Quantitative analysis of autoinducing peptide I (AIP-I) from *Staphylococcus aureus* cultures using ultrahigh performance liquid chromatography–high resolving power mass spectrometry

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Junio, H.A., Todd, D. A., Ettefagh, K. A., Ehrmann, B. M., Kavanaugh, J. S., Horswill, A. H., & Cech, N. B. (2013). Quantitative analysis of autoinducing peptide I (AIP-I) from Staphylococcus aureus cultures using ultrahigh performance liquid chromatography – high resolving power mass spectrometry. *Journal of Chromatography B, 930(1)*, 7-12. doi: 10.1016/j.jchromb.2013.04.019

Made available courtesy of Elsevier: http://dx.doi.org/10.1016/j.jchromb.2013.04.019

Abstract:

Staphylococcus aureus infections acquired in hospitals now cause more deaths per annum in the US than does HIV/AIDS. Perhaps even more alarming is the rise in community associated methicillin-resistant *S. aureus* (CA-MRSA) infections, which have spread out of hospital settings and are infecting otherwise healthy individuals. The mechanism of enhanced pathogenesis in CA-MRSA remains unclear, but it has been postulated that high activity in the *agr* quorum-sensing system could be a contributing factor. The purpose of this study was to develop a quantitative method for analysis of autoinducing peptide I (AIP-I), the activating signal for the *agr* system in *S. aureus*. An effective method was developed using ultrahigh performance liquid chromatography (UHPLC) coupled to electrospray ionization mass spectrometry with an LTQ Orbitrap mass spectrometer. Relying on the exceptional resolving power and mass accuracy of this instrument configuration, it was possible to quantify AIP-I directly from the complex growth media of *S. aureus* cultures with a limit of detection (LOD) of 0.25 μM and a linear dynamic range of 2.6 to 63 μM. The method was then employed to monitor time-dependent production of AIP-I by *S. aureus* cultures, and it was observed that AIP-I production reached a maximum and leveled off after approximately 16 h. Finally, it was determined that virulence of *S. aureus* was correlated with AIP-I production in some (but not all) strains analyzed.

Keywords: UHPLC | Staphylococcus aureus | Virulence | AIP-I | Mass spectrometry

Article:

1. Introduction

Staphylococcus aureus as a bacterial pathogen has a major impact on the healthcare system in the US. According to one estimate, methicillin-resistant *S. aureus* (MRSA) infections caused over 100,000 infections and 18,000 deaths in 2005 [1]. Over the past decade, MRSA strains have spread to the community and are infecting otherwise healthy individuals, and these strains have been called community-associated MRSA (CA-MRSA) [2] and [3]. The CA-MRSA strains are known for being hypervirulent compared to older healthcare-associated MRSA (HA-MRSA) [4], and it has been proposed that the enhanced function of the*agr* quorum sensing system and production of virulence factors is in part responsible for the rise in CA-MRSA incidence [5], [6] and [7].

Quorum sensing in *S. aureus* is mediated by cyclic thiolactone peptides known as autoinducing peptides (AIPs) [8]. The extracellular concentration of AIP is sensed by a two-component system encoded in the accessory gene regulator (*agr*) locus. At a critical signal level, the cascade is activated, leading to the production of virulence factors that include toxins, superantigens, and exo-enzymes [9]. Considering that variation in *agr* system function has been linked to MRSA pathogenesis, methods to accurately detect and quantify AIPs would be very useful for studying virulence regulation in *S. aureus*. The goal of this paper was to develop a quantitative method for the determination of AIP type I (AIP-I) from the spent media of *S. aureus*cultures. AIP-I is produced by the outbreak USA300 strains that represent the most common lineage of CA-MRSA [10] and [11].

Chait et al. previously published the only existing report of AIP detection from *S. aureus* culture medium without pre-concentration [12]. Relying on comparisons with fragmentation patterns of a synthetic AIP-I, they effectively employed MALDI-TOF-TOF MS for rapid identification of AIP-I in spent media. However, it was not possible to detect the molecular ion for AIP-I due to interference by media components, and the method was not quantitative. No direct LC–MS methods have been published for AIP quantitation, although McDowell et al. reported approximately ~10 μM and ~14 μM of AIP-I and its sulfoxide derivative, respectively, from *S. aureus* RN6390B. For the McDowell study, solid-phase extraction of the culture supernatant followed by two stages of reversed phase HPLC purification was employed. The process was quite time-consuming, requiring bioassays of fractions to guide the isolation process [13].

The presence of other interfering compounds in the growth media (as demonstrated by Kalkum et al. [12]) combined with the low levels of AIP-I secreted by the bacteria (as demonstrated by McDowell et al. [13]) complicate its quantitative analysis. The goal of these studies was to overcome these challenges utilizing the exceptional resolution of ultrahigh performance liquid chromatography (UHPLC) coupled to high resolving power mass spectrometry. We sought to

employ these techniques to monitor the time dependent production of AIP-I by *S. aureus*, and to compare the levels of AIP-I produced by methicillin-susceptible *S. aureus* (MSSA), HA-MRSA and CA-MRSA strains [8].

2. Materials and methods

2.1. Reagents, standards and organisms

AIP-I (≥95% purity by HPLC) was obtained from AnaSpec Inc. (Fremont, CA). *S. aureus* strains were maintained in tryptic soy broth (TSB) or on tryptic soy agar (TSA) plates at 37 °C. The strains used for these studies were as follows: USA300 LAC [14], USA300 TCH1516 [15], HG001 [16], COL [17], FRI1169 [18], and four CC45 strains that included AH1886 (NRS169), AH1887 (NRS22), AH1888 (NRS27), AH1889 (NRS236). The CC45 strains were kindly provided by Dr. Barry Kreiswirth. As a control, the *Δagr*::TetM mutation was crossed from strain AH1292 [19] into RN4220 by bacteriophage 80α transduction using methods described previously [20]. The resulting strain, AH2492 (RN4220 *Δagr*::TetM), was transformed with either plasmid pEPSA5 [21] or pAgrD1 [22]. Plasmid pAgrD1 contains the *agrBD* type I genes in pEPSA5, and the expression of these genes in an *Δagr*::TetM mutant restores AIP-I production. Plasmid electroporation was performed as described previously [23] and transformants were maintained on TSA supplemented with chloramphenicol (10 μg/mL).

2.2. UHPLC-ESI-HRMS conditions

Samples were analyzed utilizing an LTQ Orbitrap XL mass spectrometer with electrospray ionization source (Thermo, San Jose, CA) coupled to an Acquity UHPLC HSS T3 (Waters, Milford, MA). A C18 column (Acquity BEH, 2.1 mm × 50 mm, 1.8 μm packing) was used. Flow rate was 0.25 mL/min, and injection volume was 3 μL. Samples were eluted at 0.25 mL/min using a two-step gradient from 80:20 to 40:60 water:acetonitrile (both containing 0.1% formic acid) from 0 to 5 min, then from 40:60 to 80:20 from 5 to 5.5 min.

The Orbitrap was operated over a scan range of *m*/*z* 300 to 2000, with resolving power of 30,000. MS–MS was conducted in data dependent scanning mode, using a collision induced dissociation (CID) activation energy of 35%. Instrument parameters were as follows, tube lens voltage, 100 V, source voltage, 4.50 kV, source current, 100.00 μA, and heated capillary voltage, 20 V.

The AIP I concentration was calculated based on a calibration curve generated as area of the relevant selected ion peak versus concentration. A mass of 961.3794 ± 0.0048 (calcd accurate mass for the $M+H^+$ion of AIP-I, ±5 ppm) was set for the selected ion chromatogram, and AIP I concentrations were calculated using the slope of the best-fit line of this calibration curve, as determined with linear regression analysis. This 5 ppm window was chosen to reduce interference from isobaric matrix components.

2.3. MRSA culture

For studies comparing AIP-I production among various MRSA strains, a single colony inoculum of the relevant strain (see Section 2.1) was incubated in tryptic soy broth (TSB) at 37 °C for 24 h. Triplicate flasks containing 1:200 sub-cultures were then incubated with shaking at 200 rpm and 37 °C until they reached OD_{600} (absorbance at 600 nm) of at least 2.0 after a 20 h minimum growth period. This medium was then vacuum filtered through a 0.22 μm surfactant-free cellulose acetate (SFCA) membrane and stored at −80 °C until time of analysis by UHPLC-ESI-HRMS. For the time course study, a 24 h culture of MRSA USA300 strain AH1263 was diluted 1:100 in triplicate flasks containing TSB and grown with shaking for 21 h. An aliquot was removed from each flask every hour, the OD_{600} was recorded, and the culture was filter sterilized and analyzed using UHPLC-ESI-HRMS as described above.

2.4. Red blood cell lysis assays

S. aureus strains were grown in TSB to an OD_{600} of 2.0, spent media was collected, and filtered through a 0.22 μm membrane (Spin-X filters, Corning Costar). Rabbit red blood cell (RBC) lysis assays to assess alpha-toxin levels were performed as previously described [24]. Association between % lysis activity and AIP I concentration was evaluated using a Pearson correlation with two-sided *p*-value using SAS software (V. 9.3).

3. Results and discussion

3.1. Identification of AIP-I from spent media

AIP-I, Tyr-Ser-Thr-c(Cys-Asp-Phe-Ile-Met), is a cyclic octapeptide with a thiolactone ring linked between the sulfhydryl group of the cysteine and the α-carboxyl group of the C-terminal methionine [25]. The major goal in this study was to develop an analytical method that would enable detection of AIP-I directly from *S. aureus*culture medium. This was achieved using ultrahigh performance liquid chromatography (UHPLC) coupled to high resolving power mass spectrometry (Fig. 1). Peaks representing the $M+H^+$ ion of the AIP-I peptide are evident in the selected ion chromatograms (961.3794 ± 0.0048) for both the spent media of CA-MRSA USA300 strain LAC (Fig. 1B) and standard AIP-I (Fig. 1C) at retention times of 3.26 min and 3.22 min, respectively. This compound was not detected in the growth medium control (tryptic soy broth) (Fig. 1A). Correct assignment of molecular formula for the AIP-I ion was confirmed by accurate mass measurements of both the putative AIP-I $[M+H]^+$ ion from the spent media (calcd 961.3794, measured 961.3797, 0.3 ppm mass error) and the standard AIP I (calcd 961.3794, measured 961. 3790, 0.4 ppm mass error). AIP-I with oxidized methionine was also detected in the supernatant (calcd 977.3743, measured 977.3747, 0.4 ppm mass error) and standard solution (calcd 977.3743, measured 977.3755, 1.2 ppm accuracy) at ~20% of the abundance of the protonated molecular ion peak for AIP (data not shown).

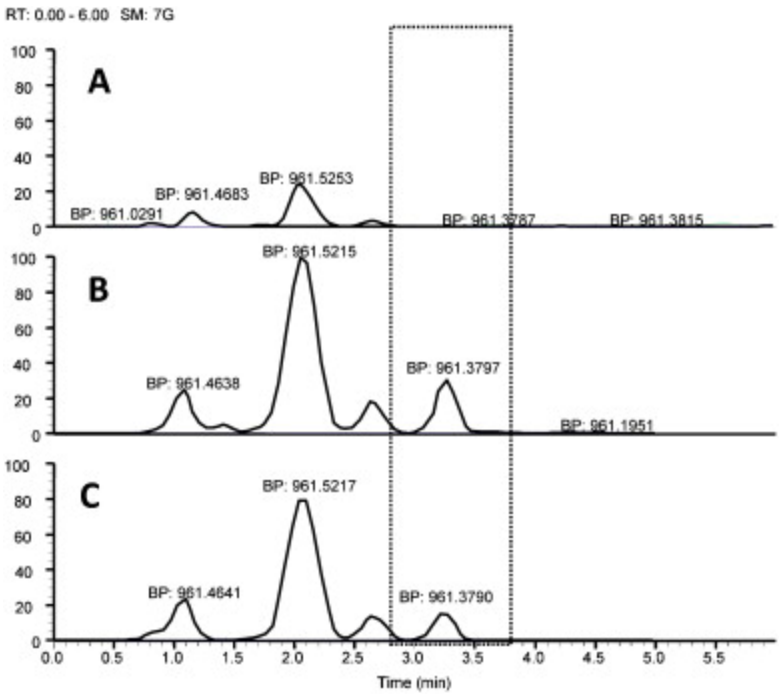

Fig. 1. Selected ion chromatogram (*m*/*z* 961.38) for tryptic soy broth (A), cell-free culture supernatant for CA-MRSA USA300 strain LAC (B), and 3.47 μM standard AIP-I in broth (C). Both the supernatant (B) and the standard (C) solution exhibited the AIP-I peak with masses of 961.3797 and 961.3790 (mass error 0.3 ppm) and 961.3790 (mass error −0.4 ppm) at retention times of 3.26 and 3.22 min, respectively. The chromatogram is normalized at 2.84×10^6 counts.

Several compounds with the same nominal mass of AIP-I were also detected in the culture medium, and are observed as additional peaks at earlier retention times in the selected ion chromatograms (Fig. 1). Thus, the use of accurate mass measurements and MS–MS fragmentation patterns was essential for the correct assignment of the AIP-I for these experiments. The MS^2 (collision induced dissociation) fragmentation spectrum using the $[M+H]^+$ ion of standard AIP-I (Fig. 2A) as the precursor (*m*/*z* 961.4) was nicely aligned with the MS^2 spectrum for the putative AIP-I from the media (Fig. 2B). The MS^2 spectrum also agreed with that previously published for AIP-I [12]. Finally, the MS^3 spectrum obtained using the most abundant MS^2fragment of the putative AIP-I, *m*/*z* 711.30, as precursor (Fig. 3) matched the MS^3 spectrum for AIP-I standard.

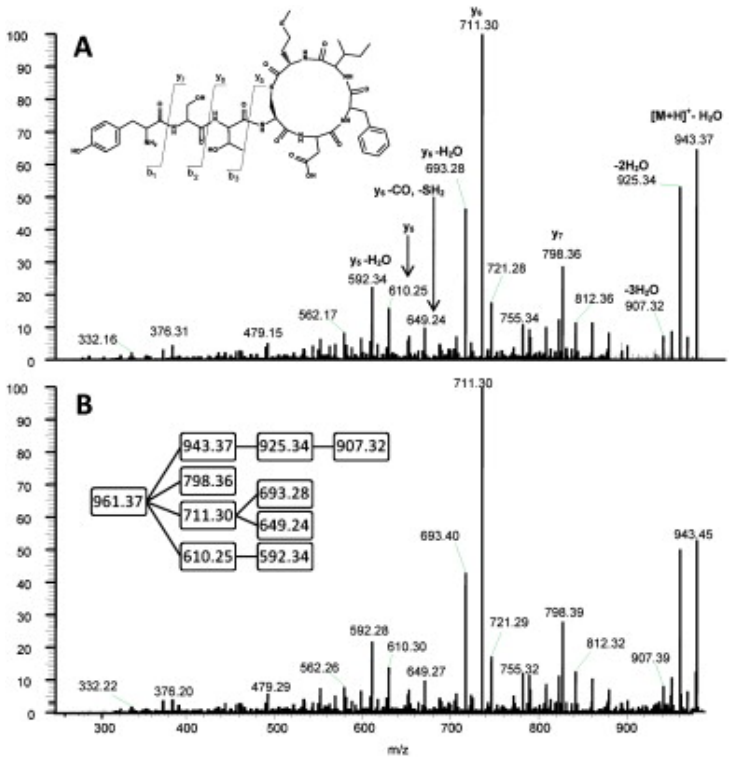

Fig. 2. Mass spectra of MS^2 fragments (with precursor ion 961.4) for AIP-I in cell-free culture supernatant from strain LAC (A) and 3.47 μM standard AIP-I in tryptic soy broth (B).

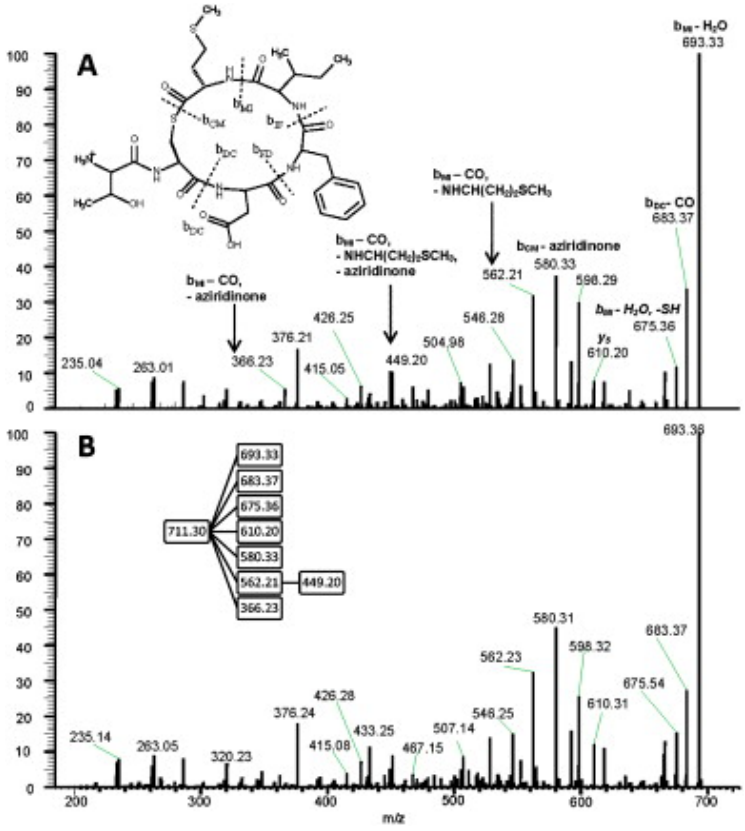

Fig. 3. Collision induced dissociation fragmentation pattern of the 711.3 fragment of the 961.4 precursor (MS^3) for AIP-I in cell-free culture supernatant from strain LAC (A) and standard AIP-I solution in TSB (B).

As further confirmation of correct identification of AIP-I, experiments were performed using an *S. aureus agr*deletion mutant (AH2492) that is unable to produce AIP-I. This strain was transformed with expression plasmid pEPSA5 [21]. As apparent from the chromatogram shown in Fig. 4A, no AIP-I was detected in the spent media from this mutant strain. When pEPSA5 containing the *agrBD* genes (pAgrD1 [22]) was transformed into the *agr* mutant, AIP-I production was restored as anticipated (Fig. 4B). The MS–MS spectrum for the putative AIP-I in the media from engineered producing strain matched the MS–MS spectrum for standard AIP-I, and accurate mass measurements confirmed the molecular formula with 0.4 ppm mass accuracy (data now shown).

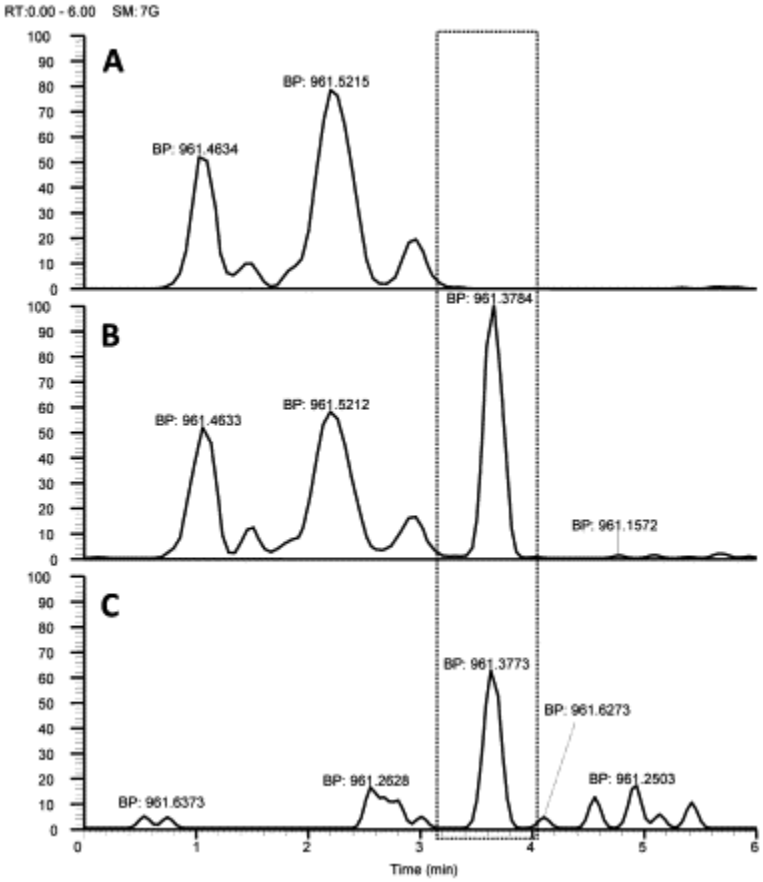

Fig. 4. Selected ion chromatograms for the mass corresponding to the $[M+H]^+$ ion of AIP I ion (961.2) for cell-free culture supernatant from a *S. aureus agr* mutant with plasmid pEPSA5 (A), cell free culture supernatant from a *S. aureus agr* mutant with plasmid pAgrD1 that produces AIP-I (B) and 3.47 μM standard AIP-I in TSB (C). All chromatograms were normalized to a signal of 9.5×10^5.

3.2. Method validation for AIP-I quantitation

Taking advantage of the excellent chromatographic resolution provided by the UHPLC, it was possible to achieve baseline resolution between the AIP-I and the interfering compounds present in the TSB (Fig. 1B). Notably, this method was also very rapid, requiring only a 6 min total UHPLC run time. The ability (relying on the high resolving power of the Orbitrap mass analyzer) to limit the window for the selected ion chromatogram to ±5 ppm was essential for quantitation, preventing signal from the interferences from being falsely assigned to the AIP I. Using this approach, a linear dynamic range for quantitation of AIP-I of 2.6–63 μM was achieved, with a limit of detection (defined as the concentration necessary to achieve signal 3 × noise) of 0.25 μM (Table 1). Below 2.6 μM and above 63 μM, the curve deviated from linearity. This deviation was attributed to components of the tryptic soy broth used to culture the bacteria (and as the matrix for standard preparation); the linear dynamic range was expanded by over an order of magnitude when the standards were instead prepared in acetonitrile (data not shown). Nonetheless, our intention with this study was to develop a method that would be applicable for rapid of analysis of peptides produced by bacteria grown in rich media. Therefore, tryptic soy broth was used as the matrix for standard preparation in all of the subsequent studies to enable analysis of samples in tryptic soy broth without concerns of inaccuracies due to matrix suppression. Even in the tryptic soy broth medium, sufficient linearity, limit of detection, limit of quantitation (Table 1), precision, and accuracy (Table 2) where achieved to enable quantitation of AIP-I in relevant biological samples.

Table 1. Regression parameters, detection limit, and limit of quantitation for analysis of AIP-I in tryptic soy broth.

Validation parameter	Result
Correlation coefficient	0.997
Standard error	3.78×10^6
y-intercept	$-1.91 \times 10^6 \pm 2.2 \times 10^6$
Slope of regression line	$3.06 \times 10^6 \pm 6.4 \times 10^4$
Number of data points	8
Range	2.6–63 μM
Limit of detection (LOD)[a]	0.25 μM
Limit of quantitation (LOQ)[b]	2.6 μM

LOD was based on a signal-to-noise ratio of 3:1

LOQ was defined as the lowest concentration for which acceptable results for precision (repeatability <20%) and accuracy (residual of <20%) were obtained.

Table 2. Accuracy and precision for quantitative analysis of AIP-I.

Theoretical concentration (μM)	Measured concentration[a](μM)	Residuals[b](%)	Repeatability[c](%)	Intermediate precision[d](%)

2.6	2.2	16	3.4	37
5.2	4.7	8.5	2.3	9.5
10.4	9.8	5.2	3.8	3.3
20.8	21.1	1.8	2.8	4.7
31.2	32.3	3.5	3.0	5.2
41.7	42.5	2.1	4.9	5.2
52.1	53.1	1.9	1.9	4.7
62.5	60.6	3.1	1.5	3.3

[a] The measured concentration is an average of the back calculated concentration of AIP-I (in tryptic soy broth medium) obtained for three triplicate analyses conducted on three different days. [b]Residuals are calculated as follows: (measured concentration − theoretical concentration)/theoretical concentration × 100. [c]Repeatabilty is expressed as the % relative standard deviation for back calculated AIP-I concentrations determined by triplicate analyses conducted on a single day. [d] Intermediate precision is expressed as the % relative standard deviation of the three back-calculated AIP-I concentrations (each an average of triplicate measurements) determined on three separate days.

3.3. Time-dependent AIP-I production and relationship between AIP production and virulence of*S. aureus* strains

The method described herein was applied to answer several biologically relevant questions regarding AIP-I production by *S. aureus*. First, our method allowed for instantaneous identification and quantification of AIP-I without tedious sample preparation. A study that focused on structure and activity of AIP-I estimated that the concentration of the thiolactone produced by *S. aureus* was in the approximate range of 10 μM [13]. However, the method used required a pre-concentration step with size exclusion filtration and solid phase extraction followed by two stages of bioassay-guided purification using reversed phase HPLC. Although the authors were able to obtain samples for activity determination, the method is not ideal to be adopted as a diagnostic tool to assess AIP production. The protracted time and expense required for sample preparation also make accurate quantitation challenging. The method described here confirms the concentration of AIP-I in spent media of *S. aureus* cultures by direct quantitative measurements. It was determined that AIP-I concentration varies depending on growth phase of a CA-MRSA USA300 strain, but is generally in the range of ~0.05 to 10 μM. Secondly, we investigated the production of AIP-I by CA-MRSA USA300 strain LAC over time (Fig. 5). Under the conditions for this study, no AIP-I was detectable in the initial culture. The peptide was, however, detected at the 4 h time point, with a concentration of 1.1 ± 0.3 μM (expressed at mean ± standard error). This concentration should be interpreted as only approximate, being above the limit of detection (0.25 μM) but below the lower limit of the linear range of the calibration curve (2.3 μM). AIP-I levels then rose steadily to a maximum concentration of 13 ± 2 μM at 16 h. After this time, the concentration leveled off until the last recorded time point at 21 h.

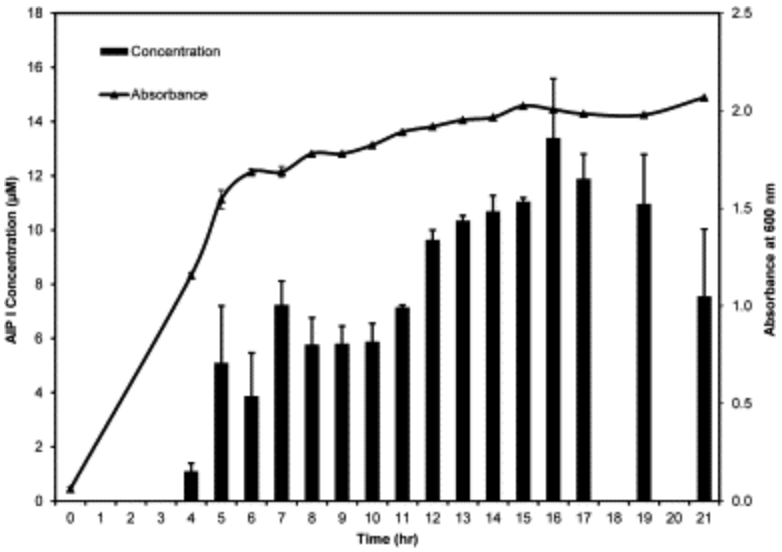

Fig. 5. Production of AIP-I by strain LAC in TSB over time. Measurements were made of from triplicate culture flasks grown with shaking and aeration. Aliquots were removed at the indicated time points, measured for OD at 600 nm, filtered, and frozen for AIP-I analysis by LC–MS. Error bars indicate ± standard error (N = 3).

Finally, literature has suggested that the activity of the *agr* quorum-sensing system can be correlated with the virulence potential of *S. aureus* strains [5], [6] and [7]. It was, therefore, of interest to evaluate the differences in AIP-I concentration produced by a series of strains representing different lineages and MRSA types, and to compare these concentrations to % rabbit red blood cell (RBC) lysis activity as a measure of virulence (Fig. 6). Rabbit RBCs are hyper-susceptible to alpha-toxin lysis, and the function of the *agr*system directly correlates with alpha-toxin production levels [9] and [24]. For purposes of comparison, we have set CA-MRSA strain LAC at 100% RBC lysis and plotted other strains relative to this level. It is apparent from visual inspection of Fig. 6 that AIP-I levels vary significantly. CA-MRSA strains are known to be hypervirulent, due in part to elevated production of *agr*-regulated toxins and exo-enzymes [4], [5] and [6]. Thus, it is not surprising that the CA-MRSA USA300 strains LAC and TCH1516 produced high levels of AIP-I (4.3 and 4.5 μM, respectively) that correlated with enhanced RBC lysis. Also as anticipated, hospital acquired methicillin-resistant *S. aureus* (HA-MRSA) strain COL has comparatively weaker *agr* system function [5], reduced pathogenesis [4], and produced low levels of AIP-I and reduced lysis (Fig. 6). As a control throughout this experiment, alpha-toxin levels were confirmed by immunoblot (data not shown).

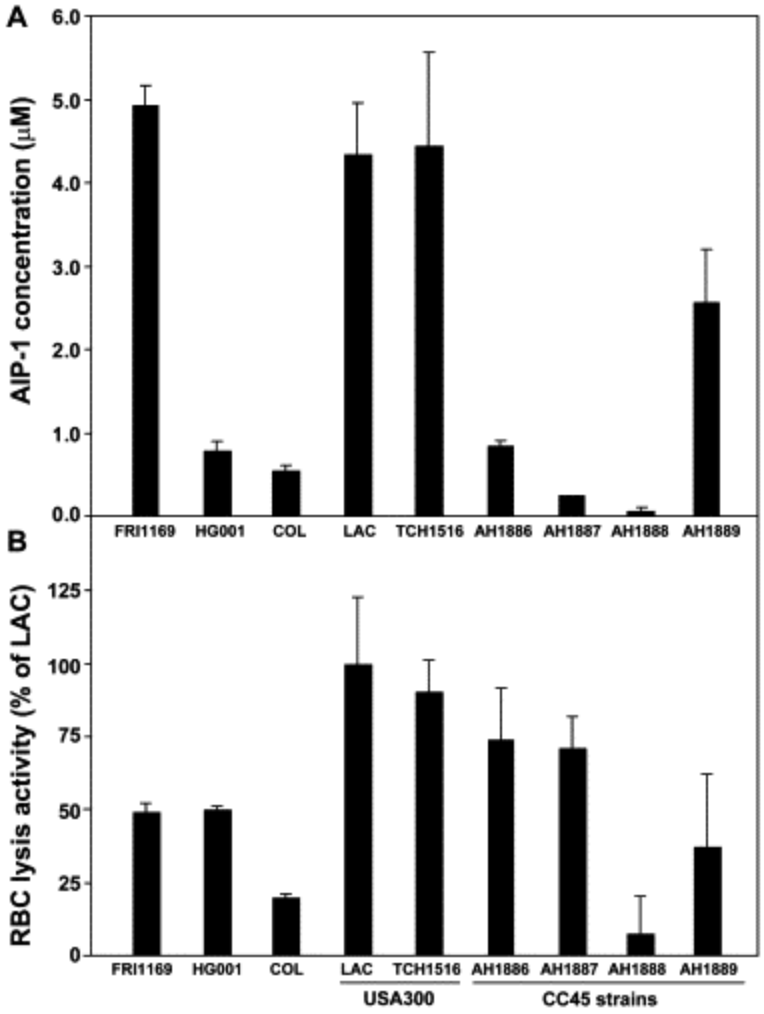

Fig. 6. Comparison of AIP-I concentrations and rabbit red blood cell (RBC) lysis activity for different strains of *S. aureus*. Strains were grown in triplicate culture flasks to an OD_{600} of 2.0, and spent media was tested for AIP-I levels and RBC lysis. (A) AIP-I analysis by LC–MS of each strain. Error bars represent standard error (N = 3). (B) RBC lysis levels of each strain normalized to USA300 LAC (set to 100%). These lysis levels correlate with alpha-toxin production, which was confirmed through immunoblot (data not shown). Error bars represent standard error (N = 3).

To further evaluate the relationship between AIP-I production and virulence, we extended the AIP-I analysis to other *agr* type I strains. We chose strains FRI1169, a known toxic shock toxin producer [18]; HG001, an engineered laboratory strain [16]; and four clonal complex 45 (CC45) strains. These strains were chose for their divergent backgrounds to gain insight on the generality of the UHPLC method application for *S. aureus*. Strain FRI1169 produced the highest levels of AIP-I (4.9 μM), but only triggered a moderate level of RBC lysis (49% of USA300 LAC). In contrast, HG001 had a similar ability to induce lysis but produced low levels of AIP-I (0.8 μM). Considerable variation was also observed in the CC45 strains. While strain AH1889 produced

moderate levels of AIP-I and RBC lysis, other CC45 strains AH1886 and AH1887 produced low levels of AIP-I but retained high RBC lysis. These outliers, combined with the relatively low number of strains evaluated, meant that there was no significant statistical correlation between AIP-I production and % RBC lysis (Pearson correlation of 0.23, $p = 0.53$). However, it should be noted that only one time point was chosen for these studies, and *agr* system dynamics will vary over time across *S. aureus* strains. Taken together, the levels of AIP-I show striking consistency with the reported virulence properties of some strains, such as CA-MRSA USA300 strains, but our additional studies demonstrate that quantifying AIP should not be considered in isolation as the only metric of *S. aureus* pathogenic potential.

4. Conclusions

This study demonstrates the power of UHPLC coupled to high resolving power mass spectrometry for quantifying biologically relevant analytes in challenging matrices. The UHPLC enabled shorter run times than were possible with other methods. Furthermore, quantitative analysis of an autoinducing peptide was possible directly from complex culture media. The quantitative method facilitated comparisons of the absolute levels of AIP-I produced by *S. aureus* strains with varying virulence. It was observed that the highest AIP-I levels were produced by the CA-MRSA USA300 strains and low AIP-I levels are produced by HA-MRSA, both observations that are supported by pathogenic assessments of these strains. Thus, measurements of AIP-I levels may have value as an indicator of *S. aureus* virulence that could be used in conjunction with more traditional pathogenesis assessments. Furthermore, this new method for quantifying AIP-I could be applied to other classes of AIP structures to assess signal production across diverse Gram-positive bacterial pathogens.

Acknowledgments

We acknowledge funding from the National Institutes of Health National Center for Complementary and Alternative Medicine (NCCAM) grant #1 R15 AT005005-01 (to NBC) and from the National Institute of Allergy and Infectious Diseases (NIAID) grant AI078921 (to ARH). Mass spectrometry experiments were conducted in the Triad Mass Spectrometry Facility at the University of North Carolina Greensboro. Assistance with statistical analyses was generously provided by Dr. Scott Richter. We thank Dr. Barry Kreiswirth for providing the CC45 strains.

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