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Effect of Light on Nonphotosynthetic Microorganisms. VI.[1] Photochromogenicity in Genera *Micromonospora*, *Microbispora* and *Actinoplanes*

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The effect of light on the pigmentation of various strains of *Micromonospora*, *Microbispora* and *Actinoplanes* was investigated. It was found that four of eight strains of *Micromonospora*, two of five strains of *Microbispora* and two of three strains of *Actinoplanes* were photochromogenic. These photochromogenic strains produced carotenoids by photoinduction. Biochemical studies on the mechanism of photochromogenicity in *Micromonospora echinospora*, *Microbispora chromogenes* and *Actinoplanes philippinensis* showed that photoinduced carotenoid synthesis in these strains consists of an initial temperature-independent photochemical reaction step and a series of dark metabolic reaction steps, which involve *de novo* protein synthesis.

Keywords——photochromogenicity; *Micromonospora*; *Microbispora*; *Actinoplanes*; photoinduced carotenoid synthesis; photoinduced protein synthesis

Among nonphotosynthetic bacteria, certain species of mycobacteria, *e.g.*, *M. kansasii*,[2a] *M. marinum*[2b-d] and a *Mycobacterium* sp.,[2e-g] as well as *Myxococcus xanthus*,[3] *Micrococcus roseus*[4] and *Flavobacterium dehydrogenans*,[5] are capable of photoinduced carotenogenesis which consists of two main steps, a photochemical reaction (temperature-independent step) and a series of dark metabolic reactions (temperature-dependent steps).

Meanwhile, our previous reports[6a,b] on the photochromogenicity in genera *Streptomyces, Nocardia, Corynebacterium, Brevibacterium* and *Flavobacterium* revealed that six of 19 species of *Streptomyces*, 30 strains of 57 strains of 21 species of *Nocardia*, two of 15 strains of *Corynebacterium*, two of 2 strains of *Arthrobacter*, six of 13 strains of *Brevibacterium* and two of 14 strains of *Flavobacterium* exhibited photochromogenicity, a diagnostic key for mycobacteria.[7] Recently, we reported on the mechanism of photoinduced pigmentation in *Brevibacterium sulfureum* AU-31, which exhibits photochromogenicity, and showed that the same mechanism as seen in the previously known photochromogenic mycobacteria,[2] is operating in *B. sulfureum*.[6c]

In view of the facts mentioned above, it may be considered that photochromogenicity is not a specific phenomenon in particular species of bacteria, but a common, widely distributed feature of various species of bacteria, at least in *Actinomycetes* and related microorganisms. To confirm this assumption, we investigated the effect of light on the pigmentation of bacteria in genera *Micromonospora, Microbispora* and *Actinoplanes*, since no work has been published on the photochromogenicity in these genera.

In this paper, we will describe the photochromogenicity in genera *Micromonospora, Microbispora* and *Actinoplanes*, and the mechanism of photochromogenicity in *Micromonospora echinispora*, *Microbispora chromogenes* and *Actinoplanes philippinensis*.

Experimental

Microorganisms——All of the test strains of *Micromonospora*, *Microbispora* and *Actinoplanes*, numbered in the KCC series, were obtained through the courtesy of KCC Culture Collection of Actinomycetes, Kaken Chemical Co., Ltd. The test strains were maintained on glycerol Kelner–Morton agar.

Media and Cultivation of Organisms——Glucose oatmeal agar (yeast extract, 1.0 g; glucose, 1.0 g; oatmeal infusion, 1000 ml; agar, 18 g; pH 7.2), glycerol Kelner–Morton agar (glycerol, 60 g; soluble starch, 10 g; glucose 2.0 g; meat extract, 3.0 g; polypepton, 5 g; K_2HPO_4, 0.8 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; potato infusion, 1000 ml; agar, 18 g; pH 7.0) and 6b broth (soluble starch, 20 g; glucose, 10 g; yeast extract, 5 g; NZ-amine, 5 g; $CaCO_3$, 1.0 g; dist. water, 1000 ml) were used. Cultivation of the organism was carried out at 27 °C with rigid light exclusion unless otherwise specified. *Micromonospora parva* and *Microbispora aerata* were incubated at 37 °C, and *Microbispora bispora* incubated at 60 °C. When organisms were transplanted, a photographic red lamp had to be used.

Procedure of Photoinduction——Photoinduction on agar plates was carried out as described in the previous paper.[6b] To determine whether or not the phenomenon was simply due to a photochemical event, the dark-grown colonies were exposed to light (10000 lux) for 8 h at 0—4 °C.

Preparation of Seed for Cultivation in Liquid Medium——After two cycles of cultivation in the dark, the bacteria were inoculated into 6b broth and incubated in the dark for 40 h. Mycelia harvested by centrifugation were transferred onto sterilized filter papers to remove water. The mycelia thus prepared were stored at −20 °C. All operations were carried out in the dark.

For inoculation, the stored mycelia were suspended in 6b broth (50 mg mycelia/ml) and homogenized in a Universal Homogenizer (Nihon Seiki Co., Ltd.) for 1 min at top speed. The homogenate was inoculated at the concentration of 1 mg mycelia/ml.

Photoinduction in Shake-Cultured Organisms——The cell suspension prepared as mentioned above was inoculated into 600 ml of 6b in a 3 l Erlenmeyer flask and incubated at 27 °C for 20 h in the dark. The culture was cooled sufficiently in an ice bath, then exposed to light (5000 lux) with gentle stirring for a stated time.

For the experiments on the light dose response at 0—5 °C or at 27 °C in *Micromonospora echinospora*, *Microbispora chromogenes* and *Actinoplanes philippinensis*, 20 ml portions of the culture which had been exposed to light in an ice bath, or at 27 °C for 15, 30, 45, 60, 90, 120, 150, 180, 240, 300 and 360 min, were transferred to 100 ml of Erlenmeyer flasks and then incubated for 20 h in the dark at 27 °C.

For the observation of the time course of carotenoid synthesis in the bacteria, a culture exposed to light for 1 h in an ice bath was incubated in the dark at 27 °C. Aliquots (20 ml) of the culture were centrifuged at 0, 15, 30, 45, 60, 75, 90, 105, 120, 140, 160, 180, 210, 240, 270 and 300 min after the initiation of re-incubation and pigments were extracted from the mycelia.

For the experiments on the inhibition of carotenoid synthesis by chloramphenicol or rifamycin, a light-exposed culture (1 h, at 0—5 °C) was re-incubated in the dark. At 0, 5, 10, 15, 20, 25, 30, 40, 60 and 90 min after the initiation of re-incubation, 20 ml of culture was transferred to a 100 ml Erlenmeyer flask, to which 1 mg of chloramphenicol or rifamycin had been added, and then incubated for 20 h.

For the experiment on the stability of photoinduction, a culture exposed to light in an ice bath for 1 h was stored in a refrigerator. After storage for 20, 30, 40, 60, and 80 h, 20 ml of culture was incubated at 27 °C in the dark for 20 h.

Induction of Carotenoid Synthesis by Hydrogen Peroxide——A cell suspension of the test organism was added to a series of 50 ml aliquots of medium in 300 ml Erlenmeyer flasks and incubated for 20 h in the dark. Hydrogen peroxide at various concentrations was added to the cultures at final concentrations of 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.4 M, and the mixtures were incubated for 30 min at 27 °C. After centrifugation, cells treated with hydrogen peroxide were washed twice with 1/15 M phosphate buffer (pH 8.0). The washed cells were suspended in the supernatant of the culture, which had been incubated in the dark for 20 h. Subsequently the suspended cells were incubated for 20 h in the dark.

Measurement of the Volume of Total Mycelia——After centrifugation ($900 \times g$, 5 min), the mycelia were transferred to a graduated centrifugation tube and centrifuged for 15 min ($1300 \times g$), then the packed volume of the total mycelia was measured.

Analysis of Pigments——After measurement of the packed volume of mycelia, pigments were extracted according to the method described by Liaaen-Jensen and Jensen.[8] The extracts dried under vacuum were dissolved in 5 ml of methanol, and OD_{470} of the solution was measured with a spectrophotometer.

Results

On the basis of the results shown in Tables I, II and III, the test organisms have been characterized as photochromogenic, scotochromogenic and nonphotochromogenic strains according to our previous definition.[6b]

TABLE I. The Pigmentation of Various Strains of Genus *Micromonospora* Grown under Illumination or in the Dark on Glycerol Kelner–Morton Agar and Glucose Oatmeal Agar at 27 °C for 7 d

Test organisms	Color of colonies grown on glycerol Kelner–Morton agar		Color of colonies grown on glucose oatmeal agar		Classification[b]
	Under illumination[a]	In the dark	Under illumination[a]	In the dark	
M. chalcea					
KCC A-0031	Orange	Orange	Orange	Orange	Scoto-
M. fusca					
KCC A-0050	Orange	Orange	Orange	Orange	Scoto-
M. melanospora					
KCC A-0063	Orange	Orange	Orange	Orange	Scoto-
M. echinospora					
KCC A-0073	Reddish orange	Light brown	Orange	Whitish	Photo-
M. purpurea					
KCC A-0074	Reddish orange	Light brown	Orange	Whitish	Photo-
M. sp.					
KCC A-0087	Orange	Pale orange	Orange	Pale orange	Photo-
M. megalomicea					
KCC A-0105	Beige	Beige	Beige	Beige	Nonphoto-
M. coerulea					
KCC A-0175	Orange	Pale orange	Orange	Yellowish white	Photo-

a) Bacteria were exposed to light under white fluorescent lamps during growth. *b*) Photo-, photochromogenic strain; scoto-, scotochromogenic strain; nonphoto-, nonphotochromogenic strain.

TABLE II. The Pigmentation of Various Strains of Genus *Microbispora* Grown under Illumination or in the Dark on Glycerol Kelner–Morton Agar and Glucose Oatmeal Agar for 7 d

Test organisms	Color of colonies grown on glycerol Kelner–Morton agar		Color of colonies grown on glucose oatmeal agar		Classification[b]
	Under illumination[a]	In the dark	Under illumination[a]	In the dark	
M. chromogenes					
KCC A-0022	Orange	Whitish	Orange	Pale orange	Photo-
M. diastatica					
KCC A-0023	Reddish-orange	Pale yellow	Pink	Whitish	Photo-
M. parva					
KCC A-0024	Pale orange	Pale orange	Pale orange	Pale orange	Scoto-
M. aerata					
KCC A-0076	Beige	Beige	Beige	Beige	Nonphoto-
M. bispora					
KCC A-0092	Whitish	Whitish	Whitish	Whitish	Nonphoto-

The strain KCC A-0022 and KCC A-0023 were incubated at 27 °C, KCC A-0024 and KCC A-0076 were incubated at 37 °C and KCC A-0082 was incubated at 60 °C. *a*, *b*) See legend to Table I.

Photochromogenicity in Genus Micromonospora

Eight strains of *Micromonospora* were tested on glucose oatmeal agar and glycerol Kelner–Morton agar plates. The colors of the substrate mycelia of the test organisms grown under illumination and in the dark are shown in Table I. Four of 8 strains were photochromogenic, three were scotochromogenic and one was nonphotochromogenic.

When the photochromogenic strains, *M. echinospora, M. purpurea, M.* sp. and *M.*

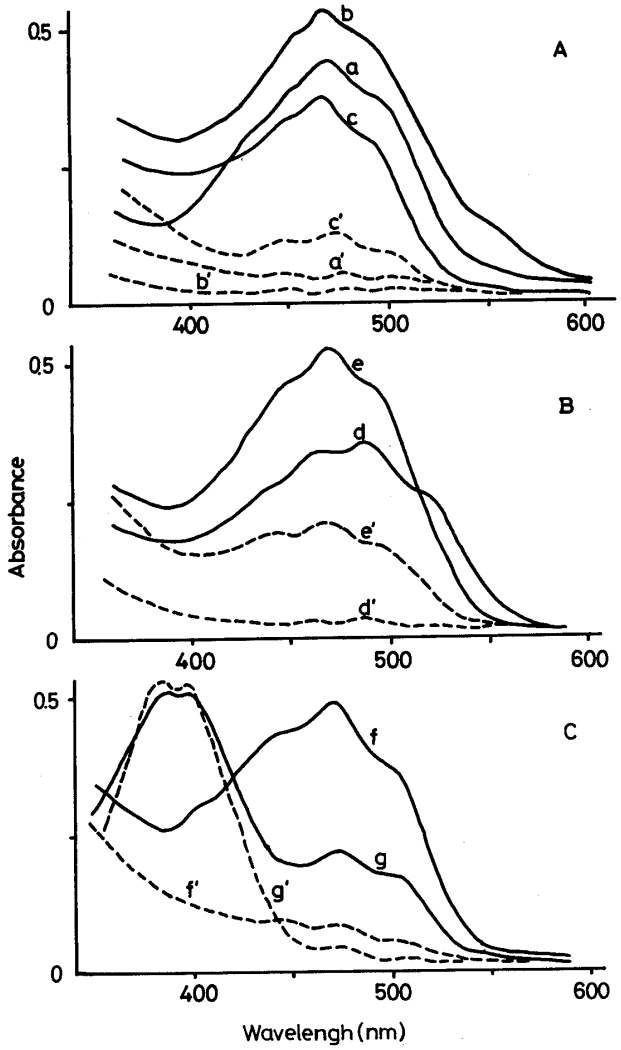

Fig. 1. Visible Light Absorption Spectra of the Extracts from Light-Exposed and Dark-Grown Cells of Photochromogenic *Micromonospora*, *Microbispora* and *Actinoplanes*

(——), extracts from light exposed cells; (– – – –), extracts from dark grown cells. a and a′, *M. echinospora* KCC A-0073; b and b′, *M. purpurea* KCC A-0074; c and c′, *M.* sp. KCC A-0087; d and d′, *M. chromogenes* KCC A-0022; e and e′, *M. diastatica* KCC A-0023; f and f′, *A. philippinensis* KCC A-0001; g and g′, *A.* sp. KCC A-0043.

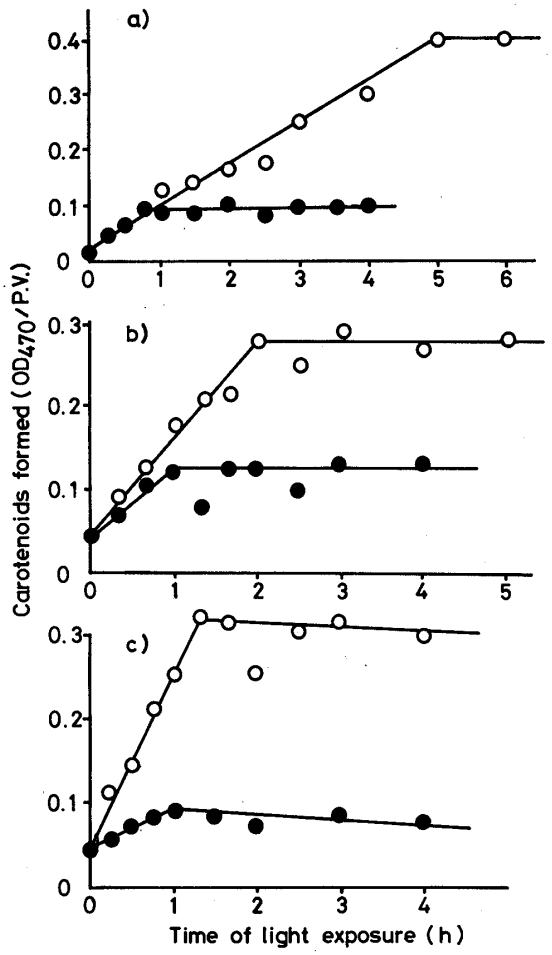

Fig. 2. Dependence of Carotenogenesis on the Duration of Light Exposure

The organisms preincubated in the dark for 20 h were exposed to light in an ice bath or at 27 °C. After exposure to light for designated periods, 20 ml portions of the culture were transferred to 100 ml Erlenmeyer flasks at each time, then incubated in the dark for 20 h. The pigments extracted were dried *in vacuo* and dissolved in 5 ml of methanol. The amount of carotenoid was measured at OD_{470} and expressed as OD_{470}/packed volume of the mycelia (P.V.). (○), exposed to light at 27 °C; (●), exposed to light in an ice bath. a) *M. echinospora* KCC A-0073, b) *M. chromogenes* KCC A-0022, c) *A. philippinensis* KCC A-0001.

coerulea were incubated in 6b broth under illumination, the organisms acquired orange to reddish-orange pigmentation. A large amount of pigments was produced in the light-exposed cells (assumed to be a mixture of carotenoids from the characteristic visible light absorption spectra of the pigments extracted[6c,8]), whereas only a small amount of carotenoids was produced in the dark-grown cells (Fig. 1A).

Photochromogenicity in Genus Microbispora

Five strains of *Microbispora* were tested. As shown in Table II, two of 5 strains were photochromogenic, one strain was scotochromogenic and two were nonphotochromogenic.

TABLE III. The Pigmentation of Various Strains of Genus *Actinoplanes* Grown under Illumination or in the Dark on Glycerol Kelner–Morton Agar and Glucose Oatmeal Agar at 27 °C for 7 d

Test organisms	Color of colonies grown on glycerol Kelner–Morton agar		Color of colonies grown on glucose oatmeal agar		Classification[b]
	Under illumination[a]	In the dark	Under illumination[a]	In the dark	
M. philippinensis KCC A-0001	Orange	Pale orange	Orange	Yellowish-white	Photo-
A. sp. KCC A-0043	Orange	Brown	Orange	Yellowish-white	Photo-
A. armeniacus KCC A-0070	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white	Scoto-

a, b) See legend to Table I.

The visible light absorption spectra of the extracts from the light-exposed and dark-grown cells of *M. chromogenes* and *M. diastatica* are shown in Fig. 1B; they indicate that these photochromogenic strains are also capable of photoinduced carotenogenesis.

Photochromogenicity in Genus Actinoplanes

Three strains of *Actinoplanes* were tested. As shown in Table III, two of three strains were photochromogenic and one was scotochromogenic.

The visible light absorption spectra of the extracts from the light-exposed and dark-grown cells of *A. philippinensis* and *A.* sp. are shown in Fig. 1C. These photochromogenic strains also produced carotenoids on exposure to light. *A.* sp. KCC A-0043 constitutively produced the pigment which exhibited peaks at 385 and 398 nm in visible light absorption spectra, but an increased amount of carotenoids was produced in the light-exposed cells (g and g′ in Fig. 1).

Mechanism of Photoinduced Carotenogenesis

All the photochromogenic strains mentioned above were induced to synthesize carotenoid by exposure to light. The dark-grown cells of the photochromogenic strains also produced a small amount of carotenoids constitutively, and the amount of carotenoids produced in the dark-grown cells varied from strain to strain. To obtain clear results, photochromogenic strains *Micromonospora echinospora* KCC A-0073, *Microbispora chromogenes* KCC A-0022 and *Actinoplanes philippinensis* KCC A-0001, which produced relatively small amounts of carotenoids in the dark, were selected.

Photoinduction

The photochromogenic strains, *M. echinospora, M. chromogenes* and *A. philippinensis* were shake-cultured in 6b broth for 20 h in the dark, and then the cultures were exposed to 5000 lux of light in an ice bath or at 27 °C for designated periods. Figures 2a, b and c show that the amount of carotenoids produced was directly proportional to the duration of light exposure in each strain. When the organisms were exposed to light in an ice bath (Fig. 2, closed circle), over 1 h of exposure did not increase the amount of carotenoid in any strain.

These results showed that photoinduced carotenogenesis in *M. echinospora, M. chromogenes* and *A. philippinensis* was induced by exposure to light at 0—5 °C, and that exposure to light (5000 lux) at 0—5 °C for 1 h was sufficient to induce carotenoid synthesis to the full. However, the organisms exposed to light for over 1 h at 27 °C accumulated far more carotenoids than those exposed to light in an ice bath (Fig. 2, open circles).

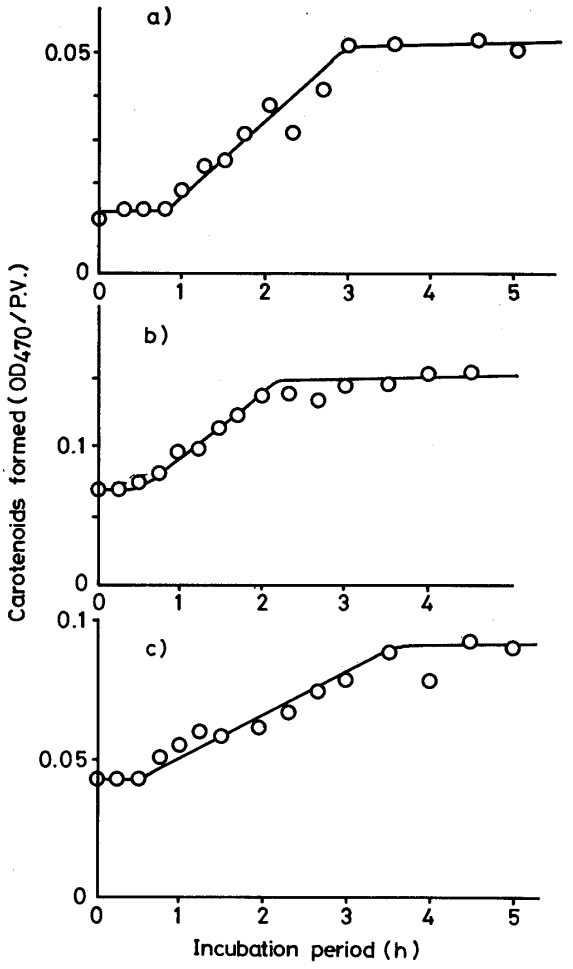

Fig. 3. Time Dependence of Carotenogenesis after Photoinduction

The culture exposed to light for 1 h in an ice bath was re-incubated in the dark at 27 °C. Portions (20 ml) of the culture were collected at the indicated times after the initiation of re-incubation. The cells were harvested by centrifugation and the pigments produced were determined. a) *M. echinospora* KCC A-0073, b) *M. chromogenes* KCC A-0022, c) *A. philippinensis* KCC A-0001.

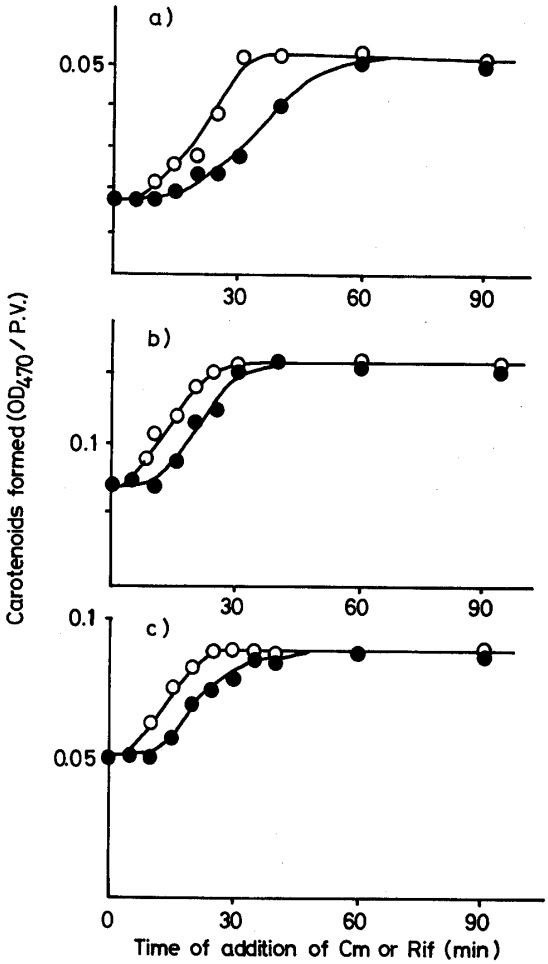

Fig. 4. Inhibition of Carotenogenesis by Chloramphenicol or Rifamycin during Dark Incubation Following Photoinduction

The culture exposed to light for 1 h in an ice bath was re-incubated in the dark at 27 °C. Portions (20 ml) of the culture were transferred to 100 ml Erlenmeyer flasks to which 1 mg of Cm or Rif had been added, at the indicated time after the initiation of re-incubation. After 20 h, dark incubation following the addition of Cm or Rif, cells were harvested and the amount of carotenoids formed was measured. (●), Cm was added; (○), Rif was added. a) *M. echinospora* KCC A-0073, b) *M. chromogenes* KCC A-0022, c) *A. philippinensis* KCC A-0001.

Time Dependence of Carotenoid Synthesis after Photoinduction

The cultures of each strain were exposed to light for 1 h in an ice bath, then the cultures were re-incubated in the dark. Carotenoids were accumulated in the cells after 40 to 60 min of dark incubation. The amount of carotenoids accumulated in the cells increased as incubation was continued but reached a plateau after 2 to 3 h incubation in the dark (Fig. 3a, b and c).

Since the amount of carotenoid increased not immediately, but with a time lag, the dark-reaction steps may involve *de novo* protein synthesis.

To obtain evidence that protein synthesis is induced by light exposure, chloramphenicol (Cm, final conc. 50 μg/ml) was added to the culture, which was then exposed to light and re-incubated in the dark. Addition of Cm at time 0 to 10 min to the culture of each strain completely inhibited the induced carotenogenesis. As Cm addition was delayed, carotenoid synthesis increased. At half to 1 h after the initiation of re-incubation, no inhibition was

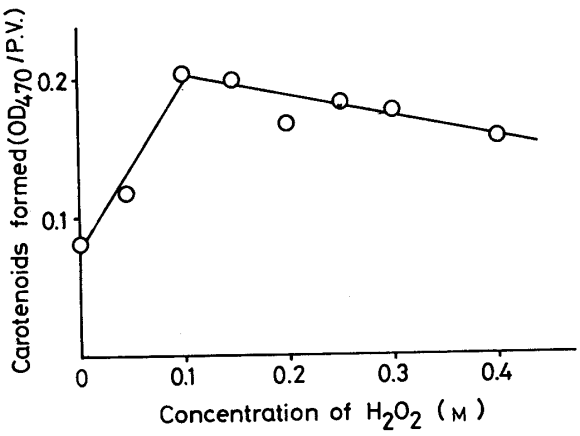

Fig. 5. Induction of Carotenogenesis by Hydrogen Peroxide

A. philippinensis was incubated in the dark for 20 h, and 50 ml portions of the culture were transferred to a series of 300 ml Erlenmeyer flasks. Various conc. of H_2O_2 were added to a series of the cultures (final conc. 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.4 M) and incubated for 30 min in the dark. The cells in each culture were washed and then suspended in the supernatant of the culture, which had been incubated in the dark for 20 h. After a further 20 h incubation in the dark at 27 °C, cells were harvested and carotenoids produced were measured.

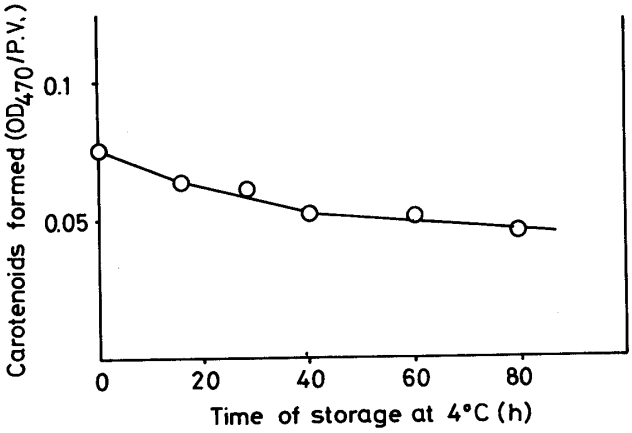

Fig. 6. Stability of Photoinduction in *A. philippinensis*

After exposure to light in an ice bath for 1 h, 20 ml portions of the culture were transferred to a series of 100 ml Erlenmeyer flasks and allowed to stand in the dark at 4 °C for indicated periods. Thereafter, each culture was incubated in the dark at 27 °C for 20 h. The amount of carotenoids extracted was measured.

observed (Fig. 4a, b and c, closed circles).

By the same procedure, the effect of rifamycin (Rif) on the induced carotenoid synthesis was investigated. As shown in Fig. 4a, b and c (open circles), Rif completely inhibited the induced carotenoid synthesis if added to the culture at time 0 to 5 min, and the degree of inhibition decreased with increasing incubation time. Half an hour after the initiation of re-incubation, addition of Rif did not inhibit the carotenoid synthesis in any strain.

Induction of Carotenogenesis by Hydrogen Peroxide

It was postulated that a metabolite produced light-independently was oxidized in the photochemical reaction steps, and the reaction product acted as an inducer of carotenoid synthesis. To confirm this assumption, *A. philippinensis* was incubated in the dark for 20 h, then treated with various concentrations of hydrogen peroxide. As shown in Fig. 5, hydrogen peroxide induced carotenoid synthesis of *A. philippinensis* in the same manner as light exposure, but increase in the concentration of hydrogen peroxide to over 0.1 M resulted in a decrease in the amount of carotenoid subsequently synthesized.

Stability of the Photoinduction

Dark-grown cells of *A. philippinensis* were exposed to light in an ice bath for 1 h. The culture exposed to light was stored in the dark in a refrigerator for various periods, then incubated in the dark for 20 h. As shown in Fig. 6, when the light-exposed cells were stored at 4 °C, the amount of carotenoid to be synthesized decreased in proportion to the period of storage. After 60 h of storage, the effect of light on the carotenoid synthesis had almost disappeared.

Discussion

According to the definition of photochromogenicity proposed by Runyon,[7a] a gradual

change or deepening in the color of colonies in light is not attributable to photochromogenicity but is a different phenomenon. In the previous paper,[6d] we reported on the effect of light on the pigmentation of strains of *Mycobacterium smegmatis* which exhibited enhancement of carotenoid production, *i.e.*, deepening of the color of colonies under illumination, pointing out that the enhancement of carotenoid production in these strains took place in the same manner as in previously known photochromogenic mycobacteria, *e.g.*, *M. kansasii*,[2a] *M. marinum*[2b-d] and a *Mycobacterium* sp.[2e-g] From these results, we proposed that a microorganism showing a gradual change or deepening in the color of colonies in light should be classified as a photochromogenic organism if the organism can be photoinduced to synthesize carotenoids. As stated in the present paper, light apparently enhanced pigmentation (deepening of the color of colonies) in growing cells of *Micromonospora* sp. KCC A-0087 and *Microbispora diastatica* KCC A-0023, producing carotenoids in addition to those constitutively produced in the cells. On the basis of the above findings, these strains, KCC A-0023 and KCC A-0087, were classified as photochromogenic strains.

The present study revealed that photochromogenic strains were distributed in the genera *Micromonospora, Microbispora* and *Actinoplanes*. This, when considered with our previous results,[6] shows that photochromogenesis is not specific but is a common, widely distributed phenomenon in *Actinomycetes* and related microorganisms.

The mechanism of photochromogenesis in *M. echinospora, M. chromogenes* and *A. philippinensis* could be divided into two main steps, as in the previously known photochromogenic strains.[2-6]

In the photochemical reaction steps of *M. echinospora, M. chromogenes* and *A. philippinensis*, light exposure at 27 °C had a higher efficiency than that at lower temperature, where bacterial growth did not take place. Two factors might account for this difference. One is the amount of a metabolite produced independently of light exposure; such a metabolite may serve as a substrate of the photochemical reaction, and the photochemical reaction product may derepress the carotenoid synthesis. With light exposure in an ice bath, the amount of the metabolite does not increase. However, organisms exposed to light at 27 °C continue to synthesize the metabolite during the exposure. The difference in the amount of the metabolite would reflect the difference in the effect of light exposure on induced carotenoid synthesis. The other factor is the stability of the reaction product of photochemical reaction. Cells of *Mycobacterium* sp.[2f] and *Brevibacterium sulfureum*[6c] exposed to light at 0—5 °C could synthesize carotenoid after prolonged storage in the dark at 4 °C. On the other hand, *A. philippinensis* did not show induced carotenoid synthesis after 60 h of storage at 4 °C. It was considered that the photochemical reaction product would be very unstable and would need to react with its target as soon as possible for the derepression of carotenoid synthesis. This stability of photoinduction varied from strain to strain. The most unstable example is the case of photochromogenic strains of *Streptomyces*,[6f] that is *S. canus, S. massasporeus* and *S. violaceoruber* were not induced to synthesize carotenoid by exposure to light at 0—5 °C though they were induced to do so at 27 °C.

Possible involvement of oxidation in the photochemical reaction steps of the metabolite produced light-independently was supported by the results that dark-grown cells of *A. philippinensis* treated with hydrogen peroxide were induced to synthesize carotenoid.

In the course of dark metabolic reactions, carotenoids were synthesized after a lag period, and the addition of Cm or Rif immediately after the termination of light exposure completely inhibited the induced carotenoid synthesis. These results indicated that some proteins such as carotenogenic enzymes were induced *via* transcription and translation by light exposure in *M. echinospora, M. chromogenes* and *A. philippinensis*, as in the previously known photochromogenic strains[2-6] except for *Rhodotorula minuta*,[9] in which photoinduced carotenoid

synthesis is a post transcriptional event.

Biochemical studies on photochromogenicity in bacteria including our previous studies revealed that all photochromogenic strains produced carotenoid by photoinduction, and the mechanism of photoinduced carotenogenesis, as far as investigated, was fundamentally the same.

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