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# Investigation of Evolutionary Events in Corynebacteriaceae Involving Prenyltransferases in Corynebacterium glutamicum

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# Investigation of Evolutionary Events in Corynebacteriaceae Involving Prenyltransferases in *Corynebacterium glutamicum*

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## Abstract and Background

***Abstract:*** Terpenoids, a class of compounds with 30,000 unique structures, are known to include antitumor compounds, pigments, vitamins, antibiotics, and flavor molecules. Prenyltransferases are the primary enzyme responsible for assembling the carbon backbone of these compounds from five carbon isoprene units. Bioinformatic analysis has indicated that an evolutionary event has resulted in the acquisition of an additional prenyltransferase in *Corynebacterium glutamicum* (*C. glutamicum*) compared to the majority of the *Corynebacteriaceae*. This result suggests that *C. glutamicum* may produce a non-essential terpenoid compound, or secondary metabolite. Sequencing has confirmed the successful cloning of the aforementioned genes. Present work includes expressing these genes in *Escherichia coli*, isolating the protein product, and assessing the function in vitro. This data will be used to expand our understanding of the evolutionary path of *C. glutamicum* compared to other members of the genus. Continuing work will shed light on the function of the third gene for C. glutamicum, and understanding the role of terpenoids in the family *Corynebacteriaceae* and greater *Actinobacteria*.

***Background:*** The phylum *Actinobacteria* is diverse and contains many species with applications to human and animal health or industrial uses for the production of macromolecules. Within the phylum are a diverse group of genera. *Mycobacterium* hosts known pathogens such as *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Mycobacterium leprae*[1]. The genus *Nocardia* is home to species that produce bioactive molecules that serve as antibiotics, industrial enzymes or provide multiple drug resistances to the species[1]. Finally, *Streptomyces* is the source of many or our current antibiotics[1]. Due to the diversity present in this phylum and recently available genetic data, a closer look was warranted.

Terpenoids, are a class of compounds with 30,000 unique structures[2], that exhibit a wide range of biological functions due to their chemical diversity[3]. These compounds are of interest as antibiotics, hormones, anticancer drugs, insecticides, pigments, and flavor molecules[2-4]. A terpenoid is a hydrocarbon formed from 5-carbon isoprene units, assembled through condensation reactions that are enzymatically catalyzed by prenyltransferases[3]. These elongated chains can then be acted on by other enzymes to form cyclic structures.

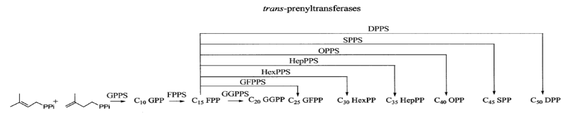


***Figure 1.*** Schematic diagram showing how 5C isoprene subunits are added linearly by different enzymes that are usually very specific in their substrate and product requirements[5]. Isopentenyl diphosphate (IPP) Dimethylallyl diphosphate (DMAPP) condense to form Geranyl diphosphate (GPP, C10), which condense with IPP or DMAPP again to form Farnesyl diphosphate (FPP, C15), which repeats the process to form Geranylgeranyl diphosphate (GGPP, C20). To form carbon chains above C20, FPP and GPP combine to make Geranylfarnesyl diphosphate (GFPP, C25). This process continues to build chains of any length, utilizing previously completed substrates[3,5].

## Experimental Methods

***Bioinformatics:*** A basic local sequence alignment (BLAST) was performed using the protein sequence from *M. tuberculosis* that encodes the Rv0562 prenyltransferase. Within the phylum, families were identified to be of interest based on the number of matches compared to this sequence. After identifying specific families with genetic data, the number of hits for the prenyltransferases sequence was examined to find species with higher than the average number of copies for the family, based on the available data. Literature was then searched for existing research to identify a single species with sufficient genetic data, and room for novel exploration. Percent identities were calculated[6].

***cDNA and Expression:*** *Corynebacterium glutamicum ATCC 13032* (ATCC, Manassas, VA, USA) was rehydrated and grown in liquid media to make a working stock using standard sterile techniques. Colonies were isolated from plates and RNA was extracted by Ribozol RNA Extraction kit (AMRESCO, Solon, OH, USA) according to the standard procedure. The extracted RNA was used to construct a cDNA stock by reverse transcriptase reaction. A standard PCR reaction using the KAPA HiFi HotStart kit (KAPA Biosystems, Boston, Massachusetts, USA) was carried out to amplify the specific genes of interest. The results were visualized by gel electrophoresis.

## Experimental Methods Cont.

***Cloning:*** *C. glutamicum* was grown to saturation in liquid culture and a phenol/chloroform extraction was used to gather a stock of genomic DNA (gDNA). Based on available genetic data, forward and reverse primers were designed to amplify the three prenyltransferase genes of interest, *Cglut1* (NCgl0456), *Cglut2* (NCgl2092), and *Cglut3* (NCgl0600). A PCR reaction was performed using the KAPA HiFi HotStart kit and the PCR products were visualized and purified by gel electrophoresis and recovered using gel extraction to create a stock. The products were then inserted into a pENTR vector using the procedures described in the Directional TOPO cloning kit (Invitrogen, *Carlsbad*, CA, USA), and the vector was inserted into chemically competent TOP10 *Escherichia coli* (*E. coli*) cells. The three cell lines were cultured and the plasmid was harvested using a miniprep system prepared in the lab. The prepared plasmid was sequenced (Iowa State University DNA Facility, Ames, Iowa, USA) to confirm successful cloning into the pENTR vector.

## Results

### Bioinformatics

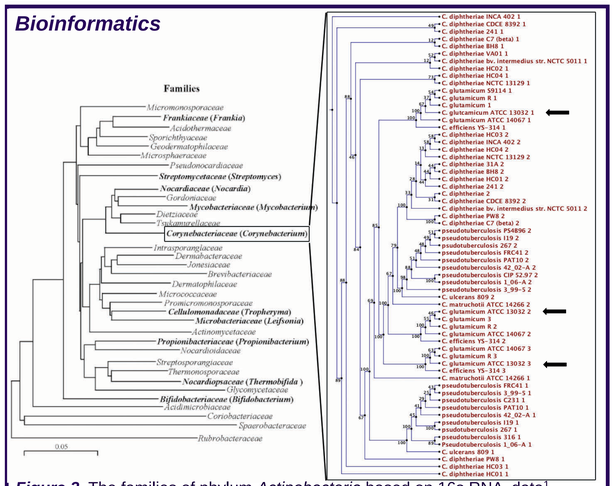


***Figure 2.*** The families of phylum *Actinobacteria* based on 16s RNA data[1]. Translated prenyltransferases based on genomic data was gathered from data available as of December 2012 for all genus in *Corynebacterium*. These sequences were aligned (gap open costs = 10.0, gap extension cost = 1.0) and a phylogenetic tree built (neighbor joining method, bootstrap = 100 replicates) to illustrate relationships between the prenyltransferase gene products. Based on the initial version of this tree, similarity between proteins was analyzed and a new tree constructed removing gene products with 100% similarity within species and clads (neighbor joining method, Jukes-Cantor distance measure, bootstrap = 100 replicates). Alignments and phylogenetic trees created in CLC Sequence Viewer 6 (http://www.clcbio.com)

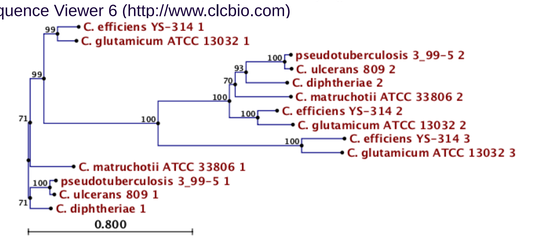


***Figure 3.*** Phylogenetic tree of putative prenyltransferase encoding genes from *Corynebacteria* species *diphtheriae, ulcerans, pseudotuberculosis, matruchotii, efficiens* and *glutamicum*. These results were interesting as there is noticeable conservation between all versions of Cglut1 with the same being true for Cglut2. However, Cglut3 which was unique to *C. glutamicum* and similar only to *C. efficiens 3* is separated from the other proteins.

The majority of *Corynebacteria* have only two copies of the prenyltransferase gene, while *C. glutamicum* has three. *C. glutamicum* is nonpathogenic and easy to work with, so it was decided to pursue exploration of why there was a third gene, and whether it was expressed or had been relegated to a non-coding region.

## Results Cont.

***Complementary DNA and Expression:*** The RNA extracted from *C. glutamicum* after an overnight culture yielded 142.32 ng/µl which was diluted and used for the reverse transcriptase reaction. This reaction was carried out in duplicate and resulted in yields of 1,653 ng/µl and 2,124 ng/µl.

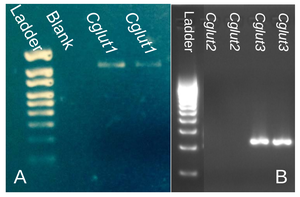


***Figure 4.*** Confirmation of gene expression by *C. glutamicum* was achieved by PCR using the cDNA template. A demonstrates amplification of *Cglut1* (1,053 bp). Repeating the experiment using the same cDNA stock presented similar results. B demonstrates the amplification *Cglut3* (1,119 bp), while *Cglut2* (1,116 bp) fails to amplify. These results were duplicated in a second experiment.

***Genomic DNA and Cloning:*** Genomic DNA was extracted with a concentration of 278 ng/µl, which was diluted and used for standard PCR reactions to produce fragments for use in downstream application of cloning.

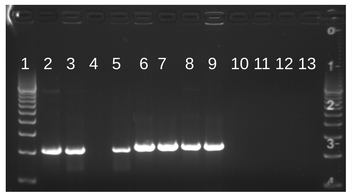


***Figure 5.***


***Figure 6.***

PCR amplification of the desired fragments was carried out. *Cglut1* and *Cglut3* presented no problem in amplification using standard procedures (Figure 6) where they were readily amplified and of the proper size. Lane 1 is a 500bp ladder. *Cglut1* in lanes 2-4, *Cglut3* in lanes 6-9 and the 16s control which didn't amplify in lanes 10-13. *Cglut2* required optimization of the PCR reaction which was achieved using a temperature gradient and then by increasing the number of cycles. The end result is visible in Figure 6, with a 500bp ladder in lane 1, and *Cglut2* in lanes 2 and 3.

These PCR products were purified successfully and transferred into pENTR vectors, which were then transformed into *E. coli* and grown for stocks. The presence of the genes were confirmed by sequencing, utilizing a total of 4 primers to complete the read, a promoter, the forward and reverse primers and a midpoint primer 300bp 3' from the forward primer.

After the contigs were assembled, the results indicated no abnormal results from the expected genetic sequences except in *Cglut1*. *Cglut1* had a point mutation at 761 C>T, resulting in P254L, which was confirmed in two separate cloning events (Figure 7). Using these pENTR vectors, pDEST17 vectors were prepared with the inserts of interest for later expression.

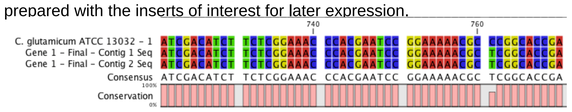


***Figure 7. Cglut1* Sequencing Results**


***Figure 8. pDEST17 PCR Results***

Figure 8 shows the results of the PCR using the pDEST17 vectors as the substrate, confirming the presence of *Cglut1* (lane 2), *Cglut2* (lanes 3, 4, 5) and *Cglut3* (lanes 6, 7, 8). *Cglut1* however showed banding, and was retransformed with greater success before making a stock.

## Discussion

The early bioinformatics work revealed that the majority of *Corynebacterium* have two copies of the putative prenyltransferase gene. This pattern was also true across *Actinobacteria*. The third prenyltransferase identified in *C. glutamicum* then hints at some evolutionary event that resulted in it gaining a third copy of the gene. Cglut1 shares 65%-82% similarity with homologs in other species as indicated in Figure 3. Similarly Cglut2 shares 53%-75% similarity. Cglut3 shares only 22-28% identity with any of the other proteins except for a homolog in *C. efficiens*, which appears to be its closest relative. Cglut3 may have resulted from an earlier evolutionary event that other species have since removed from their genome, or have occurred more recently where only a common ancestor to *C. glutamicum* and *C. efficiens* acquired this gene.

From these experiments (Figure 4a and b) it can be concluded that *Cglut2*, is expressed at much lower levels than *Cglut1* or *Cglut3*, if at all. This is interesting, as it has been shown in recent literature that both Clugt2 and Cglut3 produce C20 products *in vitro*[7]. Thus it seems more likely that one may be expressed only conditionally. Additionally the point mutation in Cglut1 is also interesting, as analysis showed that the residue resides between critical functional motifs which could result in a change of function compared to expected results.

Recently Cglut1 has been shown to produce a C45 – C50 product[7], which likely plays a role in quinone production. However, Clugt1 is also labeled as a geranylgeranyl pyrophosphate synthase, so there is still discrepancy among the literature. Cglut2 and Cglut3 both produce a C20 – C25 product[7] indicating there is a viable gene product but that it isn't being expressed in our cultures.

## Future Work

- Assay of Cglut1 wild type and point mutation mutants has been carried out. Please see poster A191 in this session for functional characterization and analysis.
- Examine *C. glutamicum* through metabolomic studies by creating knockout subspecies.
- Follow up lack of negative of *Cglut2* by exploring potential inducers of gene expression in *Corynebacterium*.
- Explore effects on product size or substrate specificity of the P254L mutation.

## Acknowledgements

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***Hover over fill-able fields for additional guideline and completion information.***

Student Name: Michael Allen

Student Email: mallen12@winona.edu

Student Major: ACS Biochemistry

Faculty Sponsor: Dr. Francis Mann

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Title of Project: Investigation of Evolutionary Events in Corynebacteriaceae Involving Prenyltransferases in Corynebacterium glutamicum

Project Abstract:

Terpenoids, a class of compounds with 30,000 unique structures, are known to include antitumor compounds, pigments, vitamins, antibiotics, and flavor molecules. Prenyltransferases are the primary enzyme responsible for assembling the carbon backbone of these compounds from five carbon isoprene units. Bioinformatic analysis has indicated that an evolutionary event has resulted in the acquisition of an additional prenyltransferase in Corynebacterium glutamicum (C. glutamicum) compared to the majority of the Corynebacteriaceae. This result suggests that C. glutamicum may produce a non-essential terpenoid compound, or secondary metabolite. Sequencing has confirmed the successful cloning of the aforementioned genes. Present work includes expressing these genes in Escherichia coli, isolating the protein product, and assessing the function in vitro. This data will be used to expand our understanding of the evolutionary path of C. glutamicum compared to other members of the genus. Continuing work will shed light on the function of the third gene for C. glutamicum, and understanding the role of terpenoids in the family Corynebacteriaceae and greater Actinobacteria.

The student-authored final report **MUST** include each of the following (check boxes to verify inclusion of each component):

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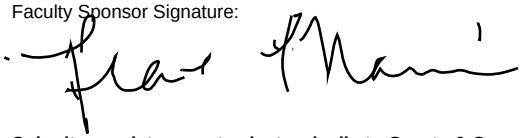

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