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# Effects of Methionine and $Cu^{2+}$ on the Expression of Tyrosinase Activity in *Streptomyces castaneoglobisporus*

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***Streptomyces castaneoglobisporus*** **HUT6202 expresses an enzyme, tyrosinase, responsible for the production of melanin-like pigments. The present study revealed that the tyrosinase synthesis by the microorganism was induced about 80-fold, when young cells cultured for 6 h were incubated with methionine (Met) to the mid-log phase of growth, in comparison to without this amino acid. The Met-induced tyrosinase synthesis was inhibited by the addition of rifampicin and chloramphenicol, suggesting that transcriptional and translational events are necessary for the induction. We found that the addition of $Cu^{2+}$ to the culture medium brings forward the period of expression of Met-induced tyrosinase activity.**

**Key words: $Cu^{2+}$-carrier protein, induction by methionine, melanin pigment, *S. castaneoglobisporus*, tyrosinase from *Streptomyces*.**

Tyrosinase, a copper-containing monooxygenase, catalyzes the oxidation of tyrosine (Tyr) *via* L-dihydroxyphenylalanine (L-DOPA) to dopaquinone, which then is oxidized spontaneously and polymerizes to form a melanin pigment. The ability to synthesize a melanin pigment has been used as a criterion for the taxonomic classification of *Streptomyces* species. A gene encoding tyrosinase has been cloned from a few *Streptomyces* species (*1-4*), and is used as a selective marker in *Streptomyces* plasmid vectors (*1*). We have noticed that *Streptomyces castaneoglobisporus* HUT-6202 has higher ability to synthesize a melanin pigment than other *Streptomyces* species (*5*). To elucidate the mechanism underlying this higher ability, we have cloned the melanin-synthesizing gene, designated as the *mel* operon, from the *S. castaneoglobisporus* chromosome (*5*). As expected, the tyrosinase activity due to the cloned gene, expressed in *S. lividans* as a host, was about 110-fold higher than that of the same host carrying the *S. antibioticus* gene (*5*). On sequence analysis we found that the *mel* operon from *S. castaneoglobisporus* contains an additional ORF consisting of 378 nucleotides (designated as ORF378) upstream of the tyrosinase gene, which exhibits 84% homology to ORF438 from *S. antibioticus*. The protein encoded by ORF438 has been reported to act as a copper-carrier protein (*1*). In a previous paper (*5*), we showed that the tyrosinase activity due to the *mel* operon of *S. castaneoglobisporus* is higher than that of *S. antibioticus* expressed under the control of the same promoter. As a reason, we have shown that ORF378 is superior to the corresponding ORF, designated as ORF438, of *S. antibioticus* in inducing the expression of tyrosinase (*5*). The ORF378-encoded protein, which acts as a superior $Cu^{2+}$-carrier protein, may efficiently facilitate the incorporation of copper into apotyrosinase.

Met has been reported to serve as an inducer of tyrosinase production in *S. antibioticus* (*6, 7*) and *S. glaucescens* (*8*). However, in another *Streptomyces* species, *S. michiganensis*, Met is ineffective in tyrosinase induction, instead, $Cu^{2+}$ induces the enzyme activity (*9*). On the contrary, $Cu^{2+}$-induced tyrosinase synthesis is not observed in *S. antibioticus* (*6, 7*), suggesting that different induction mechanisms for tyrosinase activity might exist among *Streptomyces* species.

To determine the reason for the high level tyrosinase production in *S. castaneoglobisporus*, we have investigated, in the present study, the conditions for the induction of tyrosinase activity.

## MATERIALS AND METHODS

*Bacteria and Media*—*S. castaneoglobisporus* HUT6202 was grown in GMP medium (1% glucose, 0.4% polypeptone, 0.2% yeast extract, 0.2% meat extract, 0.5% NaCl, 0.025% $MgSO_4 \cdot 7H_2O$, pH 7.0) at 28°C for 48 h. The cells (5 mg as dry cell weight) were suspended in 10 ml of 5% glycerol and then stored at −20°C until use as a seed culture, after washing twice with saline (*6*). The main culture for expression of tyrosinase activity by *S. castaneoglobisporus* was performed at 28°C for a given time, after inoculating the seed culture (100 μl) into 10 ml of a chemically defined medium (CDM) (*6*) [0.2% Glu, 0.1% Asn, 0.1% Pro, 1% glucose, 0.1% $K_2HPO_4$, 0.005% $MgSO_4 \cdot 7H_2O$, 0.0025% $ZnSO_4 \cdot 7H_2O$, 0.0025% $CaCl_2 \cdot 2H_2O$, 0.0025% $FeSO_4 \cdot 7H_2O$, 0.000078% (=3.13 μM) $CuSO_4 \cdot 5H_2O$].

The increase in cell mass, as a result of growth, was determined as the total protein (μg) in cell-extracts obtained by sonication after washing of the cells with TMDP

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Abbreviations: L-DOPA, L-dihydroxyphenylalanine; *mel*, an operon for synthesis of melanin pigment; Met, methionine; ORF, open reading frame.



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buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM DTT, 0.4 mM phenylmethyl sulfonyl fluoride].

*Assaying of Tyrosinase Activity*—Extracellular and intracellular tyrosinase activities were measured as described previously (*6*). The former activity was determined with an aliquot of the supernatant fluid obtained from the culture broth by centrifugation. The latter activity was measured using cell-extracts obtained by sonication of cells washed with TMDP buffer. One unit of tyrosinase activity is defined as the amount of enzyme which converts 1 $\mu$mol L-DOPA to dopachrome per min at 30°C. Dopachrome formation was measured at 475 nm with a spectrophotometer (Shimadzu UV-180, Tokyo) using 10 mM L-DOPA as a substrate dissolved in 0.1 M sodium phosphate buffer (pH 6.2) supplemented with 5 $\mu$M copper sulfate. Specific activity was expressed as units per $\mu$g of protein determined according to the method described previously (*10*).

*Northern Hybridization Analysis*—The cell mass (0.5 g as wet cell weight) washed with buffer I (0.3 M sucrose, 50 mM Tris-HCl, 5 mM EDTA, pH 8.0) was incubated for 1 h at 37°C with the same buffer containing 6 mg/ml lysozyme. RNA was isolated according to the method described previously (*11*). The incubation mixture was supplemented with 4 ml of a denaturation buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), and then vortexed for 1 min. After removing proteins from the solution with phenol and chloroform/isoamyl alcohol, RNA was precipitated with ethanol. The precipitate was resuspended into 0.3 ml of the denaturation buffer and then reprecipitated with ethanol. The precipitated RNA was dissolved in sterilized water, treated with diethyl pyrocarbonate, and then stored at −70°C until use. Northern hybridization analysis was principally carried out according to the method described previously (*12*). A nylon membrane (Hybond-N+; Amersham) onto which total RNA from *S. castaneoglobisporus* had been transferred using an alkaline transfer buffer was washed with 2×SSPE for 60 s. After RNA hybridization had been performed at 65°C for 20 h in a solution (5× SSPE, 0.5% SDS, 5×Denhardt's solution, 50% formamide, 20 $\mu$g/ml sheared calf thymus DNA) supplemented with part (a fragment consisting of about 700-bp) of the tyrosinase structural gene from *S. castaneoglobisporus*, nick-labeled with 740 kBq of [$\alpha$-$^{32}$P]dATP, the membrane was washed with 2×SSPE containing 0.1% SDS for 20 min and then with 1×SSPE containing 0.1% SDS at 65°C for 15 min, rinsed with 0.1×SSPE containing 0.1% SDS for 20 min at 65°C, and finally exposed to an X-ray film for autoradiography.

## RESULTS

*Induction of Tyrosinase by Met in S. castaneoglobisporus*—Met serves as an effective inducer for tyrosinase production in *S. antibioticus* and *S. glaucescens* (*6-8*). To determine the influence of hydrophobic amino acids, including Met, on the induction of the tyrosinase activity in *S. castaneoglobisporus* HUT6202, the microorganism was grown in CDM supplemented without or with each amino acid. The sum of the intracellular and extracellular tyrosinase activities of cells grown in the presence of Met was 16.9-fold higher, than that in the absence of the added amino acid. Under the same conditions, the enhancement of the tyrosinase activity by Leu, Phe, and Trp was 6.9-fold, 4.0-fold, and 2.1-fold, respectively. However, Tyr and Val

TABLE I. **Induction of total tyrosinase activity by Met or $Cu^{2+}$ in *S. castaneoglobisporus* HUT6202.** Met (10 mM) or copper sulfate (3.13 $\mu$M) was added to cells grown for 6 or 28 h in CDM without added $Cu^{2+}$, followed by incubation for the given times. The results are expressed in relative tyrosinase activity as the ratio between the tyrosinase activity of cells grown in the presence of the added Met or $Cu^{2+}$ to that in the absence of a supplement (=1.0). Total activity is expressed as the sum of the intracellular and extracellular tyrosinase activities.

| Time of addition (h) | Cultivation time after addition (h) | Relative tyrosinase activity | |
|---|---|---|---|
| | | Met | $Cu^{2+}$ |
| 6 | 24 | 81 | 2.4 |
| | 38 | 3.7 | 2.1 |
| 28 | 4 | 13.4 | 1.9 |
| | 12 | 4.4 | 2.6 |

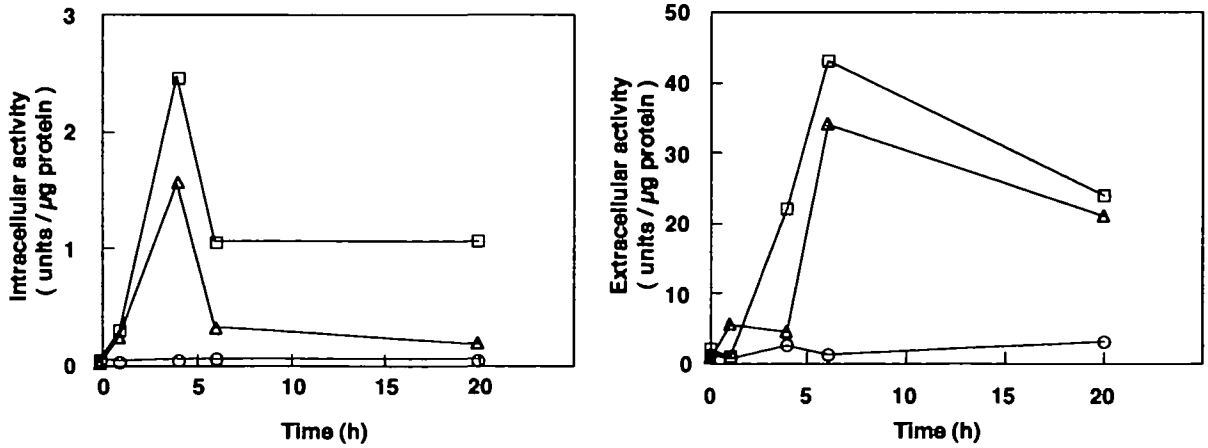


Fig. 1. **Time courses of the intracellular and extracellular tyrosinase activities induced on the addition of Met to the medium at concentrations of 0.1 and 10 mM.** After *S. castaneoglobisporus* cells had been grown in CDM at 28°C for 16 h, Met was added to the culture, followed by incubation for the given times. □, △, and ○ represent the time courses of tyrosinase activity with 10 and 0.1 mM Met, and without Met, respectively.



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repressed the expression of the tyrosinase activity (0.14-fold and 0.64-fold, respectively). The Met-induced tyrosinase activity expressed extracellularly by *S. castaneoglobisporus* was about 20-fold higher than that expressed intracellularly. In addition, the tyrosinase induction was significantly higher with 10 mM Met than 0.1 mM Met (Fig. 1), whereas that in *S. antibioticus* has been shown to be optimal at 0.1 mM (*7*).

In contrast, the tyrosinase activity in *S. michiganensis* is induced by $Cu^{2+}$, but not by Met (*9*). Table I shows the results of an experiment on the Met-induced tyrosinase activity in *S. castaneoglobisporus*, which was grown in CDM without added $Cu^{2+}$. The induction attained was 81-fold, when Met was added at the beginning (6 h) of the log-phase, followed by incubation for 24 h, in comparison to without Met. But, when Met was added at the log-phase (28 h), followed by incubation for 4 h, the Met-induced tyrosinase activity decreased to 13.4-fold. In addition, even if Met was added at the beginning of the log-phase (6 h), the induction of tyrosinase activity significantly decreased on continual cultivation of the cells for a long period (Table I).

To determine the influence of the culture age of the *S. castaneoglobisporus* cells on the induction, Met (10 mM) was added at given stages of culture, followed by incubation for 4 h. The induction of the tyrosinase activity was 25-fold (18.5 h culture), 14-fold (28 h culture), and 1.1-fold (48 h culture) higher than that in the absence of Met.

Figure 1 shows the tyrosinase activity after induction by Met. The intracellular tyrosinase activity became maximum at 4 h after the addition of Met, and then decreased. On the other hand, the extracellular tyrosinase activity reached the maximum level at 6 h after the addition of Met, and then decreased.

TABLE II. **The effects of inhibitors of translation and transcription on the induction of total tyrosinase by Met or $Cu^{2+}$.** + and − represent with and without supplementation, respectively. Met (10 mM), copper sulfate (3.13 $\mu$M), or Met (10 mM) and copper sulfate (3.13 $\mu$M) were added to cells, grown for 24 h in CDM without added $Cu^{2+}$, respectively, followed by incubation for 4 h. Rifampicin and chloramphenicol were used at the final concentrations of 100 $\mu$g/ml and 35 $\mu$g/ml, respectively. Total activity is expressed as the sum of the intracellular and extracellular tyrosinase activities.

| Inhibitor | | Specific activity of tyrosinase (units/$\mu$g protein) | | | |
|---|---|---|---|---|---|
| | $Cu^{2+}$ | − | − | + | + |
| | Met | − | + | − | + |
| − | | 0.30 | 6.29 | 0.57 | 8.80 |
| Rifampicin | | 0.29 | 0.31 | 0.34 | 0.36 |
| Chloramphenicol | | 0.31 | 0.32 | 0.36 | 0.45 |

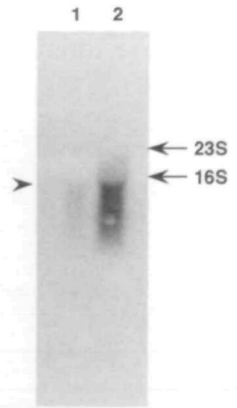


Fig. 2. **Effect of Met on the transcriptional activity of the tyrosinase gene.** *S. castaneoglobisporus* cells, grown in CDM without added $Cu^{2+}$ for 24 h at 28°C, were incubated without or with 10 mM Met for 4 h. A fragment of the tyrosinase gene was used as a probe for expression of the *mel* operon. Total RNA (20 $\mu$g) from cells grown in the absence (lane 1) or presence (lane 2) of Met was used for Northern hybridization analysis. The hybridization was carried out at 65°C for 20 h. The arrowhead indicates the transcript (approximately 1.5 kilobases) derived from the *mel* operon consisting of the ORF378 and tyrosinase genes.

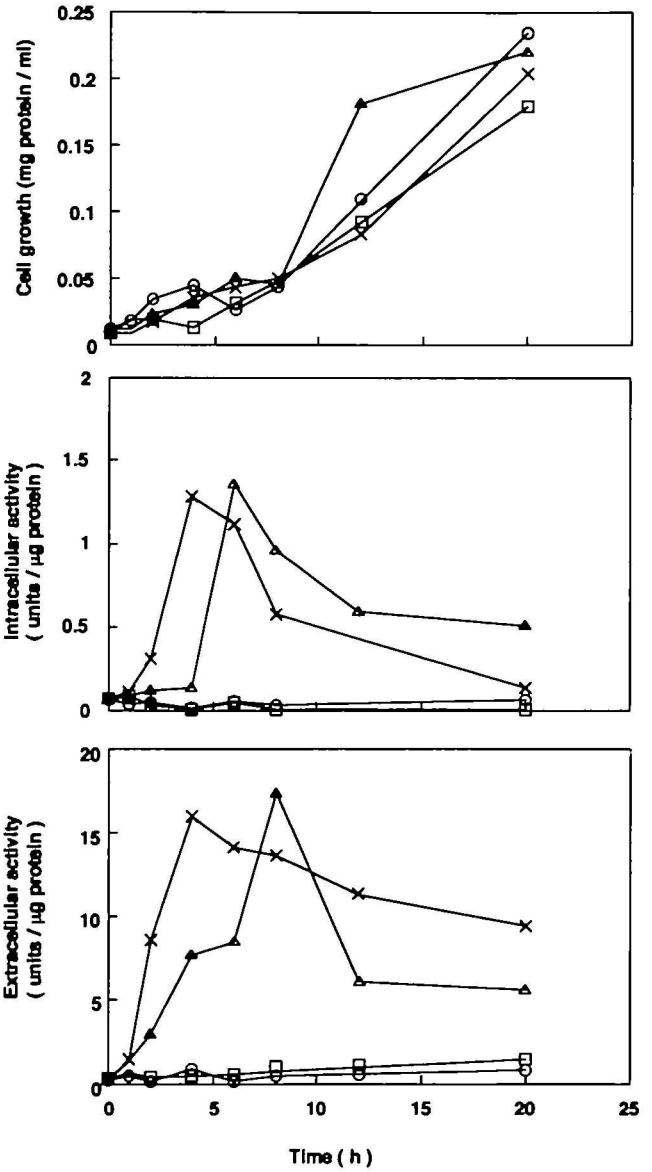


Fig. 3. **Influence of $Cu^{2+}$ on the period of expression of Met-induced tyrosinase activity.** *S. castaneoglobisporus* cells, grown for 24 h in CDM without added $Cu^{2+}$ or CDM, were incubated with Met (10 mM) at 28°C for the given times. The supernatant fluid and cell mass obtained on centrifugation of the culture broth were used to assay the extracellular and intracellular activities of tyrosinase, respectively. ○ and △ represent the time courses of tyrosinase activity produced by *S. castaneoglobisporus* cells grown in CDM without added $Cu^{2+}$ and without or with Met, respectively. □ and × represent the time courses of tyrosinase activity produced by the organism grown in CDM without or with Met, respectively.

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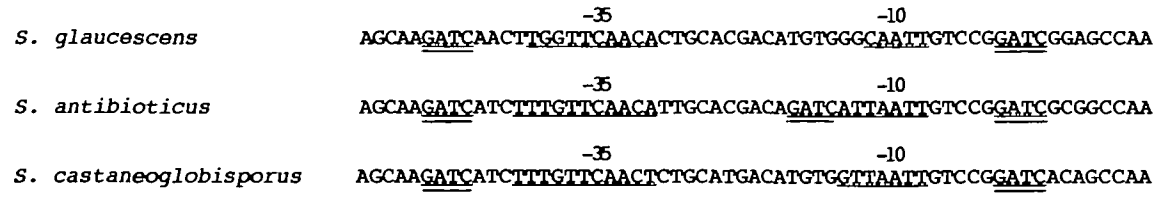


Fig. 4. **Comparison of the GATC sequence located near the promoter region in various *mel* operons.** Underlining denotes the −35 and −10 regions. Double underlining shows GATC sequences. The promoter sequences in the *S. castaneoglobisporus*, *S. antibioticus* and *S. glaucescens mel* operons have been sequenced by Ikeda *et al.* (*5*), Bernan *et al.* (*13*), and Huber *et al.* (*14*), respectively.

To determine the mechanism underlying the induction of tyrosinase activity by Met, we used rifampicin and chloramphenicol as inhibitors of transcription and translation, respectively. After *S. castaneoglobisporus* cells had been cultured in CDM without added $Cu^{2+}$ at 28°C for 24 h, rifampicin (100 μg/ml) or chloramphenicol (35 μg/ml) was added to the culture medium together with or without 10 mM Met, followed by incubation for 4 h. The tyrosinase expression induced with 10 mM Met was repressed by the addition of each inhibitor, the activity being almost the same as that without supplementation of Met (Table II). This suggests that both transcriptional and translational events are necessary for the Met-induced tyrosinase expression. Northern hybridization analysis also suggested that Met increases the transcription level of the *mel* operon (Fig. 2).

*Effect of $Cu^{2+}$ on Tyrosinase Activity*—Since $Cu^{2+}$ is essential for the synthesis of the catalytically active form of tyrosinase, we examined the effect of $Cu^{2+}$ on the expression of tyrosinase activity. When cells grown for 6 h in CDM were incubated for 24 h, total tyrosinase activity, determined as the sum of intracellular and extracellular tyrosinase activities, was significantly enhanced, *i.e.* 2.4-fold, in comparison to that in CDM without added $Cu^{2+}$ (Table I). Table II also shows an increase of tyrosinase activity of about 2-fold on the addition of $Cu^{2+}$. The Met-induced tyrosinase activity in cells grown in the presence of $Cu^{2+}$ increased to more than that in the absence of the metal ion, suggesting that $Cu^{2+}$ might enhance the tyrosinase synthesis or stabilize the enzymatic structure through its incorporation into the protein molecule. We found that when Met was added to cells grown for 24 h in CDM or CDM without added $Cu^{2+}$, followed by incubation for a given time, the Met-induced extracellular tyrosinase activity in the presence of $Cu^{2+}$ became maximum at 3 h, but that in the absence of the metal ion did so at 6-8 h (Fig. 3). The maximal activity of the Met-induced intracellular tyrosinase in the presence of the metal ion also shifted to an earlier stage of the incubation time. This suggests that $Cu^{2+}$ brings forward the expression period of Met-induced tyrosinase activity in *S. castaneoglobisporus*.

## DISCUSSION

The mechanism underlying the induction of the *S. castaneoglobisporus* tyrosinase activity by Met is likely to resemble to that in the case of *S. antibioticus* or *S. glauscescens* (*6-8*). In fact, the promoter sequence of the *mel* operon is almost the same in the three microorganisms. Figure 4 shows that GATC sequences are present upstream of the −35 region and downstream of the −10 region in the *mel* operon of *S. castaneoglobisporus*, as in the case of *S. antibioticus*. The methylation of specific GATC sequences by methylase has been reported to affect the expression of genes in *Escherichia coli* and the Mu phage (*15*). The methylation of GATC sequences near the promoter of the *mel* operon may result in activation of the promoter, with Met as a donor for the methylation, as proposed by Katz *et al.* (*6*), although the validity of this hypothesis must be examined in future experiments.

Since tyrosinase is a metallo-protein and contains copper in its active site, the addition of $Cu^{2+}$ to the assay mixture for measurement of the enzyme activity is necessary. Chen *et al.* (*16*) examined the tyrosinase activity in *S. lividans* cells harboring the *mel* operon from *S. antibioticus*, which was cultured in the presence or absence of added $Cu^{2+}$. Enzyme activity was undetectable on culturing in the absence of the ion. However, the tyrosinase activity was restored on the addition of $Cu^{2+}$ to the assay mixture to a similar level to as for culturing in the presence of $Cu^{2+}$. This suggests that the tyrosinase is present in the apoform if cells are grown in a medium without added $Cu^{2+}$, and that the *S. antibioticus* tyrosinase activity is not increased by $Cu^{2+}$ supplemented during the culture. The present study confirmed that the maximal production of enzyme activity in *S. castaneoglobisporus* occurs with over 5 μM added copper sulfate in the assay mixture and without a delay after its addition. Under these assay conditions, we observed that the tyrosinase activity was obviously enhanced 2-fold when *S. castaneoglobisporus* cells were cultured for several hours in a medium supplemented with $Cu^{2+}$. This observation is significantly different from in the case of *S. antibioticus* (*16*).

In addition, we found that the period of expression of Met-induced tyrosinase activity is brought forward by the addition of $Cu^{2+}$, but that the total activity is not significantly changed. This led us to speculate that the added $Cu^{2+}$ might promote post-transcriptional events for the Met-induced expression of the tyrosinase gene.

Recently, the ORF438 product was suggested to function as a *trans*-activator which facilitates the incorporation of copper into apotyrosinase, and the *trans*-activation has been demonstrated to be initially mediated *via* a binary complex formed from these two proteins, followed by the incorporation of $Cu^{2+}$ (*16*). As a hypothesis, it is considered that if the ORF378 protein in *S. castaneoglobisporus* cells is involved in the transfer of $Cu^{2+}$ to apotyrosinase *via* the formation of a binary complex from these two proteins, the metal ion may promote the binding of ORF378 to apotyrosinase or may promote a change in the stereoscopic conformations of these proteins leading to high expression of tyrosinase activity. This step might develop during cultur-



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ing in the medium supplemented with $Cu^{2+}$. This hypothesis should be confirmed by *in vitro* binding assays with ORF378 and tyrosinase gene products purified to homogeneity.

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