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Gene Encoding of Corneybacterium Glutamicum

Joseph Labeots
Winona State University

Michael Allen
Winona State University

Francis Mann
Winona State University

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Appendix C

RESEARCH / CREATIVE PROJECT ABSTRACT / EXECUTIVE SUMMARY
FINAL REPORT FORM

Title of Project
Gene Encoding of Corneybacterium glutamicum

Student Name Joseph Labeots and Michael Allen

Faculty Sponsor Dr. Francis Mann

Department Chemistry

Abstract
Antibiotics have been of great scientific and medical interest since the discovery of penicillin from a fungus. The research detailed below was done in order to determine similarities between genes of interest and to express them in order to determine their characteristics. Terpenoids are a class of compounds that have been shown in the past to exhibit antibiotic qualities such as is the case with terpentecin. First, bioinformatics was performed to determine similarities in genes that commonly encode for formation of terpenoids to those known to exist in the family Actinobacteriaceae. A BLAST analysis was done against the entire family with known proteins of interest. Corynebacteriaceae jumped out as a potential candidate with a vast number of hits as well as a diverse family. Further probing was done to determine what species within Corynebacteriaceae had sufficient genomic data as well as propensity to produce secondary metabolites of interest. Six species were found to be possible contenders, of which half were found to be pathogenic. Corneybacterium glutamicum(C. glutamicum) was found to be a worthy candidate due to its complete genome being sequenced as well as its production of terpenoids. The DNA of C. glutamicum was successfully extracted and purified. The genes of interest were then successfully amplified by PCR and confirmed by gel electrophoresis.

The end product of this project in electronic format has been submitted to the Provost/Vice President for Academic Affairs via the Office of Grants & Sponsored Projects Officer (Maxwell 161, npeterson@winona.edu).

Student Signature _____ Date _____

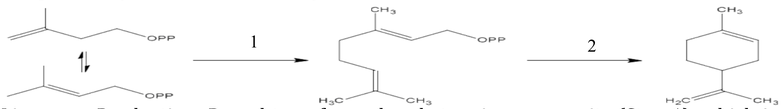
Faculty Sponsor Signature _____ Date _____

Gene Encoding of Corynebacterium glutamicum

Joseph Labeots, Michael Allen, and Francis Mann
Department of Chemistry
Winona State University

Introduction

Actinobacteriaceae are a family of bacteria that have been used, in the past, to produce antibiotics. Within this family, the genus, *Streptomyces* have been the most notable natural producer of antibiotics. They have accounted for the production of antibiotics such as tetracycline, daptomycin and streptomycin. Antibiotics have been of great scientific and medical interest since the discovery of penicillin from a fungus. Scientists discovered that penicillin was a secondary metabolite of a species of Penicillium. This discovery, around 1945, and many others since then has set the precedent for the vast use of antibiotics today[1]. The problem that many people in the scientific and medical community are now facing is that bacterial infections are starting to become resistant to many, if not all, antibiotics[1]. The discovery of new antibiotics exploded after the discovery of penicillin, however; since 1968, discovery of novel antibiotics has ceased. We propose that the reason for this is that they didn't have the necessary information about genome sequences that are available today. Additionally existing low throughput techniques slow the discovery of novel antibiotics that occur in small concentrations naturally. Finally, existing chemical libraries fail to capture the complexity of naturally occurring molecules[2]. With the greater availability of genetic data, it has become possible to search for antibiotic precursors by looking for specific, conserved, genetic sequences. Prenyl transferase, Class I cyclase and Class II cyclase are of particular interest, as they are responsible for building terpenoids, which are known to act as antibiotics. Prenyl transferase builds a terpenoid backbone, five carbons at a time, from isoprene units. The chain that results from this process will vary in length depending on the prenyl transferase used, and can then be further modified into cyclic compounds by Class I and Class II cyclases.



Limonene Production: Prenyl transferase bonds two isoprene units (Step 1), which is then cyclized with a Class I Cyclase (Step 2)[3].

It is known that antibiotics, steroids and other compounds are common metabolites of terpenoids. The purpose of this research was to use the now available genomic information and investigate terpenoids within a species of interest. Bioinformatics as well as sequence and functional analysis were used to determine the involvement of putative isoprenoid biosynthetic enzymes in the metabolism of terpenoids in *Actinobacteriaceae*.

Methods

Bioinformatics:

The BLASTp protein database was used to identify the genus *Corynebacterium* of the family *Actinobacteriaceae* as containing a large number of matches to the known prenyl transferase Rv0562. The CLC Sequence Viewer 6 software package was used to apply bioinformatic techniques to construct alignments and phylogenetic trees to identify species of Corynebacteria for further study, by examining the number of prenyl transferase genes present and species relations.

gDNA Extraction, Purification and, Replication:

C. glutamicum ATTC #13032 cells were acquired and rehydrated per ATTC instructions. The genomic DNA was then extracted and purified using of a 50:50 phenol:chloroform extraction protocol. This method results in a lower organic phase that captures the proteins and upper aqueous phase that captures the DNA. The DNA was then precipitated using ethanol to decrease the dielectric point which causes the DNA to bind cations[4]. UV-VIS spectroscopy was then used for qualitative as well as quantitative analysis of the DNA. Primers, 30 nucleotide long for each gene sequence, were created in forward and reverse fashion for use in a PCR. The primers were then diluted and used in a KAPA HiFi PCR (25µL reaction) for amplification of the genes

Reagent	5X KAPA HiFi Buffer	10mM dNTP mix	Forward Primer	Reverse Primer	Template DNA	KAPA HiFi DNA Polymerase
Final Concentration	1X	0.3 mM	0.3 µM	0.3 µM	50ng	0.5U/rxn

Methods Cont.

Thermo cycler temperatures were chosen according to melting point values of the primers. Gel electrophoresis was then performed to identify success or failure of the amplification. Once amplification was confirmed, competent E. *coli* cells were made for recombination. The genes were cloned into the E. *coli* DNA vectors using a Gateway TOPO cloning reaction. These recombinant DNA vectors were then plated for further analysis.

RNA Extraction and cDNA Replication:

A two-step process was used to generate cDNA from bacterial cells. The first step utilized Ribozol RNA Extraction reagents and procedures. Cells were lysed while suspended in Ribozol RNA Extraction Reagent (phenol solution). Chloroform was added to create distinct phases, allowing the extraction of RNA. Isopropyl Alcohol was used to precipitate the RNA, which was washed with a 75% Ethanol solution. The RNA was quantified using nanodrop spectroscopy. In the second step, a reverse transcription reaction was carried out. A 20µL reaction was set up with the following reagent concentrations, and sterile water was added to achieve volume:

Reagent	$MgCl_2$	RT 10X Buffer	dNTP	Random Primers	RNA	Reverse Transcriptase
Final Concentration	25mM	10X	10mM	500µg/mL	1µg	0.5µL

The cDNA produced from this process was then quantified by nanodrop, and treated with RNAse by overnight incubation. The RNAse was broken down by with the addition of proteinase and a stock of 50ng/µL cDNA was prepared. A 25µL reaction was setup using the same KAPA HiFi concentrations. The PCR reactions were carried out utilizing the cDNA recovered from cultured cells to test for the expression of the three genes of interest, as well as the 16s RNA gene for control purposes. Gel electrophoresis was again utilized to analyse the success of this procedure.

Results

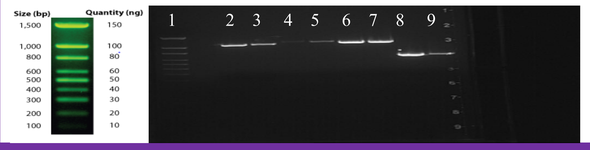
A prenyltransferase from *Mycobacterium tuberculosis*, Rv0562, which is known for its role in terpenoid elongation, was used for the comparison to *Actinobacteriaceae*. As stated in the introduction, many genera came back with hits for the protein production within their Genome[5]. the genera *Corynebacterium* was found to be a great candidate with a diverse family, resulting in 226 raw hits for closely conserved matches to the Rv0562 protein, of which, 48 were unique. The available data through BLASTp pointed to *C. diptheriae*, *C. efficiens*, *C. glutamicum*, *C. matruchotii*, *C. pseudotuberculosis* and *C. ulcerans* as species with enough information to warrant investigation. *C. glutamicum* was chosen to work with in the lab due to accessibility, lack of pathogenic traits, availability of culturing media, and the presence of three genetic regions that code for prenyl transferases[5]. Once C. *glutamicum* was selected, a representative cell line was ordered (ATCC #13032), rehydrated and a successful culture was grown in the lab. The gDNA extraction using the phenol-chloroform procedure resulted the successful acquisition of a suitably pure (Table 2) gDNA stock for use in further experiments.

Table 2: Nanodrop Spectroscopy Results-gDNA Extraction

260/280	260/230	Concentration
1.79	1.70	312.5ng/µL

The first gel that was run to confirm success of the PCR showed success for genes 1 and 3 but a slight failure at amplifying gene 2. Gene 2 completely failed in one of the lanes and only showed up slightly on the other. Gene 2 was put at a lower temp and

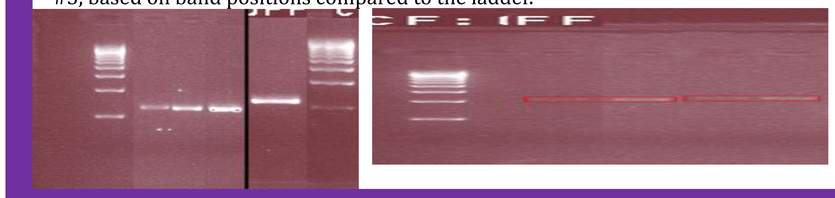
the successful PCR is shown by the gel below:



Lane #	Content
1	Ladder
2 and 3	gene 1 (1053bp)
4 and 5	gene 2 (1116bp)
6 and 7	gene 3 (1119bp)
8 and 9	RNA

All ran the gel similar to the 1000 base pair sample on the ladder which corresponds well with the size of all genes. An attempt was made at cloning the genes into a competent E. *coli* vector for plating and analysis purposes but was unsuccessful due to procedure error.

The extraction of RNA from a *C. glutamicum* culture was similarly successful. The results from the nanodrop analysis indicates successful extraction of RNA followed by the successful reverse transcriptase reaction to build cDNA. The resulting cDNA was then used to run a PCR reaction to confirm the expression of the genes of interest, which resulted in mixed success. The PCR reaction was run on four different samples of cDNA. The presence of strong 16s RNA bands in the UV-Vis of the gel allows the confirmation of *C. glutamicum* genetic material. Additionally, it can be concluded that gene #1 was amplified, as well as at least one of either gene #2 or #3, based on band positions compared to the ladder.



Conclusion

Much was learned about the terpenoid related genes of interest for C. *glutamicum*. They were successfully extracted and purified with phenol:chloroform extraction and ethanol precipitation. They were also successfully amplified using PCR and this was confirmed with gel electrophoresis. In addition, 2 out of the 3 genes were confirmed to be expressed by reverse transcription of RNA and analysis of the cDNA. An attempt was also made at introducing them to E. *coli* cloning vectors but was unsuccessful due to a procedure error. It is not known if the strain of bacteria is actually toxic to E. *coli* or if it is feasible to use E. *coli* vectors.

Further work

Further research could be done to determine what type of terpenoids are being produced here. It could also be researched as to the use of these terpenoids in the secondary metabolism of C *glutamicum*.

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Acknowledgments

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