AN ATTEMPT AT GROUPING OF THE GENUS *PSEUDOMONAS*

HIROSHI IIZUKA and KAZUO KOMAGATA¹

Institute of Applied Microbiology, University of Tokyo, Tokyo

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The generic name of *Pseudomonas* was given by MIGULA (1) in 1894 to the group of bacteria which were GRAM negative rods possessing polar flagella. *Ps. aeruginosa* was chosen as the type species. The beautiful greenish yellow fluorescent pigments are produced by some species. Pseudomonads are widely distributed in nature, and are isolated from many sources, such as river water (2,3), sewage, beer (4), maple sap (5), dairy products (6), cereals (7), petroleum (8), natural gas brine (8), etc.

The generic concept of *Pseudomonas* has been mainly based upon cell morphology, flagellation, and GRAM stain, and it includes the largest number of species in *Pseudomonadaceae*. MIGULA (9) divided this genus into five groups based upon the characteristics of pigmentation. In both BERGEY'S (10) and KRASSILNIKOV'S manuals (11), the production of pigments is used for the differential key of this genus. However, the pigmentation was chosen merely for the sake of convenience, and its taxonomical value was not critically evaluated. Although the significance of some taxonomical characteristics of this genus has been reported by several investigators (12-16), the number of strains and species studied was too limited to cover the whole generic group. In the present paper, the authors describe the results of comparative and systematic studies on the physiological, biochemical and ecological features of *Pseudomonas* strains, which led to the conclusion that taxonomically the genus should be divided into three groups on the basis of the characteristics of pigmentation. Details on the systematic of this genus will be reported elsewhere.

MATERIALS AND METHODS

Microorganisms. Pseudomonads tested were both those freshly isolated from cereals, petroleum, natural gas brine, etc. by the authors, and those obtained from the type culture collections of several institutions including the Institute of Applied Microbiology, University of Tokyo. Those comprised 202 strains belonging to 28 species. In Table 1 are shown the number of strains and species tested and the sources of their isolation. Marine and plant pathogenic pseudomonads were excluded from this study.

1) Present address: Central Research Laboratory, Ajinomoto Co., Inc., 2964, Suzuki-cho, Kawasaki, Japan.

Table 1. Species of *Pseudomonas* and sources of isolation

	Cereals	Brine of petroleum and natural gas	Assimilators of hydrocarbons	Assimilators of aromatic compounds	Others	Strains preserved at type culture collections	No. of strains
Fluorescent group							
<i>Ps. aeruginosa</i>					11	17	28
<i>Ps. azotoformans</i>	1	7			12		20
<i>Ps. chlororaphis</i>						2	2
<i>Ps. pavonacea</i>	6						6
<i>Ps. schuylkilliensis</i>	7	4		1	9		21
<i>Ps. aureofaciens</i>						1	1
<i>Ps. cinnamona</i>	3						3
<i>Ps. straminea</i>	1						1
<i>Ps. nitroreducens</i>		5	1				6
<i>Ps. ovalis</i>	3	1		6	16	1	27
<i>Ps. rugosa</i>	3						3
<i>Ps. fulva</i>	6						6
Achromogenic group	30	17	1	7	48	21	124
<i>Ps. putrefaciens</i>		2			1	1	4
<i>Ps. alcaligenes</i>			5				5
<i>Ps. stutzeri</i>		5			1		6
<i>Ps. rathonis</i>		6					6
<i>Ps. denitrificans</i>			4				4
<i>Ps. desmolytica</i>	1		6	3	1		11
<i>Ps. dacunhae</i>				3			3
<i>Ps. riboflavina</i>						1	1
<i>Ps. iners</i>		3					3
<i>Ps. diminuta</i>						1	1
Chromogenic group	1	16	15	6	3	3	44
<i>Ps. perlurida</i>	7						7
<i>Ps. trifolii</i>	3					1	4
<i>Ps. cerealis</i>	4						4
<i>Ps. melanogenum</i>	4						4
<i>Ps. lacunogenes</i>	13						13
<i>Ps. ochracea</i>	2						3
	33					1	34
Total	64	33	16	13	51	25	202

Experimental methods used were mainly those described in the "Manual of Microbiological Methods" (17). All the tests except liquefaction of gelatin were carried out at 30°.

Morphological characteristics. Cell form, motility, size, and GRAM stain were observed on the cells cultured on nutrient agar slants for 18 to 24 hr. The capsule formation was examined after 4 to 5 days' incubation. The irregular form was observed after 7 to 10 days' incubation. The flagellation was confirmed by the staining method of NISHIZAWA and SUGAWARA (18) and by electron microscopy.

Cultural characteristics. The growth, surface structure, pigmentation, etc. of nutrient agar and glutamate agar plate cultures were observed after 5 to 7 days' incubation. On nutrient agar and glutamate agar slants, the same characteristics were noted after 3 to 5 days' incubation. Formation of pellicle, pigments, turbidity, etc. were observed in liquid cultures. Cultures on nutrient gelatin were incubated at 20° and 30° for a month. For the milk test B.C.P was used as pH indicator.

Biochemical and physiological characteristics.

a) Production of pigments

Water-soluble pigments were produced in the cultures in glutamate broth and the other synthetic media but not in those in nutrient broth. The following medium, composed of 10 g glycerol, 2.5 g ammonium lactate, 5.0 g sodium glutamate, 1.0 g K_2HPO_4 , 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g KCl, and 1,000 ml of dist. water (pH 7.2), was used for the detection of formation of watersoluble pigments. The production of non-water-soluble pigments was tested on slant or plate cultures of nutrient agar and glutamate agar.

b) Reduction of nitrate

Nutrient broth with 0.2% of potassium nitrate, and succinate-nitrate broth (19) were used for the tests of reduction of nitrate. Nitrite was detected by applying α -naphthylamine and sulfanilic acid after 1, 3, and 5 days' incubation.

c) Nitrate respiration

Anaerobic growth occurring with the performance of nitrate respiration was examined by the following procedure. The cells grown on nutrient agar slant were inoculated in a nutrient broth containing 1% of potassium nitrate, and the cultures was immediately sealed with sterilized liquid paraffine. For controls, unsealed cultures in nutrient broth with or without potassium nitrate and sealed cultures in nutrient broth without potassium nitrate were also run. After 1 to 2 days' incubation, growth, turbidity, and bubbling of gas were observed.

d) Production of indole

Indole was detected by using KOVAC's reagent after 1, 3, and 5 days' incubation in 1% tryptone (Difco) water.

e) Production of hydrogen sulfide

Nutrient broth containing 0.05% cysteine was used for the test of production of hydrogen sulfide. Hydrogen sulfide was detected by using the lead acetate paper strip.

f) Hydrolysis of starch

Streak cultures on nutrient agar plates containing 0.2% of soluble starch were treated with iodine solution after 1, 3, and 5 days' incubation.

g) Catalase

Young cells grown on nutrient agar slants for 18 to 24 hr were dipped in 3% aqueous solution of hydrogen peroxide, and production of bubbles was examined.

h) Relation to temperature

Growth at temperatures of 5°, 10°, 25°, 30°, 37°, and 42° were compared using nutrient broth as culture medium.

RESULTS

The experimental results are summarized in Tables 2 and 3.

Morphological characteristics

The strains tested were all motile except two and all GRAM negative. In no case was the staining positive, variable or irregular. Cells were invariably straight rods and not curved. They occurred singly or in pairs, but not in long chains. In older cultures (about 7 to 10 days' incubation) there appeared cells bulged at terminal, and cells over 40 microns in length on rare occasions. All the motile strains showed polar flagellation, with no indication of the generation of lateral flagella. The number of flagella varied with species and strains. Two non-motile varieties found in the group produced water-soluble greenish yellow fluorescent pigments. All the strains tested were non-acid fast, and almost all of them formed no capsule. No particular relation was recognized between the morphological characteristics and the production of pigments.

Cultural characteristics

The cultural characteristics of *Pseudomonas* are not so distinct as those of other bacteria or of yeasts and molds. Colonies on nutrient agar are mostly circular, smooth or rough with textures that are in most cases butyrous and rarely membranous. Pigmentation and surface structure of colonies are more distinctive in the colonies on glutamate agar than in those on nutrient agar. The slant cultures showed characteristics similar to those of agar colonies. On glutamate agar colonies of *Ps. lacunogenes* and *Ps. stutzeri* had markedly wrinkled surface and colonies of *Ps. perlurida* and *Ps. trifolii* showed metallic sheen. These characteristics of colonies appeared less marked when the organisms were grown on nutrient agar. Some strains formed pellicle but some others not. About half of the species tested liquefied gelatin. *Ps. straminea*, *Ps. lacunogenes*, and

Table 2. Cultural characteristics

	No. of strains	Color of nutrient agar colonies	Water-soluble pigments	Nutrient gelatin stab	B.C.P. milk
Fluorescent group					
<i>Ps. aeruginosa</i>	28	brown	green to brown	liquefied	alkaline, peptonized.
<i>Ps. azotoformans</i>	20	pale brown	greenish yellow	liquefied	alkaline, peptonized.
<i>Ps. chlororaphis</i>	2	yellowish brown	yellow	liquefied	alkaline, peptonized.
<i>Ps. pavonacea</i>	6	pale brown	greenish yellow	liquefied	alkaline, peptonized.
<i>Ps. schuylkilliensis</i>	21	pale brown	greenish yellow	liquefied	alkaline, peptonized.
<i>Ps. aureofaciens</i>	1	orange brown	orange yellow	liquefied	alkaline, peptonized.
<i>Ps. cinnamomea</i>	3	orange yellow	dark orange	liquefied	alkaline, peptonized.
<i>Ps. straminea</i>	1	yellow	greenish yellow	liquefied	alkaline, peptonized.
<i>Ps. nitroreducens</i>	6	pale brown	greenish yellow	not liquefied	alkaline
<i>Ps. ovalis</i>	27	pale brown	greenish yellow	not liquefied	alkaline
<i>Ps. rugosa</i>	3	pale brown	greenish yellow	not liquefied	acid
<i>Ps. fulva</i>	6	yellow	greenish yellow	not liquefied	alkaline
Achromogenic group					
<i>Ps. putrefaciens</i>	4	reddish brown	not produced	liquefied	alkaline, peptonized.
<i>Ps. alcaligenes</i>	5	yellowish brown	not produced	liquefied	alkaline, peptonized.
<i>Ps. stutzeri</i>	6	pale brown	not produced	not liquefied	alkaline
<i>Ps. rathonis</i>	6	pale brown	not produced	not liquefied	alkaline
<i>Ps. denitrificans</i>	4	pale brown	not produced	not liquefied	alkaline
<i>Ps. desmolytica</i>	11	pale brown	not produced	not liquefied	alkaline
<i>Ps. dacunhae</i>	3	pale brown	not produced	not liquefied	alkaline
<i>Ps. riboflavina</i>	1	brownish gray	not produced	not liquefied	unchanged
<i>Ps. iners</i>	3	pale brown	not produced	not liquefied	unchanged
<i>Ps. diminuta</i>	1	brownish gray	not produced	not liquefied	unchanged
Chromogenic group					
<i>Ps. pertwida</i>	7	yellow	not produced	liquefied	acid, coagulated.
<i>Ps. trifolii</i>	4	yellow	not produced	liquefied	acid, coagulated.
<i>Ps. cerealis</i>	4	yellow	not produced	liquefied	alkaline, peptonized.
<i>Ps. melanogenum</i>	4	pale yellow	not produced	liquefied	alkaline, peptonized.
<i>Ps. lacunogenes</i>	13	yellow	not produced	liquefied	alkaline, peptonized.
<i>Ps. ochracea</i>	2	pale yellow	not produced	liquefied	alkaline

Table 3. Physiological and biochemical characteristics

	No. of strains	Reduction of nitrate	Nitrate respiration	H ₂ S	Indole	Hydrolysis of starch	Growth at			Catalase
							30°	37°	42°	
Fluorescent group										
<i>Ps. aeruginosa</i>	28	+	+	-	-	-	+	+	+	+
<i>Ps. azotofromans</i>	20	+	+	-	-	-	+	-	-	+
<i>Ps. chlororaphis</i>	2	+	+	-	-	-	+	±	-	+
<i>Ps. pavonacea</i>	6	-	-	-	-	-	+	+	-	+
<i>Ps. schuylkilliensis</i>	21	-	-	-	-	-	+	±	-	+
<i>Ps. aureofaciens</i>	1	+	-	-	-	-	+	±	-	+
<i>Ps. cinnamona</i>	3	-	-	+	-	-	+	±	-	+
<i>Ps. straminea</i>	1	-	-	+	-	-	+	+	-	+
<i>Ps. nitroreducens</i>	6	+	+	-	-	-	+	±	-	+
<i>Ps. ovalis</i>	27	-	-	-	-	-	+	+	-	+
<i>Ps. rugosa</i>	3	-	-	-	-	-	+	-	-	+
<i>Ps. fulva</i>	6	-	-	±	-	-	+	+	-	+
Achromogenic group										
<i>Ps. putrefaciens</i>	4	+	-	++++	-	+	+	-	-	+
<i>Ps. alcaligenes</i>	5	+	-	++++	-	-	+	+	-	+
<i>Ps. stutzeri</i>	6	+	+	-	-	+	+	+	-	+
<i>Ps. rathonis</i>	6	+	+	-	-	+	+	+	-	+
<i>Ps. denitrificans</i>	4	+	+	++	-	-	+	+	-	+
<i>Ps. desmolytica</i>	11	+	-	+	-	-	+	+	-	+
<i>Ps. dacunhae</i>	3	-	-	+	-	-	+	+	-	+
<i>Ps. riboflavina</i>	1	-	-	+	-	-	+	-	-	+
<i>Ps. iners</i>	3	-	-	-	-	-	+	-	-	+
<i>Ps. diminuta</i>	1	-	-	-	-	-	+	+	-	+
Chromogenic group										
<i>Ps. perlurida</i>	7	-	-	+	+	-	+	+	-	+
<i>Ps. trifolii</i>	4	+	-	+	-	-	+	-	-	+
<i>Ps. cerealis</i>	4	-	-	+	-	+	+	+	+	+
<i>Ps. melanogenum</i>	4	+	-	++	-	-	+	-	-	+
<i>Ps. lacunogenes</i>	13	-	-	+	-	-	+	+	±	+
<i>Ps. ochracea</i>	2	-	-	+	-	-	+	+	±	+

Ps. ochracea scarcely liquefied gelatin at 20°, but rapidly at 30°. The reaction to milk was generally alkaline, and almost half of the species peptonized casein. Prior to peptonization, some strains coagulated milk

giving neutral or alkaline reaction, a rennet-like coagulation. *Ps. perlurida* and *Ps. trifolii* compactly coagulated milk causing acidification, acid coagulation.

Biochemical and physiological characteristics

a) Production of pigments

According to the characteristics of pigment formation, the strains tested were divided into the following three groups: (1) those producing water-soluble greenish-yellow-fluorescent pigments, (2) those producing neither water-soluble nor non-water-soluble pigments, and (3) those producing non-water-soluble yellow pigments. The representative of Group (1) is *Ps. aeruginosa* which produces as major pigment the well-known compound pyocyanine. Members of the same group are: *Ps. azotoformans*, *Ps. chlororaphis*, *Ps. pavonacea*, *Ps. schuykilliensis*, *Ps. aureofaciens*, *Ps. cinnamona*, *Ps. straminea*, *Ps. nitroreducens*, *Ps. ovalis*, *Ps. rugosa*, and *Ps. fulva*. In general characteristics of this group, the following species may also be included in this group, although they produce, besides water-soluble pigments, some non-water-soluble yellow pigments: *Ps. cinnamona*, *Ps. straminea*, and *Ps. fulva*. Group (2) comprises *Ps. putrefaciens*, *Ps. alcaligenes*, *Ps. stutzeri*, *Ps. rathonis*, *Ps. denitrificans*, *Ps. desmolytica*, *Ps. dacunhae*, *Ps. riboflavina*, *Ps. iners*, and *Ps. diminuta*, and Group (3): *Ps. perlurida*, *Ps. trifolii*, *Ps. cerealis*, *Ps. melanogenum*, *Ps. lacunogenes*, and *Ps. ochracea*. Among these, *Ps. melanogenum* had a specific character of changing the color of nutrient agar containing tyrosine dark brown, which seems to be due to the formation of melanine.

b) Reduction of nitrate

In nitrate broth, some species reduced nitrate to nitrite. Almost all the strains which grew in succinate-nitrate broth accumulated nitrite in the presence of nitrate. Reduction of nitrate accompanied by production of gaseous nitrogenous compounds was observed with *Ps. aeruginosa*, *Ps. azotoformans*, *Ps. nitroreducens*, *Ps. stutzeri*, *Ps. rathonis*, *Ps. denitrificans*, etc. Some of the strain tested, when grown for 5 to 7 days in nitrate broth, produced only a trace of nitrite (less 3 γ /ml), while they produced a large amount of nitrite in the early stage of growth in succinate-nitrate broth, indicating that their mode of assimilating and reducing nitrate is different according to the composition of the medium applied. In our description of experimental results, the production of nitrite in amount of more than 5 γ /ml in the early culture stage was recorded as being positive in regard to the test of nitrate reduction.

c) Nitrate respiration

A considerable number of species of *Pseudomonas* were found to be capable of growing anaerobically by performing nitrate respiration. These were, when classified according to their characteristics of pigment formation:

Ps. aeruginosa, *Ps. azotoformans*, *Ps. nitroreducens*, and *Ps. chlororaphis* of Group (1); *Ps. stutzeri*, *Ps. rathonis*, and *Ps. denitrificans* of Group (2). Noteworthy was the fact that there was no species possessing the ability of nitrate respiration in Group (3). Almost all the strains which grew anaerobically by performing nitrate respiration showed a property of producing gaseous nitrogenous compounds from nitrate.

d) Production of indole

Indole was produced only by *Ps. perlurida* which belongs to Group (3).

e) Production of hydrogen sulfide

Nearly all the strains belonging to Groups (2) and (3) produced hydrogen sulfide abundantly, while those belonging to Group (1) scarcely produced it.

f) Hydrolysis of starch

Starch was hydrolyzed by *Ps. stutzeri*, and *Ps. rathonis* of Group (2) and *Ps. cerealis* of Group (3). The strains belonging to Group (1) were all incapable of hydrolyzing starch.

g) Catalase

All the strains belonging to the three groups contained catalase, although some strains belonging to Group (2), namely *Ps. putrefacines*, *Ps. alcaligenes*, and *Ps. riboflavina* showed weaker catalase activity compared with other species.

h) Relation to temperature

Generally, the optimum temperature for the growth ranged from 25° to 30°. Several strains of Group (1) grew well at 5° and 10°. *Ps. aeruginosa* grew well at 42°.

DISCUSSION

According to the characteristics of pigmentation the strains belonging to the genus *Pseudomonas* were divided into three groups: (1) Those producing water-soluble greenish yellow fluorescent pigments, sometimes accompanied by non-water soluble pigments; (2) those producing neither water-soluble nor non-water-soluble pigments; and (3) those producing only non-water-soluble yellow pigments. Compared with the strains belonging to Groups (2) and (3), those belonging to Group (1) are biochemically more active, being, in most cases, capable of growing on simple synthetic media. However, they do not produce hydrogen sulfide nor hydrolyze starch. Nearly all the strains belonging to Group (2) produce hydrogen sulfide. Among the strains of this group, *Ps. riboflavina*, *Ps. alcaligenes*, *Ps. iners*, and *Ps. diminuta* do not grow on simple synthetic media. The third group is characterized by their lack of capacity for growing anaerobically by performing nitrate respiration. *Ps. lacunogenes* of this group forms membranous colonies on nutrient agar and glutamate agar, which was never observed with other strains tested.

It has already been reported by the present authors that for various strains of *Pseudomonas* we can find certain parallel relations between the

characteristics of pigmentation and other biochemical properties, such as accumulation of amino acids (20), oxidation of steroid (21), and nutritional requirements (19). Interesting relations were also found in ecological characteristics on the organisms in question: namely, as shown in Table 1, the species of the first group were mainly those isolated from soil, sewage, water, etc, the species of the second group were chiefly those found in oil-brine (8), and the organisms belonging to the third group were those specifically found in cereal grains (7).

Based on these facts the authors propose the grouping of the genus *Pseudomonas* according to the characteristics of pigmentation, calling the first group Fluorescent group, the second group Achromogenic group, and the third group Chromogenic group. The following is the taxonomic description of three groups.

1. Fluorescent group

Produce water-soluble yellowish green to orange pigments, usually fluorescent. Some species produce non-water-soluble pigments besides water-soluble ones. Good growth on glutamate agar. A number of species reduce nitrate to nitrite in succinate-nitrate broth. Aerobe. Some species grow anaerobically by performing nitrate respiration. Hydrogen sulfide is hardly produced. Starch is not hydrolyzed. Widely distributed in water, sewage, soil, etc. *Ps. aeruginosa* is the type species.

2. Achromogenic group

Do not produce either water-soluble or non-water-soluble pigments. Some species grow on glutamate agar. Reduction and assimilation of nitrate vary with species. Some species grow anaerobically by performing nitrate respiration. Hydrogen sulfide is produced. Indole is not produced. The nutritional requirements are more complex than those of the fluorescent group. Widely distributed in water, sewage, especially in oil-brine. *Ps. putrefaciens* is the type species.

3. Chromogenic group

Produce non-water-soluble pigments. Some species grow on glutamate agar. Reduction and assimilation of nitrate vary with species. Aerobe. No strains was found to grow anaerobically by performing nitrate respiration. Hydrogen sulfide is produced. Starch is hydrolyzed by one species. Indole is produced by *Ps. perlurida*. The reaction to milk is usually alkaline. *Ps. perlurida* and *Ps. trifolii* coagulated milk. The nutritional requirements are more complex than those of the fluorescent group. Widely distributed in cereal grains. *Ps. lacunogenes* is the type species.

SUMMARY

Taxonomical studies on the genus *Pseudomonas* were carried out with 28 species and 202 strains, including those obtained from several type culture collections as well as those isolated from various sources by the

authors. The cultural, biochemical, physiological and ecological properties of these organisms were found to be more or less closely correlated to the characteristics of pigmentation. In respect to the pigmentation characteristics, distinction was made of three groups which were named: Fluorescent group, Achromogenic group and Chromogenic group.

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