Metabolic Engineering 29 (2015) 208–216

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

# Identification and elimination of metabolic bottlenecks in the quinone modification pathway for enhanced coenzyme $Q_{10}$ production in *Rhodobacter sphaeroides*

Wenqiang Lu [a], Lidan Ye [a,b], Xiaomei Lv [a], Wenping Xie [a], Jiali Gu [a], Zhaofeng Chen [c], Yongqiang Zhu [a], Aipeng Li [a], Hongwei Yu [a,*]

[a] Institute of Bioengineering, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China
[b] Key Laboratory of Biomass Chemical Engineering of Ministry of Education, Zhejiang University, Hangzhou 310027, China
[c] Group of Bioengineering, ZheJiang NHU Company Limited, Shaoxing 312521, China

ARTICLE INFO

*Article history:*
Received 8 November 2014
Received in revised form
9 February 2015
Accepted 18 March 2015
Available online 26 March 2015

*Keywords:*
Quinone modification pathway
$CoQ_{10}$
*Rhodobacter sphaeroides*
Fusion expression
Bottleneck removal
Intermediate elimination

ABSTRACT

In this report, UbiE and UbiH in the quinone modification pathway (QMP) were identified in addition to UbiG as bottleneck enzymes in the $CoQ_{10}$ biosynthesis by *Rhodobacter sphaeroides*. The $CoQ_{10}$ content was enhanced after co-overexpression of UbiE and UbiG, however, accompanied by the accumulation of the intermediate 10P-MMBQ. UbiH was then co-overexpressed to pull the metabolic flux towards downstream, resulting in an elevated $CoQ_{10}$ productivity and decreased biomass. On the other hand, the expression levels of UbiE and UbiG were tuned to eliminate the intermediate accumulation, however at the sacrifice of productivity. To alleviate the detrimental effect on either productivity or cell growth, we tried to fuse UbiG with UbiE and localize them onto the membrane to elevate intermediate conversion. By fusing UbiE and UbiG to pufX, $CoQ_{10}$ was accumulated to $108.51 \pm 2.76$ mg/L with a biomass of $12.2 \pm 0.9$ g/L. At last, we combined the optimized QMP and the previously engineered 2-methyl-D-erythritol-4-phosphate pathway (MEP) to further boost $CoQ_{10}$ biosynthesis, resulting in a strain with $138 \pm 2.64$ mg/L $CoQ_{10}$ production.



## 1. Introduction

Coenzyme $Q_{10}$ (2,3-dimethoxy, 5-methyl, 6-decaisoprene para-benzoquinone) is a high-added-value natural compound. Aside from its function as an electron transporter in the electron transport chain of aerobic respiration, it acts as an antioxidant by scavenging the free radicals, which are harmful to the membrane phospholipids and proteins (Cluis et al., 2007). In addition, $CoQ_{10}$ is well-known for its benefits in heart and vascular health, as well as in treating diabetes, Parkinson's disease, Alzheimer's disease and mitochondrial diseases (Beal, 2004; Cluis et al., 2007; Dhanasekaran and Ren, 2005). Hence, $CoQ_{10}$ is in growing demand in the pharmaceutical, food and cosmetic additive industries.

Currently, $CoQ_{10}$ is mainly produced by fermentation of the natural $CoQ_{10}$ producers due to the advantages of the bio-produced $CoQ_{10}$ compared to that of chemical source. Hence, a high-yield producer is essential for the commercial purpose in the fermentation process. In the past decades, enormous efforts have been made to construct high-titer-$CoQ_{10}$ producers by metabolic engineering of the workhorse bacterium *Escherichia coli* (Cheng et al., 2010; Choi et al., 2009; Cluis et al., 2011; Huang et al., 2011; Kim et al., 2006; Koo et al., 2010; Park et al., 2005; Zahiri et al., 2006). However, most were encountered with problems such as low fermentation titers, and emergence of byproducts like $CoQ_8$ and $CoQ_9$ (Cluis et al., 2011; Okada et al., 1998; Park et al., 2005). As an alternative route, taking natural $CoQ_{10}$ producers as the starting host for metabolic engineering could overcome these obstacles (Zhang et al., 2007). And among the $CoQ_{10}$ natural producers, *Rhodobacter sphaeroides* is a promising microbe for fermentative production of $CoQ_{10}$ (Choi et al., 2005). $CoQ_{10}$ is synthesized through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, the shikimate pathway and the quinone modification pathway (QMP) (Fig. 1). In our previous studies, we explored the metabolic regulation of *R. sphaeroides* and successfully improved the $CoQ_{10}$ production through engineering of the MEP pathway (Lu et al., 2013, 2014). In this study, we focused our efforts on systematic engineering of QMP in *R. sphaeroides* for breeding higher-titer $CoQ_{10}$ producing strain. Since the removal of the bottleneck nodes in the pathway is an efficient strategy for production improvement, as an essential preparative step, the bottleneck enzymes in the pathway should be identified before initiating metabolic engineering. To the best of our knowledge, information about

* Corresponding author. Fax: +86 571 87951873.
*E-mail address:* yuhongwei@zju.edu.cn (H. Yu).

http://dx.doi.org/10.1016/j.ymben.2015.03.012
1096-7176/

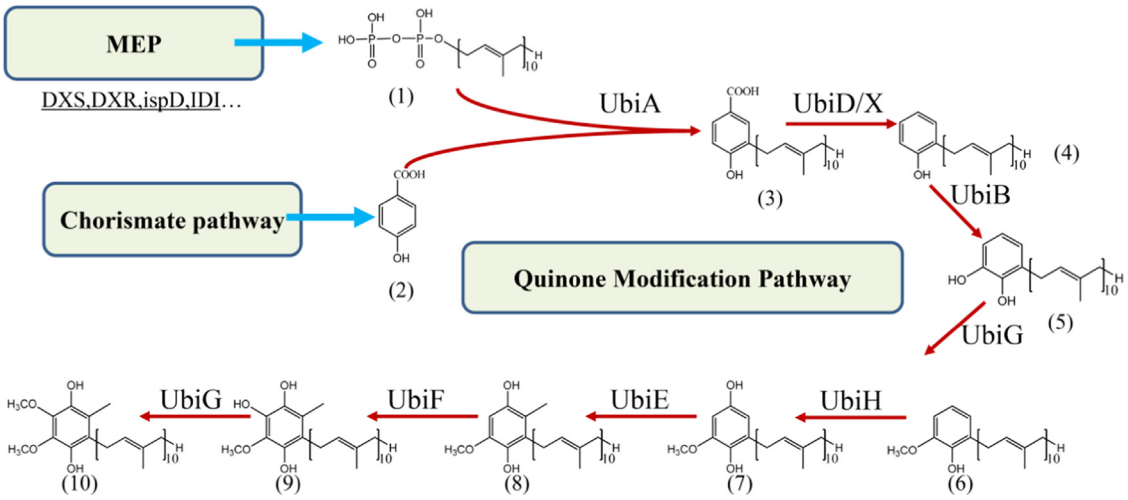


**Fig. 1.** The *R. sphaeroides* quinone modification pathway in coenzyme $Q_{10}$ biosynthesis. MEP: 2-C-methyl-D-erythritol 4-phosphate pathway; (1) decaprenyl diphosphate; (2) para-hydroxybenzoic acid; (3) 3-decaprenyl-4-hydroxybenzoate; (4) 2-decaprenylphenol; (5) 2-decaprenyl-6-hydroxyphenol; (6) 2-decaprenyl-6-methoxyphenol; (7) 2-decaprenyl-6-methoxy-1,4-benzoquinol; (8) 2-decaprenyl-3-methyl-6-methoxy-1,4-benzoquinol; (9) 2-decaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol; (10) coenzyme $Q_{10}$.

the enzymes in the pathway aside from the gene sequences in this host is limited (Lu et al., 2013) and remains to be further explored.

During metabolic engineering towards enhanced pathway flux, the newly emerged bottleneck nodes resulting from overexpression of upstream enzymes often lead to accumulation of intermediates, which can cause waste of carbon source and/or toxicity to the host (Anthony et al., 2009; Lee et al., 2013; Lu et al., 2014; Martin et al., 2003). Two strategies have been proposed to circumvent this problem: enhancing the metabolic flux downstream of the bottleneck node to improve the intermediate conversion, or attenuating the metabolic flux upstream of the bottleneck node to balance the intermediate supply. In terms of the first strategy, the common workflow is to first identify the rate-limiting enzymes and then elevate their expression levels accordingly (Cluis et al., 2011; Wang et al., 2011). As for the second strategy, many methods have been developed, such as tuning the strength of promoters, ribosomal binding site sequences (RBS), or terminators (Ajikumar et al., 2010; Zelcbuch et al., 2013), and constructing gene clusters in the form of monocistron or polycistron (Pitera et al., 2007; Wang et al., 2011). However, we need to be careful with both strategies. The first has the risk of excessive expanding the pathway flux, leading to exhaust of the carbon source which results in reduced cell growth, while the second is prone to optimize cell growth but often with a compromise in the pathway flux. An alternative strategy proposed recently is fused expression of the rate-limiting enzymes (Conrado et al., 2012; Dueber et al., 2009; Wang et al., 2011). Fusing multi-enzymes to form a chimeric multifunctional enzyme can construct a substrate channel and prevent the intermediates from diffusing into the cytoplasm, thus increasing the utility of the intermediate.

In the present study, we first screened for the candidates of rate-limiting enzymes in the quinone modification pathway by overexpression of individual Ubi enzymes at different levels. Based on these results, we engineered the pathway by co-expression of the bottleneck enzymes to improve the $CoQ_{10}$ production. Subsequently, the three above-mentioned strategies were investigated to eliminate the metabolic bottleneck by balancing the metabolic flux or improving the catalytic efficiency in the pathway for further production enhancement. At last, the unimpeded QMP was combined with the previously engineered MEP employing the self-regulation strategy (Lu et al., 2014) to further boost $CoQ_{10}$ synthesis in *R. sphaeroides*.

## 2. Materials and methods

### 2.1. Bacterial strains and culture media

*E. coli* JM109 was used for plasmid construction and propagation. *E. coli* S17-1 was used for di-parental conjugation. *E. coli* BL21 (DE3) was used as the template for cloning the *UbiF* gene. *R. sphaeroides* 2.4.1 was used for $CoQ_{10}$ production and its genome was used as the template for cloning of other *Ubi* 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 genome preparation and routine cultivation, *R. sphaeroides* was cultivated in medium A, which contains 2 g/L NaCl, 3 g/L glucose, 8 g/L yeast extract, 1.3 g/L $KH_2PO_4$, 0.125 g/L $MgSO_4$, 1 mg/L thiamine hydrochloride, 15 μg/L biotin, 1 mg/L nicotinic acid and 15 g/L agar, supplemented with 25 μg/mL kanamycin and 2.5 μg/mL nalidixic acid when necessary. For shake-flask cultivation, *R. sphaeroides* was cultivated in a rich medium B, which contains 40 g/L glucose, 4 g/L corn steep liquor, 3 g/L sodium glutamate, 2.8 g/L NaCl, 3 g/L $(NH4)_2SO_4$, 3 g/L $KH_2PO_4$, 6.3 g/L $MgSO_4$, 2 g/L $CaCO_3$, 1 mg/L thiamine hydrochloride, 15 μg/L biotin, 1 mg/L nicotinic acid, and 25 μg/mL kanamycin and 2.5 μg/mL nalidixic acid.

### 2.2. Construction of plasmids and recombinants

General DNA manipulation was conducted according to standard protocols. T4 ligase was purchased from Thermo Inc. (ThermoScientific, USA). PrimerStar DNA polymerase with high GC buffer used in polymerase chain reaction (PCR) and restriction endonucleases were purchased from Takara (Takara, China). Primer sequences are listed in Table S1. Plasmids were constructed as described in the Supplementary materials and the structures of the plasmids are shown in Fig. 2. All the plasmids used are kanamycin resistant.

### 2.3. Di-parental conjugation

Conjugation mating was carried out as described in a previous report (Lu et al., 2014). The donor was *E. coli* S-17 transformed with the target plasmid and the receptor was *R. sphaeroides* 2.4.1. The two strains were mixed at a donor/receptor ratio of 1:10 for conjugation and the positive colonies were screened on LB agar