### **Lycopene34 Ortholog Scan**

### This experiment evaluates two *ispA* orthologs in the lycopene-producing plasmid pLYC72, the top-performing construct from lycopene32. The *ispA* gene encodes farnesyl diphosphate synthase (FPPS), a key enzyme in the isoprenoid biosynthesis pathway that catalyzes the formation of FPP from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) [1]. FPP serves as a central precursor to multiple cellular metabolites, including quinones, dolichols, and carotenoids such as lycopene [2]. In engineered systems, lycopene production acts as a sensitive proxy for pathway flux and FPPS functionality. When *ispA* is expressed in a strain co-producing the carotenogenic enzymes *crtE*, *crtB*, and *crtI*, lycopene accumulates as a red pigment, allowing for visual and spectrophotometric quantification which is used in the assay portion of this experiment.

Four orthologs were selected:

* *Enterobacter hormaechei* (GenBank: CP115689)
* *Citrobacter amalonaticus* (CP041362.1)
* *Salmonella enterica* (CP074309.1)
* *Klebsiella electrica* (CP112887.1)

The functionality of the *ispA* gene in E.coli MG155 specifically encodes for farnesyl diphosphate synthase (FPPS), meaning our ortholog search was filtered to only *ispA* genes encoding for this enzyme. The four orthologs selected are a part of the same family as *E. coli*, the *Enterobacteriaceae* family. The goal of choosing orthologs from this common group of Gram-negative bacteria was to achieve high functionality success in the orthologs, since similar species will likely have smaller mutations.

Twenty different orthologs from an OrthoDB query from different bacteria families were tested using the BLASTp method for querying and sequence identity against the *ispA* *E.coli MG155* strain. Out of the queried organisms, the four selected fit the desired range of 70-95% identity matching and matched for the specific coding of FPPS. Since *ispA* is part of a large prenyltransferase family that has undergone extensive gene duplication and functional diversification, homologs can perform similar reactions but with different substrates. It was crucial to ensure our orthologs encoded for the same FPPS enzyme to optimize functionality results.

The *K. electrica ispA* gene contains an internal *BsaI* site, which prevents direct cloning via Golden Gate. To address this, a codon-modified version was synthesized using the *removeSites* function in C6-Tools and delivered in a gBlock fragment to then be assembled via Gibson. In contrast, the other three genes lack internal restriction sites and were amplified directly from genomic DNA. Both genes were inserted into the pLYC72 backbone in place of the native *E. coli ispA* to generate new lycopene pathway constructs.

**Constructs:**

* pLYC78: *E. hormaechei ispA* inserted via PCR
* pLYC79: *C.amalonaticus* *ispA*, inserted via PCR
* pLYC80: *S. enterica ispA*, inserted via PCR
* pLYC81: codon-modified *K. electrica ispA* gBlock*,* inserted via Gibson Assembly

All constructs were assembled using Golden Gate and transformed into *E.coli* *Mach1*. Resulting strains were tested for function based on colony pigmentation.

**Assay:**

1. **Transformation:**
   * The four ortholog minipreps were used to transform into *E.coli* *Mach1* again to assess against a positive control, the pLYC72 backbone, and negative control, unmodified, wild-type *Mach1* cells
2. **Visual Screening of Lycopene Production:**
   * Plates to be incubated at 28°C for 24 hours to allow lycopene accumulation.
   * Observe against a white background under ambient light conditions.
   * Red or deep pink pigmentation is an indicator of *ispA*-mediated FPP synthesis and lycopene yield.
3. **Quantitative Lycopene Assay with Spectrophotometry:**
   * Overnight cultures are grown in 2XYT containing ampicillin
   * Cells are harvested by centrifugation, and lycopene is extracted using an acetone:ethanol:hexane (1:1:2) mixture.
   * After phase separation, collect the top hexane layer and measure at 472 nm using a spectrophotometer [3].
   * Record OD600 measurements of each culture and normalize lycopene levels
4. **Data Analysis:**
   * Absorbance values and OD600 readings were used to calculate normalized lycopene output
   * Compare across different *ispA* ortholog constructs.

**Next Steps:** To interpret the results of lycopene production, variation likely reflects differences in enzyme efficiency, expression levels, or stability due to sequence divergence.To improve this ortholog scan for future experiments, more evolutionary diverse orthologs can be picked from different bacterial families to express a larger manifold, using a lower sequence identity hit threshold.

**References:**

1. Fujisaki, S., et al. (1990). Farnesyl diphosphate synthase: the key enzyme for isoprenoid biosynthesis. *J Biochem*, 108(6), 995–1000.
2. Rohmer, M. (1999). The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat Prod Rep*, 16(5), 565–574.
3. Kim, S.W., et al. (2016). Lycopene production via metabolically engineered *E. coli* using the MVA pathway. *Biotechnol Bioeng*, 113(9), 2103–2112.