TECHNICAL REPORT
NATICK/TR-17/009

AD _____

DEVELOPMENT OF PURIFICATION PROTOCOL SPECIFIC FOR BACTERIOCIN 105B

by
Robert Stote
Jennifer M. Rego
and
Courtney M. Cowell

February 2017

Final Report
November 2013 – August 2014

U.S. Army Natick Soldier Research, Development and Engineering Center
Natick, Massachusetts 01760-5020

DISCLAIMERS

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

Citation of trade names in this report does not constitute an official endorsement or approval of the use of such items.

DESTRUCTION NOTICE

For Classified Documents:

Follow the procedures in DoD 5200.22-M, Industrial Security Manual, Section II-19 or DoD 5200.1-R, Information Security Program Regulation, Chapter IX.

For Unclassified/Limited Distribution Documents:

Destroy by any method that prevents disclosure of contents or reconstruction of the document.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
09-02-2017	Final	November 2013 – August 2014

4. TITLE AND SUBTITLE

DEVELOPMENT OF PURIFICATION PROTOCOL SPECIFIC FOR BACTERIOCIN 105B

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Robert Stote, Jennifer M. Rego, and Courtney M. Cowell

5d. PROJECT NUMBER
13-115b

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

U.S. Army Natick Soldier Research, Development and Engineering Center
ATTN: RDNS- TMS
General Green Avenue, Natick, MA 01760-5020

8. PERFORMING ORGANIZATION REPORT NUMBER

NATICK/TR-17/009

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This report summarizes work conducted to establish a purification procedure specific to a narrow-spectrum antimicrobial peptide. This antimicrobial agent, termed a bacteriocin, was evaluated in previous work and determined to exhibit activity against a pathogenic organism of interest to the Army, *Bacillus anthracis* Sterne, a surrogate of the active form of *Bacillus anthracis*. As the current application of broad-spectrum antimicrobials promotes the development of multi-drug resistant microorganisms, targeted antimicrobials are being investigated as a suitable alternative. Although bacteriocins have exhibited great promise as narrow-spectrum antimicrobial agents, their wide-spread adaptation has been limited in part due to the time-consuming and costly processes employed for development. The work cited in this report presents a robust purification procedure for an individual bacteriocin to maximize the yield of the antimicrobial peptide for employment in future assays and development into a platform, such as a textile, that relays antimicrobial activity.

15. SUBJECT TERMS

105B	PROCEDURES	CULTURES(BIOLOGY)	ANTIMICROBIAL PEPTIDES
YIELD	PREPARATION	NARROW-SPECTRUM	TARGETED ANTIMICROBIALS
ASSAYS	PURIFICATION	BACILLUS ANTHRACIS	DRUG-RESISTANT MICROORGANISMS
PEPTIDES	BACTERIOCINS	TEST AND EVALUATION	
BACTERIA	CELLS(BIOLOGY)	ANTIMICROBIAL AGENTS	

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Robert Stote
U	U	U	SAR	20	**19b. TELEPHONE NUMBER** *(include area code)* 508-233-4629

This page intentionally left blank

Table of Contents

List of Figures

Preface

This report documents work by the Biological Sciences and Technology Team (BSTT) of the Warfighter Directorate at the Natick Soldier Research, Development and Engineering Center (NSRDEC) during the period of November 2013 – August 2014. The objective of this work was to establish a purification protocol for a specific narrow-spectrum antimicrobial agent, a bacteriocin, to facilitate its future incorporation into platforms, such as textiles, to impart antimicrobial functionality. This work leveraged previous work that established a general bacteriocin purification procedure ("Protocol for Initial Purification of Bacteriocin", 2015) and an investigation that isolated an environmental bacteriocin (sample 105b) with targeted activity against *Bacillus anthracis* Sterne, a surrogate of anthrax, which is a pathogen of interest to the DoD ("Isolation, Characterization and Identification of Environmental Bacterial Isolates with Screening for Antagonism Against Three Bacterial Targets", *in press* 2017).

DEVELOPMENT OF SPECIFIC PURIFICATION PROTOCOL FOR BACTERIOCIN 105B

1 Introduction

The Biological Sciences and Technology Team (BSTT), Warfighter Directorate, of the Natick Soldier Research, Development and Engineering Center (NSRDEC) is investigating the use of narrow-spectrum antimicrobials in order to fabricate textiles using non-traditional methods for Warfighter protection against harmful bacteria. Work was performed from November 2013 to August 2014. This investigation has several parts; one focus of the effort is to produce a sufficient quantity and purity of a bacteriocin, a class of narrow-spectrum antimicrobial peptide. This purified bacteriocin would ultimately be applied toward the fabrication of a textile that exhibits antibacterial functionality. Previous work by this research group has established a universal protocol for the purification of bacteriocins to facilitate their rapid screening for activity ("Protocol for Initial Purification of Bacteriocin" 2015). Leveraging this foundation, a purification protocol for a specific bacteriocin, an environmental bacteriocin termed 105b, was developed to provide a sufficient yield and purity for future applications. The objective of this technical report was to describe the development of a purification method specific to bacteriocin 105b and the resulting characterization of the peptide through the purification procedure.

The wide-spread use of broad-spectrum antimicrobial agents has led to the development of drug resistant strains of pathogenic bacteria (Levy, 2002). BSTT has initiated investigations into alternative solutions for protection against pathogenic bacteria to prevent or overcome resistance. One hypothesis that accounts for the evolution of drug resistance proposes that broad-spectrum antimicrobials promote the growth of bacteria that is resistant to the antimicrobial (Levy *et al*. 2004). The resistance to the antimicrobial arises when broad-spectrum antimicrobials are employed and the entire bacteria population is not killed. Bacteria that possess resistance genes or have developed resistance to the antimicrobial through genetic transfer will survive. Benign microorganisms that facilitate the control of drug-resistant pathogenic strains are eradicated, resulting in the unconstrained growth of the resistant strains (Levy *et al*. 2004, Gulberg *et al*. 2011). After a few exposures to a broad-spectrum antimicrobial agent, the population of bacteria becomes primarily composed of resistant strains, rendering the broad-spectrum antimicrobial ineffective. Often when this occurs, the ineffective antimicrobial agent is replaced with another broad-spectrum antimicrobial agent in attempts to combat the resistant pathogenic strains (Knobler *et al*. 2003). This strategy may provide an opportunity for the bacterial pool to be further selected, which leads to the development of multi-drug resistant bacteria, or "super bugs". As concern about the emergence of multi-drug resistant bacteria increases, new strategies are being investigated to inhibit the development of resistant strains. One promising strategy is to implement narrow-spectrum antimicrobials, such as bacteriocins, as alternatives to broad-spectrum agents.

Bacteriocins are narrow-spectrum bacterial toxins secreted by bacteria to kill other closely related bacteria that are competing for the same resources. Many bacteriocins have very specific activity spectrums, as they only target one or two species. This selectivity offers a paradigm shift of applying antimicrobials where instead of indiscriminately killing as many species of

bacteria as possible, only specific pathogens could be targeted, leaving beneficial bacteria unaffected and capable of thriving (Abt *et al*. 2014). Bacteriocins are generally considered a green alternative to currently used antimicrobials and only a few species have demonstrated adverse effects on eukaryotic cells (Cotter *et al*. 2005, Cox *et al*. 2005, Galvez *et al*. 2011). Greater understanding of the mechanisms of activity of bacteriocins as well as bioengineering of the antimicrobial peptide may eliminate the development of bacteriocin-resistant bacteria. Further, the efficacy of individual bacteriocins can readily be enhanced via combination with other antimicrobials (Cotter *et al*. 2013). These characteristics make bacteriocins an appealing option to supersede broad-spectrum antimicrobials.

Although bacteriocins have been studied for several decades, their utilization in industrial applications has been limited. Extensive time is required to produce, purify and characterize the activity of individual bacteriocins. Because the structure and molecular characteristics of bacteriocins are heterogeneous, a universal method for purifying sufficient quantities of bacteriocins for characterization is not readily available (Heng *et al*. 2007). A purification procedure must be developed for each unique bacteriocin, which may not necessarily translate to other bacteriocins of interest. Previous work documented in the technical report "Protocol for Initial Purification of Bacteriocins" (2015) focused on developing a universal purification protocol that could yield sufficiently pure material to facilitate the rapid evaluation of the bacteriocin's activity against target pathogenic microorganisms. In this protocol, bacteriocins were isolated from cellular extracts using tangential flow filtration, which is similar to purification methods carried out in industry (personal conversation, Immucell, Portland, ME 2015). Three filters with differing molecular weight cutoffs (MWCO) were used sequentially. Conversely, purification methods developed in the laboratory are small-scale and include ammonium sulphate precipitation (Pingitore *et al*. 2007). While purification of bacteriocin is achieved using this process, ammonium sulphate precipitation is an expensive and labor intensive process when scaled-up to industrially required quantities. Because of this, applying tangential flow filtration in conjunction with ion exchange column chromatography was chosen as a practical foundation for developing the purification protocol.

Employing a bacteriocin for applications to protect the Warfighter from pathogenic bacteria requires one that demonstrates robust activity against an Army relevant pathogenic microorganism and can be consistently and readily produced in high quantities and purities. Bacteriocin 105b is one such bacteriocin that exhibits strong activity against *Bacillus anthracis* Sterne, a surrogate for the active, live agent producing strain of *Bacillus anthracis*. Purification of bacteriocin 105b was achieved using the universal protocol cited previously, which enabled its activity to be easily assessed. However, the yield of bacteriocin 105b acquired through this protocol could be substantially improved.

The objective of the work described in this technical report was to develop a purification protocol tailored specifically for bacteriocin 105b to improve its yield so that it could be readily produced to impart antimicrobial activity to protective constructs for the Army. The universal purification protocol previously developed was used as a starting point. The results obtained from the previous technical report, "Protocol for Initial Purification of Bacteriocins" were carefully analyzed to determine steps in the process that could be targeted for improvement to increase the yield of bacteriocin 105b. This analysis provided insight into the results of the

universal purification protocol on the purification of bacteriocin 105b and provided the launching point for the investigation carried out in the current task.

2 Materials and Methods

2.1 Preparation of Target Bacteria

Bacteriocin 105b demonstrates strong activity against *Bacillus anthracis* Sterne (BAS), a surrogate for the live, active toxic agent producing strain of *Bacillus anthracis*. BAS was stored at -80 °C until needed. A fresh culture of BAS was prepared by inoculating a single colony from a streak plate into 10 mL nutrient broth. The culture was incubated on a shaker incubator at 37 °C for 4 h at 220 rpm, or until the optical density (OD) measured 1 absorbance units (AU) at 600 nm. To prepare soft agar overlays for activity tests, 40 µL from this culture was added to 7 mL of soft agar, which was then poured over TSB media and allowed to harden.

2.2 Production of Bacteriocin 105b

A fresh culture of the bacteriocin 105b producer strain *Bacillus subtilis* was prepared by inoculating a single colony from a streak plate into 10 mL of tryptic soy broth (TSB). The culture was incubated on a shaker incubator at 37 °C for 4 h, or until the OD measured 1 AU at 600 nm. A 1 L flask of TSB was inoculated and incubated with agitation for 16 h at 37 °C.

2.3 Purification of Bacteriocin 105b

To isolate bacteriocin 105b from the cellular extract, the culture described in Section 2.2 was first centrifuged at 10,000 rpm for 30 min to separate the cells. The supernantant was filtered through a 0.22 µm filter. Tangential flow filtration was then used to filter the resulting filtrate. The filtrate from the 0.22 µm filter was subject to tangential flow filtration using a 30k MWCO filter. The retentate was washed twice with 600 mL of 25 mM Tris buffer at pH 8.5. A 1 mL aliquot of both the retentate and filtrate were concentrated using a SpeedVac to $1/10^{th}$ the volume (100 µL). To characterize the presence of bacteriocin, both the retentate and filtrate were probed for activity using an activity drop test on a target overlay. Results of the activity test indicated bacteriocin 105b is retained in the retentate of the 30k MWCO filter. The retentate was then passed through a diethylaminoethyl (DEAE) Sephadex A-25 (GE Healthcare) column equilibrated with 25 mM Tris buffer pH 8.5 to further eliminate contaminating proteins from bacteriocin 105b. The column was washed with 25 mM Tris buffer pH 8.5 containing 150 mM or 1M NaCl to ensure any potential bacteriocin 105b was removed from the column. To isolate the presence of bacteriocin 105b, aliquots of the flow-through and wash fractions were evaluated for activity using an activity drop test on a target overlay.

2.4 Evaluation of Protein Purity

To evaluate the purity and query the yield of bacteriocin 105b collected from the DEAE Sephadex fractions, gel electrophoresis was used. Bis-Tris polyacrylamide gel electrophoresis (PAGE) gels were prepared either using 40% acrylamide, tetramethylethylenediamine (TEMED), sodiumdodecyl sulfate (SDS) and ammonium persulfate as a 10-12% gel or gels were purchased pre-poured as 4-12% gels from Invitrogen. Fractions resulting from 150 mM and 1M NaCl washes were desalted to prevent interference when run on the gel. The fractions were desalted using dialysis or G10 Sephadex columns against 25 mM Tris buffer pH 8.5. Samples were run in duplicate lanes on the same gel. At the end of electrophoresis the gel was cut in half. One half of the gel was stained using SimplyBlue Coomassie stain (Life Technologies) to visualize the protein bands. The other half of the gel was assessed for activity. The gel was

overlayed onto media and a soft agar overlay inoculated with the target microorganism was poured over the gel and incubated overnight at 37 °C. Pictures of the stained and overlayed gels were compared to identify the active peptide band represented by a zone of clearing in the target overlay.

3 Results and Discussion

3.1 Isolation of Bacteriocin 105b

The development of a universal purification protocol to purify bacteriocins ("Protocol for Initial Purification of Bacteriocin", *in press*), sequentially implemented 30k, 10k and 1k MWCO filters for tangential flow filtration, where the filtrate of the preceding filter was applied as the flow-through for the next filter. Bacteriocin 105b was initially purified from its host using the universal protocol. During this study, the molecular weight of bacteriocin 105b was determined to be around 4 kilodaltons (kDa) using SDS-PAGE. Due to its molecular weight, bacteriocin 105b was predicted to be isolated in the retentate of the 1k MWCO filter. However, the resulting retentate collected from the 1k filter provided a poor yield of bacteriocin 105b. To increase the yield of bacteriocin 105b achieved through purification, a purification protocol specific to 105b was developed, building upon the findings of the previously employed universal purification method.

The purification of bacteriocin 105b through the process of tangential flow filtration using three different MWCO filters was evaluated to determine where the antimicrobial peptide is retained. Aliquots for the retentate and filtrate of each filter were collected and activity tests were conducted at each filter step to assess the presence of bacteriocin 105b. Figure 1 shows the activity of the respective retentates and filtrates for each filter size. As seen in Figure 1, zones of clearing indicative of activity induced from the presence of bacteriocin 105b are only present in the crude sample (no tangential flow filtration, panel A) as well as the retentate remaining from tangential flow filtration over the 30k MWCO filter (panel B). This result indicates that bacteriocin 105b does not pass through the 30k MWCO filter and affirms that additional processing using smaller MWCO filters in tangential flow filtration is not necessary to purify bacteriocin 105b. As a result, the utilization of 10k and 1k MWCO filters for tangential flow filtration was eliminated from the purification process specific for bacteriocin 105b. Also of note, the small colonies observed in the zone of clearing in the aliquot of the 30k retentate suggest the presence of contaminating bacteria resistant to bacteriocin 105b. This serves as a reminder that clean laboratory techniques must be executed to ensure contaminating microorganisms are restrained.

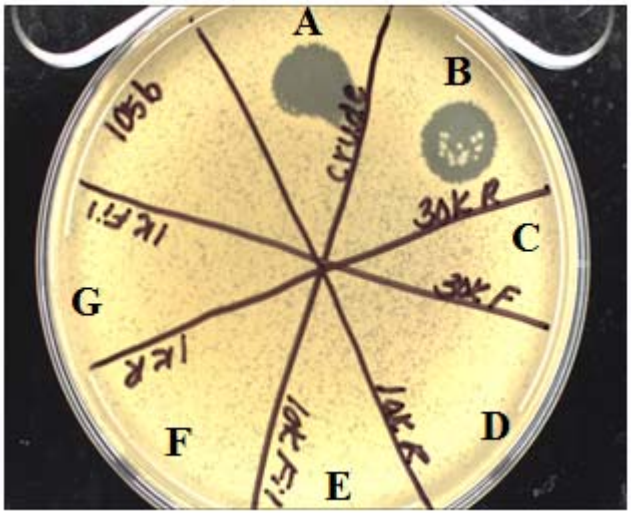

Figure 1. Isolation of Bacteriocin 105b Using Tangential Flow Filtration. A.) Crude bacteriocin 105b sample not subjected to tangential flow filtration; B.) retentate from 30k MWCO filter; C.) filtrate from 30k MWCO filter; D.) retentate from 10k MWCO filter; E.) filtrate from 10k MWCO filter; F.) retentate from 1k MWCO filter; G.) filtrate from 1k MWCO filter.

Isolation of bacteriocin 105b through the tangential flow filtration process was confirmed by testing the activity using a drop test of an aliquot of collected retentates and filtrates against a target field. The presence of bacteriocin 105b was confirmed by the observation of a zone of clearing indicative of activity.

Because the proposed size of bacteriocin 105b is much smaller than the expected size range for retention using the 30k MWCO filter, this result was unanticipated. Many conditions associated with the tangential flow filtration process may affect the retention of a protein. One hypothesis that may explain why bacteriocin 105b does not flow through the 30k MWCO filter is that the antimicrobial peptide interacts with other solutes such as polyglutamic acid, a biopolymer also excreted by *Bacillus subtillus*. Due to this interaction, bacteriocin 105b may not exist as a free peptide in solution, which may account for the antimicrobial activity observed in the retentate of the 30k MWCO tangential flow filtration filter. If bacteriocin 105b does not remain a free peptide in solution, this justification may also suggest that molecular interactions are necessary for bacteriocin 105b to retain its stability when extracted from the cell. The nature of the proposed interaction between polyglutamic acid and/or other proteins in the cellular extract is not known. Additional studies are required to fully comprehend the interaction of bacteriocin 105b with other excreted molecules and their effects on the stability and activity of bacteriocin 105b. Polyacylamide gel electrophoresis in conjunction with an activity overlay assay would evaluate the size of the potential protein complex containing bacteriocin 105b. Additional assays to dissociate the complex and characterize the associated proteins would also be necessary.

3.2 Purity of Bacteriocin 105b

Once bacteriocin 105b was isolated from the cellular extract using tangential flow filtration, additional steps were performed to remove remaining contaminants. The retentate collected from the 30k MWCO filter was passed through anion exchange column chromatography using DEAE Sephadex. The collected fractions were assessed for activity by activity drop test and evaluated for purity using polyacrylamide gel electrophoresis (PAGE) to visualize the number of protein bands. Figure 2 shows a representative activity drop test that evaluates the activity of fractions collected from DEAE Sephadex column chromatography to isolate the presence of bacteriocin 105b. Figure 2 shows zones of clearing only for the 30k MWCO filter retentate that was applied to the ion exchange column chromatography and the aliquot collected of the resulting flow-through. Activity is not observed after washing the column with 150 mM NaCl in 25 mM Trish buffer at pH 8.5. This result suggests that bacteriocin 105b does not interact with the DEAE Sephadex column. Instead, the antimicrobial peptide passes through the column and is eluted in the flow-through. Again, contaminating bacteria colonies resistant to bacteriocin 105b are observed in the zones of clearing observed in Figure 2.

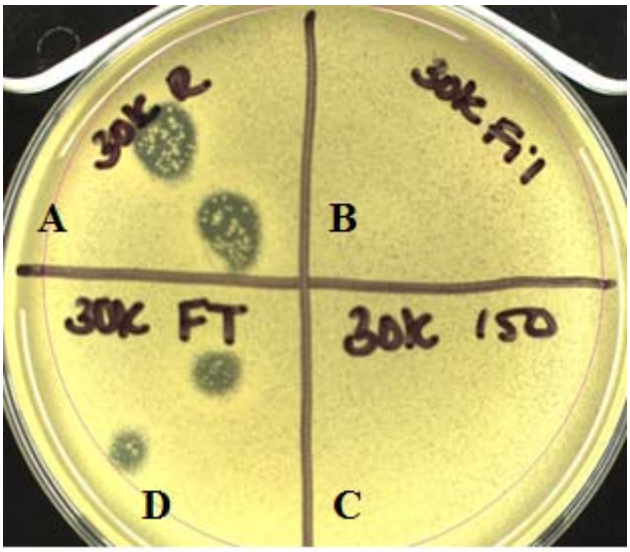

Figure 2. Isolation of Bacteriocin 105b from Purification with Ion Exchange Column Chromatography. Representative activity drop test assay evaluating activity of fractions collected from ion exchange column chromatography to isolate bacteriocin 105b. Dropped samples: A.) 30k MWCO filter retentate; B.) 30k MWCO filter filtrate; C.) flow-through after applying 30k MWCO retentate to ion exchange column; D.) wash fraction collected after washing column with 25 mM Tris buffer 150 mM NaCl.

Polyacrylamide gel electrophoresis was carried out to assess the purity of bacteriocin 105b in the flow-through collected after retentate from the 30k MWCO filter was applied to an ion exchange column. Figure 3 shows a representative PAGE gel with samples collected from ion exchange column chromatography. Lane 1 shows the Novex Sharp Pre-Stained Protein Standard. Lane 2 shows the flow-through collected after passing the 30k MWCO retentate over the column. Lanes 3 and 4 show the fractions collected after washing the column with 250 mM NaCl and 1 M NaCl, respectively. As seen in Figure 3, the greatest amount of protein is present in the fraction collected after washing the column with 250 mM NaCl (Lane 3). Substantially less protein is

observed in the flow-through collected from ion exchange column chromatography, where activity tests indicate bacteriocin 105b is retained (Lane 1). This result suggests that most of the extraneous protein in the 30k retentate interacts with the column and is only eluted after a salt wash. This result also shows minimal contaminants were present in the fraction of the flow-through collected (Lane 2), where antimicrobial activity was observed when the fraction was evaluated for activity (Figure 2).

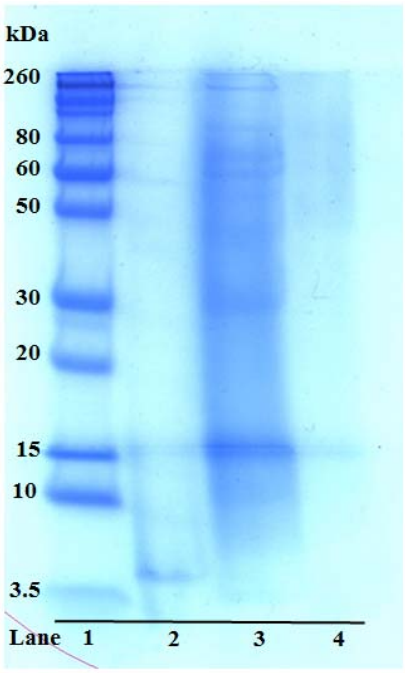

Figure 3. Purification of Bacteriocin 105b after Ion Exchange Column Chromatography. Representative PAGE gel with samples collected from column chromatography using DEAE Sephadex. Lane 1: Novex Sharp Pre-Stained Protein Standard. Lane 2: flow-through collected after passing retentate from 30k MWCO filter through ion exchange column. Lane 3: fraction collected after washing column with 250 mM NaCl in 25 mM Tris buffer at pH 8.5. Lane 4: fraction collected after washing column with 1M NaCl in 25 mM Tris buffer at pH 8.5.

To confirm the presence of bacteriocin 105b in the flow-through fraction analyzed by PAGE, a gel analyzing the flow-through collected from ion exchange column chromatography was assessed for antimicrobial activity. Figure 4A shows a representative PAGE gel with the flow-through collected after applying the 30k retentate to the DEAE Sephadex column (Lanes 1 and 2). Only one protein band is observed around 4 kDa. Figure 4B shows the results of an activity overlay assay of the gel. Two zones of clearing are observed which relate to the two protein bands observed in Figure 4A. This result suggests that the protein band at 4 kDa is bacteriocin 105b, which was eluted from the column during the flow-through. The presence of contaminating proteins in the flow-through fraction is not observed, which may suggest the purity of bacteriocin 105b is high. However, contaminating proteins may be present in the purified 105b sample but may not be visible by the PAGE analysis due to their low abundance. To confirm the absence of contaminating proteins, a higher concentration of the purified 105b

sample could be evaluated by PAGE and/or characterized using a more sensitive visualization technique, such as silver staining. The purified 105b sample could also be characterized by Fast Protein Liquid Chromatography (FPLC) to determine proteins present.

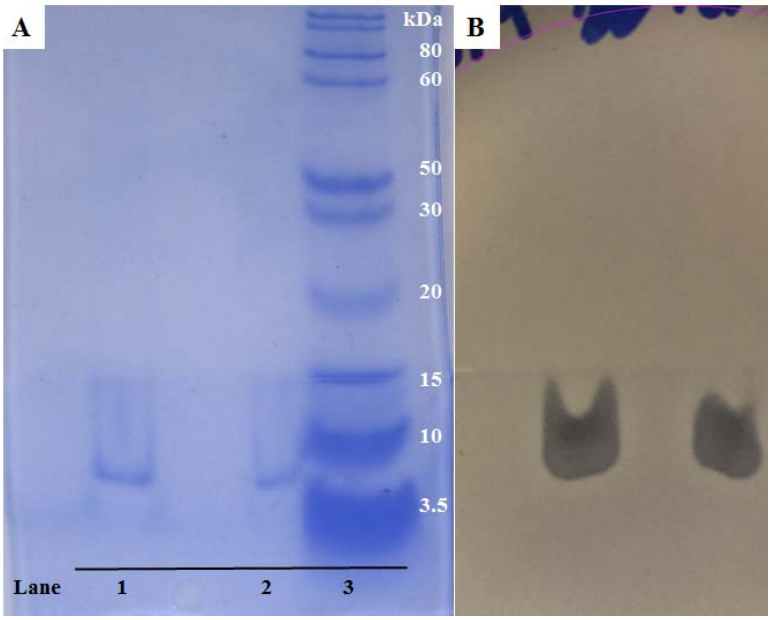

Figure 4. Confirmation of bacteriocin 105b in the flow-through of ion exchange column chromatography. A.) SDS-PAGE gel stained with Coomassie Blue. Lanes 1 and 2: flow-through collected after applying 30k retentate to the DEAE Sephadex column. Lane 3: Novex Sharp Pre-Stained Protein Standard. Only one protein band around 4 kDa is present in Lanes 1 and 2. B.) Results of activity overlay of duplicate gel pictured in Figure 4A. Two zones of clearing relating to the two protein bands observed in Lanes 1 and 2 in Figure 4A are observed.

To comprehensively evaluate the developed purification protocol specific for bacteriocin 105b, the quantified yield of bacteriocin 105b must be considered. Ideally, the developed purification protocol should generate a high quantity of the antimicrobial peptide at a high purity. Work is currently ongoing to elucidate the quantity of bacteriocin 105b that is produced using this method. The bicinchoninic acid (BCA) assay will be applied to assess the total protein concentration obtained in the sample of purified 105b. Because the crude extract from the bacteriocin 105b producing host is subjected to less processing using tangential flow filtration than in the universal purification protocol, it is expected that the total protein concentration will be greater. Liquid chromatography methods such as FPLC and/or High Performance Liquid Chromatography (HPLC) will be utilized to isolate bacteriocin 105b and characterize its concentration to determine the yield of antimicrobial peptide obtained.

4 Conclusions

Previous work developing a universal purification protocol for quickly characterizing bacteriocins was leveraged as the foundation for this work, similar to purification methods employed by industry (Immucell, Portland, ME). This purification method employed tangential flow filtration to rapidly yield a crude bacteriocin sample with enough purity to evaluate activity. This process is advantageous to purification procedures developed in the laboratory, which predominantly employ precipitation by ammonium sulfate (Pingitore *et al*. 2007) and do not readily translate to large-scale industrial applications due to high cost and labor. As detailed in this report, building upon the universal purification protocol, a purification protocol specific to bacteriocin 105b was achieved. Tangential flow filtration in conjunction with anion exchange column chromatography was employed. Initial characterization of the ensuing sample of antimicrobial peptide suggests a high yield of bacteriocin 105b was acquired. Further evaluation is necessary to confirm the purity and establish the quantity produced of bacteriocin 105b. The heterogeneous nature of bacteriocins as well as the numerous and varying protocols employed to purify bacteriocins makes it difficult to compare the results of this purification protocol to others (Carolissen-Mackay *et al*. 1997). Furthermore, bacteriocin 105b is an environmental isolate whose identity is currently unknown.

In future studies regarding bacteriocin 105b, the antimicrobial peptide should be sequenced to ascertain if the antimicrobial peptide is novel or has been previously characterized in literature. The use of bacteriocin 105b is envisioned in a textile that demonstrates antimicrobial activity to protect the Warfighter from pathogenic bacterial threats. To accomplish this goal, the stability of bacteriocin 105b in a textile must be evaluated and supplemented as necessary to ensure the antimicrobial peptide remains active. Long-term stability and activity of bacteriocin 105b should be established to determine the effects of potential storage conditions of the antimicrobial textile in an employable environment. Additionally, the activity of bateriocin 105b against commensal flora, as well as the identity of any remaining impurities in the extract of bacteriocin 105b, must be evaluated to ensure the safety of the Warfighter. The development of a purification protocol specific to bacteriocin 105b to yield a highly pure antimicrobial peptide with narrow-spectrum activity is a significant step towards advancing a novel construct to provide antimicrobial protection to the Warfighter.

This document reports research undertaken at the U.S. Army Natick Soldier Research, Development and Engineering Center, Natick, MA, and has been assigned No. NATICK/TR- 17/009 in a series of reports approved for publication.

5 References

Abt, M.C. and E.G. Pamer. “Commensal Bacteria Mediated Defenses against Pathogens”. *Curr. Opin. Immunol*. 29C: 16-22 (2014).

Carolissen-Mackay, V., G. Arendse, and J.W. Hastings. “Purification of Bacteriocins of Lactic Acid Bacteria: Problems and Pointers”. *Int. J. Food Microbiol*. 34:1-16 (1997).

Cotter, P.D., C. Hill, and R.P. Ross. “Bacteriocin Developing Innate Immunity for Food”. *Nat. Rev. Microbiol.* 3:777-78 (2005).

Cotter, P.D, Ross, R. P., Hill, C. “Bacteriocins – A Viable Alternative to Antibiotics?” *Nat. Rev. Microbiol*. 11:95-105 (2013).

Cox, C.R., P.S. Coburn, and M.S. Gilmore, M. S. “Enterococcal Cytolysis: a Novel Two Component Peptide System that Serves as a Bacterial Defense Against Eukaryote and Prokaryote Cells*”. Curr. Protein Pept. Sci.* 6:77-84 (2005).

Galvez, A., H. Abriouel, R.L. Lopez, and N.B. Omar. “Bacteriocin-based Strategies for Food Biopreservation”. *Int. J. of Food Microbiol*. 120:51-70 (2007).

Gulberg, E., S. Cao, O.G. Berg, C. IIback, L. Sandegren, D. Hughes, and D.L. Andersson. “Selection of resistant bacteria have very low antibiotic concentrations”. *PLOS pathogens* 7(7):1-9 (2011)

Heng, N.C.K, P.A. Wescombe, J.P. Burton, R.W. Jack, and J.R. Tagg. “The Diversity of Bacteriocins in Gram-Negative Bacteria”. *Bacteriocins: Ecology and Evolution* Eds. M.A. Riley, M.A. Chavan Heidelberg: Springer-Verlag Berlin Heidelberg, 2007. 45-92.

Levy, S.B. “Factors Impacting on the Problem of Antibiotic Resistance”. *J. Antimicrob. Chermother.* 49(1): 25-30 (2002).

Levy, S.B. B. and Marshall. “Antibacterial resistance worldwide: causes, challenges and response”. *Nat. medicine*. 10(12):S122-S129. (2004).

Knobler, S.L., S.M Lemon, M. Najafi, *et al*., editors; “The Resistance Phenomenon in Microbes and Infectious Disease Vectors: Implications for Human Health and Strategies for Containment: Workshop Summary”. *Washington DC: National Academies Press (US)*; (2003) Available from: http://www.ncbi.nlm.nih.gov/books/NBK97126

Pingitore, E.V., E. Salvucci, F. Sesma, and M.E. Nader-Macias. “Different Strategies for Purification of Antimicrobial Peptides from Lactic Acid Bacteria (LAB)”. *Communicating Current Research and Education Topics and Trends in Applied Microbiology* Ed. A. Mendez-Vilas. Spain: Formatex, 2007

Stote, R. E. (2015). *Protocol for Initial Purification of Bacteriocin*. (Tech. Rep. NATICK/TR-16/002). Natick, MA: U.S. Army Natick Soldier Research, Development and Engineering Center.