Research Article

Farnesol production in *Escherichia coli* through the construction of a farnesol biosynthesis pathway – application of PgpB and YbjG phosphatases

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Abbreviations: DMAPP, dimethylalyl diphosphate; **FPP,** farnesyl diphosphate; **GC,** gas chromatography; **MVA,** mevalonate; **PGP,** phosphatidylglycerol phosphate; **UPP,** undecaprenyl diphosphate; **UP,** undecaprenyl phosphate

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Abstract

Farnesol is a sesquiterpenoid alcohol that has important industrial and medical potential. It is usually synthesized from farnesyl diphosphate (FPP) by farnesol synthase in plants. FPP accumulation can cause up-regulation of phosphatases capable of FPP hydrolysis, resulting in farnesol production in *Escherichia coli*. We found that PgpB and YbjG, two integral membrane phosphatases, can hydrolyze FPP into farnesol. Overexpression of FPP synthase (IspA) and PgpB, along with a heterologous mevalonate pathway, enabled recombinant *E. coli* to produce 526.1 mg/L of farnesol. This result indicates that the phosphatases PgpB and YbjG can be used to construct a novel farnesol synthesis pathway for mass production in *E. coli*.

1 Introduction

The emergence of metabolic engineering enables the intentional redesign of entities for the production of many valuable chemicals. This engineering usually relies on the construction of metabolic pathways in a tractable host organism (e.g., *Escherichia coli*), where many known enzymes catalyzing canonical metabolic reactions are combined to produce industrially interesting chemicals, including pharmaceuticals, cosmetics, and biofuels [1, 2]. In most cases, a major challenge in the construction of the desired metabolic pathways is to find or create the effective enzymes responsible for the reactions of interest. To this end, mining the wealth of genomic databases and the evolution of existing enzymes are commonly used to explore new enzymes and activities [3, 4]. Exploring the moonlight functions of existing enzymes, which may appear in atypical metabolic environments of engineered organisms, is also promising. For example, the ADP phosphatase NudF and its paralogs can be induced upon the excessive accumulation of isopentenyl diphosphate (IPP) and dimethylalyl diphosphate (DMAPP) to catalyze their depyrophosphorylation in *E. coli*, which has been successfully used to construct isopentenol synthesis pathway [4, 5].

Farnesol is an important C_{15} isoprenyl alcohol with potential industrial applications as a fragrance, a medicine, and a biofuel. This sesquiterpenoid alcohol can be synthesized from the hydrolysis of farnesyl diphosphate (FPP) by phosphatases [6, 7]. By enhancing the FPP synthesis, farnesol of 145.7 mg/L has been produced in *Saccharomyces cerevisiae* via a long-time (7 days) fermentation process [8]. Farnesol is also produced in *E. coli* engineered to over-accumulate FPP, but the relevant phosphatases have not been isolated

from *E. coli* [7]. It is assumed here that the intracellular FPP accumulation (exotic stress) acts as a trigger for the expression of endogenous phosphatases, which hydrolyze FPP as their moonlight function to reduce FPP toxicity. Based on this assumption, the potential phosphatases of FPP hydrolysis can be discovered by exploring the transcriptional changes of phosphatases upon FPP accumulation. A recent DNA microarray analysis reported the transcriptome of *E. coli* upon FPP accumulation, in which amorpha-4,11-diene synthase was inactivated to eliminate the production of amorpha-4,11-diene [9]. In this study, several phosphatase candidates that were up-regulated upon FPP accumulation were evaluated for farnesol production in an engineered *E. coli* over-producing FPP (Fig. 1). Two integral membrane phosphatases, undecaprenyl (C_{55}) diphosphate (UPP) phosphatase (YbjG) and phosphatidylglycerophosphatase B (PgpB) were determined to have significant FPP hydrolysis activity for farnesol production. These results offer the prospect of renewable production of this valuable sesquiterpenoid alcohol from biomass using a microbial process.

2 Materials and methods

2.1 Plasmid constructions

E. coli DH5α (F$^-$, λ$^-$, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *recA*1, *endA*1, *hsdR*17(r_K^- m_K^+), *phoA*, *supE*44, *thi*-1) was used for all cloning and plasmid constructions. Fourteen phosphatase genes *agp*, *hisI*, *nudE*, *ostB*, *ybjG*, *yedP*, *ygiF*, *yqaB*, *bacA*, *pgpA*, *pgpB*, *pgpC* and *yeiU* were amplified from the genomic DNA of *E. coli* MG1655 using PCR and their corresponding primer sets with restriction sites of *Bam*HI (or *Bgl*II) and *Sal*I (or *Xho*I)

flanking the Shine-Dalgarno sequences and stop codons (Supplementary Table S1). The resulting PCR products were inserted into the *Bam*HI/*Sal*I sites of pT-ispA to yield the following plasmids: pTispA-agp, pTispA-hisI, pTispA-nudE, pTispA-ostB, pTispA-ybjG, pTispA-yedP, pTispA-ygiF, pTispA-yqaB, pTispA-bacA, pTispA-pgpA, pTispA-pgpB, pTispA-pgpC and pTispA-yeiU. The homologs of *pgpB* and *ybjG* from four bacterial species, *Enterobacter aerogenes*, *Klebsiella pneumonia*, *Pantoea ananatis* and *Salmonella enterica*, were amplified from their genomic DNA with appropriate primer sets (Supplementary Table S1); the PCR products were cloned into the *Bam*HI/*Sal*I sites of pT-ispA to yield the following plasmids: pTispA-pgpB$_{Ea}$, pTispA-pgpB$_{Kp}$, pTispA-pgpB$_{Pa}$, pTispA-pgpB$_{Se}$ pTispA-ybjG$_{Ea}$, pTispA-ybjG$_{Kp}$, pTispA-ybjG$_{Pa}$, and pTispA-ybjG$_{Se}$. All the plasmids are listed in Supplementary Table S2.

2.2 Cell culture for farnesol production

E. coli DH5α was used as the host strain to transform with the entire mevalonate (MVA) pathway plasmid pSNA and one of farnesol synthesis plasmids for production. The resulting strains (NA_IPh1 - IPh24, Supplementary Table S3) were cultivated at 30 °C in 2YT medium (16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl) containing 2% (v/v) glycerol and 0.1 mM IPTG, unless otherwise specified. The culture was performed using a decane overlay to harvest the farnesol product [7]. Ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL) were added as required. All of the cultivations were performed in duplicate.

2.3 *pgpB* and *ybjG* knock-outs

Knock-outs of *pgpB* and *ybjG* in *E. coli* DH5α were conducted using the RED/ET recombination method (Gene Bridges GmbH, Germany). Briefly, the kanamycin cassettes for the knock-outs of *pgpB* and *ybjG* were amplified from plasmid pKD13 by PCR reaction with primer sets pgpB-Del-F/pgpB-Del-R and ybjG-Del-F/ybjG-Del-R (Supplementary Table S1), respectively. The resulting PCR products were electro-transformed into *E. coli* DH5α competent cells containing plasmid pRED/ET to generate *E. coli* Δ*pgpB* and *E. coli* Δ*ybjG*. The double mutant *E. coli* Δ(*pgpB*, *ybjG*) was created by electro-transformation of the *pgpB* deletion PCR product into *E. coli* Δ*ybjG*. Before electro-transformation, the kanamycin cassette in the genome of *E. coli* Δ*ybjG* was removed by plasmid pCP20. To analyze the phenotype changes in farnesol synthesis, these three mutants were transformed with plasmids pT-ispA and pSSN12Didi encoding the bottom portion of the MVA pathway to generate strains MUT1_SN, MUT2_SN and MUT3_SN (Supplementary Table S3). By supplying a given amount (5 mM) of mevalonate, FPP can be synthesized in a moderate manner to avoid the possible deleterious effects of FPP accumulation.

2.4 Site-directed mutagenesis of *pgpB* and *ybjG*

The *pgpB*^{H207A} and *ybjG*^{H145A} variants were generated using a PCR-based QuickChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA), according to the manufacturer's instructions. pTispA-pgpB^{H207A} was generated from pTispA-pgpB using the primers pgpB^{H207A}-F and pgpB^{H207A}-R (Supplementary Table S1) to mutate the 207th histidine (H) of PgpB to alanine (A). pTispA-ybjG^{H145A} was generated from pTispA-ybjG

using ybjG^{H145A}-F and ybjG^{H145A}-R primers to mutate the 145th histidine (H) of YbjG to alanine (A). The mutated genes *pgpB*^{H207A} and *ybjG*^{H145A} were sequenced by the Solgent Corp. (Daejeon, Korea) to confirm the incorporation of appropriate mutations.

2.5 Analysis of farnesol production

Farnesol production was quantified using gas chromatography (GC) as previously described [7]. Briefly, the decane layer of each culture was harvested and subjected to gas chromatograph (Agilent Technologies 7890A) equipped with a flame ionization detector and an auto-sampler. Each sample (1 μL) was injected into an HP-5 column (19091J-413) at a split ratio of 1:10. The oven temperature was initiated at 50 °C for 1 min and increased at a rate of 25 °C/min to 150 °C and at a rate of 10 °C/min until 260 °C, where it was held for 1 min. Nitrogen was used as the carrier gas at an inlet pressure of 39 *psi*. The detector was maintained at 260 °C.

3 Results and discussion

3.1 Screening of endogenous phosphatases for the hydrolysis of FPP to farnesol

Many phosphatases non-specifically hydrolyze a myriad of substrates, such as phosphorylated proteins and small phosphoesters, for the regulation of cellular physiology and the assimilation of phosphorous nutrients. We speculate that some endogenous phosphatases can promiscuously hydrolyze FPP to farnesol; and their expression may be up-regulated by the accumulation of toxic FPP. Thus, farnesol synthesis-associated phosphatases were probed from a total of 67 endogenous phosphatases known to

hydrolyze small phosphoesters (Supplementary Table S4) in *E. coli*. The DNA microarray analysis indicated that the expression of 9 phosphatase genes, *agp*, *hisI*, *nudE*, *OstB*, *ybjG*, *yedP*, *ygiF*, *yihX* and *yqaB*, were up-regulated ($\log_2$ (exp) > 0.5) by FPP accumulation (Supplementary Fig. S1). These phosphatases were consequently selected as the FPP phosphatase candidates, and were assembled with FPP synthase gene (*ispA*) to construct 9 farnesol synthesis operons (*ispA-phosphatases*) to evaluate their ability in synthesis of farnesol from FPP (Fig. 1). An exogenous mevalonate (MVA) pathway was used to effectively supply IPP and DMAPP for FPP synthesis (Fig. 1) [10]. Among the strains NA_IPh1 - IPh9 (Supplementary Table S3), over-expression of the *ispA-ybjG* operon resulted in strain NA_IPh5 producing 3.3-fold more farnesol than the reference strain (REF) that did not over-express any of these phosphatases; a less than 2-fold improvement in farnesol production was observed from strains over-expressing the other eight farnesol synthesis operons (Fig. 2A).

3.2 PgpB and YbjG are two phosphatases capable of farnesol synthesis

YbjG is an undecaprenyl (C_{55}) diphosphate (UPP) phosphatase that catalyzes the dephosphorylation of UPP to undecaprenyl phosphate (UP), a polyisoprenoid carrier lipid involved in *E. coli* envelope synthesis [11-14]. There are three other UPP phosphatases, BacA, YeiU and PgpB, which catalyze UPP hydrolysis in *E. coli* (Supplementary Table S4 and Fig. S2). These three phosphatases were also evaluated for farnesol synthesis in the same manner as YbjG. Interestingly, the strain NA_IPh12 over-expressing the *ispA-pgpB* operon resulted in a 6.3-fold increase in the farnesol synthesis capacity over the reference strain,

while the over-expression of the *ispA-bacA* or -*yeiU* operons failed to cause any significant improvement in the farnesol synthesis capacity (Fig. 2A). PgpB is also known to catalyze the dephosphorylation of phosphatidylglycerol phosphate (PGP) to phosphatidylglycerol, an essential cellular membrane phospholipid [15]. *E. coli* has two other PGP phosphatases, PgpA and PgpC (Supplementary Table S4 and Fig. S2) [16-18]. However, over-expression of the *ispA-pgpA* or -*pgpC* operons did not improve the farnesol synthesis capacity (Fig. 2A). Taken together, these results suggested the moonlight function of the phosphatases PgpB and YbjG in the hydrolysis of FPP to farnesol in *E. coli*.

Their FPP hydrolysis activity was further confirmed in *E. coli* Δ*pgpB* and *E. coli* Δ*ybjG* by the deletions of *pgpB* and *ybjG*. *E. coli* DH5α with over-expression of the MVA pathway and the IspA (REF_SN) is capable of farnesol production; this ability was significantly reduced by the deletion of *pgpB* or *ybjG* (Fig. 2B). The farnesol production in strains MUT1_SN and MUT2_SN was reduced to 34.5% and 25.4%, respectively, when compared with the production in the reference strain REF_SN. A further decrease in farnesol production (13.8% of the farnesol production of the reference strain REF_SN) was observed in the strain MUT3_SN with double deletions of *pgpB* and *ybjG*. PgpB and YbjG significantly affect FPP hydrolysis, and their moonlight function of FPP hydrolysis is desirable for the engineering of *E. coli* for mass production of farnesol.

The farnesol production of *E. coli* strains over-expressing the farnesol synthesis operons *ispA-pgpB* or -*ybjG* alongside the MVA pathway was next measured over time. After 60 h of culture, the over-expression of PgpB and YbjG, respectively, yielded 526.1 mg/L and 154 mg/L of farnesol, which represented a 7.1-fold and 2.1-fold increase compared with the

production(74.3 mg/L) in the reference strain (REF) (Fig. 3A). Cell growth retardation was observed in the culture of the reference strain, which could be attributed to the toxicity of the FPP accumulation because no growth inhibition was observed in the recombinant strains over-expressing PgpB and YbjG (Fig. 3B).

3.3 Farnesol synthesis by PgpB and YbjG is dependent on the histidine (H) residues of the C3 motifs

PgpB and YbjG are the integral membrane proteins catalyzing UPP dephosphorylation to recycle the UP carrier lipids in *E. coli* [12, 13]. Both phosphatases belong to the type II phosphatidic acid phosphatase (PAP2) family, which has three conserved motifs, C1 (KX_6RP), C2 (PSGH) and C3 (SRX_5HX_3D) [19] (Fig. 4A). These motifs are widely conserved in a myriad of microorganisms. The *pgpB* and *ybjG* homologs from four bacterial species *E. aerogenes*, *K. pneumonia*, *P. ananatis* and *S. enterica* were cloned and assembled with *ispA* to generate 8 farnesol synthesis operons similar to *E. coli pgpB* and *ybjG*. Over-expression of these operons alongside the MVA pathway in *E. coli* (strains NA_IPh15 - IPh22) resulted in farnesol production comparable to those over-expressing *E. coli pgpB* or *ybjG* counterparts (strains NA_IPh5 and _IPh12) (Fig. 4B). The conserved histidine (His) residue of the C3 motifs of PgpB and YbjG plays a critical role in UPP hydrolysis by attacking the diphosphate group of UPP substrate [19, 20]. Two mutated variants, $ybjG^{H145A}$ and $pgpB^{H207A}$, were thus created to assess the role of the conserved C3 motif in FPP hydrolysis. The farnesol production of the strains NA_IPh22 and _IPh23 harboring the mutated *ispA-*$pgpB^{H207A}$ and -$ybjG^{H145A}$ operons was comparable to that of the reference strain (REF),

and never reached a comparable level produced by strains harboring the unmutated *ispA-pgpB* and -*ybjG* counterparts (Fig. 4B). These results suggest that the moonlight FPP hydrolysis function of PgpB and YbjG originates from their UPP hydrolysis activity via the C3 motif and is dependent on it.

The integral membrane proteins PgpB and YbjG consist of multiple transmembrane helixes and a PAP2 catalytic site that is oriented towards the periplasmic side of the inner membrane (Supplementary Fig. S3) [12, 21]; these features are consistent with a UP carrier lipid recycling function in periplasm. Given the catalytic site location of PgpB and YbjG, it is assumed that lipophilic FPP is translocated to either the periplasm across the inner membrane or the periplasmic face of the inner membrane where the non-native FPP substrate is dephosphorylated by PgpB and YbjG (Fig. 5). Some bacterial translocators are able to transport UPP-linked oligosaccharides across the inner membrane for O-antigen synthesis and protein glycosylation [22, 23]. Short-chain isoprenyl diphosphates of IPP and geranyl (C_{10}) diphosphate can be transported across plastid membranes during metabolic cross talk between the cytosolic and plastidial pathways of isoprenoid synthesis in plants [11, 24, 25]. Thus, the elucidation of the machinery and mechanisms responsible for the membrane bilayer passage of FPP in *E. coli* would greatly improve farnesol production by PgpB and YbjG.

4 Concluding remarks

The exploration of new enzymes with desirable activities for metabolic engineering heavily relies on our ability to mine the wealth of genomic databases or tailor any existing

enzyme. However, these efforts are often hampered by a lack of high-throughput screening approaches or our poor understanding of the enzymes. To construct a novel farnesol biosynthesis pathway, two integral membrane phosphatases YbjG and PgpB, which are natively involved in UP lipid carrier recycling, have been identified in this study. Over-expression of the farnesol synthesis operon *ispA-pgpB* and the MVA pathway in *E. coli* resulted in the farnesol production of 526.1 mg/L in a test-tube culture, which is a 3.6-fold increase over the reported titer (145.7 mg/L) via a long-time fermentation in *S. cerevisiae*. The results suggest that "old" phosphatases with moonlight functions can be used to construct a novel farnesol synthesis pathway. Moreover, FPP is most likely hydrolyzed by PgpB and YbjG in the periplasm to generate farnesol. It would be interesting to elucidate the mechanism of FPP translocation across membranes because this information could be used in the metabolic engineering of *E. coli* for further improvement in farnesol production.

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Conflicts of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

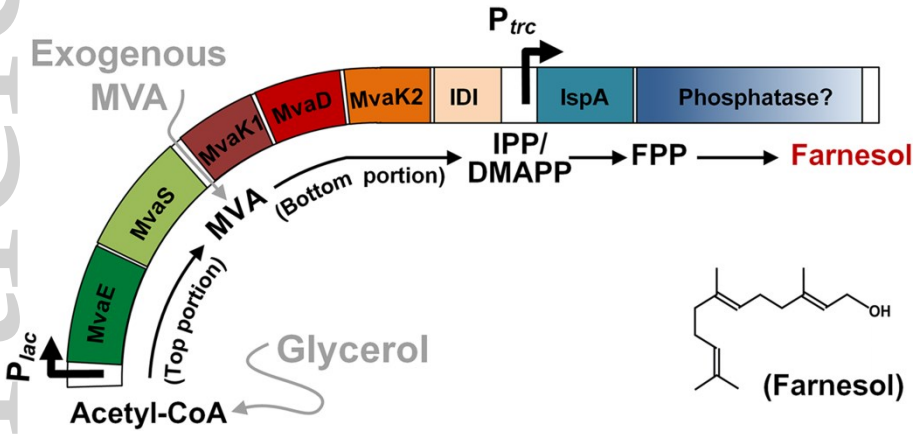

Figure 1. Scheme of the heterologous farnesol biosynthetic pathway constructed in *E. coli*. The fanesol biosynthetic pathway is modularized into two portions; the first portion is the MVA pathway leading to the synthesis of IPP and DMAPP, and the second portion is the farnesol synthesis from IPP and DMAPP via FPP. MvaE (bifunctional acetoacetyl-CoA thiolase and hydroxymethylglutaryl-CoA reductase), MvaS (hydroxymethylglutaryl-CoA synthase), MvaK1 (mevalonate kinase), MvaK2 (phosphomevalonate kinase), MvaD (mevalonate diphosphate decarboxylase) and IDI (IPP isomerase) enzymes constitute the entire MVA pathway, which catalyzes the synthesis of IPP and DMAPP from acetyl-CoA. The bottom portion of the MVA pathway can also synthesize IPP and DMAPP from exogenously supplemented mevalonate. The farnesol synthesis portion consists of IspA (FPP synthase) that catalyzes the condensation of DMAPP and IPP to FPP and a phosphatase that hydrolyzes FPP to farnesol.

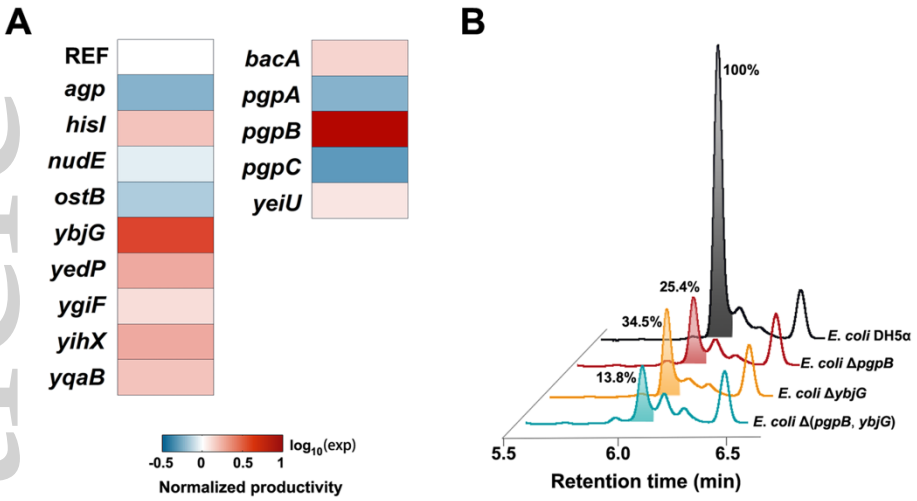

Figure 2. Phosphatases hydrolyze FPP to farnesol. (A) Relative FPP hydrolysis and farnesol production capacity of different phosphatase candidates. The phosphatase candidate genes were assembled with *ispA* to construct the farnesol synthesis operons, which were over-expressed along with the MVA pathway in recombinant *E. coli* DH5α. The resulting strains NA_IPh1 - IPh14 were grown in 2YT media containing 2% (v/v) glycerol at 30 °C for 48 h. The farnesol production (mg/L/OD_{600}) from each recombinant strain was normalized to that of the reference strain (REF) that had no over-expression of phosphatases. The normalized results (fold changes) represent the FPP hydrolysis capacity of the recombinant strains over-expressing the candidate phosphatases. **(B)** Deletions of *ybjG* and *pgpB* validate their FPP hydrolysis activity. The bottom portion of the MVA pathway and IspA were co-expressed in the reference strain *E. coli* DH5α (black, REF_SN), and the null mutants *E. coli* Δ*pgpB* (red, MUT1_SN), *E. coli* Δ*ybjG* (orange, MUT2_SN) and *E. coli* Δ(*pgpB*, *ybjG*) (cyan, MUT3_SN). The culture was performed using 2YT media with 2% (v/v) glycerol and 5mM mevalonate at 30 °C for 48 h. The farnesol peak area of each mutant strain was normalized to the farnesol peak area of the reference strain *E. coli* DH5α (100%).

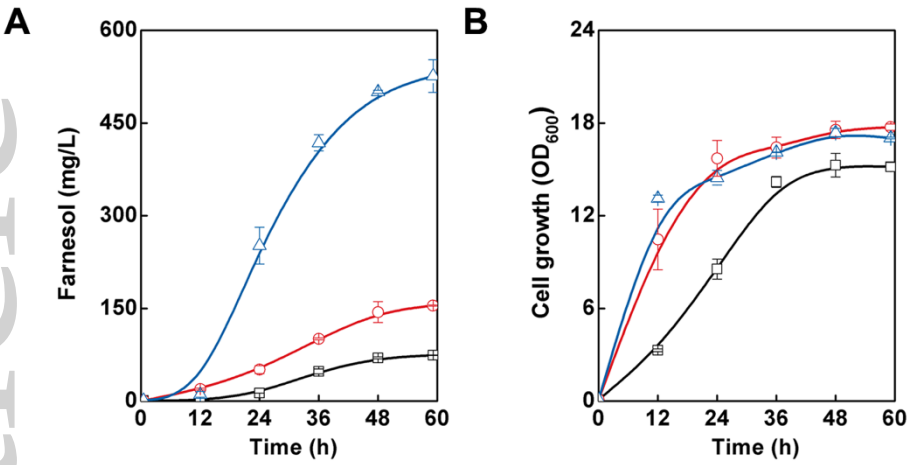

Figure 3. Farnesol production of recombinant *E. coli* expressing *ispA-ybjG/-pgpB* operons and the MVA pathway. (A) Farnesol production. **(B)** Cell growth. *E. coli* DH5α strains over-expressing the farnesol synthesis operons *ispA-pgpB* (blue △, NA_IPh12), *ispA-ybjG* (red ○, NA_IPh5) and *ispA* only (black □, REF) along with the MVA pathway were grown in 2YT media with 2% (v/v) glycerol at 30 °C. Error bars represent the standard deviation of two biological replicates.

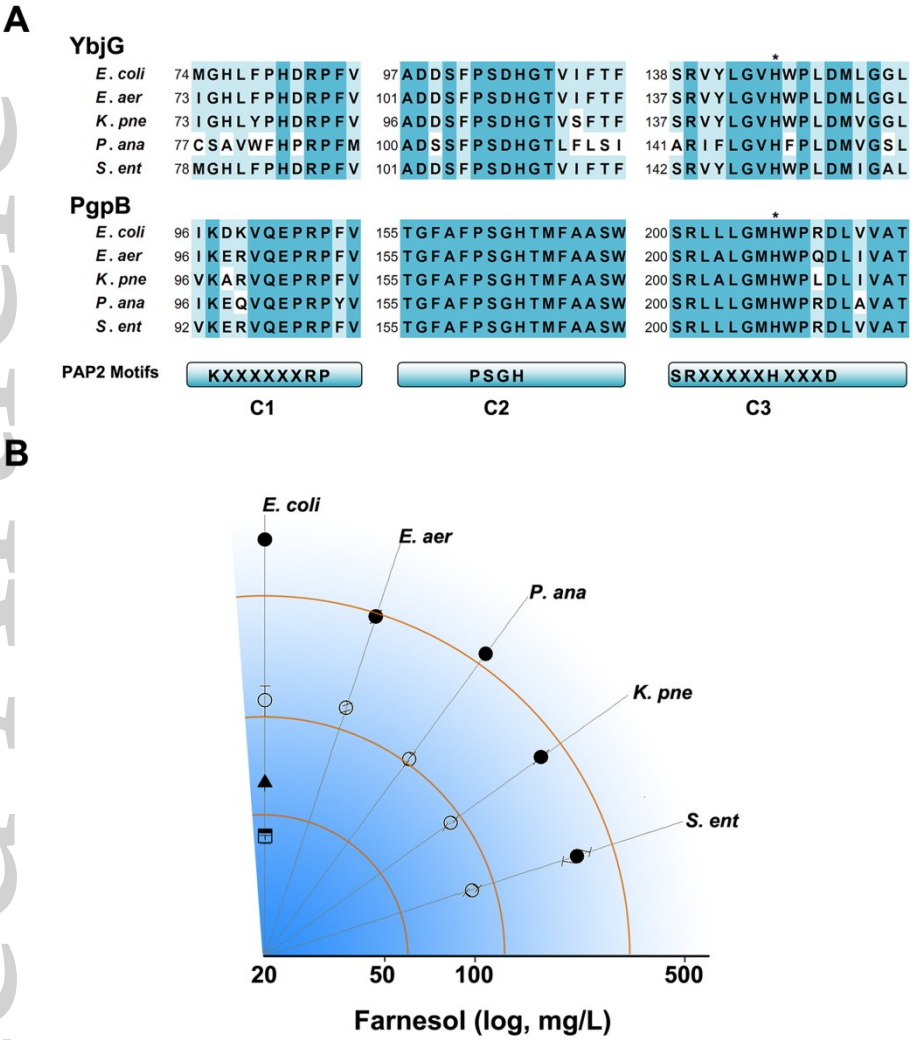

Figure 4. Analysis of the critical residue of PgpB and YbjG for the hydrolysis of FPP to farnesol. (A) Alignments of the three signature motifs of PgpB and YbjG homologs. The C1 (KX_6RP), C2 (PSGH) and C3 (SRX_5HX_3D) motifs were aligned between the PgpB and YbjG homologs from *E. coli*, *Enterobacter aerogenes* (*E. aer*), *Klebsiella pneumonia* (*K. pne*), *Pantoea ananatis* (*P. ana*) and *Salmonella enterica* (*S. ent*). The asterisks (*) indicate the conserved residues, His^{207} of PgpB and His^{145} of YbjG. **(B)** Farnesol production by the PgpB and YbjG homologs from *E. aer*, *K. pne*, *P. ana* and *S. ent* (NA_IPh15-IPh22) and the mutants from *E. coli* (NA_IPh23, and _IPh24). The farnesol synthesis operons of the *pgpB* homolog genes (●) and mutated *pgpB* (■), and the *ybjG* homolog genes (○) and mutated *ybjG* (□)

were over-expressed along with the MVA pathway in recombinant *E. coli* DH5α. Cells were grown in 2YT media with 2% (v/v) glycerol at 30 °C for 48 h. The REF strain (▲) was also cultured as a control. Error bars represent the standard deviation of two biological replicates.

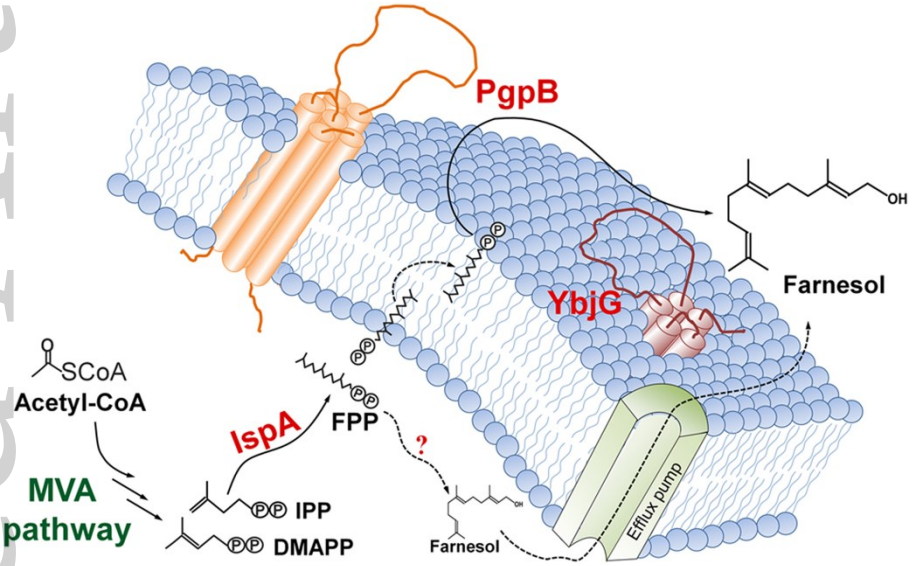

Figure 5. Scheme of the farnesol synthesis in the recombinant *E. coli*. The PgpB and YbjG phosphatases are integrated into the inner membrane. A surplus amount of FPP is synthesized in the cytosol via the MVA pathway and IspA. There are two presumed routes of FPP hydrolysis resulting in farnesol production: i) FPP is transported to the periplasm by a yet unknown mechanism and hydrolyzed by PgpB and YbjG; and ii) FPP hydrolysis is conducted in the cytosol by unknown phosphatases (?) and farnesol is expelled by unknown efflux pumps. The solid and broken arrows indicate the known and unknown reactions, respectively.