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Enhanced synthesis of Coenzyme Q_{10} by reducing the competitive production of carotenoids in *Rhodobacter sphaeroides*

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ABSTRACT

In *Rhodobacter sphaeroides*, synthesis of coenzyme Q_{10} (CoQ_{10}) shares the same precursor geranylgeranyl diphosphate (GGPP) with carotenoids. Therefore, suppression of carotenoids synthesis is supposed to pose positive effects on accumulation of CoQ_{10}. In this paper, the carotenogenesis pathway was repressed using two different strategies, deletion of carotenogenic genes and overexpression of *ppsR*, a transcriptional regulator for photosynthesis genes, respectively. Knockout of carotenoids synthesis (*crt*) genes resulted in undetectable carotenoids as expected. However, the production of CoQ_{10} and biomass were both decreased to half as compared to the wild-type strain. In contrast, upon overexpression of *ppsR*, the production of carotenoids was decreased from 15.7 mg/L to 2.2 mg/L, and the CoQ_{10} production and content were enhanced by 28% and 34.2%, respectively. To further enhance the production of CoQ_{10}, *crtE* was constitutively co-overexpressed with *ppsR* to improve the supply of GGPP as a key precursor for the isoprenoid side chain of CoQ_{10}. The CoQ_{10} production and content of the resulting strain RspPE were increased to 73.2 mg/L and 5.67 mg/g, respectively, representing 47% and 55% improvement as compared to the wild type. This result demonstrated that appropriate reduction of carotenoids rather than complete blocking could enhance the CoQ_{10} production in *Rhodobacter. sphaeroides*.

1. Introduction

As an oil-soluble quinone with decaprenyl side chain, Coenzyme Q_{10} is an antioxidant, with the capability of scavenging free radicals. CoQ_{10} is also an electron transporter in the electron transport chain of aerobic respiration [1,2]. Moreover, CoQ_{10} is involved in the regulation of several genes, some of which play important roles in cholesterol metabolism or inflammatory responses [3,4]. The benefits of CoQ_{10} supplementation to patients with cardiovascular diseases, hypertension and Parkinson's diseases have been documented [1,5,6]. Besides, CoQ_{10} can also be supplemented in cosmetics for its function of reducing wrinkle formation [7]. Therefore, there is a growing demand from the pharmaceutical and cosmetic industries, leading to increasing research interest into its synthesis.

CoQ_{10} is formed by adding the hydrophobic tail of ten repeated isoprene units to the quinone head, and its synthesis pathway consists of the 2-C-methy-D-erythritol 4-phosphate (MEP) pathway synthesizing the isoprene side chain, the shikimate pathway and the ubiquinone pathway. The flux through MEP pathway is known to be limiting [8,9] and is expected to become rapidly restrictive in a system where the carbon flow is pulled towards CoQ_{10} synthesis [10]. Currently, three kinds of strategies have been adopted to enhance the side chain flux in prokaryotic cells. The first strategy is overexpression of 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which is a known rate-limiting enzyme of MEP pathway [11,12]. The second strategy for increasing flux of isoprenoids is heterologous expression of a typical eukaryotic mevalonate pathway [1,13]. The third strategy is based on addition of exogenous isoprene precursor into the fermentation medium [14]. Considering the significant content of carotenoids in *R. sphaeroides*, the production of which shares the MEP pathway and its downstream steps up to GGPP synthesis as the upper section of synthesis pathway [15], reducing the flux to carotenoids might be an alternative means to divert the GGPP precursor to CoQ_{10} synthesis.

Deletion or downregulation of the competing pathway is a common approach to enhanced flux of the target pathway. For

Abbreviations: *R.sphaeroides*, *Rhodobacter sphaeroides*; CoQ_{10}, Coenzyme Q10; GGPP, geranylgeranyl diphosphate; MEP, 2-C-methy-D-erythritol 4-phosphate; *crt*, carotenoids synthesis; *E. coli*, *Escherichia coli*.

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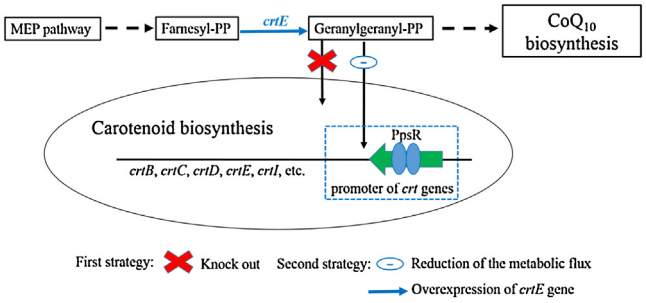

Fig. 1. The biosynthesis pathways of CoQ_{10} and carotenoids and the regulation strategies. First strategy is the deletion of *crtB*, *crtC*, *crtD* and *crtI*. Second strategy is the suppression of *crt* genes by *ppsR* overexpression and overexpression of *crtE* at the same time.

example, the synthesis pathway of oxaloacetic acid (OAA), acetate and ethanol is the competitive pathway for lactic acid synthesis. Knockout of the key genes for OAA, acetate and ethanol synthesis (*ppc*, *ackA* and *adhE*) led to 1.6-fold higher lactic acid production [16]. Considering that most of the enzymes involved in the biosynthesis of carotenoids are encoded by *crt* genes, deletion of the *crt* genes (*crtB*, *crtC*, *crtD* and *crtI*) downstream of GGPP is expected to block the carotenogenesis pathway, thereby diverting the isoprene precursor to CoQ_{10} (Fig. 1).

Aside from gene knockout, down-regulation of the competing pathway is an alternative route to enhance the production of target metabolite, especially when the competing pathway is essential to cell health. For example, repression of squalene synthesis by replacing the ERG20 promoter with a weaker promoter resulted in 49-fold enhancement of isoprene production in *saccharomyces cerevisiae* [17]. Other methods for down-regulating gene expression include SiRNA, σ factor, etc. [18,19]. In *R. sphaeroides*, PpsR is specifically involved in the transcriptional regulation of photosynthesis (PS) genes such as bacteriocholorophyll synthesis (*bch*), carotenoids synthesis (*crt*), pigment-binding proteins of light harvesting complex II (*puc*), and polypeptides of the reaction centers (*puf*). Under aerobic conditions, PpsR represses gene transcription by cooperative binding to two palindromic sites in its target promoter, and its repressive action can be antagonized by the redox-sensitive protein AppA in the dark [20,21]. Overexpression of *ppsR* to enhance the ratio of PpsR/AppA is anticipated to repress the transcription of *crt* genes and thus the competitive synthesis of carotenoids.

In the present study, the supply of isoprene precursor to CoQ_{10} was improved by deletion and PpsR-mediated transcriptional repression of the carotenogenic genes, respectively. Considering the repression of GGPP synthesis catalyzed by *crtE* upon overexpression of *ppsR*, *crtE* was co-overexpressed to ensure GGPP supply in the latter case. The influences of these strategies on carotenoids synthesis, biomass and CoQ_{10} production were comparatively analyzed and discussed.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture media

All the strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 was used for construction and propagation of plasmids. *E. coli* S17-1 was used for diparental conjugation. *R. sphaeroides* 2.4.1 was used for CoQ_{10} production and its genome was used as the template for cloning of *ppsR*, *crtD*, *crtE* and *crtI* genes.

E. coli strains were cultivated at 37 °C in Luria-Bertani medium (LB, tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) supplemented with 50 μg/ml of kanamycin or 100 μg/ml of ampicillin when necessary. For routine cultivation and genome preparation, *R. spaeroides* was cultivated in medium A containing 8 g/L yeast extract, 3 g/L glucose, 2 g/L NaCl, 1.3 g/L KH_2PO_4, 0.125 g/L $MgSO_4$, 15 mg/L biotin, 1 mg/L nicotinic acid, 1 mg/L thiamine hydrochloride and 15 g/L agar, supplemented with 2.5 μg/mL nalidixic acid and 25 μg/mL kanamycin when necessary. For shake-flask cultures, *R. sphaeroides* was cultivated in medium B containing 40 g/L glucose, 6.3 g/L $MgSO_4$, 4 g/L corn steep liquor, 3 g/L sodium glutamate, 3 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 2 g/L $CaCO_3$, 1 mg/L nicotinic acid, 1 mg/L thiamine hydrochloride, 15 μg/L biotin, and 2.5 μg/mL nalidixic acid, supplemented with 25 μg/mL kanamycin or 50 μg/mL ampicillin.

For deletion of *crt* genes, the recombinant cells were cultivated in medium SMM (pH 6.9) containing 20 mL/L CB mother liquor (20 g/L nitrilotriacetic acid, 29.2 g/L $MgSO_4$, 3.4 g/L $CaCl_2$, 18.4 mg $(NH_4)_2Mo_2O_7{\cdot}4H_2O$, 400 mg/L $FeSO_4{\cdot}7H_2O$, 16 g/L KOH), 4 g/L DL-malic acid, 3.5 g/L K_2HPO_4, 0.5 g/L $(NH_4)_2SO_4$, 0.5 g/L NaCl, 2 g/L KOH, 8 μg/L biotin, 0.4 mg/L nicotinic acid, 0.2 mg/L niacin thiamine.

Table 1
Plasmids and strains used in this study.

Plasmids and strains	Description	Reference or source
Plasmids		
pGEX-4T1	P_{tac} expression vector, pBR322 origin, Ampicillin resistance	Amersham Pharmacia Biotech
pTrc99a	Expression vector, ampicillin resistance	Pharmacia
pBBR1MCS2	Cloning vector, kanamycin resistance, Broad-Host-Range Plasmid	[22]
pBMTT	Fragment containing terminator from pTrc99a and *tac* promoter from pGEX-4T1 cloned into pBBR1MCS2	This study
pBMTTPpsR	Fragment containing *ppsR* from *R. sphaeroides* 2.4.1 cloned into pBMTT	This study
pBMTTPE	Fragment containing *ppsR* and *crtE* with RBS cloned into pBMTT	This study
pK18mobsacB	Suicide vector	NCBI accession No. FJ437239.1
pK18caroI	Fragment containing partial *crtI* cloned into pK18mobsacB	This study
pK18sacBΔCaro	Fragment containing partial *crtD* and *crtE* cloned into pKcaroI	This study
Strains		
E. coli JM109	*endA1*, *gyrA96*, *hsdR17*(rk^-mk^+), *recA1*, *relA1*, *supE44*, *thi-1*, del(*lac-proAB*), *e14*$^-$(*McrA*$^-$), F'[*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZdelM15*]	
E. coli S-17	*recA*, harbors the *tra* genes of plasmid RP4 in the chromosome; *proA*, *thi-1*	[23]
R. sphearoides 2.4.1	Wild-type *Rhodobacter sphaeroides*, nalidixic acid resistance	ATCC BAA-808
Rsp261	*R. sphaeroides* 2.4.1 with *crtB, crtC, crtD and crtI* deleted	This study
RspPpsR	*R. sphaeroides* 2.4.1 harboring pBMTTPpsR	This study
RspPE	*R. sphaeroides* 2.4.1 harboring pBMTTPE	This study