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Metabolic engineering for synthesis of aryl carotenoids in *Rhodococcus*

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Abstract *Rhodococcus erythropolis* naturally synthesizes monocyclic carotenoids: 4-keto-γ-carotene and γ-carotene. The genes and the pathway for carotenoid synthesis in *R. erythropolis* were previously described. We heterologously expressed a β-carotene desaturase gene (*crtU*) from *Brevibacterium* in *Rhodococcus* to produce aryl carotenoids such as chlorobactene. Expression of the *crtU* downstream of a chloramphenicol resistance gene on pRhBR171 vector showed higher activity than expression downstream of a native 1-deoxyxylulose-5-phosphate synthase gene (*dxs*) on pDA71 vector. Expression of the *crtU* in the β-carotene ketolase (*crtO*) knockout *Rhodococcus* host produced higher purity chlorobactene than expression in the wild-type *Rhodococcus* host. Growth of the engineered *Rhodococcus* strain in eight different media showed that nutrient broth yeast extract medium supplemented with fructose gave the highest total yield of chlorobactene. This medium was used for growing the engineered *Rhodococcus* strain in a 10-l fermentor, and ~18 mg of chlorobactene was produced as the almost exclusive carotenoid by fermentation.

Introduction

Aryl carotenoids (aromatic carotenoids) are naturally present in a few actinomycetes and photosynthetic green bacteria as mixtures with their intermediates and/or derivatives (Liaaen-Jensen et al. 1964; Kohl et al. 1983; Takaichi et al. 1997). These bacteria contain carotenoid synthesis genes to synthesize carotenes with one β-ionone ring (γ-carotene) or two β-ionone rings (β-carotene). The *crtU* gene, encoding the carotene desaturase, desaturates the β-ionone ring (s) and simultaneously migrates a methyl group to form an aromatic ring(s). The *crtU* gene has been identified and characterized in the actinomycetes including *Streptomyces*, *Mycobacterium*, and *Brevibacterium* (Krugel et al. 1999; Krubasik and Sandmann 2000; Viveiros et al. 2000). The CrtU in the actinomycetes acts on β-carotene substrate and synthesizes β-isorenieratene and isorenieratene. *Brevibacterium linens* synthesizing isorenieratene and the hydroxy derivatives are commonly used as food colorant for cheese industry. Chlorobactene is an asymmetric aryl carotenoid that has one of the aromatic rings compared to isorenieratene. Chlorobactene may potentially be used as another colorant.

This class of asymmetric aryl carotenoids derived from γ-carotene was reported in photosynthetic green bacteria such as *Chlorobium*. Recent genomic sequencing of *Chlorobium tepidum* (Eisen et al. 2002) identified a putative carotene desaturase gene (*crtU*), which might be responsible for the synthesis of the native chlorobactene and derivatives. Function of the putative γ-carotene desaturase was proposed based on the phenotype of the *crtU* knockout mutant of *Chlorobium* (Frigaard et al. 2004).

Rhodococcus belongs to the group of *Actinomycetales*, which is closely related to the native actinomycete hosts of CrtU. *Rhodococcus* exhibits diverse metabolic capability and potential to synthesize secondary metabolites. The carotenoids synthesized by *Rhodococcus erythropolis* were previously characterized to be 4-keto-γ-carotene as the major carotenoid and sometimes γ-carotene as the minor carotenoid (Tao et al. 2004). It is likely that the CrtU from actinomycetes might also act on other substrates in addition to β-carotene, such as converting γ-carotene to chlorobactene. We heterologously expressed the *crtU* gene from *Brevibacterium* in *Rhodococcus* and produced chlorobactene as the predominant carotenoid (>90%) by fermentation.

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Materials and methods

Growth conditions

Escherichia coli cells were grown in Luria–Bertani (LB) with appropriate antibiotics at 37°C with shaking overnight. *Rhodococcus* cells were grown at 30°C with shaking (250 rpm) for 1–2 days in NBYE medium (0.8% nutrient broth, 0.5% yeast extract, 0.05% Tween 80), unless otherwise indicated. The antibiotics used for selection in *E. coli* were 10 μg/ml tetracycline or 100 μg/ml ampicillin. The antibiotics used for selection in *Rhodococcus* were 10 μg/ml tetracycline and/or 40 μg/ml chloramphenicol.

Construction of pDCQ140

Plasmid pDCQ23 containing the *Rhodococcus dxs* gene cloned on pRhBR171 was described previously (Kostichka et al. 2003). A unique *Msc*I site on pDCQ23 was identified immediately downstream of *dxs* into which the *crtU* gene was cloned. The *crtU* gene with its native ribosome binding site was amplified from genomic DNA of *B. linens* (ATCC 9175) by PCR, using forward primer 5′-GTGCTCATGCT GTGGCAGTGGCAA-3′ and reverse primer 5′-TCATCG ACGTCTCCTGATGAGCCCGAGCACT-3′. The 1,554-bp PCR product was first cloned in the pCR2.1-TOPO cloning vector (Invitrogen), and the 1.6-kb-*Eco*RI fragment containing the *crtU* gene was filled in by Klenow DNA polymerase and ligated to the *Msc*I site in pDCQ23. In the resulting construct pDCQ139, the *crtU* gene was in the same orientation as the *dxs* gene. The *crtU* gene presumably was cotranscribed with the *dxs* gene, since they were separated by 131 bp of DNA sequence containing no apparent transcription termination site. The pDCQ139 construct contained a tetracycline resistant marker, which was the same marker as the one used to create the *crtO* knockout host (Tao and Cheng 2004). To provide selection in the *Rhodococcus crtO* knockout mutant, the 6.0-kb-*Hin*dIII fragment containing the *dxs* and *crtU* gene cluster was cut out from pDCQ139 and ligated to the *Hin*dIII site of *Rhodococcus* shuttle vector pDA71 (ATCC 77474), which has a chloramphenicol resistant marker (GenBank Accession # AJ308376). The resulting construct, pDCQ140, was verified by sequencing.

Construction of pDCQ143

The Cm^r gene including 500 bp of its upstream region was PCR amplified from pDA71 plasmid DNA, using forward primer 5′-ccatggcgaagtaccgt**cacgtg**cac-3′ and reverse primer 5′-ccatgg*caattg***tca**ggctgggacggtttcct-3′. The forward primer spanned the *Bbr*PI site (bold). The reverse primer contained the complementary sequence of a stop codon of the gene (bold). Both primers contained *Nco*I sites (underlined), and the reverse primer contained a *Mfe*I site (italicized). The 1,640-bp PCR product was cloned in the pCR2.1-TOPO cloning vector (Invitrogen), and the 1.6-kb-*Nco*I fragment was subcloned into the *Nco*I site in pRhBR171 (Kostichka et al. 2003) to construct the new *E. coli–Rhodococcus* shuttle plasmid pDCQ142 with the Cm^r gene. The *Eco*RI fragment containing the *crtU* gene and its ribosomal binding site was subcloned into the unique *Mfe*I site immediately downstream of Cm^r gene in pDCQ142 in the same orientation. The two genes on the resulting construct pDCQ143 were separated by 62 bp DNA without an apparent transcriptional termination site. The *crtU* gene could presumably be cotranscribed with the Cm^r gene by its promoter.

Rhodococcus transformation

Rhodococcus cells were grown in NBYE medium at 30°C with shaking (250 rpm) to an optical density OD_{600} of about 1.0. Electrocompetent cells were prepared as described for *E. coli* (Sambrook et al. 1989). Electroporation was performed with 0.2–2 μg of plasmid DNA using settings similar to the ones used for *E. coli* electroporation. The transformed cells were incubated at 30°C for 4 h before plating on NBYE plates with appropriate antibiotics.

Carotenoid analysis

Carotenoids were extracted from *R. erythropolis* and *E. coli* cell pellets with acetone. The extracted carotenoids were analyzed using high-pressure liquid chromatography (HPLC) with diode array detector, and molecular weight of carotenoids was determined by liquid chromatography/mass spectrometry (LC/MS) as described previously (Tao et al. 2004). Concentrated carotenoids extracts were also spotted on a 250-μm thickness Silica Gel 60 plate (EM Separations Technology, Gibbstown, NJ, USA) and separated in 7.5% acetone+92.5% hexane for thin layer chromatography (TLC). 4-keto-γ-carotene in the wild-type ATCC 47072, γ-carotene in ATCC 47072 (*crtO*-), and chlorobactene in $crtO^-$ (pDCQ143) were previously confirmed by LC/MS.

Shake flask studies

Freshly grown *Rhodococcus* cells were diluted 1:200 in 200 ml LB or NBYE medium alone or supplemented with 1% fructose or glycerol. The *Rhodococcus* cells were also grown in minimal S12 medium (Bramucci et al. 2002) with 1% fructose or citrate as the sole carbon and energy source. The engineered traits of the *Rhodococcus* strain were maintained with 10 μg/ml tetracycline and 40 μg/ml chloramphenicol. The cells were grown at 30°C with shaking for 3 days. Cells were harvested and the wet weight of the cell pastes was measured. The cell pastes were extracted with 10 ml acetone twice, and the pigment yield was measured by absorption at OD_{458}, which is the characteristic λ_{max} of chlorobactene.

Fermentation conditions

Fed-batch fermentation was performed with chlorobactene-producing *Rhodococcus* strains in a 10-l (working volume) stirred tank bioreactor (B. Braun Biotech International GmbH, Melsungen, Germany) with temperature, pH, dissolved oxygen (DO), pressure, and airflow controls. A seed culture was started from a 1 mL frozen glycerol stock prepared from cells grown from a single colony. The seed culture was grown in 500 ml NBYE medium supplemented with 1% fructose, 10 μg/ml tetracycline, and 40 μg/ml chloramphenicol in a 2-l Erlenmeyer flask at 28°C with shaking for 18–24 h. This seed culture (OD_{600} between 1.5 and 4) was used to inoculate a 10-l stirred tank with 8.5 l starting volume of the same medium as the seed culture. Mazu antifoam was added at 1 g/l. Vessel operating parameters were set at agitation, 350 rpm; airflow, 0.3 vvm; temperature, 28°C; DO, 150% (probe calibration at 100% at atmospheric pressure); pressure, 0.5 bar; and pH 6.8. Off gases were monitored by MS. Neither pH nor DO was controlled during fermentation. After approximately 17 h of fermentation, 1 L of fed-batch medium (10× initial medium) was added. Fermentation continued for additional 40 h after growth stopped as determined by OD of culture and mass spectrometer carbon dioxide evolution rate (CER). Vessel was chilled and cell paste was collected using a continuous flow centrifuge at 15,000 rpm. This protocol was later successfully scaled to 200-l fermentation.

Results

Comparison of different expression plasmids for expression of the *Brevibacterium crtU* gene

Since aryl carotenoids from *B. linens* is used as a colorant for cheese industry, the *crtU* gene encoding the β-carotene desaturase from *B. linens* (Krubasik and Sandmann 2000) was chosen for expression in *R. erythropolis* to produce aryl carotenoids such as chlorobactene. The two different *crtU* expression plasmids (Fig. 1) were compared in the CrtO mutant of *R. erythropolis* ATCC 47072 (see below for host comparison).

Initially, the *Brevibacterium crtU* gene was cloned downstream of a *Rhodococcus* 1-deoxyxylulose-5-phosphate synthase gene (*dxs*) (Kostichka et al. 2003) for two reasons. First, the *dxs* promoter fragment had been demonstrated to be functional in *Rhodococcus*. Second, the *dxs* gene could be used to increase carotenoid titer since the *Rhodococcus* strain overexpressing the *dxs* gene had been shown to produce twice as much carotenoid. Plasmid pDCQ140 has the pDA71 backbone with the *crtU* gene expressed downstream of the *dxs*. ATCC 47042 (*crtO*-) carrying the pDA71 vector control produced γ-carotene eluting at 15.4 min by HPLC. In the ATCC 47042 (*crtO*-) carrying pDCQ140, majority of the carotenoids produced was γ-carotene. A new carotenoid likely chlorobactene representing 7% of the total carotenoids eluted at 15.1 min.

The CrtU activity on pDCQ140 appeared to be low because of the observed low conversion (7%) to chlorobactene. One possible reason was that the *dxs* promoter was weak, since *dxs* had been shown to be a limiting step for isoprenoid synthesis (Albrecht et al. 1999). The promoter of a Cm^r gene on pDA71, which was previously localized to the 1.8-kb-*Bbr*PI-*Stu*I fragment (De Mot et al. 1997), was tested to express the *crtU*. Plasmid pDCQ143 has the pRhBR171 backbone with the *crtU* expressed downstream of the Cm^r gene. Plasmid pRhBR171 was previously shown to be very stable in *Rhodococcus*, and almost 100% of the population maintained the plasmid after 30 generations even without antibiotic selection (Kostichka et al. 2003). ATCC 47042 $crtO^-$ carrying the vector control produced γ-carotene eluting at 15.3 min. 47042 $crtO^-$ carrying pDCQ143 produced a carotenoid at 15.0 min, which has the same absorption spectra as γ-carotene (λ_{max}=435, 458, and 486 nm). No carotenoid was observed eluting at 15.3 min from 47042 $crtO^-$ carrying pDCQ143. Approximately equal amount of carotenoids extracted from the vector control strain and the strain carrying pDCQ143 was mixed and loaded on HPLC. Two carotenoid peaks were observed with 41% eluting at 15.0 min and 59% eluting at 15.3 min. This strongly suggested that the carotenoid

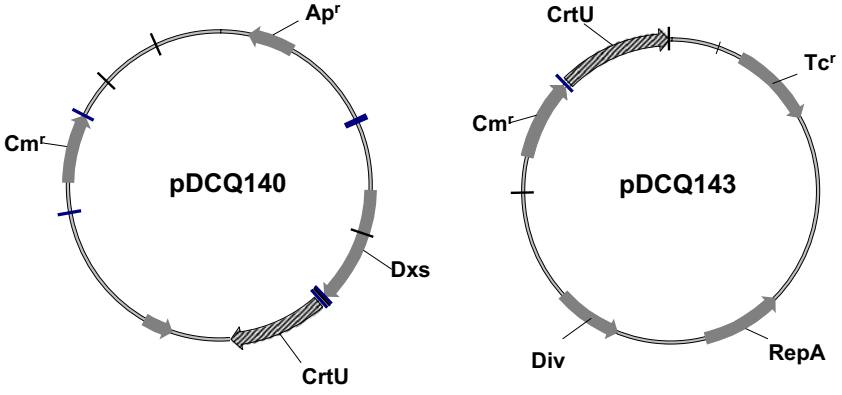

Fig. 1 *E. coli–Rhodococcus* shuttle plasmids for expression of *Brevibacterium crtU* in *R. erythropolis*. pDCQ140 has pDA71 vector backbone. Ap^r allows ampicillin selection in *E. coli*. Cm^r allows chloramphenicol selection in *Rhodococcus*. Dxs was from *R. erythropolis*. pDCQ143 has pRhBR171 vector backbone with plasmid replication protein (*RepA*) and plasmid stability protein (*Div*). Tc^r allows tetracycline selection in both *E. coli* and *Rhodococcus*. Cm^r allows selection in *Rhodococcus*

produced from the strain containing pDCQ143 was different from the γ-carotene produced from the vector control strain. The *crtU* expressed downstream of *Cm*^{r} gene on pDCQ143 achieved over 90% conversion of γ-carotene to the new carotenoid.

Molecular weight of the carotenoids from different *Rhodococcus* strains was determined by LC/MS (Fig. 2). Wild-type ATCC 47072 produced 4-keto-γ-carotene with molecular weight of 550 Da, and ATCC 47072 (*crtO*-) produced γ-carotene with molecular weight of 536 Da. ATCC 47072 (*crtO*-) containing pDCQ143 produced the new carotenoid with molecular weight of 532 Da. The 4-Da difference of molecular weight between γ-carotene and the new carotenoid was consistent with the aromatization of the β-ionone ring of the γ-carotene by two desaturation steps. The new carotenoid produced in 47072 (*crtO*-) containing pDCQ143 was chlorobactene as confirmed by LC/MS.

Comparison of different *Rhodococcus* hosts for expression of the *Brevibacterium crtU* gene

Both *R. erythropolis* strain AN12 and ATCC 47072 were characterized to produce 4-keto-γ-carotene as the major carotenoid and sometimes γ-carotene as the minor carotenoid (Tao et al. 2004). Strain ATCC 47072 was used as the production host because of higher transformation efficiency. A mutant of ATCC 47072 that inactivated the CrtO ketolase was previously constructed and shown to accumulate γ-carotene (Tao and Cheng 2004). We postulated that this CrtO mutant might be a preferred host to produce chlorobactene (Fig. 3) since CrtO competes with CrtU for the γ-carotene substrate. Expression of *crtU* on pDCQ143 was compared in the wild-type ATCC 47072 and the CrtO mutant of ATCC 47072. In the CrtO mutant containing pDCQ143, chlorobactene was produced as the predominant carotenoid (>90%), and a small amount of γ-carotene was sometimes also present. In the wild type containing pDCQ143, 4-keto-γ-carotene was produced in addition to γ-carotene and chlorobactene. 4-keto-γ-carotene (λ_{max}= 465 nm) eluted at 14.5 min was estimated to contribute to at least 10% of total carotenoids. Samples were also concentrated and analyzed by TLC (Fig. 4). TLC result, which was consistent with the HPLC result, showed that higher purity of chlorobactene was produced in the CrtO mutant of ATCC 47072.

Comparison of different growth substrates for chlorobactene production

The CrtO mutant of ATCC 47072 containing pDCQ143 was growing on different substrates in shake flasks to evaluate for chlorobactene production (Table 1). Since chlorobactene was produced as the predominant caroten-

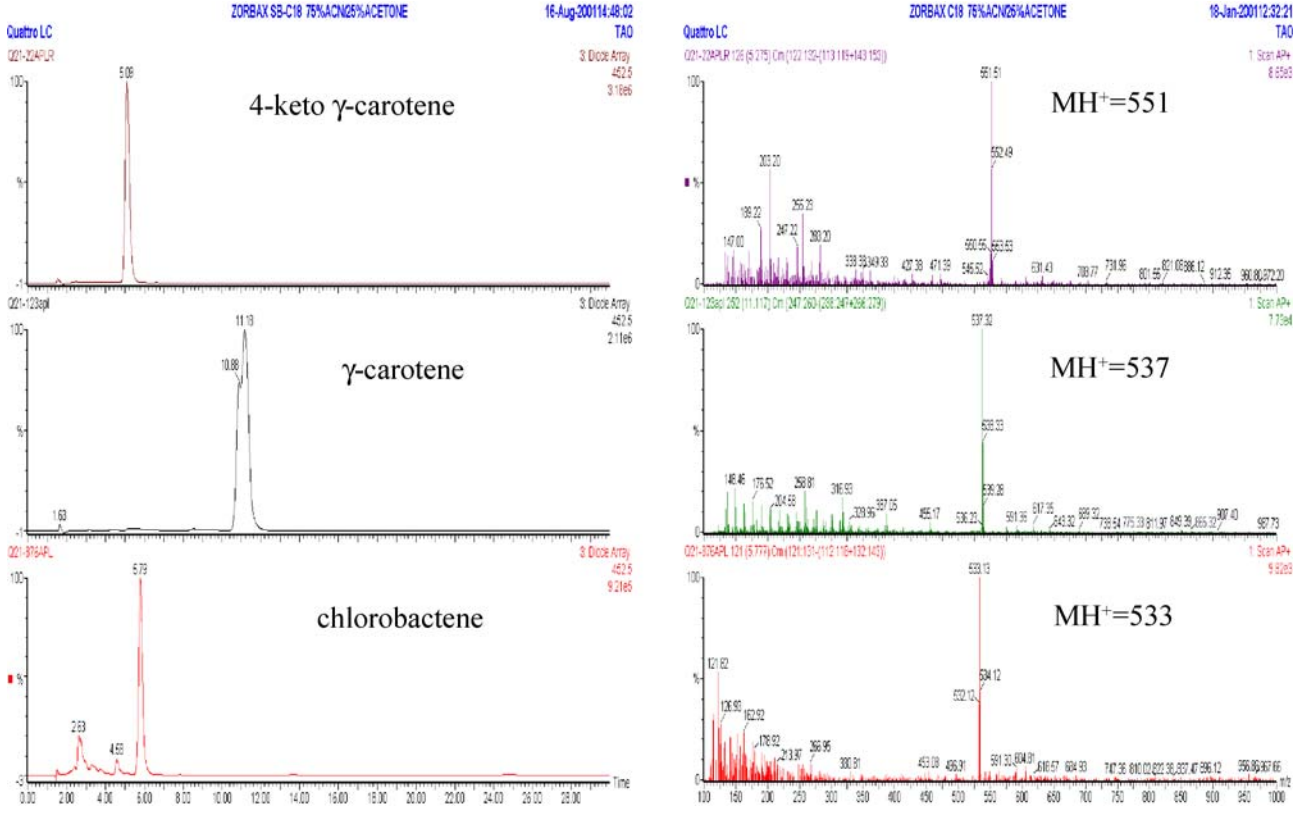

Fig. 2 LC/MS analysis of carotenoids from *R. erythropolis* ATCC47072 (*top*), ATCC47072 CrtO mutant (*middle*) and ATCC47072 CrtO mutant carrying pDCQ143 (*bottom*). *Left panels* show the LC elution profiles. *Right panels* show the MS spectra of the major carotenoid peaks

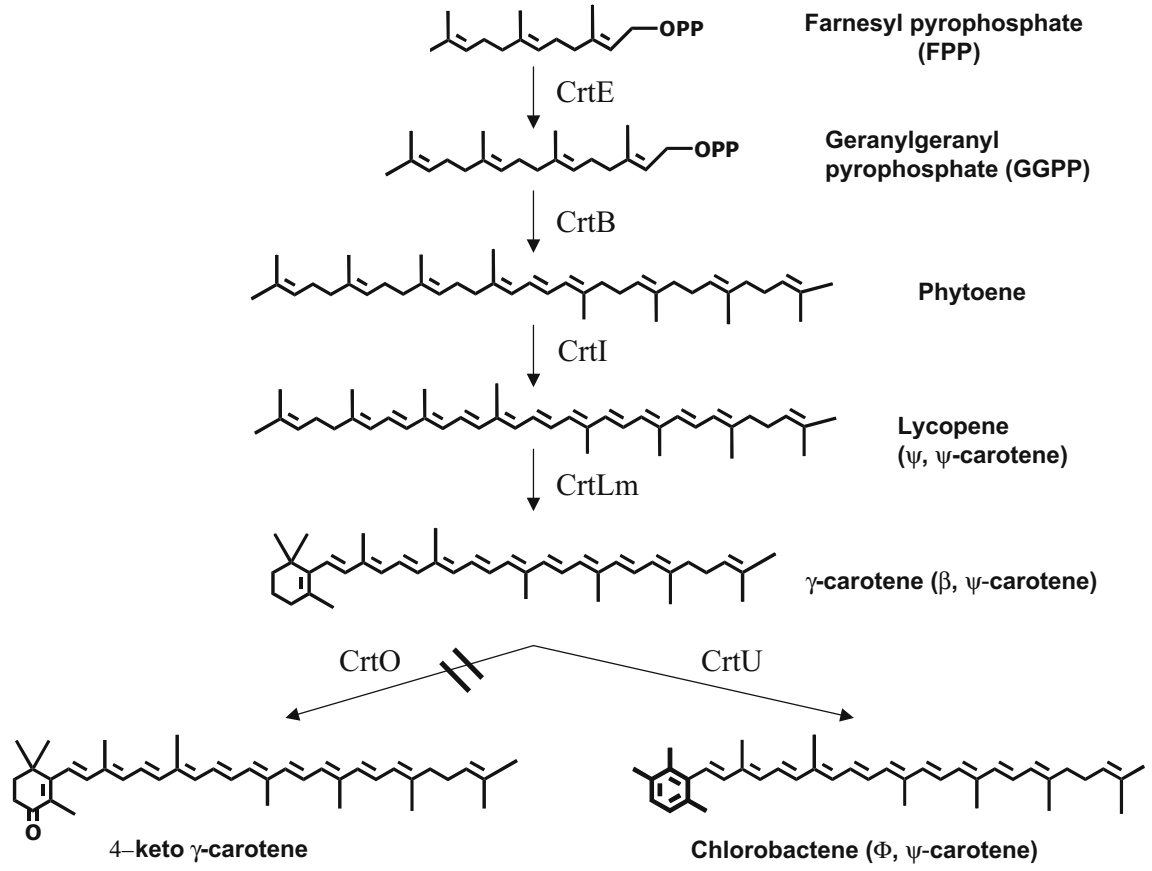

Fig. 3 Strategy for producing chlorobactene in *R.erythropolis*. CrtE, geranylgeranyl pyrophosphate synthase; CrtB, phytoene synthase; CrtI, phytoene dehydrogenase; CrtLm, lycopene β-cyclase; CrtO, β-carotene ketolase; CrtU, β-carotene desaturase. All enzymes are native to *Rhodococcus erythropolis* except that the CrtU is from *Brevibacterium linens*. The *double lines* on the arrow indicate the genetic block of the step in the *R. erythropolis* host

oid (>90%) in this strain and no other contaminant absorbs in the same wavelength region, spectrophotometric measurement was used for chlorobactene quantitation using the observed λ_{max}=458 nm of chlorobactene. Growing in NBYE medium gave the highest specific pigment yield per gram of wet cell paste (10.9 μg pigment/g cell). Growing in NBYE supplemented with 1% fructose (200-ml medium) gave the highest total pigment yield (50.3 μg) due to more biomass generated.

Production of chlorobactene by fermentation

Chlorobactene was produced from the ATCC 47072 CrtO mutant containing pDCQ143 by fed-batch fermentation in a 10-l stirred tank bioreactor. NBYE medium supplemented with 1% fructose was chosen as the fermentation medium based on the shake flask study. After 17 h of fermentation, cell growth slowed down as indicated by the OD, the increase in DO, and decrease in CER. A fed-batch medium was added when cells reached OD_{600} of 18.4. Fermentation continued for additional 40 h after cell growth stopped. The final pH rose to 6.74 after dropping to around 6 during fermentation from the starting pH 7. The final DO returned to the initial 150% after it dropped during fermentation. The final OD_{600} reached 45 and 370 g wet cell paste was obtained. A fraction of the cell paste was extracted and HPLC analysis showed that chlorobactene was almost the exclusive carotenoid produced from the ATCC 47072 CrtO mutant containing pDCQ143. A 10-l fermentation yielded 18 mg of chlorobactene.

Fermentation of wild-type ATCC 47072 containing pDCQ143 was also performed, since it showed higher growth rate than the CrtO mutant containing pDCQ143, and 4-keto-γ-carotene by-product was present in small amount (10%) in shake flasks. Consistent with the better growth phenotype observed in shake flasks, larger quantity of cell paste was obtained for the wild-type host from the 10-l fermentation. However, the cell paste appeared pink/orange color compared to the yellow-colored cell paste from the CrtO mutant host. HPLC analysis showed that the wild-type ATCC 47072 containing pDCQ143 produced approximately equal amount of 4-keto-γ-carotene and chlorobactene during fermentation.

Discussion

Members of the genus *Rhodococcus* have been successfully used as production hosts for commercial production

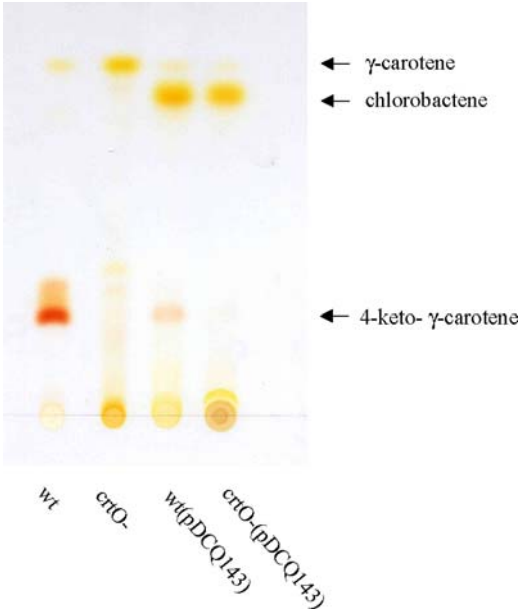

Fig. 4 Comparison of chlorobactene production in the wild type and the CrtO mutant of *R. erythropolis* ATCC47072 by thin layer chromatography (TLC). *Rhodococcus* cultures (200 ml) were grown at 30°C for 2 days, and cell pellets were extracted twice with 10 ml acetone. The extraction was concentrated in 0.5 ml of acetone/hexane (v/v 7.5:92.5), and the insoluble material was removed by centrifugation at 16,000×*g* for 2 min. Approximately 20 μl of the concentrated extract was loaded and separated on the TLC by acetone/hexane (v/v 7.5:92.5)

of a variety of compounds including acrylamide (Yamada and Kobayashi 1996), nicotinamide, and polyhydroxy alkanoic acids. They exhibit robust growth on many substrates such as sugars and fatty acids. Besides their diverse metabolic capabilities, resistance to many solvents offers another advantage for process development and certain applications (Bouchez-Naitali et al. 2004). *Rhodococcus*

Table 1 Chlorobactene production by *R. erythropolis* ATCC 47072 CrtO mutant containing pDCQ143 growing on different substrates

Medium	Supplement[a]	Wet cell weight[b] (g)	OD_{458}[c]	Pigment yield (μg/g cell)[d]	Total pigment yield (μg)
S12	Fructose	4.34	0.466	7.1	30.8
	Citrate	3.79	0.352	6.1	23.1
LB	None	2.29	0.168	5.8	13.3
	Fructose	3.04	0.284	6.2	18.9
	Glycerol	2.94	0.224	5.0	14.7
NBYE	None	2.89	0.479	10.9	31.5
	Fructose	5.92	0.761	8.5	50.3
	Glycerol	5.44	0.675	8.2	44.6

[a]The carbon source was supplemented as 1% final concentration in the media
[b]Wet cell weight was measured from the 200-ml culture grown in flasks
[c]OD_{458} was the observed λ_{max} of chlorobactene
[d]Pigment yield was calculated using specific extinction coefficient of 3,040 as reported for chlorobactene in petroleum ether

belongs to the actinomycetales, which include the native CrtU-containing hosts such as *Streptomyces*, *Mycobacterium*, and *Brevibacterium*. *Rhodococcus* was thus chosen as the host to express the *crtU* gene from *Brevibacterium* for producing chlorobactene.

Metabolic engineering in *Rhodococci* is generally hampered by the lack of genetic tools. Expression plasmids and promoters are rather limited. The Cm^r gene on pDA71 has been used as an antibiotic marker cassette for selection in *Rhodococcus*. We used the promoter of the Cm^r gene to cotranscribe the downstream *crtU* gene on pDCQ143. Plasmid pDCQ143 is a pAN12-based shuttle plasmid, which showed similar copy number as the pDA71-based shuttle plasmid (Kostichka et al. 2003). The higher CrtU activity observed with pDCQ143 was likely due to the stronger promoter of the Cm^r gene. Another advantage of using the Cm^r gene promoter for heterologous gene expression in *Rhodococcus* is that this Cm^r gene only expresses in *Rhodococcus* and not in *E. coli*, which circumvents potential toxicity or stability problems in *E. coli* during subcloning steps. It is likely that stronger Cm^r gene promoter variants could be obtained by mutation and selected using higher concentration of antibiotics.

The 10-l fermentation yielded 18 mg chlorobactene (~200 ppm) from *Rhodococcus*, which is comparable to the amount of carotenoid produced from an un-engineered *E. coli* strain expressing carotenoid synthesis genes. The rate-limiting steps for carotenoid synthesis in *Rhodococcus* are likely in the upstream isoprenoid pathway (*dxs* and *idi*) as reported for *E. coli* (Albrecht et al. 1999). The chlorobactene titer could be further improved by engineering the isoprenoid pathway or the precursor supply in *Rhodococcus* in a similar way as been done in *E.coli* (Kajiwara et al. 1997; Albrecht et al. 1999; Harker and Bramley 1999; Wang et al. 1999; Farmer and Liao 2000, 2001; Kim and Keasling 2001).

Two types of CrtU enzymes were reported: the β-carotene desaturase CrtU in the actinomycetes that synthesizes isorenieratene from β-carotene (Krugel et al. 1999; Krubasik and Sandmann 2000; Viveiros et al. 2000) and the γ-carotene desaturase CrtU in the photosynthetic bacterium *Chlorobium* that synthesizes chlorobactene from γ-carotene (Frigaard et al. 2004). The CrtU enzymes from actinomycetes are 510–520 amino acids in length and share 45–55% sequence identities. The CrtU from *Chlorobium* has 647 amino acids in length and share 23–25% sequence identities with the actinomycetes CrtU. Other CrtU homologues have been identified in cyanobacteria (Frigaard et al. 2004); however, their functions have not been demonstrated. Interestingly, no aryl carotenoids were reported in these cyanobacteria, implying other functions for these CrtU homologues. The *Chlorobium* CrtU and the cyanobacteria CrtU homologues contain a Rieske iron–sulfur domain of 116 amino acids in the middle of the sequence. This domain is not found in the CrtU from actinomycetes. Heterologous expression of the *Brevibacterium crtU* in *E. coli* was reported and formation of isorenieratene was detected (Lee et al. 2003). We showed that the *Brevibacterium* CrtU enzyme also act on γ-carotene substrate and

produced chlorobactene. Heterologous expression of the *Chlorobium crtU* has not been reported, and our attempts to express it in *E. coli* failed, likely due to the problem of assembly the iron–sulfur center of the *Chlorobium* CrtU in *E. coli*. It is likely that the *Chlorobium* CrtU could also act on β-carotene as substrate. Similar case was reported for the *Rhodococcus* β-carotene ketolase CrtO (Tao and Cheng 2004). *Rhodococcus* CrtO was shown to add both keto groups to β-carotene to synthesize canthaxanthin in *E. coli*, although it adds only one keto group to γ-carotene to synthesize 4-keto-γ-carotene in the native *Rhodococcus* host.

The β-carotene ketolase CrtO catalyzes a competition reaction for the CrtU to produce chlorobactene from γ-carotene in *Rhodococcus*. In shake flasks, the CrtO appeared to be less active and 4-keto-γ-carotene by-product was present as ~10% of the total carotenoids. However, the CrtO was more active in the fermentor condition and 4-keto-γ-carotene by-product contributed to ~50% of the total carotenoids. It is likely that the high DO in the fermentor stimulated the CrtO ketolase activity, which requires molecular oxygen as the substrate. The CrtO mutant that inactivated the ketolase was a suitable host for chlorobactene production.

Aryl carotenoids are commonly used as food colorants in cheese industry. The typical orange color of cheese originates from isorenieratene and its hydroxy derivatives synthesized by *B. linens* in the cheese-ripening flora (Dufosse et al. 2001). Chlorobactene is an asymmetric aryl carotenoid that has similar structure as isorenieratene. It may potentially be used as a colorant that imparts a different shade of color. Production of chlorobactene by fermentation provides a source of chlorobactene to be evaluated in potential applications.

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