Chem. Pharm. Bull. 29(1) 176—180 (1981)

Effect of Light on Nonphotosynthetic Microorganisms. III.[1] Photoinduced Carotenogenesis in *Brevibacterium sulfureum*[2]

Yasumasa Koyama,*,[3] Yoshitaka Yazawa, Fumio Kato,[3] and Saburo Yamagishi

Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho, Chiba, 280, Japan

(Received July 7, 1980)

Brevibacterium sulfureum is capable of carotenoid synthesis when grown under illumination, but not in the dark. The carotenogenesis consists of two steps, an initial photochemical reaction and a series of metabolic reactions (dark reactions) which lead to carotenoid synthesis *de novo*. Inhibition experiments with chloramphenicol showed that the dark metabolic reactions include the process of *de novo* synthesis of carotenogenic enzymes.

These results show *B. sulfureum* to be a typical photochromogen.

Keywords——*Brevibacterium sulfureum*; photochromogenicity; photoinduced carotenogenesis; carotenoid; photoinduced enzyme synthesis

In the previous paper,[4] we reported that colonies of *Brevibacterium sulfureum* developed a rich yellow pigment when grown under illumination, but were entirely unpigmented in the dark, and we proposed on the basis of this observation that the organism should be classified as a photochromogen, a term proposed by Runyon in 1959 for mycobacteria.[5] It is known that carotenoids can be photoinduced in a number of bacteria.[6] Considerable progress has been made in biochemical studies of photochromogenesis, and it has been demonstrated that the mechanism of photoinduced carotenoid synthesis consists of two main reactions. The first is photochemical, while the second is independent of light and has the characteristics of inducible systems. However, previous investigations on the mechanism of photochromogenicity in *Actinomycetes* and related organisms have dealt exclusively with mycobacteria and no attention has been paid to the other bacteria belonging to this group. Therefore, it is of interest to investigate the mechanism of pigmentation of *B. sulfureum*, as an example of the coryneform group of bacteria.

The purpose of this paper is to describe the main features of the photochromogenic response and the results of experiments on the mechanism of carotenogenesis in *B. sulfureum*.

Experimental

Microorganism——*B. sulfureum* strain No. AU31 was kindly supplied by Dr. U. Shimizu, The Research Institute of Chemobiodynamics, Chiba University. Stock culture was maintained on nutrient agar slant.

Medium and Cultivation of Organism——Nutrient broth was used in all experiments. The cultivation of bacteria was carried out at 27° 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 in the dark, the bacteria were inoculated into 100 ml of medium placed in a 500 ml shaking flask and incubated for 12 hr. The bacterial suspension thus preincubated was inoculated ($OD_{560}=0.1$) into 100 ml Erlenmeyer flasks containing 20 ml of the medium and shake-cultured for a designated time. To prepare samples for column chromatography, the bacteria were incubated in the medium (3 liters) in a jar (6 liter) for 72 hr under continuous illumination (4200 lux) with aeration (1.5 liters of air/min) and agitation (250 rpm).

Photoinduction——Photoinduction of bacteria was performed by illumination with 30 W white fluorescent lamps.

Extraction of Pigments——After incubation for a designated time, the bacterial cells were harvested by centrifugation, washed twice with water and lyophilized. The dry cell mass was weighed and extracted with acetone–methanol (1 : 3 v/v) until no more pigment was visible. The extracts were evaporated to

dryness and the residue was dissolved in methanol. Insoluble material was removed by filtration. The filtrate was concentrated *in vacuo* and dissolved in methanol (10 ml). The optical density of the pigment extract was measured at 440 nm with a Hitachi 340 spectrophotometer. The amount of pigments in the cells was expressed in terms of OD_{440}/g dry weight of bacteria.

Column Chromatography——The bacterial cells were extracted as described above. The extracts were concentrated *in vacuo* and extracted with *n*-hexane. The *n*-hexane extract was concentrated and poured onto the top of a silica gel 60 (Merck) column (2 by 30 cm). The column was developed with benzene (200 ml) and benzene–methylethylketone (5: 1 v/v, 100 ml; 3: 1 v/v, 150 ml) to give three fractions. The first fraction eluted from the silica gel column with benzene (1st 50 ml) was rechromatographed on a similar column to yield a colorless oil that was identical with phytoene[7] in terms of the absorption spectra (absorption maxima: 275, 285, 297 nm). The second fraction eluted with benzene (50—200 ml) was rechromatographed on a Woelm alumina (grade I) column (1 by 10 cm). The column was developed initially with *n*-hexane and then with increasing concentrations of acetone (1—40%) in *n*-hexane, from which fractions A, B and C were obtained. Fraction A was identical with phytofluene[7] in terms of the absorption spectrum (absorption maxima: 330, 348, 367 nm). Fraction B had an absorption spectrum (absorption maxima: 376, 399, 424 nm) identical with that of ζ-carotene.[7] Fraction C had an absorption spectrum (absorption maxima: 440, 470, 502 nm) identical with that of lycopene.[7] The third fraction eluted from the silica gel column with benzene–methylethylketone (3: 1 v/v) was rechromatographed on a Woelm alumina column to give a yellow syrup that was identical with neurosporene[7] in terms of the absorption spectra (absorption maxima: 417, 438, 467 nm). Meanwhile, the *n*-hexane extract was chromatographed on a Woelm alumina column (1 by 15 cm), which was initially eluted with 1% acetone in *n*-hexane. The acetone concentration was then increased to 100%, and a yellow fraction was obtained from the acetone eluate as a single band. The fraction had an absorption spectrum [absorption maxima: 340, 428 (shoulder), 449, 478 nm] identical with that of β-carotene.

Results

Tentative Identification of Carotenoids Synthesized

As reported in our previous paper,[4] the extract of light-exposed cells of *B. sulfureum* exhibited absorption maxima at 417, 439 and 470 nm in the visible light absorption spectrum. Upon silica gel and alumina column chromatography of the extracts, several pigments were eluted from the columns, many of which appeared in only trace amounts or in mixtures. For the purpose of this work, tentative identification of only a few of these compounds was attempted, and the presence of phytoene, phytofleune, neurosporene, lycopene, ζ-carotene and β-carotene was revealed. Thus, the pigments that accumulated in the cells under illumination appeared to be a mixture of carotenoids.

Photoinduction of Carotenoid Synthesis

The bacteria were incubated either under continuous illumination (4200 lux) or in the dark. As shown in Fig. 1, the amount of carotenoids accumulated in the light-exposed cells increased roughly in line with the bacterial growth after an initial lag period, and reached a plateau a short time after the bacterial growth had ceased. However, no carotenoids were produced in the dark-incubated cells throughout their growth. It is of interest that the growth curve displays an inflection when the bacteria are incubated in nutrient broth, and the curve of pigment formation also displays an inflection at the same time of incubation (Fig. 1).

The bacteria actively growing in the dark were shifted to 0—5°, exposed to light (10000 lux) for a certain time and shifted back to 27°. Subsequently, the illuminated bacteria were incubated in the dark for 24 hr, and the pigments were extracted and assayed colorimetrically. The amount of carotenoids produced was proportional to the light dose below saturation, as shown in Fig. 2. When the illuminated bacteria were allowed to stand for 24 hr in an ice bath, where bacterial growth did not take place, no carotenoid was formed. However, incubation in the dark and at higher temperature (27°) resulted in carotenoid production. The data clearly show that light triggers caroteniod synthesis by this organism.

On the other hand, when the actively growing dark-incubated cells were placed in an atmosphere of nitrogen and shake-cultured under continuous illumination, little or no carotenoid was synthesized.

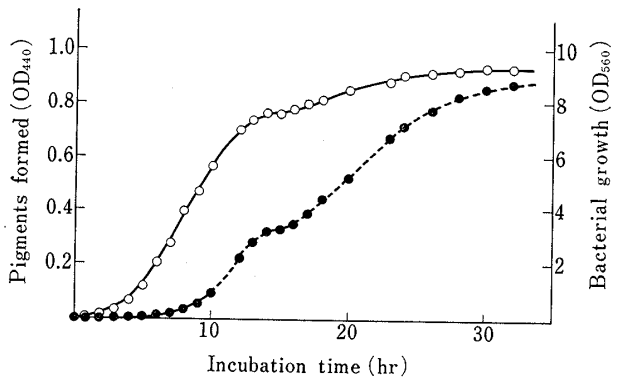

Fig. 1. Growth and Carotenoid Synthesis of *B. sulfureum* under Illumination

—○—, carotenoids formed; ---●---, bacterial growth. The orgainsm were incubated under illumination (4200 lux) in nutrient broth at 27°. The bacterial growth is shown in terms of optical density at 560 nm. The cells were harvested at the indicated time and extracted with acetone–methanol (1:3). The extracts were concentrated *in vacuo* and dissolved in methanol. OD_{440} of the methanol solution was measured.

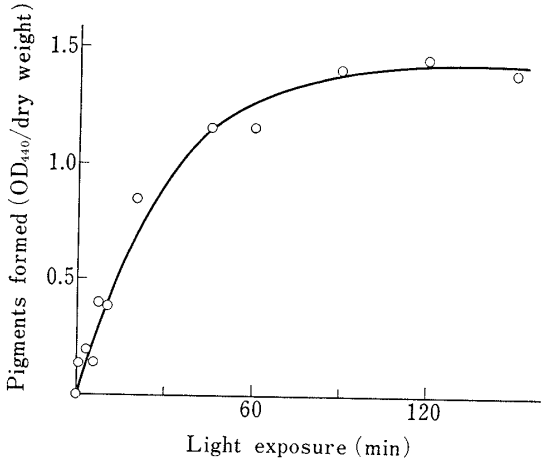

Fig. 2. Dependence of Carotenoid Synthesis of *B. sulfureum* on the Duration of Light Exposure

After incubation for 12 hr in the dark, the cultures were exposed to light (10000 lux) for a certain time in an ice bath. The cultures were then incubated at 27° in the dark for 24 hr.

Carotenoid Synthesis after Photoinduction

The actively growing dark-incubated cells were exposed to light (5000 lux) in an ice bath for 60 min followed by incubation in the dark at 27°. The time course of carotenoid production as a function of time of dark-incubation following light exposure is shown in Fig. 3. After a lag period of about 15 min the rate of carotenoid production became maximal for 4 hr and then tapered off. If the dark-grown cells were exposed to light after the maximal growth had been attained, only a small amount of carotenoids was produced in the cells. We can now conclude that the photoinduction is followed by a series of dark metabolic reactions which lead to the synthesis of carotenoids *de novo*. That this is indeed the case is demonstrated by experiment shown in Fig. 4.

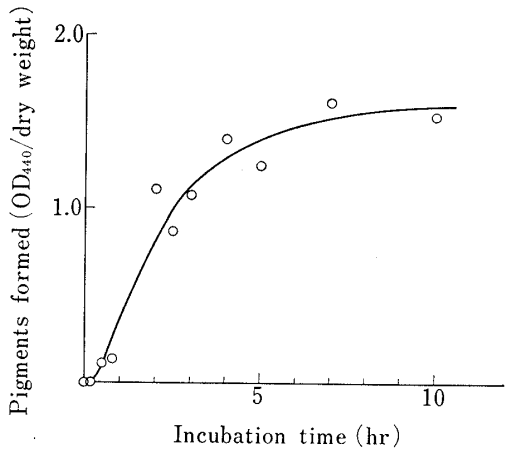

Fig. 3. Time Dependence of Carotenoid Synthesis of *B. sulfureum* after Photoinduction

After incubation for 12 hr in the dark, the cultures were exposed to light (5000 lux) for 60 min in an ice bath, then incubated in the dark for a designated period.

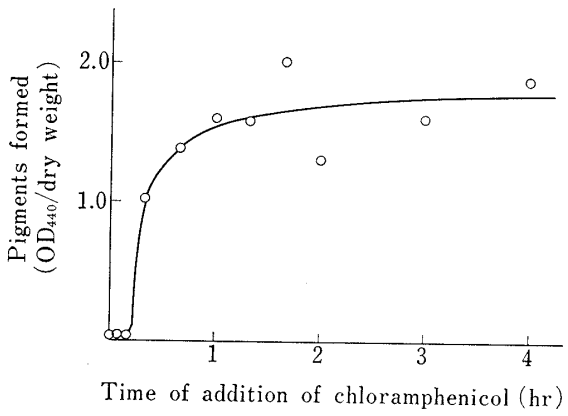

Fig. 4. Inhibition of Carotenoid Synthesis by Chloramphenicol during Dark Incubation of the Photoinduced *B. sulfureum*

After incubation for 12 hr in the dark, the cultures were exposed to light (10000 lux) for 60 min in ice bath, then incubated at 27° in the dark for 24 hr. Chloramphenicol (5 μg/ml) was added at various times during the first 4 hr of the incubation.

Chloramphenicol was added at certain times during the first 4 hr of dark incubation following photoinduction, and the amount of carotenoids synthesized after 24 hr of dark incubation was assayed. If chloramphenicol was added immediately after photoinduction, carotenoid production of this organism was completely inhibited. When the addition was delayed, increasing amounts of carotenoids were synthesized. The carotenoid synthesis after photoinduction was sensitive to the addition of chloramphenicol up to 60 min. The results of this experiment can be interpreted as follows. When the dark-grown cells were illuminated, derepression of the genetic site resulted. The derepression led to the synthesis of enzymes necessary for carotenogenesis. When the cells given initial illumination were incubated in the dark, a repression of further synthesis of carotenogenic enzymes sets in after 60 min. Consequently, we can conclude that the dark metabolic reactions following photoinduction involve *de novo* synthesis of carotenogenic enzymes.

As mentioned above, the synthesis of carotenogenic enzymes continued for about 60 min after photoinduction. On the other hand, the synthesis of carotenoids continued for about 4 hr after photoinduction, as shown in Fig. 3. Thus, there is a time lag between the enzyme synthesis and the carotenoid synthesis, and this may be explained by assuming that the continuing synthesis of carotenoids is due to residual enzyme activity and not to a continuing enzyme synthesis.

Thus, the pigmentation of *B. sulfureum* was characterized as photoinduced carotenogenesis.

Discussion

The results of the present investigation revealed that carotenogenesis in *B. sulfureum* consists of two steps, a photochemical reaction and a series of dark metabolic reactions.

The photochemical reaction was fast and temperature-independent. As shown in Fig. 2, a brief exposure to light was enough to photoinduce carotenogenesis in this organism. Once exposed to light, the organism was able to produce carotenoids after a prolonged delay, at least after 24 hr, when the illuminated cells were stored at 0—5°, indicating that the effect of the photochemical event was maintained in a stable state. However, it seems that the stability of the effect of the photochemical event varies with the organism. Rilling reported with a *Mycobacterium* sp.[6g] that the effect was so stable that the organism was able to produce carotenoids after delay of as much as three months when stored at −15°. With *Streptomyces canus*,[8] on the other hand, the effect was extremely unstable, so photoinduction was carried out during bacterial growth at higher temperature, since no detectable carotenoid was formed when the photoinduction was carried out in an ice bath followed by incubation in the dark at 27°.

As emphasized in the cases of previously known photochromogenic bacteria,[6] the photochemical step required both light and oxygen. As in the known photochromogenic bacteria, oxygen was essential to the photoinduction of *B. sulfureum*, which suggests that the photochemical event may be a photooxidative reaction with a metabolite presented in the dark-grown cells. The photooxidative reaction presumably resulted in the generation of a new compound, which could then serve as an inducer of carotenogenic enzyme synthesis. An alternative interpretation might be possible, which is that exposure to light resulted in the photooxidation of the repressor substance present at the genetic site of carotenoid synthesis. In such a case, the effect of the photochemical event might be irreversible, and the regeneration of the repressor would be achieved only after bacterial growth accompanied by carotenoid synthesis had occurred. In view of the observations with *Stm. canus*, the latter possibility seems to be unlikely. In either case, however, derepression of the genetic site can take place as a result of a photochemical event.

We will next consider the dark metabolic reactions which lead to the synthesis of carotenoids *de novo*.

In the previously known photochromogenic bacteria[6] and fungi,[9] it was demonstrated that the dark metabolic reactions involved the *de novo* synthesis of carotenogenic enzymes *via* the classical scheme of transcription and translation. Since carotenoid synthesis occurs with a difinite lag period after exposure to light (Fig. 3) and since it never appears without bacterial growth, it may be suggested that the *de novo* synthesis of carotenogenic enzymes takes place during this lag period, followed by the carotenoid synthesis. This was confirmed by the results of the inhibition experiment with chloramphenicol (Fig. 4), indicating that the same mechanism as observed in the previously studied microorganisms[6,9] was operating in the bacteria investigated here.

In previous papers,[1,4] we reported that a number of bacteria belonging to *Actinomycetes* and related organisms exhibited pigmentation in response to light exposure, and proposed that, in the absence of any biochemical evidence, photochromogenicity might not be a specific phenomenon in particular species of bacteria including certain strains of mycobacteria,, *Myxococcus xanthus*[6c,d] and *Micrococcus roseus*,[6i] but a common, widely distributed phenomenon in various species of bacteria. As mentioned in the beginning of this paper, however, previous investigations on the mechanism of photochromogenicity in *Actinomycetes* and related organisms have dealt exclusively with mycobacteria and no attention has been paid to other bacteria of this group. As shown here, *B. sulfureum* is the first photochromogen in the coryneform group of bacteria for which the mechanism of photoinduced carotenogenesis has been elucidated.

Acknowledgement The authors are grateful to Dr. Ushio Shimizu, The Research Institute of Chemobiodynamics, Chiba University, for supplying *Brevibacterium sulfureum*.

References and Notes

1) Part II: Y. Koyama, F. Kato, S. Oshibi, T. Takamatsu, and S. Yamagishi, *Jpn. J. Microbiol.*, **19**, 387 (1975).
2) A part of this study was presented at the International Symposium on *Nocardia* and *Streptomyces*, Warsaw, Oct. 1976.
3) Present address: *School of Pharmaceutical Science, Toho University, 2-2-1, Miyama-cho, Funabashi, 274, Japan.*
4) Y. Koyama, Y. Yazawa, S. Yamagishi, and T. Arai, *Jpn. J. Microbiol.*, **18**, 49 (1974).
5) E.H. Runyon, *Med. Clin. North America*, **43**, 274 (1959).
6) *a*) P.P. Batra and H.C. Rilling, *Arch. Biochem. Biophys.*, **107**, 485 (1964); *b*) P.P. Batra, R.M. Gleason, and J. Jenkins, *Biochim. Biophys. Acta*, **117**, 124 (1969); *c*) R.P. Burchard and M. Dworkin, *J. Bacteriol.*, **91**, 535 (1966); *d*) R.P. Burchard and S.B. Hendricks, *ibid.*, **97**, 1165 (1969); *e*) L.D. Hugo, *J. Bacteriol.*, **119**, 527 (1974); *f*) M.M. Mathews, *Photochem. Photobiol.*, **2**, 1 (1963); *g*) H.C. Rilling, *Biochim. Biophys. Acta*, **60**, 548 (1962); *h*) *Idem*, *ibid.*, **79**, 464 (1964); *i*) O.C. Thierry and J.J. Cooney, *J. Microbiol.*, **12**, 691 (1966); *j*) C.D. Howes, P.P. Batra, and C.F. Blakeley, *Biochim. Biophys. Acta*, **189**, 298 (1969).
7) S. Liaaen-Jensen and A. Jensen, "Methods in Enzymology," Vol. 23, ed. by S.P. Anthony, Academic Press, New York and London, 1971, pp. 586—602.
8) Y. Koyama and F. Kato, unpublished data.
9) D. Eberhard, W. Rau, and C. Zehender, *Planta*, **56**, 302 (1961); W. Rau, *ibid.*, **72**, 14 (1967); *idem*, *ibid.*, **75**, 263 (1967); *idem*, *ibid.*, **80**, 309 (1969); *idem*, *ibid.*, **84**, 30 (1969); E. Bindle, E. Lang, and W. Rau, *ibid.*, **94**, 156 (1970); M. Zalokar, *Arch. Biochem. Biophys.*, **50**, 71 (1954); *idem*, *ibid.*, **56**, 318 (1955).