# *Arthrobacter polychromogenes* nov.spec., its Pigments, and a Bacteriophage of this Species

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SCHIPPERS-LAMMERTSE, A. F., MUIJSERS, A. O. and KLATSER-OEDEKERK, K. B. 1963. *Arthrobacter polychromogenes* nov. spec., its pigments, and a bacteriophage of this species. Antonie van Leeuwenhoek **29**: 1–15.

The morphological and physiological properties of a new blue pigments producing bacterium, *Arthrobacter polychromogenes* nov. spec. are described. One of the pigments is identical with the water-insoluble indigoidine, the other pigments are water-soluble.

The new *Arthrobacter* did not fit very well into the determinative key of the genus, prepared by Lochhead, because citrate is assimilated, but inorganic nitrogen only after addition of biotin.

A bacteriophage of *Arthrobacter polychromogenes* has been isolated, some characteristics of which are described.

## INTRODUCTION

In the summer of 1960 we observed on a peptone-glucose agar plate a colony of an airborne infection, which attracted our attention because of its bluish colour. Microscopical examination showed this colour to be due to blue-black crystals which occurred in the colonies in large amounts. Further cultivation demonstrated that pigment formation was not restricted to crystals in the colonies as also the agar turned blue. Since bacterial production of blue pigments is rather rare, the bacterium was isolated and further investigated.

From the morphological and physiological properties it was concluded that the bacterium is a hitherto undescribed species of the genus *Arthrobacter*. The pigments produced by the bacterium have been studied more closely.

During the experiments plaques were observed which by various means were shown to be due to a bacteriophage. This was confirmed by electron microscopy.

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## DESCRIPTION OF *Arthrobacter polychromogenes* NOV. SPEC.

### *Morphology*

The bacterium shows a pronounced pleomorphism, and all morphological characters of the genus *Arthrobacter* could be observed. Growth studies performed on this organism in the yeast-soil extract broth of Lochhead and Burton (1955) indicated that there is a distinct morphological pattern during the growth cycle. This was established by means of the method of Stevenson (1961); the yeast-soil extract medium was inoculated with 2 ml of a culture of *A. polychromogenes* in the same medium and after various intervals the culture was examined under the microscope. For photomicrographs, smears were made on glass slides at set times during the growth period and these were stained with crystalviolet. Figs. 1–4 represent the results of this experiment. At 0 time (24 hr inoculum) there are only typical, small rods (Fig. 1), which after 5 hr have changed into long rods (Fig. 2). These rods show already cross-walls delineating the new, small cells they will be divided in after 9 hr (Fig. 3). After 24 hr the cells are again of the same size as the coccoid cells of the inoculum (Fig. 4).

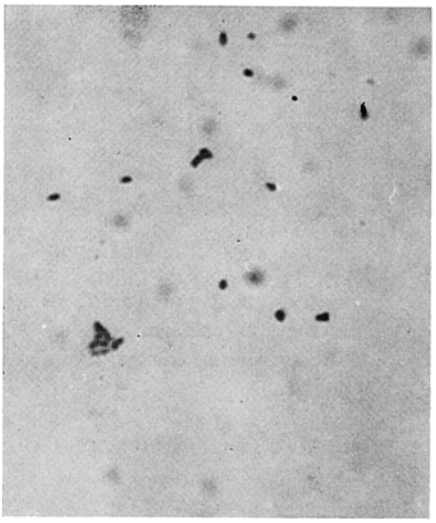
Figs. 5–9 show how a large "cystite" germinates and gives rise to two rods. After cell division the rods will turn until a V formation is reached (Figs. 10 and 11). X or square formations are also often observed (Fig. 12). As to the origin of V forms in *Arthrobacter atrocyaneus* three possibilities have been described by Starr and Kuhn (1962). The germination of adjacent cocci as well as the angular growth occurred in our cultures of *A. polychromogenes*, but a snapping post-fission movement has never been observed.

Especially in rich liquid media the "myceloid" stage of long irregular, often branched rods may occur (Fig. 13).

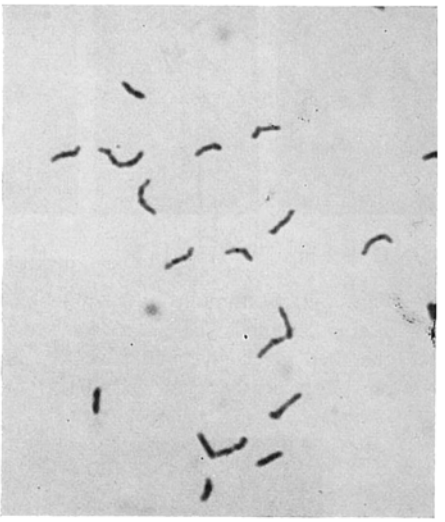
The blue organism is Gram-variable. In any phase there are distinctly positive and distinctly negative forms, the former usually predominating. Also Gram-negative bacteria with positive granules appear (Fig. 14). The myceloids are Gram-negative with positive bands or granules; the cystites are invariably Gram-positive.

Motility has never been observed and the results of flagella-staining (Leifson, 1960) were negative.

In old cultures and also in phage infected ones often R-colonies occur: irregular colonies with a wrinkled umbonate dull surface and erose margin (Fig. 15).



0 hr



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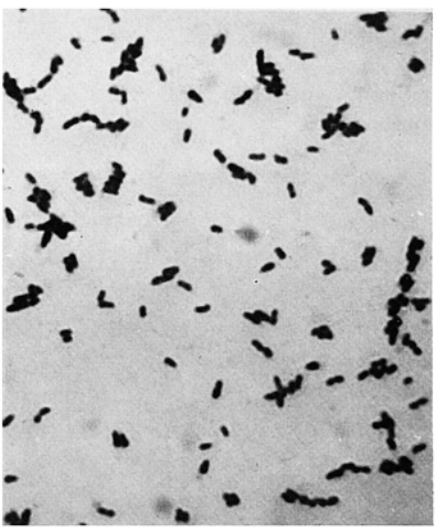
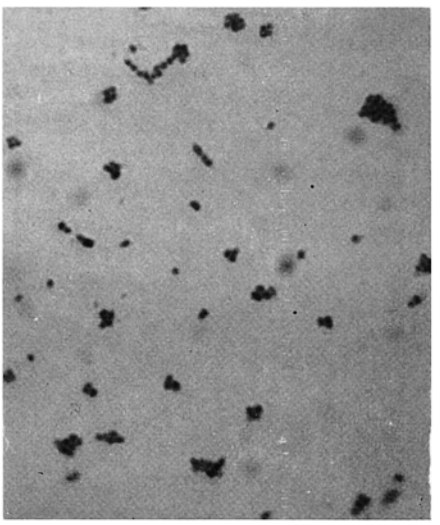
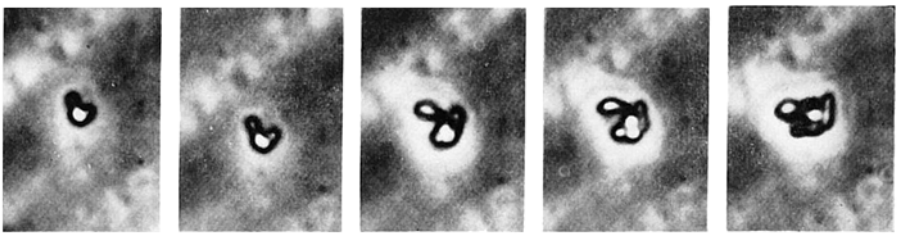


9 hr



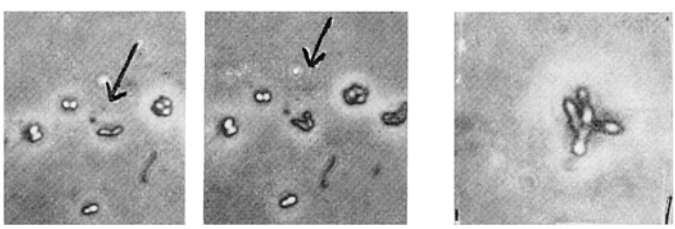
24 hr

Figs. 1-4. Growth cycle of *A. polychromogenes*; crystalviolet stain; 1500×.



H + 1 hr H + $1\frac{3}{4}$ hr H + $3\frac{1}{4}$ hr H + 4 hr H + $4\frac{3}{4}$ hr

Figs. 5-9. Continuous phase-microscopic observation of the "germination" of a large "cystite". The culture was grown on a thin layer of peptone agar in a slide chamber. Fig. 5 gives the situation 1 hour after two rods began to emerge from a previously nearly round coccoid cell (H hr).



Figs. 10 and 11. Two rods turning until V formation, 1500 ×.
Fig. 12. Typical X formation, 3000 ×.

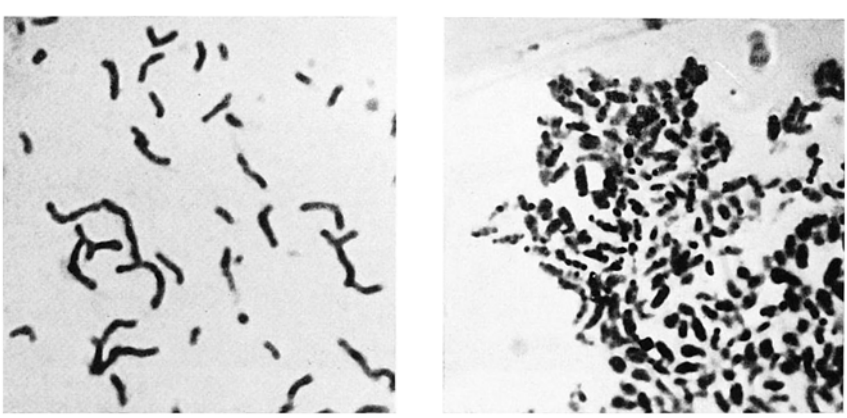


Fig. 13. "Myceloid" stage in glucose broth stained with crystalviolet, 2500 ×.
Fig. 14. Gram-negative bacteria with positive granules, 1750 ×.

*Cultural and physiological characteristics*[1])

*Peptone gelatin stab:* rather slow liquefaction, crateriform. Under these conditions the slowness of liquefaction is due to the strongly aerobic properties of the bacterium. On gelatin plates the liquefaction is rapid, the liquid remaining rather clear with colonies and flaky sediment floating in it.

*Nutrient agar:* good growth, colonies white, circular, smooth, convex, up to 4 mm, edge entire.

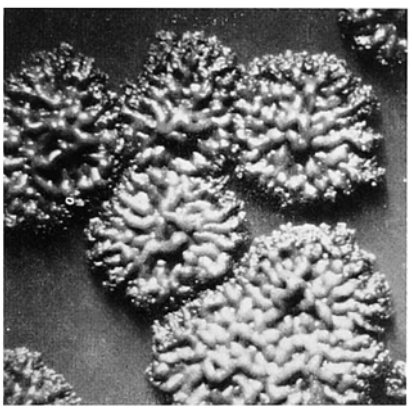


Fig. 15. R-colony on PKGl agar, 3 ×.

*Agar slant:* filiform.

*Asparagine agar:* no growth; + *biotin:* growth.

*Nicotine agar* (Sguros, 1955): very scant growth.

*Yeast extract-dextrose-chalk agar* (*YDC-agar*): colonies large (5 mm), circular, convex, entire, deep blue with numerous crystals. The agar around the colonies turns blue.

*Peptone agar:* colonies white or grayish, 1 mm.

*Peptone-glucose agar* (Sabouraud's): same appearance as on YDC-agar.

*Nutrient broth:* turbid; streaky sediment.

*Potato:* colonies slimy, green. Around the colonies the potato becomes bluish.

*Litmus milk:* no change after several weeks.

*Indol production:* negative.

*Hydrogen sulfide production:* negative on the three following media: motility sulfide medium, containing l-glycine (Difco, B 450); lead acetate agar (Difco, B 88); peptone iron agar, containing ferric ammonium citrate (Difco, B 89).

[1]) Further details will be given in the section *Taxonomy and Discussion.*

*Acid from sugars:* little or no acid production from sugars. On many carbohydrate-containing peptone media a blue colour is produced.

*Acid from glycerol:* positive.

*Acetoin production:* negative.

*Starch hydrolysis:* positive.

*Nitrate reduction:* reduction to nitrite. No denitrification.

*Urease:* negative. The following media were tried: urea broth (Difco, B 272); urea agar base (Difco, B 283).

*Catalase:* strongly positive.

Strongly aerobic.

Ammonium or nitrate without biotin is not sufficient as nitrogen source. Sodium citrate is sufficient as sole carbon source, also in the absence of biotin.

Excellent growth and much pigment is obtained on a medium of peptone 1%, glycerol 2%, KCl 0.6%, agar 2%, pH = 9 (PKGl). The bacterium also grows well, though colourless, when vitamin-free casamino acids are substituted for peptone in the last mentioned medium; hence the bacterium does not need vitamins for growth. The colour appears in the vitamin-free medium after addition of biotin; so biotin is necessary for the formation of the pigments.

The temperature optimum for growth and pigment production is about 25 C. Most often growth but no colour is observed at 37 C. No growth at 41 C; growth at 10 C. A colourless culture, grown at 37 C, soon becomes coloured at room temperature.

At a pH of about 5 no growth occurs. Optimum pigment formation occurs at the rather high pH value of 9 to 10. The maximum for growth and pigment production is at pH = 11 or above. No pigment formation at a pH value below 6.

*Source*: airborne infection.

*Habitat:* unknown.

## THE PIGMENTS OF *Arthrobacter polychromogenes*

### *Conditions affecting pigmentation*

As to the pigment production, the organism showed considerable variation as was clearly shown by the frequent formation of colonies with differently coloured sectors (Fig. 16). This was often the case in a green variant (also an airborne infection), probably identical with the new *Arthrobacter* species. This green strain formed all sorts of yellow-green, white and blue sectors in one giant colony. From this green organism blue mutants arose constantly, which could not be distinguished in any respect from the original blue one. The latter strain

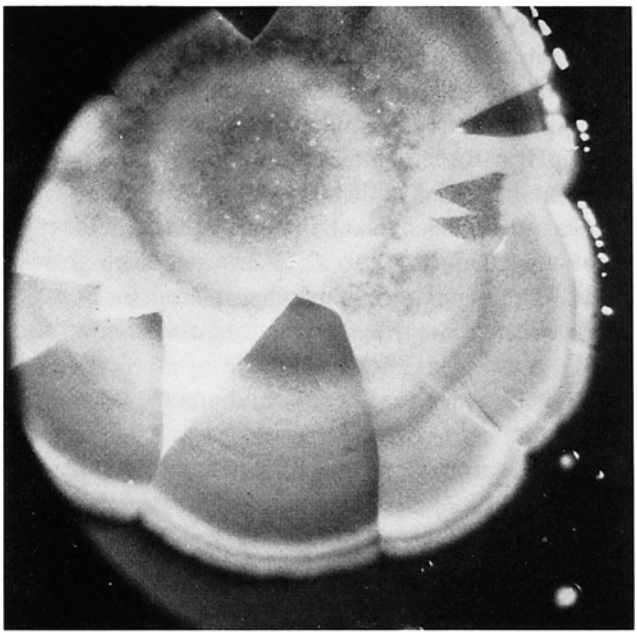


Fig. 16. Giant colony showing differently coloured sectors, 6 ×.

did not show visible variation to the same extent, although lighter and darker colonies regularly appeared.

Although the original colony was only faintly coloured, it turned out to be possible, by means of selection of the darkest mutants, to obtain strains with a very intensive pigment production. The colonies on a suitable agar medium became dark blue and showed a green golden metallic lustre caused by countless water-insoluble pigment crystals; in addition blue purple soluble pigments had penetrated into the agar, lending the entire plate a dark violet colour.

The bacterium had been cultivated on yeast extract-dextrose-chalk agar until it turned out that a 1% peptone medium with 2% glycerol and 0.6% KCl and 2% agar gave a much higher yield of pigment. KCl was preferable to NaCl or $KNO_3$.

When in the PKGl medium vitamin-free casamino acids are substituted for peptone the organism grows colourless. Addition of a vitamin solution (Wickerham and Burton, 1948) gives the usual pigment production. Further investigation showed that only biotin was responsible for this fact, the colour appearing with biotin concentrations exceeding $2 \times 10^{-5}$ $\mu$g/ml.

Besides glucose and glycerol also fructose, lactose, saccharose, mannose, raffinose and mannitol, when added to the peptone medium, afforded a good

pigment production. Much less pigment was formed with Ca-gluconate, salicin, and galactose. With maltose, xylose, ribose, sorbose, arabinose, rhamnose, erythritol, starch and Na-pyruvate little or no pigment was formed. On glycerol agar the pigment production was stimulated by increasing the pH of the medium to 9 or 10. At a pH of 6 the colonies showed only a faint blue colour. The quantity of pigment increased with the pH.

Temperature has also a significant influence. At 25 C the plates become much deeper coloured than at 20 or 30 C. The organism grows at 37 C, but always colourless. The plates turn blue when placed for some time at room temperature, as is also the case with *Arthrobacter atrocyaneus* (Kuhn and Starr, 1960).

On a blue plate held under anaerobic conditions the colonies become much lighter, nearly beige.

On solid media much more pigment is formed than in liquid media. A culture in peptone water, glycerol 2%, KCl 0.6%, pH 10, which is constantly shaken, turns eventually dark blue-brown to black. This colour is caused by insoluble pigment crystals and degradation products of the soluble pigments.

## ISOLATION AND PROPERTIES OF THE PIGMENTS

A superficial observation of the coloured plates had shown that there were at least two pigments, i.e. insoluble black crystals in the colonies and a violet soluble pigment penetrating the agar. The pigments were examined separately.

### *The insoluble pigment*

As the pigment crystals considerably exceed the bacteria in size, the method already used for indigoidine (Elazari Volcani, 1939) can be applied.

The bacterial colonies are scraped from the agar, suspended in water, and carefully centrifuged at low speed. In this manner it is possible to separate the crystals while most bacteria remain in the liquid. By repeating this process several times, followed by washing with various solvents, the crude pigment is obtained; however, it still contains a certain amount of bacteria.

The insolubility of the pigment in the majority of the common solvents is a striking property. So were tried in vain: ethanol, dioxane, carbon disulfide, acetone, methanol, butanol, benzene, toluene, petroleum ether, ether, carbon tetrachloride and chloroform. The crystals are soluble in hot quinoline, producing a red colour, in formamide and pyridine with a bright violet colour, but the amount of pigment in solution was very small. The formamide-solution is decomposed within 24 hr to an orange-brown solution. In 96% sulfuric acid

the pigment produces an orange colour. Upon dilution with water the solution becomes blue; after some time a violet precipitate is formed. In 0.1 N NaOH the crystals dissolve rapidly with a violet colour, but within a few minutes the solution turns brown and later on decolorizes. In 5 N HCl hardly any pigment is dissolved; when heated a yellow colour is produced.

As recrystallization was difficult because of the poor solubility, purification of the pigment was tried along other lines. To remove the bacteria from the crystals the crystal suspension was treated with lysozyme and with hemicellulase but with little success. Yet this process combined with washing the crystals with NaCl and Tween 60 solutions produced a somewhat purified pigment. Quantitative analysis of the purified pigment gave the following results: carbon 47.04%, hydrogen 3.94%, nitrogen 18.80% and oxygen 27.84%. In a Kofler melting point microscope the crystals were heated to 320 C without melting or decomposition phenomena. It was not possible to register the ultraviolet spectrum because of the strong absorption of pyridine, the only suitable solvent. Only the spectrum in visible light was recorded and this showed a peak at 580.8 m$\mu$. To obtain an infrared spectrum the method of the KBr plate had to be used.

Comparing the properties and the spectrum with those published by Sommer, Silver and Vining (1961) and others, it could be concluded that the pigment crystals are identical with indigoidine. Only the visible absorption peak at 580.8 m$\mu$ in pyridine differs from that of indigoidine (605 m$\mu$).

### *The soluble pigment*

In contrast to the colony crystals, the material which is responsible for the blue colour of the agar is readily soluble. The solubility in water is obvious because the colour rapidly diffuses through the whole plate. It is also possible to extract the pigment from the nutrient medium with alcohol, methanol, glycerol, glycol, acetone, propanol or pyridine. The same holds for acetic anhydride, acetic acid, mesityloxide or formic acid, but in these cases the solution is not stable and decolorizes rather quickly. The pigment is less soluble in methylethylketone and *sec*-butanol and insoluble in carbon disulfide, carbon tetrachloride, benzene and other non-polar solvents. The pigment is extracted from the agar with alcohol after the colonies are scraped off. The remaining bacteria clot together in the alcohol and can be removed by filtration over paper. A deep violet solution is obtained which is red in transmitted light, but which also contains many other substances originating from the agar.

Attempts to concentrate the solution by evaporation in vacuo with gentle

heating led to decomposition of the rather unstable pigment. The alcoholic solution also slowly decolorizes when air is bubbled through and the bottle is placed in artificial light for some days.

Upon treatment of the alcoholic solution with alcoholic NaOH a blue salt precipitates and the liquid becomes light green. This salt is soluble in water, glycerol, glycol, propyleneglycol, lower carboxylic acids and their anhydrides, but not in alcohol, ether, acetone, $CS_2$, $CCl_4$, benzene and ethylacetate. By recrystallization the purity of the salt was not much improved. Treatment with an Amberlite cation-exchanger to replace $Na^+$ by $H^+$ resulted in a strong fall of the pH with subsequent destruction of the pigment. The salt dissolves in 96% sulphuric acid with an orange colour, just like the crystals from the colonies.

Microanalysis proved that the salt contains both nitrogen and sulphur. By means of a method given by Feigl (1960) it was shown that the sulphur is probably present as a sulphonic acid group; this explains the water-solubility of the pigment. The infrared spectrum of the (impure) salt was very obscure. The violet solution obtained by extracting the agar with alcohol or water becomes immediately colourless upon treatment with bromine water, an indication of the presence of olefinic double bonds.

Several bacterial pigments have initially been supposed to consist of only a simple compound whilst further investigation showed them to be a mixture of closely related compounds. *Serratia marcescens* with its numerous pigments furnishes a beautiful example (Williams, 1956).

Starting from the violet alcoholic solution it was tried to determine chromatographically the number of pigments present. After some unsuccessful efforts at last a suitable chromatographic separation method was found. The applied techniques were combinations of column adsorption chromatography and ion-exchange. It was not always easy to judge which of these two processes was the more important in a given case. By these methods it could be shown that we were dealing with a pigment mixture of at least three components. Two typical experiments will be described here.

In the first one, adsorption chromatography was mainly applied.The alcoholic pigment solution was dried over anhydric sodium sulphate and 10 ml was poured on a column of about 20 cm length, prepared from diethylaminoethylcellulose (DEAE) suspended in absolute alcohol. By elution for some time with absolute alcohol light yellow impurities were separated and driven out of the column. These impurities showed a strong fluorescence in ultraviolet light. The elution was continued with alcohol with an increasing percentage of water, then with pure water and subsequently with water, containing an increasing amount of pyridine. In the course of this elution at least three different blue

and violet zones were observed, moving downwards one after another with the various elution liquids.

In the second experiment the anion-exchanging capacities of the DEAE played a more dominating role. The DEAE, a weak base, was brought in the form of the HCl-salt with 0.01 N HCl. The excess HCl was removed by washing; the anion-exchanger was precipitated by centrifugation and again washed until a negative $Cl^-$ reaction had been obtained. The column was filled and the aqueous pigment solution was poured upon it. Elution was accomplished by an acetate buffer solution (pH 5) or with a 0.1 N NaCl solution. Shortly after the beginning of the elution at least three violet and blue bands were observed. With more efficient columns the coloured zones might possibly have been separated into still more components.

Although the presence of several pigments in the agar is likely, still the possibility must not be excluded that there is only one soluble pigment, the others being degradation products formed by decomposition reactions during the extraction process.

## TAXONOMY AND DISCUSSION

The morphological and physiological characters indicate rather clearly that this organism has to be classed with the genus *Arthrobacter*. In the VIIth Edition of Bergey's Manual of Determinative Bacteriology, Lochhead has subdivided this genus into two groups; the first group includes the species which can grow on a medium with an inorganic nitrogen source and besides on a medium with citrate as sole carbon source. The organisms of the other group lack these properties. Following this classification it is impossible to place our organism in one of the groups as it attacks citrate even in the absence of vitamins, while nitrate is only used as sole nitrogen source when biotin has been added.

Our experiments have proved that all species of the first group can actually grow on inorganic nitrogen and citrate without addition of vitamins.

Recently Kuhn and Starr (1960) have described a blue microbe, *Arthrobacter atrocyaneus*, which produces the pigment indigoidine. Studying an authentic culture of *A. atrocyaneus* we observed that citrate was not attacked (in accordance with the description) even with added vitamins. In the absence of vitamins *A. atrocyaneus* does not grow with inorganic nitrogen, but the development is good on the same medium with biotin (not mentioned in the original description). This makes it obvious, that *A. atrocyaneus* is more closely related to the second group of the nutritionally more exacting species than our organism. E. G. Mulder (Wageningen) has informed the authors (personal communi-

cation), that he too, has isolated *Arthrobacter* species, which cannot be classified by means of the present key of the genus *Arthrobacter*. Thus it is clear that a revision of the determinative key is necessary.

Other properties have been compared with those of *Arthrobacter atrocyaneus* since this species most closely resembles ours. We have found that, just like our organism, *A. atrocyaneus* needs biotin for the production of indigoidine. Our organism differs from *A. atrocyaneus* in liquefying gelatin, in being non-motile, in growing at 10 C and in producing the diffusable pigments. This last fact is illustrated in Fig. 17, showing growth of *A. atrocyaneus* and of

Fig. 17. Comparison of *A. atrocyaneus* (left) and *A. polychromogenes* (right) on YDC agar.

*A. polychromogenes* on YDC-agar. The same result was obtained on the PKGl medium, pH 9. On the media urea broth (Difco, B 272), and urea agar base (Difco, B 283), for the demonstration of urease, *A. atrocyaneus* is strongly positive (contrary to the original description), and our organism negative. Remarkably enough, the new blue organism can grow with added vitamins on urea as nitrogen source, so urease is yet present. Serologically it has been established with the antiserum of the new species, that the latter differs from *A. atrocyaneus*. It was difficult to make an antiserum of *A. atrocyaneus*, for this organism gave a cross-reaction with the normal sera of all tested rabbits till a dilution of 1 : 500.

The second blue pigmented *Arthrobacter* species is *A. oxydans* (Sguros, 1955), which produces only on nicotine agar a diffusable blue pigment which turns red to yellow-brown with age. Some differences between our strain and *A. oxydans* are the absence, with our strain, of a reaction with litmus milk and the very scant growth on nicotine agar.

The organism described here exhibits a number of properties that clearly distinguish it from the previously named *Arthrobacter* species, so we are of the opinion that this blue microbe is a new species for which we propose the name *Arthrobacter polychromogenes.*

## A BACTERIOPHAGE OF *Arthrobacter polychromogenes*

When cultivating the bacterium in large amounts for the isolation of the pigments, we had repeatedly difficulties owing to the appearance of plaques (Fig. 18).

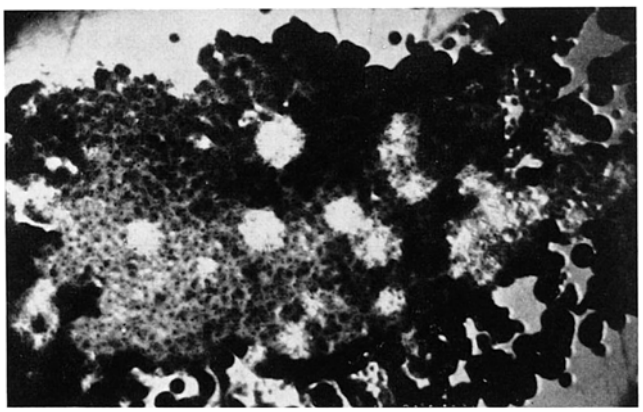


Fig. 18. Plaques caused by bacteriophage in bacterial culture on PKGl agar. Photograph in transmitted light. Where the colonies have disappeared only the indigoidine crystals are left as little black dots.

Bacteriophages of members of the genus *Arthrobacter* have been reported already twice. Robinson and Corke (1959) observed plaques when soil perfusates were plated with *Arthrobacter* species and Gillespie (1960) isolated two bacteriophages of *Arthrobacter globiformis*, but did not study their morphology.

Experiments dealing with the properties of the bacteriophage of *Arthrobacter polychromogenes* and some information about its morphological structure will be presented in the following.

### *Methods*

Bacteriophage preparations were made by suspending several of the lytic areas in PKGl-liquid. After some shaking, these suspensions were Seitz-filtered; the filtrates were stable in the refrigerator for at least ten months.

The titre of the phage filtrates was determined by the agar-layer method of Adams (1950).

The indicator, i.e. sensitive strains were the offspring of the spontaneous plaque-forming cultures. To detect clearly sensitive strains the spray-technique of Stolp (1957) was used. By means of an asthma-sprayer the bacteriophage filtrate was sprayed over a 24 hr plate-culture of the bacterium.

Filtrates with a titre of $10^8$–$10^9$ particles/ml for electron microscopy were prepared in the following way. Lytic areas were scraped from the agar and suspended in PKGl-liquid; ammonium sulphate was added to a concentration of 3 M. Subsequently the phages were precipitated by centrifuging at ± 15000 rev/min. The pellet was resuspended in 0.01 M ammonium acetate, pH 7.4 and adsorbed on a diethylaminoethylcellulose anion exchange column. The phages were eluted from the column by 0.1 M ammonium acetate (Hall et al., 1959).

## *Results and discussion*

Although differently formed plaques were sometimes distinguished, these turned out to be caused by the same bacteriophage. On testing several strains of *Arthrobacter polychromogenes* many strains proved to be resistant, perhaps lysogenic, and a very small number were sensitive strains. The latter were always those strains which produced pigment most strongly whereas the resistant strains were much less coloured.

The "resistant" strains were investigated on lysogenicity by means of the above mentioned method of Stolp. 24 hr old liquid cultures of the "resistant" strains were sprayed with an asthma-sprayer on 24 hr old cultures of the indicator strains. As never any plaques appeared it seemed possible, that the "resistant" strains contained not enough free particles to be detected in this manner. Therefore we tried to concentrate them.

Great quantities of liquid cultures freed from bacteria by Seitz-filtration were centrifuged at ± 15000 rev/min for 0.5 hr. When the pellets were tested on the indicator strains by the method of Stolp, never any plaques were formed. Possibly the number of bacteria in these strains, which go into spontaneous lysis, is so small, that also in this way no plaques can be found.

Lastly ultraviolet light was applied to induce the "resistant" strains (Lwoff et al., 1950). The strains to be irradiated were grown for 24 hr in PKGl-liquid. The cultures were diluted to $10^{-3}$ and 2–3 ml of these suspensions were spread in Petri dishes, which were irradiated for 100, 150 or 200 sec. The dishes were shaken during the irradiation at a distance of 25 cm from a Philips TUV 25 W lamp. Several fractions were tested again by the method of Stolp on the in-

dicator strains. With the last technique it turned out, that some of the tested strains were actually lysogenic.

When a phage filtrate was sprayed on a culture of *Arthrobacter atrocyaneus* plaques were never observed.

The results of electron microscopy have clearly demonstrated the presence of a bacteriophage. A detailed description and pictures are presented in the following article by Daems (1963).

The chief features of the bacteriophage of *Arthrobacter polychromogenes* are the hexagonal prismatic head ($\pm$ 750Å $\times$ $\pm$ 630Å), the tail sheath with a length of $\pm$ 2200Å, on which a cross striation is visible (spacing $\pm$ 35Å). At the end of the tail sheath an anchor-like structure can be observed.

The authors are indebted to Prof. Dr. T. Y. Kingma Boltjes for his help and valuable suggestions. We thank Mr. H. van Noord for his assistance with the serology.

*Received* 3 *August* 1962

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