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Targeted and Untargeted Analysis of Secondary Metabolites to Monitor Growth and Quorum Sensing Inhibition for Methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

Drug resistant infections are an increasing problem world-wide, responsible for an estimated 700,000 annual mortalities. The use of antibiotics to treat such infections has resulted in the development of resistant bacterial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA). One potential alternative strategy for treating drug resistant bacterial infections is to inhibit the production of toxins, thereby making the bacteria less harmful to the host, a so called “anti-virulence” approach. In MRSA, the *agr* quorum sensing system is one of the major regulators of toxin production, and quorum sensing inhibitors that target this system are a promising anti-virulence strategy. With this study, we developed a method that enables the activity of quorum sensing inhibitors to be measured using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS). This method is an improvement over existing methods because it can be employed to distinguish antimicrobial activity from quorum sensing inhibition activity based on the UPLC-MS data. This is possible by simultaneously tracking production of metabolites regulated by the *agr* quorum sensing system (AIP-I and formylated δ -toxin) and a metabolite that appears not to be *agr* regulated under the conditions of this study (aureusimine B). The newly developed method provides more nuanced indication of how metabolite production changes over time and in response to quorum sensing or growth inhibition than is possible with commonly employed spectroscopic methods.

Keywords

Metabolomics; virulence; mass spectrometry; aureusimine B; selectivity ratio; quorum sensing

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Conflict of interest

The authors declare that they have no conflict of interest.

1. Introduction

Staphylococcus aureus is a Gram-positive bacterium that colonizes the mucosal membranes and epithelial surfaces of more than 30% of the healthy adult population (Jenul et al., 2018, Wertheim et al., 2005). *S. aureus* causes infections in both healthy and immunocompromised individuals (Gajdacs, 2019, Grumann et al., 2014), and methicillin-resistant *S. aureus* (MRSA) is one of the most problematic multi-drug resistant pathogens, responsible for more than 10,000 hospital-acquired annual mortalities in the US alone (CDC, 2019, Gajdacs, 2019). While MRSA was originally observed primarily in hospital settings, over the past two decades, there has been an increase in community-acquired MRSA infections (DeLeo et al., 2010, Gajdacs, 2019). The MRSA strains most commonly associated with community acquired infections are characterized by their hyper-virulence, which results from their production of high levels of toxins that cause inflammation and tissue damage (Otto, 2014). Thus, it has been suggested that a promising strategy for combating MRSA infections would be targeting toxin production, a so-called “anti-virulence” approach.

Toxin production in MRSA is controlled by two-component regulatory systems, most importantly the accessory gene regulatory (*agr*) system (Thoendel et al., 2009, Thoendel et al., 2011). Activation of this system occurs in a cell-density dependent fashion when an autoinducing peptide (AIP) secreted by the bacteria binds to the extracellular receptor AgrC, a membrane-bound histidine kinase. Binding of AIP to AgrC induces changes in gene expression that ultimately lead to the production of multiple virulence factors, including the phenol soluble modulins (PSMs) (Table 1).

Previous investigations have sought to track virulence in MRSA by measuring quorum sensing regulated metabolites. For example, our group and others have detected the AIPs directly from bacterial culture media (Gless et al., 2019, Junio et al., 2013, Kalkum et al., 2003, Todd et al., 2016, Williams et al., 2019). Others have reported the detection of outputs of the quorum sensing system such as phenol soluble modulins (PSMs) (Diep et al., 2008, Hodille et al., 2016, Laabei et al., 2014, Wu et al., 2018) including delta toxins (Hodille, et al., 2016, Quave et al., 2014, Somerville et al., 2003). The limitation of measuring these metabolites to monitor virulence is that it is difficult to disentangle anti-virulence activity from anti-microbial activity; inhibition or delay in bacterial growth will have the same effect (decrease in metabolite production) as inhibition of quorum sensing. Prior to the current work, this problem has been addressed by simultaneously monitoring quorum sensing and tracking bacterial density with OD₆₀₀ readings (Todd et al., 2017, Todd, et al., 2016). With the current study, we sought to develop an alternative strategy, the application of a mass spectrometric method that could simultaneously monitor multiple classes of secondary metabolites produced by MRSA, including metabolites controlled by the *agr* system (as an indicator of virulence) and those that are not (as an indicator of growth).

In conducting this study, we predicted that the aureusimines might be useful metabolites to employ for the purpose of monitoring *S. aureus* growth. Production of aureusimines by *S. aureus* has been independently discovered by the Fischbach and Magarvey research groups (Wyatt et al., 2010, Zimmermann et al., 2010). Aureusimine biosynthesis is dependent on the *ausAB* operon, also referred to as *pznAB* operon, which encodes a nonribosomal peptide

synthetase (*ausA*) and 4'-phosphopantetheinyltransferase (*ausB*). An early report (Wyatt, et al., 2010) had suggested aureusimines to be major virulence regulators in *S. aureus*, but a more recent study showed that these regulatory effects were due to a secondary mutation in the SaeRS two-component system (Sun et al., 2010). Therefore, the biological role of aureusimines has yet to be determined. With this study, we endeavored to test the utility of tracking aureusimine production as a way to monitor MRSA growth.

2. Materials and Methods

2.1. MRSA culture for monitoring metabolite production over time

Methicillin-resistant *Staphylococcus aureus* (LAC USA300) was used for these experiments (Junio, et al., 2013). A 24 hr culture was grown in Mueller Hinton Broth (MHB) at 37°C with shaking at 200 rpm using a New Brunswick Scientific shaker/incubator series I-26. The culture was diluted into triplicate borosilicate glass Erlenmeyer flasks containing 150 mL of MHB at a dilution of 3.7×10^8 CFU (colony forming units). Every 2 hr, a 500 µL aliquot was removed from each flask and the OD₆₀₀ was measured. Also, at each time point a second aliquot was removed, filtered with a 0.22 µm centrifugal filter with a polyvinylidene difluoride (PVDF) membrane, and the filtrate was retained for analysis with ultraperformance liquid chromatography-mass spectrometry (UPLC-MS).

2.2. MRSA culture for growth and quorum sensing inhibition

A slight modification of a previously described method was used to evaluate the influence of inhibitors on MRSA metabolite production (Todd et al., 2017). Briefly, MRSA (LAC USA300) was cultured at 37 °C in tryptic soy broth (TSB) for 24 hr with shaking at 200 rpm in a New Brunswick Scientific shaker/incubator series I-26. This culture broth was diluted 1:100 in TSB and cultured with shaking at 200 rpm at 37 °C for 2 hr, at which point the OD₆₀₀ was measured to be in the range of 0.08–0.1. A 96-well tissue culture treated flat bottom plate (Corning Incorporated) was inoculated with 245 µL of this diluted bacterial inoculum and 5 µL of inhibitor or control in each well. The inhibitors included ambuic acid (Adipogen life Sciences) and chloramphenicol (Sigma Aldrich) at assay concentrations of 0.77 µM to 100.00 µM and 2.34 µM to 300.00 µM, respectively. Stock solutions of ambuic acid and chloramphenicol were prepared in DMSO and assay content of DMSO was 2% in all wells. Assays were performed in triplicate for each treatment and control. The vehicle control consisted of 2% DMSO. The plate was incubated at 37 °C and shaken at 1000 rpm in a Stuart Microtitre Shaker Incubator (SI505). OD₆₀₀ was measured at 1 hr intervals using a Synergy H1 Plate Reader and the experiment was considered complete at OD₆₀₀ of 2.0 (~4–6 hr). At completion of the incubation time, the culture broth containing bacteria was transferred to a clear sterile 96-well, 0.22 µm hydrophilic plate with low protein binding durapore membrane (MultiScreen®) and filtered under vacuum. The spent media were then analyzed by UPLC-MS.

2.3. MRSA culture for experiments with deletion mutants

Three strains of MRSA were used in these experiments, a clinical isolate referred to as MRSA LAC USA300 (AH1263) (Boles et al., 2010) and a genetic deletion mutant engineered to knock out aureusimine production (AH2137), and a genetic deletion mutant

engineered to knock out the *agr* quorum sensing system (AH1292) (Somerville, et al., 2003). Triplicate samples of a diluted 24 hr inoculum (prepared as described in Section 2.2) of each strain (30 mL total volume) were cultured in MHB in 50 mL falcon tubes for 24 hr with shaking at 200 rpm at 37 °C. At the 24 hr time point, each culture was filtered with a centrifugal filter (PVDF) and the spent medium was analyzed by UPLC-MS.

To construct the AH2137 *ausA* deletion plasmid, homology arms (~1100 bp) upstream and downstream of *ausA* were amplified with primer pairs (IDT) CLM508/CLM512 and CLM510/CLM511 (Table S1). The PCR products were column purified with the QIAquick PCR purification kit (Qiagen) and fused in a second PCR with primers CLM508 and CLM511. The PCR product was gel purified with the QIAquick gel extraction kit (Qiagen), digested with SacI and SalI (New England BioLabs Inc), and ligated into plasmid pJB38 (Wormann et al., 2011) digested with the same restriction enzymes to generate plasmid pCM44. The plasmid was electroporated (Löfblom et al., 2007) into strain RN4220 (Nair et al., 2011) and clones carrying pCM44 were selected on TSA plates containing chloramphenicol (Cm, 10 µg/ml) at 30 °C. The plasmid was recovered with the QIAprep Spin Miniprep Kit (Qiagen) from overnight cultures grown at 30 °C in TSB containing Cm (10 µg/ml) and sequenced with primers SEQ, REVCOMPSEQ and SEQ62D. The plasmid was then electroporated into USA300 strain LAC, clones carrying pCM44 were selected on TSA plates containing Cm (10 µg/ml) and individual colonies were subsequently streaked on TSA plates containing Cm (10 µg/ml) and incubated at 42°C to select for integration into the chromosome. Single colonies were grown in TSB at 30°C with shaking and diluted 1:500 in fresh media for four successive days before diluting to 10⁻⁶ and plating on TSA containing 200 ng/ anhydrotetracycline to select for loss of the plasmid. Colonies were screened for resistance to Cm, and Cm^S colonies were screened by PCR for deletion of *ausA*.

2.4. UPLC-MS analysis

An Acquity UPLC system (Waters, Corporation, Milford, MA) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA) was used for UPLC-MS analyses. For the samples collected in the 24 hr growth analysis (Section 2.1) a 3 µL injection of each sample was eluted from the column (Acquity UPLC BEH C18 1.7 µm, 2.1 × 50 mm, Waters Corporation) at a flow rate of 0.3 mL/min with solvent A consisting of water (Optima LC-MS grade) with 0.1% formic acid added and solvent B consisting of methanol (Optima LC-MS grade). The gradient was initiated with an isocratic composition of 60:40 (A:B) for 0.5 min, increasing linearly from 0.5 min to 7.50 min to 30:70 (A:B) followed by an isocratic hold for 0.5 min. From 8.0–8.5 min the gradient increased linearly to 0:100 with a 0.5 min isocratic hold. The gradient returned to initial starting conditions from 9.0–9.5 min and was held for 0.5 min. The mass spectrometer was operated in positive ion mode for the scan range of 150–1500 with the following settings: capillary voltage of 5 V, capillary temperature of 300 °C, tube lens offset of 35 V, spray voltage of 3.80 kV, sheath gas flow of 35, and auxiliary gas of 20. The same method was employed for samples from the growth inhibition studies (Section 2.2) and deletion mutant experiments (Section 2.3) except that a different column (Acquity UPLC BEH SHIELD RP C18 1.7 µm, 2.1 × 50 mm, Waters corporation) was used.

To identify selected MRSA metabolites, MS-MS analysis was conducted using collision-induced dissociation (CID) with a collision energy of 35.0. Fragmentation patterns, retention times, and accurate masses were compared between putative metabolite signals in the MRSA culture medium and data for standards for AIP-I (Anaspec), δ -toxin (Anaspec) and aureusimine B (Cayman chemicals). Data dependent acquisition was employed with an inclusion list for the masses of 961.3798 (AIP-I), 229.1332 (aureusimine B) and 752.4135 (δ -toxin). A calibration curve was also collected for the AIP-I standard to establish the linear range of instrument response, using a concentration range of 1.0×10^{-4} to $1.0 \times 10^1 \mu\text{g} \times \text{mL}^{-1}$.

All mass spectrometric data have been made publicly available through MassIVE (MassIVE ID: MSV000085457, doi: [10.25345/C5WT5R](https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?accession=MSV000085457), <https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?accession=MSV000085457>).

2.5. Untargeted metabolomics procedures

Untargeted metabolomics data analysis was conducted on the mass spectrometric data for samples generated in the growth inhibition studies (Section 2.2). Mass spectrometric data were analyzed, aligned and filtered with MZmine 2.2 software (<http://mzmine.sourceforge.net/>). The parameters can be found in Table S3. Briefly, peak detection in MZmine was obtained above a baseline of 1.2×10^6 and a chromatogram was constructed for each of the m/z values that spanned longer than 0.1 min. The deconvolution parameters for peak detection included noise level (absolute value) at 1.2×10^6 , minimum peak duration 0.5 min, tolerance for m/z variation 0.001 and tolerance for m/z intensity variation 10%. The join aligner algorithm was used to create a compiled peak table by setting the balance between m/z and retention time at 10.0 each, m/z tolerance at 0.05, and retention time tolerance as 2 min. The spectral data matrix was imported to Excel (Microsoft, Redmond, WA, USA) and the OD₆₀₀ data were included to form a final data matrix. Using optical density (OD₆₀₀) as the dependent variable, we conducted multivariate statistical analysis to construct 4-component partial least squared scores and loadings plots. From the univariate model, selectivity ratio analysis was conducted using Sirius 10.0 (Pattern Recognition Systems AS, Bergen, Norway). This approach was used to identify which features of the dataset (detectable ions/independent variables) were correlated with growth.

3. Results and Discussion

3.1. Untargeted metabolomics of a growth curve to detect MRSA metabolites

To inform the selection of analytes for targeted mass spectrometric analysis, we first conducted untargeted mass spectrometry metabolomics experiments. Critical in these experiments was the application of a data analysis tool, the selectivity ratio, which enables complex mass spectrometric datasets to be simplified by identifying features associated with a given dependent variable (Caesar et al., 2019, Kellogg et al., 2016, Kvalheim et al., 2011). Using untargeted metabolomics with selectivity ratio analysis, we sought to identify specific MRSA metabolites that could be detected as increasing in abundance with increasing OD₆₀₀.

Untargeted metabolomics analysis of MRSA cultures is complicated by high levels of background introduced by the rich culture medium. This high level of background makes it difficult to distinguish the signals for MRSA metabolites of interest from spurious signals resulting from the culture medium. To resolve this issue, we analyzed a time-dependent series of MRSA cultures using UPLC-MS and conducted untargeted metabolomics analysis of the combined dataset. We then employed selectivity ratio analysis (Caesar et al., 2018) to determine which features in the metabolomics dataset were correlated with bacterial growth (as measured by OD₆₀₀) (Fig. 1). The selectivity ratio serves as a ranking tool, indicating which features are more likely to be associated with the dependent variable (in this case growth). For a given study, the investigator may select a selectivity ratio cut-off value for which features to include in the dataset. This cut-off value can be adjusted up or down depending on the research question being asked. For the studies described herein, a selectivity ratio cut off of 0.36 was selected because all of the known MRSA metabolites had selectivity ratios greater than or equal to this value.

In Fig. 1, features (*m/z* retention time pairs) that correlate with growth (OD₆₀₀) are indicated by positive selectivity ratios. Of the 2090 features detected above a signal intensity of 1.2×10^6 with untargeted UPLC-MS metabolomics across the twelve timepoints of the growth experiment, only 5% had selectivity ratios above 0.36, more than half of these (49 ions) were putatively identified as isotopes, multiply charged ions, or adducts associated with seven known MRSA metabolites based on accurate mass values reported in the literature (Table 1). The known metabolites for which ions were detected in the untargeted metabolomics analysis include aureusimines A and B, AIP-I, formylated and deformylated δ – toxin, phenol soluble modulins (PSM) α -2 and PSM α -3 (see ions color coded and labeled in Fig. 1). In addition to the expected known MRSA metabolites, a number of additional features were detected among the top 5% of ions with highest selectivity ratios (shown unlabeled in grey in Fig. 1). These features could be unidentified adducts, fragments, or charge states of known metabolites or could correspond to hitherto unidentified MRSA metabolites. Future studies to assign structures to the unidentified features would be of interest. The data in Fig. 1 illustrate the utility of selectivity ratio analysis as a methodology for interpreting untargeted metabolomics data; it is far simpler to find 49 relevant features from among 87 features, than from the total of 2090 features. Additionally, the number of potential relevant unidentified metabolites is greatly reduced using the selectivity ratio analysis.

3.2. Confirmation of detection of known MRSA metabolites

The structural assignments indicated in Fig. 1 are only tentative, relying on accurate mass measurement alone (Table S2). To provide a more rigorous confirmation of structure, correct identification of several of the known MRSA metabolites labeled in Fig. 1 was confirmed by comparing accurate mass (Table S2), retention time (Fig. 2), and MS-MS fragmentation data (Fig. S1, S2 and S3) to that of available standards and previous literature. Additional confirmation was provided by comparison with an *agr* deletion mutant and a *ausA* deletion mutant (Fig. 2). The *ausA* gene encodes a nonribosomal peptide synthetase that is essential for the biosynthesis of aureusimines (Wyatt, et al., 2010, Zimmermann, et al., 2010) hence the *ausA* deletion mutant is deficient in aureusimine production. The data for the mutants display the expected findings, AIP-I and δ -toxin are not produced in the *agr* mutant, and

aureusimine B is not produced in the *ausA* deletion mutant. The findings summarized in Fig. 2 provide confirmation of correct structural assignment for the subset of metabolites indicated in Table 1 for which relevant standards and mutants were available.

3.2. Comparison of OD₆₀₀ and mass spectrometric methods for monitoring bacterial growth

The newly developed method for detecting MRSA metabolites was employed to track metabolite production over time (Fig. 3). Bacterial growth was monitored using the standard method of OD₆₀₀ (optical density at 600 nm) measurements (Fig. 3A) while at the same time peak areas for metabolites of interest, AIP-I (Fig. 3B), aureusimine B (Fig. 3C) and formylated δ -toxin (Fig. 3D) were also measured. Growth studies show that as the bacterial population increases (as measured by increase in OD₆₀₀), abundance of individual known MRSA metabolites from different classes also increases (Fig. 3). It is furthermore apparent that the time point at which each metabolite is first detected varies among the metabolites, AIP-I and aureusimine B were first detected at the four hr time point (Fig. 3B and C), whereas formylated δ -toxin (Fig. 3D) was not detected until 12 hr. The differences in onset time for production of AIP-I and δ -toxin are expected based on gene regulation with the *agr* system in MRSA, which has been well described in the literature ((Thoendel, et al., 2011)). The promoter driving AIP-I biosynthesis functions at a low constitutive level, and the promoter driving δ -toxin expression strictly depends on AIP-I signal accumulation.

It is also apparent from Fig. 3 that metabolite production levels off in the latter part of the time course experiment. This is presumably due to reduced metabolite production, although degradation of the metabolite would also cause an apparent leveling in production and cannot be ruled out. Differences in the time at which signal levels off is observed between the various metabolites (Fig. 3). While AIP-I levels saturate quickly (after just 6 hr) (Fig. 3B), aureusimine B (Fig. 3C) production continues to increase up to 16 hr, even after saturation has been observed in OD₆₀₀ (Fig. 3A). The reason for the observed saturation in AIP response is biological (decreased production by bacteria or degradation of signal) not methodological (response in the mass spectrometer saturated at high concentrations). A calibration curve of mass spectrometric peak area versus AIP concentration is linear up to a peak area of 1×10^8 (Fig. S4), while saturation in AIP signal is observed in Fig. 3B at a response in the range of 8×10^5 .

3.3 Targeted analyses of known metabolites in response to treatment with an antimicrobial or quorum quencher

Building on the demonstrated ability of the newly developed method to simultaneously track the production of several metabolites associated with virulence of MRSA (AIP-I and formylated δ -toxin), we sought to demonstrate its applicability to track *agr* regulated quorum sensing inhibition. Ambuic acid has previously been shown to act as an inhibitor of the *agr* system (Todd, et al., 2017), while chloramphenicol is a well-known bacterial protein synthesis antibiotic (Newman et al., 2012). Therefore, we monitored the influence of these compounds on metabolite production by MRSA across a range of concentrations. Consistent with its antimicrobial activity, chloramphenicol treatment inhibited the production of the three measured metabolites (Fig. 4 B, D, and F). Also as expected, ambuic acid inhibited

production of AIP-I by MRSA in a dose-dependent fashion (Fig. 4A). Suppression of AIP-I production should also result in decreased production of other secondary metabolites that are outputs of the quorum sensing system, including δ -toxin. This expected result is observed in Fig. 4C. Consistent with previous literature (Todd, et al., 2017), the observed suppression of AIP-I and formylated δ -toxin by ambuic acid treatment was not due to growth inhibition or growth delay. These experiments were conducted using a short incubation time (6 hr) established previously (Todd, et al., 2017), which achieves rapid MRSA growth by shaking at a high RPM. As demonstrated in the growth curves that accompany Fig. 3 (Fig. S5), stationary phase was reached in the 6 hr incubation period, and ambuic acid caused no decrease in OD₆₀₀ up to 100 μ M (Fig. S4).

In tandem with the quorum sensing inhibition measurements, we sought to employ the mass spectrometric method to monitor growth inhibition. Several ions were identified based on the untargeted metabolomics experiments that could serve as candidates for tracking growth. These ions were detected at m/z values of 198.0868, 229.1332, 245.1285, 815.4288 and 871.4870 in positive ion mode mass spectrometry. Among these, only aureusimine B (m/z 229.1332) and aureusimine A (m/z 245.1285) were identified as a known constituents of MRSA. While the other ions appear to be MRSA constituents, their identities are currently unconfirmed. The MS signal for aureusimine A was much lower than that of aureusimine B, making it less desirable for tracking MRSA growth. Therefore, we focused these studies on aureusimine B. Under the conditions employed herein, it appeared that aureusimine B was not strongly regulated by the *agr* system (Fig. 4E).

The data in Fig. 4E suggest that aureusimine B production could be used as a means to track MRSA growth, and that, at least under the conditions employed in this study, the production of this metabolite appears not to be altered in the presence of a quorum sensing inhibitor. Thus, by tracking *agr* regulated metabolites and aureusimine B simultaneously with UPLC-MS, it is possible to measure quorum sensing inhibition and rule out growth interference.

Conclusion

With this study, we demonstrate that UPLC-MS can be employed to simultaneously monitor production of multiple secondary metabolites produced by MRSA. To our knowledge, this is the first study to use a single mass spectrometric method to track both growth and *agr* regulated virulence. We show that aureusimine B production is correlated with bacterial growth, and that by monitoring aureusimine B production in tandem with the production of *agr*-regulated metabolites, it is possible to distinguish the effect of *agr* quorum sensing inhibitors and compounds from those that simply inhibit bacterial growth. The method developed herein could be of benefit to researchers who seek to obtain a more nuanced representation of how metabolite production changes over time or in response to the addition of inhibitors than is provided by OD₆₀₀ data alone. Additionally, this approach could be expanded to have utility in situations where OD₆₀₀ readings are not possible, for example in measurements of bacterial growth *in situ* on surfaces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Histogram showing selectivity ratio (SR) for each feature in the untargeted metabolomics dataset for the 24-hour growth experiment shown in Fig. 2. The MRSA was sampled every two hr and the metabolite profiles for each time point collected using UPLC-MS. OD₆₀₀ measurements (indicating bacterial growth) at each timepoint were used as the dependent variable in calculating the selectivity ratio. The x-axis represents the feature (identified by its *m/z*) detected in the metabolomics dataset. Features are shown in order of increasing *m/z*. Peaks are numbered to correspond with known metabolites that were detected as shown in Table 1. Higher selectivity ratio indicates stronger correlation with growth (as measured by OD₆₀₀). 87 features had selectivity ratios above 0.36, 49 of which were putatively identified as ions associated with 5 known MRSA metabolites.

A: AIP-I **B: Formylated δ -toxin** **C: Aureusimine B** **Fig. 2.**

Comparison of selected ion chromatograms from targeted UPLC-MS analysis of MRSA metabolites (numbered as in Table 1). Data are shown for a clinical isolate of MRSA (LAC USA 300 strain AH1263), a *ausA* non-ribosomal peptide synthetase (NRPS) deletion mutant (AH2137), and an *agr* gene deletion mutant (AH1292). The data in (A) compare retention time and mass for an AIP-I standard (m/z 961.3798, $[M+H]^+$) with the putative AIP-I ion in the MRSA cultures. The data shown in (B) compares formylated δ -toxin production (m/z 752.4135 for $[M+4H]^{4+}$) across all strains of MRSA and demonstrate agreement in retention time and mass with the standard. Panel (C) shows a comparison of aureusimine B production (m/z 229.1332 for $[M+H]^+$) across all strains. As expected, the same metabolites were detected for the aureusimine gene deletion mutant with the exception

of aureusimines, which are absent. For the *agr* gene deletion mutant, metabolites regulated by the *agr* system (AIP and δ -toxin) are not detected.

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Fig. 3.

Change in abundance of metabolites produced by a USA 300 clinical strain of MRSA over time. MRSA growth was measured by turbidity (OD₆₀₀) (A). In parallel, abundance of each metabolite was measured every two hr over a 24 hr time period using UPLC-MS. Signal for each metabolite was measured using the area under the curve of the selected ion chromatogram for the relevant ion, AIP-I (B), with m/z of $[M+H]^+$ at 961.3798, aureusimine B (C) with m/z of $[M+H]^+$ at 229.1332 m/z , formylated δ -toxin (D) with m/z of $[M+4H]^{4+}$ at 752.4122.

Fig. 4.

Logarithmic dose inhibitory response of known MRSA metabolites to treatment with the quorum sensing inhibitor ambuic acid (A,C, and E) or the antimicrobial chloramphenicol (B, D and F). Masses of the individual ions are indicated in Table 1. All peak areas were calculated as the average of triplicate cultures. Error bars represent the standard error of the mean. All metabolites decrease in abundance when treated with the antimicrobial chloramphenicol, while only the *agr* regulated metabolites (AIP-I and formylated δ -toxin) respond to treatment with ambuic acid. Data were all collected when the bacterial cultures had reached stationary phase after a 6 hr incubation period. Accompanying growth curves

are provided in Fig. S5. IC_{50} values indicate the concentration at which the measured response (peak area) is reduced by 50%.

Table 1:

Known secondary metabolites produced by MRSA indicating structure and function. All compounds were tentatively identified in the MRSA cultures based on accurate mass measurement (Table S2), as shown in Fig. 1.

Name	Id Number	Structure/Sequence	Monoisotopic Mass	Function	Reference
Autoinducing Peptide-I (AIP-I)	1	YSTc(CDFIM)	960.3720	signaling peptide for the <i>agr</i> system	(Arvidson et al., 2001, Johnson et al., 2015, Todd, et al., 2016, Vasquez et al., 2017)
δ -toxin	2	MAQDIISTIGDLVKWIIDT-VNKFTKK	2977.6277	<i>agr</i> -regulated virulence factor	(Hodille, et al., 2016, Laabei, et al., 2014, Somerville, et al., 2003, Wu, et al., 2018)
Formylated δ -toxin	3	fMAQDIISTIGDLVKWIIDT-VNKFTKK	3005.6297	<i>agr</i> -regulated virulence factor	(Hodille, et al., 2016, Laabei, et al., 2014, Somerville, et al., 2003, Wu, et al., 2018)
Aureusimine A	4		244.1207	function undetermined	(Wilson et al., 2013, Zimmermann, et al., 2010)
Aureusimine B	5		228.1257	function undetermined	(Secor et al., 2012, Zimmermann, et al., 2010)
PSM α -2	6	fMGIIAGIIFKFKGLIEKFTGK	2305.3685	<i>agr</i> -regulated virulence factor	(Diep, et al., 2008, Hodille, et al., 2016, Wu, et al., 2018)
PSM α -3	7	fMEFVAKLFKFFKDLLGKFLGNN	2633.4080	<i>agr</i> -regulated virulence factor	(Diep, et al., 2008, Hodille, et al., 2016, Wu, et al., 2018)