Published in final edited form as:

J Microbiol Methods. 2016 August ; 127: 89–94. doi:10.1016/j.mimet.2016.05.024.

Hybrid Quadrupole-Orbitrap Mass Spectrometry for Quantitative Measurement of Quorum Sensing Inhibition

Daniel A. Todd, David B. Zich, Keivan A. Etefagh, Jeffrey S. Kavanaugh, Alexander R. Horswill, and Nadja B. Cech

Abstract

Drug resistant bacterial infections cause significant morbidity and mortality worldwide, and new strategies are needed for the treatment of these infections. The anti-virulence approach, which targets non-essential virulence factors in bacteria, has been proposed as one way to combat the problem of antibiotic resistance. Virulence in methicillin-resistant *Staphylococcus aureus* (MRSA) and many other Gram-positive bacterial pathogens is controlled by the quorum sensing system. Thus, there is excellent therapeutic potential for compounds that target this system. With this project, we have developed and validated a novel approach for measuring quorum sensing inhibition *in vitro*. Ultrapformance liquid chromatography coupled to mass spectrometry (UPLC-MS) was employed to directly measure one of the important outputs of the quorum sensing system in MRSA, auto-inducing peptide I (AIP I) in bacterial cultures. The method for AIP detection was validated and demonstrated limits of detection and quantification of range of 0.0035 μM and 0.10 μM , respectively. It was shown that the known quorum sensing inhibitor ambuic acid inhibited AIP I production by a clinically relevant strain of MRSA, with an IC_{50} value of $2.6 \pm 0.2 \mu\text{M}$. The new method performed similarly to previously published methods using GFP reporter assays, but has the advantage of being applicable without the need for engineering of a reporter strain. Additionally, the mass spectrometry-based method could be applicable in situations where interference by the inhibitor prevents the application of fluorescence-based methods.

Keywords

Quorum sensing; anti-virulence; autoinducing peptide; mass spectrometry; *Staphylococcus aureus*

1. Introduction

The economic impact of antibiotic resistant bacterial infections is estimated by the Centers for Disease Control to be \$55 billion dollars per annum in the US alone (2013). One of the most problematic of these pathogens, Methicillin-resistant *Staphylococcus aureus* MRSA, now causes more deaths each year than HIV/AIDs (Klevens et al., 2007, Spellberg et al., 2011). In light of the severity of the problem of drug resistance, there is increasing interest in development of new ways to treat bacterial infections. For example, The White House announced a *National Strategy for Combating Drug Resistant Infections* in September 2014,

Conflict of interest

The authors declare that they have no conflict of interest.

for which one of the five major goals is to “accelerate basic and applied research and development for new antibiotics, other therapeutics, and vaccines.” (Fact Sheet, 2014)

The so-called anti-virulence approach is one promising alternative strategy against drug resistant bacterial infections (Cech and Horswill, 2013). The concept behind the anti-virulence approach is to target bacterial pathogenesis, thereby reducing the severity of the impact of the pathogen on the host, and facilitating clearance of the infection (Khodaverdian et al., 2013, Rasko and Sperandio, 2010). Although anti-virulence therapies have shown promise in animal models (Sully et al., 2014), they have yet to be applied in a clinical setting. To capitalize on the full potential of anti-virulence therapeutics, there is a need to develop increased understanding of virulence mechanisms and inhibition.

In Gram-positive bacteria, virulence is regulated by a quorum sensing system known as the accessory gene regulator (*agr*) system, which has previously been described in detail (Thoendel et al., 2011). Activation of this system is accomplished by cyclic peptides known as autoinducing peptides or AIPs. AIP is not only an activator of the *agr* system, but also a direct output, making it an appealing biomarker for *agr* system activity. If the activity of the *agr* system is reduced, for example by the addition of an inhibitor, an associated decrease in AIP production should be observed. Thus, with these studies, we sought to quantify AIP production as a means of monitoring quorum sensing inhibition.

Existing methods for monitoring quorum sensing activity involve reporter strain assays or protein readouts for the exo-proteins (e.g. alpha toxin) controlled by the system (Quave and Horswill, 2014). The disadvantage of reporter strain assays is that the desired reporter genes must be engineered into each bacterial strain of interest. Furthermore, for reporter strain measurements, spectral interference can occur if the inhibitor quenches fluorescence at the wavelength of emission by the reporter (Brown et al., 2015, Hudson et al., 2009, Matsuura et al., 2002). Toxin blots are an effective alternative to reporter assays (Quave and Horswill, 2014), but they are limited for quantitative purposes. To supplement the existing methods, the goal of this study was to develop a robust, sensitive, and quantitative method for measurement of quorum sensing inhibition relying on direct measurement of AIP concentration using mass spectrometry.

Until very recently, AIP detection with mass spectrometry has been a cumbersome task requiring extensive sample cleanup (Ji et al., 1995, Nakayama et al., 2001, Olson et al., 2014). One method was previously developed that allowed direct measurement of AIPs from several Gram-positive bacterial species using matrix-assisted laser desorption ionization mass spectrometry (MALDI) (Kalkum et al., 2003). However, this approach was inherently limited to qualitative assessments (identification but not quantification). Another method developed by our laboratory could be used to quantify AIP in MRSA cultures with a limit of quantification of 2.6 μ M (Cech et al., 2012). This method relied on measurements obtained using a hybrid ion trap Orbitrap mass spectrometer (the LTQ Orbitrap). Recently, a new generation of high resolving power hybrid mass spectrometers has been developed that employs a combination of a segmented quadrupole and Orbitrap mass analyzer (the Q-Exactive). This instrument design facilitates measurement with even better sensitivity and dynamic range than was possible with earlier generation Orbitrap instruments such as the

LTQ Orbitrap. With this study, we sought to demonstrate the applicability of measurements with UPLC coupled to a Q-Exactive Orbitrap mass spectrometer (UPLC-MS) to develop the first mass spectrometric method for measuring quorum sensing inhibition.

2. Methods and Materials

2.1 Instrumentation

Optical density readings were performed using a Synergy H1 Multi-Mode Reader (Biotek Instruments, Inc., Winooski, VT). Liquid chromatography-mass spectrometry was performed using an Acquity ultra-high performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA) coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Unless otherwise stated, all solvents used for chemical analyses were purchased from Thermo Fisher Scientific (Waltham, MA).

2.2 Identification and Detection of auto-inducing peptides

A single isolated colony of Erm-sensitive CA-MRSA USA300-0114 (AH1263) (Boles, Thoendel, Roth and Horswill, 2010) was cultured overnight at 37 °C in Tryptic Soy broth (TSB, Sigma Aldrich, St. Louis, MO). Overnight cultures were diluted 1:200 (bacterial culture:broth) and shaken for at least 16 hr at 200 rpm and 37 °C. Cells were pelleted by centrifugation at $6,000 \times g$ for 5 min and removed by 0.22- μ m filtration. AIP was detected directly in spent media filtrate using UPLC-MS.

A 7 μ L injection of each sample was eluted from the column (Acquity UPLC BEH C18 1.7 μ m, 2.1 $\times$ 50 mm, Waters Corp.) at a flow rate of 0.3 mL/min using the following binary gradient with solvent A consisting of water (Optima LC/MS grade) with 0.1% formic acid additive and solvent B consisting of acetonitrile (Optima LC/MS grade) with 0.1% formic acid additive. The gradient initiated at 90:10 (A:B) and increased linearly from 0.0–8.0 min. to 40:60 (A:B), followed by an isocratic hold at 40:60 (A:B) from 8.0–8.5 min, gradient returned to starting conditions of 90:10 (A:B) from 8.5–9.0 min, and was held at this composition from 9.0–10 min.

The mass spectra were collected using two scan events: the first scan event was a positive mode full scan with a mass range from 300 – 2000 and a resolution of 35,000. The second scan event was positive mode selected ion monitoring for the calculated mass of *S. aureus* AIP I (m/z 961.37994) with an isolation window of 4 Da. The mass spectrometer was operated using a heated electrospray ionization source with the following setting: capillary temperature set at 300°C, S-Lens RF level set at 80, spray voltage set at 4.0 kV, sheath gas flow set at 50, and auxiliary gas flow set at 15.

Confirmation of correct AIP identification was achieved through MS-MS analysis. Calculated AIP m/z value was selected as precursor ion and subjected to high-energy collision dissociation (HCD) at a stepped normalized collision energy of 20, 23 and 25. Resulting spectra were compared to predicted fragmentation pattern of AIP ions. Fragmentation prediction was performed using Mass Frontier 7.0 (HighChem, Ltd., Slovak Republic).

2.3 Quantitative measurements of AIP production

A synthetic standard of *Staphylococcus aureus* AIP-I was purchased from Anaspec, EGT (Fremont, CA). Accurate mass, retention time, and fragmentation pattern of this peptide were used to confirm the correct identification of detected AIP. LC-MS analysis was performed using method described above. A standard calibration curve of synthetic AIP was prepared in two-fold dilutions from 104 μM to 0.40 nM in TSB. The selected-ion chromatogram for m/z 961.37994 was plotted, and a calibration curve was constructed as area of this chromatogram versus concentration AIP. AIP concentration in the spent *S. aureus* broth was determined by a $1/\times^2$ weighted Least-squares (WLS) linear regression of this calibration curve.

2.4 Mass spectrometric measurements of AIP production inhibition

Evaluation of inhibition of AIP production by ambuic acid was performed in a 96-well plate format and setup in the following manner. Overnight cultures were diluted 1:200 (culture:broth) and shaken (200 rpm) at 37 °C for two hours. Bacteria culture (200 μL) was combined with 45 μL broth and 5 μL dimethyl sulfoxide (DMSO) containing ambuic acid (Adipogen International, San diego, CA) in concentrations from 20 μM to 5 mM prepared in two-fold dilutions. Final concentration of ambuic acid in cultures ranged from 390 nM to 100 μM , with a final DMSO concentration of 2% (v/v) in all wells. Cultures were shaken at 1000 rpm at 37 °C using a Stuart S1505 microtitre plate shaker (Bibby Scientific Limited, Staffordshire, U.K.). Cell growth was monitored using an optical density of 600 nm measured at one hour intervals. Cells were grown to the end of the log-phase and were removed via vacuum filtration. Filtrate was analyzed using the LC-MS method described above. Selected ion chromatograms for the identified AIP m/z values were plotted and peak area values were used for relative AIP concentration comparison. IC_{50} values were determined from a four parameter logistic function (4PL) of the AIP peak area transformed into units of percent vehicle using the drc extension package for **R** (Ritz et al., 2015, Ritz and Streibig, 2005).

2.5 Quorum sensing inhibition assay with reporter strain

For comparison with the mass spectrometry data, the quorum quenching activity of ambuic acid (Adipogen, San Diego, CA) was quantified using the *agrP3*-sGFP (*agr* type I) fluorescent reporter strain AH1677 (Kirchdoerfer et al., 2011), as previously described (Quave et al., 2015). Briefly, AH1677 cultures grown in tryptic soy broth were treated with ambuic acid ranging in concentrations from 0.39–100 μM , with a final DMSO concentration of 1% (v/v) in all wells. Fluorescence (top reading, 493 nm excitation, 535 nm emission, gain 60) and optical density (OD) readings at 600 nm were recorded at 30 min increments using a Tecan Systems (San Jose, CA) Infinite M200 plate reader. Dose-response curves, for 8 hours of incubation, were generated using the drc extension package for **R** (Ritz, Baty, Streibig and Gerhard, 2015, Ritz and Streibig, 2005) and IC_{50} 's were obtained by subjecting fluorescence data (that had been transformed in to units of percent of vehicle) to four-parameter logistic fits (4PL).

3. Results and Discussion

3.1 Structure elucidation of AIPs

An important first step in the application mass spectrometry to monitor quorum sensing inhibition is confirming correct identification of the AIP signaling molecule. To accomplish this structure elucidation, possible AIP structures (and their corresponding masses) are predicted by inspection of sequence data for the *agrD* gene (Thoendel, Kavanaugh, Flack and Horswill, 2011). The longest possible AIP structure coincides with the entire length of the AgrD sequence, and other possible structures can be anticipated by subtracting amino acids one-by-one from the N-terminus. On the basis of these predictions, the UPLC-MS data is filtered to identify ions with m/z ratios corresponding to possible AIP structures.

Once ions with m/z values corresponding to putative AIP structures are detected in the spent bacterial broth, structures can be verified in several ways. If a standard synthetic peptide is available (or can be synthesized), retention times can be compared between the standard and the unknown. However, synthesizing all of the possible AIP peptides without *a priori* knowledge of likely AIP structure can be a time consuming and expensive enterprise. Thus, it is useful to first obtain a prediction of the likely AIP structure based on molecular formula and fragmentation pattern. The use of fragmentation patterns, most often obtained with collision-induced dissociation (CID), is a common approach for peptide identification (Paizs and Suhai, 2005). CID on a LTQ-Orbitrap mass spectrometer has been employed previously to identify AIPs. A limitation of CID for this purpose, however, is that it does not directly produce fragments of the cyclized portion (Junio et al., 2013) of the AIP. With this study, we sought to instead employ stepped high energy collision induced dissociation (HCD) on the Q-exactive Orbitrap mass spectrometer for AIP identification. HCD tends to produce richer fragmentation patterns than CID, and the fragments are measured with a mass error less than 10 ppm. For these reasons, we expected HCD would prove useful for confirming the structure of both the linear and cyclic portions of the AIP peptide.

As a specific example of the applicability of HCD for AIP identification, the proposed structure for the *S. aureus* AIP I (Figure 1) was supported by the detection of an ion with m/z 961.38043 in a MRSA spent bacterial culture analyzed via UPLC-MS in the full scan positive ion mode. This mass corresponds to the 8-residue sequence YSTCDFIM with an 5-residue cyclic thiolactone (CDFIM) on the C-terminus (Calc. $[M+H]^+$ m/z 961.37994, mass error of 0.51 ppm). HCD fragmentation was performed, and the resulting product ions matched the masses of multiple predicted fragments for this peptide as well as the fragmentation pattern for a synthetic standard of AIP I. Notably, the use of stepped normalized collision energy (NCE) HCD enabled collection of a fragmentation spectrum that included the protonated precursor ion (961.37189), a series of y ions, and a number of fragments of the ring. These fragments were all measured with mass accuracy of <10 ppm, and matched the masses predicted using Mass Frontier 7.0 (HighChem, Ltd., Slovak Republic) fragment predictor based on the proposed AIP I structure (Figure 1).

3.2 Method Validation

Our ultimate intention was to apply AIP I identification as a means to monitor quorum sensing inhibition. As an intermediate step, it was of interest to validate the analytical capabilities of the method for quantitative analysis of AIP I. Using a series of matrix-matched standards (synthetic AIP I in tryptic soy broth), it was determined that the method was linear over a concentration range of 2.6 orders of magnitude (Table 1). The lower limit of quantitation (LOQ) for the new method was 0.10 μM , which is more than 10 times lower than that previously reported using an LTQ Orbitrap mass spectrometer (Junio et al., 2013). Furthermore, the new method exhibited an impressive limit of detection of 0.0035 μM (Table 1), more than 100 times lower than that previously reported (Junio et al., 2013). Finally, the Q-exactive method showed excellent accuracy and precision (Table 2). Very close agreement was obtained between theoretical and measured concentration of standards, and the method demonstrated repeatability and intermediate precision values of less than 6% across the entire linear range (Table 2).

3.3 Quantitative measurements of AIP I as an indicator of quorum sensing inhibition

The ability to detect and quantify AIP I at low levels in spent media enabled us to devise a simple workflow for monitoring quorum sensing inhibition (Figure 2). With this approach, bacteria cultures are grown in a 96 well plate in the presence of an inhibitor, the plate is filtered, and the resulting spent media is analyzed directly with UPLC-MS. Orthogonal hourly measurements of OD₆₀₀ collected with a plate reader enable collection of a series of growth curves to monitor potential growth inhibition.

As a test case for evaluating the method described in Figure 2, we measured AIP I production by MRSA in the presence of varying concentrations of the fungal metabolite ambuic acid (Figure 3A). Ambuic acid is a known quorum sensing inhibitor (Nakayama et al., 2009), and, as such, AIP I production by MRSA should be reduced in the presence of ambuic acid. As predicted, the addition of ambuic acid suppressed AIP I production by a clinical isolate of MRSA (USA300 LAC) (Boles et al., 2010) in a dose-dependent fashion (Figure 3A), with an IC₅₀ value of $2.6 \pm 0.2 \mu\text{M}$. For the purpose of comparison with the new method, we also employed the published GFP reporter approach (Kirchdoerfer et al., 2011) to measure a dose-response curve for ambuic acid against the same strain of MRSA (Figure 3B). Quorum sensing inhibition was also observed with the reporter strain method, although the IC₅₀ value was somewhat higher than that observed with the mass spectrometry-based approach ($25.4 \pm 1.7 \mu\text{M}$). Importantly, the inhibition demonstrated in Figure 3 was not due to growth inhibition or growth delay; ambuic acid had no influence on bacterial growth up to a concentration of 100 μM using either the USA300 LAC strain of MRSA (AH1263) (Figure 4) or the GFP reporter strain of USA300 (AH1677) (data not shown).

The lack of agreement in IC₅₀ values between the two assay methods (Figure 3) is not entirely unexpected. The reporter assay is based on the *agr*P3 promoter controlling RNAPIII expression, while AIP production is controlled by the P2 promoter (Thoendel 2011). Thus, while both the mass spectrometry and GFP reporter strain methods measure inhibition of the quorum sensing system, the apparent potency of the inhibitor differs depending on which

output is being quantified. On a practical level, either method would be effective for comparing potency among a series of inhibitors, as long as comparisons were not made between data collected with different methods.

4. Conclusion

This study is one of many to show the power of mass spectrometry as a quantitative tool for molecular biology. The applicability of mass spectrometers in this field has been facilitated by the advent of Orbitrap mass analyzers, and the development of hybrid quadrupole Orbitrap instruments with exceptional robustness, sensitivity and mass accuracy further advance our capabilities in this arena. As demonstrated here, the Q-exactive can be employed to make quantitative measurements of biologically relevant compounds with little or no sample preparation. This study is just one example of the power of such measurements for obtaining chemical information about biologically important systems.

Our study is the first to demonstrate the applicability quantitative measurements of AIP production for monitoring quorum sensing inhibition. The approach described here could be employed for future studies aimed at screening for inhibitors, or studying mechanism of action of known inhibitors. An advantage of the mass spectrometry-based approach is that it can be conducted on any organism for which AIP can be detected and quantified. The additional step of engineering a GFP reporter strain is not necessary when mass spectrometry is used to monitor inhibition.

Acknowledgments

Partial funding for this research was provided by the National Center for Complementary and Integrative Medicine, a component of the National Institutes of Health (grant number 1R01 AT006860).

References

- Boles BR, Thoendel M, Roth AJ, Horswill AR. Identification of genes involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation. PLoS One. 2010; 5:e10146. [PubMed: 20418950]
- Brown AR, Etefagh KA, Todd D, Cole PS, Egan JM, Foil DH, Graf TN, Schindler BD, Kaatz GW, Cech NB. A mass spectrometry-based assay for improved quantitative measurements of efflux pump inhibition. PLoS ONE. 2015; 10
- Cech NB, Horswill AR. Small-molecule quorum quenchers to prevent *Staphylococcus aureus* infection. Future Microbiol. 2013; 8:1511–1514. [PubMed: 24266350]
- Cech NB, Junio HA, Ackermann LW, Kavanaugh JS, Horswill AR. Quorum quenching and antimicrobial activity of goldenseal (*Hydrastis canadensis*) against methicillin-resistant *Staphylococcus aureus* (MRSA). Planta Med. 2012; 78:1556–1561. [PubMed: 22814821]
- Center for Disease Control and Prevention. Antibiotic resistance threats in the United States. 2013
- Fact Sheet: Obama administration takes actions to combat antibiotic-resistant bacteria. <https://www.whitehouse.gov/the-press-office/2014/09/18/fact-sheet-obama-administration-takes-actions-combat-antibiotic-resistance>
- Hudson SA, Ecroyd H, Kee TW, Carver JA. The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds. FEBS Journal. 2009; 276:5960–5972. [PubMed: 19754881]
- Ji G, Beavis RC, Novick RP. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci U S A. 1995; 92:12055–12059. [PubMed: 8618843]

- Junio HA, Todd DA, Ettefagh KA, Ehrmann BM, Kavanaugh JS, Horswill AR, Cech NB. Quantitative analysis of autoinducing peptide I (AIP-I) from *Staphylococcus aureus* cultures using ultrahigh performance liquid chromatography-high resolving power mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2013; 930:7–12.
- Kalkum M, Lyon GJ, Chait BT. Detection of secreted peptides by using hypothesis-driven multistage mass spectrometry. *Proc. Natl. Acad. Sci*. 2003; 100:2795–2800. [PubMed: 12591958]
- Khodaverdian V, Pesho M, Truitt B, Bollinger L, Patel P, Nithianantham S, Yu G, Delaney E, Jankowsky E, Shoham M. Discovery of antivirulence agents against methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2013; 57:3645–3652. [PubMed: 23689713]
- Kirchdoerfer RN, Garner AL, Flack CE, Mee JM, Horswill AR, Janda KD, Kaufmann GF, Wilson IA. Structural basis for ligand recognition and discrimination of a quorum-quenching antibody. *J Biol Chem*. 2011; 286:17351–17358. [PubMed: 21454495]
- Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *J Am Med Assoc*. 2007; 298:1763–1771.
- Matsuura N, Aradate T, Sasaki C, Kojima H, Ohara M, Hasegawa J, Ubukata M. Screening system for the Maillard reaction inhibitor from natural product extracts. *J Health Sci*. 2002; 48:520–526.
- Nakayama J, Cao Y, Horii T, Sakuda S, Akkermans AD, de Vos WM, Nagasawa H. Gelatinase biosynthesis-activating pheromone: a peptide lactone that mediates a quorum sensing in *Enterococcus faecalis*. *Mol Microbiol*. 2001; 41:145–154. [PubMed: 11454207]
- Nakayama J, Uemura Y, Nishiguchi K, Yoshimura N, Igarashi Y, Sonomoto K. Ambuic acid inhibits the biosynthesis of cyclic peptide quorumones in Gram-positive bacteria. *Antimicrob Agents Chemother*. 2009; 53:580–586. [PubMed: 19015326]
- Olson ME, Todd DA, Schaeffer CR, Paharik AE, Van Dyke MJ, Büttner H, Dunman PM, Rohde H, Cech NB, Fey PD, Horswill AR. *Staphylococcus epidermidis* agr quorum-sensing system: Signal identification, cross talk, and importance in colonization. *J Bacteriol*. 2014; 196:3482–3493. [PubMed: 25070736]
- Paizs B, Suhai S. Fragmentation pathways of protonated peptides. *Mass Spectrom Rev*. 2005; 24:508–548. [PubMed: 15389847]
- Quave CL, Horswill AR. Flipping the switch: tools for detecting small molecule inhibitors of staphylococcal virulence. *Front Microbiol*. 2014; 5:706. [PubMed: 25566220]
- Quave CL, Lyles JT, Kavanaugh JS, Nelson K, Parlet CP, Crosby HA, Heilmann KP, Horswill AR. *Castanea sativa* (european chestnut) leaf extracts rich in ursene and oleanene derivatives block *Staphylococcus aureus* virulence and pathogenesis without detectable resistance. *PLoS One*. 2015; 10:e0136486. [PubMed: 26295163]
- Rasko DA, Sperandio V. Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov*. 2010; 9:117–128. [PubMed: 20081869]
- Ritz C, Baty F, Streibig JC, Gerhard D. Dose-response analysis using R. *PLoS One*. 2015; 10
- Ritz C, Streibig JC. Bioassay analysis using R. *Journal of Statistical Software*. 2005; 12:1–22.
- Spellberg B, Blaser M, Guidos RJ, Boucher HW, Bradley JS, Eisenstein BI, Gerding D, Lynfield R, Reller LB, Rex J, Schwartz D, Septimus E, Tenover FC, Gilbert DN. Combating antimicrobial resistance: Policy recommendations to save lives. *Clin Infect Dis*. 2011; 52:S397–S428. [PubMed: 21474585]
- Sully EK, Malachowa N, Elmore BO, Alexander SM, Femling JK, Gray BM, DeLeo FR, Otto M, Cheung AL, Edwards BS, Sklar LA, Horswill AR, Hall PR, Gresham HD. Selective chemical inhibition of agr quorum sensing in *Staphylococcus aureus* promotes host defense with minimal impact on resistance. *PLoS Pathog*. 2014; 10:e1004174. [PubMed: 24945495]
- Thoendel M, Kavanaugh JS, Flack CE, Horswill AR. Peptide signaling in the Staphylococci. *Chem Rev*. 2011; 111:117–151. [PubMed: 21174435]

Fig 1.

Structure elucidation for AIP I from *Staphylococcus aureus* using HCD fragmentation patterns collected on a Q-Exactive Orbitrap mass spectrometer. Labeled fragments were all measured within a 10 ppm mass error or their predicted m/z , and ions labeled with an asterisks (*) are predicted fragments of the thiolactone ring.

Fig 2.

Flow diagram for the newly developed mass spectrometry-based assay for monitoring quorum sensing inhibition. 1. Bacteria are cultured in the presence of inhibitors or controls in triplicate wells in a 96 well plate format. 2. OD₆₀₀ is monitored for the bacterial cultures at 1 hr intervals to check for growth inhibition or growth delays. 3. Bacterial cultures are filtered (using a 96-well plate compatible filter) and the filtrate (spent broth) is analyzed directly using an ultraperformance liquid chromatograph coupled to a Q-exactive Orbitrap

mass spectrometer (UPLC-MS). 4. Plots of AIP peak area versus time are used to quantitatively measure quorum sensing inhibition.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig 3. AIP production is inhibited by ambuic acid, as measured using mass spectrometric detection of AIP (A) and a GFP reporter strain (B). The mass spectrometry based assay (A) employed biological assays conducted in triplicate, and the error bars represent the standard deviation of these measurements. The GFP reported based assay was performed with four replicates, and also shown with error bars indicating standard deviation.

Fig 4.

Ambuic acid showed no effect on the growth up to a concentration of 100 µM using the USA300 LAC strain of MRSA (AH1263) as monitored by absorbance at 600 nm (OD₆₀₀). The error bars represent the standard error of measurements made on triplicate wells (biological replicates).

Table 1

Regression parameters, detection limit, and limit of quantification for *Staphylococcus aureus* auto-inducing peptide I (AIP I)

Validation parameter	Result ^a
Correlation coefficient	0.999
Standard error	8.6×10^6
y-Intercept	$(-2.2 \pm 2.0) \times 10^6$
Slope of regression line	$(7.178 \pm 0.035) \times 10^7$
Limit of detection (LOD)	$0.0035 \mu\text{M}^b$
Lower limit of quantitation (LLOQ)	$0.10 \mu\text{M}^c$
Upper limit of quantitation (ULOQ)	$26 \mu\text{M}$

^aCalculations were based on triplicate analyses of nine standards of *S. aureus* Auto-inducing peptide I using UPLC-MS.

^bLOD was defined as the concentration that provided a signal to noise ratio of 3:1 with noise determined by injection of a solvent blank (tryptic soy broth with 2% dimethyl sulfoxide).

^cLLOQ was defined as the lowest concentration of standard in the calibration curve that provided a residual of less than 15% (Table 2).

Table 2Precision data for *Staphylococcus aureus* auto-inducing peptide I (AIP I)

Theoretical concentration (μM)	Measured concentration ^a (μM)	Residuals ^b (%)	Repeatability ^c (%)	Intermediate precision ^d (%)
0.102	0.107	5.7	0.5	1.9
0.203	0.191	-6.3	0.2	2.3
0.407	0.359	-12	1.9	5.7
0.813	0.822	1.0	0.8	2.6
0.163	1.65	1.2	0.7	1.5
3.25	3.27	0.4	1.0	2.0
6.51	6.69	2.8	0.6	2.4
13.0	13.7	5.0	1.9	2.0
26.0	26.5	1.8	3.0	2.5

^a The measured concentration is an average of back calculated concentration of *S. aureus* auto-inducing peptide I obtained from triplicate analyses on three different days.

^b (Measured concentration-theoretical concentration) / theoretical concentration × 100.

^c Repeatability is expressed as the percentage relative standard deviation for triplicate analyses conducted on a single day.

^d Intermediate precision is expressed as the percentage relative standard deviation of the three back-calculated *S. aureus* auto-inducing peptide I concentrations (each an average of triplicate measurements) determined on three separate days.