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Strain-Dependent Carotenoid Productions in Metabolically Engineered *Escherichia coli*

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Abstract Seven *Escherichia coli* strains, which were metabolically engineered with carotenoid biosynthetic pathways, were systematically compared in order to investigate the strain-specific formation of carotenoids of structural diversity. C30 acyclic carotenoids, diaponeurosporene and diapolycopene were well produced in all *E. coli* strains tested. However, the C30 monocyclic diapotorulene formation was strongly strain dependent. Reduced diapotorulene formation was observed in the *E. coli* strain Top10, MG1655, and MDS42 while better formation was observed in the *E. coli* strain JM109, SURE, DH5a, and XL1-Blue. Interestingly, C40 carotenoids, which have longer backbones than C30 carotenoids, also showed strain dependency as C30 diapotorulene did. Quantitative analysis showed that the SURE strain was the best producer for C40 acyclic lycopene, C40 dicyclic β-carotene, and C30 monocyclic diapotorulene. Of the seven strains examined, the highest volumetric productivity for most of the carotenoids structures was observed in the recombinant SURE strain. In conclusion, we showed that recombinant hosts and carotenoid structures influenced carotenoid productions significantly, and this information can serve as the basis for the subsequent development of microorganisms for carotenoids of interest.

Keywords Carotenoids · *E. coli* · Strain dependency · Metabolic engineering · Pathway engineering

Introduction

Carotenoids, derivatives of isoprenoids, belong to an important class of natural pigments used in biotechnology applications. They are classified according to C30, C40, or C50

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carbon backbones and dicyclic, monocyclic, or acyclic features [1]. These structurally diverse pigments have different biological functions including coloration, photoprotection, light harvesting, and precursors for many hormones [2]. Carotenoids are commercially used as food colorants, animal feed supplements, and, more recently, as nutraceuticals and cosmetic and pharmaceutical compounds [1]. Currently, only a few carotenoids can be produced commercially by chemical synthesis, fermentation, or isolation from a few abundant natural sources [3]. The rising importance of carotenoids in industries has led to greater efforts aimed at developing bioprocesses for large-scale production of a range of carotenoids through metabolic engineering, including lycopene, β-carotene, as well as more structurally diverse carotenoids [4–9].

Despite the recent accomplishments in metabolic engineering of *Escherichia coli* cells for carotenoid production [1], the production levels are not yet competitive to commercial carotenoid levels presently produced through fermentation, synthesis, or isolation [3, 10]. As a way to increase carotenoid production levels in recombinant *E. coli*, precursor pool engineering has been carried out by several groups [11–13]. In these studies, most common C40 carotenoid lycopene or β-carotene was used as an indicator for precursor pool engineering [14, 15]. Generally, carotenoids of different structures tend to incorporate or localize differently in the membrane according to their own structural property [16]. The different localization of carotenoids subsequently affects cellular membrane fluidity and membrane function, resulting in suboptimal conditions for cell growth. Besides carotenoid structures, it has been reported that heterologous formation of carotenoid such as lycopene [17] was strain dependent in recombinant *E. coli*. Therefore, target carotenoid structures and heterologous *E. coli* strains should be carefully tested and considered for the expression of carotenoids. However, there has been no systematic study on the effect of *E. coli* strains on the formation of structurally diverse carotenoids although the strains can dramatically affect carotenoid formation. In this study, seven *E. coli* strains were metabolically engineered with C30 and C40 carotenoid biosynthetic pathways, and the production levels and profiling of structurally diverse carotenoids were systematically compared.

Materials and Methods

Bacterial Strains and Plasmids

Seven *E. coli* strains JM109, SURE, XL1-Blue, MG1655, TOP10, MDS42, and DH5α were chosen, and their brief genotypes were shown in Table 1. Four carotenogenic plasmids pACM-E_{BL}-B_{BL}-I_{BL}, pACM-M_{SA}-N_{SA}, pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}, and pACM-M_{SA}-NyYt were used for metabolically engineering the *E. coli* strains to produce lycopene, diapolycopene/diaponeurosporene, β-carotene, and diapotorulene, respectively (Table 1). The plasmid was electrotransformed into seven *E. coli* strains according to the manufacture' s protocol (Bio-Rad Laboratories, Inc), and the recombinant *E. coli* strains producing carotenoid were selected on Luria–Bertani (LB) plates containing 50 μg ml^{-1} chloramphenicol as a selection marker.

Culture Growth and Isolation of Carotenoids

For carotenoid production, recombinant *E. coli* cells harboring carotenogenic plasmids were cultivated in 100 ml of LB medium supplemented with chloramphenicol (50 μg ml^{-1}) and glycerol (5 g l^{-1}) at 30 °C and 250 rpm for 48 h [18]. Cells were pelleted by centrifugation

Table 1 Strains and plasmids used in this study.

Strains or plasmid	Relevant characteristics	Source or reference
E. coli		
JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14− [F′ traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17($r_K^-m_K^+$)	
SURE	endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC:: Tn5 uvrC e14− Δ(mcrCB-hsdSMR-mrr)171 F′[proAB$^+$ lacI^q lacZΔM15 Tn10]	
XL1-Blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F′[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	
MG1655	F$^-$ λ$^-$ ilvG− rfb-50 rph-1	[23]
Top10	F$^-$ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$	
MDS42	A chromosome that is 14.3% smaller than that of its parental *E. coli* strain MG1655	[21]
DH5α	F$^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ$^-$	
Plasmid		
pACYC184	Cloning vector, Cm^r	NEB
pACM-E_{BL}-B_{BL}-I_{BL}	Constitutively express *crtE*, *crtB*, and *crtI* genes of *B. linens* on pACYC184 to produce lycopene	Kim et al. (submitted for publication)
pACM-M_{SA}-N_{SA}	Constitutively express *crtM* and *crtN* genes of *S. aureus* on pACYC184 to produce Diapolycopene	Unpublished data
pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}	Constitutively express *crtE*, *crtB*, *crtI*, and *crtY* genes of *B. linens* on pACYC184 to produce β-carotene	Kim et al. (submitted for publication)
pACM-M_{SA}-NyYt	Constitutively express *crtM*, *mutant crtNy* of *S. aureus*, and mutant *crtYt* genes of *B. linens* on pACYC184 to produce diapotorulene	Unpublished data

(4 °C, 3,000×*g*) and extracted repeatedly with a total volume of 15 ml acetone until all visible pigments were extracted. After centrifugation (4 °C, 3,000×*g*), the colored supernatants were pooled and re-extracted with an equal volume of hexane after the addition of an equal volume of double distilled water. All hexane extracts were passed through a column packed with anhydrous sodium sulfate (BioBasic) for dehydration, dried under nitrogen gas, and then resuspended with 0.5 ml acetone [4].

HPLC and Mass Spectrometry

Twenty microliters of the acetone extracts were applied to a Zorbax eclipse XDB-C18 column (4.6×150 mm, 5 μm; Agilent Technologies) and typically eluted under isocratic conditions with a solvent system containing 80% acetonitrile, 15% methanol, and 5% isopropanol (*v*/*v*/*v*) at a flow rate of 1 ml min^{-1} using an Agilent 1200 HPLC system equipped with an photodiode array detector. Mass fragmentation spectra were monitored in a mass range of *m*/*z* 200–800 on a UPLC–mass spectrometry system (Quttro-Micro, Waters) equipped with an electron spray ionization interface. For structural elucidation,

carotenoids were identified by a combination of HPLC retention times, absorption spectra, and mass fragmentation spectra.

Quantification of Carotenoid

The total content of carotenoid accumulated in *E. coli* transformants was quantified by measuring the absorbance at a specific wavelength using a Smart Plus 3255PC spectrophotometer: lycopene at 474 nm, β-carotene at 454 nm, diapolycopene at 470 nm, and diapotorulene at 450 nm. The equation used to calculate the total carotenoid content was:

$$\frac{A \times \mathrm{Vol(ml)} \times 10^4}{E^{1\%}_{1\,\mathrm{cm}} \times \mathrm{wet\,cell\,weight(g)}}$$

where A is absorbance, a wet cell weight (WCW) is weight of a drained cell pellet, $E^{1\%}_{1\,\mathrm{cm}}$ is defined as the theoretical absorbance of a solution of 1% (*w*/*v*) concentration (i.e., 1 g in 100 ml) in a cuvette of 1 cm path length. $E^{1\%}_{1\,\mathrm{cm}}$ for lycopene is 3,410, $E^{1\%}_{1\,\mathrm{cm}}$ for β-carotene is 2,443, $E^{1\%}_{1\,\mathrm{cm}}$ for diapolycopene is 3,410, and $E^{1\%}_{1\,\mathrm{cm}}$ for diapotorulene is 2,500 [19].

Results and Discussion

Effect of C40 Acyclic Lycopene Formation on the Growth and Production Level in *E. coli* Strains

As shown in Fig. 1, carotenoids have structural diversity [20] and can be subclassified into cyclic, e.g., β-carotene, and acyclic, e.g., lycopene. They are further divided into C30, C40, and C50 carotenoids according to the length of the backbone isoprene unit [2]. Because structurally diverse carotenoids differentially affect recombinant host microorganisms, suitable *E. coli* hosts should be carefully determined for the maximal production of the carotenoid of interest. Therefore, we selected four basic carotenoid structures—acyclic C40, cyclic C40, acyclic C30, and cyclic C30 (Fig. 1)—and seven *E. coli* strains—JM109, SURE, XL1-Blue, MG1655, TOP10, MDS42, and DH5α (Table 1)—and then investigated the effects of carotenoid with different structures on cell growth and carotenoid production level.

Lycopene was chosen as a representative for C40 acyclic carotenoids, and the lycopene-producing plasmid pACM-E_{BL}-B_{BL}-I_{BL} (Table 1) was transformed into the seven *E. coli* strains: JM109, SURE, XL1-Blue, MG1655, TOP10, MDS42, and DH5α. When the resulting recombinant *E. coli* strains were grown in LB+5 g l^{-1} glycerol [11], DH5α grew much slower than the other six strains and reached the lowest cell density (Fig. 2a). The SURE strain produced the highest specific amount of lycopene (12.3 μg g^{-1} WCW), which was 2.7 times higher than the MDS42 strain (Table 2) and the highest volumetric amount of lycopene (8 μg ml^{-1}; Fig. 3a). Even though MDS42 is known to grow robustly under normal laboratory conditions and even better under high-cell density fermentation conditions [21], this *E. coli* strain may not be a good host for lycopene production. Instead, the SURE strain was found to be the best host for acyclic C40 carotenoid production. Even though the XL1-Blue strain produced a higher volumetric amount of lycopene than DH5α (Fig. 3a), the specific amount of lycopene produced by XL1-Blue was much lower than DH5α (Table 2).

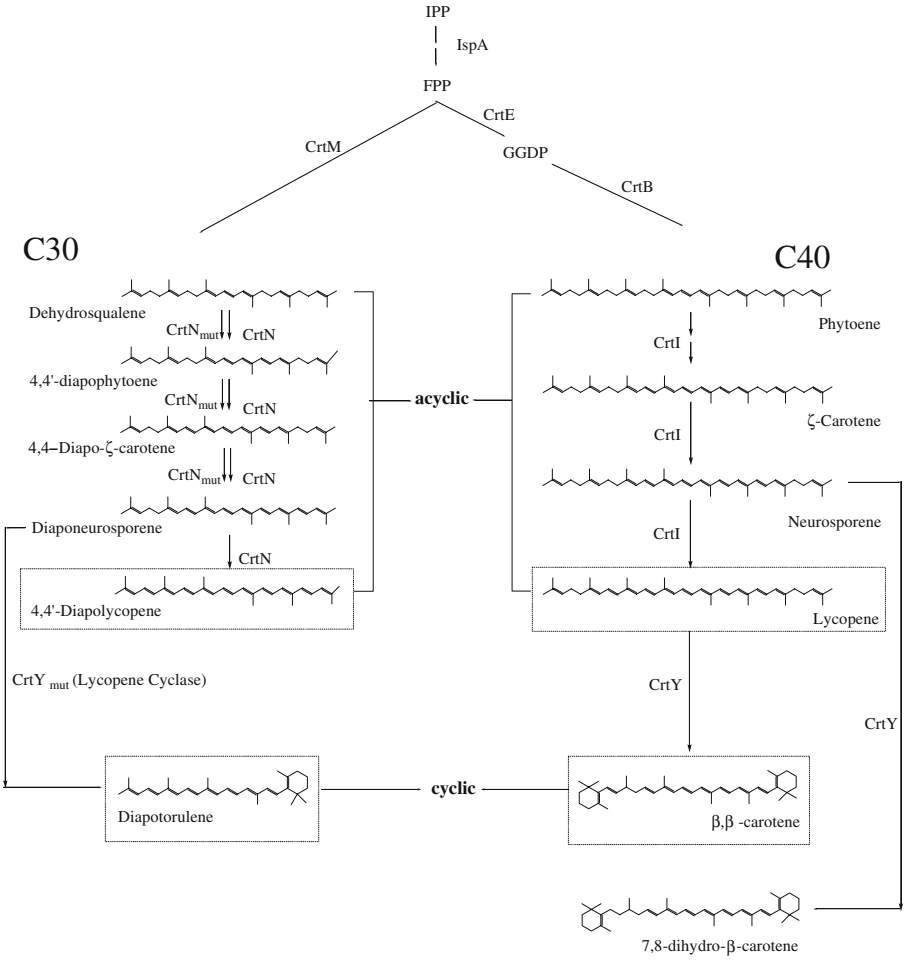

Fig. 1 Engineered biosynthetic pathways of C30 acyclic/cyclic and C40 acyclic/cyclic carotenoids in the heterologous host *E. coli.* The enzymes used are *IspA*, FPP synthase; *CrtM*, dehydrosqualene synthase; *CrtN*, diapophytoene synthase; *CrtNmut*, directed evolved diapophytoene synthase; *CrtE*, GGPP synthase; *CrtB*, phytoene synthase; *CrtI*, phytoene desaturase; *CrtY*, lycopene cyclase; and *CrtYmut*, directed evolved lycopene cyclase. *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *FPP* farnesyl diphosphate, *GGPP* geranylgeranyl diphosphate

Functional coordination of pathway enzymes is essential for proper carotenoid production especially in heterologous hosts [8]. Imbalanced assembly of pathway enzymes can cause intermediate accumulation and also a low production level of the final structure of isoprenoids [22]. Therefore, the profile of carotenoids accumulated in the seven *E. coli* strains were investigated by HPLC and LC–mass spectrometry. As shown in Fig. 3b, there were no significant changes in the carotenoid profiles in the *E. coli* strains, indirectly indicating that carotenogenic enzymes are functionally coordinated even though the production level of lycopene was strongly dependent on the *E. coli* strain. At this point, it is not clear why carotenoid production was strongly linked to host strains.

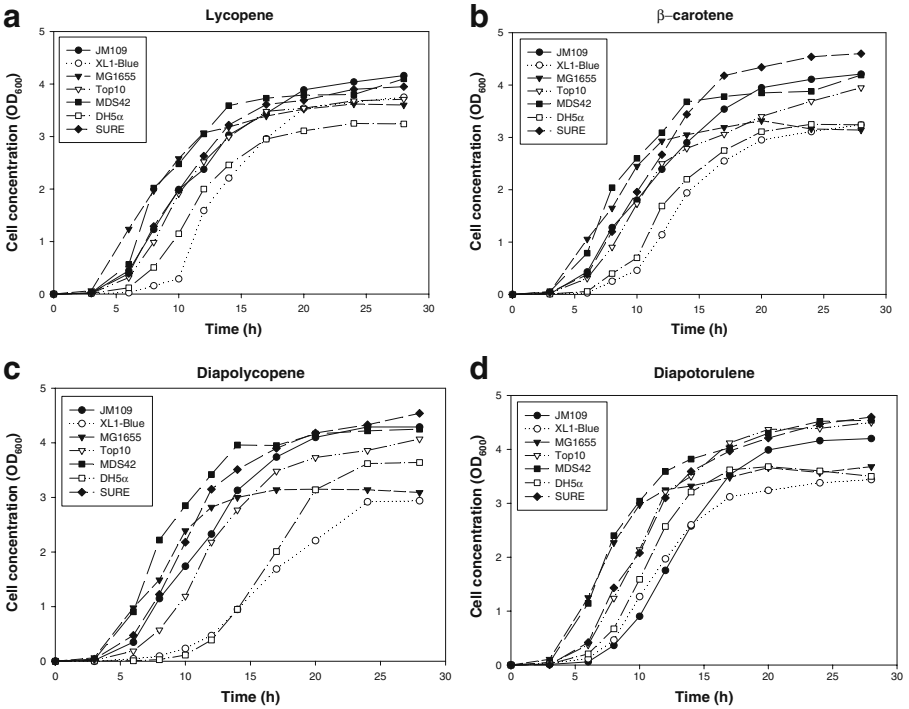

Fig. 2 Cell growth of seven *E. coli* strains expressing C40 acyclic lycopene (**a**), C40 dicyclic β-carotene (**b**), C30 acyclic diapolycopene (**c**), or C30 monocyclic diapotorulene (**d**)

Table 2 Specific amount of carotenoids produced in seven recombinant *E. coli* strains.

Lycopene	β-Carotene	Diapolycopene	Diapotorulene
SURE, 12.3 μg g^{-1} (2.7)[a]	SURE, 14.4 μg g^{-1} (4.3)	JM109, 49.4 μg g^{-1} (11.5)	SURE, 38.8 μg g^{-1} (8.5)
DH5a, 10.2 μg g^{-1} (2.3)	XL1-Blue, 12.5 μg g^{-1} (3.7)	SURE, 35.3 μg g^{-1} (8.2)	JM109, 35.4 μg g^{-1} (7.7)
MG1655, 6.0 μg g^{-1} (1.3)	JM109, 7.7 μg g^{-1} (2.3)	MG1655, 19.7 μg g^{-1} (4.6)	XL1-Blue, 15.3 μg g^{-1} (3.3)
XL1-Blue, 6.0 μg g^{-1} (1.3)	DH5a, 6.5 μg g^{-1} (1.9)	DH5a, 17.8 μg g^{-1} (4.1)	Top10, 9.4 μg g^{-1} (2.0)
Top10, 4.8 μg g^{-1} (1.1)	MG1655, 4.9 μg g^{-1} (1.5)	XL1-Blue, 12.8 μg g^{-1} (3.0)	MG1655, 7.3 μg g^{-1} (1.6)
JM109, 4.8 μg g^{-1} (1.1)	Top10, 3.8 μg g^{-1} (1.1)	Top10, 12.4 μg g^{-1} (2.9)	DH5a, 6.5 μg g^{-1} (1.4)
MDS42, 4.5 μg g^{-1} (1.0)	MDS42, 3.4 μg g^{-1} (1.0)	MDS42, 4.3 μg g^{-1} (1.0)	MDS42, 4.6 μg g^{-1} (1.0)

[a] Fold relative to the lowest amount

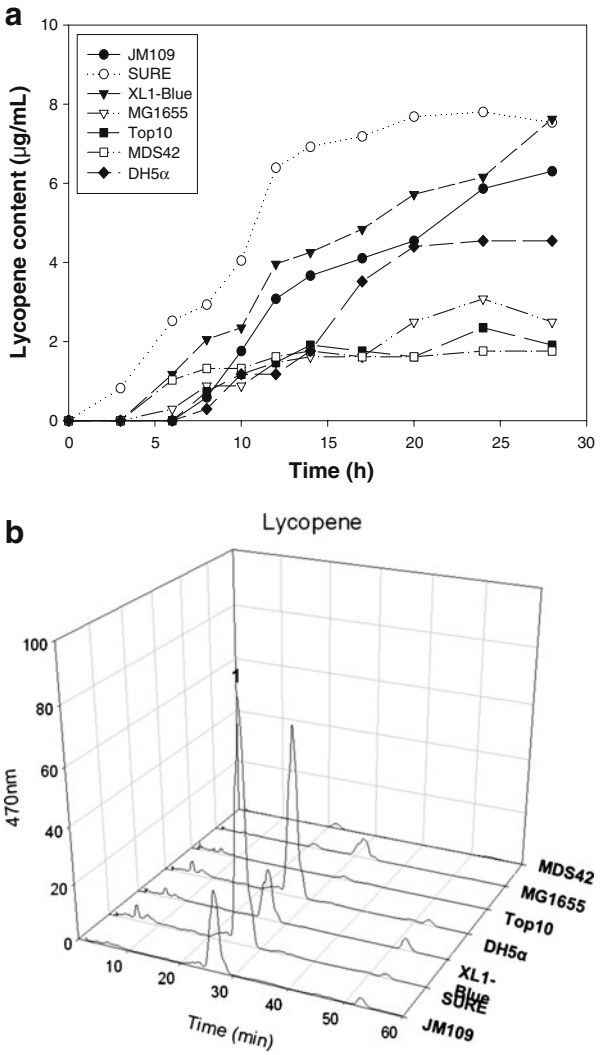

Fig. 3 C40 acyclic lycopene production (**a**) and HPLC analysis (**b**) of acetone extracts from recombinant *E. coli* strains harboring pACM-E_{BL}-B_{BL}-I_{BL}. Peak 1 refers to lycopene

Effect of C40 Cyclic β-Carotene Formation on Growth and Production Level in *E. coli* Strains

Next, the production of the C40 cyclic carotenoid was investigated with the same *E. coli* strains used for the C40 acyclic lycopene. β-Carotene was chosen and quantified to examine the relationship between β-carotene formation and *E. coli* host strains. β-Carotene-producing *E. coli* strains were prepared by transforming with pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL} (Table 1) and were grown in LB+5 g l^{-1} glycerol. XL1-Blue and DH5α grew comparably slower than the others and reached the lowest cell density (an OD_{600} of 3.1) after 28 h (Fig. 2b). As observed for lycopene, the SURE strain produced the highest specific amount of β-carotene (14.4 μg g^{-1} WCW), which was 4.3 times higher than

MDS42 strain (Table 2), and the highest volumetric amount of β-carotene (11 μg ml^{-1}; Fig. 4a). While *E. coli* MDS42 proved to be the worst host for β-carotene production, the SURE strain was the best host for β-carotene production as was the case for lycopene.

When the profile of the β-carotene pathway in the seven *E. coli* strains was investigated, a high amount of 7,8-dihydro-β-carotene (λ_{max}=403, 429, 453; $[M+H]^+ = 539.5$) was detected along with β-carotene in the acetone extracts of strains DH5α and XL1-Blue

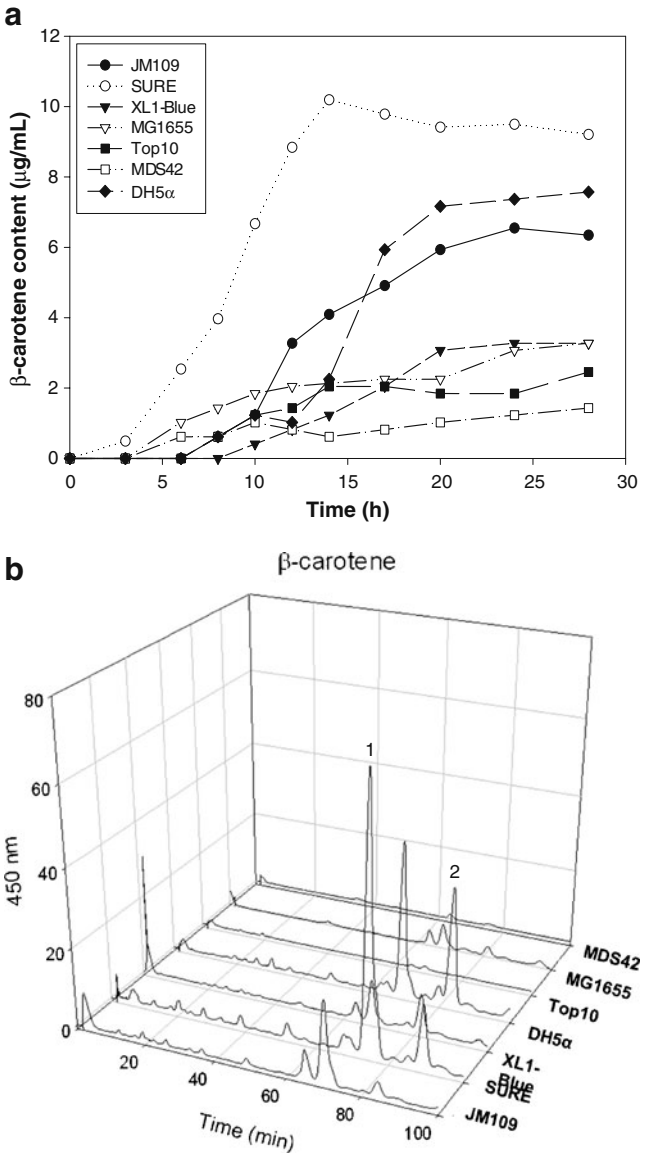

Fig. 4 C40 cyclic beta-carotene production (**a**) and HPLC analysis (**b**) of acetone extracts from recombinant *E. coli* strains harboring pACM-E_{BL}-B_{BL}-I_{BL}-Y_{BL}. Peak 1 refers to beta-carotene; peak 2 refers to dihydro-beta-carotene

(Fig. 4b). The recombinant SURE strain also produced 7,8-dihydro-β-carotene. It is rare for lycopene cyclase CrtY to actively act on neurosporene to generate 7,8-dihydro-β-carotene in nature. Thus, it seems that CrtY of *Brevibacterium linens* has a wider substrate specificity or promiscuity for substrates. MG1655, TOP10, and MDS42 produced very small amounts of β-carotene.

Effect of C30 Acyclic Diapolycopene Formation on Growth and Production Level of *E. coli* Strains

The diapolycopene pathway of *Staphylococcus aureus* [4] was chosen as a representative for C30 acyclic carotenoids (Fig. 1), and the carotenogenic plasmid pACM-M_{SA}-N_{SA} (Table 1) was transformed into seven *E. coli* strains. When the resulting diapolycopene-producing *E. coli* strains were grown in LB+5 g l^{-1} glycerol, the DH5α and XL1-Blue strains grew much slower than the other strains and XL1-Blue had the lowest cell density with an OD_{600}=2.8 (Fig. 2c). Interestingly, the JM109 strain produced the highest specific amount of diapolycopene (49.4 μg g^{-1} WCW; Table 2) and the highest volumetric amount of lycopene (28 μg ml^{-1}; Fig. 5a). The MDS42 strain produced the lowest amount of diapolycopene.

When the profile of the diapolycopene pathway in the seven *E. coli* strains was investigated, the ratio of diapolycopene to diaponeurosporene was significantly affected by *E. coli* strains (Fig. 5b). Structurally diaponeurosporene has one less conjugated double bond than diapolycopene (Fig. 1). Reconstructed *S. aureus* C30 diapolycopene pathway produced diaponeurosporene as well as diapolycopene in heterologous *E. coli* strain (unpublished data). Its ratio was between 2:1 and 3:1 in *E. coli*. Among the seven recombinant strains, JM109 produced diapolycopene and diaponeurosporene at a ratio of 2:1, and the others produced more diapolycopene than diaponeurosporene at ratios ranging from 4:1 to 6:1. As shown in Fig. 1, diaponeurosporene can be converted into diapolycopene by introducing a conjugated double bond, which is catalyzed by diapophytoene desaturase CrtN. Therefore, more diapolycopene formation suggests that CrtN acts on more diaponeurosporene and introduces one more double bond to generate more diapolycopene under the specific conditions in *E. coli*.

Effect of C30 Cyclic Diapotorulene Formation on Growth and Production Level of *E. coli* Strains

To investigate the C30 monocyclic diapotorulene production levels and cell growth of recombinant *E. coli* strains, seven *E. coli* strains were transformed with pACM-M_{SA}-Ny-Yt (Table 1) and then cultured in LB–glycerol medium. The plasmid pACM-M_{SA}-Ny-Yt expresses wild-type CrtM of *S. aureus*, mutant CrtN of *S. aureus*, and mutant CrtY of *B. linens* simultaneously (unpublished data), and thus, functional assembly of these enzymes can produce monocyclic diapotorulene in recombinant *E. coli* (Fig. 1). Because dicyclic C30 carotenoid diapo-β-carotene has not yet been reported, monocyclic C30 diapotorulene was chosen as an alternative.

Like C30 acyclic carotenoids, XL1-Blue grew relatively slower and reached the lowest cell density (an OD_{600} of 3.2) after 28 h (Fig. 2d). However, even though JM109 produced the most acyclic diapolycopene, the SURE strain produced the highest specific amount of diapotorulene (38.8 μg g^{-1} WCW; Table 2) and volumetric amount (20 μg ml^{-1}; Fig. 6a). Both SURE and JM109 were found to be good producers for cyclic C30 carotenoid.

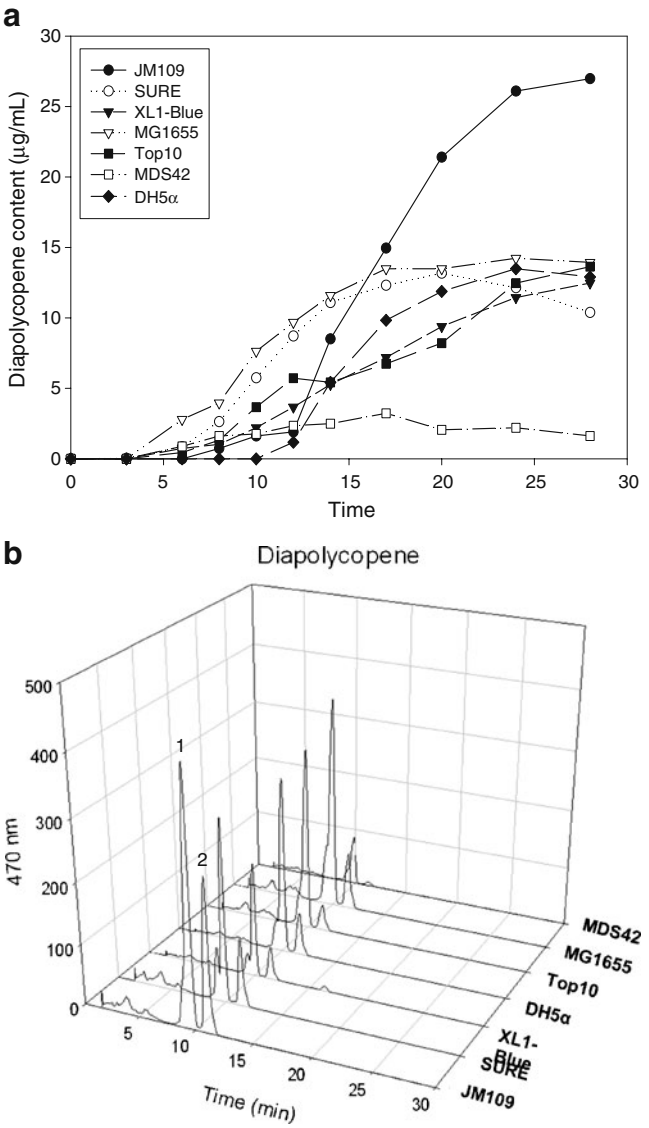

Fig. 5 C30 acyclic diapolycopene/diaponeurosporene production (**a**) and HPLC analysis (**b**) of acetone extracts from recombinant *E. coli* strains harboring pACM-M_{SA}-N_{SA}. *Peak 1* refers to diapolycopene; *peak 2* refers to diaponeurosporene

When the profile of the diapotorulene pathway in the seven *E. coli* strains was investigated, no diapolycopene accumulation in SURE and JM109 was observed (Fig. 6b), indicating that, in the SURE and JM109 strains, the mutant CrtY of *B. linens* has a higher affinity for diaponeurosporene than the mutant CrtNy. Interestingly, diapolycopene accumulation was observed in DH5α, indicating that DH5α did not provide a good environment for the mutant CrtNy.

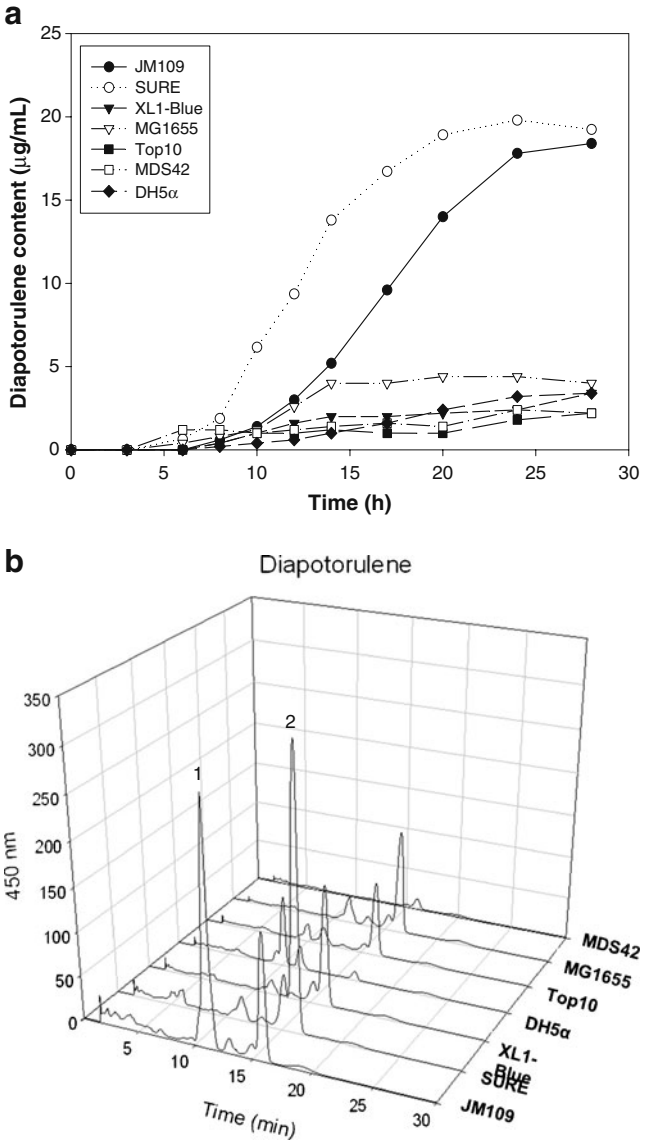

Fig. 6 C30 cyclic diapotorulene production (**a**) and HPLC analysis (**b**) of acetone extracts from recombinant *E. coli* strains harboring pACM-M_{SA}-NyYt. *Peak 1* refers to diaponeurosporene; *peak 2* refers to diapotorulene

In conclusion, we showed that recombinant hosts and carotenoid structures significantly influenced carotenoid productions. Heterologous carotenoid formation was strongly dependent on *E. coli* strains. Among the seven *E. coli* strains used in this study, the SURE strain was found to be the best producer for carotenoids. It is not clear why SURE was the best strain for the production of carotenoids with chemically diverse structures. The difference in carotenoid production was not due to a pathway plasmid

instability (data not shown). Different precursor pools or different cellular environments for functional assembly of enzymes are one probable explanation for this strain dependency. Metabolomic and proteomic study is being carried out to explain this dependency at system level.

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