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Examination of Isoprenoid Chain-Length Control in Corynebacterium glutamicum

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Title of Project: Examination of Isoprenoid Chain-Length Control in Corynebacterium glutamicum

Project Abstract:

Prenyltransferases catalyze the polymerization condensation reaction of isopentenyl diphosphate (IPP) and allylic acceptors of varying lengths. Throughout the prenyltransferases enzymes found in the family, Actinobacteria, there exist two catalytic DDxxD motifs in the amino acid sequence. The 4th and 5th amino acid residues before the first motif classically determine chain length of the isoprenoid product and, through manipulation of those amino acids, the resulting chain length can be altered based on the size of the amino acid residue. Corynebacterium glutamicum contains the conserved DDxxD motifs, but the upstream amino acids vary across the three predicted prenyltransferases. Additionally, the presence of a third prenyltransferase is uncommon in the genus. The goal of the project is to explore the naturally occurring variation encoded in the C. glutamicum genome. The one specific, well-conserved gene in C. glutamicum was cloned and manipulated using site-directed mutagenesis to model the paralogs. The mutant sequences were confirmed, and recombinantly expressed in Escherichia coli for the determination of the isoprenoid product, compared to the wild-type's 20-carbon chain product, geranylgeranyl pyrophosphate, or GGPP.

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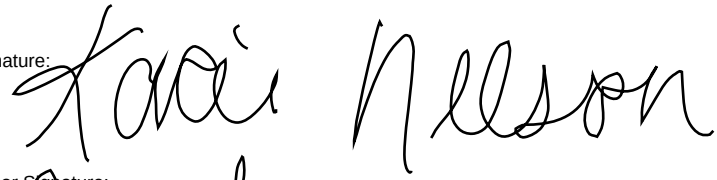
Applicant Signature: 

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Examination of Isoprenoid Chain-Length Control in *Corynebacterium glutamicum*

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Abstract

Prenyltransferases catalyze the polymerization condensation reaction of isopentenyl diphosphate (IPP) and allylic acceptors of varying lengths. Throughout the prenyltransferases enzymes found in the family, Actinobacteria, there exist two catalytic DDxxD motifs in the amino acid sequence. The 4th and 5th amino acid residues before the first motif classically determine chain length of the isoprenoid product and, through manipulation of those amino acids, the resulting chain length can be altered based on the size of the residue. *Corynebacterium glutamicum* contains the conserved DDxxD motifs, but the upstream amino acids vary across the three predicted prenyltransferases. Additionally, the presence of a third prenyltransferase is uncommon in the genus. The goal of the project is to explore the naturally occurring variation encoded in the *C. glutamicum* genome. The one specific, well-conserved gene in *C. glutamicum* was cloned and manipulated using site-directed mutagenesis to model the paralogs. The mutant sequences were confirmed, and recombinantly expressed in *Escherichia coli* for the determination of the isoprenoid product, compared to the wild-type's 20-carbon chain product, geranylgeranyl pyrophosphate, or GGPP.

Introduction

Isoprenoids are natural products with many important biological functions in Eukarya, Bacteria, and Archaea.[1] Some of the most common isoprenoids include steroids, carotenoids and rubber. They are made of five-carbon units, isopentenyl diphosphate (IPP) and its isomer, dimethyl-allyl diphosphate (DMAPP).[2] Prenyltransferases are enzymes that catalyze reactions involving IPPs and DMAPPs, resulting in isoprenoid products.[2]

Isoprenoids are formed by combining IPP units to DMAPP units, or other allyic acceptors, creating long chain products consisting of various numbers of five-carbon units.[3] The chemical reaction for the production of isoprenoids is a pyrophosphate ionization carbocation cascade reaction[4] as shown in Figure 1.[8]

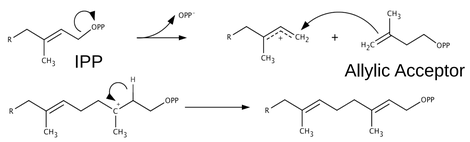


Figure 1

Prenyltransferases have two highly conserved catalytic motifs responsible for these reactions. The 4th and 5th amino acid residues before the first DDxxD motif determine product length, while the second DDxxD motif is responsible for IPP binding. It has been shown that manipulating the 4th and 5th amino acid residues before the DDxxD motif results in altered chain lengths based on the size of the amino acid present. The smaller the size of the amino acid residue, the longer the chain length of the product of the reaction.[2] For example, FPP synthase from species *Bacillus stearothermophilius* with tyrosine residue substitution results in 10-carbon chains. Also, heptaprenyl diphosphate synthase from species *Bacillus stearothermophilius* with alanine substitution will result in 40-carbon chains.[1]

Not all bacterial prenyltransferases follow these patterns for the determination of chain length. For example, *Mycobacterium tuberculosis (M. tuberculosis)* prenyltransferases do not necessarily contain the expected amino acid residues for an expected chain length of isoprenoid product.[5] *M. tuberculosis* is a member of the phylum *Actinobacteria,* which raises the question whether this is true for all *Actinobacteria*. *C. glutamicum* is also a member of the phylum *Actinobacteria* and possesses a prenyltransferase gene in excess compared to other members of the genus *Corynebacterium.* Being that a third prenyltransferase gene does not appear across the genus, *C. glutamicum* was chosen to explore these gene differences due to the potential for evolution of new functions. Examining the roles of the 4th and 5th amino acid residues prior to the first DDxxD motif in *C. glutamicum* will allow for determining whether other *Actinobacteria* exhibit similar trends compared to *M. tuberculosis.*

Experimental Methods

Site-Directed Mutagenesis. Using CLC Sequence Viewer (Qiagen, Germantown, MD, USA) protein sequences of *C. glutamicum* were analyzed. The 4th and 5th amino acid residues before the first DDxxD motif were manipulated during primer design to control the chain length of the gene. A polymerase chain reaction (PCR) using the designed primers was conducted in order to amplify the DNA using the KAPA HiFi DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA), creating a mutant sequence. A gel electrophoresis was used to determine whether the PCR reactions were successful. Samples were run in 1% agarose for approximately 45 minutes at 120 volts. A 500 bp DNA standard ladder, Lonza DNA ladder (Lonza Rockland, Inc., Rockland, ME, USA), was used for a comparative analysis of band size of PCR products. After confirmation that the PCR product was successful, DPN1 was added to digest the methylated wild-type DNA leaving only mutant DNA.

Transformation. The PCR product was transformed into chemically competent *Escherichia coli (E. coli)* cells in a pENTR vector with Kanamycin resistance.

Mini-Prep. A mini-prep procedure was used to purify and quantify DNA present. The transformed colonies were grown up in c5 mL cultures overnight, resuspended with a buffer composed of glucose, EDTA, and Tris-HCl. The cells were lysed with a buffer composed of 0.2 N NaOH and 1% sodium dodecyl sulfate. The samples were then neutralized with a sodium acetate buffer. Binding resin composed of 6M guanidine HCl and Diatomaceous Earth was used to collect the DNA. A wash buffer composed of 200mM NaCl, 20mM Tris-HCl, 5mM EDTA, and 50% ethanol was used to further purify the DNA. The resulting DNA products were quantified by Nanodrop (Thermo Fisher Scientific Inc., Waltham, MA, USA) data.

Sequencing. The mutant sequences were sent for sequencing (Iowa State University, Ames, IA, USA) to confirm the amplified DNA sequences.

LR Clonase Reaction. The two most conserved mutants, and the wild-type, were cloned into pDEST 14 and pDEST 17 vectors using LR Clonase II (Life Technologies, Waltham, MA, USA), adding proteinase K following the reaction to digest any proteins. The products were transformed into competent cells, followed by a mini-prep procedure to purify the DNA.

Transformation into C41 PLS Cells. The mutants and the wild-type, in each destination vector, were transformed into C41 PLS cells (*E. coli* cells) in pDEST 14 and pDEST 17 vectors with carbenicillin resistance.

Growth Cultures. These bacteria cells were grown in 5 mL NZY media cultures to saturation, and then transferred to 50 mL cultures. The 50 mL cultures were grown up until their absorbance, read at 600 nm, reached a value between 0.600 and 0.800. IPTG was added to each culture to induce protein expression. The cultures shook at 200 RPM at 16˚C for three days.

Hexane Extraction.[9] After 3 days, a hexane overlay was added and stored in a refrigerator for one day. The hexane layer was then extracted and dried. The samples were brought up to 100 µL volume for analysis on the GC/MS. The GC/MS method injected 5.0 µL of sample beginning at 50˚C for 5 minutes and increasing by 20˚ every minute thereafter, holding for 3 minutes at 150˚C, 200 ˚C, 250˚C, and 280 ˚C. The column was a 30 m x 0.250 mm, 0.25 µM (Agilent Technologies, Santa Clara, CA, USA).

Results

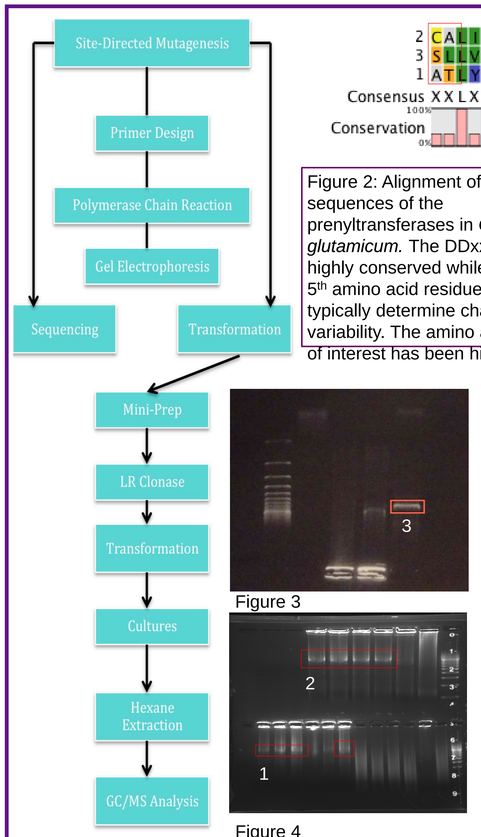


Figure 2: Alignment of the protein sequences of the prenyltransferases in *C. glutamicum.* The DDxxD motif is highly conserved while the 4th and 5th amino acid residues prior typically determine chain length variability. The amino acid sequence of interest has been highlighted.

Figure 3

Figure 4

Figure 3: The gel electrophoresis product from the original PCR, demonstrating that the DNA amplified with A118S mutation was present.

Figure 4: The gel electrophoresis product from the PCR with incorporated temperature gradient, demonstrating the DNA amplified with the A117C mutation and T118L mutation were present.

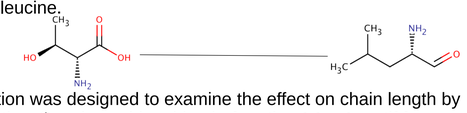
Results

Gene	4th Residue	5th Residue	Product[9]
1	Threonine	Alanine	20 Carbon
2	Alanine	Cysteine	N/A
3	Leucine	Serine	20-25 Carbon

Table 1: The wild-type's 4th and 5th amino acid residues before the catalytic DDxxD motif.

The 1st mutation was designed to examine the effect on chain length by manipulating the 5th amino acid residue before the DDxxD motif from alanine to cysteine.

The 2nd mutation was designed to examine the effect on chain length by manipulating the 4th amino acid residue before the DDxxD motif from threonine to leucine.



The 3rd mutation was designed to examine the effect on chain length by manipulating the 5th amino acid residue before the DDxxD motif from alanine to serine.

Conclusion

GC/MS data provided confirmation of the GGPP wild-type product in *C. glutamicum* prenyltransferase 1, while mutation of this gene to *C. glutamicum* prenyltransferase 1 T118L resulted in FPP. The FPP product result confirms the original hypotheses that the isoprenoid product chain length would shorten as a result of a larger amino acid substitution in the wild-type prenyltransferase protein sequence. The demonstration of GGPP product in the wild-type is interesting as other *Actinobactera* adhere to chain length specifications that would have us believe the chain length of the product would be much longer than 20-carbons long, which would also result in longer mutated sequences, when an amino acid substitution resulted in FPP, 15-carbons long.

Acknowledgements

I would like to acknowledge Dr. Francis M. Mann for guidance and assistance of this project for the past 2 years. I would like to thank and acknowledge Michael D. Allen for cloning the gene that was used throughout the entirety of the project. I would also like to acknowledge Winona State University's Chemistry Department for the use of their facilities and equipment. Lastly, I would like to thank Winona State University for the grant money that allowed me to conduct this research.

Results

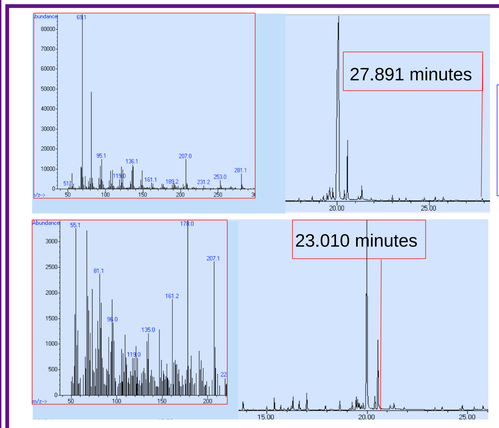


Figure 5:The resulting spectra of the wild-type *C. glutamicum* prenyltransferase 1 product, confirming the GGPP product.[7]

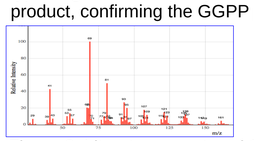


Figure 6: Literature spectra of GGPP.[7]

Figure 8: The resulting spectra of the *C. glutamicum* prenyltransferase 1 mutation T118L, resulting in the product, FPP, a 15-carbon chain.[7]

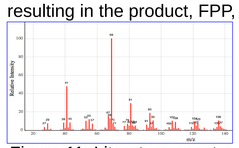


Figure 11: Literature spectra of FPP.[7]

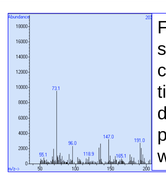


Figure 7: The resulting spectra of the C41 PLS cells at the same retention time, 27.891 minutes, demonstrating the GGPP product resulted from the wild-type.

Figure 9: Wild-type at 23.004 minutes.

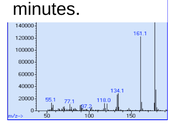


Figure 10: C41 PLS cells at 23.004 minutes.

Figures 9 and 10 demonstrate FPP as a product of the mutant, not made by the wild-type or the cells.

References

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