THESIS

ARCHAEAL ISOPRENOID BIOSYNTHESIS

Submitted by

Lie Stefanus Geraldy Liman

Department of Biochemistry and Molecular Biology

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Master's Committee:

Advisor: Thomas Santangelo

Paul Laybourn
Christie Peebles

ABSTRACT

ARCHAEAL ISOPRENOID BIOSYNTHESIS

Many high value natural products - including artemisinin, squalene, and farnesene – are isoprenoids. Efforts to commercially produce isoprenoids are often complicated by low concentrations of isoprenoid precursors and the toxicity of isoprenoids in common production platforms (i.e. bacteria and yeasts). Archaeal-based production platforms provide a potential solution to the precursor toxicity problems as archaea produce isoprenoids in large quantities to generate their unique membrane hydrocarbon chains. One roadblock to commercial archaeal isoprenoid production platforms is the uncharacterized pathway leading to isoprenoid precursor synthesis. This project details, genetically and biochemical, the first three steps in the proposed pathway of archaeal isoprenoid biosynthesis - from acetyl-CoA to mevalonate - in *Thermococcus kodakarensis.*

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INTRODUCTION

Isoprenoids – also known as terpenes or terpenoids – are a highly diverse structural class of products composed of polymerized isoprene unit(s), isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DAMPP) (Figure 1). The diversity of form is indicative of the diversity of function, with many high-value products, including artemisinin (anti-malarial drug), β-Santalol (fragrance), and farnesene (jet fuel replacement), all stemming from these two isoprene units. Most isoprenoids are generated in low or moderate quantities in their native host, and overexpression systems are typically necessary to generate isoprenoids in commercial scale. Several limitations have hampered expression of many valuable isoprenoids and raised costs associated with production of others. Alternative platforms need to be developed in parallel with improvements to existing production hosts to maximize the commercial opportunities of isoprenoids.

Each Domain of life has the capability of isoprenoid production, though two radically different synthesis routes are found in nature. Bacteria and chloroplasts typically encode a 2C-methyl-D-erythritol 4-phosphate pathway (MEP pathway), while eukaryotes and archaea typically encode a mevalonic acid (MVA) pathway (Matsumi, Atomi, Driessen, & van der Oost, 2011). Both systems can be upregulated to produce higher volumes of isoprenoids (Leavell, McPhee, & Paddon, 2016), but toxicity of isoprenoid precursors at high concentration (Sivy, Fall, & Rosenstiel, 2011) often limits overall production.

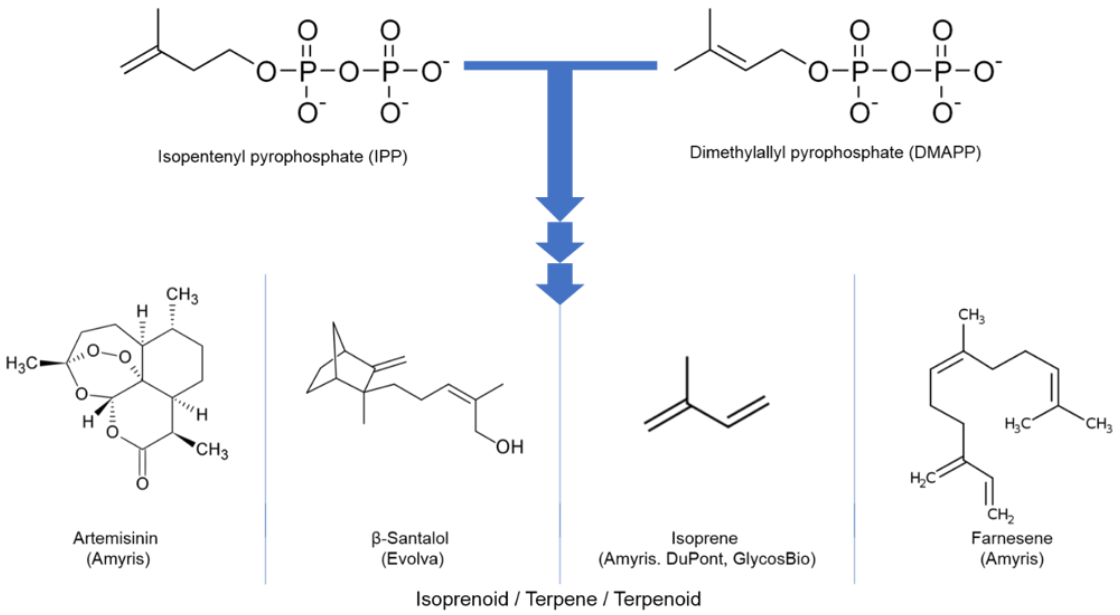


Figure 1. Two isoprene units forming Isoprenoid compounds.

The archaeal domain of life has a unique lipid membrane composition compared to their bacterial and eukaryotic counterparts. Four main unique characteristics of archaeal membrane lipids (Figure 2) include: 1) different glycerol moiety stereo-structure; 2) ether linkage connecting archaeal hydrocarbon chains compared to the ester linkage in bacterial and eukaryotic lipids; 3) potential production of a lipid monolayer in archaea instead of the usual lipid bilayer; and 4) isoprenoid hydrocarbon chains in archaeal lipid membranes instead of long fatty acid hydrocarbon chains (Valentine, 2007). Archaeal species dependency on the production of isoprenoid precursors for their lipid membranes offer a potentially better host for high valued isoprenoid product biosynthesis. However, the exact pathway(s) used in archaeal isoprenoid biosynthesis has yet to be fully identified and characterized. Current hypothesis of archaeal isoprenoid biosynthesis focusses on the MVA pathway with some branched pathway unique to archaeal domain (Figure 3). A study also proposed the presence of leucine or lysine shunt (Figure 4) in some archaeal organisms as an effective pathway of mevalonate production bypassing the use of Acetyl-CoA (Yamauchi, 2010). This project focuses on the first part of isoprenoid biosynthesis pathway in archaea.

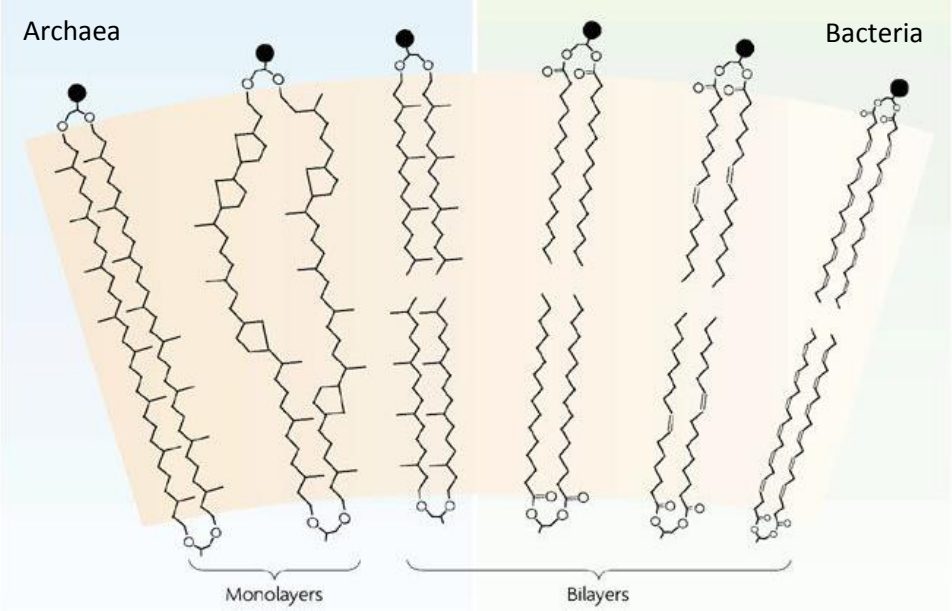


Figure 2. Archaeal plasma membrane compared to bacterial plasma membrane (Valentine, 2007).

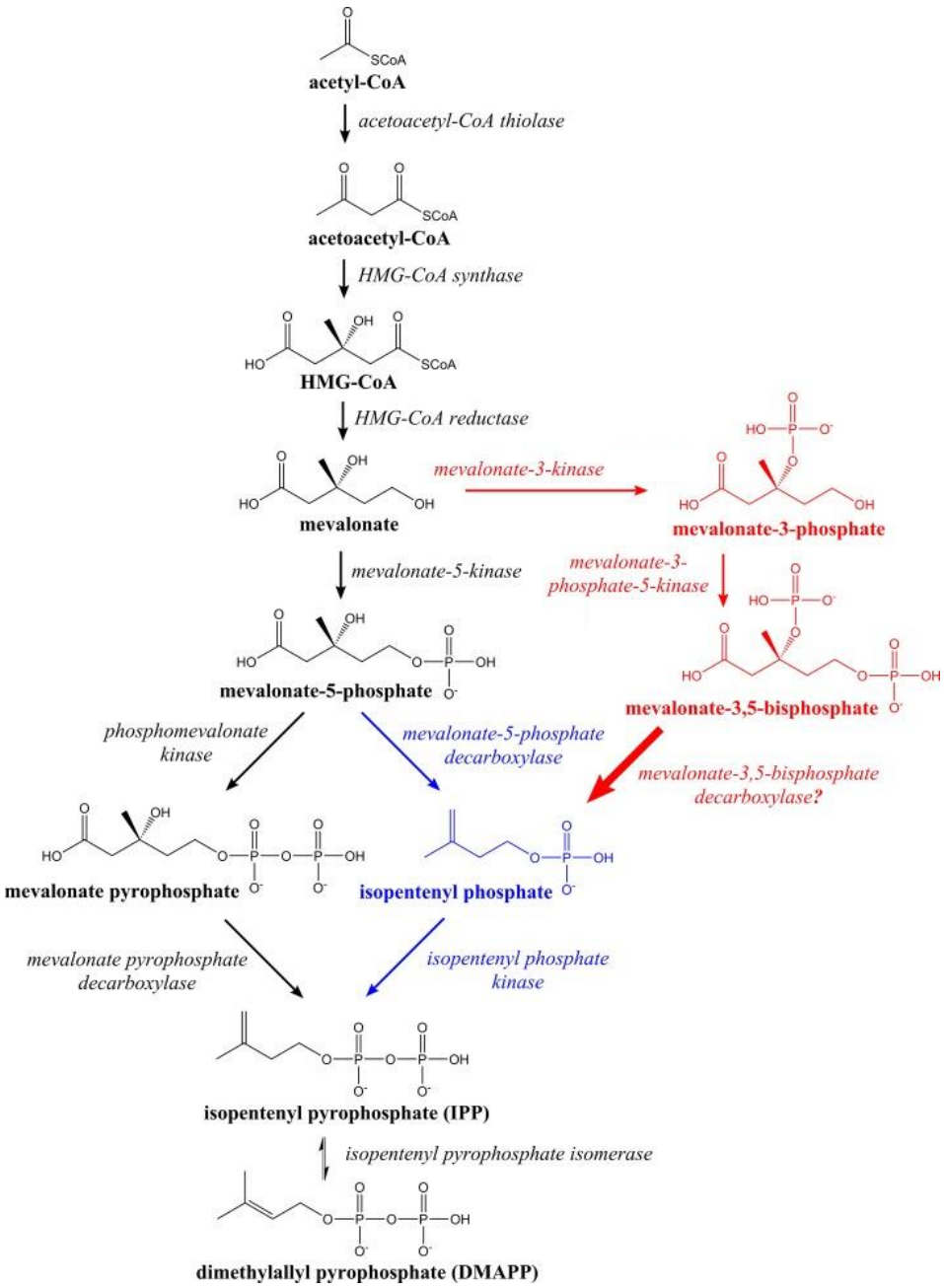


Figure 3. MVA pathway in eukaryote (black) and archaea (blue and red) (Vinokur, Korman, Cao, & Bowie, 2014).

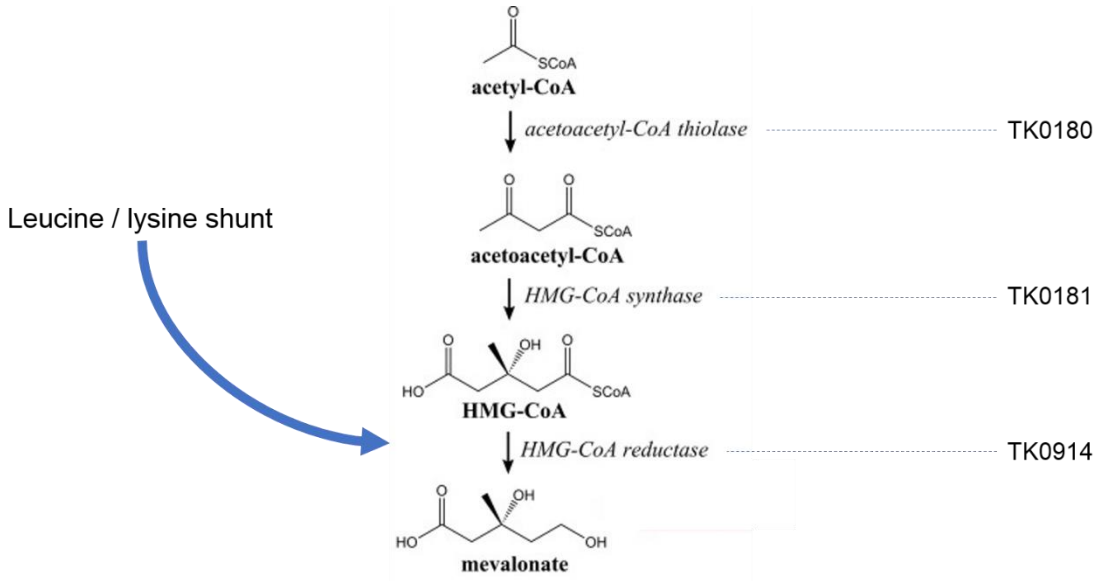


Figure 4. First part of mevalonate pathway and proposed leucine shunt (adapted from (Vinokur, Korman, Cao, & Bowie, 2014).

To investigate the pathway for isoprenoid biosynthesis in archaea, a two-pronged approach was used: 1) Using bioinformatics to identify, then genetic techniques to delete homologs of known genes involved in mevalonate pathway to determine the essentiality of the gene and possibility of biologically viable secondary pathway of isoprenoid production. 2) Characterizing the encoded enzymes to confirm their activity. *Thermococcus kodakarensis* is chosen as the model organism. The main reasons for choosing *T. kodakarensis* are: 1) short generation time (~40 minutes); 2) natural DNA transformation competency; and 3) available genetic techniques to generate markerless genetic alteration through homologous recombination (Figure 5) (Hileman & Santangelo, 2012). We will use *T. kodakarensis'* TS559 parental strain (ΔpyrF; ΔtrpE::pyrF, ΔTK0664, ΔTK0149) and pTS700 derived plasmid, carrying selectable markers, including TK0149 (agmatine biosynthesis) and TK0664 (purine scavenging pathway), complementary to the parental strain. Since mevalonic acid production is assumed to be essential, the growth media is supplemented with mevalonate/mevalonic acid.

MATERIALS AND METHODS

T. kodakarensis growth

T. kodakarensis strains were grown in artificial sea water medium supplemented with yeast extract, tryptone, pyruvate and elemental sulfur (ASW-YT-pyr-S˚) anaerobically at 85˚C (Farkas, Picking, & Santangelo, 2013; Hileman & Santangelo, 2012; Sato, Fukui, Atomi, & Imanaka, 2003), with or without the addition of 1mM mevalonic acid. Optical density at 600 nm was used to monitor growth of *T. kodakarensis* for growth curve experiment. Gelrite or gelzan-solidified media was used for *T. kodakarensis* growth on plate as previously mentioned (Santangelo, Cubonová, James, & Reeve, 2007; Santangelo, Cubonová, & Reeve, 2010), incubated anaerobically at 85˚C. Visualization of colonies on solid media is done by blotting with PVDF followed by staining with commassie brillant blue.

Mevalonate synthesis

Generation of mevalonate was done by hydrolyzing 500mg (±)-Mevalonolactone in 10 ml 0.5M KOH at 37˚C for one hour. Adjustment to the pH is done by adding HCl to the reaction until pH read 7.2.

Plasmid construction for deletion strains

Plasmid containing deletion of the gene TK0180, TK0181, and TK0914 were constructed from parental plasmid pTS700 (Hileman & Santangelo, 2012). Genes were ampified along with ~500 bp of flanking DNA on the 5’ and 3’ end of the target gene from genomic DNA of *T. kodakarensis.* Resulsting amplicons were cloned into pTS700 to generate pOSU-0180A, pOSU-0181A, and pOSU-0914A, followed with Quikchange mutagenesis to delete the coding region of the gene of interest from each plasmid generating pCSU-0180B, pCSU-0181B, and pCSU-0914B.

TK0180, TK0181, and TK0914 deletion strains

Plasmids incapable of autonomous replication in *T. kodakarensis* containing deletion of TK0180, TK0181, and TK0914 were individually transformed into *T. kodakarensis* TS559 (ΔTK0149; ΔTK0664; ΔTK0254::TK2276; ΔTK2276) (Farkas et al., 2013; Hileman & Santangelo, 2012; Santangelo et al., 2010). Pop-in/pop-out, Selection/counter-selection method by first eliminating agmatine auxotrophy through homologous recombination of plasmid into TS559 genome and followed by 6-methyl purine (6MP) counter-selection to select for cells with plasmid sequence excised out of the genome (Farkas et al., 2013; Hileman & Santangelo, 2012). Cells with incorporated plasmid in its genome were selected into liquid ASW-YT-pyr-S° after 48 hour growth on ASW-YT-S° plates lacking agmatine supplementation. Confirmation of locus specific recombination of pCSU-0180B, pCSU-0181B, and pCSU-0914B into TS559 parental strain were done using diagnostic PCR. Strains with confirmed plasmid incorporation were grown overnight in liquid media (ASW-YT-pyr-S°) supplemented with agmatine and mevalonate (1mM), then plated onto ASW-S° plates containing 20 amino acids, 6MP, agmatine, and mevalonate. Cells that are resistant to 6MP counter-selection were isolated and cultured overnight in liquid culture (ASW-YT-pyr-S°) containing agmatine and mevalonate (1mM). Diagnostic PCR were used on each deletion strains Δ0180, Δ0181, and Δ0914 to confirm locus specific deletion of the target genes.

Plasmid construction for tagged TK0914 protein

pQE80L plasmid was used as parental plasmid for tagged TK0914 enzyme expression and digested with EcoRI. Primer pairs containing C-terminal 6-his tag, ribosomal binding site, EcoRI palindromic recognition site, and homology sequence for both pQE80L and the gene TK0914 was used to generate gene block fragments for insertion was amplified using pOSU-TK0914A as template DNA. pQE80L-TK0914 was generated using In-fusion HD protocol to insert the gene fragment into digested pQE80L, followed by transformation into Stellar cells, and

confirmed via colony PCR. Once confirmed, pQE80L-0914 plasmid is transformed into *E. coli* Rosetta 2 cells via heat pulse method at 42 °C for 45 seconds.

Tagged TK0914 protein purification

Biomass of Rosetta cell containing pQE80L-0914 was grown in 4-liter LB liquid media containing ampicillin and chloramphenicol. Biomass was induced with IPTG (275 µM) at OD600 of 0.4 and incubated for four more hours after induction to recombinantly express the protein of interest (TK0914). Then cells were pelleted using centrifugation.

Purification of 6-His tagged TK0914 protein was done by first sonication of the cells followed by heat treatment at 85°C for 30 minutes. Clarified, heat treated cell lysate was loaded into Ni^{2+} affinity column chromatography and fractionated with increasing imidazole concentration. Fractions imaged on SDS-PAGE to check the relative size of the protein isolated then pooled and dialyzed.

Recombinant TK0914 enzyme kinetics

Spectrophotometry activity assays were done on Beckman Coulter DU 800 HMG-CoA reductase reaction. In 200 µl total reaction, 25 mM Tris-HCl pH 8.0, 100 mM NaCl, and 1mM DTT, 1 mM NADPH, purified recombinant TK0914 (10nM), and varied amount of HMG-CoA (10 – 5000 µM) was incubated in 85°C. Absorbance at 320 nm was measured at t=0 and t=5 minutes. Data were then plotted into Lineweaver-Burk plot to get Km and V_{max}.

RESULTS

PCR confirmation of Δ0180, Δ0181, and Δ0914 strains show deletion

T. kodakarensis Δ0180, Δ0181, and Δ0914 strains contain complete markerless genetic deletion of the presumptive acetoacetyl-Coenzyme A thiolase (TK0180), 3-hydroxy-3-methylglutaryl Coenzyme A synthase (TK0181), and HMGCR encoding gene (TK0914), respectively. All deletion strains, Δ0181, Δ0180, and Δ0914, showed dependency on exogenous mevalonic acid supplementation for growth. Confirmation of deletion strains is done by three methods; PCR (with sequencing) for genetic construct confirmation, challenge plates for exogenous mevalonate dependency on solid media growth, and growth curve for mevalonate dependency on liquid media growth.

Δ0180, Δ0181, and Δ0914 strains genetic construct confirmation are done through PCR against parental strain, TS559, as control. Primers used in the reactions are located both internally and externally to the gene (Figure 5A, 6A, 7A). External primers show smaller products consistent with the size of the deleted genes compared to TS559. As expected, PCR with any combination of internal primers only showing amplification on TS559 but not the deletion strains.

Growth challenge on solid media show necessity of mevalonate supplementation

All strains, Δ0180, Δ0181, Δ0914, and TS559 grown on variable solid media. Plates are made with mavalonate (1mM) supplementation or without mavalonate supplementation. Serial dilutions of the strains are made before plating onto the solid media made with gelzan, agmatine, vitamins, 6MP, polysulfides, 20-amino-acids, and incubated at 85°C for four days. Plates without mevalonate showing growth only on TS559 parental strains but not on the deletion strains (Figure 5B, 6B, and 7B). Deletion strains can grow only on plates with 1mM mevalonic acid supplementation. Plates are blotted using polyvinylidene fluoride (PVDF) membrane stained with commassie brillant blue to visualize the T. kodakarensis colonies.

Growth curves with deletion construct show reliance on mevalonate for Δ0180, Δ0181, and Δ0914

Δ0180, Δ0181, Δ0914 and TS559 strains are grown in liquid media to show dependency to mevalonate and essentiality of the genes. Liquid media containing ASW-YT-S, agmatine, vitamins, and either with 1mM mevalonic acid or without mevalonic acid were used to inoculate the strains. Strains were washed three times with 0.8x ASW to clean carry-over mevalonic acid from previous media. The growth curve showed deletion strains Δ0180, Δ0181, and Δ0914 (figure 5C, 6C, and 7C) can grow to the same optical density as TS559 parental strains only if supplemented with exogenous mevalonate. However, all deletion strains showed no growth on liquid media lacking mavalonate unlike TS559 parental strain which is independent to mmavalonate supplementation.

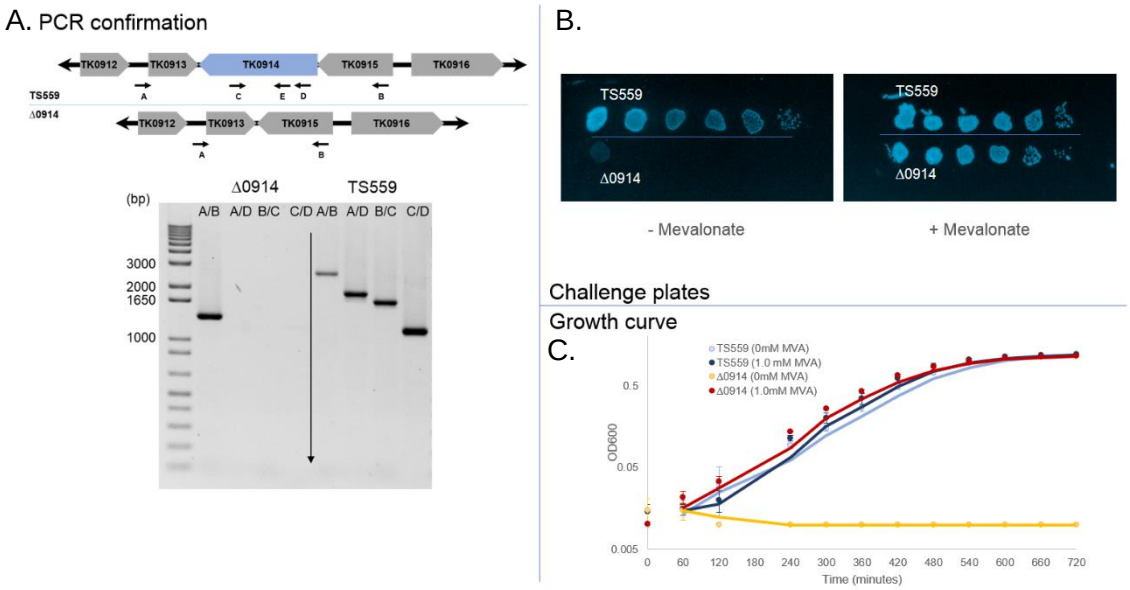


Figure 5. TK0914 is involved in the mevalonate pathway. (A) Diagnostic PCR was done on genomic DNA of Δ0914 strain and TS559 with external primers and internal primers (annealing locations showed). (B) Challenge plates with and without 1mM mevalonate were done on Δ0914 strain and TS559. (C) Growth curve on liquid media with or without 1mM mevalonate were done on TS559 and Δ0914 strain.

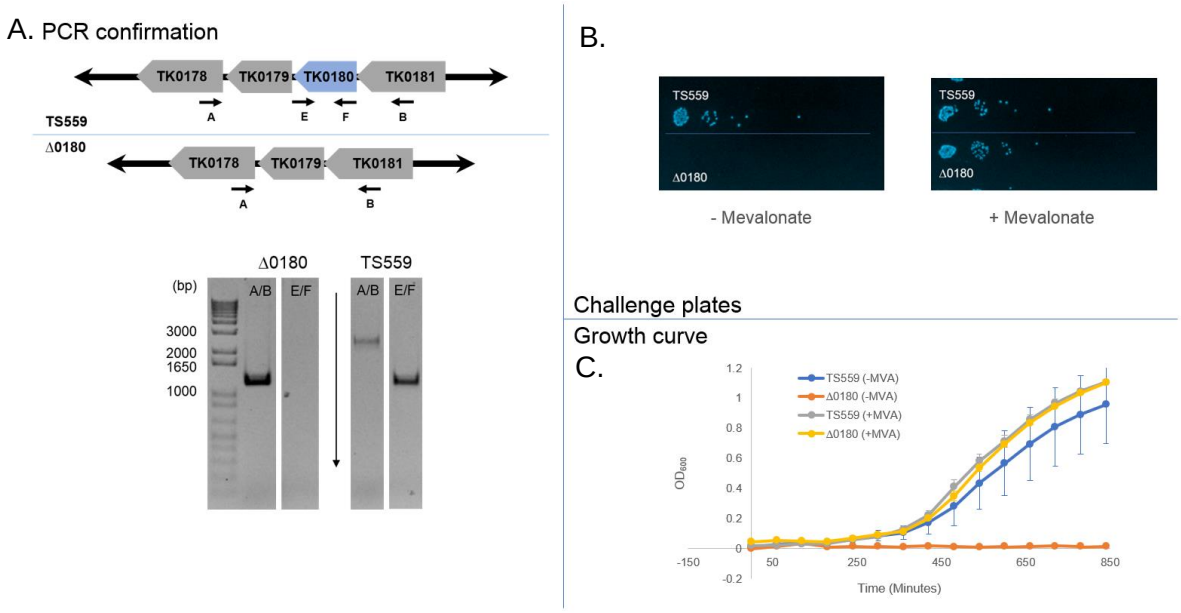


Figure 6. TK0180 is involved in the mevalonate pathway. (A) Diagnostic PCR was done on genomic DNA of Δ0180 strain and TS559 with external primers and internal primers (annealing locations showed). (B) Challenge plates with and without 1mM mevalonate were done on Δ0180 strain and TS559. (C) Growth curve on liquid media with or without 1mM mevalonate were done on TS559 and Δ0180 strain.

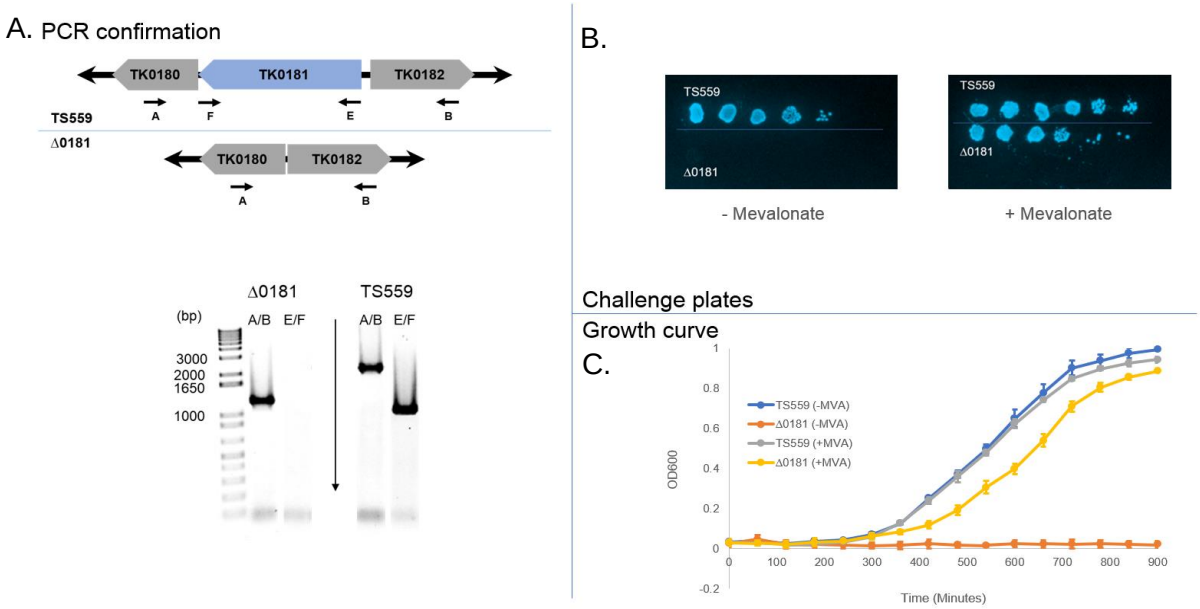
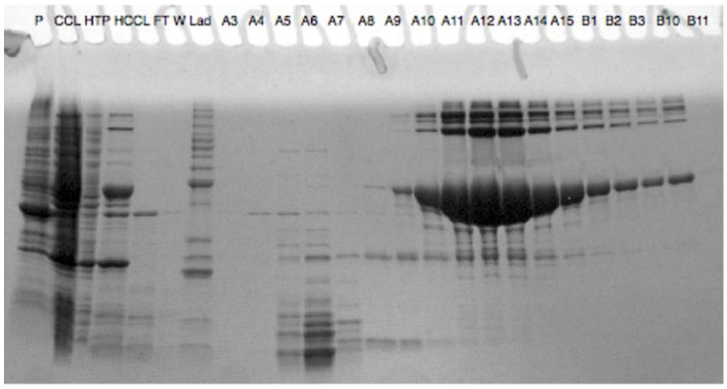


Figure 7. TK0181 is involved in the mevalonate pathway. (A) Diagnostic PCR was done on genomic DNA of Δ0181 strain and TS559 with external primers and internal primers (annealing locations showed). (B) Challenge plates with and without 1mM mevalonate were done on Δ0181 strain and TS559. (C) Growth curve on liquid media with or without 1mM mevalonate were done on TS559 and Δ0181 strain.

TK0914 from T. kodakarensis is a 3-hydroxy-3-methylglutaryl Coenzyme A reductase

In vitro enzymology study on recombinant enzyme TK0914 predicted to be a HMGCR in T. kodakarensis shows the correct activity (Figure 8). Recombinant TK0914 was reacted with HMG-CoA with the presence of either NADPH or NADH cofactor, with the prior showing higher activity. Enzymatic activity of the recombinant TK0914 is monitored by measurement of absorbance at 320 nm correlating to the disappearance of NADPH from the reaction. Measurements are taken at t=0 minute and t=5 minutes to get the difference of absorbance and natural decay of NADPH was subtracted from each of the absorbance reading.

A.



B.

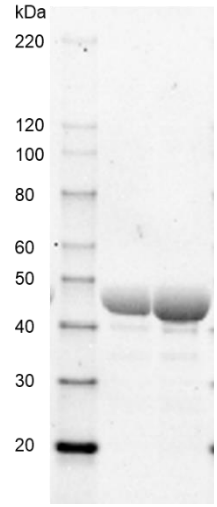
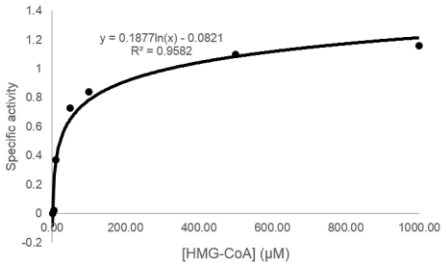


Figure 8. TK0914 purification samples ran on SDS-PAGE. (A) Samples were taken after every treatment and ran on SDS-PAGE. Fraction A10 through B10 were pooled and dialyzed. (B) Purified and dialyzed TK0914 ran on SDS-PAGE.

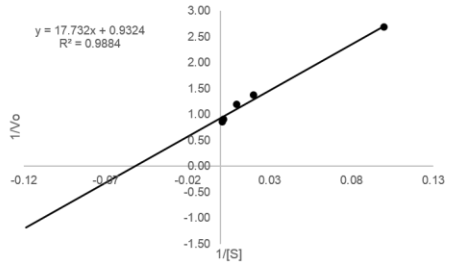
A. **(S)-3-hydroxy-3-methylglutaryl-CoA** + 2 NADPH = **(R)-mevalonate** + CoA + 2 $NADP^+$

A320nm

B.



C.



D.

HMG-CoA reductase	Km (μM)
T. kodakarensis	19
Sulfolobus solfataricus	17
Haloferax volcanii	60
Syrian hamster	4.3

Figure 9. TK0914 is a HMG-CoA reductase. (A) Chemical reaction catalyzed by TK0914 and followed by monitoring the decrease of absorbance of NADPH at 320 nm. (B) Specific activity of TK0914 changes depending on the concentration of HMG-CoA substrate. (C) Lineweaver-Burk plot used to derive Km of TK0914 towards HMG-CoA. (D) Km value of characterized HMG-CoA reductase compared to Km value of TK0914.

CONCLUSIONS

In summary, isoprenoid production are highly sought by biomanufacturing companies. However, there are challenges in producing these high valued isoprenoid compounds in common host platforms such as yeast and *E. coli* in high concentration. Two main challenges include 1) low isoprenoid precursor availability (Jongedijk et al., 2016) and 2) precursor toxicity at higher concentration (Sivy et al., 2011). Archaeal platforms gained some traction recently and presented as an interesting option as a potential host for industrial isoprenoid production host. Phytoene, an isoprenoid compound used as a carotenoid precursor, production in hyperthermophilic archaea (Fuke, Sato, Jha, Tansengco, & Atomi, 2018) and geraniol production in methanogenic archaea (Lyu et al., 2016) have recently been shown to be viable. Although synthetic enzymes have been successfully introduced into archaeal organisms, the exact biochemical pathway for isoprenoid biosynthesis in archaea is still unknown. Some of the steps involved in archaeal isoprenoid biosynthesis have been shown in this work. Thus, using archaeal platforms for isoprenoid production could be profitable in an industrial scale by targetting the enzymes used in this study.

We have succedded in deleting genes encoding the enzymes catalyzing the first three reactions in the mevalonate biosynthesis pathway. Based on the PCR, solid media challenge plates, liquid media growth curve results for Δ0180, Δ0181, and Δ0914 strains, the genes proposed to be encoding for acetoacetyl-Coenzyme A thiolase (TK0180), 3-hydroxy-3-methylglutaryl Coenzyme A synthase (TK0181), and experimentally characterized HMGCR encoding gene (TK0914) can be deleted from the genome of *T. kodakarensis* only under the presence of mevalonic acid. These results suggest that the three enzymes are indeed involved in the mevalonate pathway in *T. kodakarensis*.

To test the idea of leucine or lysine shunt, we have also tried to grow deletion strains under 5 and 10 times the normal amount of leucine and lysine and cells are still not showing

significant growth (Data not shown). The results of these experiments are consistent with the idea that the three genes corresponding to enzymes in the first three steps of the mevalonate pathway are essential for *T. kodakarensis* growth and that there is no leucine or lysine shunt. The probability of physiologically critical secondary pathway for isoprenoid biosynthesis involving the MEP pathway and leucine shunt in *T. kodakarensis* is unlikely, given that acetoacetyl-Coenzyme A thiolase (TK0180), 3-hydroxy-3-methylglutaryl Coenzyme A synthase (TK0181), and HMGCR encoding gene (TK0914) are essential. Based on these results, TK0180, TK0181, and TK0914 are the enzymes that should be upregulated to increase isoprenoid production in *T. kodakarensis.* Based on the human isoprenoid biosynthesis pathway, the production of mevalonate is the rate limiting step (Friesen & Rodwell, 2004). Thus, we propose that these enzymes are likely the best choice to upregulate for isoprenoid over-production.

This work has verified the metabolic steps and genes encoding the enzymes responsible in *T. kodakarensis* to get from Acetyl-coA up to mevalonate. However, the rest of the pathway remains largely uncharacterized. Through bioinformatics, we have identified potential genes encoding the enzymes responsible for catalyzing the remaining steps of the isoprenoid systhesis pathway. Initial analysis of TK1477 predicted to encode phosphomevalonate decarboxylase is not essential. This finding points to a potential for novel isoprenoid biosynthesis pathway used by *T. kodakarensis* to get from mevalonate to Isopentenyl pyrophosphate. Thus, more research is required to identify enzymes responsible for the remaining steps in isoprenoid synthesis pathway in *T. kodakarensis.*

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