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Membrane engineering - A novel strategy to enhance the production and accumulation of β-carotene in *Escherichia coli*

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ABSTRACT

Carotenoids are a class of terpenes of commercial interest that exert important biological functions. While various strategies have been applied to engineer β-carotene production in microbial cell factories, no work has been done to study and improve the storage of hydrophobic terpene products inside the heterologous host cells. Although the membrane is thought to be the cell compartment that accumulates hydrophobic terpenes such as β-carotene, direct evidence is still lacking. In this work, we engineered the membrane of *Escherichia coli* in both its morphological and biosynthetic aspects, as a means to study and improve its storage capacity for β-carotene. Engineering the membrane morphology by overexpressing membrane-bending proteins resulted in a 28% increase of β-carotene specific producton value, while engineering the membrane synthesis pathway led to a 43% increase. Moreover, the combination of these two strategies had a synergistic effect, which caused a 2.9-fold increase of β-carotene specific production value (from 6.7 to 19.6 mg/g DCW). Inward membrane stacks were observed in electron microscopy images of the engineered *E. coli* cells, which indicated that morphological changes were associated with the increased β-carotene storage capacity. Finally, membrane separation and analysis confirmed that the increased β-carotene was mainly accumulated within the cell membrane. This membrane engineering strategy was also applied to the β-carotene hyperproducing strain CAR025, which led to a 39% increase of the already high β-carotene specific production value (from 31.8 to 44.2 mg/g DCW in shake flasks), resulting in one of the highest reported specific production values under comparable culture conditions. The membrane engineering strategy developed in this work opens up a new direction for engineering and improving microbial terpene producers. It is quite possible that a wide range of strains used to produce hydrophobic compounds can be further improved using this novel engineering strategy.

1. Introduction

Carotenoids are a class of terpenes of commercial interest that exert important biological functions. β-Carotene functions as an antioxidant and has protective properties against cancer, in addition to stimulating the immune system in humans (Palozza and Krinsky, 1992). It is also the precursor of vitamin A and has many applications in pharmaceuticals, nutraceuticals, cosmetics and foods (Das et al., 2007; Ajikumar et al., 2008). Although 90% of commercially available β-carotene is produced through chemical synthesis, microbial cell factories have drawn increasing interest due to their various advantages (Lee and Schmidt-Dannert, 2002).

Extensive research has been carried out to develop β-carotene producer strains of *Escherichia coli* (Yuan et al., 2006; Yoon et al., 2009; Albermann et al., 2010). For example, the native 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway was overexpressed in various forms to increase the supply of isoprenoid synthesis precursors (Jin and Stephanopoulos, 2007; Choi et al., 2010). In a similar study, a heterologous mevalonate (MVA) pathway was introduced into *E. coli* to increase the isopentenyl pyrophosphate (IPP) supply (Martin et al., 2003; Yoon et al., 2007). At the global metabolic network scale, systematic and combinatorial methods were employed to identify gene knockouts or gene overexpression targets for improving β-carotene production (Alper et al., 2005; Choi et al., 2010). Central metabolic modules were also engineered to increase the supply of ATP and NADPH to improve β-carotene production (Zhao et al., 2013).

However, to our best knowledge, no engineering work has been done to improve the storage of hydrophobic terpene products in the

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heterologous microbial hosts. Unlike fatty acids, polyhydroxybutyrate or lipids, which are accumulated in the cytoplasm, hydrophobic terpene products are thought to accumulate in the membrane compartment of *E. coli* (Ahrazem et al., 2016). Thus, expanding the membrane surface area and increasing the amount of membrane structures might be a workable strategy to increase the accumulation of β-carotene in *E. coli* cells. Overexpression of a number of endogenous integral membrane proteins, i.e. fumarate reductase (Weiner et al., 1984), ATP synthase (Von Meyenburg et al., 1984), ATP synthase b-subunit (Arechaga et al., 2000), mannitol permease MtlA (Van Weeghel et al., 1990), and chemotaxis receptor Tsr (Herskovits et al., 2002), was reported to induce the formation of membrane stacks or tubules in the cytoplasm, which were close to and continuous with the inner (cytoplasmic) membrane. Furthermore, the glycosyltransferase enzyme monoglucosyldiacylglycerol synthase (Almgs), which is responsible for the synthesis of non-bilayer glucolipid-glucosyl-diacylglycerol (GlcDAG), was overexpressed in *E. coli* and induced the widespread formation of intracellular membrane vesicles (Eriksson et al., 2009). From a metabolic engineering aspect, these proteins might be employed as novel tools for the modification of the cell membrane. We therefore proposed to engineer the membrane using these functional proteins to change its morphology, increase its surface area and consequently accumulate greater amounts of hydrophobic terpene products.

On the other hand, membrane synthesis is another aspect that can be modulated and improved. Glycerophospholipids and phosphoglycerides are glycerol-based phospholipids, which are structural components of biological membranes. Therefore, modulating the diacylglycerol-3-phosphate synthesis pathway to increase the amount of membrane building blocks might be a valid strategy to increase the total membrane content, and consequently the accumulation of hydrophobic terpene products (Röttig et al., 2015).

In this work, we aimed to engineer the cell membrane of *E. coli* by introducing membrane bending proteins and enhancing the membrane-synthesis pathway, in order to improve the production and accumulation of β-carotene (Fig. 1).

2. Methods

2.1. Strains, media and culture conditions

Bacterial strains used for DNA manipulation and β-carotene production in this study are listed in Table 1. The β-carotene-producing strains CAR015 and CAR025 were used as the parent strains for membrane engineering. For strain construction, cultures were grown aerobically at 30 °C or 37 °C in Luria broth (per liter: 10 g tryptone, 5 g yeast extract and 10 g NaCl). For β-carotene production, single colonies were picked from LB plates and transferred into 15 mm × 100 mm tubes containing 4 ml of LB with or without 34 mg/L of chloramphenicol, and cultured at 37 °C and 250 rpm overnight. The resulting seed cultures were used to inoculate 100 ml flasks containing 10 ml of fermentation medium to an initial OD_{600} of 0.05, and grown at 30 °C and 250 rpm. For strains bearing the Ptrc promoter, 0.1 mM IPTG was added 3 h after inoculation, which was followed by 45 h of growth. The fermentation medium was LB containing 2% glycerol with or without chloramphenicol. After 48 h of growth, the cells were collected for the measurement of β-carotene production.

Table 1
Strains and plasmids used in this work.

Strains and plasmids	Relative characteristics	Resources
Strains		
CAR005	ATCC 8739, M1–37::*dxs*, M1–46::*idi* M1–93::*Crt*, M1–46::*SucAB*, M1–46::*sdh*, M1–46::*talB*	Zhao et al. (2013)
CAR015	CAR005, ispG-mRSL-4, ispH-mRS-14	Lab collection
CAR025	CAR015, replacing the promoter of *crtEYIB* with Ptrc promoter	Lab collection
CAR015–37Almgs	CAR015, M1–37::*Almgs*	This work
CAR015–46Almgs	CAR015, M1–46::*Almgs*	This work
CAR015–93Almgs	CAR015, M1–93::*Almgs*	This work
CAR025–37Almgs	CAR025, M1–37::*Almgs*	This work
Plasmids		
pACYC184-M	cat; replace *tet* with *lacI* and Ptrc of pTrc99A-M	Zhao et al. (2013)
pCas9	Cas9	Zhao et al. (2016)
pAlmgs	pACYC184-M with Ptrc controlled *almgs*	This work
pMtlA	pACYC184-M with Ptrc controlled *mtla*	This work
pTsr	pACYC184-M with Ptrc controlled *tsr*	This work
pPlsb	pACYC184-M with Ptrc controlled *plsb*	This work
pPlsb-plsc	pACYC184-M with Ptrc controlled *plsb* and *plsc*	This work
pPlsb-plsc-dgka	pACYC184-M with Ptrc controlled *plsb*, *plsc* and *dgka*	This work

2.2. Construction of plasmids and recombinant strains

All plasmids used in this study are listed in Table 1. Plasmids were assembled using the Golden Gate method (Hillson et al., 2012). To construct the plasmids for membrane bending, *almgs* from *Acholeplasma*

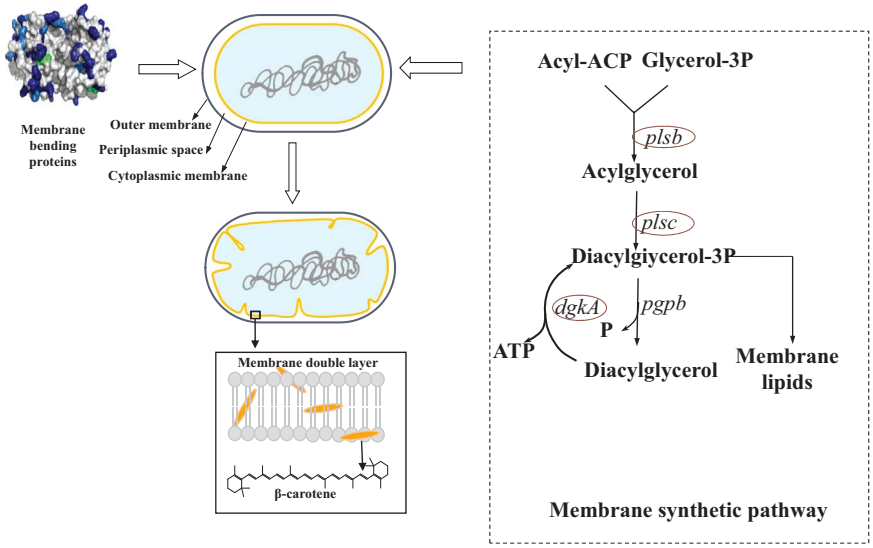

Fig. 1. Membrane engineering strategies for increasing β-carotene production and accumulation. Membrane morphology and the membrane-synthesis pathway were engineered to improve the production and accumulation of β-carotene.