U.S. ARMY

U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER
ABERDEEN PROVING GROUND, MD 21010-5424

DEVCOM CBC-TR-1855

Characterization and Production of the *Coxiella burnetii* Specific O-Antigen Carbohydrate Virenose in Engineered *Escherichia coli*

Nathan D. McDonald
Sarah E. Katoski
RESEARCH AND OPERATIONS DIRECTORATE

Erin E. Antoshak
EXCET, INC.
Springfield, VA 22150-2519

October 2023

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED	
		START DATE	END DATE
XX-10-2023	Final	Oct 2019	Sep 2021

4. TITLE AND SUBTITLE
Characterization and Production of the *Coxiella burnetii* Specific O-Antigen Carbohydrate Virenose in Engineered *Escherichia coli*

5a. CONTRACT NUMBER	5b. GRANT NUMBER	5c. PROGRAM ELEMENT NUMBER
		ILIR PE 0601101A Project 91A
5d. PROJECT NUMBER	**5e. TASK NUMBER**	**5f. WORK UNIT NUMBER**

6. AUTHOR(S)
McDonald, Nathan D.; Katoski, Sarah E. (DEVCOM CBC); and Antoshak, Erin E. (Excet)

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Director, DEVCOM CBC, ATTN: FCDD-CBR-BM, APG, MD 21010-5424
Excet, Inc.; 6225 Brandon Avenue, Suite 360, Springfield, VA 22150-2519

8. PERFORMING ORGANIZATION REPORT NUMBER
DEVCOM CBC-TR-1855

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)	11. SPONSOR/MONITOR'S REPORT NUMBER(S)
U.S. Army Combat Capabilities Development Command Chemical Biological Center In-house Laboratory Independent Research Program; 5183 Blackhawk Road, Aberdeen Proving Ground, MD 21010-5424	DEVCOM CBC ILIR	

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution Statement A. Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The Gram-negative bacterium *Coxiella burnetii* is the causative agent of Q fever and has been long regarded as a potential biological warfare agent. Only the phase I strains of *C. burnetii* are considered pathogenic, and these strains bear a unique O-antigen in the lipopolysaccharide. This O-antigen contains the carbohydrate virenose, which is only found in phase I *C. burnetii* strains, making it a unique biomarker for Q fever. Despite this, the O-antigen carbohydrate virenose remains poorly understood. Virenose serves as a valuable target for detection assays as well as a potential antigen in *C. burnetii* vaccine development. Although comparative proteomics analysis studies for the biosynthesis of D- or L-enantiomers of virenose in silico have identified a putative biosynthetic route, the complete biosynthetic pathway of virenose has yet to be experimentally determined. We hypothesize that L-virenose biosynthesis can be validated using a non-native host through synthetic engineering of the predicted L-virenose pathway. Here we leverage synthetic biology to characterize the virenose biosynthesis pathway in *E coli* and assess the ability of this engineered strain to produce the carbohydrate.

15. SUBJECT TERMS
Synthetic biology *Coxiella* Virenose Lipopolysaccharide

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES
a. REPORT	b. ABSTRACT	C. THIS PAGE		
U	U	U	UU	24

19a. NAME OF RESPONSIBLE PERSON	19b. PHONE NUMBER *(Include area code)*
Renu B. Rastogi	(410) 436-7545

STANDARD FORM 298 (REV. 5/2020)
Prescribed by ANSI Std. Z39.18

Blank

PREFACE

The work described in this report was authorized by the U.S. Army Combat Capabilities Development Command Chemical Biological Center (Aberdeen Proving Ground, MD) and funded via the in-house Laboratory Independent Research Program. The work was started in October 2019 and completed in August 2021.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

Acknowledgments

The authors extend appreciation to Dr. Christopher Boddy (University of Ottawa; Ottawa Canada) for generously providing pMGX plasmids in support of this technical effort.

Blank

CONTENTS

FIGURES

TABLES

Characterization and Production of the *Coxiella burnetii* Specific O-Antigen Carbohydrate Virenose in Engineered *Escherichia coli*

1. INTRODUCTION

Lipopolysaccharides (LPSs) are a component of the outer membrane of Gram-negative bacteria and play a key role in pathogenesis. The LPS molecule can generally be divided into three distinct domains, namely, the lipid A, core oligosaccharide, and O-antigen, each of which possess unique characteristics and properties (Figure 1). Although there are exceptions, one lipid A unit is composed of a linked D-glucosamine disaccharide backbone that is phosphorylated at positions 1′ and 4′ of the carbohydrates.[1] The backbone is acylated with branching fatty acid chains of varying lengths and substitutions, depending on the species. This main unit of lipid A can be further modified by various additions including phosphates, carbohydrates, and other small molecules, which can alter the overall charge. Together, it is the lipid A unit that is responsible for the endotoxic properties of LPSs by activation of the innate immune system via recognition by toll-like receptor TLR4.[1]

Moving outward, linked to the lipid A is the core oligosaccharide domain of the LPS. The function of the core oligosaccharide is to provide increased rigidity and structure to the outer leaflet of the Gram-negative bacteria through cationic interactions. Gram-negative bacteria link the core oligosaccharide to the lipid A via a common carbohydrate, Kdo. The majority of Gram-negative bacteria possess a heptose–heptose–Kdo trisaccharide unit as the core composition; however, exceptions to this have been identified. The core oligosaccharide, much like the lipid A, can also be further modified with substitutions. The most common substitutions to the core oligosaccharide include phosphates, additional carbohydrates, and in some cases, amino acids. The addition of these small molecules can dictate various interactions between the core oligosaccharide and the environment. Together, the lipid A and the core oligosaccharide represent the minimal components necessary for bacterial survival.

The O-antigen is the final, most outwardly facing unit of LPS. It is composed of monosaccharides assembled into units that can be repeated between 1 and over 100 times, making it the most genetically and structurally diverse component.[1] The structural diversity of O-antigens has allowed for researchers to classify strains within a species by serotyping, which can result in hundreds of variants, as is the case with *Escherichia coli* and *Vibrio cholerae*. The structural heterogeneity is a result of dramatic variations within the O-antigen biosynthesis gene clusters of these bacteria. In many cases, the bacteria can regulate the expression of O-antigen genes and thereby cause structural changes upon exposure to certain environments, such as during infection of a human host. This is known as phase variation. Alternatively, prophages can carry biosynthetic pathways and insert within the genome, rapidly changing display structures.

Together, the overall structure of the LPS in each organism can have profound effects. This macromolecule can itself serve as a virulence factor by (a) masking a pathogen to evade the immune response, (b) rendering the bacterial cell resistant to antimicrobials both natural and synthetic, (c) causing a dramatic immune response that can lead to rapid septic

shock, and (d) distinguishing the bacteria for identification and detection. Because of each of these features, the LPS has been characterized for numerous pathogens, including many of those that are select agents associated with biological warfare threats. In addition, because of the significance of the LPS, the genetics and biogenesis pathways for each of the domains have been well defined across hundreds of species.

Coxiella burnetii is a Gram-negative, intracellular pathogen that causes the disease Q fever. Because of its pathogenicity, the lack of medical countermeasures, and its previous development as a bioweapon, *C. burnetii* is considered a select agent by the Centers for Disease Control and Prevention (Atlanta, GA). The unique mechanisms of immunity and pathogenicity of *C. burnetii* are not fully understood, hindering advances in early diagnosis, treatment, and prevention of infection. One specific virulence factor known to be associated with the pathogenesis of *C. burnetii* is the presence of a unique LPS. The *C. burnetii* LPS has the ability to undergo irreversible antigenic (or phase) variations, defined as phase I and phase II strains (Figure 1).[2,3] These phase variations present a unique antigen or immunogen shift that is particularly valuable for differentiation between the chronic, less infectious phase II strain and the more acute, infectious phase I strain.[2,3]

The outer carbohydrates of the LPS, known as O-antigens, represent the more-variable section of the LPS and are implicated in *C. burnetii*'s antigenic specificity.[2] The rare O-antigen carbohydrates virenose and dihydrohydroxystreptose are major virulence determinants unique to *C. burnetii*. Virenose, an O-antigen carbohydrate found only in the virulent phase I strain of *C. burnetii*,[4] is a methylated-6-deoxyhexose that serves as a unique biomarker for the disease (Figure 1).

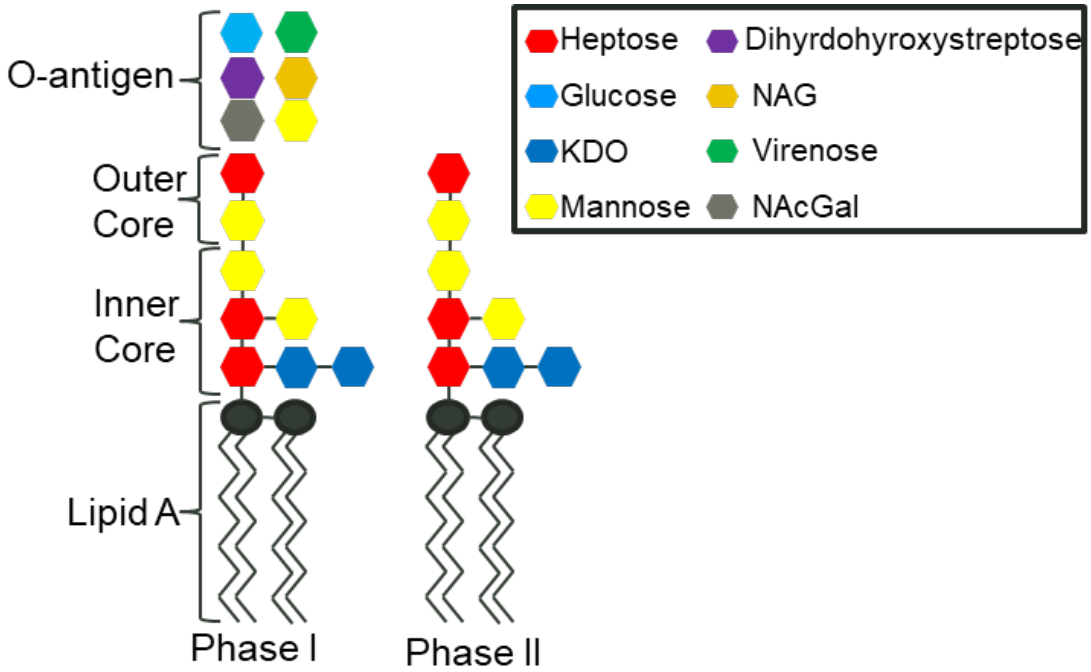

Figure 1. LPS structure of phase I and phase II *C. burnetii.* NAG, *N*-acetylglucosamine; KDO, 3-deoxy-d-manno-oct-2-ulosonic acid; and NAcGal, *N*-acetylgalactosamine.

Only the virulent phase I isolates of *C. burnetii* bear this O-antigen; however, despite its uniqueness, the complete biosynthetic pathway of virenose has yet to be experimentally determined.

In chromosomal deletion studies of key LPS biosynthesis genes of phase II isolates of *C. burnetii,* nine genes were identified that are predicted to be involved in the biosynthesis of virenose and dihydrohydroxystreptose.[4–6] Additionally, a study using comparative proteomics in silico analysis showed the biosynthesis of D- or L-enantiomers of virenose is likely composed of five enzymatic steps. Based on the genes associated with the chromosomal changes in phase II strains of *C. burnetii*, the pathway predicted was most likely that of L-virenose (Figure 2).[6] We hypothesize that the putative *C. burnetii* L-virenose pathway can be synthetically engineered into *E. coli* to validate the biosynthetic pathway and provide a source of recombinant virenose as a marker for *C. burnetii* detection.

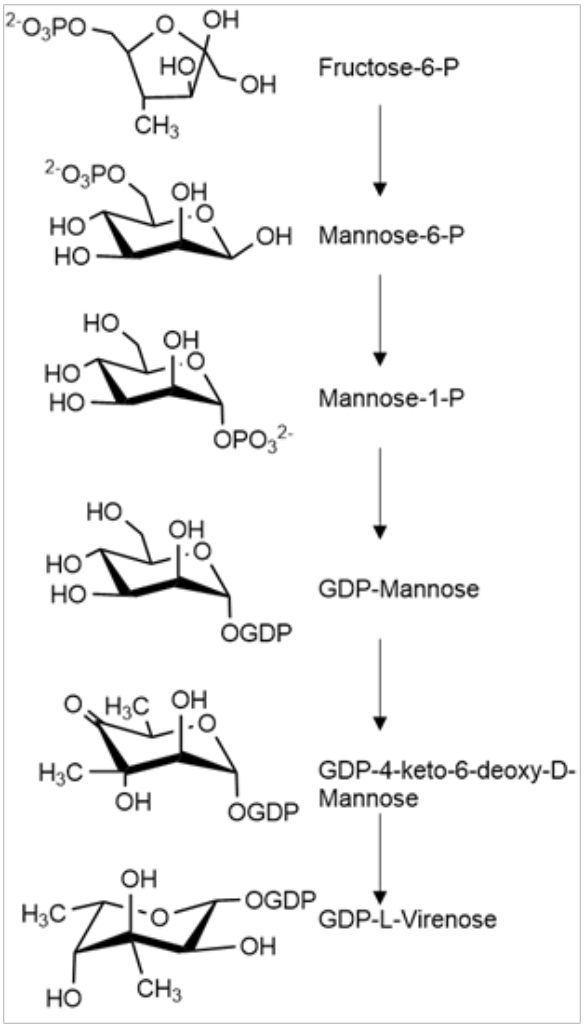

Figure 2. Predicted biosynthetic pathway of guanosine diphosphate (GDP)-L-virenose.

Work in the fields of synthetic biology and genome engineering has facilitated the identification and characterization of biosynthetic pathways of countless molecules of interest. The ability to produce non-native products in engineering-conducive organisms such as *E. coli* or *Saccharomyces cerevisiae* has been described in many cases.[7,8] The use of engineered biosynthesis pathways can be cost effective, as these are often more efficient than complex multistep organic synthetic routes. Furthermore, many microbial species are being engineered as biofactories for the production and characterization of rare carbohydrates, which can have significant applications across many fields.[8–10] Here, we leverage synthetic biology to study the biosynthesis and recombinant production of virenose.

2. MATERIALS AND METHODS

2.1 Obtaining and Amplifying Virenose Biosynthesis Genes

The sequence information for each of the five genes predicted to be involved in the biosynthesis of L-virenose was obtained from the NCBI database using the locus tags provided in Table 2. The sequences were codon optimized for expression in *E. coli* using the web-based tool available from Integrated DNA Technologies (IDT; Coralville, IA). The codon-optimized sequences were purchased as gBlocks fragments (IDT) and were routinely used as templates in polymerase chain reaction (PCR) amplification assays. The PCR assays were conducted utilizing Phusion high-fidelity DNA polymerase (New England Biolabs; Ipswich, MA) in accordance with the manufacturer's recommended protocol. The thermocycling conditions for the PCR reactions were as follows: 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 90 s, and a final extension was held at 72 °C for 10 min. The amplified DNA was separated via gel electrophoresis run at 100 V on a 1% agarose gel. DNA was visualized with ethidium bromide. The amplified PCR products were purified using a QIAquick gel extraction kit (Qiagen; Germantown, MD) in accordance with the manufacturer's recommended protocol. The purified gene PCR products were used for downstream cloning procedures. All primers used in this study are listed in Table 1.

Table 1. Primers Used in This Study

Primer Name	Sequence
pMGX_F	GCGGATAACAATTCCCCTCT
pMGX_R	ACCCCTCAAGACCCGTTTAG
CBU0688_fwd	AGTGGTGGTGGTGGTGGTGCTCGAGCGCATTTTTTTCAATACCTTC
CBU0688_rev	CTTTAAGAAGGAGATATACATATGCAAAAAGATGCCCCTATTTTTC
CBU0688_Xhol F	GTGCTCGAGCGCATTTTTTTCAATACCTTC
CBU0688_BamHI R	TCGGGATCCTATGCAAAAAGATGCCCCTAT
F_CBU0688_pMGX	CCGCATATGCAAAAAGATGCCCCTAT
R_CBU0688_pMGX	CCGGGATCCTTACGCATTTTTTTCAATAC
CBU0671_F	AGTGGTGGTGGTGGTGGTGCTCGAGTAAAGAGGTAGATTCGACAAC
CBU0671_R	CTTTAAGAAGGAGATATACATATGGAGGAGTGTATCGTTCCG
F_CBU0671_pMGX	CAGCATATGGAGGAGTGTATCGTTCC
R_CBU0671_pMGX	CACGGATCCTCATAAAGAGGTAGATTCGA
CBU0671_Xhol_F	GTGCTCGAGTAAAGAGGTAGATTCGACAAC
CBU0671_BamHI_R	TCGGGATCCTATGGAGTGTATCGTTCC
CBU0689_F	AGTGGTGGTGGTGGTGGTGCTCGAGCTTCAGGCCCTCAATGCAAAC
CBU0689_R	CTTTAAGAAGGAGATTATACATATGCGTAAAAAAGCGTTTATCACAGG
CBU0689_Xhol_F	GTGCTCGAGGCCCTCAATGCAAAC
CBU0689_BamHI_R	TCGGGATCCTATGCGTAAAAAAGCGTTTAT
F_CBU0689_pMGX	CCGCATATGCGTAAAAAAGCGTTTATCCGGGATCCTTA
R_CBU0689_pMGX	CCGGGATCCTTACTTCAGGCCCTCAATGC
F_CBU0294_pMGX	CAGCATATGGCCGCACAGGGGAAGAT
R_CBU0294_pMGX	CCGGGATCCTTAAAATGGAAGCTCCAAGG
CBU0294_Xhol_F	GTGCTCGAGAAATGGAAGCTCCAAGGCGTT
CBU0294_BamHI_R	TCGGGATCCTATGGCCGCACAGGGGAAGAT
F_CBU0691_pMGX	CCGCATATGACCTCATACCGTCAAAT
R_CBU0691_pMGX	CCGGGATCCCTAAATGATACGGACTTCCG
CBU0691_Xhol_F	GTGCTCGAGAATGATACGGACTTCCGGAAA
CBU0691_BamHI_R	TCGGGATCCTACCTCATACCGTCAAATCAC

2.2 Cloning the Virenose Biosynthesis Genes

Purified PCR gene products were used to clone into either the protein purification vector pET21-B+ or the gene expression plasmid pMGX-HK. Both the PCR products and the plasmids were digested using either XhoI/NdeI for pET-21b+ or NdeI/BamHI for pMGX-HK. The restriction digests were carried out at 37 °C for 2 h. The digested DNA products were purified in accordance with the QIAquick gel extraction kit manufacturer's protocol. The digested and purified DNA products were used for ligation reactions with T4 DNA ligase (New England Biolabs). The ligation reactions were carried out in accordance with the manufacturer's suggested protocol and were incubated at 16 °C overnight. Following the ligation, the reactions were used to transform *E. coli* Dh5α or BL21. The transformants were validated by either restriction digestion or sequencing.

2.3 Expression Test for Protein Purification

To determine the expression levels of the virenose biosynthetic enzymes for purification, an expression test was conducted. *E. coli* transformants harboring the protein expression plasmids were grown overnight in Overnight Express Instant TB medium (Merck; Darmstadt, Germany) at 37 °C. Following incubation, the bacterial cultures were pelleted at 13,000 ×*g* for 5 min. Bacterial pellets were resuspended in an equal volume of Laemmli buffer and boiled at 98 °C for 5 min. The samples were separated on a polyacrylamide gel, and proteins were stained using the Simplyblue safe stain (Invitrogen; Carlsbad, CA) in accordance with the manufacturer's recommended protocol.

2.4 Mass Spectroscopy Analysis of Recombinant Virenose

The *E. coli* strain BL21+pMGX_0691_0688 was grown overnight in Gibco M9 minimal media (Thermo Fisher Scientific; Waltham, MA). The expression of the genes was induced via addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside. After growth, the cells were harvested and lysed via successive freeze–thaw cycles. The lysed cells were clarified via centrifugation. The clarified lysate predicted to contain the virenose was collected for high-performance liquid chromatography (HPLC)–mass spectrometry (MS) analysis. The samples were purified on the Waters preparative HPLC–MS system using a 20 mL/min flow rate; eluent (A) was 0.1% formic acid in Millipure H_2O, and eluent (B) was 0.1% formic acid in HPLC-grade acetonitrile. The inlet file ratios of (A)/(B) were as follows: 0 min at 95/5, 4 min at 5/95, 4.5 min at 5/95, and 4.8–5 min at 95/5. The product was collected based on [M–H]– electrospray ionization (ESI) negative ions.

3. RESULTS

3.1 Generating Biosynthetic Pathway DNA Fragments

To test our hypothesis and attempt to fully characterize the biosynthesis pathway of virenose from *C. burnetii*, we first needed to optimize and obtain the genes encoding the predicted enzymes of the pathway. In a previous study, a bioinformatics investigation of the *C. burnetii* genome was used to predict the enzymes involved in the biosynthesis of virenose.[6] In this study, several potential routes for virenose biosynthesis in *C. burnetii* were identified, and one route was expected to be the most likely.[6] This pathway was predicted to involve six enzymatic steps catalyzed by five unique enzymes (Table 2).

Table 2. Genes Predicted to Biosynthesize Virenose

Gene Name	Predicted Function	Locus Tag	Size (bp)
rfbA	Mannose-1-phosphate-guanylytransferase/ mannose-6-phosphate isomerase	CBU_0671	1433
N/A	Phosphomannomutase	CBU_0294	1415
gmd	GDP-mannose 4,6 dehydratase	CBU_0689	1046
N/A	Methyltransferase	CBU_0691	1238
wcaG	GDP-L-fucose synthase	CBU_0688	998

N/A, not applicable.

In our attempt to recapitulate virenose biosynthesis in a non-native host, we chose to test the function of these five enzymes. We first obtained the DNA sequence for these five genes from the National Center for Biotechnology Information (NCBI) database and chose standard laboratory *E. coli* as the non-native host to evaluate virenose biosynthesis. Because *C. burnetii* and *E. coli* are distantly related bacteria, we needed to ensure that the genes from *C. burnetii* were optimized for expression in *E. coli*. Using a bioinformatics tool available through Integrated DNA Technologies, the coding regions for each of the five genes were optimized for the codon usage in *E. coli*. After the genes were optimized, they were obtained as gBlocks fragments (IDT) and amplified via PCR for cloning into the expression vectors of interest (Figure 3).

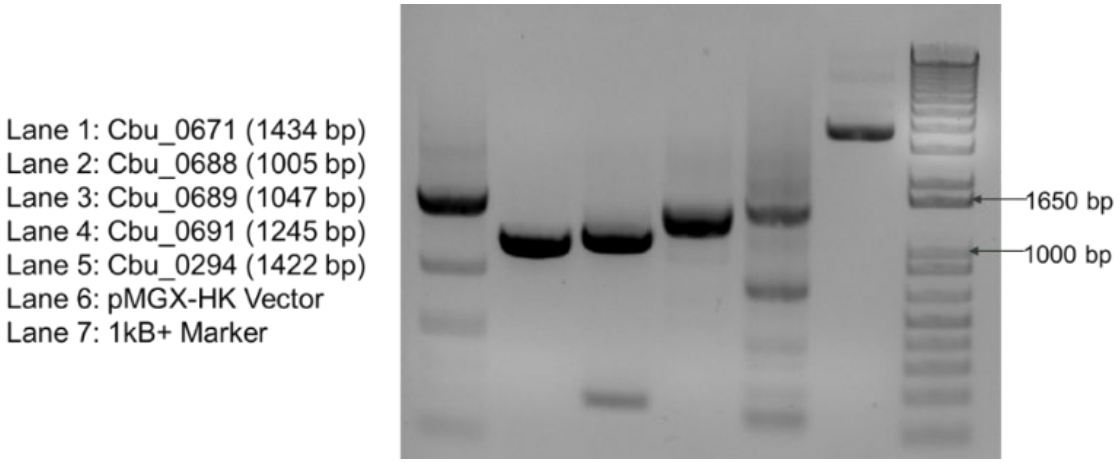

Figure 3. Amplification of putative virenose biosynthesis genes. Each of the procured gene gBlocks was amplified using PCR with Phusion high-fidelity polymerase and the primers listed in Table 1. PCR products were separated by gel electrophoresis and visualized by ethidium bromide staining.

3.2 Generating Multi-Gene Expression Pathway with Synthetic Genes

After successfully obtaining the optimized genes from *C. burnetii*, we wanted to take two approaches to characterize their potential role in virenose biosynthesis. Our first approach was to assemble each gene into a polycistronic operon under the control of a single inducible promoter that functions in *E. coli* (Figure 4).

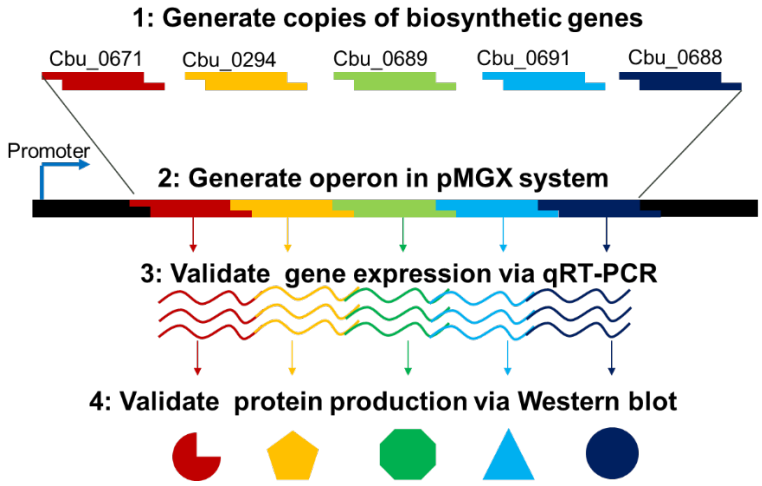

Figure 4. Generating virenose biosynthetic pathway operon workflow. qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

We used a previously described plasmid expression system that has been used to characterize carbohydrate biosynthetic pathways.[10] Each of the five genes was cloned individually into the pMGX-HK vector to be systemically cloned into a single operon under the control of a single inducible promoter. However, in attempting to subclone each of the five genes into a single polycistronic unit, we were only able to achieve a vector containing two genes (Figure 5). PCR and gel electrophoresis analyses confirmed that we successfully ligated the putative methyltransferase (CBU0691) and GDP-L-fucose synthase (CBU0688) genes into our plasmid (Figure 5). Specifically, two *E. coli* clones, nos. 1 and 4, contained the pMGX and CBU0691 and CBU0688 genes in one plasmid (Figure 5).

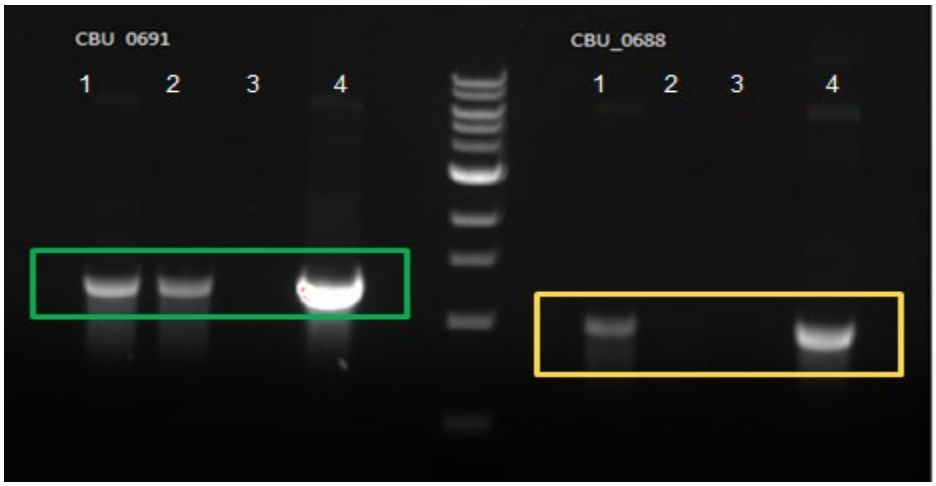

Figure 5. Confirmation of individual genes by PCR and gel analysis. Two of the putative virenose biosynthesis genes we co-cloned into the pMGX-HK background and transformed into *E. coli*. Positive clones were screened by colony PCR, and products were analyzed by gel electrophoresis. Bands within green and yellow boxes represent CBU0691 and CBU0688, respectively.

3.3 Evaluation of Recombinant Virenose Biosynthesis

Having successfully generated the inducible construct containing the genes CBU0691 and CBU0688, we could next screen our *E. coli* strain for virenose biosynthesis. Although our construct only contained two of the five genes predicted in the virenose biosynthesis pathway, these two genes were expected to be specifically required for the biosynthesis (Figure 2). Because of this, we predicted that expression of these two genes could hijack the common intermediates of hexose biosynthesis in *E. coli*, specifically, GDP-4-keto-6-deoxy-D-mannose (Figure 2). The engineered *E. coli* strain was grown in minimal media to remove background interference from the culture conditions, and the cells were lysed to release any virenose that had been recombinantly produced prior to HPLC–MS analysis. Evaluation with MS further confirmed that our engineered strain was not expressing masses that corresponded to GDP-virenose or virenose (Figure 6).

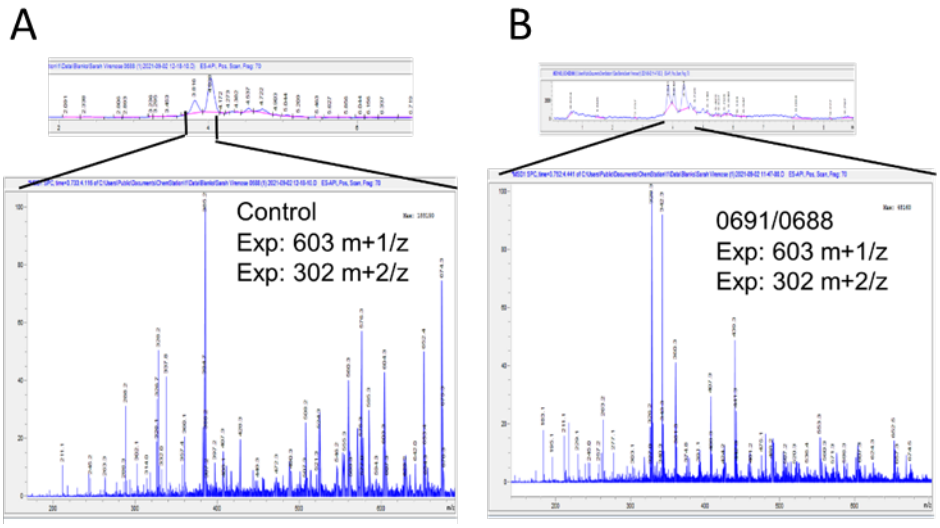

Figure 6. HPLC–MS of *E. coli* +pMGX_CBU0691/CBU0688 recombinant strain. Cellular lysates from wildtype *E. coli* or *E. coli* +pMGX_CBU0691/0688 were separated by reverse-phase HPLC, and components were analyzed by ESI–MS. Expected masses for GDP-virenose of *m/z* 603 or virenose without the GDP nucleotide of *m/z* 302 were not observed in the expected strain, and the spectra between the sample and the control were not significantly different.

3.4 Purification of Predicted Virenose Biosynthesis Enzymes

Our second approach to determine whether the *C. burnetii* enzymes can catalyze virenose biosynthesis was to evaluate the function of each enzyme in vitro. Purifying each of the enzymes and assessing the functions in vitro would allow us to determine the role of virenose biosynthesis outside of the context of the non-native host background. The five genes were individually cloned into the protein expression vector pET-21b+, which also included a 6× histidine tag on the proteins, and were used for affinity chromatography. After generating the constructs, we tested the expression of the proteins prior to the purification (Figure 7).

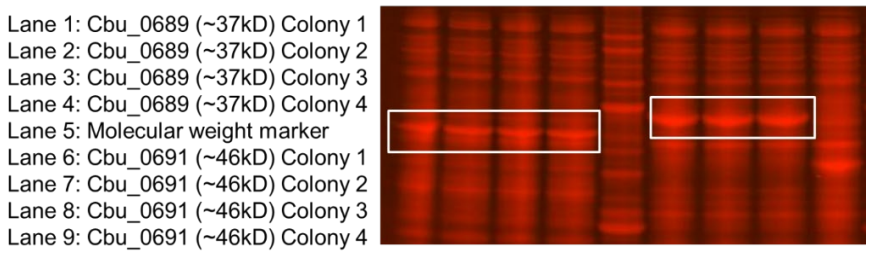

Figure 7. Representative expression test of Cbu0689 and Cbu0691. Four colonies containing pET_CBU0689 or pET_CBU0691 were evaluated for recombinant protein expression. Expression for each colony was evaluated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and protein staining with Coomassie blue. Expected proteins are shown within white boxes.

Although both proteins expressed well in small-scale expression tests (Figure 7), attempts at large-scale immobilized metal-affinity chromatography purification were unsuccessful. The protein yields obtained were insufficient for in vitro characterization.

4. CONCLUSIONS

We set out to characterize the biosynthetic pathway of virenose, a key carbohydrate marker in the O-antigen of the pathogen *C. burnetii*. Using advances in synthetic biology, we obtained and cloned the genes encoding the predicted virenose biosynthesis enzymes. After expressing two genes predicted to be essential in generating virenose in *E. coli*, we were unable to confirm successful biosynthesis of the carbohydrate. We have identified several explanations as to why this approach was unsuccessful. One explanation for the failure to generate virenose recombinantly in *E. coli* is that the carbohydrate was degraded internally by endogenous catabolic pathways. This internal degradation of recombinant carbohydrate production has been previously observed.[10] Future work is needed to examine carbohydrate degradation pathways in *E. coli* to determine whether any specific pathways need to be mutated for identification of virenose. The second explanation as to why virenose was not produced in our recombinant strain is that the predicted enzymes evaluated are not actually involved in the biosynthesis. This explanation would be particularly true for CBU0691, a predicted methyltransferase. The *C. burnetii* genome has multiple methyltransferases across the genome that may be involved in the biosynthesis of virenose. Future work is needed to use both in silico prediction models and functional assays to evaluate predicted enzymes in the virenose biosynthesis pathway.

LITERATURE CITED

1. Knirel, Y.A.; Valvano, M.A., Eds. *Bacterial Lipopolysaccharides: Structure, Chemical Synthesis, Biogenesis and Interaction with Host Cells*; Springer: Vienna, 2011.

2. Abnave, P.; Muracciole, X.; Ghigo, E. *Coxiella burnetii* Lipopolysaccharide: What Do We Know? *International Journal of Molecular Sciences* **2017,** *18* (12), 2509.

3. Hackstadt, T. Antigenic Variation in the Phase I Lipopolysaccharide of *Coxiella burnetii* Isolates. *Infection and Immunity* **1986,** *52* (1), 337–340.

4. Beare, P.A.; Jeffrey, B.M.; Long, C.M.; Martens, C.M.; Heinzen, R.A. Genetic Mechanisms of *Coxiella burnetii* Lipopolysaccharide Phase Variation. *PLoS Pathogens* **2018,** *14* (3), e1006922.

5. Toman, R.; Škultéty, L.T.; Ftáček, P.; Hricovíni, M. NMR Study of Virenose and Dihydrohydroxystreptose Isolated from *Coxiella burnetii* Phase I Lipopolysaccharide. *Carbohydrate Research* **1998,** *306* (1–2), 291–296.

6. Flores-Ramirez, G.; Janecek, S.; Miernyk, J.A.; Skultety, L. In Silico Biosynthesis of Virenose, A Methylated Deoxy-Sugar Unique to *Coxiella burnetii* Lipopolysaccharide. *Proteome Science* **2012,** *10* (1), 67.

7. Keasling, J.D. Synthetic Biology and the Development of Tools for Metabolic Engineering. *Metabolic Engineering* **2012,** *14* (3), 189–195.

8. Li, Z.; Gao, Y.; Nakanishi, H.; Gao, X.; Cai, L. Biosynthesis of Rare Hexoses Using Microorganisms and Related Enzymes. *Beilstein Journal of Organic Chemistry* **2013,** *9* (1), 2434–2445.

9. Granström, T.B.; Takata, G.; Tokuda, M.; Izumori, K. Izumoring: A Novel and Complete Strategy for Bioproduction of Rare Sugars. *Journal of Bioscience and Bioengineering* **2004,** *97* (2), 89–94.

10. Hassan, M.I.; Lundgren, B.R.; Chaumun, M.; Whitfield, D.M.; Clark, B.; Schoenhofen, I. C.; Boddy, C.N. Total Biosynthesis of Legionaminic Acid, a Bacterial Sialic Acid Analogue. *Angewandte Chemie* **2016,** *128* (39), 12197–12200.

Blank

ACRONYMS AND ABBREVIATIONS

DEVCOM CBC	U.S. Army Combat Capabilities Development Command Chemical Biological Center
ESI	electrospray ionization
HPLC	high-performance liquid chromatography
IDT	Integrated DNA Technologies
LPS	lipopolysaccharide
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction

DISTRIBUTION LIST

The following individuals and organizations were provided with one electronic version of this report:

U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC)
FCDD-CBR-BM
ATTN: McDonald, N.
Basi, K.

DEVCOM CBC Technical Library
FCDD-CBR-L
ATTN: Foppiano, S.
Stein, J.

Defense Technical Information Center
ATTN: DTIC OA

U.S. ARMY

U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER