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Effect of Light on Nonphotosynthetic Microorganisms. IV.[1] Photoinduced Carotenogenesis in *Mycobacterium smegmatis*

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Although *Mycobacterium smegmatis* produces carotenoids when grown in the dark, the carotenoid production of this organism is enhanced by exposure to light. This enhancement consists of an initial photochemical reaction and a series of metabolic reactions (dark reactions) which lead to the enhancement of carotenoid synthesis. Inhibition experiments with chloramphenicol showed that the dark metabolic reactions following light exposure include the process of photoinduced protein synthesis. Thus, *M. smegmatis* is capable of light-independent and photoinduced carotenogenesis.

Keywords——*Mycobacterium smegmatis*; photochromogenicity; photoinduced carotenogenesis; carotenoid; light-independent carotenogenesis; photoinduced protein synthesis

As described in Bergey's manual of determinative Bacteriology,[2] no pigments were produced at least in young cultures of *Mycobacterium smegmatis* on egg-media, but pigmented colonies may be observed in older cultures and more commonly on non-egg-media. Besides the pigments constitutively produced in mature colonies grown in the dark on non-egg-media, we have repeatedly observed that colonies of this organism developed a rich orange color (deepening of color of the colonies) when grown under illumination with visible light.

According to the definition proposed by Runyon[3] in 1959 for grouping of mycobacteria, photochromogens have been defined as follows. 1) Pigmentation is little or none if the strains are grown in the dark, but bright yellow to orange or brick-red if grown in continuous light. 2) Young, actively growing, nonpigmented colonies will become yellow in the dark 6 to 12 hr after exposure to light for 1 hr (45 cm from a 30 W lamp). 3) Light reaction of mature colonies is slow or none. 4) Many other mycobacteria will show some gradual change or deepening of color in light, but this is a different phenomenon. 5) Photochromogenicity is a pronounced and rapid change. Thus, *M. smegmatis*, which shows deepening of the color of colonies, has not been recognized as a photochromogen. In fact, previously known photochromogenic mycobacteria,[4] *e.g.*, *M. kansasii*[4d] and *M. marinum*[4b,f] produced carotenoids only in response to light exposure and remain creamy white when grown in the dark.

In spite of extensive studies of photoinduced carotenogenesis in photochromogenic mycobacteria,[4] little is known about the nature of pigmentation of bacteria showing a gradual change or deepening of the color of colonies in the light. Therefore, it seemed of interest to investigate the effect of light on the pigmentation of various strains of *M. smegmatis*.

This paper describes the results of experiments on the mechanism of pigmentation of *M. smegmatis* and shows that the pigmentation is a result of photoinduced carotenogenesis and light-independent carotenogenesis.

Experimental

Microorganisms——The test strains of *M. smegmatis* numbered T-202 to T-211 were supplied by Dr. M. Tsukamura, The National Sanatorium, Chubu Chest Hospital, the strains T-212 and T-213 were from Dr. Y. Mizuguchi, National Institute of Health, Tokyo, and the strains T-201 and T-2053 were from Dr. T. Arai,

The Institute of Food Microbiology, Chiba University. Except for strain T-207, the properties of the test strains of *M. smegmatis* were consistent with those described in Bergey's manual[2] in the following tests, *i.e.*, carbon utilization, arylsulfatase test (−), nitrate reduction test (+), growth at 45° (+), growth at 52° (−) and survival at 60° for 4 hr (−). The properties of strain T-207 were consistent with those described in Bergey's manual,[2] except for the growth test at 45° (−). Stock cultures of the test organisms were maintained on 1% Ogawa egg medium (Eiken Chemical Co.).

Media——The following media were used. TB broth (yeast extract, 2.0 g; polypeptone, 4.0 g; $Na_2HPO_4 \cdot 12H_2O$, 2.5 g; KH_2PO_4, 1.0 g; Na-citrate, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; glycerol, 10.0 g; dist. water 1000 ml, pH adjusted to 7.0), Kirchner medium [KH_2PO_4, 4.0 g; $Na_2HPO_4 \cdot 12H_2O$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; Na-citrate, 2.5 g; asparagine, 5.0 g; malachite green, 2 mg; glycerol, 20 ml; TB medium albumin (Eiken Chemical Co.), 100 ml; dist. water 900 ml, pH 6.2], tryptosoy broth (tryptone, 17 g; soypeptone, 3.0 g; glucose, 2.5 g; K_2HPO_4, 2.5 g; NaCl, 5.0 g; dist. water 1000 ml, pH adjusted to 7.3), Dubos' medium (KH_2PO_4, 1.3 g; $Na_2HPO_4 \cdot 12H_2O$, 2.2 g; asparagine, 2.0 g; peptone, 5.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2$, 0.5 mg, ferric ammonium citrate, 10 mg; $ZnSO_4$, 0.1 mg; $CuSO_4 \cdot 5H_2O$, 0.1 mg; Tween 80, 0.5 g; malachite green, 2 mg; TB medium albumin, 100 ml; dist. water 900 ml, pH 6.2), Karlsson's medium [glycerol, 20 g; $(NH_4)_2SO_4$, 1.0 g; $FeSO_4 \cdot 7H_2O$, 20 mg; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 10 mg; KH_2PO_4, 1.5 g; $Na_2HPO_4 \cdot 12H_2O$, 4.0 g; dist. water 1000 ml, pH adjusted to 7.0], synthetic medium I [$Na_2HPO_4 \cdot 12H_2O$, 4.0 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; Na-citrate, 2.5 g; $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 10 mg; H_3BO_4, 10 μg; MoO_3, 20 μg; $CuCl_2$, 10 μg; $MnCl_2$, 2 μg; $ZnCl_2$, 200 μg; $CoCl_2$, 10 μg; asparagine, 5.0 g; glucose, 20 g; thiamine-HCl, 0.4 mg; dist. water 1000 ml, pH 6.2], synthetic medium II (add 0.2 g of cysteine to synthetic medium I) and 1% Ogawa egg medium (Eiken Chemical Co.).

Cultivation of Organisms——The cultivation of bacteria was carried out at 37° with rigid light exclusion unless otherwise specified. When the organism was transplanted, a photographic red lamp had to be used. After two cycles of cultivation on slants (1% Ogawa egg medium) in the dark, each test strain was shake-cultured in 100 ml of TB broth in a 500 ml Erlenmeyer flask for 48 hr, harvested by centrifugation, homogenized and suspended in 5 ml of phosphate buffer (1/15 M, pH 7.2). In all experiments, the bacterial suspension thus prepared was used for inoculation.

For examination of photochromogenicity (Figs. 1 and 2, Tables I and II), the preincubated bacterial suspension was inoculated ($OD_{600}=0.1$) into 500 ml Erlenmeyer flasks each containing 100 ml of medium. For the experiments shown in Fig. 1 and Tables I and II, each of two inoculated flasks was shake-cultured for 72 hr, the one under continuous illumination (4600 lux) and the other in total darkness. For the experiment shown in Fig. 2, the bacteria were shake-cultured either under continuous illumination (4600 lux) or in the dark for a designated time. For elucidation of the mechanism of photoinduced carotenogenesis (Figs. 3—5), the preincubated bacterial suspension was inoculated ($OD_{600}=0.1$) into 100 ml Erlenmeyer flasks each containing 20 ml of TB broth and shake-cultured for 24 hr in the dark. The cultures were shifted to low temperature (0—5°), exposed to light (12000 lux) for a certain time, shifted back to darkness and incubated again at 37° for a designated time.

Photoinduction——Photoinduction of bacteria was performed by illumination with white fluorescent lamps.

Extraction of Pigments——After incubation for a designated time, the bacterial cells were harvested by centrifugation, washed with 1/15 M phosphate buffer (pH 7.2), suspended in distilled water and lyophilized. The dry cell mass was weighed and repeatedly extracted with acetone–methanol (1 : 3 v/v) until no more pigment was visible. The extracts were combined, evaporated to dryness *in vacuo* and then dissolved in methanol (5 ml for Tables I and II, Figs. 3 and 4; 10 ml for Figs. 1, 2 and 5). Optical density at 450 nm of the methanol solutions was measured with a Hitachi-340 spectrophotometer. For the experiments shown in Tables I and II, the carotenoid content of the methanol solution was determined as recommended by Liaaen-Jensen and Jensen.[5] For thin-layer chromatography, silica gel without fluorescent indicator (Merck) was used as the absorbent and 5% acetone in *n*-hexane was used as the solvent system.

Results

Carotenoid Content in Light-Exposed and Dark-Grown Bacteria

As shown in Fig. 1, the extracts from the light-exposed and dark-grown cells of *M. smegmatis* T-211 exhibited the same absorption maxima at 425 (shoulder), 450 and 478 nm (shoulder) in the visible light absorption spectra, suggesting that the light-exposed and dark-grown cells of this strain produced the same pigments. Thin layer chromatograms of the extracts from the light-exposed and the dark-grown cells showed the same principal pigment spots with *Rf* 0.59, 0.06 and 0.00, though minor pigment spots with *Rf* 0.53 and 0.44 were also detectable only in the extracts from light-exposed cells.

From the spectral data shown in Fig. 1 and on the basis of the results previously reported on the pigments of this organism,[6] the pigments were assumed to be a mixture of carotenoids.

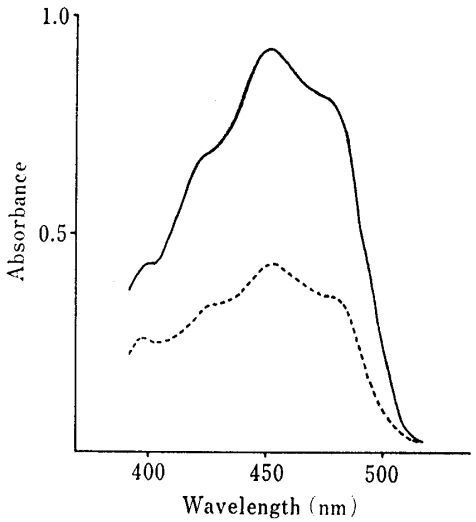

Fig. 1. Visible Light Absorption Spectra of the Extracts from Light-exposed and Dark-grown Cells of *M. smegmatis* T-211

The organism was inoculated ($OD_{600}=0.1$) into two 500 ml Erlenmeyer flasks each containing 100 ml of TB broth. One of the two flasks was incubated at 37° under continuous illumination (4600 lux) and the other in the dark. After 72 hr, the cells were harvested and extracted with acetone-methanol (1: 3 v/v). The methods for cultivation of the organism and extraction of the pigments are described in the text. The extracts from the light-exposed and the dark-grown cells were each concentrated *in vacuo*, dissolved in methanol (10 ml) and subjected to visible light absorption spectroscopy.
—— : extracted from the light-exposed cells,
---- : extracted from the dark-grown cells.

Fig. 1 also indicates that the amount of carotenoid in the cells apparently increased as a result of light exposure.

In order to clarify whether the phenomenon mentioned above is common to strains of *M. smegmatis*, 14 strains belonging to this species were arbitrarily selected and tested. In 13 out of 14 strains incubated in TB broth, it was found that the amount of pigments accumulated in the light-exposed cells was greater than that of the dark-grown cells (Table I). As shown in Table II, a similar enhancement of carotenoid production under illumination was also observed in strain T-204 incubated in seven different media.

Time Course of Carotenoid Synthesis

The amount of carotenoids accumulated in the cells as a function of time of incubation is shown in Fig. 2. Under continuous illumination, the carotenoid began to accumulate after an initial lag period and increased throughout the incubation until bacterial growth ceased. On the other hand, the dark-grown cells also produced carotenoids, but much less of the pigment than the light-exposed cells.

When actively growing cells were shifted to 0—5° and exposed to light (12000 lux) for various periods, followed by incubation in the dark for 24 hr, the amount of carotenoids accumulated was proportional to the light dose below saturation, as shown in Fig. 3. When the illuminated bacteria were allowed to stand for 24 hr in an ice bath, where bacterial growth did not take place, no increase of carotenoid production was observed. The time course of caro-

Table I. Carotenoid Content of Light-exposed and Dark-grown Cells of *M. smegmatis*

Strain number	Source	Carotenoid content (μg/g dry weight)	
		Light-exposed cells	Dark-grown cells
T-201	T. Arai	13.96	3.40
T-202	17001, M. Tsukamura	4.92	3.72
T-203	17002, M. Tsukamura	1.00	1.12
T-204	17003, M. Tsukamura	3.72	2.16
T-205	17004, M. Tsukamura	9.96	5.36
T-206	17005, M. Tsukamura	10.12	4.16
T-207	17021, M. Tsukamura	15.08	3.80
T-208	17022, M. Tsukamura	13.96	5.36
T-209	17023, M. Tsukamura	11.28	3.92
T-210	17024, M. Tsukamura	8.96	3.36
T-211	17027, (ATCC 14468*), M. Tsukamura	7.28	3.20
T-212	strain Lacticola, Y. Mizuguchi	9.60	2.76
T-213	strain Jucho, Y. Mizuchi	13.84	4.03
T-2053	T. Arai	13.76	3.36

The experimental procedure is described in the text.
*suggested neotype strain

TABLE II. Carotenoid Content of Light-exposed and Dark-grown Cells of *M. smegmatis* Strain T-204 incubated in Different Media

Medium	Carotenoid content (μg/g dry weight)	
	Light-exposed cells	Dark-grown cells
TB broth	3.80	2.20
Kirchner medium	4.40	1.00
Tryptosoy broth	2.80	1.60
Dubos' medium	3.04	1.28
Karlsson's medium	3.08	1.00
Synthetic medium I	3.92	2.44
Synthetic medium II	5.70	1.60

The experimental procedure is described in the text.

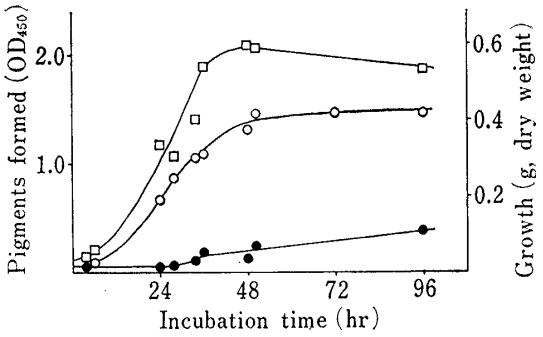

Fig. 2. Growth and Carotenoid Synthesis of *M. smegmatis* T-212 grown under Illumination or in the Dark

The organism was inoculated (OD_{600}=0.1) into a series of 500 ml Erlenmeyer flasks each containing 100 ml of TB broth. Each of the flasks inoculated was incubated at 37° either under continuous illumination (4600 lux) or in the dark. The bacterial growth is shown in terms of g dry weight. The cells were harvested at the indicated time and extracted with acetone-methanol (1: 3 v/v). The extracts were concentrated *in vacuo* and dissolved in methanol (10 ml). OD_{450} of the methanol solution was measured.

—○—: carotenoid formed in the light-exposed cells;
—●—: carotenoid formed in the dark-grown cells;
—□—: bacterial growth

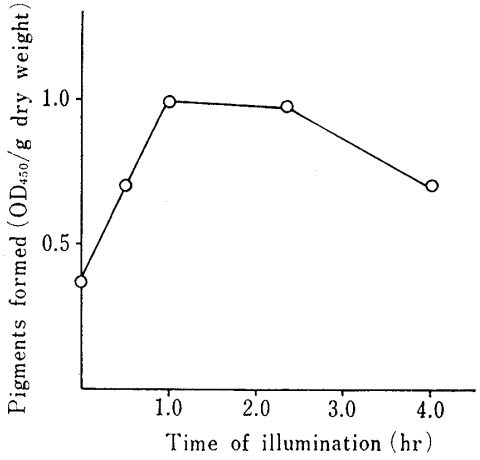

Fig. 3. Dependence of Carotenoid Production of *M. smegmatis* T-212 on the Duration of Light Exposure

The organism was inoculated (OD_{600}=0.1) into a series of 100 ml Erlenmeyer flasks each containing 20 ml of TB broth. After incubation for 24 hr in the dark, each of the cultures was exposed to light (12000 lux) for a certain time (0.5, 1.0, 2.4 or 4.0 hr) in an ice bath. The cultures were then incubated at 37° in the dark for 24 hr. Then, carotenoids formed in the harvested cells were extracted with acetone-methanol (1: 3 v/v), concentrated *in vacuo* and dissolved in methanol (5 ml). OD_{450} of the methanol solution was measured.

tenoid synthesis as a function of time of dark incubation following light-exposure is shown in Fig. 4. The carotenoid production in non-photoinduced cells gradually and consistently increased, while that in photoinduced cells sharply increased from 0.5 hr until about 2 hr of dark incubation after light-exposure and then paralleled the former.

Apparently an initial photochemical reaction is followed by a series of dark metabolic reactions which lead to the photoinduced synthesis of carotenoids.

Effect of Chloramphenicol on the Time Course of Pigment Production

To gain a better insight into the dark metabolic reactions, chloramphenicol was added at various times during the first 3 hr of dark incubation following photoinduction and the amount of carotenoid accumulated after 24 hr of dark incubation was assayed (Fig. 5). If chloramphenicol was added immediately after photoinduction, the enhancement of carotenoid production caused by exposure to light was completely inhibited. When the addition of

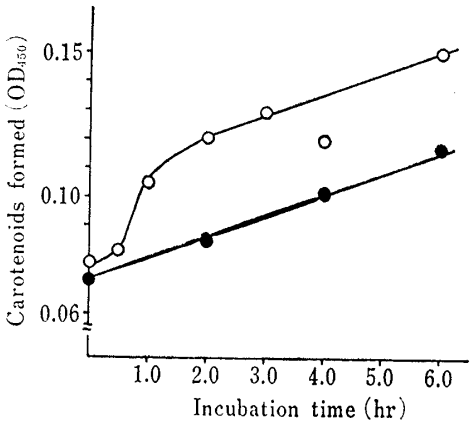

Fig. 4. Time Dependence of Carotenoid Synthesis of *M. smegmatis* T-212 after Photoinduction

The organism was inoculated (OD_{600}=0.1) into a series of 100 ml Erlenmeyer flasks each containing 20 ml of TB broth. After incubation for 24 hr in the dark, the cultures were exposed to light (12000 lux) for 2 hr in an ice bath. Thereafter, the cultures were incubated in the dark at 37°. The cells were harvested at the indicated time. Carotenoids formed in the cells (—○—) were extracted with acetone-methanol (1: 3 v/v), concentrated *in vacuo* and dissolved in methanol (5 ml). OD_{450} of the methanol solution was measured. As a control, the time dependence of carotenoid production of non-photoinduced cells is also shown (—●—).

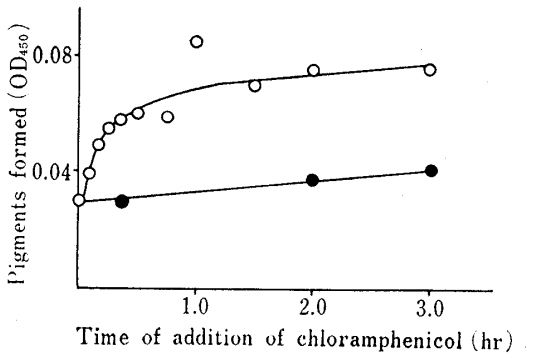

Fig. 5. Inhibition of Carotenoid Synthesis by Chloramphenicol during Dark Incubation of Photoinduced *M. smegmatis* T-212

The organism was inoculated (OD_{600}=0.1) into a series of 100 ml Erlenmeyer flasks each containing 20 ml of TB broth. After incubation for 24 hr in the dark, the cultures were exposed to light (12000 lux) for 2 hr in an ice bath. Thereafter the cultures were incubated in the dark for 24 hr. Chloramphenicol (100 μg/ml) was added to the cultures at a certain time (0, 5, 10, 15, 20, 30, 45, 60, 90, 120, or 180 min) during the first 3 hr of the dark incubation following light exposure. Carotenoids formed in the cells (—○—) were extracted with acetone-methanol (1: 3 v/v), concentrated *in vacuo* and dissolved in methanol (10 ml). OD_{450} of the methanol solution was measured. As a control, the inhibition of carotenoid synthesis of the non-photoinduced cells by chloramphenicol is also shown (—●—).

chloramphenicol was delayed, increasing amounts of carotenoids were formed. After about 1.5 hr, however, the sensitivity to chloramphenicol diminished, and the effect of chloramphenicol on carotenoid production in photoinduced cells became slight, paralleling that in non-photoinduced cells. The results showed that the dark metabolic reactions following the initial photochemical reaction include the process of protein synthesis.

Discussion

As revealed in the present investigation, *M. smegmatis* produced carotenoids, even if the organism was incubated in total darkness, but the accumulation of carotenoids in the dark-grown cells was less than that in the light-exposed cells (Figs. 1 and 2; Tables 1 and 2). As shown in Tables 1 and 2, the amounts of carotenoids accumulated in the dark-grown and light-exposed cells varied depending upon the strain and media, except for strain T-203. Thus, it became clear that *M. smegmatis* was capable of light-dependent carotenoid production together with light-independent carotenoid production. It has been reported by Rilling[4h] that a photochromogenic *Mycobacterium* sp. is capable of synthesizing small quantities of three kinds of carotenoids, phytoene, phytofluene and a epiphastic fraction independently of light, though the carotenogenesis is markedly light-dependent. However, he also mentioned that this slight light-independent carotenogenesis is qualitatively different from the photoinduced synthesis. In contrast to the photochromogenic *Mycobacterium* sp.,[4h] *M. smegmatis* produces almost the same carotenoids in both light-exposed and dark-grown cells (Fig. 1). Small amounts of light-independent carotenogenesis have also been observed in photochromogenic fungi, *Neurospora crassa*[7a] and *Fusarium aquaeductuum*.[8a]

The present investigation also revealed that the light-dependent carotenoid production in *M. smegmatis* took place in essentially the same manner as photoinduced carotenogenesis in typical photochromogenic mycobacteria, e.g., *M. marinum*,[4b,f] *M. kansasii*[4d] and a *Mycobacterium* sp.,[4a,e,g,h] *Myxococcus xanthus*,[9] *Brevibacterium sulfureum*,[1] *Neurospora crassa*[7] and *Fusarium aquaeductuum*.[8]

Fig. 3 clearly shows that light triggers the enhancement of carotenoid production. Even if the illumination was performed at low temperature (0—5°), where bacterial growth did not take place, once exposed to light the organism was able to enhance its carotenoid production on subsequent incubation at higher temperature (37°) in the dark. The data currently available do not fully explain why prolonged exposure to light (4 hr) causes a decrease in the amount of carotenoids in the photoinduced cells (Fig. 3). This decrease can be ascribed in part to the photochemical degradation of carotenoids previously produced in the dark-grown cells. The results of the experiments shown in Figs. 3 and 4 indicate that the enhancement of carotenoid production in this organism under illumination consists of an initial photochemical reaction and a series of temperature-dependent dark metabolic reactions.

It was demonstrated that the dark metabolic reactions in known photochromogenic bacteria[1,4,9,10] and fungi[7,8] involve the *de novo* synthesis of carotenogenic enzymes *via* the classical scheme of transcription and translation. Since enhancement of carotenoid synthesis occurs with a lag period after exposure to light (Fig. 4), and also since it never appears without bacterial growth, it may be suggested that the synthesis of carotenogenic enzymes takes place during this lag period, followed by the carotenoid synthesis. This view was supported by the results of the inhibition experiment with chloramphenicol (Fig. 5). Since the amount of carotenoids synthesized after addition of chloramphenicol may be regarded as an index of the concentration of carotenoid-synthesizing enzymes, present in the cells at the time of chloramphenicol addition, the results obtained from this experiment might be interpreted as follows: when the dark-grown cells are illuminated, derepression of a genetic site results, and this derepression leads to the synthesis of protein, probably carotenogenic enzymes. After 1.5 hr of dark-incubation, the lack of effect of chloramphenicol on the enhancement of carotenoid production caused by exposure to light (Fig. 5) is probably due to the onset of repression of the photoinduced protein synthesis. Thus, the results of the inhibition experiment with chloramphenicol (Fig. 5) suggest that the mechanism observed in the previously studied microorganisms[1,4,7,8,9] may also be operating in *M. smegmatis*.

As mentioned in the beginning of this paper, *M. smegmatis* is not regarded as a photochromogen according to Runyon's definition[3] for mycobacteria, since the organism only shows deepening of the color of colonies when grown under illumination. From the standpoint of the mechanism of photoinduced carotenogenesis elucidated in known photochromogenic bacteria,[1,4,9] we proposed in the previous paper[10] that in the absence of biochemical evidence, a microorganism showing a gradual change or deepening of the color of colonies under illumination should be recognized as a photochromogenic microorganism if the organism can be photoinduced to synthesize pigment-forming enzymes subsequently shows enhancement of its pigment production. That proposal is supported by the results of the present investigation with *M. smegmatis*.

The data currently available do not explain why *M. smegmatis* is capable of light-independent and photoinduced carotenogenesis. Two possibilities can be considered. The first is that the genetic sites of constitutive and photoinducible carotenogenesis are different in *M. smegmatis*. (This implies that the exceptional strain T-203 might be a mutant at the gene(s) for photoinducible carotenogenesis.) The second is that carotenogenesis might not be very stringent in terms of regulation and that the exceptional strain T-203 might be a constitutive mutant. Further investigations are in progress to elucidate the genetic control of photoinduced and nonphotoinduced carotenogenesis in *M. smegmatis*.

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