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Staphylococcus aureus is an opportunistic pathogen that has become an increasing issue over the years due to its acquired resistance to different antibiotics such as methicillin[1]. The development of resistance has sparked interest in finding new ways to combat *S. aureus*, and other pathogens. Recent hypotheses suggest that different types of commensal bacteria in the skin are able to cross-inhibit virulence in *S. aureus*, by suppressing the production of its virulence factors responsible for disease[10]. Cross-inhibition is believed to involve autoinducing signaling molecules that are unique to different bacterial species. These signaling molecules are responsible for self-regulating functions in a bacterium that produces them and for potentially disrupting similar processes in other species[10,13]. More knowledge is needed about how these signaling molecules from commensal bacteria can affect quorum sensing in the bacterial pathogen *S. aureus*, however, in order to further study these interactions, the properties of the signaling molecules must be known, starting with their molecular structure. The goal of this project is to detect and elucidate the structures of unknown signaling molecules from different commensal staphylococcal species that have previously displayed cross-inhibition activity against quorum sensing in *S. aureus*.

IDENTIFICATION OF COMMENSAL BACTERIA AUTOINDUCING PEPTIDES WITH ULTRAHIGH PERFORMANCE LIQUID CHROMATOGRAPHY – HIGH RESOLUTION MASS SPECTROMETRY

By

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

1.1 Methicillin Resistant *Staphylococcus aureus* (MRSA)

Antibiotic resistance pathogens have become an increasing issue in recent years. Antibiotics discovered and developed in the past are not as effective as they once were against the pathogens they were intended to target. According to the Center for Disease Control and Prevention, methicillin resistant *Staphylococcus aureus* (MRSA), caused over 80,000 severe infections and 11,000 deaths in the United States in 2015 alone, and it has been responsible for more deaths than HIV/AIDs in recent years[1-4]. The development of new antibiotics is not only costly and time consuming but is potentially a short-term solution, as pathogens could quickly evolve resistance to the new antibiotics. This problem highlights the need to develop new approaches to treat MRSA as well as other emerging cutaneous pathogens, which have been on the rise[1,2]. A promising approach is to develop therapeutics that will target virulence rather than killing bacterial pathogens to break the cycle of resistance[4]. The first task to do this is to develop an understanding of the systems that regulate virulence in bacteria pathogens. Virulence in Gram-positive bacteria, such as *Staphylococcus aureus*, is regulated via quorum sensing by a system called the accessory gene regulator (*agr*) system. The *agr* system is a cell density dependent system, which is activated by a small cyclic peptide known as the autoinducing peptide or AIP. Quorum sensing regulates cell population density and controls the production of virulence factors such as the toxins that are responsible for the negative effects of *S. aureus* infections[5]. Due to the involvement of the agr system in virulence, finding ways to disrupt this process could influence the pathogenicity of MRSA.

1.2 Commensal Bacteria and Cross-Communication with *S. aureus*

Many types of commensal bacteria that reside in and on the human body can play an important role in human health by protecting against pathogenic bacteria. Recent studies focused on screening spent media from different commensal bacteria against MRSA have led to the hypothesis that some species of commensal bacteria inhibit quorum sensing by pathogens sucas *S.* aureus[6]. Signaling molecules called autoinducing peptides (AIPs) from some species in the *Staphylococcus* genus have been shown to hinder the production of virulence factors from S. aureus by directly competing for the binding of the sites involved in this self-regulation process[6]. However, the mechanisms involved in this process are not fully understood, making quorum sensing inhibition an area with a big knowledge gap. To acquire a better understanding of cross-inhibition, all of the factors involve must be accounted for and understood as much as possible. This is why AIPs are of great importance into understanding how this process is regulated and/or inhibited. This project was focused on identifying the structures of previously unknown AIPs from different staphylococcal species that have shown to influence quorum sensing of *S. aureus*. Focusing on MRSA and on these species is especially of great importance, given that staphylococci are the most abundant bacteria colonizing the skin[13]. The strains listed on **Table 1** were selected to study cross-inhibition with *S. aureus*. These strains were selected due to previous studies suggesting their involvment in cross-inhibition against *S. aureus* and MRSA[27,28].

1.3 Quorum Sensing Regulated by Autoinducing Signaling Peptides

Staphylococcal species use quorum sensing to communicate with one another and to regulate gene expression. *Staphylococcus aureus* uses quorum sensing to produce a broad array of virulence factors that contribute to its pathogenesis[5]. The production of these virulence factors is regulated via the accessory gene regulator (*agr*) quorum-sensing system[5,7,8]. This system is an auto-inducing system found in many staphylococcal species, but best understood in *S. aureus.* As illustrated in **Figure 1[9]**, the *agr* operon (gene cluster) encodes four proteins, Agr A – D of which AgrC and AgrA form a two-component regulatory system. AgrD consists of three regions: an amphipathic N-terminal section which localizes AgrD into the inner membrane, a pro-peptide

which is the linear precursor of its AIP, and a C-terminal recognition component that is recognized by AgrB, a membrane-bound endopeptidase, which is responsible for cleaving the amphipathic N-terminal and C-terminal recognition regions from AgrD and cyclizing the AIP precursor into the final AIP structure into the extracellular medium[5,7-9]. When the AIP concentration in the extracellular matrix exceeds a critical threshold, AIP binds to AgrC, a membrane bound histidine kinase that will phosphorylate AgrA, an intracellular transcription factor. AgrA then binds to the P2 promoter which upregulates the *agr* operon that will then increase the production of AgrA – D and therefore, maximizing the quorum sensing cycle. Simultaneously, AgrA upregulates the P3 promoter which is responsible for upregulating the transcription of the virulence factors responsible for illness in the host[5, 7-9]. AIPs of different staphylococcal species share similarities in structures. Typically, they consist of a linear amino acid sequence with a 5-residue cyclic thiolactone ring linked between the sulfhydryl group of the cysteine and the α-carboxyl group of the C-terminal methionine (Figure 1), however, different species and strains vary in the amino acid sequence and length[9]. Although gene sequence analysis of the agr locus has made it possible to predict the amino acid sequence of AgrD, the precise mechanism that takes places when AgrD binds to AgrB in order to undergo cyclization and transformation into the final AIP structure is not well understood[7,9,26]. It is known that SpsB, a signal peptidase, is involved in the biosynthesis of AIP and that it plays a role in splicing the N-terminal region from AgrD after it has been cyclized, however, the lack of knowledge of this process makes it difficult to predict the structure of the AIP structure with precission. There are regions in the AgrD sequence which appear to be conserved and indicative of where the precursor peptide will be cyclized, and which region of the sequence will most likely include the AIP structure, however, analysis of known AIPs from different bacterial species and strains have demonstrated inconsistencies on which region of the AgrD N-terminal tail is selected by AgrB to splice it[7,9,27].

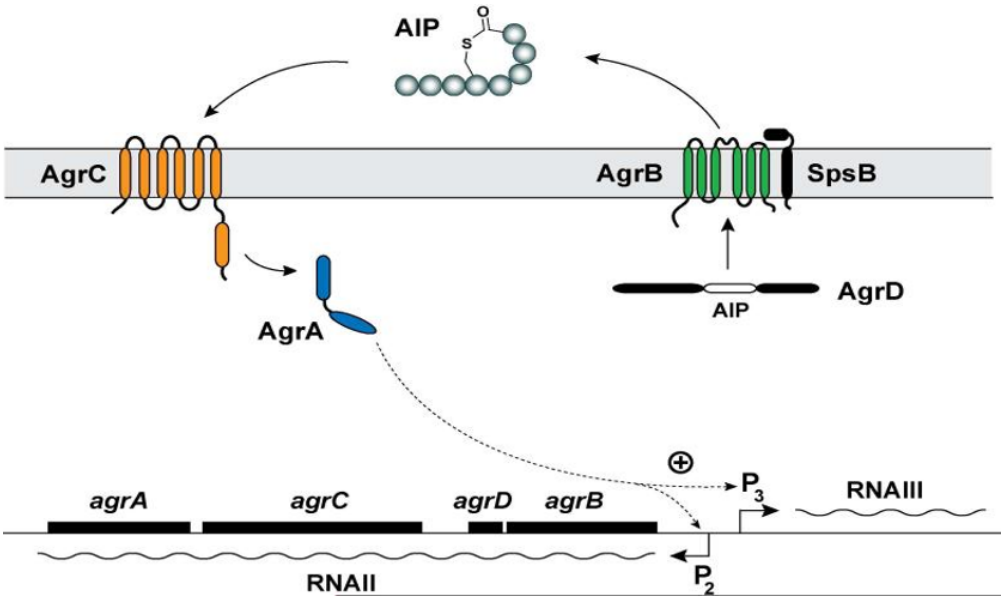


Figure 1[9]. Quorum Sensing System in *S. aureus* and Production of AIP I by the Agr System. The Agr system consists of four genes (Agr A-D). The AgrD is the precursor to AIP I, and upon binding to AgrB, it is cyclized to form a marcrocyclic thiolactone (AIP I) that is then transferred through the cytoplasmic membrane. AIP I then binds to the AgrC protein causing the phosphorylation of the AgrA, resulting in the activation of either the P2 or P3 promoter[4,6-8]. This figure is an adaption of the version reported by Thoendel et al. Copyright 2009 The American Society for Biochemistry and Molecular Biology, Inc.

1.4 Discovery and Structure Elucidation of Signaling Peptides from Staphylococcal Species via Semi-Targeted and Targeted LC-MS Analysis

The lack of understanding of the mechanism between AgrB and AgrD leads us to the need for effective strategies to determine the structures of unknown AIPs. A first approach towards this goal of identifying new AIPs is by directly screening the spent media filtrate from bacterial cultures of the staphylococcal species of interest, via liquid chromatography and mass spectrometry analysis[11]. This is accomplished by using procedures previously established by our laboratory to culture bacteria in favorable conditions for the production of AIPs. Using the known genetic information of AgrD from each species of bacteria being studied, predictions can be made about the amino acid sequence of the AIPs, as well as the masses for each peptide sequence. These predictions allow for the screening of these peptides using mass spectrometry analysis[11]. However, biological variations concerning the production of AIPs from different bacteria and

variations in chemical interactions from different AIP structures may influence their final concentration in the spent media, which could make the direct analysis of spent media a challenge if the concentration of AIPs is too low. To overcome this limitation, sample preparation methods were used to increase the AIP concentration from the spent media samples. These methods involve the use of a sample purification chromatography technique called solid-phase extraction, which is used to separate and purify compounds from a matrix based on their chemical properties[24].

CHAPTER II

METHODS

2.1 Analysis of Autoinducing Peptides from Commensal Staphylococcal Strains

The precursor peptide AgrD is comprised of three components, an N-terminal amphipathic helix, a middle region that makes up the AIP and a charged C-terminal[9]. Some studies in the past have focused on investigating the importance of each component of AgrD for the biosynthesis of the AIP by sequentially deleting amino acid residues from both the C-terminal tail and the N-terminal helix. It has been found that the entire amphipathic N-terminal helix and the first 10 residues of the C-terminal tail are necessary for AIP production, however, only a region referred as the middle region is transformed into the AIP[7,9]. The middle region is determined by conserved residues found in the C-terminal tail and N-terminal helix such as an aspartate, glutamine, and leucine in the C-terminal tail and a glycine in the N-terminal helix[9] as well as the cysteine that is responsible for forming the thiolactone ring in the AIP[7,9]. The amino acid sequence for the middle region that can be determined has additional residues that are not part of the AIP and there is no clear indication of which residues are lost when forming the AIP without understanding the mechanism that takes place in the AgrB, This sets the opportunity for the use of UPLC-MS to screen for the possible AIP masses based on the known middle region of the AgrD of a given species. **Table 1** shows the staphylococcal strains and their corresponding middle AgrD sequences that were analyzed in this project to search for their AIPs and ultimately elucidate their structure.

Table 1. Commensal Staphylococcal Strains Studied

Species	Strain Number	Middle AgrD sequence
S. warnerii	AH 4548	FVAGYSPCTNFF
	AH 5013	
S. simulans	AH 4549	NLAKYNPCLGFL
	AH 5532	NLAKYYPCWGYF
	AH 5534	NLAKYNPCWGYF
S .hominis	AH 4553	TLASYSPCATF

2.2 UPLC-MS for Peptide Analysis

Mass spectrometry in tandem with liquid chromatography (LC-MS) is an effective technique for identification of compounds present in complex mixtures, including the analysis of peptides and proteins. One of the most efficient and broadly used techniques for this process is through collision-induced dissociation (CID), which employs gas phase collision at low energy to break a molecule into fragments[11]. The fragments are then measured with a second stage mass spectrometric analysis. This combination of first and second stage mass analysis is often referred as MS^2. This method is ideal for peptide detection and structure elucidation, given that peptides are made up of amino acids which fragment readily at their amide bonds, consequently creating a cascade of ion fragments that represent the amino acid sequence of the initial peptide[16]. By relying on accurate mass measurements of intact peptides and the ion fragments that result from CID, unknown AIP structures can be identified[11,16]. The Q-Exactive, a high resolving power hybrid mass spectrometer, was used for this study. It includes a segmented quadrupole and Orbitrap mass analyzer, allowing for the improvement in sensitivity and dynamic range in analysis. This makes it possible to detect masses in complex matrices without prior sample clean up and for better quantification in comparison to other instruments[11].

The Q-Exactive has allowed for effective detection and quantification of AIP produced by some staphylococcal species, including MRSA and other *S. aureus* strains, through the direct analysis of the spent media filtrate obtained from large-scale growth cultures. The workflow for this process is illustrated in **Figure 2.** The middle region of the *AgrD* sequence (**Table 1**) is used to calculate the possible mass of an AIP by process of elimination. By omitting an amino acid at a time from the AgrD sequence and calculating its mass sequentially, we can screen for previously unidentified AIPs based on their single charged (z=1) and double charged (z=2) mass to charge ratios. This allows for the detection of the molecule in its entirety and the ions it produces when fragmented, down to the 5-membered thiolactone ring[11]. *S. aureus* AH1263 has been subject to these growth and filtration techniques and analyzed via LC-MS using a Q-Exactive mass spectrometer as mentioned. The extensive work done with *S. aureus* in our lab has allowed for the opportunity to apply these methods on other bacteria in order to identify their corresponding AIPs.

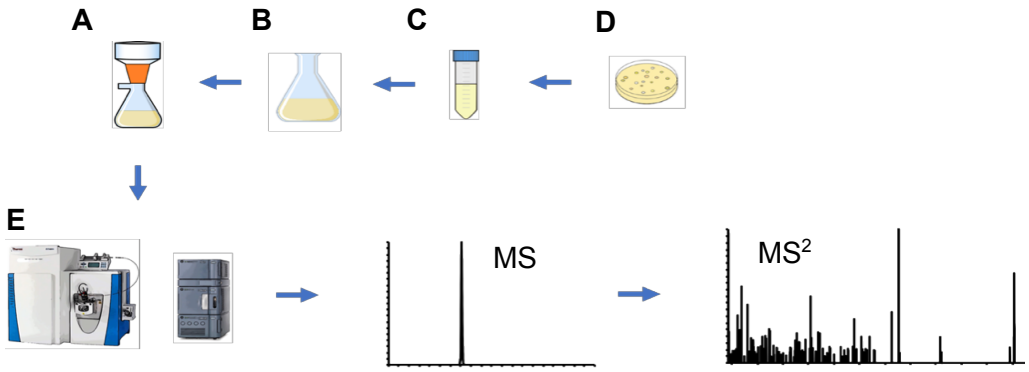
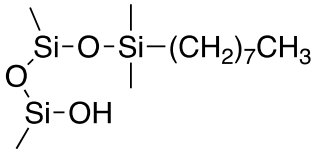
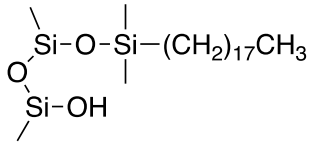
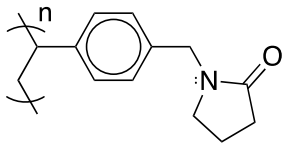
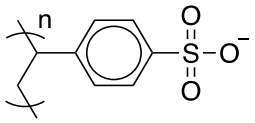
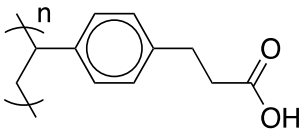


Figure 2. Experimental Workflow for Direct Analysis of Bacterial Culture Filtrate. Bacteria are subject to various growth techniques. (A) Bacterial culture is grown on agar plate, (B) a single colony is grown in liquid tryptic soy broth overnight, (C) overnight culture is diluted 1:200 in tryptic soy broth (200 mL) and grown for 17-24 hours, (D) spent media is filtered from the culture, (E) spent media is analyzed using an LC-MS analysis strategy involving a Thermo Scientific Q Exactive Plus Mass Spectrometer coupled to an Acquity Ultra-High Performance Liquid Chromatography System to elucidate the AIP structure.

2.3 Peptide Purification via Solid Phase Extraction

The direct analysis of spent media from a bacterial culture does not always allow for detection of AIPs. Some bacterial strains may not produce AIPs in a high enough concentration to meet the threshold and limit of detection of the Q-Exactive mass analyzer which is around 0.0035 μM.[11] Also, the spent media in which the bacteria is cultured is very complex and contains many compounds that could interfere with detection of the AIPs[18]. If the AIPs are low in abundance in a sample, it can be challenging to detect them using LC-MS without prior sample clean-up. To resolve this issue, solid phase extraction (SPE), a widely used technique for the preparation of samples prior to LC-MS analysis can be used[18]. SPE is a method commonly applied to the extraction and clean-up of small molecules from biological samples such as serum, plasma, silica, and urine, which are very complex matrices[18,19]. In essence, SPE is a chromatography technique that allows for the removal of components that may cause interference during analysis of an analyte of interest. SPE involves the use of a stationary phase (sorbent) and a sequence of different mobile phase washes with varying polarities in order to selectively retain and extract compounds from a matrix sample based on their physicochemical properties[19-22]. This process is referred to as the solvent switching method[21,22]. Traditionally, SPE for peptide analysis consists of the five steps highlighted in **Figure 3.** This process can be very effective for exploiting the hydrophobic, basic and acidic characteristics of different peptides to be purified. Conventionally, the most commonly used SPE columns are reversed phased columns consisting of organosilicon groups bonded to 8-carbon (C8) chains or bonded to 18-carbon (C18) chains as sorbents. These have proven to be effective tools for selectively separating oligopeptide from different types of matrices, as well as for the fractionation of small peptides into basic, neutral and acidic pools[23].

Table 2. SPE Column Types and Properties

Phase	Sorbent Type	Functional Group	Sorbent pKa	Target Analytes
C8-bonded silica	Octyl		~4.0	Non-polar & highly hydrophobic
C18-bonded silica	Octadecyl		~4.0	Highly polar & hydrophobic
Polymeric Reversed Phase	Polymer-based		~14.7	Neutral & aromatic
Strong Cation Mixed Mode	Polymer-based		~0	Weakly basic
Weak Cation Mixed Mode	Polymer-based		~4.5	Highly basic

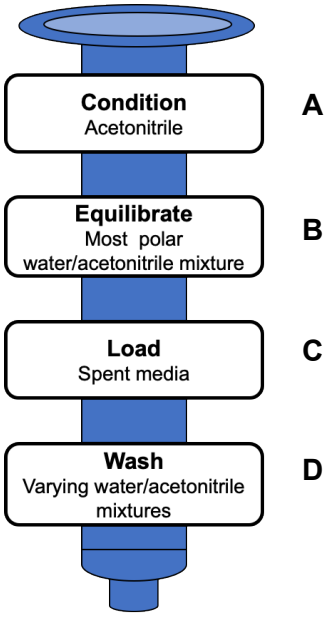


Figure 3. Five Step Sample Purification in Solid-Phase Extraction.(A) Column is conditioned and (B) equilibrated with the starting solvents. (C) The spent media is then loaded and flushed out to waste. (D) The solvent selected based on polarity is loaded and (E) eluted out to be collected.

Different bacteria may follow different patterns in growth and output of AIPs, making it possible that a given SPE method will not be equally effective across all strains without further experimentation and method development. For this, different strains were tested on the different columns listed on **Table 2.** There are SPE columns available beyond the conventional C8 and C18 columns which range in selectivity based on their sorbent structure and functional groups which ultimately influence their affinity for different analytes (**Table 2**)[24]. The SPE columns chosen for this study were based on analyte selectivity, determined by the sorbent size, structure, and polarity, which correlate to the sorbents pKa values. Additionally, the chemical properties and interactions of peptides vary depending on their amino acid sequence, which will ultimately influence how they interact with in different columns. This is important to consider in order to identify the most suitable columns and solvents needed to optimize extraction and purification of AIPs from the sample matrix.

CHAPTER III

RESULTS

3.1 *S. aureus* as a Control

S. aureus was used as a control through multiple experiments focused on identifying unknown AIPs to confirm the validity of the methods used. **Table 3** shows the calculated monoisotopic protonated masses from the mid-AgrD region of AIP-I produced by S. *aureus*.

Table 3. Calculated Mass to Charge Ratios Using AgrD Sequence from *S. aureus*

Middle region of AgrD	***m/z1***	***m/z2***
NIAAYSTCDFIM	1330.58116	665.290581
IAAYSTCDFIM	1216.53824	608.269118
AAYSTCDFIM	1103.45417	551.727086
AYSTCDFIM	1032.41706	516.208529
YSTCDFIM	961.37994	480.689972
STCDFIM	798.31662	399.158312
TCDFIM	711.28460	355.642298
CDFIM	610.23692	305.118458

Full-scan MS analysis was used to detect $[M+H]^+$ ion of 961.3774 corresponding to the peptide sequence YSTCDFIM (**Figure 4**). MS^2 analysis of the precursor ion detected was conducted to detect the masses corresponding to the fragments down to the thiolactone ring (CDFIM). A synthetic AIP standard with the corresponding sequence (YSTCDFM) was ordered from Anaspec EGT (Fremont, CA), it was analyzed via UPLC-MS analysis and compared with

spent media from *S. aureus*. The [M+H+] ion 961.3773 for AIP-I was detected on both the spent media and the standard as shown in **Figure 4,** with close retention time.

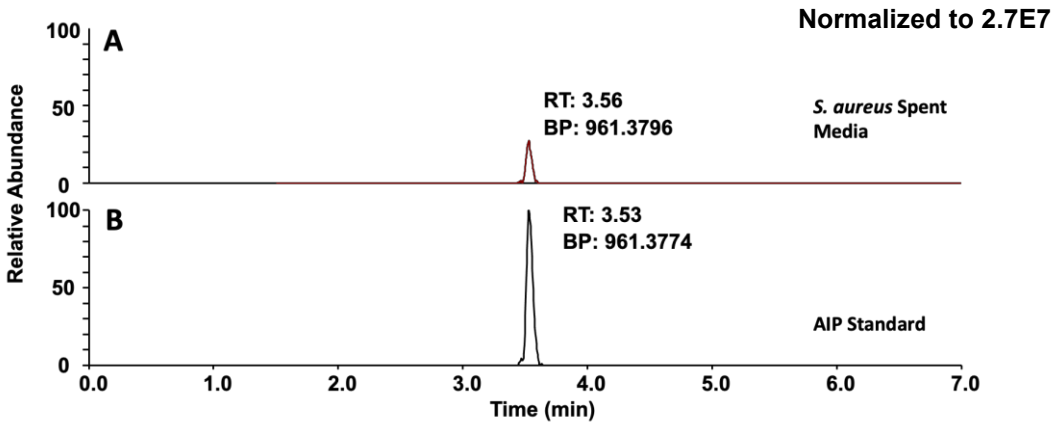


Figure 4. Comparison of Selected Ion Chromatograms (*m/z* 961.3799) *S. aureus* and synthetic AIP (YSTCDFIM) standard in tryptic soy broth. (A) *S. aureus* spent media RT = 3.56) displayed a peak with mass of 961.3796 and (B) AIP standard (RT = 3.53) displayed a peak with mass of 961.3774.

MS^2 fragmentation analysis was performed on the base peak ions detected for the $[M+H]^+$ 961.37994 for the spent media and the synthetic AIP standard samples to produce the fragmentation spectra shown in **Figure 5.** The fragmentation spectra for both samples display masses very close to the theoretical masses calculated from the AgrD sequence of AIP-I. Spectra comparison of the spent media and AIP standard for the AIP mass provides a high level of confidence necessary for the determination of a given AIP in the spent media.

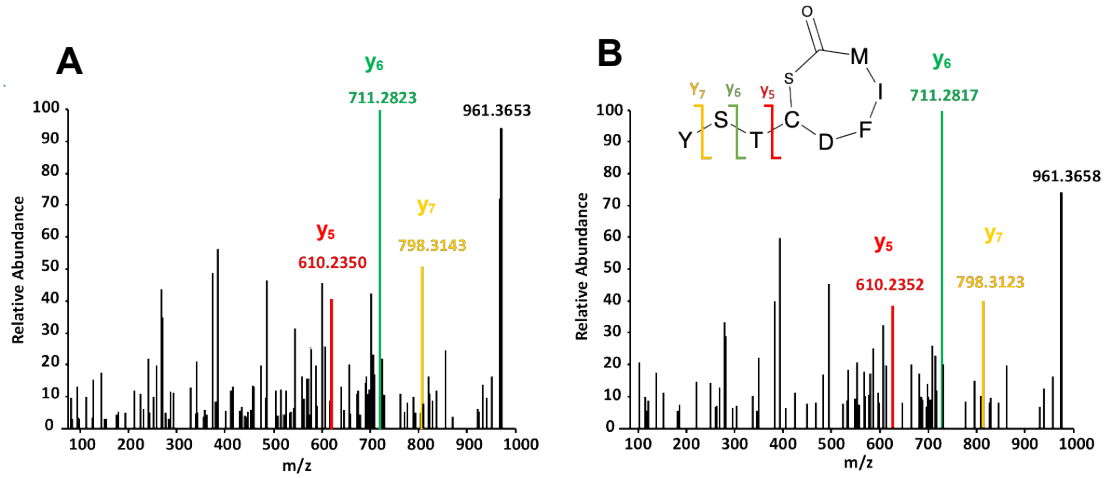


Figure 5. Comparison of MS^2 Fragmentation for [M+H]$^+$ ion 961.379) corresponding to (A) *S. aureus* spent media and (B) synthetic AIP (YSTCDFIM) standard in tryptic soy broth.

3.2 Direct UPLC-MS Analysis of Spent Media

The experimental methodology and analysis described in **Figure 2** and section **3.1** were used to analyze the spent media from every bacterial strain in this project. Multiple attempts made it possible to detect distinct masses corresponding to AgrD sequence and fragments of it for each strain. Results varied greatly among different strains and experimental runs, with varied abundance of AIPs detected in some runs and no detection of AIP in other runs. On average, the abundance of AIPs detected across multiple runs were from low to none, with NL levels for the intensity of the peaks as low as E3, nearing the limit of detection. *S. simlans* AH4549 was the first strain screened for the detection of AIPs. The direct analysis of its spent media was sufficient to detect [M+H]$^+$ ion of 1036.52899 corresponding to peptide sequence KYNPCLGFL. A synthetic AIP matching this peptide sequence and the spent media of AH4549 were subject to targeted analysis to compare the detection of this base peak (**Figure 6)** and the fragments produced under CID analysis (**Figure 7**).

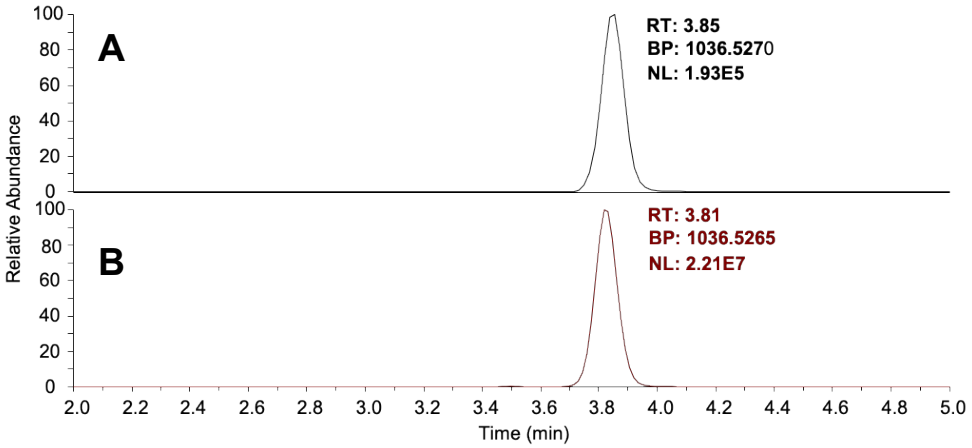


Figure 6. Comparison of Selected Ion Chromatograms (*m/z* 1036.5290) of *S. simulans* AH4549 and synthetic AIP (KYNPCLGFL) standard in tryptic soy broth. (A) AH4549 (RT = 3.85) displayed a peak with mass of 1036.5270, shown at normalization level of 1.93E5. (B) AIP standard KYNPCLGFL (RT = 3.81) displayed a peak with mass of 1036.5265, shown at normalization level of 2.21xE7.

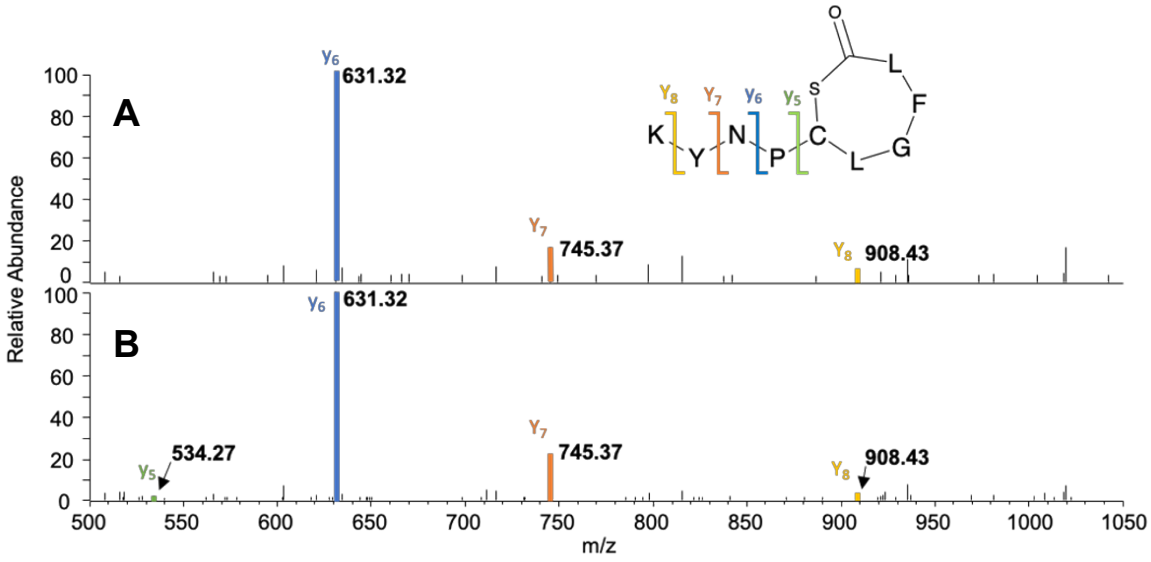


Figure 7. Comparison of MS^2 Fragmentation for Ion [M+H]$^+$ 1020.4137 corresponding to (A) *S. simulans* AH4549 spent media and (B) synthetic AIP (KYNPCLGFL) standard in tryptic soy broth.

After multiple runs of the direct analysis of AIPs from *S. simulans* strains AH5532 and AH5534, masses corresponding to peptide sequences KYYPCWGYF and KYNPCWGYF respectively, were detected and used to compare the spent media against synthetic AIP standards with the corresponding sequences. Although only one amino acid residue differentiated the AIPs for AH5532 and AH5534, detection of AIPs for AH5534 was of much less abundance across every run. When detected at higher abundances, the abundance of AIPs for AH5534 was detected close to 6.7 times less than that of AH5532 based on NL as shown in **Figures 8 and10**, with NL of 1.95E5 and 2.87E4 respectively.

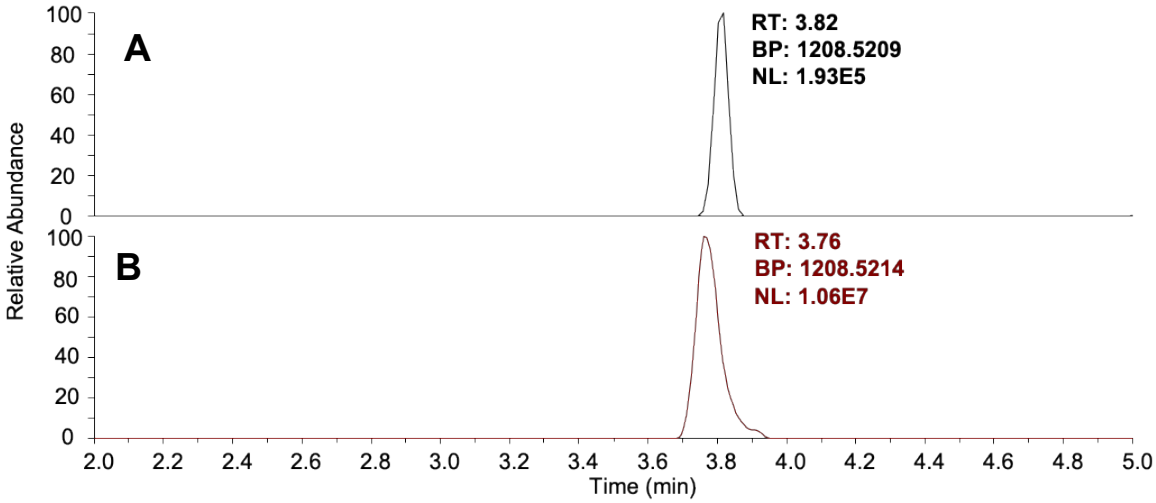


Figure 8. Comparison of Selected Ion Chromatograms (*m/z* 1208.5239) of *S. simulans* AH5532 and synthetic AIP (KYYPCWGYF) standard in tryptic soy broth. (A) AH5532 (RT = 3.82) displayed a peak with mass of 1208.5209, shown at normalization level of $1.93x10^5$. (B) AIP standard KYYPCWGYF (RT = 3.76) displayed a peak with mass of 1208.5214, shown at normalization level of $1.06x10^7$.

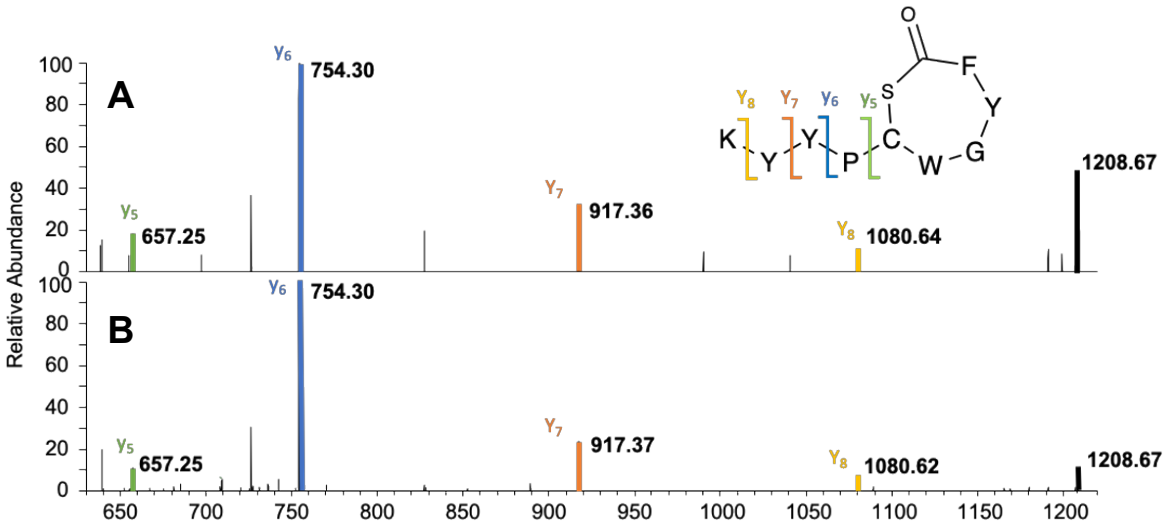


Figure 9. Comparison of MS^2 Fragmentation for Ion [M+H]$^+$ 1208.5239 corresponding to (A) *S. simulans* AH5532 spent media and (B) synthetic AIP (KYYPCWGYF) standard in tryptic soy broth.

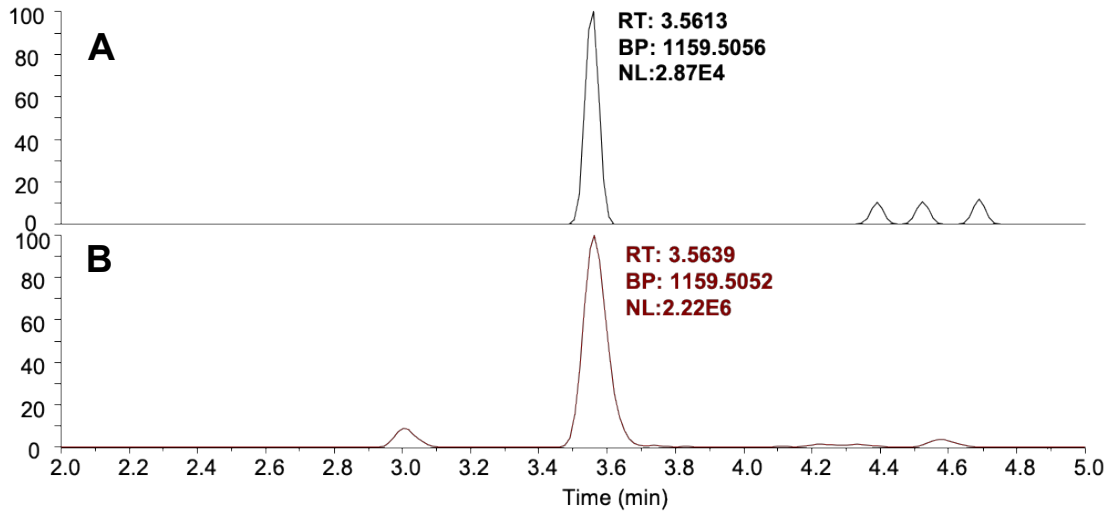


Figure 10. Comparison of Selected Ion Chromatograms (*m/z* 1159.5035) of *S. simulans* AH5534 and synthetic AIP (KYNPCWGYF) standard in tryptic soy broth. (A) AH5534 (RT = 3.56) displayed a peak with mass of 1159.5056 shown at normalization level of 2.87X10^4. (B) AIP standard KYNPCWGYF (RT = 3.56) displayed a peak with mass of 1159.5035, shown at normalization level of 2.22x10^6.

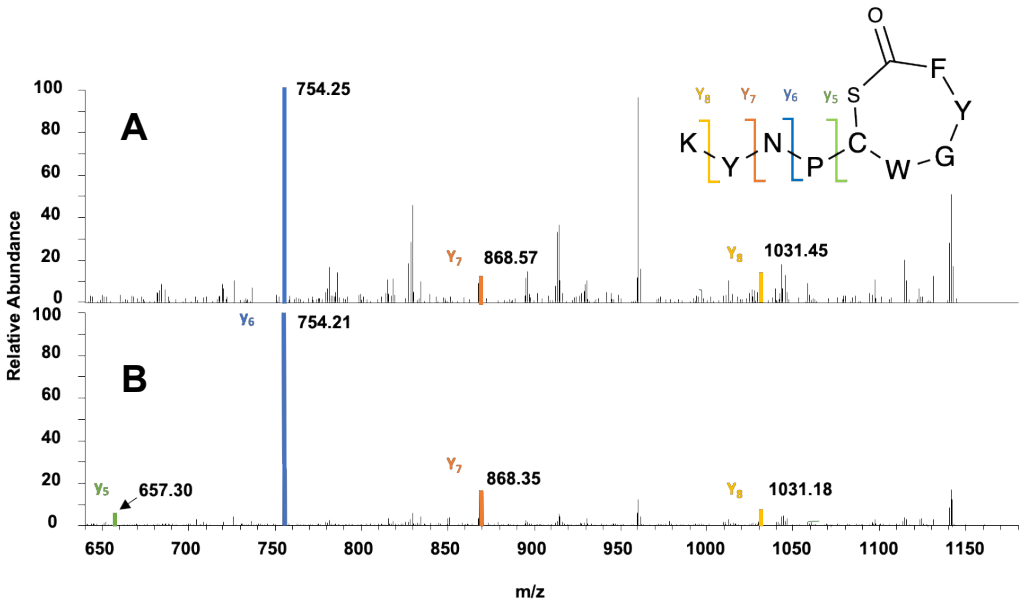


Figure 11. Comparison of MS^2 Fragmentation for Ion $[M+H]^+$ 1159.5035 corresponding to (A) *S. simulans* AH5534 spent media and (B) synthetic AIP (KYNPCWGYF) standard in tryptic soy broth.

A mass corresponding to calculated peptide sequence YSPCTNFF was detected for both *S. warnerii* strains AH 4548 and AH5013. Results for both strains were consistent with one another and consistently aligned with the results of an AIP standard with the same sequence.

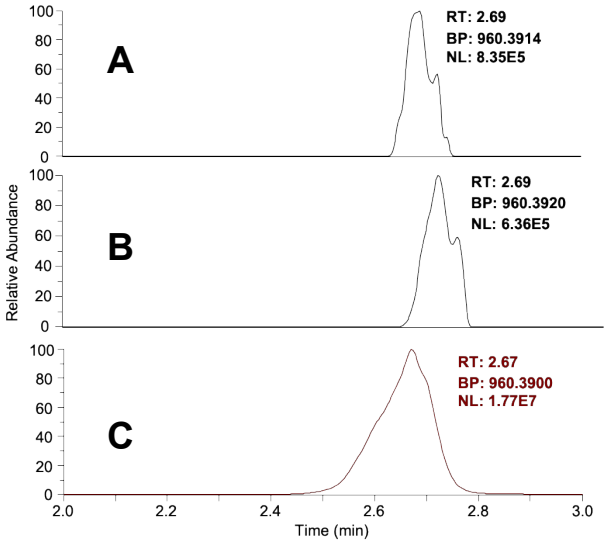


Figure 12. Comparison of Selected Ion Chromatograms (*m/z* 960.3926) of *S. warnerii* AH4548, *S. warnerii* AH5013 spent media and synthetic AIP (YSPCTNFF) standard in tryptic soy broth. (A) AH4548 (RT = 2.69) displayed a peak with mass of 960.3914, shown at normalization level 8.35x10^5. (B) AH5013 (RT = 2.69) displayed a peak with mass of 960.3920, shown at normalization level of 8.35x10^5. AIP standard GYSPCTNFF (RT = 2.67) displayed a peak with mass of 960.3900, normalization level 1.77x10^7.

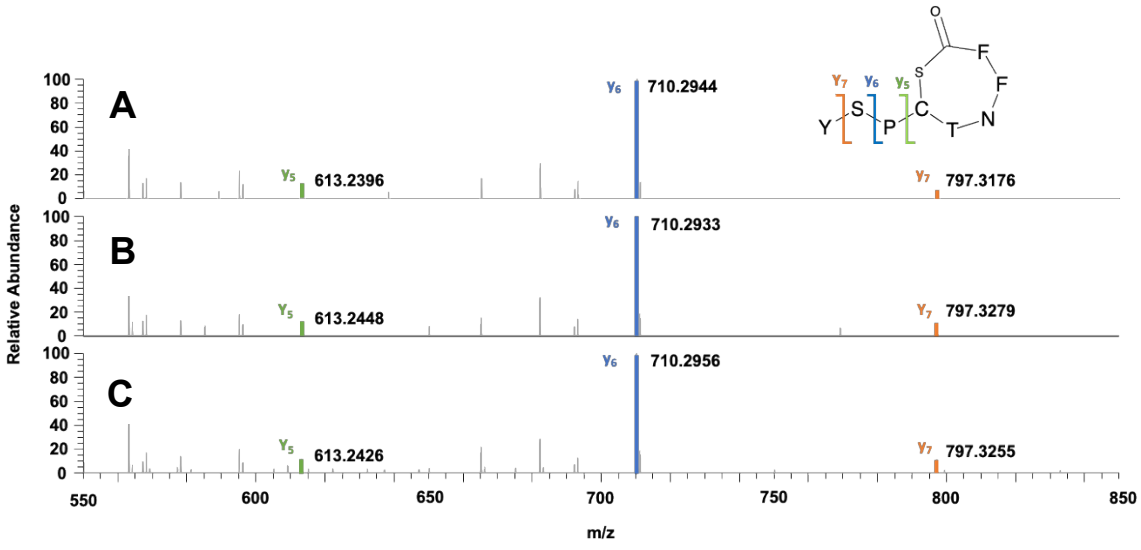


Figure 13. Comparison of MS^2 Fragmentation for Ion [M+H]$^+$ 9603926 corresponding to (A) *S. warnerii* AH4548 spent media, (B) *S. warnerii* AH5013 spent media and (C) synthetic AIP (GYSPCTNFF) standard in tryptic soy broth.

3.3 Solid Phase Extraction to Detect AIPs

Low abundance of AIPs in spent media samples made it difficult to detect them, indicating the need for sample preparation techniques that could help minimize this issue. In this this project, SPE proved to be a working technique to solve the issue of low abundance of peptides in a sample. The spent media of bacteria being studied in this project were subject to SPE methods prior to LC-MS analysis and compared against untreated spent media. As a control, AIP-I from *S. aureus* spent media and its synthetic standard was analyzed. Among the five column types used (**Table 2**) the strong cation mixed mode and the C-18 bonded columns were the only two that demonstrated favorable results. A notable achievement accomplished by the use of SPE was the detection of the AIP corresponding to *S. hominis* strain AH4553. AH4553 was not detected in spent media after multiple runs but was solely detected in the pure methanol fraction of the C-18 bonded column. **Figure 14** shows the comparison of the methanol fraction sample against the crude spent media and different fractions of the strong cation column, which was favorable in other experiments. No peaks were detected in any other sample, nor in the crude spent media but was detected at a NL of 1.61E7 in the methanol fraction which further highlights the efficiency of this method for purifying samples and concentrating peptides such as AIPs.

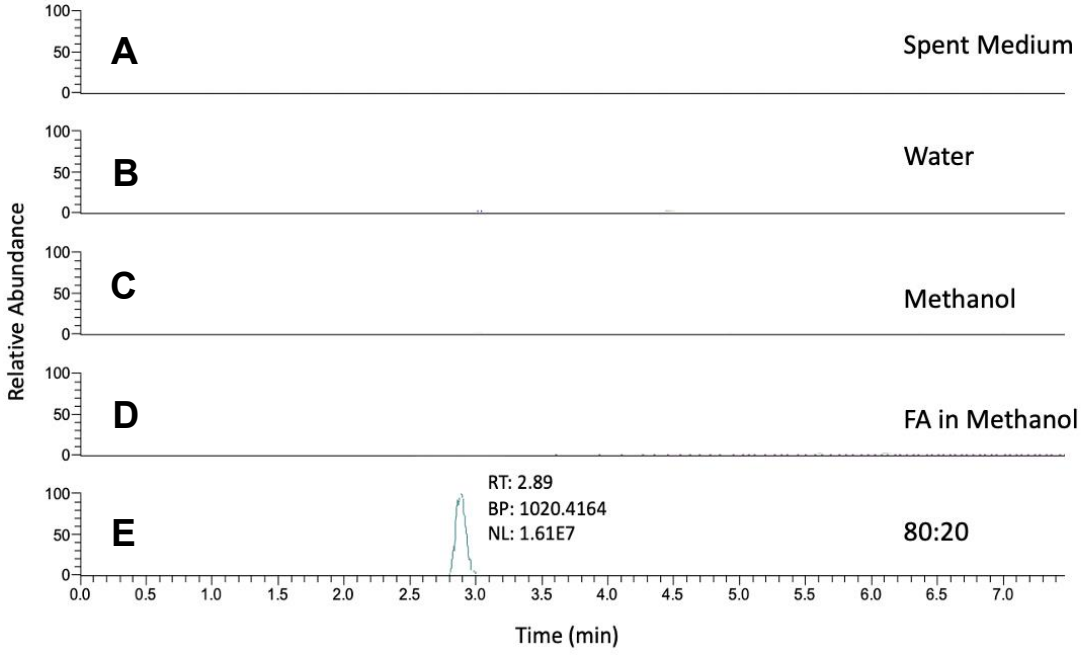


Figure 14. Comparison of Selected Ion Chromatograms (*m/z* 1020.41368) from SC and C18 Column Fractions used on *S. hominis* AH 4553**.** (A) selected ion peak signal absent from the culture filtrate without any SPE treatment, (B) pure water fraction, (C) pure methanol fraction (D) 5% FA in methanol fraction, (E) Pure methanol fraction. The selected ion peak is only present in the pure methanol fraction as outlined in green. All chromatograms were normalized to a signal of 1.61E7.

A separate experiment using the same SPE methods was reproduced for the methanol fraction from the C-18 column and a synthetic AIP standard to confirm the predicted AIP sequence of SYSPCATYF for *S. hominis* AH4553. This AIP was not detected in any other fraction from any other column used, indicating the specificity of each column and the variability of chemical properties from different AIPs. It is important to note that this AIP was prepared at the same concentration as every other AIP standard (10 μM) but demonstrated up to a ten-fold increase in concentration in comparison (E7 to E8 normalization levels).

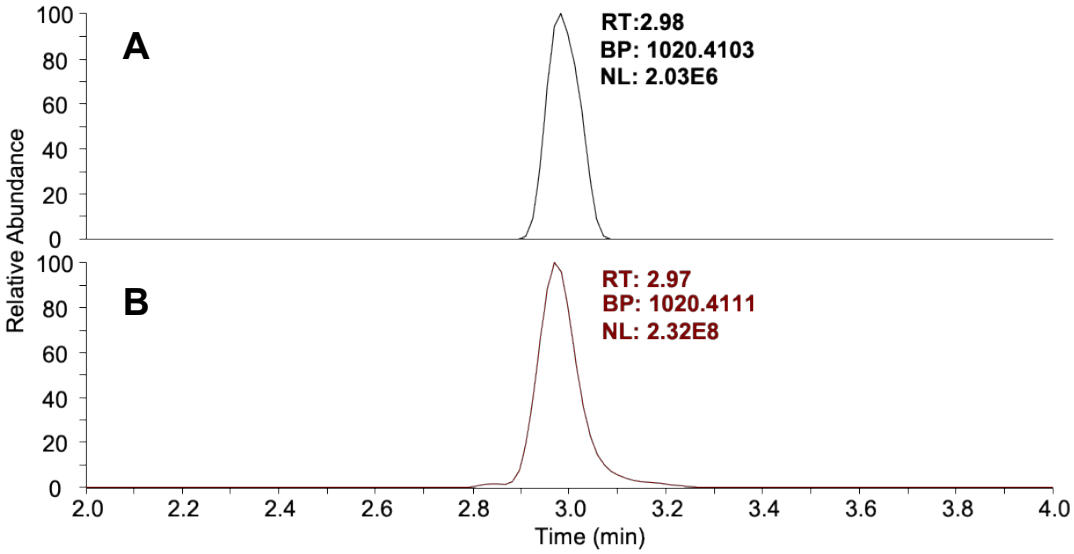


Figure 15. Selected Ion Chromatograms (*m/z* 1020.4134) of Methanol Fraction from *S. hominis* AH4553 and synthetic AIP (SYSPCATYF) in 80/20, water/methanol. (A) AH4553 (RT = 2.98) displayed a peak with mass of 1020.4103 shown at normalization level of $2.03X10^6$. (B) AIP standard SYSPCATYF (RT = 2.97) displayed a peak with mass of 1020.4111, shown at normalization level of $2.32x10^8$.

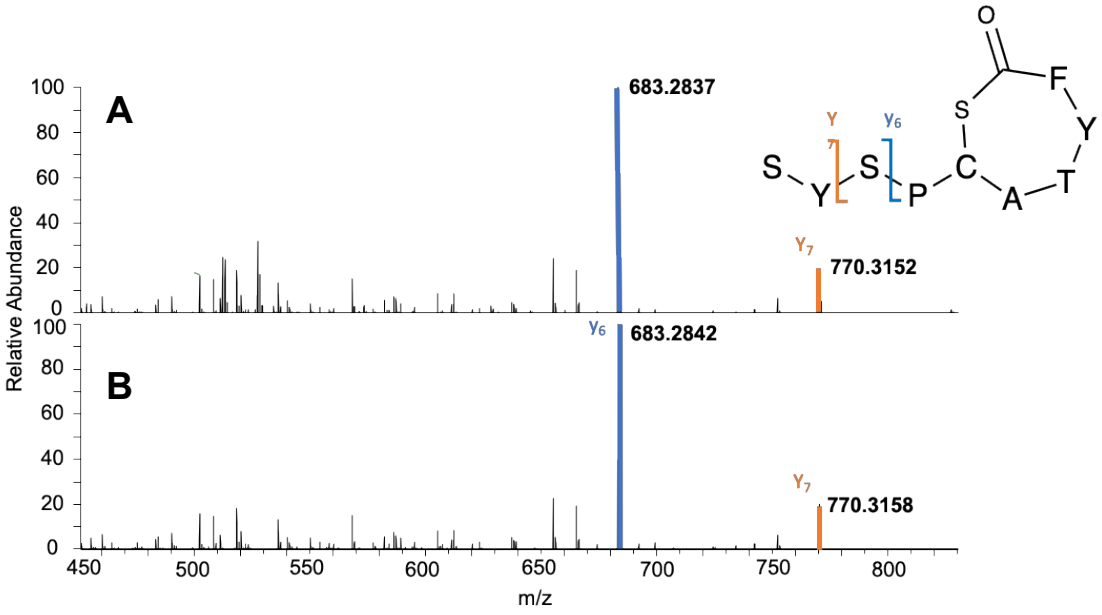


Figure 16. MS^2 Fragmentation for Ion $[M+H]^+$ 1020.4134 of Methanol fraction corresponding to (A) *S hominis* AH4553 SPE methanol fraction (B) synthetic AIP (SYSPCATYF).

Similarly, to *S. simulans* AH 5532, the strong cation column was not as reliable as the C18 column for purifying and detecting AIPs from *S. warnerii* AH5013. However, the fraction that was effective in concentrating AH5013 AIPs from the spent media was in the 80/20, water/methanol fraction. **Figure 17** demonstrates not AIP detection in the spent media of AH5013 and a signal of 8.10E6 for the 80/20, water/methanol fraction in the same run. Although AIP from AH5013 was detected in separate runs as shown in **Figure 12**, SPE made it possible to detect AIP YSPCTNFF for the first time and consistently demonstrated at least a ten-fold increase in AIP abundance when compared to the spent media samples.

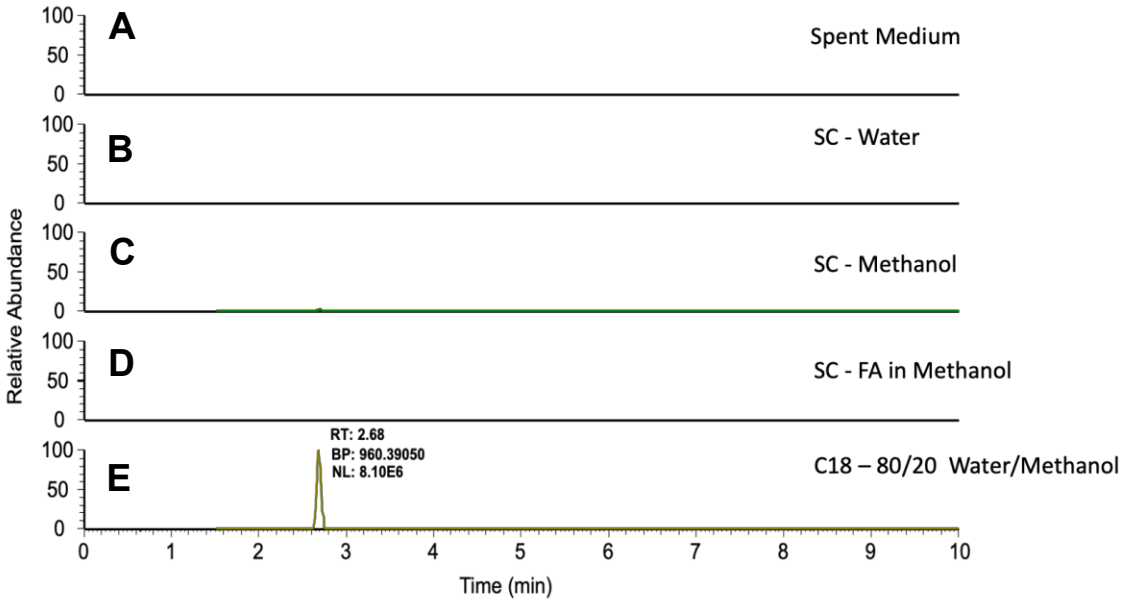


Figure 17. Comparison of Selected Ion Chromatograms (*m/z* 960.39256) of different fractions from the SC column and C18 columns used on *S. warnerii* AH5013**.** (A) SC selected ion peak signal absent from the culture filtrate without any SPE treatment, (B) SC pure water fraction, (C) SC pure methanol fraction (D)SC 5% FA in methanol fraction, (E) C18 methanol fraction ion peak corresponding to sequence YSPCTNFF. All chromatograms were normalized to a signal of $8.10x10^6$.

The C18 bonded column did not prove to be as effective with concentrating AIPs from the *S. simulans* strains. However, in contrast to AIPs from AH4553 and H5013, detection of AIPs from *S. simulans* AH5532 was achieved with the use of pure methanol and the SC column. This combination of column and solvent suggested the specificity of the AIPs, given that no AIPs were detected in spent media and different fractions, except for the pure methanol fraction as shown in **Figure 18.** Multiple runs were required for the detection of AIPs in spent media for AH5532 shown in **Figure 8** with no detection of AIPs being common. In contrast, the use of the SC column to purify the spent media from AH5532 allowed for the detection of AIP with sequence KYYPCWGYF in multiple runs.

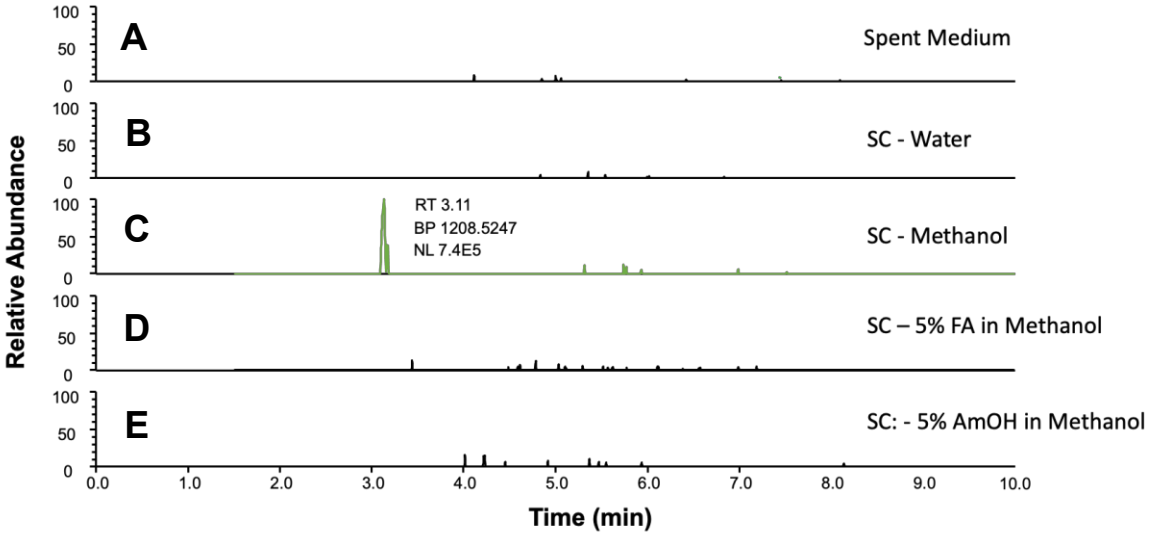


Figure 18. **Selected Ion Chromatograms (*m/z* 1208.5239) from Different SC Fractions** used on *S. simulans* AH 5532**.** (A) selected ion peak signal absent from the culture filtrate without any SPE treatment, (B) pure water fraction, (C) pure methanol fraction (D) 5% FA in methanol fraction, (E) 5% AmOH in methanol fraction. The selected ion peak is only present in the pure methanol fraction as outlined in green. All chromatograms were normalized to a signal of 7.4E5.

Table 4. Confirmed AIPs from studies

Species	Strain Number	AgrD sequence	Confirmed AIP Sequence	Monoisotopic protonated mass
S. warnerii	AH 4548	FVAGYSPCTNFF	YSPCTNFF	960.39256
	AH 5013			
S. simulans	AH 4549	NLAKYNPCLGFL	YNPCLGFL	1036.52899
	AH 5532	NLAKYYPCWGYF	KYYPCWGYF	1208.52389
	AH 5534	NLAKYNPCWGYF	KYNPCWGYF	1159.50350
S. hominis	AH 4553	TLASYSPCATYF	SYSPCATYF	1020.41368

CHAPTER IV

CONCLUSION

The AIPs for six different commensal staphylococcal strains were identified using semi-targeted and targeted UPLC-MS analysis of their spent media. Limitations of the direct analysis of spent media were due to low abundance of AIPs in the spent media, below the limit of detection of the MS instrument. This limitation was addressed by using SPE techniques to purify the samples and concentrate the AIPs. SPE proved to be effective in increasing the concentration of AIP in spent media and for *S. hominis* AH4553 in particular. It made the detection and structure elucidation of its AIP possible, given that it was not detectable in spent media across different experimental runs. Having solved the AIP sequence for these commensal bacteria will allow for further analysis of cross-inhibition against *S. aureus.* Knowing the structure of these AIPs allows for the use of standards that could be used to study the mechanistic approaches of this cross-inhibition as well as to monitor the concentrations required to do so. Ultimately, having the structures for these AIPs allows for the monitoring of these concentrations in cross-inhibition experiments that will also help us understand how these bacteria behave in the human body, how they play a role in health and for their potential in treatments against MRSA and other aggressive pathogens.

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