Chapter 9

Isolation and Light-Stimulated Expression of Canthaxanthin and Spirilloxanthin Biosynthesis Genes from the Photosynthetic Bacterium *Bradyrhizobium* sp. Strain ORS278

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Abstract

Some aerobic photosynthetic bacteria produce a cocktail of carotenoids, some of them being of a high economic value. A good example is the photosynthetic *Bradyrhizobium* sp. strain ORS278, which synthesizes, in addition to the photosynthetic carotenoid spirilloxanthin, large amounts of canthaxanthin. Here, we describe the procedures that have been successfully used to isolate the different *crt* genes involved in the synthesis of both carotenoids in this bacteria. The synthesis of these carotenoids is stimulated under far-red light by a bacteriophytochrome. The procedure we developed to study the effect of the light on carotenoids synthesis is also described. Finally, we describe a procedure to genetically transform photosynthetic *Bradyrhizobium* strain ORS278 for improvement of canthaxanthin production.

Key words: Canthaxanthin, Spirilloxanthin, Carotenoid, *crt* genes, Photosynthetic *Bradyrhizobium*, Bacteriophytochrome, LED, Light regulation, Genetic engineering

1. Introduction

Aerobic phototrophic bacteria display the remarkable feature to synthesize an unusually diverse variety of carotenoids. Besides the synthesis of photosynthetic carotenoids like spirilloxanthin or spheroidenone, they often produce large amount of bicyclic carotenoids (β-carotene and hydroxyl derivatives), which act probably as oxygen singlet scavengers (1, 2). Among them, the *Bradyrhizobium* sp. ORS278 strain is certainly one of the most studied at the genetic and physiological levels. In addition to spirilloxanthin, this bacterium produces important quantities of canthaxanthin (3). This orange-red-colored pigment is used as a food

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additive (4, 5). In cosmetology and pharmacology, it is also combined with β-carotene for use as a dermal photo-protector (6).

The first three steps of canthaxanthin and spirilloxanthin biosynthesis pathways are common up to the formation of lycopene through the successive action of CrtE, CrtB, and CrtI enzymes. The synthesis of canthaxanthin from lycopene necessitates two additional enzymes (7): CrtY which catalyzes the cyclization of lycopene leading to β-carotene, and CrtW which oxygenates β-carotene to form canthaxanthin (Fig. 1). The synthesis of spirilloxanthin involves three additional enzymes CrtC, CrtD, and CrtF which catalyze the successive reactions of hydratation, desaturation, and methylation of lycopene (Fig. 1) (8, 9).

Our research over the past decade has permitted to characterize the different *crt* genes involved in canthaxanthin and spirilloxanthin biosynthesis and to understand the molecular mechanism of their regulation by light. Unexpectedly, ORS278 strain contains two distinct *crt* gene clusters, one dedicated for spirilloxanthin synthesis and the other for canthaxanthin synthesis (10, 11). Interestingly, each cluster contains the first genes, *crtE*, *crtI*, and *crtB*, necessary for carotenoids' synthesis (Fig. 1). Despite this redundancy, the biosyntheses of both carotenoids are strongly interconnected at the level of the common precursor lycopene (11). Indeed, the up-regulation of *crt* spirilloxanthin genes by light stimulates the synthesis of both carotenoids. This light control is due to the action of a bacteriophytochrome, which, depending on the light conditions, antagonizes the repressive action of the photosynthesis-specific regulator PpsR (11–13).

Here, we describe the strategies we used to isolate and characterize the two *crt* gene clusters present in ORS278 strain. A method to precisely study the effect of the light quality on carotenoids' biosynthesis is also shown. Finally, we propose a procedure to genetically transform ORS278 strain to stimulate the production of canthaxanthin, a valuable carotenoid.

2. Materials

2.1. Isolation of Spirilloxanthin and Canthaxanthin Biosynthesis Genes

1. *Bradyrhizobium* sp. strain ORS278 (LMG P-12187).
2. crtIf primer: 5′-GTNGGNGCRGGCACNCAYCC-3′.
3. crtBr primer: 5′-TCGCGRGCRATRTTSGTSARRTG-3′.
4. pufLf primer: 5′-TTYTAYGTNGGNTTYTTYGG-3′.
5. pufMr primer: 5′-CCCATNGTCCANCGCCARAA-3′.
6. SuperCos1 cosmid vector kit (Stratagene, La Jolla, CA).

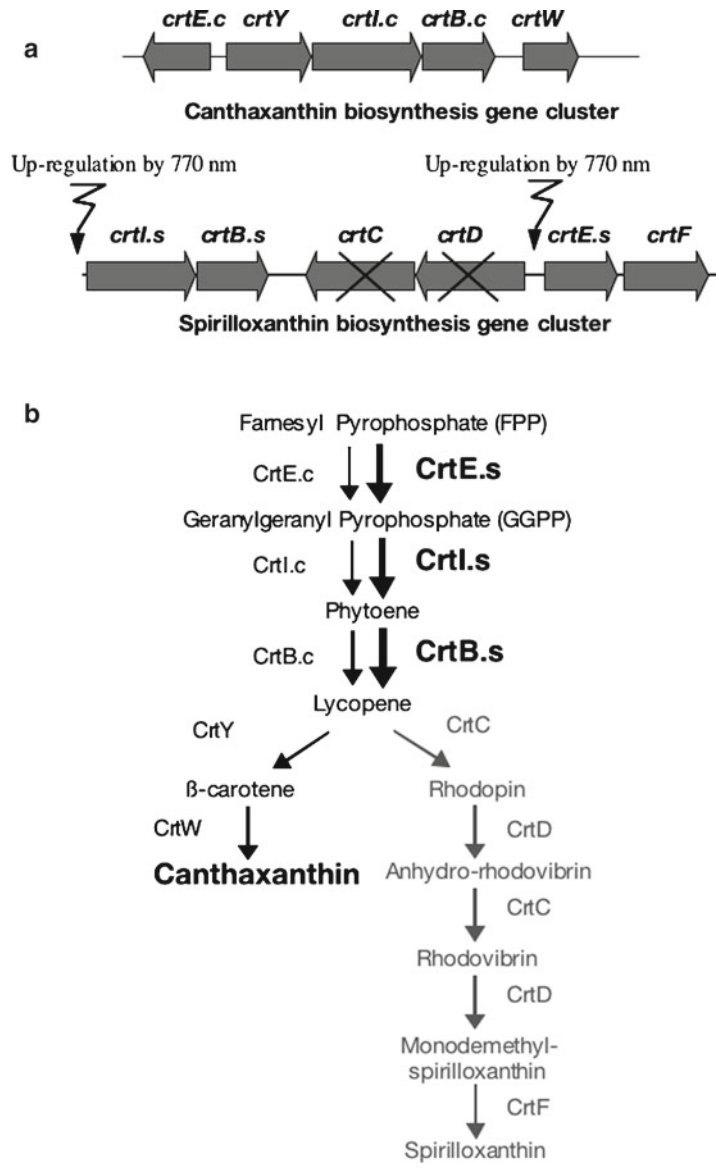

Fig. 1. General strategy to improve canthaxanthin production in *Bradyrhizobium* sp. strain ORS278. Note that the *crtE*, *crtI*, and *crtB* genes are present in each cluster. To distinguish them, we designate the genes from the spirilloxanthin *crt* cluster *crtE.s, crtI.s,* and *crtB.s* and those from the canthxanthin gene cluster *crtE.c, crtI.c,* and *crtB.c*. To stimulate the production of canthaxanthin in ORS278 strain, the *crtC* and *crtD* genes are deleted and the expression of the *crtE.s, crtI.s,* and *crtB.s* is up-regulated by far-red light illumination at 770 nm. (**a**) Organization of the canthaxanthin and spirilloxanthin gene clusters of ORS278 strain. (**b**) Canthaxanthin and spirilloxanthin biosynthesis pathways. In grey is presented the part of the spirilloxanthin pathway that is shut down. The lycopene produced principally through the successive action of CrtE.s, CrtI.s, and CrtB.s enzymes is rerouted towards the CrtY and CrtW enzymes for a maximal canthaxanthin biosynthesis.

7. Gigapack® III Gold Packaging Extract kit (Stratagene, La Jolla, CA).
8. QIAquick PCR extraction kit (QIAGEN GmbH, Hilden, Germany).
9. Minipreps DNA Purification kit (Promega, Madison, WI).
10. Luria–Bertani (LB) broth: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl.
11. LB-agar: LB and 16 g/L agar.
12. Kanamycin stock solution: 50 mg/mL.
13. Omnitrays Numc (Thermo Fisher Scientific, Rochester, NY).
14. TSP Numc (Thermo Fisher Scientific, Rochester, NY).

2.2. Light Control of Carotenoids' Biosynthesis

1. YM-modified medium: 2.5 g sodium glutamate, 2 g yeast extract, 0.66 g K_2HPO_4, 0.05 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2$, and 0.004 g $FeCl_3$. Add water to a volume of 900 mL. Mix and adjust to pH 6.8. Make up to 1 L with water.
2. YM-modified agar medium: YM-modified medium and 16 g/L agar.
3. Illumination apparatus. Light-Emitting Diodes (LEDs) are made by a large number of manufacturers. Emission wavelengths can be found from 370 to 950 nm, the LED's bandwidth usually lies between 20 and 30 nm, and intensities up to 100 µE/m^2/s can be obtained.
4. Acetone/methanol: 7 mL acetone and 2 mL methanol.
5. Hypersil C18 column: 5 µm, 250 by 4.6 mm (Phenomenex, Torrance, CA).
6. Acetonitrile/methanol/isopropanol: 40 mL acetonitrile, 50 mL methanol, and 10 mL isopropanol.
7. Photodiode array detector 996 (Waters, Milford, MA).
8. Canthaxanthin standard (Aventis, Paris, France).

2.3. Engineering of Bradyrhizobium ORS278 Strain to Improve Canthaxanthin Production

1. crtDf primer: 5′-TAGTCGACGCAATGGCGCGCCACGATCTATC-3′.
2. crtCr primer: 5′-ACAGTCGACCGGTCTTGGAGCGGTGATAATG-3′.
3. pGEM-T vector (Promega, Madison, WI, USA).
4. pKOK5 (14).
5. pJQ200mp18 (15).
6. *E. coli* S17-1 λpir (Biomedal, Sevilla, Spain).
7. Nalidixic acid stock solution: 35 mg/mL.

3. Methods

3.1. Isolation of Spirilloxanthin and Canthaxanthin Biosynthesis Genes

The level of identity among *crt* genes is relatively low (for example, the CrtE, CrtI, and CrtB paralogs identified in ORS278 display between 35 and 50% of amino acid identity). This could constitute an obstacle for the isolation of the *crt* genes, in particular for a bacterial species phylogenetically distant from those already characterized. In the case of the photosynthetic *Bradyrhizobium* strain ORS278, two different strategies were used for the isolation of spirilloxanthin and canthaxanthin *crt* gene clusters. The first one is based on the use of degenerated primers defined from consensus sequences identified from the alignment of CrtI and CrtB proteins (see Note 1). The second is based on the observation that, in purple bacteria, the *crt* genes involved in the synthesis of photosynthetic carotenoids have been always found associated to other photosynthesis genes (*bch*, *puf*, etc.) in the so-called photosynthesis gene cluster (PGC) of around 45 kb (16). Among them, the *pufLM* genes that encode the major polypeptides of the photosynthetic reaction center are well conserved. They could, therefore, be used as a probe to identify cosmids or clones containing the PGC and consequently the *crt* genes. The following protocol, designed for the isolation of the two *crt* gene clusters from *Bradyrhizobium* ORS278 strain, could be a useful starting point for designing isolation strategies for *crt* genes from other species. Depending on the photosynthetic character or not of the carotenoid studied, one or the other strategy should be privileged to obtain a specific probe of the corresponding *crt* genes.

1. Amplify by polymerase chain reaction (PCR) a specific region of *crtI/crtB* genes or *pufL/pufM* genes using the degenerated primers crtIf/crtBr or pufLf/pufMr, respectively. Use a touchdown PCR program as follows: initial denaturation at 94°C for 5 min followed by 20 cycles consisting of a 30-s denaturation at 94°C, 30-s annealing temperature from 60 to 50°C, and 1-min primer extension at 72°C, followed by 15 cycles consisting of a 30-s denaturation at 94°C, 30-s annealing temperature at 50°C, and 1-min primer extension at 72°C.
2. Purify the corresponding PCR product using a QIAquick PCR extraction kit and sequence it.
3. Design specific primers from the obtained sequence. These primers will be used to screen by PCR a DNA genomic library.
4. Construct a DNA genomic library of the considered strain using the SuperCos1 cosmid vector (see Note 2). Briefly, the genomic DNA is partially digested with *Sau*3AI, dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIAP), and ligated into the SuperCos1 vector previously double digested

with *Xba*I/*BamH*I. The resulting high-molecular-weight concatemers are encapsidated using the Gigapack III Gold Packaging Extract kit.

5. Pick the clones and array them into 96-well plates, each plate containing 120 µL of LB medium supplemented with kanamycin (50 µg/mL) (see Note 3). After overnight culture at 37°C, add 50 µL of 70% glycerol per well and store the library at −80°C.
6. Subculture each 96-well plate onto an LB-agar plate (Omnitrays Numc) with the help of a 96-pin lid (96 plots TSP Numc).
7. Pool the colonies from an agar plate with 10 mL of water and extract the plasmids using the Minipreps DNA Purification kit.
8. Screen by PCR the different plasmid pools using the specific primers defined in step 3 in order to identify the positive plates (see Note 4).
9. Subculture the positive plates and screen again by PCR directly on colonies in order to identify a positive clone containing the wanted *crt* genes (see Note 5).
10. Sequence completely the cosmid or a subclone containing the *crt* genes identified by PCR or Southern-blot hybridization in order to characterize the *crt* gene cluster.

3.2. Light Control of Carotenoids' Biosynthesis

Light stimulation of carotenoids' biosynthesis has been reported in numerous organisms, including plants, fungi, and bacteria (17). In higher plants, this regulation is mediated by a phytochrome that controls the level of the phytoene synthase expression (18). This class of light sensor has been identified in photosynthetic and non-photosynthetic bacteria (19). Phytochromes and bacteriophytochromes are light sensor chromoproteins that display two photo-interconvertible forms, a red-absorbing form (Pr) and a far-red absorbing form (Pfr) (20). Depending on the (bacterio)phytochromes, the Pr or the Pfr form could be the active form (20). It has been also reported that (bacterio)phytochromes could control carotenoids' biosynthesis, such as deinoxanthin in the non-photosynthetic bacteria *Deinococcus radiodurans* (19), and spirilloxanthin and canthaxanthin in ORS278 strain (11). The simplest way to determine whether a carotenoid biosynthesis is under the control of a bacteriophytochrome is to check the effect of light quality in the red/far-red region on its synthesis. Below, we describe the procedure that we used for ORS278 strain.

1. Prepare a fresh culture of the ORS278 strain. Grow the bacteria in Falcon tubes of 50 mL containing 20 mL of YM-modified medium. Incubate the culture in the dark at 37°C under agitation (170 rpm) for 5 days.
2. Inoculate homogeneously the bacteria onto round Petri dishes (diameter 9 cm) containing YM-modified agar medium. Prepare one Petri dish per each light condition tested.

3. Place each Petri dish under an illumination apparatus equipped of LED of different peak wavelengths. The LED is fixed on the cover of a Petri dish placed on the top of a plastic pipe whose diameter is slightly larger than the diameter of the Petri dishes. This pipe of 20-cm high is coated with a reflecting paint to funnel the light and its extremity covered by a piece of tracing paper to homogenize the illumination. The wavelength peaks of the LEDs are chosen in order to span the two spectral forms of bacteriophytochromes, typically between 550 and 800 nm (see Note 6).
4. After 1 week of incubation at 37°C, resuspend the cells grown at the surface of the Petri dishes in 6 mL of water containing 9 g/L NaCl.
5. Harvest the cells by centrifugation (3,200 × *g* for 30 min at 4°C) and resuspend the pellets with 1 mL of cold acetone/methanol to extract carotenoids.
6. Place the samples in a rotary agitator and incubate for 30 min at 4°C in the dark.
7. Centrifuge (3,200 × *g* for 30 min at 4°C), keep the supernatant, and repeat twice the extraction procedure on the pellets.
8. Pool the supernatants and analyze the carotenoids' content by HPLC. For sipirilloxanthin and canthaxanthin analysis, use the following conditions: Hypersil C18 column, acetonitrile/methanol/isopropanol as eluent, and a flow rate of 0.8 mL/min. Monitor the eluted fractions with a photodiode array detector, scanning from 270 to 600 nm every 2 s. Identify carotenoids by their retention times and by comparison of the spectral features with those of pure compounds or with reported data. Determine the amount of canthaxanthin from the area of the peak detected at 480 nm using a calibration curve obtained with a canthaxanthin standard. If you lack of spirilloxanthin standard, estimate the amount of spirilloxanthin from the area of the peak detected at 494 nm using the canthaxanthin correlation coefficient. Determine the normalized quantity of carotenoid with respect to the dry weight of the pellet.
9. Plot the amount of carotenoids produced according to the illumination wavelengths (see Notes 7 and 8).

3.3. Engineering of Bradyrhizobium ORS278 Strain to Improve Canthaxanthin Production

As previously stated, a cross talk between the canthaxanthin and spirilloxanthin biosynthesis pathways exists at the level of the common precursor lycopene. The stimulation of canthaxanthin production by far-red light observed in the ORS278 strain results, in fact, to the overexpression of the spirilloxanthin *crt* genes cluster. This enhances the production of lycopene and, as a consequence, the canthaxanthin synthesis. A strategy to further increase canthaxanthin production is to construct a deletion mutant in the

crtC and *crtD* genes that act downstream in the spirilloxanthin biosynthesis pathway. This completely reroutes the lycopene towards the CrtY and CrtW enzymes for maximal canthaxanthin synthesis (Fig. 1). Such engineering strategy to improve the synthesis of the desired carotenoid could be applied to other aerobic photosynthetic bacteria that are known to produce a cocktail of photosynthetic and non-photosynthetic carotenoids. However, the characterization of the different *crt* genes as described in Subheading 3.1 is needed. Below, we describe the methodology for the construction of a *crtCD* deletion mutant of the ORS278 strain and its use to overproduce canthaxanthin.

1. Amplify by PCR the DNA region (3.5 kb) encompassing the *crtC* and *crtD* genes using the primers crtDf and crtCr.
2. Clone the PCR product into the pGEM-T vector.
3. Delete a 2-kb region inside the *crtC* and *crtD* genes by *Xho*I digestion and insert in place the 4.7-kb *Sal*I pKOK5 cassette containing a kanamycin resistance gene and a *lacZ* reporter gene (14) (see Note 9).
4. Remove the DNA region containing the mutated *crt* genes by *Sal*I digestion and clone the fragment into the *Xho*I site of the pJQ200mp18 suicide vector (15) (see Note 10).
5. Transfer by electroporation the pJQ200mp18 plasmid containing the mutated *crt* genes into *E. coli* S17-1 λpir to permit its mobilization by bipartite conjugation in the ORS278 strain.
6. Grow *E. coli* S17-1 λpir containing the pJQ200mp18 plasmid overnight at 37°C in LB medium supplemented with kanamycin (50 μg/mL). Grow ORS28 strain for 5 days at 37°C in YM-modified medium.
7. Harvest 2.5 mL of *E. coli* S17-1 λpir and 10 mL of ORS278 cells by centrifugation (3,200 × *g* for 30 min at 4°C).
8. Wash the pellets twice with 9 g/L NaCl.
9. Resuspend each pellet in 200 μL of 9 g/L NaCl.
10. Gently mix the two pellets and transfer to a YM-modified agar plate. Incubate for 3 days at 37°C.
11. Resuspend the mating in 1 mL of YM-modified medium, make serial dilutions until 10^{-4} into 9 g/L NaCl, and spread 100 μL of each dilution onto YM-modified agar plates containing kanamycin (50 μg/mL) and nalidixic acid (35 μg/mL) (see Note 11).
12. Incubate the plates at 37°C for at least 10 days (see Note 12).
13. Screen the colonies by PCR to check that the first event of recombination occurred.
14. Grow one recombinant strain for 1 week at 37°C in YM-modified medium supplemented with 50 μg/mL kanamycin and 35 μg/mL nalidixic acid.

15. Make serial dilutions until 10^{-4} into 9 g/L NaCl and spread 100 µL of each dilution onto YM-modified agar plates containing sucrose (7%), kanamycin (50 µg/mL), and nalidixic acid (35 µg/mL) (see Note 13).
16. Incubate the plates at 37°C for at least 1 week.
17. Screen the colonies by PCR to check that the second event of recombination occurred.
18. Store the mutant strain in glycerol (25% v/v) at −80°C.
19. Cultivate the mutant in YM-modified medium under 770-nm illumination as previously described in Subheading 3.2.
20. Extract the carotenoids as previously described in Subheading 3.2 (see Note 14).

4. Notes

1. The genes *crtI* and *crtB* encoding, respectively, phytoene synthase and phytoene desaturase, two enzymes involved in the initial steps of carotenoid biosynthesis (Fig. 1), have been isolated and characterized from various microorganisms. In all of the cases, these genes were found adjacent and with the same direction of transcription. Comparison of the deduced amino acid sequences of CrtI and CrtB proteins from *Erwinia uredovora*, *Erwinia herbicola*, *Flavobacterium* sp. ATCC21588, *Rhodobacter sphaeroides*, and *Agrobacterium aurantiacum* revealed well-conserved domains at the C-terminal end of CrtI (LVGAGTHPG) and in the central region of CrtB (QLTNIARD). These motifs were chosen for designing the degenerated primers crtIf and crtBr.
2. This is a critical step. It is recommended to follow precisely the protocols furnished by Stratagene.
3. Considering an insert size from 30 to 42 kb, about 2,000 clones are estimated to be enough to cover the entire genome of the ORS278 strain (about 7.2 Mb).
4. In our case, it was not possible to identify a positive clone by its color. This was not due to a problem of functionality of *Bradyrhizobium* CRT enzymes in *E. coli*, but to a problem of promoter recognition preventing *crt* genes' expression (10).
5. In order to limit the number of PCR reactions, the colonies present in the same line and the same column could be pooled. This diminishes the number of PCR reactions from 96 to 20.
6. To examine the light effect on carotenoids' biosynthesis in ORS278 strain, we use six different wavelengths (590, 660,

735, 770, 810, and 850 nm). They cover the absorption range of both the Pr and Pfr forms of a bacteriophytochrome.

7. Light control of carotenoid biosynthesis by a bacteriophytochrome typically should result in a bell curve with an optimum around 650 nm if Pfr is the active form, or an optimum around 750 nm if Pr is the active form. In the case of the ORS278 strain, we observed that the production of canthaxanthin increased twice under 770-nm light, whereas the production of spirilloxanthin increased three times.
8. Alternatively, the same procedure could be used to examine the effect of the light on *crt* genes' expression. In this last case, we constructed a transformant strain, where a reporter gene (such as *lacZ*, *gusA*, etc.) is under the control of the promoter region(s) of the *crt* genes. The expression of the reporter gene is then analyzed according to the different light conditions tested. According to this method, we determined that the *crt* spirilloxanthin gene cluster is only up-regulated by far-red light, whereas the synthesis of both carotenoid is stimulated (11).
9. The mutant construction strategy is designed so that the flanking regions of the cassette, which correspond to the 5′-end of *crtD* and 3′-end of *crtC*, have a size of around 700 bp in order to promote the double-crossing-over event.
10. The use of the pJQ200mp18 suicide plasmid, which does not replicate in ORS278 strain, permits to integrate the DNA region containing the deleted *crtC* and *crtD* genes into the chromosomal *crtC* and *crtD* genes due to the similarity between one of the flanking regions of the insert and the genomic DNA.
11. The ORS278 strain is resistant to nalidixic acid. The addition of nalidixic acid to the medium inhibits the growth of *E. coli* cells. The addition of kanamycin permits, on the other hand, to select the ORS278 recombinant cells in which the pJQ200mp18 vector is integrated into the chromosome.
12. *Bradyrhizobium* strains grow very slowly, the first colonies appearing between 7 and 10 days.
13. Clones in which double events of recombination occurred are selected by plating on sucrose-containing medium. Cells retaining the vector backbone are lost since the *sacB* gene on the pJQ200mp18 backbone converts sucrose to toxic levansucrose.
14. In such condition, the total amount of canthaxanthin produced by the *crtCD* mutant reached 1.5 mg of canthaxanthin per g of dry cells, against 0.3 mg/g for the wild-type strain grown in the dark (11).

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