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#### Determination of Methods to Separate and Analyze Small and Large Chain Isoprenoids from Mycobacterium tuberculosis

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### ABSTRACT

The genes responsible for the isoprenoid carrier lipids involved in cell wall biosynthesis of Mycobacterium tuberculosis (M.tb) are believed to be Rv2361c, Rv1086, and Rv0989c. These genes encode prenyltransferases catalyzing production of isoprenoids of increasing length. Rv0989c and can work in tandem with Rv1086 to create precursors to decaprenyl diphosphate, the final product of Rv2361c. Rv2361c can also work by itself to produce decaprenyl diphosphate. In addition to Rv2361c, Rv1086, and Rv0989c, M.tb encodes seven total different prenyltransferases, and in other biochemical studies it has been suggested that these genes may provide redundancy with the isoprenoids that are formed. These genes can be expressed in *Escherichia coli* and the isoprenoids extracted through lipid extraction. The samples are then dephosphorylated using alkaline phosphatase. Once dephosphorylated these samples can be analyzed using HPLC in order to separate and analyze the isoprenoid products. HPLC separation occurs with a mobile phase of water and an increasing gradient of 60:40 isopropanol:methanol at a rate of 1 mL/min. These samples are ran over a C18 column. The HPLC allows us to view the different products that may be formed during errant metabolism when alternate isoprenoid precursors are available. Continuing work with these products will allow us to see the influence of each gene with the other genes present in M.tb in the formation of decaprenyl diphosphate.

### INTRODUCTION

In *Mycobacterium tuberculosis* (M.tb) a significant portion of metabolism is devoted to building the cell wall and synthesize polyprenyl phosphate (Pol-P).<sup>2</sup> Escherichia coli (E.coli) naturally produces five isoprenoids, dimethylallyl pyrophosphate (DMAPP), isopentenyl diphosphate (IPP), farnesyl diphosphate (FPP), octaprenyl diphosphate (OPP), and undecaprenyl diphosphate (UPP).<sup>5</sup> The production of these isoprenoid products are catalyzed by prenyltransferases.

### Figure 1. Synthesis of FPP via IPP and GPP

In this research E.coli is used as a host for expression of prenyltransferases from M.tb. Prenyltransferases work together by building off of the products of the enzymatic reaction, by adding on a group of five carbons each time. Multiple enzymes can work in succession to produce isoprenoids of varying length and stereochemistry. An example of this is difference between Rv3398c and Rv1086, these are both FPP synthases in M.tb, however Rv3398c synthesizes *E*,*E*-FPP while Rv1086 synthesizes *Z*,*E*-FPP.<sup>2</sup> In addition to these genes, Rv0989c is a GPP synthase,<sup>2</sup> and both Rv0562 and Rv3383c are GGPP synthases.<sup>1</sup> IPP, DMAPP, and FPP are all short chain isoprenoids that be used as substrates for further elongation by prenyltransferases.<sup>5</sup> Rv1086 and Rv2361c produce a decaprenyl diphosphate from IPP and GPP.<sup>3</sup> However, Rv2361c can also work with other prenyltransferases, such as Rv0562, Rv3398c, and Rv0989c.<sup>1, 2</sup> Despite the apparent variety of potential reactions between prenyltransferases and substrates, M.tb has a relatively narrow range of isoprenoid products. These include: IPP, DMAPP, Z, E-FPP, E, E-FPP, GPP, E, E, E-GGPP, GGPP, farnesol. geranylgeraniol, and differing decaprenyl diphosphates.<sup>4</sup> The goal of the research is to characterize flux through isoprenoid biosynthetic pathways in M.tb. In order to perform this metabolomics analysis, new methodology for separation and identification of isoprenoid products was necessary. HPLC chromatography was used to separate the small and large chain isoprenoids that are formed by different combinations of the enzymes present in M.tb.

# **Determination of Methods to Separate and Analyze Small and** Large Chain Isoprenoids from Mycobacterium tuberculosis **Allison Guthrie and Francis M. Mann** Department of Chemistry, Winona State University, Winona, Minnesota

## **EXPERIMENTAL METHODS**

### Cell Transformation:

Cell transformations were performed on selected genes from M.tb: Rv0562, Rv1086, Rv2361c, and Rv3383c. Rv1086 were characterized and cloned by Crick et. al at Colorado State University.<sup>4</sup> Rv2361c was cloned at Winona State University by Dr. Francis Mann. Rv0562 and Rv3383c were characterized at Iowa State University by Mann et. al.<sup>1</sup> Rv2361c was in the DEST 17 vector, while Rv0562, Rv1086, and Rv3383c were in the ACYC vector. The constructs were combined in C41 PLS cells (Lucigen Corporation, Middleton WI) and left to sit for 30 min on ice. They were then heat shocked for 40 seconds at 42°C, and then fed NZY media and placed on a shaker for one hour at 37°C, after shaking the mixtures were plated on NZY agar plates containing the following antibiotics: 50 µg/mL chloramphenicol, 50 µg/mL carbenicillin, or a mixture of the two.

### Liquid Cultures:

Liquid cultures were then made by taking one colony from each plate and inoculating it with sterile NZY media and the appropriate antibiotic. These were shaken at 37°C at 220 rpm and grown to saturation for approximately 16 hours. New cultures were then created by inoculating the saturated cultures by inoculating 3 mL of the saturated culture into 100 mL of sterile NZY media, antibiotic, and 0.05% Tween-20. These were then incubated at 37°C at 220 rpm to grow to an optical density  $\sim 1$  at 600 nm. Upon completion, the samples were chilled and 0.5 mM of IPTG was added to the samples, which were then placed back on the shaker at 16°C at 220 rpm and left to shake for three days. 0.05 M sodium pyruvate was added 24 hours after protein expression was induced. After the three days, the cells were pelleted at 5000xg.

Methanol Chloroform Extraction:

The cell pellet was resuspended in 5 mL 0.5 M NaCl. The cells were then transferred into glass test tubes, and mixed with 6 mL 2:1 chloroform:methanol. The samples were then centrifuged for 10 min at max speed. Once centrifuged the top methanol layer was extracted. The extraction was repeated with 2 mL of methanol. The methanol layers were combined and dried to completion. Alkaline Phosphatase:

After the samples were dry, 3 mL sterile H<sub>2</sub>O, 0.3 mL optizyme buffer, and 10 µL alkaline phosphatase (provided by Fisher BioReagents, Loughborough, UK) was added to the dried samples. The samples were then incubated at 37°C for 16 hours while being shielded from the light.

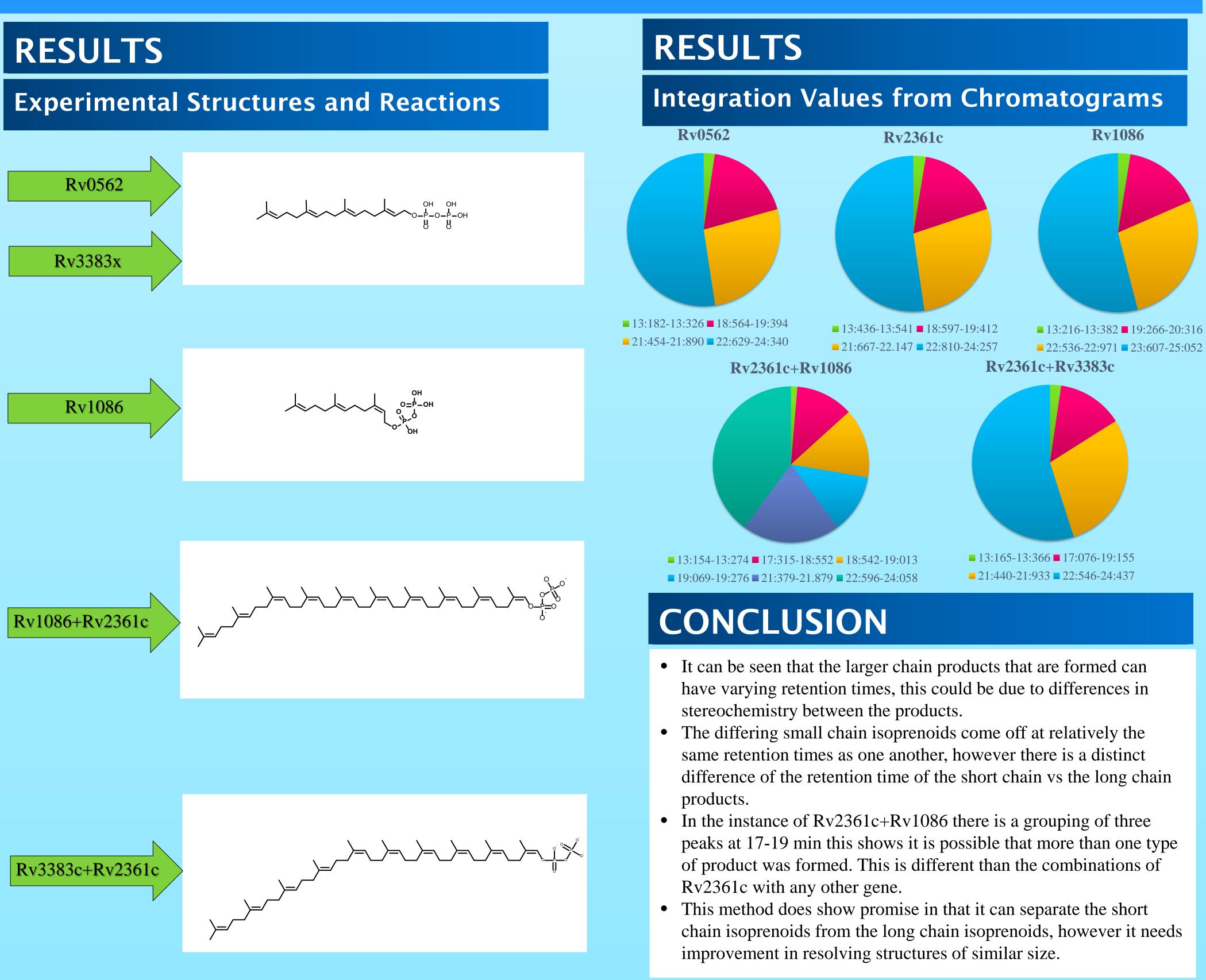
Hexane Extractions:

The samples were then washed with hexanes five times each. The hexane layers were then dried to completion. After drying, 1 mL of methanol was placed into each sample and vortexed. The methanol samples were then analyzed using HPLC chromatography.

#### HPLC Method:

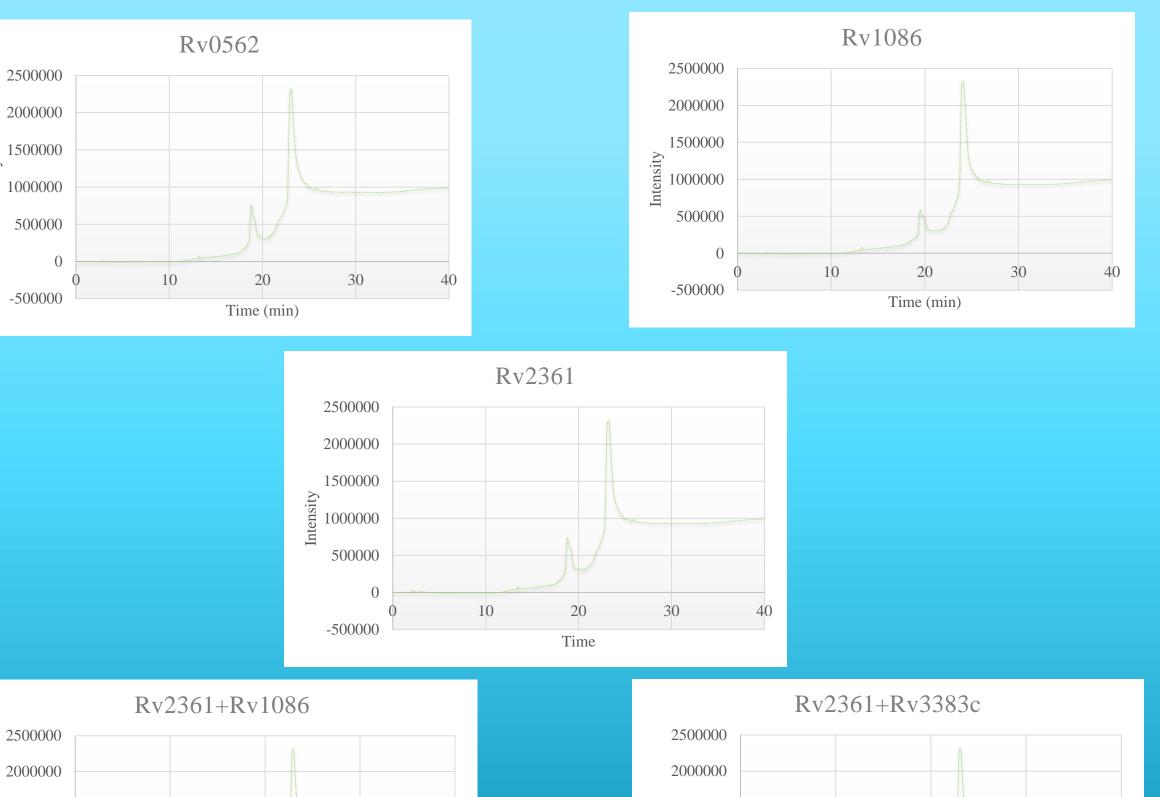
The instruments that comprise the HPLC used are: DGU-14 A Shimadzu Degasser, LC-10 AT Shimadzu Liquid Chromotography, SPD-10AV VP Shimadzu UV-Vis Detector, and SCL-10A VP System Controller. The column used is an Agela Technologies column, 5  $\mu$ m, 100 Å, 4.6×250 nm with a detection at 210 and 280 nm.

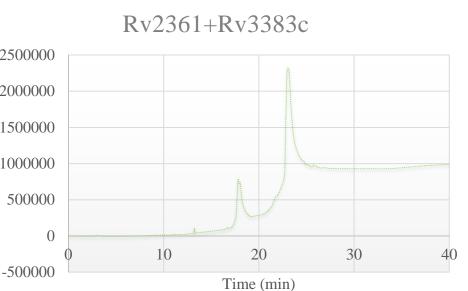
HPLC Method				
Pump A	Filtered H <sub>2</sub> O	Time	Command	Value
		Program		
		0.01	Pump B	0
			Conc.	
Pump B	60:40	5.00	Pump B	0
	Isopropanol:Methanol		Conc.	
		20.00	Pump B	100
			Conc.	
Injection	300 µL	40.00	Pump B	100
Volume			Conc.	
SPD-10 Avpλ	210 and 280			



### **Reaction Chromatograms**

Time (min)







## ACKNOWLEDGEMENTS

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