

Formation of the Blue Pigment Indigoidine by Phytopathogenic *Erwinia*

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ABSTRACT

STARR, MORTIMER P. (University of California, Davis), GLADYS COSENS, AND HANS-JOACHIM KNACKMUSS. Formation of the blue pigment indigoidine by phytopathogenic *Erwinia*. *Appl. Microbiol.* 14:870-872. 1966.—Most cultures belonging to the "*Erwinia chrysanthemi* group" of soft-rot bacteria form traces of a blue, extracellular, water-insoluble pigment. This pigment is generally not found in cultures of the other members of the genus *Erwinia*. The blue substance has been isolated and purified from three members of the *E. chrysanthemi* group; it has been identified as indigoidine, 5,5'-diamino-4,4'-dihydroxy-3,3'-diazadiphenone-(2,2').

Indigoidine is the characteristic blue pigment produced by the alfalfa wilt pathogen *Corynebacterium insidiosum* (18). This pigment, which was first found in *Pseudomonas indigofera* (3), is produced also by *Arthrobacter atrocyaneus* (7) and *A. polychromogenes* (16). The pigments formed by the aforementioned organisms have been shown to be identical, and the structure of indigoidine was recently proven to be 5,5'-diamino - 4,4' - dihydroxy - 3,3' - diazadiphenone-(2,2') (8, 9).

We have often noted the formation of a small amount of extracellular blue pigment in cultures of certain pectinolytic *Erwinia*. This group of cultures could be called the "*Erwinia chrysanthemi* group," since the majority appear to be similar to *Erwinia chrysanthemi* (1). The nomenclature of this group is in a state of confusion, and members of it are variously designated: *Erwinia chrysanthemi* f. sp. *philodendroni* (11); *Erwinia carotovora* f. sp. *parthenii* var. *dianthicola* (4); *Erwinia cytolytica* (2); the corn stalk rot pathogen called *Erwinia carotovora* f. sp. *zeae* (13, 14, 15) or *Erwinia maydis* (6); the pathogen(s) of *Dieffenbachia* which is referred either to *Erwinia dieffenbachiae* (10) or to *Erwinia chrysanthemi* (12); and *Erwinia carotovora* f. sp. *parthenii* (17), which is considered by some workers to be in this group.

It is the purpose of the present communication to report that this extracellular insoluble blue pigment produced by these pectinolytic *Erwinia* cultures is indigoidine, identical to that formed by taxonomically unrelated coryneform and pseudomonad bacteria.

CULTURES STUDIED

Forty-eight cultures broadly representative of the "*Erwinia chrysanthemi* group" were included in this study; 11 of these cultures were *E. carotovora* f. sp. *parthenii*, which is not universally accepted as belonging to this group. These cultures, labeled with the several names given above, are part of the International Collection of Phytopathogenic Bacteria (ICPB, Department of Bacteriology, University of California, Davis, M. P. Starr, Curator). Every one of the 48 cultures is motile and each is able to liquefy a calcium pectate gel (17).

A blue pigment is formed by three of four isolates from Malayan pineapple (5) labeled *Erwinia carotovora* and possibly belonging to that species rather than to *E. chrysanthemi*. With this possible exception, only members of the *E. chrysanthemi* group have ever been observed to form a blue pigment in the course of routine examinations of the hundreds of old and new accessions of *Erwinia* spp. in the International Collection of Phytopathogenic Bacteria. However, not every strain belonging to the *E. chrysanthemi* group forms the blue pigment. In the present study, every one of the 11 strains of *E. carotovora* f. sp. *parthenii* [all from a single outbreak of the guayule disease (17)], 3 of the 23 strains labeled *E. chrysanthemi*, and 2 of the 5 strains of *E. dieffenbachiae* did not produce pigments. Each of the remaining 32 cultures of the *E. chrysanthemi* group produced a small amount of an extracellular, water-insoluble, blue to gray substance when grown on YDC agar [Difco yeast extract,

1.0%; glucose, 2.0%; calcium carbonate (USP light powder no. 4052 of Mallinckrodt), 2.0%; Difco agar, 1.5%]. In most cases, the blue pigment occurred transiently only in young cultures and soon disappeared. This behavior is in contrast to the increase in pigment with the age of the culture seen in the indigoidine-producing coryneform and pseudomonad bacteria (7-10). Possible explanations for these differing kinetics might be sought in differing biosynthetic pathways (M. P. Starr and C. A. Westby, *Bacteriol. Proc.*, p. 17, 1964) or in differing control of pigment degradation via the soluble, and presumably metabolizable, leucoform of the pigment (9).

From this group of 32 cultures which form traces of extracellular insoluble blue pigment, three cultures were selected for further study on the basis of above average pigment production. It must be emphasized, however, that the quantity of blue pigmentation in any of these cultures is very small—an order of magnitude below that of ordinary wild-type cultures of *C. insidiosum* (18).

PRODUCTION OF CRUDE PIGMENT

The three cultures intensively studied were ICPB-EM108, *Erwinia maydis*, isolated from corn stalk rot by Kelman in 1957; ICPB-EC176, *Erwinia chrysanthemi*, isolated from slow wilt of carnations by Bakker and Scholten in Holland; and ICPB-EC207, *Erwinia cytolytica*, isolated by Lazar from dahlia in Roumania in 1962.

Culture media. *E. maydis* was grown on YDC agar poured in thick layers into 100-mm petri dishes. *E. chrysanthemi* and *E. cytolytica* were grown on potato-glucose-peptone-agar prepared as follows. Sound potatoes were scrubbed, peeled, sliced, and diced; 200 g of the diced potatoes was placed in 1,000 ml of distilled water and cooked in an autoclave for 1 hr at 5 psi of steam pressure. The potato infusion was filtered through cheesecloth, and the volume was restored to 1,000 ml. To 1,000 ml of potato infusion were added 10 g of glucose, 10 g of peptone (Difco), and 20 g of agar (Difco). The pH was adjusted to 7.2, and the medium was sterilized by autoclaving. The medium was then poured in thick layers into 100-mm petri dishes.

Inocula. Selected pigmented colonies of the three organisms were suspended in sterile distilled water in test tubes. Six to eight streaks were made on each plate with a transfer loop.

Incubation. Plates inoculated with *E. maydis* were incubated at room temperature (ca. 23 to 25 C) for 4 days. (Longer incubation resulted in production of a gritty material which centrifuged

in a layer under the pigment and made separation of the pigment difficult.) *E. chrysanthemi* and *E. cytolytica* were incubated at room temperature for 3 or 4 days, and the plates were then stored in a refrigerator at 4 C for 24 hr. The plates were scraped, allowed to incubate at room temperature, and then scraped a second time. This procedure tended to increase the meager yield with these two strains.

Pigment harvest. The pigmented cell mass was scraped from the medium with a bent glass rod. The material was suspended in water and agitated on a rotary shaker for 5 to 10 min. The extracellular pigment was then separated from the bacterial cells by centrifugation in a Servall SS-3 centrifuge, with the use of a GSA head and 250-ml bottles. This centrifuge, used for the initial separation of a large amount of cells from a small quantity of pigment, has the advantage of reaching its maximal speed slowly. The first centrifugation reached a final speed of 6,000 rev/min in a period of about 15 min. The cells were washed away, and the small quantities of pigment were washed with water into 40-ml centrifuge tubes. The centrifugation was continued in a Servall SP-X centrifuge. The second centrifugation was started at a setting of 3,000 rev/min, and, after 15 min at that speed, the final packing was completed at the top speed of 5,000 rev/min. The cell layers were decanted and the pigment was re-suspended in water. This procedure was repeated about five times, until there was no longer an upper layer of white bacterial cells above the lower layer of blue-black pigment. The pigment was kept frozen until it was lyophilized.

Yield of crude pigment. The average yield with *E. maydis* was 0.28 mg of crude pigment per petri plate, based on a harvest of 1,120 plates. The average yield from 110 plates of *E. chrysanthemi* was 0.2 mg of extremely crude pigment per plate, and the average yield from 120 plates of *E. cytolytica* was 0.36 mg of extremely crude pigment per plate.

PURIFICATION OF PIGMENTS

The indigoidine contents of the three crude pigments were then determined. This was done by dissolving them in dimethylformamide (DMF), filtering, and estimating the concentration spectrophotometrically at 602 $m\mu$ (authentic λ_{max} , 602 $m\mu$; log ϵ , 4.37). The *E. maydis* culture gave the best quality crude pigment of the three cultures used. This is well demonstrated by a 30.0% indigoidine content in the crude pigment and a pigment production amounting to 84.0 μg per petri plate. For *E. chrysanthemi*, the percentage of

indigoidine in the crude pigment was found to be 3.5%. Calculated in terms of yield per plate, only 7.0 μg of pure indigoidine was produced per petri plate. For *E. cytolytica*, with an indigoidine content of 1.6% in the crude pigment, the pigment production amounted to only 5.7 μg per plate. The pigment particles from this latter organism were surrounded by bacterial slime, a factor which may have contributed to our lack of success in its purification.

Since the pigments from the three *Erwinia* cultures are very crude as compared with the pigment produced by the pseudomonads and coryneforms, it was necessary to modify the purification procedure used earlier (9). To minimize the time the pigment stays in the unstable leucoform, a large excess of the sodium dithionite must be avoided.

The lyophilized, pigment-containing material (ca. 500 mg) was suspended in 100 ml of saturated sodium bicarbonate solution. The sodium dithionite was added in small quantities until most of the indigoidine was dissolved. The solution was quickly filtered through silica gel; the pigment was reoxidized with oxygen, separated by centrifugation, and then washed with 1 N acetic acid.

IDENTIFICATION OF THE PIGMENTS

All visible absorption spectra were obtained with a Cary recording spectrophotometer (model 14). Infrared tracings were made with a Perkin-Elmer Infracord spectrophotometer.

E. maydis ICPB-EM108. The purified pigment was identified as indigoidine by its visible absorption spectrum in DMF [λ_{max} , 599 $m\mu$; $\log \epsilon$, 4.26; with a weak shoulder at 570 $m\mu$ due to impurities (authentic λ_{max} , 602 $m\mu$; $\log \epsilon$, 4.37)] and by its infrared spectrum. The properties of the diacetyl derivative of this substance are identical with those of authentic diacetyl indigoidine prepared by the same procedure (9). The visible absorption spectrum of the crude diacetyl derivative (yield 75%) exhibited a maximum in DMF at λ_{max} , 510 $m\mu$; $\log \epsilon$, 4.34 (authentic λ_{max} , 511 $m\mu$; $\log \epsilon$, 4.39). Although a small amount of impurities was still present, an infrared spectrum was obtained which is identical to that of the authentic diacetyl indigoidine.

E. chrysanthemi ICPB-EC176. Purification of the pigment by the dithionite method yielded only a very small amount of material. For this reason, the pigment was identified only by its visible absorption spectrum in DMF (λ_{max} , 601 $m\mu$) and by its infrared spectrum, which is identical to the authentic indigoidine.

E. cytolytica ICPB-EC207. It was impossible to purify this sample by the dithionite method because of the low content of pigment; therefore,

the pigment was identified as indigoidine only by its visible absorption spectrum in DMF (λ_{max} , 600 to 601 $m\mu$).

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LITERATURE CITED

- BURKHOLDER, W. H., L. A. MCFADDEN, AND A. W. DIMOCK. 1953. A bacterial blight of chrysanthemums. *Phytopathology* **43**:522-526.
- CHESTER, F. D. 1938. A bacteriosis of dahlia, *Erwinia cytolytica*. *Phytopathology* **28**:427-432.
- ELAZARI-VOLCANI, B. 1939. On *Pseudomonas indigofera* (Voges) Migula and its pigment. *Arch. Mikrobiol.* **10**:343-358.
- HELLMERS, E. 1958. Bacterial stunt of carnation. (*Pectobacterium parthenii* var. *dianthicola* var. n.). *Dansk Botanisk Arkiv* **18**:95-133.
- JOHNSTON, A. 1957. Pineapple fruit collapse. *Malayan Agr. J.* **40**:253-263.
- KELMAN, A., L. H. PERSON, AND T. T. HERBERT. Bacterial stalk rot of irrigated corn in North Carolina. *Plant Disease Repr.* **41**:798-802.
- KUHN, D. A., AND M. P. STARR. 1960. *Arthro-bacter atrocyaneus*, n. sp., and its blue pigment. *Arch. Mikrobiol.* **36**:175-181.
- KUHN, R., H. BAUER, AND H.-J. KNACKMUSS. 1965. Struktur und Synthesen des Bakterienfarbstoffs Indigoidin. *Chem. Ber.* **98**:2139-2153.
- KUHN, R., M. P. STARR, D. A. KUHN, H. BAUER, AND H.-J. KNACKMUSS. 1965. Indigoidine and other bacterial pigments related to 3,3'-bipyridyl. *Arch. Mikrobiol.* **51**:71-84.
- MCFADDEN, L. A. 1961. Bacterial stem and leaf rot of *Dieffenbachia* in Florida. *Phytopathology* **51**:663-668.
- MILLER, H. N., AND L. A. MCFADDEN. 1961. A bacterial disease of philodendron. *Phytopathology* **51**:826-831.
- MUNNECKE, D. E. 1960. Bacterial stem rot of *Dieffenbachia*. *Phytopathology* **50**:696-700.
- SABET, K. A. 1954. Etiological aspects of the bacterial root and stalk rot disease of maize. *Cairo Univ. Fac. Agr. Bull.* **48**:3-17.
- SABET, K. A. 1954. A new bacterial disease of maize in Egypt. *Empire J. Expt. Agri.* **22**:65-67.
- SABET, K. A., A. S. SAMRA, AND H. A. EL-SHAFFEY. 1964. On the systematic position of the coliform bacteria responsible for stalk rot of maize. *Ann. Appl. Biol.* **53**:267-274.
- SCHIPPERS-LAMMERTSE, A. F., A. O. MUISERS, AND K. B. KLATSER-OEDEKERK. 1963. *Arthro-bacter polychromogenes* nov. spec., its pigments and a bacteriophage of this species. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **29**:1-15.
- STARR, M. P. 1947. The causal agent of bacterial root and stem disease of guayule. *Phytopathology* **37**:291-300.
- STARR, M. P. 1958. The blue pigment of *Corynebacterium insidiosum*. *Arch. Mikrobiol.* **30**:325-334.