PSEUDOMONAS AUREOFACIENS KLUYVER AND PHENAZINE α-CARBOXYLIC ACID, ITS CHARACTERISTIC PIGMENT

WILLIAM C. HAYNES, FRANK H. STODOLA, JOAN M. LOCKE, THOMAS G. PRIDHAM, HOWARD F. CONWAY, VIRGIL E. SOHNS, AND RICHARD W. JACKSON

Northern Utilization Research Branch, U. S. Department of Agriculture, Peoria, Illinois

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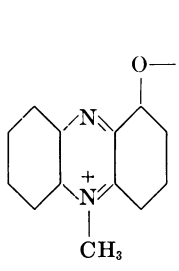
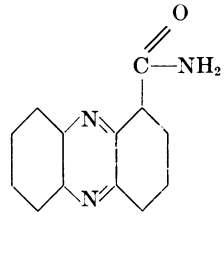
Heretofore three species of bacteria have been known to form phenazine pigments. Two of them are well established members of the genus *Pseudomonas*. One is *P. aeruginosa*, which produces pyocyanine (I) (Jensen and Holten, 1949); the other is *P. chlororaphis*, its pigment, chlororaphine, being a reduced form of oxychlororaphine (II) (Dufraisse, Etienne and Toromanoff, 1952).

The third microorganism capable of producing a phenazine pigment was originally designated *Chromobacterium iodinum* by Davis (1939). Tobie (1939) suggested that this microorganism belonged to the genus *Pseudomonas* because of its production of the phenazine pigment iodinin (III) (Clemo and Daglish, 1950; Kiprianov, Serebryanyi and Chernetskii, 1952).



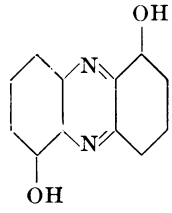
I

Pyocyanine



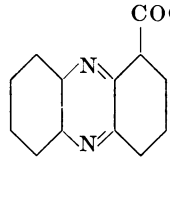
II

Oxychlororaphine



III

Iodinin



IV

Phenazine α-carboxylic acid

In this article a fourth microorganism is described which produces a phenazine as its principal pigment. Structure of the pigment has been established as phenazine α-carboxylic acid (IV). As stated in the preceding paper (Kluyver, 1956) this organism was first isolated in Prof. A. J. Kluyver's laboratory in Delft in 1936.

Without any knowledge of the work at Delft we isolated a strain of this organism in early 1953 and determined the structure of the acid produced. We first learned of Dr. Kluyver's work during his visit to our laboratory in 1954. By agreement we are presenting (1) a detailed description of the microorganism, *Pseudomonas aureofaciens* Kluyver, (2) data showing yields in flasks and in pilot plant equipment and (3) the results of a study of the action of phenazine α-carboxylic acid on various plant pathogens grown in artificial culture.

MATERIALS AND METHODS

Cultures. We have worked with four strains of the organism which, in collaboration with Dr. Kluyver, we have designated *Pseudomonas aureofaciens*. One strain was isolated in Dr. Kluyver's laboratory in 1936 from a sample of clay which had been kept under kerosene at 25 C for several weeks. We have done little with this organism (NRRL B-1576) except to compare it with our isolates. Two strains (NRRL B-1482, NRRL B-1543) were isolated in 1953 at this laboratory from soils from Iowa and Ohio. The third isolate, NRRL B-1681, was obtained from Michigan soil in 1955. All three of our isolates produce phenazine α-carboxylic acid as the major pigment.

Methods of characterization. The methods used in determining the morphology and biochemistry of these strains are in common use as described in the *Manual of Methods for Pure Culture Study of Bacteria*. The determination of gluconate oxidation, of slime formation from gluconate, and of temperature relationships was done according to the recommendations of Haynes (1951) in his study of *Pseudomonas aeruginosa*.

Pigment production in flasks and pilot plant.

A high pigment-producing culture is streaked or plated on yeast-glucose-agar of the following composition: yeast extract (Difco), 1 per cent; glucose, 1 per cent; agar, 2 per cent; distilled water; pH 7.7. After incubation at 28 C for about 3 days the colonies are large enough to pick. Three colonies are selected because of their resemblance to high yielding strains and transferred to slants of yeast-glucose-agar. Following incubation at 28 C for 3 days, growth from the slant showing the most soluble pigment is chosen to seed the inoculum flask. The medium in the inoculum flask(s) is composed of peptone (Difco), 2 per cent; glucose, 1 per cent; salt solutions A and B of Speakman (Speakman, 1923; Snell and Strong, 1939), 0.5 per cent each; and distilled water. Adjust pH to 7.7. Inoculum flasks, incubated at 28 C on a reciprocal shaker for 24 hr, are used to inoculate the production medium which contains peptone (Difco), 1 per cent; KNO_3, 0.1 per cent; NaCl, 0.5 per cent; glycerol, 2 to 8 per cent; tap water; pH 7.2. Inoculation is at a rate of 2 per cent. After inoculation, these cultures, usually 800 ml of medium in 2800 ml Fernbach flasks, are put on a rotary shaker at 28 C. The shaker operates at 200 rpm, and each flask rotates through an orbit 2¼ in diameter.

After 24 hr the medium is very turbid and has become distinctly yellow. On the second day the culture is quite orange, and the intensity of color increases during the next two to three days. By the fifth day the color has changed to a dirty yellow, and a dark brown precipitate settles out when the flasks are allowed to remain stationary for a few minutes. Maximum yield of phenazine α-carboxylic acid is usually attained in about 5 days with NRRL B-1543.

In order to accumulate enough pigment for evaluation, several pilot plant runs were made in a 60 gallon stainless steel fermentor. The culture was prepared in the same manner as for laboratory runs except that the inoculum was built up through two stages to obtain sufficient numbers of cells to inoculate 40 gals of production medium at a rate of 2 per cent. The aeration in the vat was at a rate of 1 volume of air per volume of medium per min. The agitator was set at 100 rpm and the temperature at 30 C. For foam control a solution of 0.75 per cent octadecanol in ethanol was released automatically.

Both in pilot plant runs and in flask experiments, samples are taken, usually at 24 hr intervals, until the yield appears to be maximal. The pH, viable population, packed cell volume and phenazine α-carboxylic acid content of these samples are determined. Viable populations are obtained by conventional dilution and plating on the yeast-glucose-agar. The colonies are large enough to count readily at 3 days of age. To obtain packed cell volumes, 2 to 10 ml of culture are put into Constable protein tubes and centrifuged at maximum speed for 30 min in an International table-model, clinical-type centrifuge.

Analysis for phenazine α-carboxylic acid. To 800 ml of culture liquor, 10 g of diatomaceous earth (Celite 545) and 5 ml of concentrated hydrochloric acid are added. After 1 hr the solution is filtered. The Celite pad, after drying in air, is boiled for 5 min with 50 ml of chloroform. The resulting suspension is filtered, 70 ml of chloroform being used for transferring and washing. The yellow filtrate is shaken thoroughly with 20 ml of water containing 500 mg of sodium hydroxide. After separation of the emulsion, the red aqueous solution is run off and carbon dioxide bubbled through it for 20 min to precipitate deep red, dense crystals. On this basis the product is judged to be phenolic. (At high concentrations of phenazine α-carboxylic acid its sodium salt sometimes appears as long, yellow needles which are easily recognized. In such cases enough water is added to bring the sodium salt into solution.) The phenolic precipitate is then removed by filtration, dried and weighed. The filtrate is acidified with HCl, the phenazine α-carboxylic acid filtered off, dried and weighed.

Assay procedure with plant pathogens. Agar streak tests of the sodium salt of phenazine α-carboxylic acid with a variety of bacteria and fungi were run as follows: A solution of the salt in pH 7 phosphate buffer is sterilized by filtration (UF fritted disc filter) and the filtrate incorporated in an agar medium in amounts necessary to attain the desired final concentration. This medium has the following compositon per L: asparagine, 2 g; $K_2HPO_4 \cdot 3H_2O$, 0.43 g; KH_2PO_4, 0.07 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; thiamine HCl, 0.5 mg; glucose, 40 g; corn steep liquor, 10 ml. The solution was adjusted to pH 6.5, 15 g agar added and the medium sterilized for 20 min at 121 C.

Twenty-four-hour broth cultures of the bacteria are used as inocula for streaking the surface of the test media in petri plates. Inocula for the antifungal tests consist of distilled water suspen-

sions prepared from seven-day cultures of the fungi growing on slants of the same medium. Plates inoculated with the phytopathogenic bacteria are incubated for 48 hours at 28 to 30 C. Slants inoculated with the test fungi are incubated for 72 hr at 25 C. Media inoculated with other test organisms are incubated at 37 C for 48 hr.

RESULTS

Taxonomic relationships. The four strains of *Pseudomonas aureofaciens* in our possession were isolated from soils which appear to be the normal habitat of this species. Although widely distributed it is apparently rare since it has seldom been encountered. The organism is a rod 1.3 μ in length by 0.7 μ wide and is gram negative. Fat inclusions may be observed in cells grown in the presence of glucose. All four strains were motile by means of polar flagella when first isolated, but the current strain of B-1543 is nonmotile and has no flagella. The rods occur singly or in pairs, seldom in short chains. All cultural and physiological characteristics were studied after incubation at 28 C. Nutrient and plain gelatin are rapidly liquefied, and the medium becomes orange. The most interesting colonies on agar are those of B-1543, which after about 7 days on peptone-glucose agar resemble miniature phonograph records. There are two stages of growth as inferred from the recognition of rough and smooth colonies. B-1543, B-1576 and B-1681 have been seen only as smooth colonies which grow on the surface of plates as butyrous, circular, entire, convex, opaque disks with concentric rings of color. Generally there are three rings of which the innermost is reddish brown, the outer one is yellow and the area between the two is bright orange. Such colonies may be 2 to 5 mm in diameter in 3 days and 7 to 10 mm in diameter at 7 to 10 days. The rough colonies derived from B-1482 look like the smooth colonies at first, but in 3 to 5 days they begin to show a brick-red, ground-glass surface. Apparently superimposed on the surface of some smooth colonies, a thin, dull greenish film may appear. The under sides of old colonies may show black granules which under the microscope look like bird shot. These may sometimes be in the colonies themselves.

The growth on nutrient agar slants is rapid and abundant. In 24 hours the growth is pale orange, smooth, glistening, and butyrous, and the medium is usually slightly discolored. The soluble blue-green fluorescence often noted in pseudomonad cultures is not always evident except in those of the smooth strains of B-1482. By 72 hours the agar is deeply orange close to the growth, the intensity of color diminishing with increasing distance. With smooth strains the growth remains butyrous, smooth and glistening, and becomes more luxuriant, and orange in color. The growth at the butt of the slant may be dull greenish on the surface. A dark, almost black, soluble pigment diffuses into the medium from the butt growth. Rough growth differs only in that the brick-red, ground-glass appearance develops at the top of the slant and slowly spreads downward.

In stationary nutrient broth cultures a pellicle develops, and the medium becomes deep orange. There is little turbidity or sediment if the culture is undisturbed. In shaken cultures the medium becomes markedly turbid, orange or yellow in color, and eventually a dark, granular precipitate forms. This precipitate is probably composed of a conglomeration of phenazine pigments, principally phenazine α-carboxylic acid, which have precipitated out of the medium.

Litmus milk cultures become alkaline, curdled, and eventually peptonized. The litmus is reduced.

Growth on potato is luxuriant, smooth, glistening, spreading, deep orange to dark brown, and the potato darkens. The potato tissue seems to be unaffected, except in color.

Indole is not formed.

The ability to reduce potassium nitrate in nutrient broth varies with the strain. None of our isolates reduces nitrate. The absence of nitrites or gas and the presence of residual nitrate in cultures up to 9 days of age supports this conclusion. Kluyver's strain, NRRL B-1576, on the other hand, consistently reduces nitrate to nitrite. Furthermore, and again in contrast to our isolates, it grows anaerobically in nutrient broth supplemented with potassium nitrate. The growth observed under these conditions is much less than is seen in aerobic cultures.

The organism is strictly aerobic and does not ferment sugars anaerobically. In shaken cultures it utilizes glucose and glycerol, but the products, other than phenazine α-carboxylic acid, are not known. It oxidizes potassium gluconate to a reducing compound presumed to be potassium

2-ketogluconate. In stationary culture in gluconate broth a slime is produced which resembles that formed by *P. aeruginosa* under similar conditions of growth (Haynes, 1951). It does not attack cellulose.

Acetyl methyl carbinol is not produced (Voges-Proskauer test negative).

Blood serum is not liquefied but is softened by most strains.

Cultures have a bland, rather inoffensive, odor.

The optimum temperature for growth is probably in the neighborhood of 28 to 30 C. Growth and pigmentation on slants is good at 25 C. At 37 C growth is good, but pigment is almost wholly absent. Both growth and pigmentation fail at 41 C. As with many pseudomonads, growth at 5 to 10 C, though slow, is good. At the end of two weeks it is about as good as is attainable at 28 C. Like many pseudomonads, *P. aureofaciens* grows well in media with ammonium salts or urea as nitrogen source, glucose, and essential minerals.

The distinctive characteristic of this species is the production of the pigment phenazine α-carboxylic acid in media containing glycerol, glucose or other suitable carbon sources. The rapid identification of the species, however, depends primarily upon the simultaneous formation of other pigments which are pH indicators. When a chloroform extract of the culture liquor is shaken with dilute alkali, the aqueous solution turns red; acidification of the alkaline extract gives an orange-yellow, crystalline precipitate.

It is likely that there are variants which fail to produce the characteristic pigments; in fact, this form may already be named and otherwise adequately described. If this is found to be the case, the older name will deserve priority and the form described above will be regarded as a chromogenic variant.

Characterization of phenazine α-carboxylic acid. The acid, readily obtainable in the pure state by crystallization first from dimethyl formamide and then from ether-chloroform, gave analytical figures in agreement with a $C_{13}H_8O_2N_2$ formula (Calcd: C, 69.64; H, 3.60; N, 12.49. Found: C, 69.6, H, 3.55; N, 12.7).

Its structure was established as that of phenazine α-carboxylic acid by the following constants and reactions: (1) mp 242 C (Kögl and Postowsky (1930) give 239 C). (2) Conversion by zinc dust distillation to phenazine having the correct melting point (171 C) and the reported ultraviolet absorption spectrum (Rădulescu and Ostrogovich, 1931). The phenazine also forms a resorcinol addition compound with the correct melting point (213 C) (Zerewitinoff and Ostromisslensky, 1911). (3) Reaction with thionyl chloride to give an acid chloride (Kögl and Postowsky, 1930). (4) Reaction of the acid chloride with methanol to form a methyl ester melting at

TABLE 1

Results obtained in a flask run with NRRL B-1543 in production medium containing 2 per cent glycerol

Age of Culture	pH	Packed Cell Volume	Viable Count	Phenolic Fraction	Phenazine α-Carboxylic Acid
hr		*ml/5 ml*	*billions/ml*	*mg/L*	*mg/L*
24	7.8	0.025	5.5	54	203
	8.0	0.030	3.1		
48	7.5	0.055	5.6	158	440
	7.6	0.050	7.5		
72	7.0	0.055	4.7	239	748
	7.1	0.055	6.1		
96	5.7	0.050	—	210	1001
	5.6	0.050			
120	5.2	0.045	—	251	1008
	5.4	0.045			
144	5.1	0.070*	—	94	1180
	5.3	0.115			

* Precipitated pigment accounts for the increased volume.

TABLE 2

Results of a pilot plant run using strain B-1543 in 40 gallons of production medium containing 4 per cent glycerol

Age of Culture	pH of Culture at Harvest	Packed Cell Volume	Viable Count	Phenazine α-Carboxylic Acid
hr		*ml/5 ml*	*billions/ml*	*mg/L*
24	7.90	0.030	5.2	—
48	7.10	0.070	5.9	—
72	6.30	0.050	4.1	—
96	5.10	—	—	—
120	5.00	—	—	—
144	5.10	—	—	1100

TABLE 3

Action of phenazine α-carboxylic acid on plant pathogenic microorganisms

Identity	Test Organism NRRL No.	Sodium Salt of Phenazine α-Carboxylic Acid μg/ml.			
		0	75	100	200
Corynebacterium fascians	B-190	++++*	0†	0	0
Corynebacterium flaccumfaciens	B-729	++	0	0	0
Agrobacterium tumefaciens	B-36	++++	++	++	0
Erwinia aroideae	B-134	+	+	+	0
Stemphylium sarcinaeforme	2188	++	0	0	0
Diplodia zeae	2282	++++	0	0	0
Claviceps purpurea	2386	++	0	0	0
Pythium debaryanum	A-5347	++++	+	0	0
Cercospora musae	A-5348	++++	0	0	0

* The number of plus signs indicates the relative amount of growth.

† A zero signifies complete inhibition.

123–124 C in agreement with the value reported by Birkofer and Birkofer (1952). (5) Formation of the amide (mp 241–242 C) from the acid chloride. Kögl and Postowsky's amide (1930) melted at 241 C.

The phenazine α-carboxylic acid dissolves readily in sodium hydroxide solution and slowly in sodium bicarbonate solution. The phenolic fraction dissolves in sodium carbonate solution but not in sodium bicarbonate solution.

Pigment production in flasks. In a typical flask run with NRRL B-1543 grown in a 2 per cent glycerol medium, the results shown in table 1 were obtained.

Yield of acid in a pilot plant run. Table 2 gives the results of a typical pilot plant run, using the same strain (B-1543), in 40 gallons of production medium (4 per cent glycerol). From this run 167 g of phenazine α-carboxylic acid was obtained.

Action on plant pathogens. Table 3 summarizes the results of tests of phenazine α-carboxylic acid on a number of plant pathogens grown in artificial culture.

DISCUSSION

Pseudomonas aureofaciens appears to be a rare species. If it were not, it would surely have been described before because it is readily distinguished as unusual on ordinary laboratory media. From observations of four strains we conclude that it belongs to the group of pseudomonads which liquefies gelatin and grows readily at 37 C but not at 42 C. Within this group it shows the closest affinity to *P. reptilivora*, a pathogen for rodents and reptiles. However, the production of phenazine α-carboxylic acid and other apparently related pigments serves to differentiate these two.

The pigments produced are soluble in the medium and impart to it first an orange-yellow, then a deep orange and finally a reddish-orange color. A chloroform extract of such a culture, containing principally phenazine α-carboxylic acid and phenolic compounds, becomes red when made basic and yellow when acidified. These color changes constitute a simple test which may be used for presumptive identification of strains of *P. aureofaciens* without recourse to the more lengthy isolation of phenazine α-carboxylic acid. In this regard the new organism resembles *P. aeruginosa* which may be readily identified by its formation of the indicator pigment pyocyanine. *P. iodina*, which produces iodinin, is another species in which the pigment expedites identification. In the case of *P. chlororaphis*, deposition of green crystals may be regarded as presumptive evidence of identity with this organism. Characterization of the pigment, however, is somewhat more difficult than the simple observation of the color changes undergone by the water-soluble pigments of the other three species.

Although Kluyver's paper (1956) and ours are the first to describe the biological production of phenazine α-carboxylic acid, it was synthesized in 1930 by Kögl and Postowsky and in 1934 by Clemo and McIlwain. In our work we have been able to recover about a gram per liter of culture liquor both in laboratory shake flasks and in pilot plant runs of 40 gallons each.

Our reasons for making pilot plant runs was to accumulate sufficient material for field tests of its effectiveness in controlling plant diseases. All known phenazine derivatives are biologically active, and it was anticipated that this form would be also. In fact, Schales, *et al.*, in 1945 found phenazine α-carboxylic acid to be bacteriostatic to the growth of *Staphylococcus aureus* when present at a concentration of 10 mg per 100 ml of solution. Later Birkofer and Birkofer (1949) reported that it inhibits the growth of *Mycobacterium tuberculosis* typus *gallinaceus* (*M. avium*) at a concentration of 0.01 per cent, and of *Bacillus anthracis* at higher concentrations. These results, of course, gave no clue to the effectiveness against bacteria and molds instrumental in causing diseases of plants. Our results, showing inhibition of selected plant pathogens in artificial culture, appear to be the first reported in this field.

ACKNOWLEDGMENT

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SUMMARY

A complete description of *Pseudomonas aureofaciens* Kluyver based on four strains is given. The characteristic pigment which it produces has been identified as phenazine α-carboxylic acid. This acid is produced in flasks and in pilot plant fermentors to the extent of about one gram per liter of culture liquor. The pigment is able to inhibit the growth of some plant pathogens in artificial culture.

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