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BIOTECHNOLOGY and BIOENGINEERING

Metabolic Engineering of Menaquinone-8 Pathway of *Escherichia coli* as a Microbial Platform for Vitamin K Production

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ABSTRACT: Menaquinone-8 (MK-8, vitamin K) is composed of a non-polar side chain and a polar head group. *Escherichia coli* was chosen and metabolically engineered as a microbial platform for production of MK-8. MK-8 content in *E. coli* was significantly enhanced by modulating two precursor pools, which supply a non-polar side chain and a polar head group, and further increased by blocking formation of the competitor ubiquinone-8 (Q-8). Overexpression of *E. coli* IspA, DXR, or IDI increased MK-8 content up to twofold. A similar positive effect was also observed when *E. coli* MenA, MenB, MenC, MenD, MenE, MenF, or UbiE was overexpressed. The Q-8-deficient *ubiCA* mutant enhanced MK-8 content by 30% compared to wild-type *E. coli*. When MenA or MenD was overexpressed, MK-8 content was enhanced fivefold compared with wild-type *E. coli*.

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KEYWORDS: menaquinone-8; ubiquinone-8; *E. coli*; metabolic engineering; vitamin K

Quinones are one of key components of the respiratory electron transfer chains in both prokaryotes and eukaryotes. These lipid components, containing isoprenoid side chains of various lengths, can be divided into benzoquinones and naphthoquinones, representing two major structural groups (Rothery et al., 1998). A facultative anaerobic bacterium, *Escherichia coli*, synthesizes mainly a benzoquinone-type ubiquinone-8 (Q-8) under aerobic conditions but mainly produces a naphthoquinone-type menaquinone-8 (MK-8) under anaerobic conditions (Lin and Kuritzkes, 1987; Meganathan, 1996).

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As shown in Figure 1, both Q-8 and MK-8 pathways use an isoprenoid C40 octaprenyl diphosphate (OPP) for the non-polar side chain and a chorismate for the polar head group. Q-8 is synthesized by sequential reactions catalyzed by UbiC (chorismate lyase), UbiA (4-hydroxybenzoate octaprenyltransferase), and UbiE (Q-8/MK-8 biosynthesis methyltransferase) (Olson and Rudney, 1983). MK-8 is synthesized by the sequential activity of seven pathway enzymes: MenA (1,4-dihydroxy-2-naphthoate octaprenyltransferase), MenB (naphthoate synthase), MenC (*o*-succinylbenzoate-CoA synthase), MenD (2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase/2-oxoglutarate decarboxylase), MenE (*o*-succinylbenzoic acid-CoA ligase), MenF (menaquinone-specific isochorismate synthase), and UbiE (Q-8/MK-8 biosynthesis methyltransferase). The UbiE is required for both Q-8 and MK-8 biosynthesis (Wissenbach et al., 1992).

The clinical functions of the K vitamins, including phylloquinone and menaquinone, include bone protective activities such as reducing bone loss and bone fractures in humans (Cheung et al., 2008), reducing brain diseases such as Alzheimer's Disease (Allison, 2001), antioxidant activity (Li et al., 2003), and they are a key factor in blood coagulation (Lurie et al., 2010). Therefore, the K vitamins are receiving increasing interest as nutritional supplements for humans. However, commercial production of vitamin K is mainly based on extraction or chemical synthesis. Even though fermentative production of K vitamins, such as a menaquinone-producing wild-type or mutant *Flavobacterium* sp. (Tani and Taguchi, 1989), *Bacillus subtilis*, and *Propionibacterium freudenreichii* (Furuichi et al., 2006; Sato et al., 2001) have been reported, there is no systemic metabolic engineering model for developing a microbial producer of vitamin K. Therefore, *E. coli* was selected and evaluated as a microbial platform for production of vitamin K.

In this study, endogenous MK-8, Q-8, and precursor isoprenoid pathways were metabolically engineered in a synthetic approach to construct an efficient *E. coli* platform

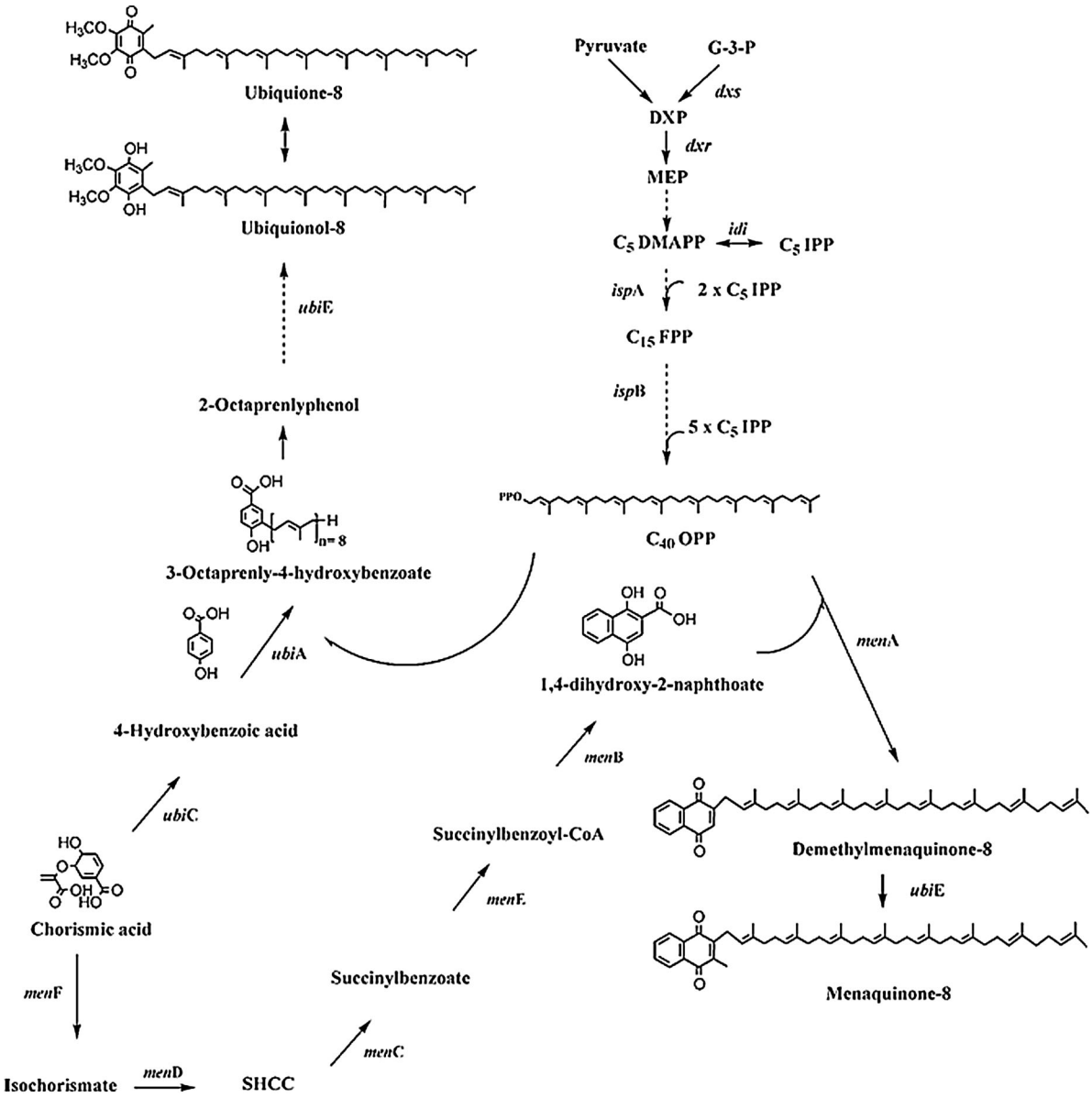

Figure 1. MK-8 and Q-8 biosynthetic pathways in *E. coli*. Genes encoding the pathway enzymes are *men*A, 1,4-dihydroxy-2-naphthoate octaprenyltransferase; *men*D, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCC) synthase/2-oxoglutarate decarboxylase; *men*F, menaquinone-specific isochorismate synthase; *men*B, naphthoate synthase; *men*C, *o*-succinylbenzoate-CoA synthase; *men*E, *o*-succinylbenzoic acid-CoA ligase; *ubi*E, ubiquinone/menaquinone biosynthesis methlytransferase; *isp*A, geranyl-transtransferase; *isp*B, octaprenyl-diphosphate synthase; *dxr*, 1-deoxy-D-xylulose 5-phosphate synthase; *ubi*C, chorismate lyase; *ubi*A, 4-hydroxybenzoate octaprenyltransferase. Q-8 pathway enzymes such as UbiD, G, H, X, and F are not shown in this figure.

to synthesize vitamin K. Furthermore, the *ubiCA* gene was knocked out to block the precursor flux toward unwanted formation of Q-8. Formation of MK-8 was evaluated in metabolically engineered *ΔubiCA* mutant cells.

To select an *E. coli* host for MK-8 production, six *E. coli* strains (JM109, Top10, DH5α, MG1655, SURE, and MDS42), which had been tested for carotenoid production (Chae et al., 2010), were microanaerobically grown in LGN media at 30°C, and then MK-8 and by-product Q-8 were quantified. The highest content of 55–57 μg MK-8/g-WCW was observed in JM109 and SURE, which was 2–3 times more MK-8 than found in the other strains (Fig. S1). It is known that MK-8 and Q-8 contents in *E. coli* vary considerably due to culture conditions and growth stages (Lin and Kuritzkes, 1987). In the metabolic pathway of *E. coli*, MK-8 and Q-8 share the same isoprenoid precursor, C5 IPP (Lee et al., 2003), which is supplied by the non-mevalonate pathway in *E. coli* (Lee and Schmidt, 2002). Therefore, we speculate that the isoprenoid precursor flux between *E. coli* strains may provide some additional explanation of quinone formation. This hypothesis is currently under study using experiments based on GC-

mass spectrometry and proteomics. JM109 was chosen for more engineering of the MK-8 pathway in the following experiments.

As shown in Figure 1, the polar head structure of MK-8 is derived from the chorismate precursor pathway and the non-polar tail structure is derived from the isoprenoid pathway. It is unknown which pathway exerts more influence on MK-8 formation in *E. coli*. Therefore, both precursor pathways were targeted for engineering to investigate the effect of the precursor pathway on MK-8 formation. All head structure biosynthesis enzymes (UBiE, MenA, MenB, MenC, MenD, MenE, and MenF) and selected tail structure biosynthesis enzymes (IspA, DXR, and IDI) were constitutively overexpressed in JM109. IspA, DXR, and IDI are known to be key enzymes in isoprenoid precursor pathways (Lee et al., 2004).

Ten recombinant *E. coli* cells were grown to give an OD_{600} of 1.5–2.3 in 28 h, which were comparable to control JM109 containing an empty plasmid pUCM (an OD_{600} of 2.0) (Fig. 2A). Overexpression of all seven head structure biosynthesis enzymes positively influenced MK-8 formation compared to control JM109. Among the seven enzymes, overexpression of MenA and MenD induced a twofold increase in MK-8 content (~150 μg MK-8/g WCW) over the control JM109 (~60 μg MK-8/g WCW), whereas Q-8 formation was not significantly enhanced in any of the 10 recombinant *E. coli* strains (~30 μg Q-8/g WCW) (Fig. 2B). This significant effect of MenA and MenD is reasonable since MenD is involved in the early precursor biosynthesis step, while MenA combines the head structure (1,4-dihydroxy-2-naphthoate) and tail structure (OPP) into an intermediate dimethylmenaquinone (DMK-8) (Fig. 1). Therefore, MenA and MenD may be the key enzymes in MK-8 formation in *E. coli*. Interestingly, DMK-8 also significantly increased in *E. coli* cells expressing MenA, MenD, or UbiE. The enhanced formation of DMK-8 by UbiE overexpression was unexpected because overexpression of UbiE rapidly converts DMK-8 into MK-8; consequently, the cellular level of DMK-8 would be expected to be less than that in wild-type JM109. Therefore, the accumulation of DMK-8 in *E. coli* cells expressing UbiE seems to be more complexly regulated.

Similar to the positive effect of MenA and MenD, overexpression of the tail-structure biosynthesis enzymes IspA, DXR, and IDI increased MK-8 content up to 150–180 μg MK-8/g WCW. It seems that IspA, DXR, and IDI enhanced the cellular level of OPP, which is rapidly transformed into DMK-8 by MenA. This is supported by the enhanced content of DMK-8 found in *E. coli* cells expressing IspA, DXR, and IDI.

We found that overexpression of MenA, MenD, IspA, DXR, or IDI significantly increased MK-8 content. To increase the MK-8 content further, we focused on balancing the biosynthesis of the head and tail structures. Therefore, the tail structure biosynthetic enzymes IspA, DXR, or IDI were co-expressed with the head structure biosynthetic enzyme, MenD. MenA was excluded from this experiment

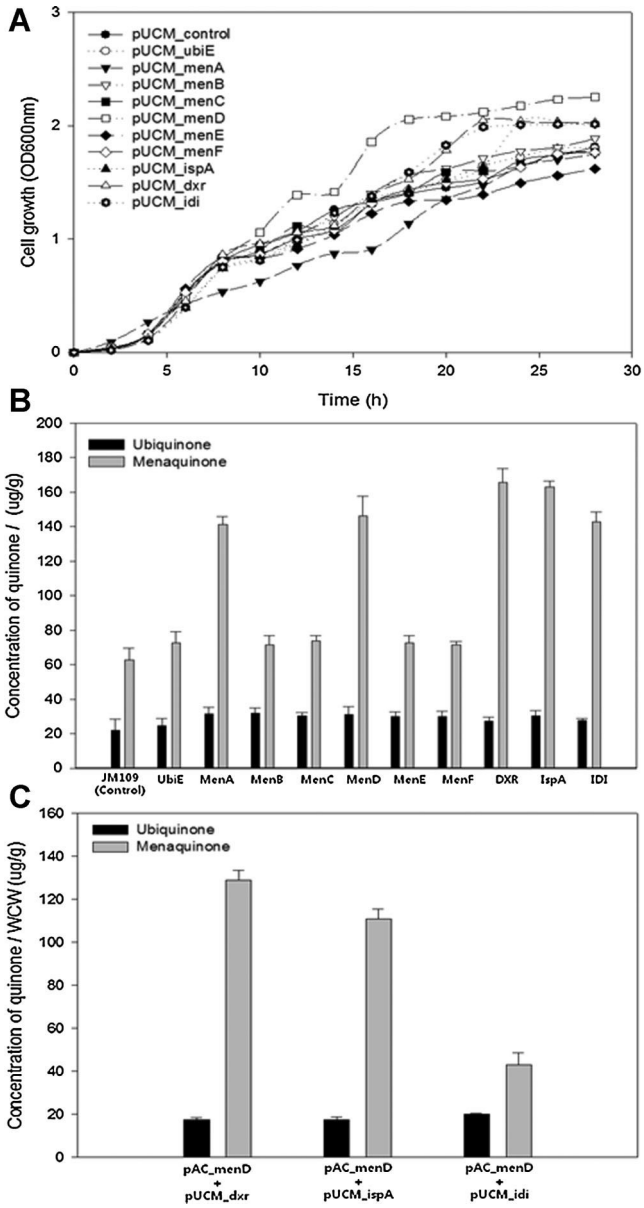

Figure 2. Effect of overexpression of head or/and tail-pathway enzymes on MK-8 and Q-8 formation in recombinant *E. coli* JM109. **A**: Growth of recombinant *E. coli* cell expressing UbiE, MenB, MenC, MenD, MenE, MenF, MenA, IspA, DXR, or IDI. **B**: MK-8 and Q-8 contents in recombinant JM109 cell expressing UbiE, MenB, MenC, MenD, MenE, MenF, MenA, IspA, DXR, or IDI. **C**: MK-8 and Q-8 contents in recombinant JM109 cell co-expressing MenD/DXR, MenD/IspA, or MenD/IDI.

because MenA is not involved in the head structure-formation step. As shown in Figure 2C, the MK-8 content (110–130 μg MK-8/g WCW) in *E. coli* co-overexpressing MenD/IspA or MenD/DXR were slightly less than that in *E. coli* overexpressing MenD alone (~150 μg MK-8/g WCW). This low MK-8 content was mainly due to the accumulation of DMK-8, indicating that considerable amounts of DMK-8 were not transformed into MK-8 in *E. coli* co-overexpressing MenD/IspA or MenD/DXR. Unexpectedly, the MK-8 content (~50 μg MK-8/g WCW)

in *E. coli* co-overexpressing MenD/IDI was similar to that in control JM109 having two empty vectors, pUCM and pACYC184 (~60 μg MK-8/g WCW). These results demonstrate that balancing the biosynthesis of the head and tail structures of MK-8 is proven an effective engineering approach for MK-8 and DMK-8 production.

Next, we focused on blocking Q-8 biosynthesis to increase the MK-8 content, since Q-8 biosynthesis competes with MK-8 biosynthesis from the same precursor isoprenoid, OPP (Fig. 1). The *ubiCA* genes encoding UbiC and UbiA, were targeted because they involve the first committed reaction for making the head structure of Q-8. The *ΔubiCA* mutant was generated by the Red/ET recombination system and deletion of *ubiCA* genes was verified by PCR of gDNA of *ΔubiCA* mutant and wild-type JM109 (Fig. S1).

The *ΔubiCA* mutant was transformed with pUCM-xxx series, expressing 10 genes (Table I), and then microanaerobically cultured to investigate the effect of overexpression of pathway enzymes. The recombinant *Δubi*CA mutants grew similarly to the recombinant JM109 having control plasmid pUCM, except for longer lag phases (Fig. 3A). This suggests that there was no significant growth inhibition caused by *ubiCA* deletion in microanarobic cultures. The *ubi*CA deletion mutant enhanced MK-8 content by 30% compared to that in wild-type (80 μg vs. 60 μg MK-8/g WCW) and suppressed Q-8 formation (Fig. 3B), indicating that the flux was redirected toward MK-8 formation. As expected, when MenA or MenD was overexpressed in *ΔubiCA* mutant, a dramatic increase in MK-8 content were observed. *ΔubiCA* mutant overexpressing MenA increased MK-8 content up to fivefold (290 μg vs. 60 μg MK-8/g WCW) compared to wild-type and up to twofold (290 μg vs. 150 μg MK-8/g WCW) compared to *E. coli* expressing MenA only (Fig. 3B). Overexpression of other enzymes (UbiE, MenB, MenC, MenE, and MenF) did not increase the MK-8 content significantly. Interestingly, accumulation of DMK-8 was significantly suppressed in *ΔubiCA* mutant overexpressing MenA or MenD. Another unexpected result was that only DMK-8 accumulated, without MK-8 formation, in the *ΔubiCA* mutant overexpressing DXR, IspA, or IDI. There is no clear explanation for this result because little information on regulation of MK-8 and Q-8 pathways exists. However, it is clear that regulation of the MK-8 and Q-8 pathways is quite complex or may be affected by expression of their enzymes or their intermediates. Additional insight might be provided through a systems-level approach such as proteomics or metabolomics.

In conclusion, modulating the expression levels of the OPP and 1,4-dihydroxy-2-naphthoate precursor pathways and blocking the competitor Q-8 pathway, is an important

Table I. *E. coli* strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or Refs.
E. coli		
JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14- [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17($r_K^- m_K^+$)	NEB
ΔubiCA	JM109 ubiC, ubiA deletion mutant	This study
Top10	F$^-$ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$	Invitrogen
DH5α	F$^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ$^-$	Invitrogen
MG1655	F$^-$ λ$^-$ ilvG- rfb-50 rph-1	Lee et al. (2009)
MDS43	A chromosome that is 14.3% smaller than that of its parental *E. coli* strain MG1655	Lee et al. (2009)
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]	Agilent Technologies
Plasmid		
pUCM	Cloning vector modified from pUC19, constitutive *lac* promoter, Ap^r	Kim et al. (2010)
pUCM-menA	pUCM constitutively expressing *men*A	This study
pUCM-menB	pUCM constitutively expressing *men*B	This study
pUCM-menC	pUCM constitutively expressing *men*C	This study
pUCM-menD	pUCM constitutively expressing *men*D	This study
pUCM-menE	pUCM constitutively expressing *men*E	This study
pUCM-menF	pUCM constitutively expressing *men*F	This study
pUCM-ubiE	pUCM constitutively expressing *ubi*E	This study
pUCM-DXR	pUCM constitutively expressing *dxr*	This study
pUCM-idi	pUCM constitutively expressing *idi*	This study
pUCM-ispA	pUCM constitutively expressing *isp*A	This study
pACYC184	Cloning vector, Cm^r	NEB
pAC-menB	pACYC184 constitutively expressing *men*B	This study
pAC-menC	pACYC184 constitutively expressing *men*C	This study
pAC-menD	pACYC184 constitutively expressing *men*D	This study
pAC-menE	pACYC184 constitutively expressing *men*E	This study
pAC_menF	pACYC184 constitutively expressing *men*F	This study
pAC-ubiE	pACYC184 constitutively expressing *men*E	This study

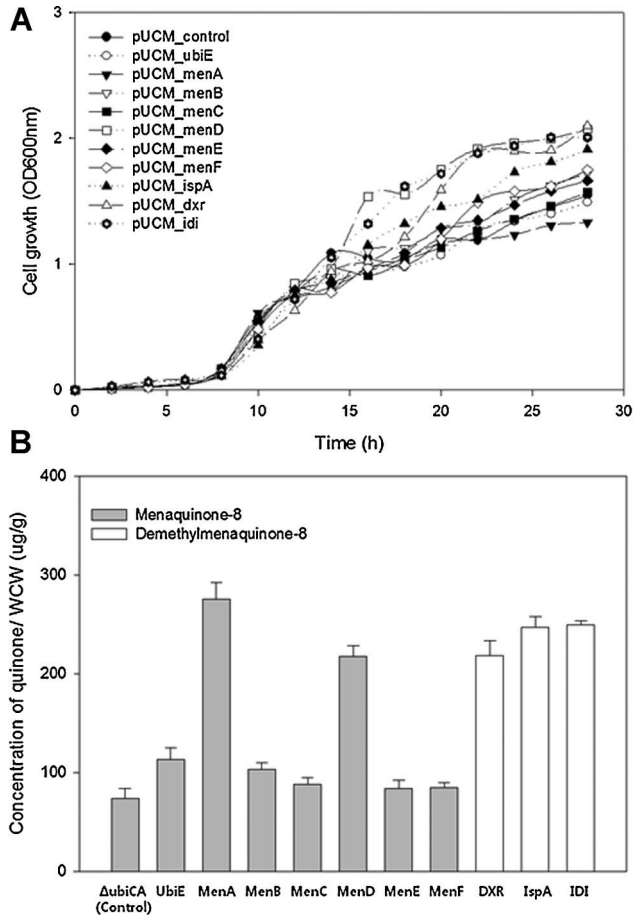

Figure 3. Effect of overexpression of head or tail-pathway enzymes on MK-8 and Q-8 formation in the Δ*ubi*CA mutant. **A**: Growth of recombinant Δ*ubi*CA cell expressing UbiE, MenB, MenC, MenD, MenE, MenF, MenA, IspA, DXR, or IDI. **B**: MK-8 and Q-8 contents in recombinant Δ*ubi*CA cell expressing UbiE, MenB, MenC, MenD, MenE, MenF, MenA, IspA, DXR, or IDI.

factor to ensure the effective channeling of the quinone flux to MK-8 biosynthesis.

Materials and Methods

Bacterial strains and plasmids used in this study are shown in Table I. *E. coli* JM109 was used for general cloning experiments and as a genomic DNA source. The genes encoding MenA, MenB, MenC, MenD, MenE, MenF, UbiE, DXR, IDI, or IspA were amplified from isolated genomic DNA of JM109 using the PCR primers listed in Table SI. The PCR products were purified, digested with restriction enzymes, and cloned into the *Xba*I and *Eco*RI sites or the *Xba*I and *Not*I sites of pUCM, a constitutive expression plasmid (Kim et al., 2010), resulting in a pUCM-xxx series (where xxx stands for a gene in Table I). To co-express pathway enzymes, each expression module, composed of promoter, structural gene, and terminator, was amplified from the corresponding pUC-xxx plasmid with gene-specific PCR primers, treated with *Bam*HI or *Hin*dIII, and then subcloned into the *Bam*HI or *Hin*dIII sites of a pACYC184 plasmid, resulting in expression vectors pAC-xxx series (where xxx stands for a gene in Table I). All DNA manipulations were carried out following standard procedures. For cloning and maintenance of plasmids, *E. coli* was aerobically grown in Luria–Bertani (LB) medium at 37°C for 16 h. For production of quinones, *E. coli* was microanaerobically grown on a table rotating at 100 rpm at 30°C for 26–28 h, in 300-mL flasks containing 200 mL Luria–Bertani–glycerol–sodium nitrate (LGN) medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 5 g/L glycerol, and 10 g/L sodium nitrate). Under anaerobic conditions, *E. coli* use nitrate (NO_3) and glycerol as an electron acceptor and a carbon source, respectively (Soballe and Poole, 1998). For aerobic cultures, *E. coli* were grown in 300-mL baffled flasks containing 100 mL LGN medium with vigorous shaking at 250 rpm. Ampicillin (100 mg/L), kanamycin (30 mg/L), chloramphenicol (50 mg/L), tetracycline (10 mg/L), or combinations thereof were added to the medium to provide selection pressure, if necessary.

Deletion of *ubi*CA in JM109 was carried out using the Red/ET recombination system (Gene Bridges GmbH, Heidelberg, Germany). Deletion of *ubi*CA in the genome was verified by PCR of genomic DNA isolated from mutants using *ubi*CA-specific primers (Table SI).

Wild-type JM109 and mutant Δ*ubi*CA were used as hosts for the production of Q-8 and MK-8. Pelleted cells were extracted with 20 mL chloroform/methanol (2:1, v/v) three times (Hu et al., 1999). The bottom layer was collected, washed with distilled water, dried under a gentle stream of nitrogen, and then dissolved in 1 mL of acetone. For the analysis of quinone compounds, 50 μL of the crude extract was applied to a C18 reverse-phase column (particle size 5 μm, 4.6 mm × 150 mm, Agilent, Santa Clara, CA), and eluted under isocratic conditions with methanol/isopropanol (7:3, v/v) at a flow rate of 1 mL/min using an Agilent 1200 HPLC system equipped with an photodiode array detector. The amounts of MK-8 and Q-8 were quantified by comparing the peak areas with those of concentrations of a standard vitamin K2 and Q-10 (Lee et al., 2008), respectively. Vitamin K2 and Q-10 were purchased from Sigma–Aldrich (St. Louis, MO). Six vitamin K2 solutions (0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/mL) and six Q-10 solutions (0.031, 0.062, 0.125, 0.25, 0.5, and 1.0 mg/mL) were prepared and used as standards. UPLC-mass spectrometry system (Quattro-Micro, Waters, Milford, MA) equipped with an atmospheric pressure chemical ionization (APCI) interface was used for structural identification of quinone compounds. The condition used was described in Supplementary Data. Cell growth was monitored by measuring the absorbance at 600 nm with a spectrophotometer (SPECTRAmaxPlus384, Molecular Devices, Sunnyvale, CA). Wet cell weight (WCW) was determined by weighing cell pellets after washing with distilled water three times.

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