Chromogenesis mirabilis in *Streptomyces griseus*

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A number of chromogenic *Streptomyces*, producing diffusible melanoid pigment on complex organic media, fail to form melanin pigment on conventionally used synthetic tyrosine agar. By means of our new melanin formation test, almost all the chromogenic streptomycetes can now be detected in chemically defined medium. In contrast to ordinary chromogenic streptomycetes, two streptomycete species of the International Streptomyces Project, *S. griseus* ISP 5236 and *S. ornatus* ISP 5307, produce melanin pigment only on synthetic tyrosine agar, without showing chromogenicity on complex organic media. From the results obtained with *S. griseus* ISP 5236 and *S. phaeochromogenes* ISP 5073, it was revealed that melanin formation by *Streptomyces*, in general, is inhibited by L-cysteine present in organic nitrogen sources incorporated into natural media. Most chromogenic species of streptomycetes produce a higher level of tyrosinase and rapidly utilize L-cysteine in the culture media which result in the manifestation of good chromogenicity on natural media. Peculiarity of chromogenicity of *S. griseus* and *S. ornatus* might be due to the lower ability to produce tyrosinase and to utilize L-cysteine in the culture medium.

The previous paper from our laboratory described a rapid and simplified method for the detection of melanin formation of streptomycetes and the results of comparative studies on the production of diffusible dark brown pigment on complex organic media such as peptone-yeast extract-iron agar and nutrient agar, synthetic tyrosine agar, and the in vitro melanin formation test (1).

According to the studies on 71 streptomycetes cultures used in the collaborative research of the International Streptomyces Project (10, 11), using the above-mentioned method, 48% of the cultures did not produce dark brown diffusible pigment on complex organic media and gave a negative reaction to the present melanin formation test with either L-tyrosine or 3,4-dihydroxyphenyl-L-alanine (L-DOPA) as a substrate, while 23% produced dark-brown soluble pigment on complex organic media as well as on synthetic tyrosine agar and gave a positive reaction to the melanin formation test with either L-tyrosine or L-DOPA as a substrate. However, a number of cultures failed to produce such pigment on synthetic tyrosine agar in spite of their chromogenicity on complex organic media. Most of these cultures gave a positive reaction to the melanin formation test when L-DOPA instead of tyrosine was employed as a substrate. On the other hand, two cultures, *S. griseus* ISP 5236 (IMRU 3464) (12) and *S. ornatus* ISP 5307 (INTA 17044) (3) exhibited chromogenicity on synthetic tyrosine agar, but not on complex organic media. Nevertheless, they gave a positive reaction to the melanin formation test with L-DOPA, as in the case of the all chromogenic species of streptomycetes. In addition to this peculiar chromogenicity, both cultures were found to be closely related to each other mycologically. Detailed comparative studies on these two species revealed that *S. ornatus* ISP 5307 is a synonym of *S. griseus*, although they have been reported to elaborate different antibiotics. The strange chromogenicity of these cultures was also ascribed to the inhibitory effect of peptone on the tyrosinase activity (7). This paper reports the results of further studies on this contradictory chromogenicity of *S. griseus*.

**MATERIALS AND METHODS**

*Test organisms and medium.* *S. griseus* ISP 5236 and *S. phaeochromogenes* ISP 5073 (ATCC 3338), maintained on glucose asparagine agar slants, were used. The complex organic media employed for the detection of diffusible dark-brown pigment (chromogenicity) were peptone-yeast extract-iron agar, and sometimes nutrient agar. The standard medium for our melanin formation test contained glycerol, 10 g; monosodium glutamate, 25 g; K₂HPO₄, 0.5 g;
MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 0.011 g; FeSO₄·7H₂O, 0.015 g; ZnSO₄·7H₂O, 0.013 g; CuSO₄·5H₂O, 0.005 g; and distilled water (pH 7.2), 1,000 ml. Medium (5 ml) was placed in a test tube, and one loopful of washed spore suspension was used as inoculum. Incubation was carried out stationarily at 27 C for 7 days. To test chromogenicity during the growth of test organism, 1% tyrosine was added in the medium. The effect of natural nitrogen sources was investigated in the same medium at the concentration of 1%.

**Chemicals.** For organic nitrogen sources, meat extract and casein (Wako Chemical Co., Tokyo), poly-peptone (Daigo Eiyo Co., Tokyo), yeast extract, vitamin-free Casamino Acid, peptone, tryptose peptone (Difco), and L-DOPA (Wako Chemical Co., Tokyo) were used.

**Melanin formation test.** The assay system for melanin formation consisted of 2 ml of the test fluid (usually culture filtrate of streptomycines in the standard medium), 2 ml of 0.1 M phosphate buffer (pH 5.9), and 1 ml of 0.4% substrate solution. L-Tyrosine or L-DOPA was used for the substrate. The reaction mixture was incubated at 37 C for 30 min for L-tyrosine and 5 min for L-dopa. Red coloration resulting from dopachrome formation was measured spectrophotometrically at 480 nm. The color changes from dark brown to black with time.

**Assay of tyrosinase.** The tyrosinase in the culture filtrates was assayed by the method developed by Pomerantz in 1964 (8). L-Tyrosine-3,5-'H was purchased from New England Nuclear Corp. Standard reaction mixture contained per tube: 0.5 ml of 10 x 10⁸ disintegrations per min of L-tyrosine-3,5-'H, 0.5 ml of 0.1 M phosphate buffer (pH 6.0), and 1 ml of culture filtrate. After incubating these mixtures at 37 C for 30 min, the reaction was stopped by the addition of 1 ml of 2 N metaphosphate. Rates obtained by assaying water-¹H were corrected for counts obtained in boiled enzyme controls. The acidified reaction mixture was added onto a 1.0-cm column packed with a mixture of 100 mg of activated carbon (Norit A) and 500 mg of celite (no. 555). The column was washed with water to aquire 20 ml of effluent liquid material. A sample (0.25 ml) was dissolved in 10 ml of dioxane scintillation fluid (2) and counted by Beckman LS-150 liquid scintillation system.

**Determination of cysteine.** The amounts of L-cysteine in the culture fluid of test organisms were determined by a Kassell and Brown photometric micromodification (5) of the Folin-Legg method. A wavelength of 720 nm was used for measuring the absorption by reduced phosphotungstic acid reagent.

**RESULTS**

The effect of peptone concentration in the melanin formation medium is shown in Table 1. Melanin formation by *S. griseus* was inhibited by the addition of peptone at concentrations of 0.75% or more. The inhibitory effects of other natural nitrogen sources are compared in Table 2. All the nitrogen sources except casein inhibited the melanin formation by the test organism. The inhibitory principle in these natural nitrogen sources was assumed to be an amino acid or a combination of amino acids. In Table 3, the effects of 20 amino acids on the growth

| Amino acid         | Growth | Chromogenicity |
|--------------------|--------|----------------|
| Glycine            | ±      | ±              |
| DL-Alanine         | +      | +              |
| DL-Valine          | +      | +              |
| L-Leucine          | +      | +              |
| L-Isoleucine       | +      | +              |
| DL-Phenylalanine   | +      | +              |
| L-Serine           | ±      | ±              |
| DL-Threonine       | +      | +              |
| L-Ornithine        | +      | +              |
| L-Lysine           | ±      | ±              |
| L-Citulline        | +      | +              |
| L-Arginine         | +      | +              |
| L-Asparagine       | +      | +              |
| L-Glutamine        | +      | +              |
| L-Cysteine         | ±      | ±              |
| L-Cystine          | +      | +              |
| DL-Methionine      | +      | +              |
| L-Proline          | +      | +              |
| L-Tryptophan       | +      | +              |
| L-Histidine        | ±      | ±              |

*Concentration of amino acid, 0.1%.
and melanin formation of the test organism are illustrated. Only poor growth was obtained with glycine, L-serine, L-lysine, or L-histidine. Melanin formation was also scant when these amino acids. L-Cysteine completely inhibited the melanin formation, even though the amino acid supported moderate growth of the test organism. The amino acid was inhibitory to melanin formation at a concentration of $10^{-4}$ and $10^{-3}$ M, and complete inhibition was observed at a concentration of $10^{-2}$ M (Table 4). *S.* griseus as well as *S.* phaeochromogenes showed extensive chromogenicity in this standard medium without L-cysteine, but *S.* griseus failed to exhibit chromogenicity when $10^{-2}$ M L-cysteine was incorporated. To determine whether L-cysteine affects the induction or the activity of the enzyme, the effect of the amino acid on the melanin formation test with the culture filtrates of *S.* griseus and *S.* phaeochromogenes incubated in tyrosine-free standard medium was observed by employing L-DOPA as substrate. L-Cysteine affects oxidation of L-DOPA, and the inhibition is not restricted to the case of *S.* griseus (Table 5). Melanin formation by *S.* phaeochromogenes, a typical chromogenic species of *Streptomyces*, which produces diffusible dark-brown pigment on complex organic media as well as on synthetic tyrosine agar, was also affected, although the inhibitory concentration was 10 times higher with *S.* phaeochromogenes than with *S.* griseus. The culture filtrate of *S.* griseus in the standard medium for melanin formation test also inhibited the tyrosinase reaction by *S.* phaeochromogenes culture filtrate (Table 6). Therefore, it was assumed that more than the inhibitory concentration of L-cysteine is left unchanged in *S.* griseus culture filtrate, whereas in the culture filtrate of *S.* phaeochromogenes, L-cysteine is metabolized. The fate of L-cysteine in the culture media of *S.* griseus and *S.* phaeochromogenes is shown in Fig. 1, and the results clearly indicate that a considerable amount of the amino acid remains in the culture fluid of *S.* griseus, whereas the amount of amino acid is only negligible in the culture fluid of *S.* phaeochromogenes. The respective amounts of the enzyme in the culture fluid of *S.* griseus and *S.* phaeochromogenes are also compared. The enzymatic activity of the culture filtrate of *S.* phaeochromogenes was approximately 10 times as high as that of *S.* griseus.

**DISCUSSION**

Chromogenicity in streptomyces denotes the production of diffusible dark brown pigment on media containing various organic nitrogen sources. In consequence, the chromogenicity of streptomyces is subject to much fructuration because these medium ingredients are considerably varied. Chemically defined synthetic media, however, did not prove to be satisfactory, and a number of streptomyces showing typical chromogenicity on complex organic media failed to produce melanin pigment on these synthetic media. The melanin formation test developed by us was a useful method in solving the above discrepancy, but there still remain some species which show peculiar chro-

| L-Cysteine (molar concn) | Chromogenicity |
|--------------------------|---------------|
| $10^{-1}$                | -             |
| $10^{-2}$                | -             |
| $10^{-3}$                | ±             |
| $10^{-4}$                | ±             |
| $10^{-5}$                | +             |
| $10^{-6}$                | +             |
| Control                  | +             |

| L-Cysteine (molar concn) | Chromogenicity |
|--------------------------|---------------|
| $10^{-2}$                | +             |
| $10^{-3}$                | +             |
| $10^{-4}$                | +             |
| Control*                | +             |

* Without L-cysteine.

| Culture filtrate (ml) | Optical density at 480 nm |
|-----------------------|--------------------------|
| 3                     | 0.100                    |
| 2                     | 0.180                    |
| 1                     | 0.280                    |
| Control               | 0.420                    |
mogenicity, producing melanin pigment on synthetic medium, while lacking the pigment on complex organic medium.

The above experimental results presented ample evidence to account for the contradictory chromogenicity of *S. griseus*. Melanin formation by *S. griseus* is inhibited by the addition of complex organic nitrogen sources because they contain L-cysteine. The inhibitory effect of L-cysteine on tyrosinase reaction is seen not only with *S. griseus* but also with other ordinary chromogenic species of streptomycetes. The failure of *S. griseus* to manifest chromogenicity on complex organic media was ascribed to the low yield of tyrosinase and the inability of this organism to metabolize L-cysteine. Melanin formation by tyrosinase (EC 1.10.3.1) was reported to be inhibited by thiol compounds by several workers (4, 6). Just after the completion of the present work, Sanada et al. (9), using mushroom tyrosinase, reported that pyrrocathechol, the initial intermediate of tyrosinase action, was conjugated with L-cysteine by tyrosinase action, producing colorless crystals, and that these conjugation products were not oxidized further by tyrosinase but gave some inhibitory effects on the melanin formation by the enzyme. In the present study, the colorless crystalline substance accumulated during the growth of *S. phaeochromogenes* in the medium containing L-cysteine. This phenomenon was not observed with *S. griseus*. Further experiments are needed to characterize this crystalline substance and to elucidate its role in melanin formation with reference to the different attitude of chromogenicity in *S. griseus* and *S. phaeochromogenes*.

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