It is well known that the genus *Pseudomonas* comprises several pigment-producing species, and that in all cases in which the constitution of these pigments has been established they have been found to be phenazine derivatives. Wrede and Strack (1929) were the first to prove this for pyocyanine, the pigment produced by *Pseudomonas aeruginosa* (P. *pyocyanea*). Pyocyanine is now usually considered to be a resonance hybrid of the mesomeric forms of 1-methyl 1-hydroxyphenazine (Hiltemann, 1938). Kögl and Postowski (1930) established that chlororaphine, the green pigment of *Pseudomonas chlororaphis*, is the semiquinone of phenazine-α-carboxylic acid amide, while Clemo and Daglish (1950) proved iodinin, the pigment of *Pseudomonas iodina*, to be 1:6-dihydroxyphenazine-di-n-oxide.

In view of these data it seems worthwhile to look out for further pigment-producing species within the genus *Pseudomonas* in order to check if still other phenazine derivatives occur as natural products. The opportunity presented itself in 1936 when Mr. H. Bouman, working in this laboratory, incidentally came across a bacterium which was easily identified as a *Pseudomonas* species, and which attracted attention by an amine formation of yellow crystals in its colonies. Mr. Bouman showed that a crystalline pigment could also be isolated from various liquid media in which the bacterium had grown.

Some years later his observations were extended, first by Mr. J. J. Ghijsen, and next by Mr. C. J. P. Spruit. They obtained convincing evidence that the pigment as first isolated by Bouman was phenazine-α-carboxylic acid, and therefore closely related to chlororaphine. At that time war interfered with the work, and the results obtained remained unpublished.

Interest in the organism in question and its products was revived a year ago when during a visit at the Northern Utilization Research Branch at Peoria, Ill., Dr. W. C. Haynes and Dr. F. H. Stodola informed the author regarding their investigations on a new pigment-producing pseudomonad for which they also had established production of phenazine-α-carboxylic acid. After exchange of cultures it soon became evident that the American and the Dutch strains were either identical or at least very closely related. The American workers and the present author then decided on simultaneous publication of the results obtained in each of the two laboratories. In the meantime there had also been an exchange of experimental data, which, of course, led to a mutual influence in the later phase of the investigation. The experimental work performed in Delft during this second period was carried out by the following students who successively devoted themselves for a short time to the problems involved: Tjoe Hin Soey, E. van der Slooten, W. Klop, Sie Hian Jang, and E. J. Vles. The following exposition should be considered as a condensed collective report on the fragmentary observations made over the whole period of almost twenty years; it is impossible to acknowledge here the separate individual contributions.

**RESULTS**

Isolation and cultural properties of the organism.

For reasons which can be left out of consideration here Mr. Bouman in 1936 made some experiments in which kerosene was treated with clay. A liter of heat-sterilized kerosene was poured into a 2-L Erlenmeyer flask and 100 g clay—from the river Maas—was added. The flask was kept in an incubator at 25 C for three weeks. The clay was suspended in the kerosene from time to time by shaking. At the end of the period the kerosene was poured off, and a suspension of the clay in sterile water was streaked on an agar plate containing 1 per cent peptone and 2 per cent glucose. After two days, incubation at 30 C several colonies...
had developed on the plate. Amongst these, colonies with a marked brownish yellow color prevailed. From one of these colonies a pure culture was easily obtained by replating on the same medium. The pure culture was maintained on plain peptone agar (1 per cent peptone). Gelatin was found to be strongly liquefied.

Microscopic examination of young cultures showed small motile rods which proved to be gram negative. Using Gray's stain, the presence of polar flagella could be demonstrated. No indications of endospore formation were ever encountered. The bacterium appeared to be strictly aerobic insofar as it did not grow in the usual media in the absence of oxygen. No sugar fermentation ever occurred in such media. In media containing sucrose, an ample production of levans took place.

However, a certain amount of anaerobic growth occurred in nutrient broth to which nitrate was added. Under these conditions nitrite accumulated. Because a considerable part of the nitrate in one per cent KNO₃ nutrient broth culture remains untouched, inhibition of growth is probably due to accumulated nitrite. Sometimes a very small quantity of gas is produced, but true denitrifying ability is evidently lacking. All these characteristics taken together leave no doubt that the organism should be classified as a species of the genus *Pseudomonas*.

In view of the present confused state of the taxonomy within the genus *Pseudomonas* a further determination of the bacterium would have been well-nigh impossible, had not its characteristic pigment production set it quite apart from the many colorless species, as well as from those species which are characterized by the production of a water soluble yellow-green fluorescent pigment.

After two to three days on peptone agar smooth round colonies of about 2 mm diameter are formed. They show a yellowish-orange color which is especially accentuated in the center and the color gradually turns brownish. The pigment also diffuses into the agar.

It was soon found that addition of glucose to the medium markedly increases pigment production. After some time the colonies on such a medium show, on observation under the microscope with low magnification, the presence of brown crystalline conglomerates either in or in the immediate vicinity of the colonies. Figure 1 gives a picture of the crystals as they appear in a streak culture on peptone agar + 2 per cent glucose.

Not all colonies are characterized by such a crystalline deposit. It has, however, been observed that in these cases the mere touching of the colony with a platinum needle often leads to a crystallization. In such a case we apparently are dealing with a disturbed supersaturation.

The next observation was that on flooding the plate with chloroform the crystals quickly dissolved. On evaporating the yellow chloroform solution, brownish-red crystals were at once obtained. These crystals also proved to be readily soluble in benzene, acetone and dilute alkali.

Conditions favoring pigment production. Since it seemed worthwhile to determine the nature of the pigment formed, numerous experiments were carried out in order to find conditions favoring pigment production.

In the first place it was established that good production of pigment could also take place in liquid media. At first a medium containing 1 per cent peptone, 0.5 per cent NaCl and 2 per cent glucose was examined. In stationary cultures in Erlenmeyer flasks it was at once evident that the color was practically confined to the upper layer of the medium, probably only indicating the organism's need of oxygen for proliferation. In the later phases of the investigation the cultivation was therefore usually carried out in Erlenmeyer flasks placed on a rotary shaker, although
sometimes it was preferred to cultivate in deeper layers of medium with continuous aeration.

Further experiments left no doubt that a temperature of 30°C was optimal for both growth and pigment production. Growth is still satisfactory at 35°C, but, remarkably, no pigment is produced.

Next attempts were made to increase pigment production by varying the composition of the medium. There is no need to sum up here the numerous media which were used in these comparative experiments. It may suffice to mention some of the results obtained.

In the first place it should be remarked that the substitution of glycerol for glucose proved to be favorable, whilst the same held for an addition of 0.1 per cent KNO₃ to the medium.

Furthermore it became clear that the type of peptone used played an essential role in pigment production. Although with all brands of peptone a good growth of the bacterium resulted, there was practically no pigment production with peptone (Witte), nor with peptone (Difco). On the contrary, the “peptone bacteriologique” and the “peptone pancréatique” of the “Usines chimiques de laboratoires français” were equally satisfactory, and tryptone (Difco) also gave excellent results. On the other hand, casamino acids (Difco) were unsatisfactory, whilst a vitamin-free peptone preparation again gave a good result. This proves that vitamins are needed neither for growth nor for pigment synthesis. Further it can be concluded that certain peptone preparations contain one or more constituents which are lacking in other brands. Attempts were made to supplement the medium containing peptone (Witte) by adding compounds of known constitution, such as various amino acids and intermediates of carbohydrate metabolism, but this remained unsuccessful. However, an addition of 0.1 per cent yeast autolysate to the peptone (Witte) restored pigment production. This was not primarily due to trace elements present in the autolysate, for the addition of ash of the autolysate was only slightly effective.

The general conclusion from the results obtained in these exploratory tests—extending over some 200 media—was that the following medium: “peptone bacteriologique,” 1 per cent; sodium chloride, 0.5 per cent; potassium nitrate, 0.1 per cent; glycerol, 2 per cent; pH adjusted to 7.2, gave the most favorable and consistent pigment production. In later experiments this medium has always been used.

It should be remarked that in these preliminary tests pigment production was always judged visually. Such a crude estimation seemed sufficiently reliable for the purpose, although it should be acknowledged that sometimes difficulties arose, because there were marked differences in the color of the various culture fluids. This color could vary from an almost pure yellow to orange, to reddish brown and even to dark brown. The differences were such that they strongly suggested the production of more than one pigment in certain media.

On the other hand, these differences might have been caused by variations in final pH or in oxidation-reduction potentials of the media. It was clear that only the isolation of the pigment—or pigments—could give an answer to these questions.

Isolation and purification of the pigments. Even in the earlier experiments it had become clear that the separation of the pigment from a well-colored medium was quite easy. The initial observation that the crystals present in a plate were readily soluble in chloroform led to attempts to extract the coloring substance from liquid media also with this solvent. It was, of course, necessary to give due attention to the pH of the medium, and it soon appeared that removal of the pigment from the water phase was attained only if the culture medium were first acidified.

This is in marked contrast to the behavior of pyocyanine, which is extracted from the culture medium of _P. aeruginosa_ as long as the medium is distinctly alkaline.

In the present case the addition of some hydrochloric acid to the medium changes the usually orange-red color into yellow, whilst very soon a yellow crystalline precipitate is formed. These crystals dissolve in chloroform, giving a bright yellow solution. The pigment is again removed from the chloroform by washing with dilute alkali; in this operation the orange-red color is restored. After re-acidification a second extraction with chloroform can be carried through. The chloroform solution thus obtained is free from lipids, since these have remained in the first chloroform batch. The final chloroform solution was dried overnight with anhydrous sodium sulfate and concentrated by distilling off the chloro-
form in vacuo. This always led to a crystalline pigment product.

The product was usually recrystallized a few times from ethanol (95 per cent) till the crystals showed a constant melting point.

It soon became evident that the products obtained by this simple procedure from various cultures showed marked differences. The rather pronounced difference in color of the culture media was also manifest in the final products; moreover, this difference was obviously correlated with differences in melting point and in crystal shape.

Amongst the isolated products there were two which apparently prevailed; pigment I, which was obtained in fine greenish-yellow needles with a melting point varying between 239 and 241 C (uncorr.) and which dissolved with a yellow color in dilute alkali, and pigment II, which showed orange-red to bright red irregular crystals which on heating sintered at 207 C and melted in the neighborhood of 225 C. However, as already mentioned, in other cases preparations were obtained with different shades and melting points.

The inevitable conclusion is that at least two, and possibly even more, pigments can be formed by the organism in question.

This view could be confirmed with the aid of column chromatography using Al₂O₃ (aluminium oxydatum, E. Merck) as an adsorbent. The Al₂O₃ was suspended in chloroform and by sucking off the liquid a solid column was prepared. A solution of a crude preparation in chloroform was then cautiously poured on the top of the column, and after a short time several differently colored bands appeared. On washing with a small quantity of pure chloroform at least six clearly separated bands could be observed. No details will be given here; the following may suffice. The top layer was of a somewhat dirty brown color, but then a broad, beautiful red zone occurred. The three lower layers all had a yellow color, although there was a difference in tinge. From the different zones the pigments were extracted with dilute potassium hydroxide (5 per cent), and after acidification again taken up in chloroform. It will only be mentioned that from the red zone, pigment II (dark red crystals, mp 224–225 C), and from the combined yellow layers, pigment I (greenish-yellow needles, mp 238–240 C) could be isolated.

Preliminary investigations on the nature of the pigments. From the very beginning the phenazine character of the pigments was deemed probable. The correctness of this idea was soon proved by carrying out a zinc dust distillation of both pigment I and pigment II in a suitably bent hard-glass tube. In both cases a crystalline sublimate was obtained in the cooled bent arm of the tube. The sublimate proved to have a melting point of 171 C, whilst a mixture of this product with a synthetic phenazine preparation (mp 170.5 C) did not show any depression in melting point. This result seems to offer sufficient evidence for the conclusion that in both pigments we are dealing with phenazine derivatives.

Identification of pigment I. The next step was determination of the elementary composition of pigment I. C and H were determined by combustion, N according to the micro-Dumas method.

The result (average of two analyses with quite satisfactory agreement) was: C, 69.4; H, 3.8; N, 12.4; O, 14.4 (by difference).

Accepting two N atoms to be present in the molecule, this result indicates an empirical formula: C₁₃H₈N₂O₂.

For this formula the calculated composition is: C, 69.6; H, 3.6; N, 12.5; O, 14.3.

The deviation between the experimental and the theoretical figures is so slight that a formula C₁₃H₈N₂O₂ for pigment I becomes extremely probable. This formula immediately suggests the configuration of a phenazine carboxylic acid, especially if we realize that chlororaphine, the pigment of P. chlororaphis, has been proved to be the amide of phenazine-α-carboxylic acid. Kögl and Postwsky (1930) have synthesized two phenazine carboxylic acids, α and β, with melting points of 239 and 292 C respectively. This at once strongly suggested the identity of pigment I and phenazine-α-carboxylic acid, and this became practically certain when it was found that a mixture of pigment I and of the synthetic preparation kindly provided to us by Professor Kögl did not show any melting point depression.

It can be added that in other respects also both products behave identically. On methylation both products yielded a monomethyl ester with identical melting point, 119 C, and a nitrogen content of 11.5 per cent (theoret.: 11.8 per cent). A determination of the molecular weight of pigment I by potentiometric titration of an alco-
honic solution yielded the value 211 (theoret. for phenazine carboxylic acid: 224).

Taking all together, the identity of pigment I and phenazine-α-carboxylic acid may be considered to be proved.

Attempts at identification of pigment II. Since the attempts at identification of pigment II have not yet led to a final result, only a very brief survey of provisional results will be given. It should be acknowledged that subsequent preparations yielded products with slightly varying properties, even after repeated crystallizations. The melting point is not sharp, usually it is near 225°C, but even at about 200°C marked discoloration occurs. Melting is accompanied by gas evolution.

Microanalysis of one of the preparations yielded the following results: C, 63.3; H, 3.3; N, 11.4; O, 21.9 (by difference).

This means that the empirical formula approaches C_{13}H_5O_3N_2 for which compound the theoretical values are: C, 65.0; H, 3.3; N, 11.7; O, 20.0. Assuming this formula to be correct, the constitution could be either that of a hydroxyphenazine carboxylic acid or a phenazine carboxylic acid n-oxide. A determination of the molecular weight by Rast's method did not yield reliable results, probably owing to decomposition in the melted camphor. A potentiometric titration did not give a sharp end point, the most probable value of the molecular weight being 259 (theoret. value for either of the two suggested configurations: 240).

Owing to the fact that the bacterial cultures have yielded only relatively small amounts of pigment II, and that subsequent preparations showed variations in properties, a further study of the chemical nature of this pigment had to be postponed.

It is only certain that pigment II is also a phenazine derivative; moreover, the presence of one carboxylic group is at least most probable, whilst the compound is oxidized as compared with phenazine carboxylic acid.

**DISCUSSION**

The isolation of a previously undescribed species of the genus *Pseudomonas* has been reported.

It has been shown that this species produces at least two pigments of the phenazine series in suitable culture media. One of these pigments has been identified as phenazine-α-carboxylic acid, while the second one may be some oxidized derivative of this acid. In many cases chromatographic analysis showed the presence of still other pigments of apparently related type. It should, however, be taken into consideration that both pigments are reversible redox systems and that chromatographic analysis may bring about a separation between the oxidized and the reduced component of each of the two systems. Moreover, in view of the situation encountered in the case of chlororaphine, it seems likely that the occurrence of semiquinones must also be taken into account (KögI and Postowsky, 1930; KögI and Tönnis, 1932; Elema, 1933). Only further investigations can elucidate these points.

It is proposed to name the new species *Pseudomonas aureofaciens* on account of the golden-yellow color it produces on some agar media, a property which led to its discovery.

Dr. Haynes and Dr. Stodola and their colleagues report their observations in the following article.

**SUMMARY**

In 1936 Mr. H. Bouman isolated a new pseudomonad strain which attracted attention by the production of a golden-yellow color on certain solid media, and by the formation of pigment crystals within its colonies. The organism has now been described in detail and given the name *Pseudomonas aureofaciens nov. spec.*

It has been shown that *P. aureofaciens* produces more than one pigment in varying proportions depending on the composition of the medium used and on external conditions. Two of these pigments have been isolated and both proved to be phenazine derivatives. One pigment was identified as phenazine-α-carboxylic acid. The second pigment apparently is a closely related compound, but its configuration still awaits elucidation.

**REFERENCES**


