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Short communication

Site-directed mutagenesis of UbiA to promote menaquinone biosynthesis in *Elizabethkingia meningoseptica*

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ABSTRACT

To increase the menaquinone (MK) content of an *Elizabethkingia meningoseptica,* site-directed mutagenesis was generated to suppress 4-hydroxybenzoate octaprenyl transferase (UbiA) activity and subsequently blocked the ubiquinone (UQ) biosynthesis pathway. Fourteen conserved residues except L174 and G211 were mutated to analyze the effect of site-directed mutagenesis. The expression of UbiA in twelve mutants was decreased in both mRNA and protein levels, which resulted in the decrease of UQ concentration. Based on MenA expression level, 12 mutants were divided into two groups. Second group such as N72A, D76A, K81A, L139A, and D198A enhanced the expression of MenA, which increased MK production by 127.1%, 87.9%, 96.2%, 109.7% and 130.0% in wt-EmUbiA, respectively. In general, blocking UQ synthesis pathway for by site-directed mutagenesis of the active site of UbiA in *E. meningoseptica* was a promising strategy to increase MK production in *E. meningoseptica*.

1. Introduction

As an indispensable fat-soluble vitamin in human metabolism, menaquinone (MK) has important functions in promoting blood coagulation [1,2], reducing bone loss and bone fractures [3,4], rescuing mitochondrial dysfunction and in alleviating Parkinson's disease [2,5,6]. These physiological functions have been discovered in recent years and published in major journals, indicating that MK has promising application prospects and potential economic value as a nutritional supplement for humans in food and the pharmaceutical industry. *Elizabethkingia meningoseptica* is thought to be a potentially suitable strain for industrial applications because of its high productivity. However, although high-producing strains, such as *E. meningoseptica*, were studied extensively from around 1980 to 1995 [7–10] the molecular technology to exploit this productivity was not then realized. A wider opportunity for strain improvement can now involve the use of modern molecular methods based on metabolic engineering, which has not been attempted so far.

MK is composed of a naphthoquinone head group and an isoprenoid side chain (Fig. 1a). The isoprenoid side chain is synthesized from dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) through the deoxyxylulose 5-phosphate (DXP) pathway in *E. meningoseptica* [11,12]. While the head group 1, 4-dihydroxy-2-naphthoate (DHNA) is derived from chorismate through the shikimate pathway. Seven consecutive enzymes are required for the formation of DHNA, and six men genes (*menF*, *menD*, *menH*, *menC*, *menE* and *menB*) are involved [13]. Ubiquinone (UQ), a natural compound in the plasma membrane of prokaryotes, has a similar molecular structure and metabolic pathway to MK (Fig. 1b and c). They compete for the isoprenoid side chain with the same synthetic pathway in *E. meningoseptica*. The difference is that DHNA prenyltransferase (MenA) is used to combine the isoprenoid side chain and DHNA in MK synthesis [14], while in UQ synthesis 4-hydroxybenzoate (PHB) prenyltransferase (UbiA) is used to combine the isoprenoid side chain and PHB [15,16]. Therefore, MenA and UbiA compete for the isoprenoid side chain for the biosynthesis of MK and UQ. In other words, it is beneficial

Abbreviations: SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic acid; DHNA, 1,4-dihydroxy-2-naphthoic acid; DMK, demethylmenaquinone; MK, menaquinone; MenF, isochorismate synthase; MenD, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase; MenH, (1R 6R)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid synthase; MenC, *o*-succinyl benzoate synthase; MenE, *o*-succinyl benzoate-CoA synthase; MenB, 1,4-dihydroxy-2-naphthoyl-CoA synthase; MenA, DHNA-octaprenyltransferase; UbiC, chorismate lyase; UbiA, 4-hydroxybenzoate octaprenyl transferase; UbiE, 2-DMK methyltransferase

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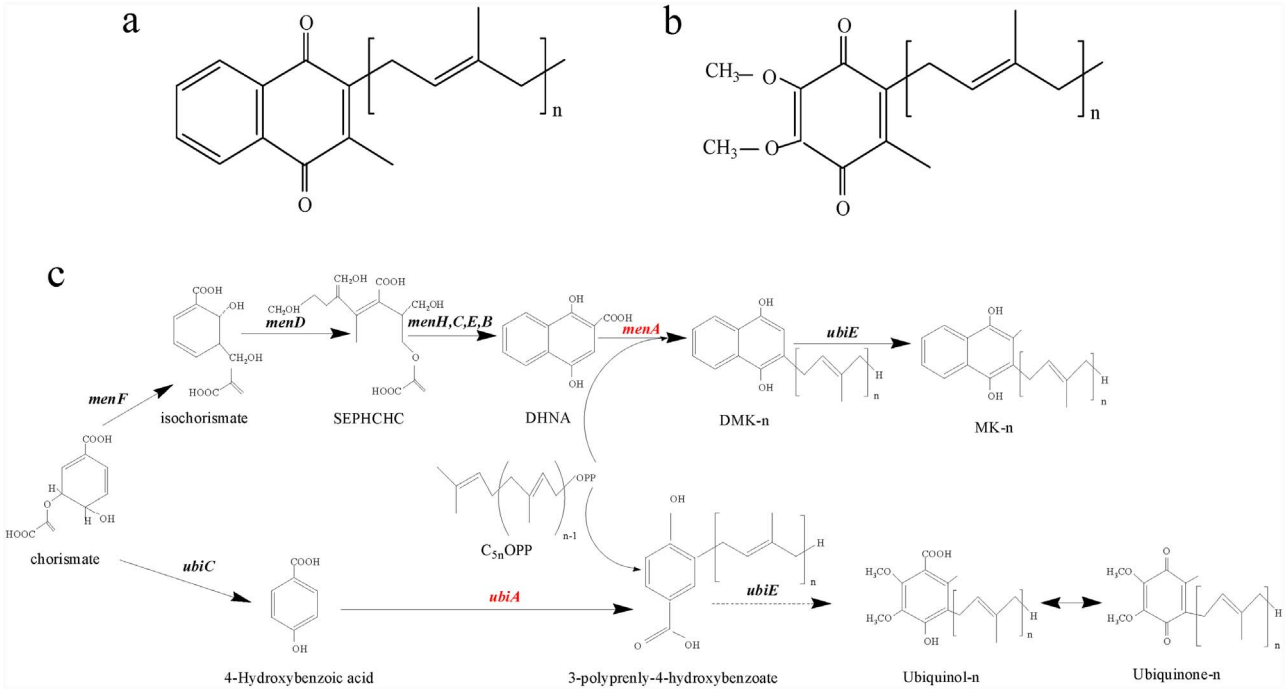

Fig. 1. The structure of menaquinone (MK) (a), Ubiquinone (UQ) (b) and their biosynthetic pathways in *E. meningoseptica* (c).

for MenA synthesis if the combined ability of UbiA with DMAPP and IPP is decreased. A previous study has showed that the over-expression of MenA lead to a fivefold increase of MK content in *Escherichia coli* [17]. Moreover, UQ content increased to 181% by deleting the *menA* gene, thereby blocking the pathway for the synthesis of MK [15]. Thus, the down-regulation of UbiA might have a similar significance in improving the yield of MK.

In recent years, there have been many methods available to regulate the catalytic activity of enzymes by protein engineering. Generally, they can be classified into three categories: rational design, semirational approach and irrational design (directed evolution). Site-directed mutagenesis, one of the most versatile methods in the rational design of protein engineering because of its high mutation rates, can achieve a systematic and detailed investigation of the targeted mutation and mutations can be induced. It has been widely applied in the modification of many industrial enzymes such as pullulanase [18], feruloyl esterase [19] and metagenomic GH11 endoxylanase [20]. In addition, as a necessary condition for site-directed mutagenesis, the structure and catalytic mechanisms of UbiA from *Escherichia coli* (EcUbiA) [21], *Aeropyrum pernix* (ApUbiA) [22] and *Archaeoglobus fulgidus* (AfUbiA) [23] have fortunately been revealed in recent years. The structures reveal UbiA contains nine transmembrane helices (TM1-9) and an extramembrane cap domain (HL23, HL45, and HL67) that surrounds a large central cavity containing the active site. Especially, two conserved Asp-rich motifs (D54***D58, D182***D186 in ApUbiA; D72***D76, D198***D202 in AfUbiA; D71***D75, D191***D195 in EcUbiA) played important roles in the acitivity of UbiA. The disclosure of these unique active sites provides a useful reference for the site-directed mutagenesis of an industrial MK strain.

In this study, for the first time, we blocked the UQ pathway by down-regulating *ubiA* to enhance the MK content in *E. meningoseptica*. Residues in the conserved region and adjacent site that might affect the activity of *E. meningoseptica* UbiA (EmUbiA) were identified based on the sequence alignment of EcUbiA, ApUbiA and AfUbiA and analysis of the ApUbiA structural model. These selected residues were mutated to investigate the influence on the mRNA expression and protein levels of UbiA, the biomass, MK production, and fermentation kinetic parameters in order to improve MK production for biotechnological applications.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *ubiA* fragment was linked to the plasmid pBBR1MCS-2 [24] by XhoI and BamHI restriction enzymes to generate the pBBR1MCS-2/*ubiA*. The recombinant plasmid (pBBR1MCS-2/*ubiA*) was used to introduce DNA manipulations, mutations, sequencing, and expression of the *ubiA* gene. *E. coli* DH5α was the host for cloning work, and *E. meningoseptica* FM2-6 [25] *ubiA* knocking out strain was the host for the expression of UbiA.

2.2. Site-directed mutagenesis

The mutants were constructed by site-directed mutagenesis. The PCR reaction was conducted using the PrimeSTAR HS DNA polymerase (Takara, Japan) and the pBBR1MCS-2-*ubiA* plasmid as the template DNA. Primers were shown in Supplementary Table 1. The PCR product was digested by DpnI (Takara, Japan) at 37 °C for 1 h. The PCR product was transformed into competent cells of *E. coli* DH5α. After the sequence verified, the extracted plasmid was transformed into *ubiA*$^-$ FM2-6 *E. meningoseptica* strain for enzyme expression and MK synthesis.

2.3. Real-time quantitative PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A 2-step real-time PCR was performed using cDNA prepared from RNA using a Superscript III First-Strand cDNA Synthesis Kit (Invitrogen) and a SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7900 instrument following the manufacturer's instructions. The thermal cycling conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, then 60 °C for 1 min. The fold change in expression levels (using GAPDH as internal control) was determined by a comparative Ct method using Equation $2^{-\Delta\Delta Ct}$, where Ct was the threshold cycle of amplification. The sequences of PCR primers were shown in