, however, a sample was removed and tested for complete adsorption with the antigen used in the adsorption process. Even if agglutination occurred in dilutions as low as 1-4 or 1-8, the adsorption procedure was repeated until no agglutination occurred in these dilutions.

When complete adsorption was achieved, the sera were subjected to agglutination tests with each antigen, including the adsorbant antigen as a final check on complete removal of specific antibodies. The titers were determined by agglutination test procedures previously outlined.

RESULTS

Biochemical Characteristics of Cultures Used

The results of the biochemical studies performed on the cultures used in this work are given in Table 4.

Table 4. Biochemical characteristics of species studied.

Strain	Species	Glucose	Sucrose	Lactose	Maltose	Gelatine Liquefaction	Indole	Nitrates Reduced	Hydrogen Sulfate	Litmus Milk	Pyocyanin
A	Pseudomonas aeruginosa	A*	-	-	-	+	-	+	-	p,r	+
B	Ps. aeruginosa	A	A	-	-	+	-	+	-	p,r	+
C	Ps. aeruginosa	A	-	-	-	+	-	+	-	p,r	+
D	Ps. aeruginosa	A	-	-	-	+	-	+	-	a	+
E	Ps. aeruginosa	A	-	-	-	+	-	+	-	p,r	+
F	Ps. chlororaphis	A	a	a	a	+	-	+	-	c	-
G	Ps. fluorescens	A	-	-	-	-	-	+	-	a	-
H	Ps. indoloxidans	A	a	a	a	-	-	+	-	n	-
I	Ps. iodinium	A	-	-	-	+	-	+	-	n	-
J	Ps. reptilovorous	A	-	-	-	+	-	-	-	a	-
K	Species X	A	A	-	-	-	-	+	-	n	-

* A, acid; a, alkaline; p, peptonization; r, reduction; n, no reaction; c, coagulation.

All strains but Pseudomonas iodinium proved to be motile in hanging drop preparations.

In the special medium of Gessard (1890) all strains of Pseudomonas aeruginosa produced pyocyanin; the other cultures did not produce it. The relative amounts produced by the five species investigated were not studied, but in all cases the presence of the blue color followed neutralization of the acid fraction with dilute alkali.

Titer of Immune Serum

As previously stated only strain A of Pseudomonas aeruginosa (N.R.R.L. #B-23) was used in the preparation of immune serum. Table 2 presents the immunization protocol used.

The original bleeding was to obtain "normal" or control serum. After the first series of injections the second bleeding was performed. At this point the antibody titer of the serum was determined using the homologous antigen. The titer was not sufficiently high, and it became necessary to continue immunization for an additional period of time as designated. When this second series of injections was completed, the third bleeding was performed. Again the antibody titer was determined and proved to be 1-1280. It was felt that this value was still too low, so immunizations were continued through a third series of injections. The fourth bleeding was performed. It was found that the titer had not risen over the previous cycle, therefore, immunizations were stopped. The maximum titer obtained from the homologous serum and antigen was 1-1280.

Cross-Agglutination Titers

Antigens prepared with the organisms investigated were subjected to agglutination tests with (1) the control serum and (2) the immune serum obtained from the fourth bleeding. The results of these agglutination tests are recorded in Table 5. The strains are listed by letter following the example of Table 1.

Table 5. Rise in titer following immunization.

Strain :	Antigen :	Control Serum :	Positive B-23 Serum
A	*Pseudomonas aeruginosa* (B-23)	20*	1280
B	*Ps. aeruginosa*	40	640
C	*Ps. aeruginosa*	20	1280
D	*Ps. aeruginosa*	20	1280
E	*Ps. aeruginosa*	80	1280
F	*Ps. chlororaphis*	32	128
G	*Ps. fluorescens*	4	16
H	*Ps. indoloxidans*	64	128
I	*Ps. iodinium*	4	16
J	*Ps. reptilovorous*	64	64
K	*Species X*	32	64

* Numbers express the reciprocal of the highest dilution at which agglutination occurred.

Agglutinin Titer Following Adsorption

In preparation for adsorption procedures, a trial-and-error method was utilized to determine complete adsorption. It was noticed that Strain A (*Pseudomonas aeruginosa* N.R.R.L. B-23) antigen standardized to Nephelometer Tube #4 added to equal amounts of immune serum failed to give complete adsorption, i.e. agglutination occurred in the first dilution following adsorption. Additional antigen was added for further adsorption until no agglutination appeared in the first dilution of adsorbed serum plus antigen. After a number of attempts, it was found that upon the addition of three parts antigen to one part positive serum, post-adsorption agglutination failed to occur. In order to begin all agglutination tests with sera diluted to the same proportions, adsorptions with the other antigens were similarly performed.

Table 6 shows the results of the titers obtained following adsorption with each antigen. The results in Table 6 are the averages of two different experiments.

Table 6. Sera titers following adsorptions

Post-adsorption Antigen		B-23 Immune serum adsorbed with strain										
Organism	Strain	A	B	C	D	E	F	G	H	I	J	K
Pseudomonas aeruginosa	A	---	---	64*	80	40	160	320	640	640	320	160
Ps. aeruginosa	B	128	---	256	80	---	128	128	256	256	320	320
Ps. aeruginosa	C	80	---	---	80	---	80	40	80	80	320	40
Ps. aeruginosa	D	160	160	640	---	160	320	1280	1280	1280	1280	640
Ps. aeruginosa	E	160	640	320	320	---	640	640	1280	1280	640	320
Ps. chlororaphis	F	16	32	64	32	8	---	---	64	64	32	64
Ps. fluorescens	G	8	8	8	---	8	---	---	16	16	16	16
Ps. indoloxidans	H	32	128	64	32	32	16	16	---	32	128	32
Ps. iodinium	I	16	8	8	8	16	8	16	---	---	16	16
Ps. reptilovora	J	16	16	32	16	8	8	64	64	64	---	8
Species X	K	---	---	8	8	32	16	16	16	16	16	---

* Numbers express the reciprocal of the highest dilution at which agglutination occurred.

DISCUSSION

Results of the biochemical analysis verified the identities of each organism. Breed et al. (1948), state that Pseudomonas aeruginosa does not ferment glucose. On the other hand the positive results obtained in this study pertaining to glucose fermentation have been observed by others previously. Munoz, et al. (1949), showed all strains of Pseudomonas aeruginosa they studied were capable of producing acid from glucose. Otherwise, the results herein reported closely parallel the characteristics reported by Breed, et al. (1948).

Because reliable results from flagella stains were not obtained, that technique was not used. Peritrichous flagellation might be determined by staining, but it is the writer's opinion that if a polar flagellum is seen, it would be difficult to determine if others were present and destroyed during the staining process or if the polar flagellum seen is in actuality the true existant characteristic of the species being examined. This one characteristic, usually considered paramount in recognition of members of the genus Pseudomonas, proves to be the largest obstacle in correctly determining members of that group.

A search of the literature failed to reveal any set of reactions useful in identifying this genus other than the presence of polar flagella, therefore, the author chose to study the antigens exhibited in common with the type species, Pseudomonas aeruginosa. One of the most distinctive characteristics of Pseudomonas aeruginosa is the production of the bluish-green, water-soluble pigment. All strains of this organism were capable of producing pyocyanin, but it evidently can not be utilized as a sole criterion in identification. Munoz, et al. (1949), reported one strain of Pseudomonas aeruginosa which was incapable of producing the characteristic pigment. They favored the idea that since the organism was also non-motile, it perhaps was a variant or a mislabelled culture.

However, Harris (1950) studying pigment production in comparison with growth stages, of 30 strains of Pseudomonas stated that 10 strains produced both the fluorescent pigment and pyocyanin, three strains produced only pyocyanin and 17 strains produced only the fluorescent pigment. Elrod and Braun (1942) investigated a plant pathogen that was capable of producing pyocyanin. Previously Clara (1930) identified it as Phytomonas polycolor. Because this organism had identical characteristics with Pseudomonas aeruginosa, Elrod & Braun (1942) identified it as such. It appears to this author that pyocyanin production is a variable characteristic controlled probably by genetics with environmental influence.

A complete comparison of the characteristics of other strains of Pseudomonas species was not undertaken, but a new classification proposed by Haynes (1953) reveals that Pseudomonas chlororaphis strains are capable of producing chlororaphine crystals in culture medium. The strain utilized in these experiments was not noticed to produce the characteristic crystals.

Species X which was obtained from Fina (1955)[1] who encountered it in methane fermentation studies. Due to the pigment produced, it was suspected of being a member of the genus Pseudomonas. From the results of the biochemical tests and the production of a fluorescent pigment, one might believe the organism to be Pseudomonas fluorescens. However, the tests indicated a different antigenic picture than that exhibited by Pseudomonas fluorescens.

At first inspection the maximum antibody titer obtained with the immunizing antigen seemed rather unsatisfactory for work of this kind. Sherwood (1926) produced immune sera with titers up to 1-5000 by injecting alternately live and dead Pseudomonas cells. This, he stated was quite low. In comparison with

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[1] Personal communication.

other species of bacteria it may be thought of as such. However, other investigations have revealed titers in more complete studies which were somewhat lower. For this investigation the titers obtained were sufficiently high to cancel any affects f non-specific antigens. Perhaps it might have been more satisfactory to use live organisms, rather than the killed suspensions for immunizing purposes, or it could possibly have been that the animal's response to this particular organism was insufficient to produce a higher titer. It is known that an animal's response to immunization varies, and the literature did not reveal the use of goats previously for antibody production against Pseudomonas aeruginosa. Rabbits seemed to be the animal of choice in all previous work reported.

The results of agglutination tests after adsorption follow the thesis that the antigenic heterogenicity of the genus Pseudomonas is characteristic. Aoki (1926), Kanzaki (1934 a,b), Elrod and Braun (1942), Mayr-Harting (1948), and Munoz, et al. (1949), have observed the same heterogenicity. Whether or not this is due to variants is unknown. The only precautions taken in obtaining a homogenous suspension was the inoculation and transferring of typically smooth colonies. Gaby (1946) investigated the possibility of such an occurrence, observing different biochemical characteristics as well as different flagellar antigen types. Mayr-Harting (1948), however, noticed that agglutination occurred at approximately the same titer regardless of the colonial type. Other investigators did not heed such characteristics and if variants were used the workers proceeded regardless.

Before studying Table 6 one must consider first the titer of the control serum and secondly the rise in titer following immunization as shown in Table 5. From a comparison of Tables 5 and 6, common antigens exhibited by the species are more easily surmised.

Based on the rise in titer, all Pseudomonas aeruginosa cultures appeared to have common antigens. Following adsorption with the different organisms, the results varied quite widely. The percentage drop from the immune titer, following adsorption for these Pseudomonas aeruginosa species ranged from 100 percent to 50 percent. The majority, however, was in the vicinity of 90 to 100 percent drop in titer. Table 7 shows the comparison of the five strains of Pseudomonas aeruginosa.

Table 7. Percent drop in original immune titer after adsorption.

Post-adsorption Antigen used	B-23 Immune serum adsorbed with strain A	B	C	D	E
Pseudomonas aeruginosa A	100	100	95.00	93.75	96.88
Ps. aeruginosa B	80.00	100	60.00	87.50	100
Ps. aeruginosa C	93.75	100	100	93.75	100
Ps. aeruginosa D	87.50	87.50	50.00	100	87.50
Ps. aeruginosa E	87.50	50.00	75.00	75.00	100

From Table 7 it can be readily seen that the five strains of organisms contain many of the same or identical antigens. The seemingly radical reaction of organisms D and E are unexplainable at this time. Sherwood (1926) however, noted similar results in his investigation, and because of the reactions of adsorption the results were not published.

For organisms other than Pseudomonas aeruginosa, the results proved equally erratic. Considering the antibody titer present in control sera, all titers were increased over the control, except those against Pseudomonas reptilovorous, by immunization with Pseudomonas aeruginosa B-23 antigen. In some cases the increase was only two-fold, but that substantiates the idea that one or more common antigens were present. However, as can be seen in Table 6 Pseudomonas reptilovorous seemed to have an affect upon the titers of the specific antigens after adsorption. Again the drop was only two-fold at maximum but at the high level titers that could raise some question. At low level titers the possibility of non-specific antigens entering into the reaction might account for such a phenomenon.

It was also noted that Pseudomonas chlororaphis and Pseudomonas fluorescens appeared to have similar antigens as shown by complete removal of antibodies by agglutination adsorptions between the two organisms. Adsorption with each of these antigens removed the antibodies against the other organism from B-23 antiserum. However, with further investigations on sera adsorbed by other antigens, results were variable. This phenomenon was also encountered in studying the results of the original titers. The control titer for Pseudomonas fluorescens was quite low, being 1-4, as compared to the 1-32 titer of Pseudomonas chlororaphis. If these particular organisms had an identical antigenic structure, one might expect the rise in titer following immunization to be the same. The results were to the contrary as shown in Table 5. In both cases the rise in titer was four-fold but the difference between the maximum titers obtained was quite high (1-128 for Pseudomonas chlororaphis and 1-16 for Pseudomonas fluorescens).

Ruzicka (1899) stated that Pseudomonas fluorescens, and Pseudomonas aeruginosa were merely extremes of the same organism. He continued in the investigation and supported his hypothesis by making the organisms assume the same characteristics. Biochemical and serological results presented in this paper fail to show any close resemblence between the two organisms. Again this may be due to variations exhibited by the organisms. Genetically the work of Ruzicka (1899) might readily be proven, since it is assumed by many that particular groups of organisms are considered to be variants of a single species. This relationship has been rather well substantiated with the genus Salmonella and to this author seems to be a logical proposal to explain the relationship between the species of the genus Pseudomonas as well as the genus Salmonella. At any rate work with the serology of Pseudomonas is not as advanced as that performed on the enteric organisms. Perhaps in the future such a similar classification will be revealed.

A system was devised to number the possible antigens present on each organism similar to the Salmonella classification scheme presented by Edwards and Ewing (1955). The scheme was set up at random and in no way does it relate to similar sets of data recorded by previous workers. It can not be used as a means of identification, however, due to the fact that no flagellar antigens were studied; thus it must be considered preliminary. The possible scheme is shown in Table 8. It must also be noted that various assumptions were made in determining the number of antigens and their distribution. For example, strain A was capable of adsorbing out practically all antibodies which are also assumed to be specific for strains B, C, D and E. Thus it was assumed that one or more common antigens were present. On the other hand strain A antigen was only capable of adsorbing out approximately 50 percent of the antibodies specific for strain G as shown in Table 7.

Table 8. Possible antigenic components of species investigated.

Strain	Organism	Somatic Antigens
A	Pseudomonas aeruginosa	I, II, III
B	Ps. aeruginosa	I, II, (III)
C	Ps. aeruginosa	I, II
D	Ps. aeruginosa	I, II, III, IV
E	Ps. aeruginosa	I, II, III, IV
F	Ps. chlororaphis	I, IV, VI
G	Ps. fluorescens	I, IV, VI
H	Ps. indoloxidans	I, VI
I	Ps. iodinium	II, VI, VII
X	Species X	I, VII

Pseudomonas reptilovorous was not included due to unexplainable results, as described previously in this thesis. No advance in titer of antibodies against Pseudomonas reptilovorous resulted due to immunizations with Pseudomonas aeruginosa B-23 antigen. However, by the process of adsorptions a drop in titer was noted.

During the course of experimentation, a brief comparison of methods was made in determining titers by agglutination procedures. This portion of the investigation was performed in order to reduce the time element involved in the procedure used. It was thought that microbial growth was occurring in the tubes during the 48 hour incubation period, thereby producing results which were falsely interpreted. A method not previously mentioned was utilized as a standard procedure by Simmons & Gentzkow (1944), and a modified method was used by Mora (1955). The method, depending on the organisms studied, suggested that the tubes be incubated at high temperatures (55° C) for two hours and then be placed at refrigerator temperature for 18 to 24 hours. Mora's (1955) method suggested incubation with shaking for 24 hours at 55° C and allowing to stand in the refrigerator overnight. Results were obtained with this latter procedure, but maximum titers were considerably different, and it was thought that since the material was adequately protected with alcohol and phenol against secondary microbial overgrowth, there was no need to change procedures. Microscopic examination confirmed the idea that mold growth had not resulted.

SUMMARY

The organisms representing the genus Pseudomonas used in this study consisted of the following species: Pseudomonas aeruginosa (5 strains), Pseudomonas chlororaphis, Pseudomonas fluorescens, Pseudomonas indoloxidans, Pseudomonas

iodinium, Pseudomonas reptilovorous and a suspected Pseudomonas species designated as Species X.

Two identified species, Pseudomonas indoloxidans and Pseudomonas iodinium were not described by Breed, et al. (1948), but a very brief notation was made concerning these organisms in the genus Bacterium. Pseudomonas iodinium proved to be non-motile as described and was placed along with Pseudomonas indoloxidans in the new classification scheme of Pseudomonas by Haynes (1953).

The results of biochemical tests followed closely those described by Breed et al. (1948). The five strains of Pseudomonas aeruginosa were capable of utilizing glucose which Breed et al. (1948), describes as variable. Many other workers, however, designate the acid reaction as characteristic.

Species X, an organism associated with methane fermentation, was suspected of being a member of the genus Pseudomonas and was very similar, biochemically, to the identified strain of Pseudomonas fluorescens. In addition pigment production by the two organisms was very similar.

Pyocyanin, the green, water-soluble pigment, was produced by all strains of Pseudomonas aeruginosa and thus also confirmed identification of this species. This particular characteristic is generally considered to be variable for strains of this species, but no variation was noted in this study. Extraction procedures for identification of pyocyanin were employed for purposes of confirmation.

Pseudomonas aeruginosa B-23 was utilized as an immunizing antigen in order to investigate similar somatic antigenic structures exhibited by other species representing the genus Pseudomonas in this study. Gradually increasing doses of the antigen, standardized at ten times Nephelometer tube number three, were injected subcutaneously on alternate days into a goat in preparing immune serum.

Following immunization, the animal was bled and the serum was extracted. Agglutination titers against antigens prepared with the other organisms as well as the immunizing antigen were determined by standard tube agglutination test procedures. The titer obtained for the homologous antigen was 1-1280 which was the maximum for this study. Titers obtained by subjecting heterologous antigens to agglutination tests with Pseudomonas aeruginosa B-23 serum varied from 1-16 to 1-1280. A rise in titer for antibodies against all species but Pseudomonas reptilovorous was experienced in a goat immunized with Pseudomonas aeruginosa B-23. It was also noted that the five strains of Pseudomonas aeruginosa, isolated from different sources, proved to have very similar antigenic patterns as evidenced by similar magnitude of maximum antibody titers obtained by immunization.

Agglutination adsorption procedures were carried out to determine the feasibility of a serological classification. The results, although erratic at points, confirmed previous reports that antigen components of Pseudomonas species are not uniform. A possible antigenic map is included.

Although injections of Pseudomonas aeruginosa B-23 antigen failed to give a rise in titer against Pseudomonas reptilovorous it had a variety of effects following adsorptions. Utilizing Pseudomonas reptilovorous as the adsorptive antigen, a drop in serum titer was experienced when tested against the other antigens including the immunizing antigen.

The other antigens proved to be equally erratic following adsorptions. Similarities were noted between strains of Pseudomonas aeruginosa, but none proved to be antigenically identical.

Although Pseudomonas fluorescens and Species X had biochemical similarities, the two organisms proved to be quite different antigenically.

Serologically there seemed to be no relationship between the two organisms.

Cross-agglutination following adsorption with Pseudomonas fluorescens and Pseudomonas chlororaphis resulted in identical antigenic pictures. Each antigen was capable of adsorbing out all antibodies for the other. On further study, reactions with other antigens were considerably different. Considering the rise in titer, due to immunization, the antigenic structure of these two organisms appeared unrelated.

Until further studies are performed, a definite classification scheme can not be organized. Any proposed schema must also include flagellar antigens. However, it can be said that since common antigens between species do occur, the possibilities of such a scheme are impressive.

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A STUDY OF THE SOMATIC ANTIGENS AND BIOCHEMICAL PROPERTIES OF SELECTED SPECIES OF THE GENUS PSEUDOMONAS

by

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Pseudomonas aeruginosa is perhaps the best known organism within the genus Pseudomonas. This is due to the fact that this organism is one of the few animal pathogens included within the genus and is pathogenic to man.

Since Pseudomonas aeruginosa is the type species according to Breed et al. (1948), this investigation was undertaken in order to determine the antigens exhibited by other species in the genus in common with the type species, as well as the exhibition of homologous or heterologus antigens by different strains of the same species.

At the same time it was felt that by the process of agglutination-adsorption techniques, an identification scheme similar to that utilized in identifying members of the genus Salmonella could be developed for the genus Pseudomonas.

The literature reveals much disagreement on the classification of the genus Pseudomonas and the differentiation of species within that genus.

Several investigators working with Pseudomonas aeruginosa and Pseudomonas fluorescens have found that heterogenic antigens appeared in various strains. On the other hand one worker states that the former organism was antigenically homogenous.

Very few workers have conducted serological studies on other organisms contained in the genus Pseudomonas. A more complete work has been performed through serological experiments on Pseudomonas aeruginosa and Pseudomonas fluorescens in addition to a number of other organisms. However, the results were quite similar, proving that heterogenic antigens were exhibited.

A similar confusion is noted in relation to the biochemical properties of organisms in the genus Pseudomonas. Variations among species have been noted, but most investigators agree that Pseudomonas aeruginosa and Pseudomonas fluorescens ferment glucose.

The organisms representing the genus Pseudomonas used in this study consisted of the following species: Pseudomonas aeruginosa (5 strains), Pseudomonas chlororaphis, Pseudomonas fluorescens, Pseudomonas indoloxidans, Pseudomonas iodinium, Pseudomonas reptilovorous and a suspected Pseudomonas species designated as Species X.

The results of biochemical tests followed closely those described by Breed et al. The five strains of Pseudomonas aeruginosa were capable of utilizing glucose which the Bergey's Manual describes as variable. Many other workers, however, designate the acid reaction as characteristic.

Two identified species, Pseudomonas indoloxidans and Pseudomonas iodinium were not described in the Bergey's Manual, but a very brief notation was made concerning these organisms in the genus Bacterium. Pseudomonas iodinium proved to be non-motile as described and was placed along with Pseudomonas indoloxidans in the new classification scheme of Pseudomonas by Haynes.

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Until further studies are performed, a definite classification scheme can not be organized. Any proposed scheme must also include flagellar antigens. However, it can be said that since common antigens between species do occur, the possibilities of such a scheme are impressive.