Production of a Naphthoquinone Pigment by a Species of Streptoverticillium and Its Accumulation by a Streptomycete

H. D. TRESNER, JEAN A. HAYES, AND D. B. BORDERS

Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York 10965

Received for publication 21 December 1970

A naphthoquinone pigment produced by a species of Streptoverticillium was accumulated by a Streptomyces sp. when the two organisms were grown in mixed cultures.

Microorganisms developing on soil dilution plates frequently exert strong competitive influences on each other. These may appear as growth inhibition, alteration of colonial morphology, or induction of crystals or abnormal pigments. An interesting influence of the latter type was observed among organisms from certain prairie soils of the midwestern United States.

Some colonies of streptomycetes and occasionally members of the Eubacteriales, grown on agar plates, were pigmented dark blue to blue-black. Usually these colonies were restricted in size and appeared in clumps as large as about 1 cm in diameter. In the center of each clump were one or more unpigmented organisms (Fig. 1) which proved to be a single species of Streptoverticillium (Lederle BL845). The dark pigmented colonies, when isolated on new media, never displayed the blue pigmentation; however, if they were inoculated in proper juxtaposition with the Streptoverticillium (i.e., within a 1-cm radius) they developed a dark bluish coloration within 48 hr that intensified for about 4 days; those nearest were pigmented most deeply. Cultures placed immediately adjacent to the Streptoverticillium sp. sometimes failed to grow.

A nonantagonistic Streptomyces sp. (Lederle BL922) was chosen for pure-mixed culture studies in liquid media with the Streptoverticillium sp. After several initial attempts failed to produce the pigment, it was learned that very precise fermentation conditions were required. Among the important influencing factors were the medium, ratio of medium volume to flask volume, preparation of the inoculum, ratio of size of the two inocula, length of fermentation, and method of agitation. The most satisfactory fermentations resulted under the following conditions. Seed inocula of the two organisms were grown separately in 35 ml of Difco AC broth in 250-ml Erlenmeyer flasks for 48 hr on a reciprocal shaker at 28 C. The resulting mycelial fragments were washed three times aseptically with saline to remove any antibiotic substances. The ratio of inocula appeared to be the most critical factor in mixed fermentations. If cells of the Streptoverticillium sp. were increased even slightly or if they were not washed free from antibiotic activity, they quickly dominated the fermentation. The two inocula were combined in fresh AC broth (35 ml per 250-ml flask) in the ratio of 1 ml of Streptoverticillium sp. (BL845; 8.0 x 10^6 growth centers/ml) to 4 ml of Streptomyces sp. (BL922; 2.3 x 10^6 growth centers/ml) and incubated under the same conditions as the seed.

After 22 hr of fermentation, the filtrate appeared as a maroon color which changed to green by 24 to 28 hr. [Green color resulted from a visual color modification of the dark blue pigmented cells of Streptomyces sp. (BL922) by the yellow color of the medium.] Pigmented cells were finely divided, typically like those of the Streptomyces sp. (BL922); a small proportion of nonpigmented pellets typical of the Streptoverticillium sp. (BL845) were also present. When the Streptoverticillium sp. was fermented alone, a maroon color was produced in the medium in small amounts; however, its production was greatly stimulated in mixed fermentations.

To isolate the pigment, cells were washed with water, suspended in 1 N HCl, and extracted by stirring the suspension with benzene for 30 min. The extract was separated from the aqueous suspension, washed with water, and evaporated to a dark residue, which was washed with ether to remove lipid impurities. The remaining material sublimed at 150 to 160 C, 0.05 mm of Hg, to give a maroon powder: melting point ~250 C sub-
VOL. 21, 1971

limes, m/e 250.0482 (calculated for C₁₂H₁₀O₆: 250.04773); ultraviolet maxima in methanol, 540 nm (log ε 3.69), 505 nm (3.87), 475 nm (3.80), 308 nm (3.89), 280 nm (3.87); δ, 3.95 (two OCH₃ singlet), 6.42 (2H singlet), 12.71 (OH singlet), 13.15 (OH singlet).

The chemical and physical properties of the pigment indicated that it was 5,8-dihydroxy-2,7-dimethoxy-1,4-naphthoquinone (1, 2), which was previously reported to be produced by a *Streptomyces* sp. (2).

**Fig. 1.** Unpigmented colony of *Streptoverticillium* sp. (Lederle BL845) in center (arrow) surrounded by several streptomycetes with dark blue coloration.

Solubility properties of the pigment and extraction procedures required to remove it from the cells suggested that it was bonded covalently to the cells. Although the pigment was soluble in dilute alkali, benzene, or chloroform, it could not be extracted from the cells with any of these agents individually or in combination. It was not significantly soluble in dilute aqueous acid, but it could be extracted from the cells into benzene or chloroform in the presence of aqueous acid. This could possibly result from release of the pigment from the cells through hydrolytic cleavage of a glycosidic-like bond linked through a phenolic hydroxyl group of the pigment which would be stable in base but unstable to mild acid.

When the *Streptomyces* sp. BL922 was grown in close proximity to crystals of the maroon compound placed on agar media, the pigment accumulated in the cells and produced the original dark blue coloration of the mixed cultures.

Other accounts of mixed-culture interactions that stimulate production of metabolites have been reported (3–5). Probably the phenomenon is not rare, but is seldom recognized for lack of detection methods.

We thank W. Fulmor and staff for spectra data.

**LITERATURE CITED**

1. Chang, C. W. J., R. E. Moore, and P. J. Scheuer. 1964. The structure of Spinochrome M. J. Amer. Chem. Soc. 86:2959.
2. Gerber, N. N., and B. Wieclawek. 1966. The structures of two naphthoquinone pigments from an actinomycete. J. Org. Chem. 31:1496.
3. Hill, J. C., and G. T. Johnson. 1969. Microbial transformation of phenazines by *Aspergillus sclerotiorum*. Mycologia 41: 452–467.
4. Patrick, Z. A., and M. Schlifer. 1970. Induction of pigmentation of *Thielaviopsis basicola* and other fungi by a bacterium isolated from soil. Can. J. Bot. 48:1879–1886.
5. Shinobu, R., and N. Kanda. 1970. *Streptomyces propurpuratus* nov. sp., a new streptomycete which produces a soluble deep purplish-red pigment in mixed culture with the other microorganisms. J. Antibiot. 23:125–130.