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ORIGINAL RESEARCH PAPER

Carotenoid production in *Bacillus subtilis* achieved by metabolic engineering

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Abstract The carotenoid synthetic genes, *crtM* and *crtN*, derived from *Staphylococcus aureus*, were introduced into *B. subtilis*, resulting in yellow pigmentation. Absorption maxima of pigments and MALDI-TOF mass spectrometry demonstrated that the pigmented strain accumulated two C_{30} carotenoids, 4,4′-diapolycopene and 4,4′-diaponeurosporene. A survival test using H_2O_2 revealed that the pigmented strain was more resistant to oxidative stress than the strain harboring an empty-vector. These findings indicate that *B. subtilis* can produce carotenoids, and the strain accumulating the carotenoids, CarotenoBacillus, will become a basal host for production of C_{30} carotenoids and evaluation of their antioxidative effects.

Keywords *Bacillus subtilis* · Carotenoid · *crtN* · Diapolycopene · Diaponeurosporene · MALDI-TOF-MS

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Introduction

Carotenoids are ubiquitous natural pigments that play important biological roles in many organisms. The most important role is their antioxidative effect. Among natural carotenoids, C_{30} carotenoids are found in a small group of bacteria, including *Staphylococcus aureus* (Marshall and Wilmoth 1981) and *Heliobacterium* sp. (Takaichi et al. 1997), and the genes responsible for their synthesis have been characterized (Pelz et al. 2005; Wieland et al. 1994). The first committed enzyme in the C_{30} carotenoid synthetic pathway is dehydrosqualene synthase CrtM. CrtM converts farnesyl pyrophosphate to dehydrosqualene. Dehydrosqualene desaturase CrtN then converts dehydrosqualene to the yellow C_{30} carotenoid, 4,4′-diaponeurosporene. In *S. aureus*, 4,4′-diaponeurosporene is finally converted to the golden pigment, staphyloxanthin. It has been shown that staphyloxanthin promotes resistance of *S. aureus* to reactive oxygen species and host neutrophil-based killing, and CrtM in the staphyloxanthin synthetic pathway could be a target site for a virulence factor-based therapy against *S. aureus* (Liu et al. 2008).

The Gram-positive bacterium *Bacillus subtilis* has long been used for the industrial production (Sauer et al. 1998), and is generally recognized as a safe (GRAS) organism (Widner et al. 2005). However, contrary to its successful applications, a carotenoid-producing *B. subtilis* has not yet been established. Only one previous report has described the transformation of

B. subtilis with *Erwinia uredovora* C_{40}-carotenoid synthetic genes (Nishizaki et al. 2007). The authors of that study concluded that the manipulation was unsuccessful. Thus, difficulty to produce carotenoids in *B. subtilis* exists. On the other hand, the genome of Gram-positive bacterium, *Staphylococcus aureus*, has a low GC content like that of *B. subtilis*. Therefore, *S. aureus* genes might be applicable to carotenoid production. Furthermore, *B. subtilis* accumulates a significant amount of isoprene, a potential precursor of carotenoids (Julsing et al. 2007). Thus, genetically engineered *B. subtilis* may produce a large amount of C_{30} carotenoids. This study was performed to construct *B. subtilis* to produce C_{30} carotenoids through the first two catalytic activities in the staphyloxanthin synthetic pathway, which might become a basal host for production of various C_{30} carotenoids and evaluation of their antioxidative effects.

Materials and methods

Bacterial strains and growth conditions

Bacillus subtilis JH642 was provided by Dr T. Sato (Tokyo University of Agriculture and Technology). *Staphylococcus aureus* NCTC 50581 and *E. coli* DH5α were used for genetic construction. All bacteria were cultivated at 37°C. Tryptone soya broth (Kanto Chemical, Tokyo) and Luria-Bertani (LB) medium were used for *B. subtilis* and *S. aureus*, respectively. Tetracycline was added at 20 μg/ml.

Construction of a carotenoid-producing plasmid

The *B. subtilis-E. coli* shuttle vector, pHY300PLK (Takara Bio, Shiga, Japan), was used to construct the carotenoid-producing plasmid. The total DNA was extracted from *S. aureus* cells using the DNeasy tissue kit (Qiagen). The ORF of *crtM* containing an artificial Shine-Dalgarno (SD) sequence and the ORF of *crtN* containing an original SD sequence were amplified from the total DNA using the primers, 5′-GC*GG ATCC*GTAAGAGAGGACTAGTATGAC (the introduced *Bam*HI site is italicized) and 5′-CGCC*GTCG AC*CGTTATGTTCAACAG (the introduced *Sal*I site is italicized), and cloned into the pGEM-T vector (Promega). The obtained plasmid was then digested with *Bam*HI and *Sal*I, generating the fragment crtMN.

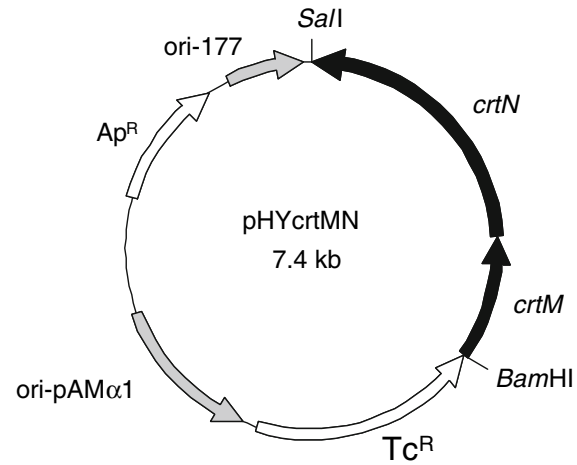

Fig. 1 Structure of the carotenoid-producing plasmid pHYcrtMN. The *crtMN* genes were inserted into the *Bam*HI/*Sal*I sites of pHY300PLK. The *arrow* indicates the direction of transcription or replication. ori-177, replication origin of plasmid pACYC177; ori-pAMα1, replication origin of plasmid pAMα1; Ap^R, ampicillin-resistance gene; Tc^R, tetracycline-resistance gene

The fragment crtMN was then inserted into pHY300PLK digested with *Bam*HI and *Sal*I, generating the carotenoid-producing plasmid pHYcrtMN (Fig. 1).

Electroporation

Two ml of *B. subtilis* overnight culture was inoculated into 32 ml LB medium containing 0.5 M sorbitol and culture was continued until OD_{600} became 0.85–0.95. The culture was incubated on ice for 10 min, then collected by centrifugation and suspended in 10 ml ice-cold solution A (10% v/v glycerol containing 0.5 M sorbitol and 0.5 M mannitol). The centrifugation and resuspension steps were repeated four times. The cell pellet was then resuspended in 0.8 ml solution A. pHYcrtMN, 200 ng, were then added to 70 μl of cell suspension and the cell-DNA suspension was transferred to an ice-cold electroporation cuvette. Electroporation was carried out using Gene Pulsar (Bio-rad) with the parameters set at 25 μF, 2 kV/mm, and 250 Ω. The cells were immediately transferred to 1 ml LB medium containing 0.5 M sorbitol and 0.38 M mannitol and cultured for 3 h. The cells were then diluted and spread onto LB agar plates for selection. The introduction of the plasmid was confirmed by PCR using several sets of primers.

Extraction of carotenoids

A single colony of transformants was inoculated into 50 ml tryptone soya broth and cultured for 24 h. Cells were collected by centrifugation and washed with 1 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). The cells were resuspended in 200 µl TE buffer. Fifty µl of 20 mg/ml lysozyme were added to the cell suspension, followed by incubation for 15 min at 37°C. The cell lysate was then transferred into a glass tube, and 500 µl acetone was added. The mixture of acetone and cell lysate was vortex-mixed for 2 min, heated for 1 min in a water bath at 55°C, and then vortex-mixed for 1 min. The extract was transferred into a new glass tube. The acetone extraction was repeated four times. The pigment extract was used immediately or stored at −20°C for HPLC or TLC.

Analyses of carotenoids

The pigment extracts were filtered and analyzed with an HPLC system equipped with a µBondapak C_{18} column (3.9 × 300 mm, 125 Å pore size, Waters). Pigments were eluted with acetonitrile/water (85:15, v/v) at 2 ml/min. The spectrum from 230 nm to 800 nm was monitored with a photodiode array detector. Pigments were identified from their absorption spectra. Pigment extracts for MALDI-TOF MS analysis were prepared with TLC. The pigment extract was separated on silica gel 60 F254 plates (Merck) with light petroleum/acetone (7:3, v/v). Colored spots were scratched off the TLC plate, and the pigments were eluted from the silica gel with 50 µl acetone. The pigment extracts were stored at −20°C until used for mass analysis. The pigment extracts were subjected to MALDI-TOF MS analysis using the Autoflex TOF/TOF (Bruker Daltonics, Germany). Dithranol [1,8-dihydroxy-9(10H)-anthracenone] was used as the matrix and dissolved in acetone. The pigment extracts was mixed with an equal volume of matrix. The droplet was air-dried at room temperature. MALDI-TOF MS spectra were obtained in the positive reflection mode. The ions were generated by irradiation with a N_2 laser emitting at 337 nm.

H_2O_2 survival test

Cells in 1.5 ml of *B. subtilis* culture at OD_{600} of 1.9–2.0 were collected by centrifugation, once washed with 1 ml PBS, resuspended in 1.5 ml PBS and then divided equally (500 µl) into three tubes. The cells in PBS were then incubated with 0, 50, or 100 mM H_2O_2 in the dark on ice. After 60 min, the cells were collected and once washed with 1 ml PBS. The cells were diluted in PBS and spread onto tryptone soya broth agar plates containing tetracycline. Colonies were counted after 24 h.

Results

Production of pigments by genetically engineered *B. subtilis*

The pHYcrtMN transformants appeared yellow compared with pHY300PLK transformants (Fig. 2a). Yellow pigment(s) could be extracted with acetone (Fig. 2b). These results indicated that the *crtMN* genes might be functional in *B. subtilis* and involved in the production of yellow pigment(s).

Identification of the carotenoids

The HPLC chromatogram of extract from *B. subtilis*(pHYcrtMN) showed two major peaks and several minor ones (Fig. 3a). The absorption maxima of the pigment eluting at 26.8 min (Fig. 3b) were identical to those of 4,4′-diapolycopene (Takaichi 2000). The absorption maxima of the pigment eluting at 28.9 min (Fig. 3c) were identical to those of 4,4′-diaponeurosporene (Takaichi 2000; Takaichi et al. 1997). The absorption spectrum of the minor carotenoid eluting at 13.8 min was identical to that of 4,4′-diaponeurosporenol (Marshall and Wilmoth 1981). These results suggested that the yellowness of the pigmented strain was mainly caused by the accumulation of 4,4′-diaponeurosporene and 4,4′-diapolycopene.

Mass spectrometric identification of carotenoids

The results of MALDI-TOF mass analysis revealed the existence of four specific compounds with the masses of 399.9, 400.9, 401.9, and 402.9 in an extract from *B. subtilis*(pHYcrtMN), as shown in Supplementary Fig. 1. Two intense mass peaks at 399.9 and 401.9 corresponded to 4,4′-diapolycopene ($C_{30}H_{40}$) and 4,4′-diaponeurosporene ($C_{30}H_{42}$), respectively.

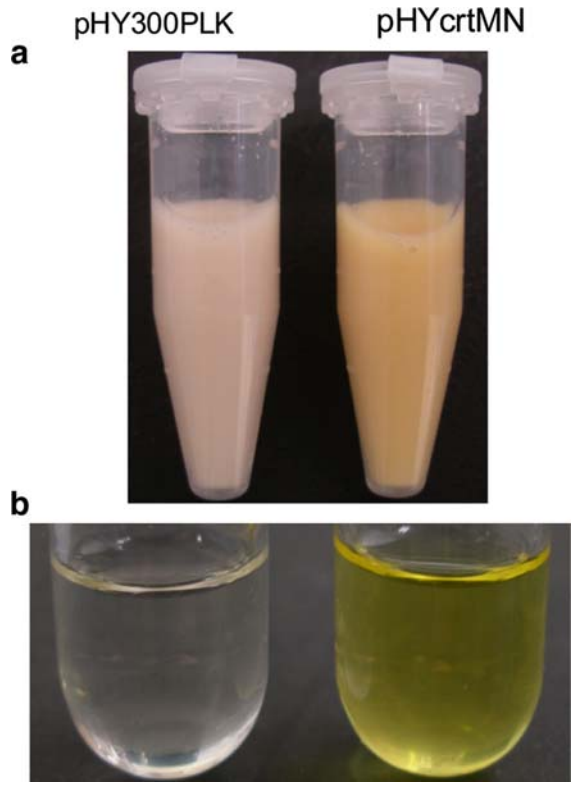

Fig. 2 Cell suspensions and pigment extracts of *B. subtilis* harboring pHYcrtMN or pHY300PLK. **a** Cells of 50 ml cultures collected in 1 ml TE buffer. **b** Pigment extracts with acetone

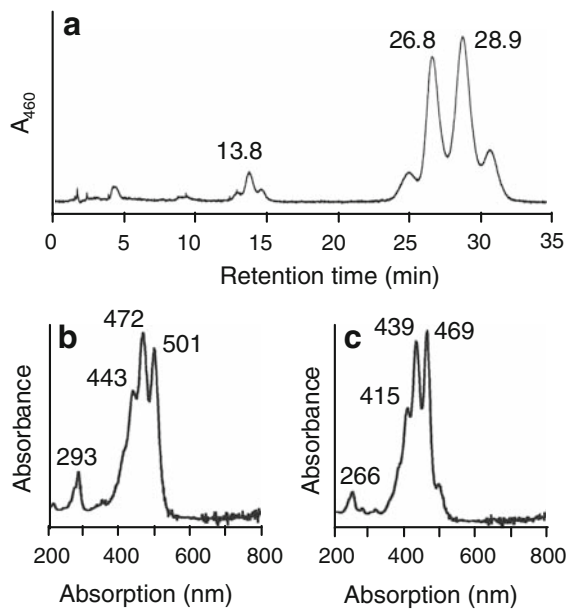

Fig. 3 HPLC analysis of pigments produced by *B. subtilis* harboring pHYcrtMN. Retention time and absorption maximum are indicated above each peak. **a** The chromatogram of pigment extract. **b** The absorption spectrum of the peak eluting at 26.8 min. **c** The absorption spectrum of the peak eluting at 28.9 min

The mass peaks at 400.9 and 402.9 corresponded to the natural isotopic 4,4′-diapolycopene and 4,4′-diaponeurosporene, respectively. These results verified that the major carotenoids produced by *B. subtilis* (pHYcrtMN) were 4,4′-diapolycopene and 4,4′-diaponeurosporene.

Effect of carotenoid production on bacterial survival in the presence of H_2O_2

4,4′-Diaponeurosporene is converted to staphyloxanthin in *S. aureus* (Marshall and Wilmoth 1981; Pelz et al. 2005). It has been suggested that staphyloxanthin possesses antioxidative effects (Clauditz et al. 2006), yet little is known about the antioxidative effects of other C_{30} carotenoids. To examine whether the carotenoids produced by *B. subtilis*(pHYcrtMN) have antioxidant activity or not, we investigated the survival of *B. subtilis* strains in the presence of H_2O_2, which is a potential source of hydroxyl radicals. The percentages of surviving cells in 50 and 100 mM H_2O_2 were higher in *B. subtilis*(pHYcrtMN) than in *B. subtilis*(pHY300PLK) (Table 1). The result indicates that the carotenoids produced by *B. subtilis*(pHYcrtMN) could contribute to protecting the cells from oxidative stress caused by H_2O_2.

Table 1 H_2O_2 survival test of *B. subtilis* harboring pHYcrtMN or pHY300PLK

	Survival (%)[a] against H_2O_2 at	
	50 mM[b]	100 mM[b]
B. subtilis(pHYcrtMN)	88 ± 2	72 ± 2
B. subtilis(pHY300PLK)	52 ± 4	41 ± 4

[a] The mean values ± SD of three independent experiments are shown

[b] Number of colonies formed from cell suspension treated with H_2O_2 at the concentrations was divided by number of colonies formed from cell suspension not treated with H_2O_2

Discussion

In the present study, we have shown the first successful production of C_{30} carotenoids, such as

4,4′-diapolycopene and 4,4′-diaponeurosporene, in *B. subtilis*. The carotenoid production indicated that the *crtMN* genes were transcribed and translated in *B. subtilis*, and thus that the carbon flux for isoprenoid biosynthesis could be directed toward the C_{30} carotenoid biosynthesis. Here, the *B. subtilis* strain transformed with the *crtMN* genes and producing the C_{30} carotenoids is designated CarotenoBacillus. Our findings will provide methods for industrial production of C_{30} carotenoids by the GRAS bacterium and/or for analyses of antioxidant activities of C_{30} carotenoids in the future.

Staphyloxanthin, the end product of the carotenoid synthesis pathway in *S. aureus*, can confer resistance to oxidative stress (Clauditz et al. 2006). In the halophilic bacterium *Halobacillus halophilus*, a pigment related to staphyloxanthin shows an antioxidant effect under peroxidative conditions (Köcher et al. 2009). The H_2O_2 survival test of the present study revealed that the carotenoids produced by CarotenoBacillus also conferred resistance to oxidative stress on *B. subtilis*. This result represents the first evidence that the intermediate carotenoids in the C_{30} pathway, such as 4,4′-diapolycopene and 4,4′-diaponeurosporene, have an antioxidant effect. It has been suggested that the antioxidant activity depends on the number of conjugated double bonds (Miller et al. 1996). The number of conjugated double bonds in 4,4′-diapolycopene (eleven) is identical to that of lycopene or β-carotene, which have been identified as strong antioxidants. 4,4′-Diaponeurosporene, which has nine conjugated double bonds, may also exhibit antioxidant effects, but to a lesser extent than 4,4′-diapolycopene. Therefore, the C_{30} carotenoids, 4,4′-diapolycopene and 4,4′-diaponeurosporene, may play a protective role under oxidative conditions in various organisms.

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