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Production of CoQ_{10} in fission yeast by expression of genes responsible for CoQ_{10} biosynthesis.

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Abbreviations: CoQ, coenzyme Q; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; Dps1, decaprenyl diphosphate synthase; Dlp1, D-less polyprenyl diphosphate synthase; DMQ, demethoxyubiquinone; DPP, decaprenyl diphosphate; E4P, erythrose 4-phosphate; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; PEP, phosphoenolpyruvate; PHB, *p*-hydroxybenzoate

Abstract

Coenzyme Q_{10} (CoQ_{10}) is essential for energy production and has become a popular supplement in recent years. In this study, CoQ_{10} productivity was improved in the fission yeast *Schizosaccharomyces pombe*. Ten CoQ biosynthetic genes were cloned and overexpressed in *S. pombe*. Strains expressing individual CoQ biosynthetic genes did not produce higher than a 10% increase in CoQ_{10} production. In addition, simultaneous expression of all ten *coq* genes did not result in yield improvements. Genes responsible for the biosynthesis of *p*-hydroxybenzoate and decaprenyl diphosphate, both of which are CoQ biosynthesis precursors, were also overexpressed. CoQ_{10} production was increased by overexpression of *Eco_ubiC* (encoding chorismate lyase), Eco_aroF^{FBR} (encoding 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase) or *Sce_thmgr1* (encoding truncated HMG-CoA reductase). Furthermore, simultaneous expression of these precursor genes resulted in 2-fold increases in CoQ_{10} production.

Introduction

Coenzyme Q (CoQ), also referred to as ubiquinone, is a component of the electron transport chain that participates in aerobic cellular respiration within eukaryotic mitochondria and, as such, is essential for ATP-dependent energy production. CoQ consists of a hydrophobic isoprenoid side chain and a quinone ring, and delivers electrons through the conversion of quinol (reduced form) to quinone (oxidized form). CoQ acts as a fat-soluble antioxidant by this oxidation-reduction reaction, which contributes to the removal of lipid peroxidation. CoQ also plays additional roles distinct from the electron transfer system and antioxidant activities. For example, CoQ acts as an electron donor during disulfide bond formation in *Escherichia coli*[1], and CoQ reduction is coupled to sulfide oxidation in *Schizosaccharomyces pombe* and other organisms[2]. CoQ is also required for *de novo* synthesis of UMP in eukaryotes[3, 4].

Living organisms possess different species of CoQ, and these are classified according to the length of the isoprenoid side chain. For example, human and *S. pombe* produce CoQ with ten isoprene units (CoQ_{10}), *E. coli* produces CoQ_8, and *Saccharomyces cerevisiae* produces CoQ_6. Side chain length is defined by polyprenyl diphosphate synthases[5, 6]. The biosynthetic pathway for the complete conversion of *p*-hydroxybenzoate (PHB) to CoQ consists of at least nine steps and eight enzymes in yeast (Fig. 1) [7, 8]. These steps include the condensation of IPP (isopentenyl diphosphate) and transfer of the isoprenoid side chain to PHB followed by hydroxylation, methylation, and decarboxylation steps. In eukaryotes, this pathway has been most comprehensively studied to date in *S. cerevisiae* and *S. pombe*. At least nine genes (*COQ1–COQ9*) in *S. cerevisiae* and ten genes (*dps1, dlp1, ppt1, coq3–coq9*) in *S. pombe* are required for CoQ biosynthesis (Fig. 1) [8]; however, the functions of the *COQ4* and *COQ9* genes have not yet been resolved.

CoQ_{10} has attracted attention in recent years as a medicine or health-promoting additive to foods and cosmetics, and the demand for CoQ_{10} has risen accordingly.

Several approaches have been used to improve the fermentative production of CoQ_{10}[9], which have relied predominantly on bacterial and yeast mutants selected for their high CoQ_{10} content[10]. Moreover, genetic engineering approaches to increase CoQ_{10} production were reported in *E. coli* (22.5 mg/l, 0.29 mg/g-dry cell weight (DCW))[11, 12], *Agrobacterium tumefaciens* (548.2 mg/l, 6.92 mg/g-DCW)[13], and *Rhodobacter sphaeroides* (93.3 mg/l, 7.16 mg/g-DCW)[14], but use of these technologies was limited in yeasts.

In this study, we aimed to improve the productivity of CoQ_{10} in *S. pombe* by expression of a panel of biosynthetic genes. Since *S. pombe* naturally produces CoQ_{10}, it is easier to manipulate gene expression to produce CoQ_{10} than other popular organisms such as *S. cerevisiae* and *E. coli*, which only produce CoQ_6 and CoQ_8, respectively. Availability of gene manipulation tools in *S. pombe* is also beneficial for using this yeast for CoQ_{10} production.

Materials and Methods

Strains, media, and genetic manipulation

The *S. pombe* strain CHP429 (*h⁻, ade6-M216, leu1-32, ura4-D18, his7-366*)[15] was used in this study. Standard yeast culture media and genetic manipulations were performed as described previously[16]. *S. pombe* strains were grown in complete YES medium (0.5% yeast extract, 3% glucose, 225 mg/l adenine, 225 mg/l leucine, 225 mg/l uracil, 225 mg/l histidine, and 225 mg/l lysine hydrochloride) or PM medium (0.3% potassium hydrogen phthalate, 0.56% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts)[17]. The appropriate auxotrophic or antibiotic supplements were added as necessary (75 mg/l adenine, 75 mg/l leucine, 75 mg/l uracil, 75 mg/l histidine, 75 mg/l lysine, and/or 0.025 μg/ml Aureobasidin A). *Escherichia coli* DH5α was used as a host strain for all plasmid manipulations and was grown in LB medium (1% bactotryptone, 0.5% yeast extract, and 1% NaCl;

pH 7.0). Standard molecular biology protocols were followed[18]. Restriction enzymes were used according to the suppliers' recommendations (TOYOBO Co. Ltd, Takara Bio Inc., and NEB Ltd.).

Plasmid construction

The plasmids constructed in this study are shown in Figure 2. The primers used for plasmid construction are listed in Table 1. pREP1-dps1-dlp1 was constructed by inserting the *dlp1* expression cassette (*nmt1* promoter - *dlp1* gene - *nmt1* terminator), which had been amplified using the nmt1-pro/PstI and nmt1-term/PstI primers from pREP1-dlp1[19], into the *Pst*I site of pREP1-dps1[19]. pREP1-HIS7-ppt1-coq5 was constructed by inserting the *coq5* expression cassette (*nmt1* promoter - *coq5* gene - *nmt1* terminator), which had been amplified using the nmt1-pro/PstI and nmt1-term/PstI primers from pREP1-coq5[19], into the *Pst*I site of pREP1-HIS7-ppt1. pREP1-HIS7-ppt1 was constructed by inserting the *ppt1* gene into the *Sal*I and *Sma*I sites of pREP1-HIS7, which was constructed by removing *LEU2* by *Hin*dIII digestion of pREP1 and inserting *his7* into the *Sac*I site. pREP2-coq3-coq6 was constructed by inserting the *coq6* expression cassette (*nmt1* promoter - *coq6* gene - *nmt1* terminator), which had been amplified from pREP1-coq6 using the nmt1-pro/PstI and nmt1-term/PstI primers[19], into the *Pst*I site of pREP2-coq3. pREP2-coq3 was constructed by inserting the *coq3* gene into the *Sal*I and *Sma*I sites of pREP2. pREP1-AUR1-coq4-coq8 was constructed by inserting the *coq8* expression cassette (*nmt1* promoter - *coq8* gene - *nmt1* terminator), which had been amplified from pREP1-coq8 using the nmt1-pro/PstI and nmt1-term/PstI primers[19], into the *PstI* site of pREP1-AUR1-coq4. pREP1-AUR1-coq4 was constructed by inserting the *coq4* gene into the *Bam*HI and *Sma*I sites of pREP1-AUR1, which was constructed by removing *LEU2* by *Hin*dIII digestion of pREP1 and inserting *aur1*[20] into the *Sac*I site. pREP1-KAN-coq7-coq9 was constructed by inserting the *coq9* expression cassette (*nmt1* promoter - *coq9* gene - *nmt1* terminator), which had been

amplified from pREP1-coq9 using the nmt1pro-n-SphI and nmt1term-c-SphI[19], into the *Sph*I site of pREP1-KAN-coq7. pREP1-KAN-coq7 was constructed by inserting the *coq7* gene[21] into the *Sal*I and *Sma*I sites of pREP1-KAN, which was constructed by removing *LEU2* by *Hin*dIII digestion of pREP1 and inserting kan^r into the *Sac*I site.

Each gene located upstream of CoQ biosynthesis was amplified using primers containing restriction sites, digested with restriction endonucleases, and then cloned into the appropriate sites of the desired pREP1, pREP2, or pREP1-HIS7 vector. A single coding mutation (Pro148Leu) was introduced into *E. coli* 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase gene, by overlapping PCR, to make the Eco_aroF^{FBR} gene[22]. In the first step, two separate PCRs were used to generate the primary PCR products, designated PCR 1a and PCR 1b. Both primary PCR products contained the desired mutation as well as overlap regions that were attached to the end of the sequence. PCR 1a was performed with primers Eco_aroF-SalI_Fw and Eco_aroF_C443T-Rv, and PCR 1b was performed with primers Eco_aroF_C443T-Fw and Eco_aroF-BamHI_Rv. These primary PCR products were overlapped in the second PCR. Overlapping PCR was also used to introduce double mutations (Asn66Lys, Ile152 Met) into mevalonate kinase from *S. cerevisiae* to make the Sce_mvk^{FBR} gene[23]. Primary PCR was performed with primers Sce_mvk-SalI_Fw and Sce_mvk_T198G-Rv, Sce_mvk_T198G-Fw and Sce_mvk_T456G-Rv, and Sce_mvk_T456G-Fw and Sce_mvk-BamHI_Rv. These primary PCR products were overlapped in the second round of PCR.

Extraction and analysis of CoQ from S. pombe

CoQ was extracted from *S. pombe* as described previously[24]. Briefly, crude lipid extracts were analyzed by normal phase thin layer chromatography using authentic CoQ_{10} (as the standard) and benzene on a Kieselgel 60 F_{254} plate. Following UV visualization, the band containing CoQ_{10} was collected from the plate and extracted

with chloroform/methanol (1:1 v/v). The samples were dried and resolved in ethanol. Purified CoQ was analyzed further by high-performance liquid chromatography (HPLC), with ethanol as the solvent.

Results

CoQ_{10} productivity of strains overexpressing individual CoQ biosynthetic genes

Of the various yeast species that produce CoQ_{10}, *S. pombe* was the best choice for genetic engineering because of the wealth of molecular tools available and the growing body of knowledge regarding *S. pombe* CoQ biosynthesis[19]. First, CoQ biosynthetic genes were individually overexpressed to determine their impact on CoQ production. Individual genes (*dps1*, *dlp1*, *ppt1*, and *coq3-coq9*) were cloned into the pREP1 vector for expression under a strong promoter (*nmt1*). The production of CoQ_{10} was determined after cell growth at 30°C for 48 hrs in PM minimal medium containing 2μM thiamine. Thiamine was added because overexpression of most of the *coq* genes under the *nmt1* promoter was thought to cause growth inhibition (Supplementary Fig. 1). The production of CoQ_{10} was evaluated per 100 ml culture volume and per 10^9 cells (Fig. 3). A slight increase in CoQ_{10} production was seen when *dps1-dlp1* or *coq3* were overexpressed, but this was minimal. In most cases the overexpression of individual genes did not increase CoQ_{10} production, with production lowered in some instances.

CoQ_{10} productivity in strains co-expressing CoQ biosynthetic genes

Next, the effect of simultaneous enhancement of multiple CoQ biosynthetic genes on CoQ productivity was assessed. Plasmids were constructed that expressed two genes on the same plasmid (Fig. 2A). The *dps*1 and *dlp1* genes were expressed on the plasmid pREP1-dps1-dlp1, *ppt1* and *coq5* on the plasmid pREP1-HIS7-ppt1-coq5, *coq3* and *coq6* on the plasmid pREP2-coq3-coq6, *coq4* and

185 *coq8* on the plasmid pREP1-AUR1-coq4-coq8, and *coq7* and *coq9* on the plasmid
186 pREP1-KAN-coq7-coq9. We initially attempted to house all these plasmids within
187 the same yeast strain, but strain maintenance proved difficult. Strains were therefore
188 transformed with PCR DNA fragments for chromosomal integration. Primers
189 PUC119L-C and ars1L-N (Table1) were used to amplify DNA fragments consisting
190 of the expression cassette and selectable marker. The amplified fragments were
191 introduced into the *S. pombe* genome by non-homologous end joining (NHEJ), and
192 transformants were screened for auxotrophic complementation and resistance to
193 antibiotics. Production of CoQ_{10} in these transformed strains was assessed in YES
194 medium, because many of strains co-expressing CoQ biosynthetic genes reduced
195 growth rate when minimal medium was used. The CoQ_{10} productivity of the wild
196 type strain did not change significantly in YES medium (0.5-0.6 μg/ml, 0.4-0.5
197 mg/g-DCW) and minimal medium (0.6-0.7 μg/ml, 0.4-0.5 mg/g-DCW). In most
198 cases, no significant increases in production were observed when two genes were
199 coexpressed; however, CoQ_{10} production per cell (but not per volume or DCW)
200 increased 2-fold, compared with wild type, in strains expressing *ppt1* and *coq5* (Fig. 4).
201 We next coexpressed four genes simultaneously and obtained 2-fold increases in CoQ_{10}
202 production per cell (but not per volume or DCW) when *ppt1, coq3*, *coq5,* and *coq6* were
203 simultaneously expressed. These increases are mainly due to changes of the cell mass
204 since we did not see significant increases of CoQ_{10} production per DCW. No production
205 increases were seen for other gene combinations. Six, eight, and ten genes were also
206 coexpressed, but no significant increases were seen in CoQ_{10} production.
207
208
209 *Manipulation of genes upstream of CoQ biosynthesis*
210 The overexpression of CoQ biosynthesis genes was only minimally successful in
211 enhancing CoQ production. Therefore, we next attempted to improve CoQ_{10}
212 productivity by increasing the supply of the CoQ_{10} precursors PHB and decaprenyl

diphosphate (DPP). Nine different biosynthetic genes from the shikimate and mevalonate pathways were overexpressed in *S. pombe* (Fig. 5). The genes encoded the following proteins: truncated HMG-CoA reductase from *S. cerevisiae* that had no inhibitory regulation in the mevalonate pathway (*Sce_thmgr1*)[25], feedback-inhibition-resistant mevalonate kinase from *S. cerevisiae* (*Sce_mvk*^{FBR})[23], phosphomevalonate kinase from *S. pombe* (*Spo_erg8*, SPAC343.01c), diphosphomevalonate decarboxylase from *S. pombe* (*Spo_mvd1*, SPAC24C9.03), feedback-inhibition-resistant DAHP synthase from *E. coli* (*Eco_aroF*^{FBR})[22], chorismate synthase from *S. pombe* (*Spo_aro2,* SPCC1223.14), chorismate mutase from *S. pombe* (*Spo_aro7*, SPAC16E8.04c), phosphoenolpyruvate synthase from *E. coli* (*Eco_ppsA*), and chorismate lyase from *E. coli* (*Eco_ubiC*). Plasmids were constructed by inserting the appropriate PCR amplicon into pREP1. Plasmids were introduced into wild type *S. pombe* and CoQ_{10} production was assessed. As shown in Figure 6, overexpression of *Eco_ubiC* or *Sce_thmgr1* gene resulted in a CoQ_{10} productivity increase of approximately 30%. Overexpression of the *Eco_aroF*^{FBR} gene increased productivity by approximately 15%. By contrast, when the *Spo_aro7* gene was overexpressed, a significant decrease in CoQ_{10} productivity was observed as a result of the accompanying reduced growth rate. These results indicated that, in some cases, overexpression of genes upstream of the CoQ_{10} pathway led to CoQ_{10} productivity improvements in *S. pombe*.

Simultaneous overexpression of genes involved in dual pathways

Three of the upstream genes that produced improvements in CoQ_{10} productivity were coexpressed and CoQ_{10} productivity was evaluated. *S. pombe* CHP429 was transformed with plasmids pREP1-Sce_thmgr1 and pREP2-Eco_aroF^{FBR}, pREP1-Sce_thmgr1 and pREP2-Eco_ubiC, pREP1-Eco_aroF^{FBR} and pREP2-Eco_ubiC, or pREP1-Sce_thmgr1, pREP2-Eco_aroF^{FBR}, and pREP1-HIS7-Ec_ubiC. CoQ_{10} production was assessed (Fig. 7) and a 2-fold increase

241 was observed in the strains harboring pREP1-Sce_thmgr1 and pREP2-Eco_aroF^{FBR},
242 and pREP1-Sce_thmgr1 and pREP2-Eco_ubiC. No further gain in productivity was
243 observed when all three plasmids (pREP1-Sce_thmgr1, pREP2-Eco_aroF^{FBR,} and
244 pREP1-HIS7-Ec_ubiC) were coexpressed. In this study, the coexpression of *S.*
245 *cerevisiae thmgr1* and *E. coli ubiC* was the most effective combination for
246 increasing CoQ_{10} productivity.
247
248
249 **Discussion**
250 In this study, we aimed to increase CoQ_{10} production in *S. pombe* by
251 overexpression of various CoQ_{10} biosynthetic genes. *S. pombe* is a widely used
252 research eukaryote alongside *S. cerevisiae*. It produces CoQ_{10} naturally, and much is
253 already understood regarding its CoQ_{10} biosynthesis[2, 19, 24, 26]; thus it was a
254 promising organism to use with this approach.
255 Industrial-scale bio-production of CoQ_{10} by microorganisms has been established
256 by several companies. Microorganisms that produce CoQ_{10}, including photosynthetic
257 bacteria and yeasts, were selected for its high yield and used for the fermentation
258 production. Until now, successful approaches for the production of CoQ_{10} have
259 relied predominantly on bacterial or yeast mutants selected for their high CoQ_{10}
260 content. However, in recent years, an application of genetic engineering to produce
261 CoQ_{10} was attempted in *E. coli*[11] and the production of CoQ_{10} reached to 22.5 mg/l.
262 In this case, decaprenyl diphosphate synthase from *Gluconobacter suboxydans* needs
263 to be expressed. On the other hand, *A. tumefaciens* and *R. sphaeroides* have been
264 used to produce CoQ_{10} and reached to 548.2 mg/l and 93.3 mg/l productivity, by
265 engineering the MEP pathway[13, 14]. These successful results in bacteria stimulated
266 our approaches to improve CoQ_{10} productivity in *S. pombe* by genetic engineering.
267 Overexpression of the genes directly involved in CoQ_{10} biosynthesis (*dps1-dlp1*,
268 *ppt1*, or *coq3-coq9*) led to only minimal increases in CoQ_{10} productivity in *S. pombe*.

One possible reason for this failure was that overexpression of the *coq* genes caused severe growth inhibition (Supplementary Fig. 1). High expression of proteins localized to the mitochondria tends to inhibit cell growth, and this might then undermine any beneficial effect of overexpression. Alternatively, individual proteins in the CoQ_{10} biosynthetic pathway might not be rate-limiting, and therefore expression of one or several genes may not be sufficient to produce an overall increase in productivity. Similarly, CoQ biosynthetic enzymes in *S. pombe* may cooperate within a large complex, as is the case in *S. cerevisiae*[27]. We therefore simultaneously enhanced the expression of multiple CoQ biosynthetic genes; however, even when the ten known CoQ biosynthetic genes were simultaneously overexpressed, CoQ_{10} productivity remained largely unimproved.

Some strains showed 2-fold increases in CoQ_{10} production per cell when *ppt1* and *coq5,* or *ppt1*, *coq3*, *coq5,* and *coq6,* were simultaneously expressed, but the changes were not observed in CoQ_{10} production per volume or per DCW. These cultures included many elongated cells (data not shown), and we therefore considered that the higher CoQ production per cell might have been due to the decrease in cell numbers resulting from delayed cell division. Such an effect might be caused by insertion of the transforming DNA into chromosomal sequences responsible for cell division.

A 3.7-fold improvement in CoQ_{10} production was previously achieved by expression of the *ppt1* gene in *S. pombe*[28]; however, we were unable to repeat this result in this study and severe growth inhibition was observed when *ppt1* was overexpressed. Differences in the CoQ productivity of the control strains between the two studies may explain this discrepancy. CoQ productivity in control strains was constant regardless of the passage of time in the previous experiment[28], but in our study CoQ expression increased over time. These differences in control CoQ production would explain the calculated difference in the production of CoQ_{10} in cells expressing *ppt1*.

We next overexpressed genes encoding CoQ_{10} biosynthetic precursors (*Sce_thmgr1*,

297 *Eco_aroF*^{FBR}, and *Eco_ubiC*). Individual overexpression of these genes led to
298 improved production of CoQ, and simultaneous coexpression yielded further gains.
299 These genes were previously reported to improve CoQ productivity in *E. coli*
300 (*Eco_aroF*^{FBR} and *Eco_ubiC*) [29, 30] and *S. pombe* (HMG-CoA reductase from *S.*
301 *pombe*) [31]. *Eco_aroF*^{FBR} and *Eco_ubiC* were previously examined only in *E. coli*,
302 and this is the first report of their effectiveness in fission yeast.
303 Overexpression of the HMG-CoA reductase gene from *S. pombe* prompted a
304 2.7-fold increase in CoQ production in a previous study[31]. A truncated HMG-CoA
305 reductase from *S. cerevisiae* was used in the present study. The difference in CoQ
306 productivity between the studies might therefore be explained by the differences in
307 the gene used, host organism, and/or culture conditions.
308 The results presented in our study indicate that the overproduction of precursors to
309 the CoQ biosynthetic pathway is an effective strategy for improving CoQ
310 productivity. As the effective genes *Sce_thmgr1, Eco_aroF*^{FBR}, and *Eco_ubiC* were
311 from organisms other than *S. pombe*, we speculate that tight regulation was lost in *S.*
312 *pombe* upon transformation with these sequences and that this led to the observed
313 increases in CoQ_{10} production.
314 In conclusion, overexpression of *dps1-dlp1* or *coq3* in *S. pombe* resulted in a slight
315 (but not significant) increase, in CoQ_{10} production. Overexpression of all ten *coq*
316 genes did not produce an increase in CoQ_{10} productivity; however, overproduction
317 of upstream biosynthetic precursors through the expression of genes such as
318 *Sce_thmgr1, Eco_aroF*^{FBR}, and *Eco_ubiC* improved CoQ_{10} production in *S. pombe*
319 2-fold.
320
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Figure legends

Figure 1. Biosynthetic pathway of coenzyme Q (CoQ) in *S. pombe*.

The pathway of CoQ biosynthesis in *S. pombe* is shown. *S. cerevisiae* protein names are shown in parantheses. At least ten genes (*dps1, dlp1, ppt1,* and *coq3-9*) are involved in coenzyme Q_{10} (CoQ_{10}) biosynthesis in *S. pombe*. Dps1 and Dlp1 form a heterotetramer and condense FPP (farnesyl diphosphate) with IPP (isopentenyl diphosphate) into DPP (decaprenyl diphosphate). Ppt1 is a *p*-hydroxybenzoate (PHB)-decaprenyl diphosphate transferase that catalyzes the condensation of PHB and DPP. Other enzymes catalyze modification of the quinone structure. DMQ_{10}, demethoxyubiquinone10.

Figure 2. Construction of expression plasmids

(A) To simultaneously overexpress multiple coenzyme Q (CoQ) biosynthetic genes, five kinds of plasmids that expressed two genes on the same plasmid were constructed. (B) Genes encoding CoQ biosynthesis precursors were inserted into the pREP1, pREP2 or pREP1-HIS7 vector under the control of the *nmt1* promoter.

Figure 3. Effect of coenzyme Q (CoQ*)* biosynthetic gene overexpression on coenzyme Q_{10} (CoQ_{10}) production.

CoQ_{10} productivity in strains expressing a single CoQ biosynthetic gene or both *dps1* and *dlp1* was compared to reference strain CHP429 (empty vector). Strains were grown at 30°C in PM minimal medium containing adenine, uracil, histidine and thiamine. Cultures were initiated at 1×10^5 cells/ml and harvested after 48 h growth. Production of CoQ_{10} was then measured by high-performance liquid chromatography (HPLC). Gray bars represent CoQ_{10} content with respect to volume (μg/ml) and open diamonds represent CoQ_{10} contents with respect to cell count (μg/1x10^9 cells). Data are represented as the mean ± SD of three measurements.

Figure 4. Effect of coexpression of multiple coenzyme Q (CoQ*)* genes on coenzyme Q_{10} (CoQ_{10}) production

CoQ_{10} productivity of strains simultaneous expressing CoQ biosynthetic genes was compared to a reference strain (CHP429). Strains were grown at 30°C in YES complete medium. Cultures were initiated at 1×10^5 cells/ml and harvested after 48h growth. Production of CoQ_{10} was then measured by HPLC. Gray bars represent CoQ_{10} content with respect to volume (%) and open diamonds represent CoQ_{10} contents with respect to cells (%). The reference strain is represented as the mean ± SD of seven measurements and the data from four individual transformants are shown for other strains.

Figure 5. Shikimate and mevalonate pathways upstream of coenzyme Q (CoQ) biosynthesis.

The shikimate pathway that leads to the synthesis of PHB and the mevalonate pathway that leads to the synthesis of DPP are indicated. *Eco, E. coli* genes*; Spo, S. pombe* genes*; Sce, S. cerevisiae* genes. *Eco_ppsA*, phosphoenolpyruvate synthase; *Eco_aroF*^{FBR}, feedback-inhibition-resistant (FBR) 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase; *Eco_ubiC*, chorismate lyase; *Spo_aro2*, chorismate synthase; *Spo_aro7*, chorismate mutase; *Sce_thmgr1*, truncated 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase 1; *Sce_mvk*^{FBR}, FBR mevalonate kinase; *Spo_erg8*, phosphomevalonate kinase; *Spo_mvd1*, diphosphomevalonate decarboxylase; *dps1-dlp1*, decaprenyl diphosphate synthase; *ppt1*, PHB-decaprenyl diphosphate transferase.

Figure 6. Enhancement of coenzyme Q (CoQ) production by overexpression of shikimate and mevalonate pathway genes.

CoQ_{10} productivity of strains expressing a single gene located upstream of CoQ biosynthesis was compared to reference strain CHP429 (empty vector). Strains were

grown at 30°C in PM minimal medium containing adenine, uracil, and histidine. Cultures were initiated at 1×10^5 cells/ml and harvested after 48h growth. Production of coenzyme Q_{10} (CoQ_{10}) was measured by HPLC. Gray bars represent relative CoQ_{10} content per volume (%) and open diamonds represent relative CoQ_{10} content per dry cell weight (DCW) (%). Data are represented as the mean ± SD of three measurements.

Figure 7. Effect of coexpression of multiple upstream genes on coenzyme Q_{10} (CoQ_{10}) production

CoQ_{10} production was compared between strains co-expressing two or three genes and reference strain CHP429 (empty vector). Strains were grown at 30°C in PM minimal medium containing adenine and histidine, or adenine. Cultures were initiated at 1×10^5 cells/ml and harvested after 48h growth. Production of CoQ_{10} was measured by HPLC. Gray bars represent relative CoQ_{10} content per volume (%) and open diamonds represent relative CoQ_{10} content per DCW (%). Data are represented as the mean ± SD of three measurements.

497 Table1, Primers used for gene amplification

Name	Sequence
nmt1-pro/PstI	5′-AACTGCAGGTCGATCGACTCTAGAG-3′
nmt1-term/PstI	5′-AACTGCAGGGATTATTTCACTTC-3′
nmt1pro-n-SphI	5′-CTATGACCATGATTACGCCAAGC-3′
nmt1term-c-SphI	5′-AAAGCATGCAGGTCGACGGATC-3′
PUC119L-C	5′-ACAGCTATGACCATGATTACGCCAAG-3′
ars1L-N	5′-AAGTCACTATGTCCGAGTGGTTAAGGAG-3′
Sce_thmgr1-SalI_Fw	5′-ACGCGTCGACAATGGCTGCAGACCAATTGGT-3′
Sce_thmgr1-BamHI_Rv	5′-ACGCGGATCCTTAGGATTTAATGCAGGTGACG-3′
Sce_mvk-SalI_Fw	5′-ACGCGTCGACAATGTCATTACCGTTCTTAACTTC-3′
Sce_mvk-BamHI_Rv	5′-ACGCGGATCCTTATGAAGTCCATGGTAAATTCG-3′
Sce_mvk_T198G-Fw	5′-GTGGTCCATCAAgGATTTCAATGCC-3′
Sce_mvk_T198G-Rv	5′-GGCATTGAAATCcTTGATGGACCAC-3′
Sce_mvk_T456G-Fw	5′-AAGCGCCTCTATgTCTGTATCACTG-3′
Sce_mvk_T456G-Rv	5′-CAGTGATACAGAcATAGAGGCGCTT-3′
Eco_aroF-SalI_Fw	5′-ACGCGTCGACAATGCAAAAAGACGCGCTGAAT-3′
Eco_aroF-BamHI_Rv	5′-ACGCGGATCCTTAAGCCACGCGAGCCGTCA-3′
Eco_aroF_C443T-Fw	5′-GGAAGCGTTAGATCtGAATAGCCCGCAAT-3′
Eco_aroF_C443T-Rv	5′-ATTGCGGGCTATTCaGATCTAACGCTTCC-3′
Eco_ubiC-SalI_Fw	5′-ACGCGTCGACAATGTCACACCCCGCGTTAAC-3′
Eco_ubiC-BamHI_Rv	5′-ACGCGGATCCTTAGTACAACGGTGACGCCG-3′
Eco_ppsA-SalI_Fw	5′-ACGCGTCGACAATGTCCAACAATGGCTCGTC-3′
Eco_ppsA-BamHI_Rv	5′-ACGCGGATCCTTATTTCTTCAGTTCAGCCAGG-3′
Spo_erg8-SalI_Fw	5′-ACGCGTCGACAATGAAAGTGTAAGTTGAAACCA-3′
Spo_erg8-BamHI_Rv	5′-ACGCGGATCCTTATTCTACGGCTAGCCCAT-3′
Spo_mvd1-SalI_Fw	5′-ACGCGTCGACAATGGACAAAAAGGTTTATCAATG-3′

Spo_mvd-BamHI_Rv	5′-ACGCGGATCCTTAATGACTTCCTATAAATTTAGGG-3′
Spo_aro2-SmaI_Fw	5′-ACGCCCCGGG ATGTCTTCCTTCGGCACTTTG-3′
Spo_aro2-SmaI_Rv	5′-ACGCCCCGGGTTATTGAGCATTGGGGAGTAG-3′
Spo_aro7-SalI_Fw	5′-ACGCGTCGACAATGAGTTTGGTTAATGAGAAGC-3′
Spo_aro7-BamHI_Rv	5′-ACGCGGATCCTTAAAGTAAGCGAGCTAACAAAT-3′

Nucleotides in lower case indicate point mutations.

Supplementary Figure 1. Growth after overexpression of coenzyme Q (CoQ) biosynthetic gene.

Five-fold serial dilutions from 1×10^7 cells of CHP429 expressing the indicated genes were spotted onto PM minimal medium or PM minimal medium containing 2 μM thiamine. Plates were incubated at 30°C for 3 days.

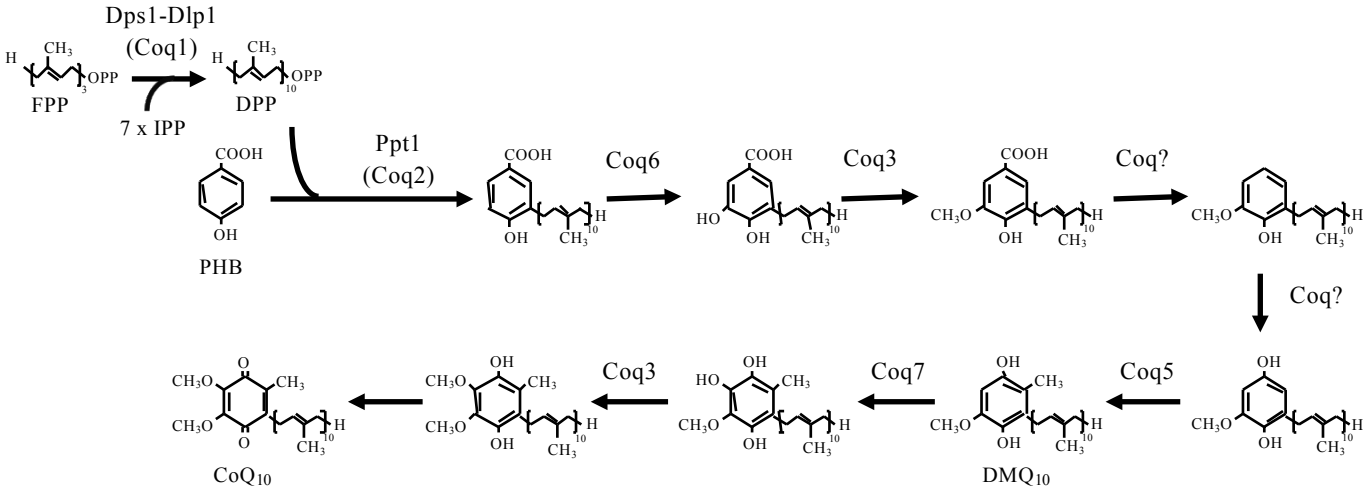
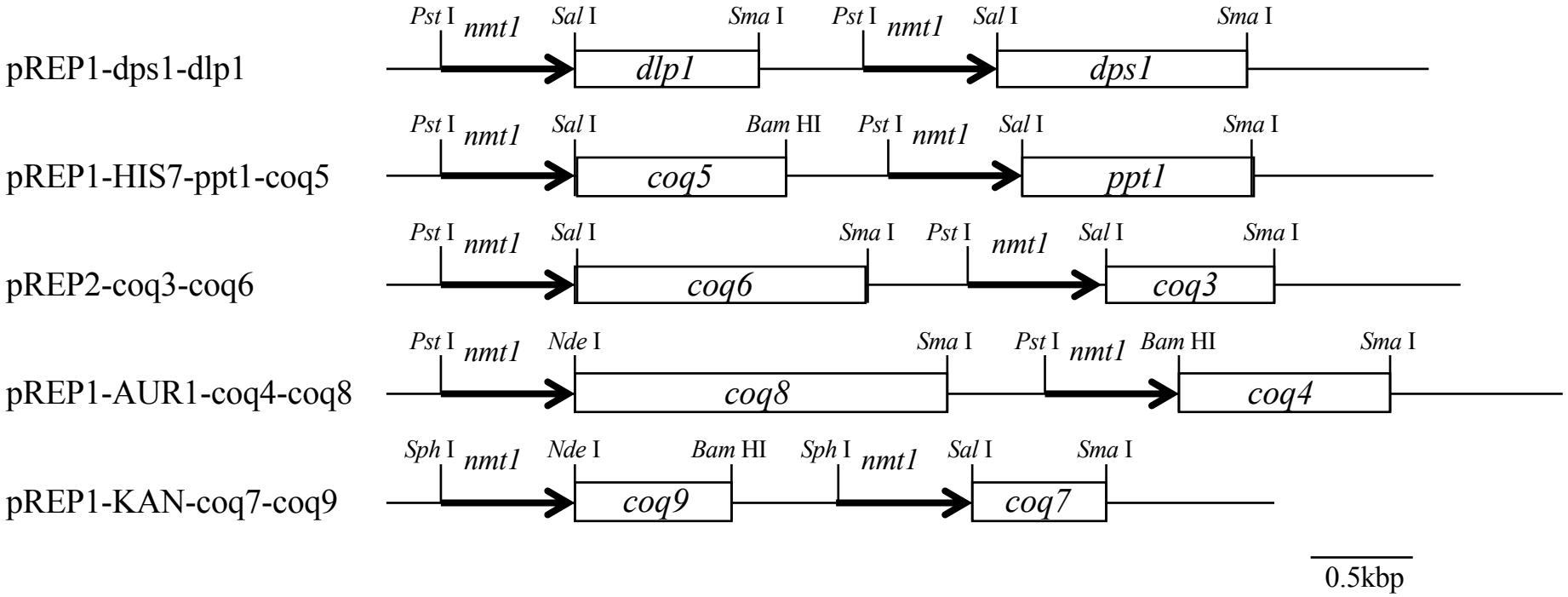

Dps1-Dlp1
(Coq1)
FPP
7 x IPP
DPP
PHB
Ppt1
(Coq2)
Coq6
Coq3
Coq?
Coq?
Coq5
Coq7
Coq3
DMQ10
CoQ10

Fig. 1

A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Sal I
Sma I
dlp1
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nmt1
Sal I
Sma I
dps1
pREP1-HIS7-ppt1-coq5
Pst I
nmt1
Sal I
Bam HI
coq5
Pst I
nmt1
Sal I
Sma I
ppt1
pREP2-coq3-coq6
Pst I
nmt1
Sal I
Sma I
coq6
Pst I
nmt1
Sal I
Sma I
coq3
pREP1-AUR1-coq4-coq8
Pst I
nmt1
Nde I
Sma I
coq8
Pst I
nmt1
Bam HI
Sma I
coq4
pREP1-KAN-coq7-coq9
Sph I
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Sph I
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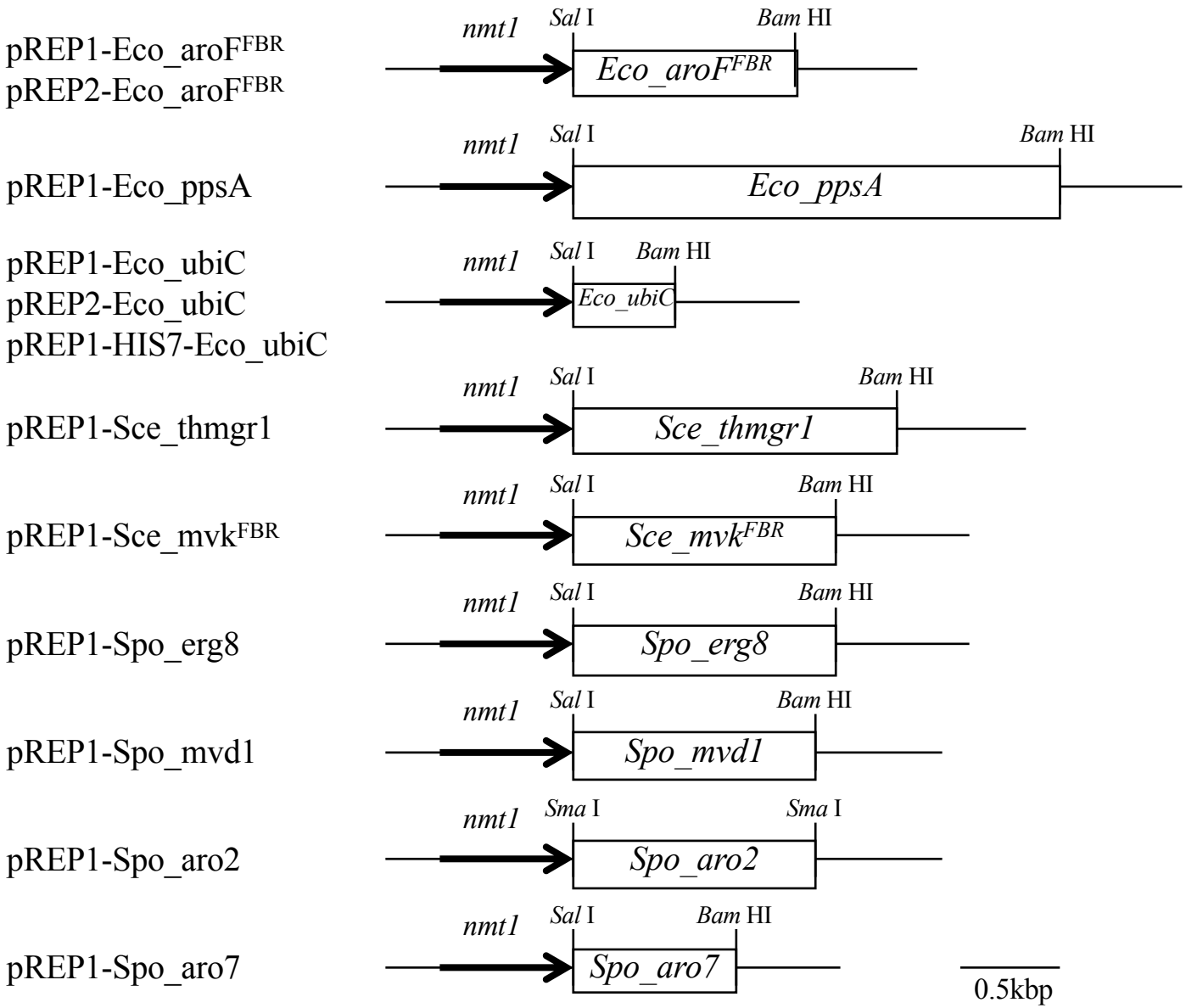
Sma I
coq7
0.5kbp

Fig. 2

B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Sal I
Bam HI
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Eco_ppsA
pREP1-Eco_ubiC
pREP2-Eco_ubiC
pREP1-HIS7-Eco_ubiC
Eco_ubiC
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Sce_thmgr1
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Sce_mvk^FBR
pREP1-Spo_erg8
Spo_erg8
pREP1-Spo_mvd1
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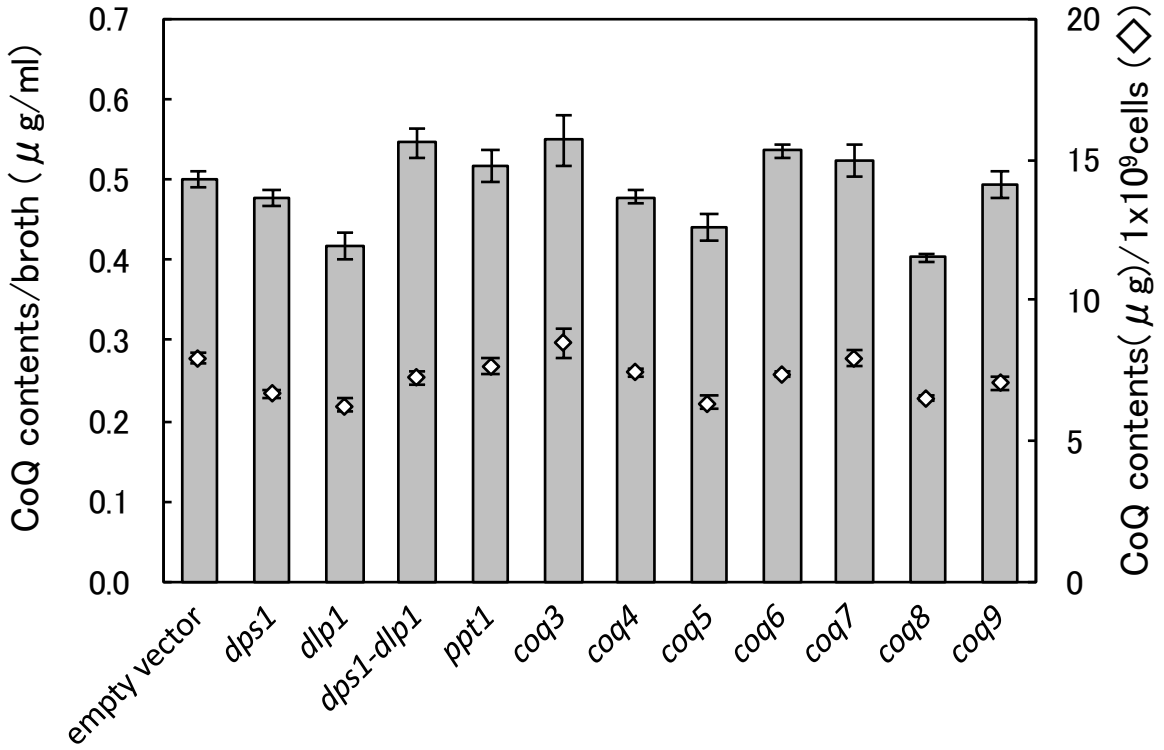
Fig. 2


CoQ contents/broth (μg/ml)
CoQ contents(μg)/1x10⁹cells (◇)
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0.4
0.3
0.2
0.1
0.0
20
15
10
5
0
empty vector
dps1
dlp1
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ppt1
coq3
coq4
coq5
coq6
coq7
coq8
coq9

Fig. 3

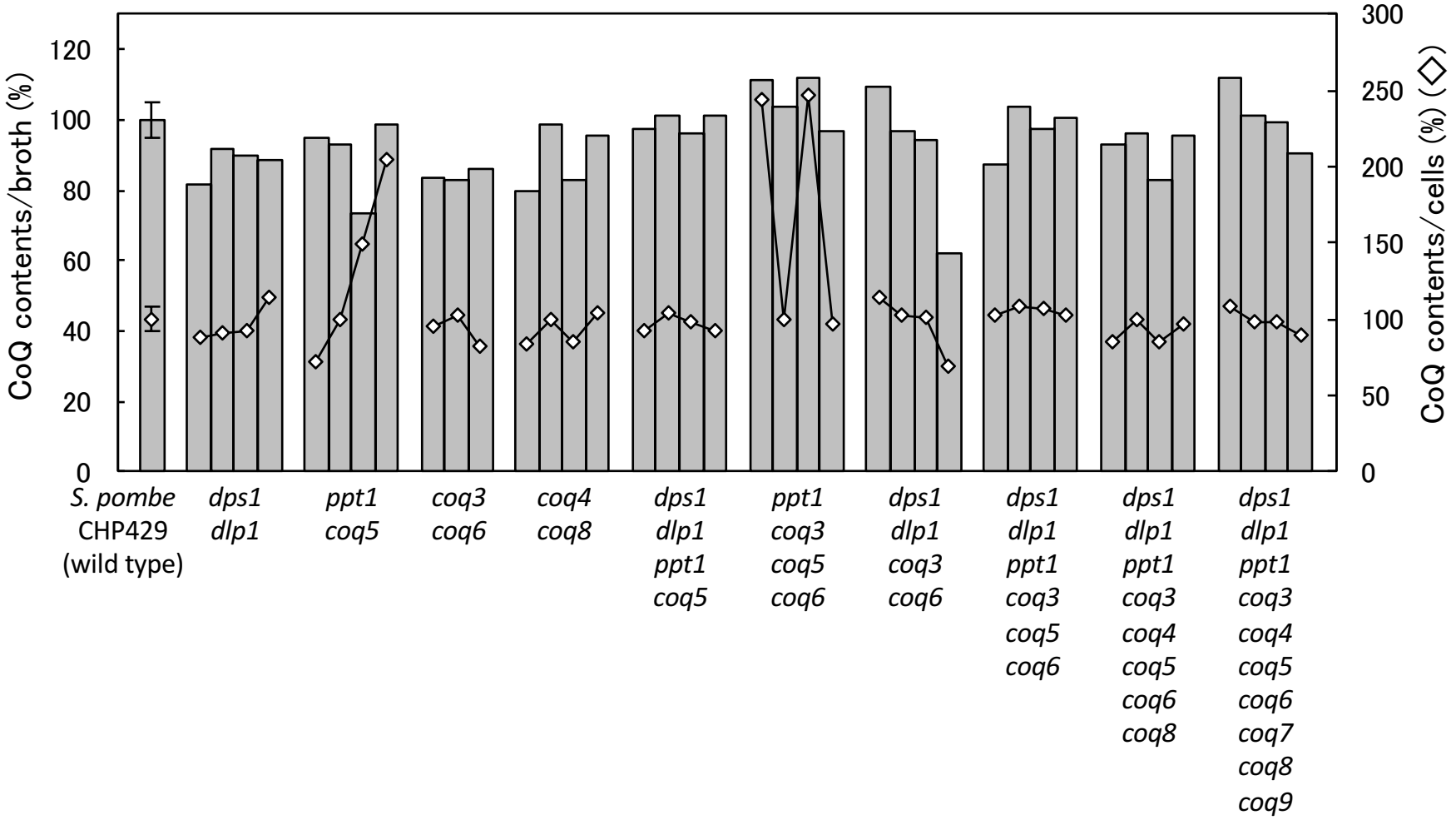
CoQ contents/broth (%)
120
100
80
60
40
20
0
300
250
200
150
100
50
0
CoQ contents/cells (%) (◇)
S. pombe CHP429 (wild type)
dps1 dlp1
ppt1 coq5
coq3 coq6
coq4 coq8
dps1 dlp1 ppt1 coq5
ppt1 coq3 coq5 coq6
dps1 dlp1 coq3 coq6
dps1 dlp1 ppt1 coq3 coq5 coq6
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Fig. 4

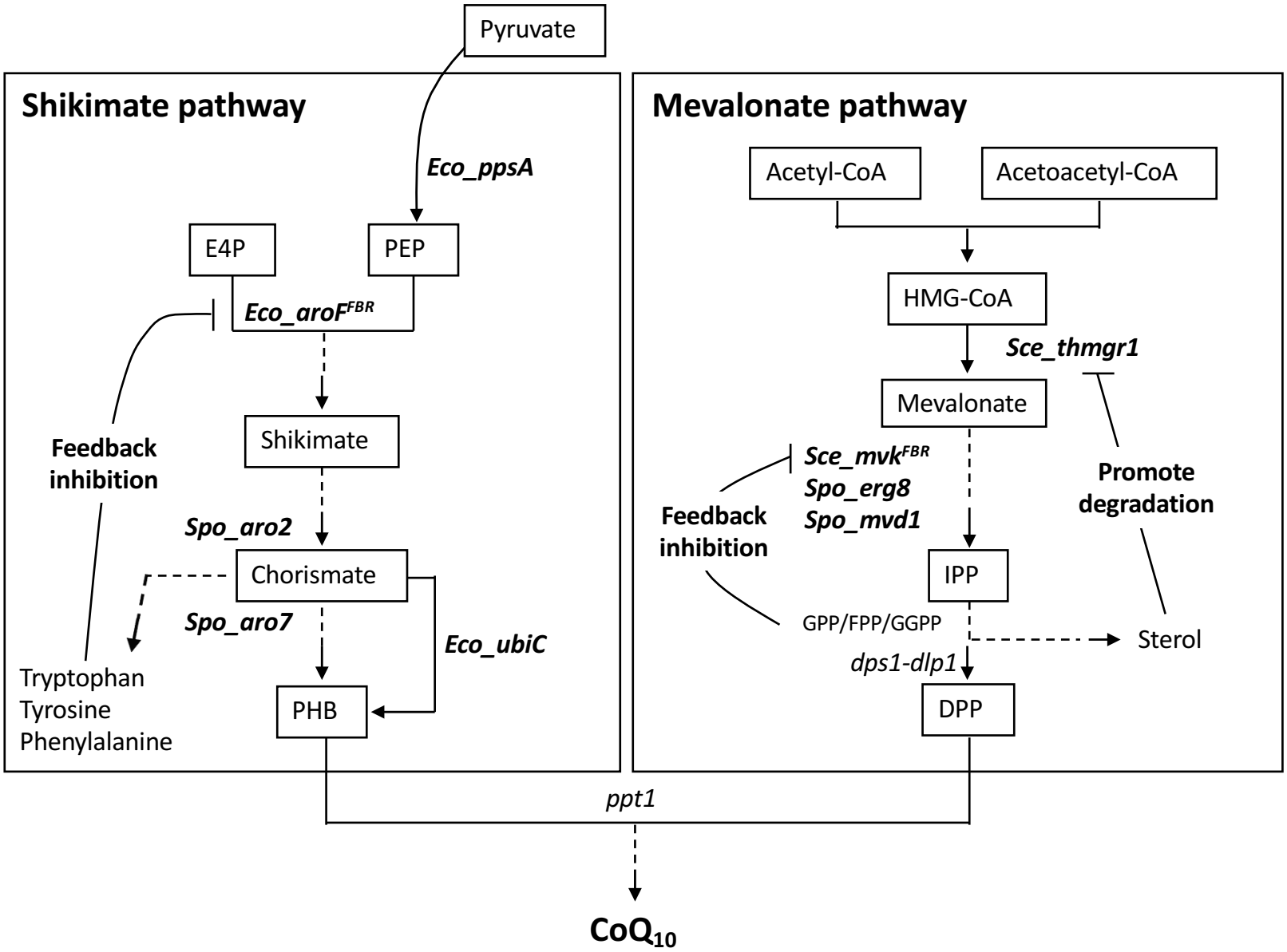
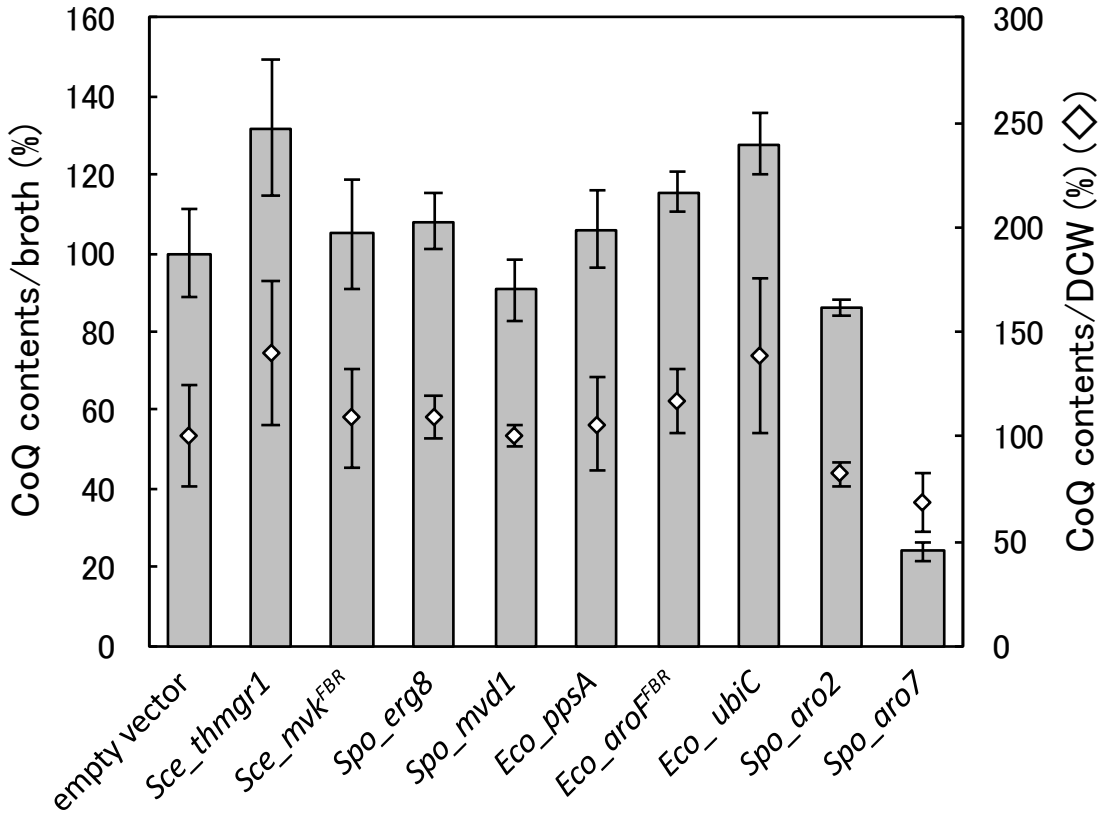

Pyruvate
Shikimate pathway
Eco_ppsA
E4P
PEP
Eco_aroF^FBR
Feedback inhibition
Shikimate
Spo_aro2
Chorismate
Spo_aro7
Eco_ubiC
Tryptophan
Tyrosine
Phenylalanine
PHB
Mevalonate pathway
Acetyl-CoA
Acetoacetyl-CoA
HMG-CoA
Sce_thmgr1
Mevalonate
Sce_mvk^FBR
Spo_erg8
Spo_mvd1
Feedback inhibition
Promote degradation
IPP
GPP/FPP/GGPP
Sterol
dps1-dlp1
DPP
ppt1
CoQ10

Fig. 5


CoQ contents/broth (%)
CoQ contents/DCW (%) (◇)
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140
120
100
80
60
40
20
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300
250
200
150
100
50
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Sce_mvk^FBR
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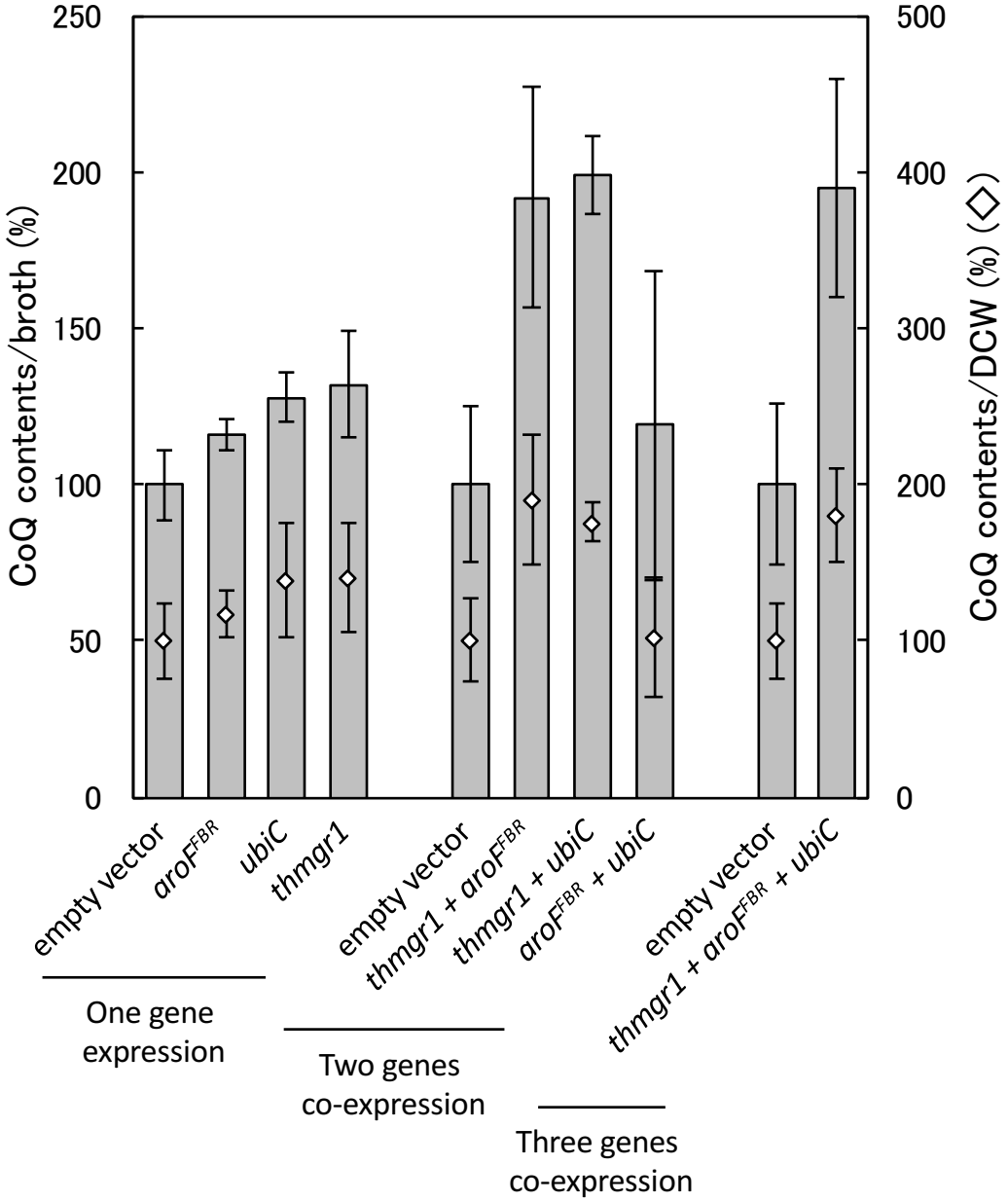
Spo_aro2
Spo_aro7

Fig. 6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CoQ contents/DCW (%) (◇)
empty vector
aroF^FBR
ubiC
thmgr1
empty vector
thmgr1 + aroF^FBR
thmgr1 + ubiC
aroF^FBR + ubiC
empty vector
thmgr1 + aroF^FBR + ubiC
One gene expression
Two genes co-expression
Three genes co-expression

Fig. 7