# Chapter 27

# Identification of Staphylococcal Quorum Sensing Inhibitors by Quantification of õ-Hemolysin with High Performance Liquid Chromatography

**Cassandra L. Quave and Alexander R. Horswill**

## Abstract

Quorum sensing plays a major role in regulation of virulence factor production by staphylococci. Chemical inhibitors that block this process and prevent the production of exotoxins and exoenzymes could have medical utility for infection prophylaxis and therapy. Here, we describe a high performance liquid chromatography method amenable to medium throughput screening for staphylococcal quorum sensing inhibitors by quantification of õ-hemolysin, a direct protein output of this system.

**Key words** Staphylococcal quorum sensing inhibitors, Accessory gene regulator, *Staphylococcus aureus*, High performance liquid chromatography, õ-Toxin

## 1 Introduction

Staphylococci perform cell-to-cell communication via the release of autoinducing peptide (AIP) molecules into the extracellular environment. When the cell density reaches a critical concentration, enough AIP has accumulated outside the cells to induce a regulatory cascade, a mechanism often called quorum sensing. The activation of this cascade induces the expression of accessory factors, including a large suite of exotoxins and exoenzymes, and hence this system was named the accessory gene regulator (*agr*) [1, 2]. The expression of many of these accessory factors is controlled by a transcript called RNAIII, and encoded within RNAIII is a low molecular weight toxin called õ-toxin or õ-hemolysin. The *agr* system plays a crucial role in the pathogenesis of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), and thus antivirulence approaches to treating staphylococcal infections have focused on disruption of this system. A number of small molecule inhibitors of *agr* have already been identified from natural product

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screening efforts [3–7]. õ-hemolysin is a direct output of the *agr* system and thus serves an ideal product to track for the discovery of effective inhibitors.

The aim of this experiment is to identify non-biocide inhibitors of virulence pathways in staphylococci. Here, we explain how a high performance liquid chromatography (HPLC) method developed for detection of õ-hemolysin in staphylococcal supernatants [8] is used to quantify õ-hemolysin in the supernatant for the purposes of identifying quorum sensing inhibitors. A major advantage of this method is that it allows for the direct injection of the bacterial supernatant into the HPLC system without any prior cleanup steps. When the HPLC is paired with an autosampler, the method is amenable to medium-throughput testing. This technique is useful for the phenotypic profiling of isolates, but it is also powerful for the identification of chemical agents with quorum sensing inhibitory activity.

## 2 Materials

Prepare all solutions with Type 1 (ultrapure) water and American Chemical Society (ACS) grade solvents or equivalent. HPLC mobile phases are prepared with HPLC grade reagents.

### 2.1 Chemical Matter

1. Chemical matter for testing, in powder form (e.g., natural product extracts or single compounds).
2. Vehicle: dimethyl sulfoxide (DMSO) or water, depending upon solubility of the chemical matter.

### 2.2 Bacterial Culture

1. *S. aureus* or *S. epidermidis* strains: High toxin producing clinical isolates are preferred for drug screening (e.g., *S. aureus* strains: LAC, NRS 225, NRS 232, NRS242, NRS249, NRS385; *S. epidermidis*: NRS101 obtained from the NARSA collection curated by BEI Resources, www.beiresources.org/).
2. Tryptic soy agar.
3. Tryptic soy broth.
4. Petri dishes.
5. 96-well plates.
6. 14 ml snap cap test tubes.
7. Microcentrifuge tubes.
8. Inoculating loop.

### 2.3 High Performance Liquid Chromatography

1. GE Healthcare Resource PHE 1-ml column.
2. Mobile Phase A: 0.1% (vol/vol) trifluoroacetic acid (TFA) in water.

3. Mobile Phase B: 0.1% (vol/vol) TFA in acetonitrile (ACN).
4. HPLC system.
5. 2 ml HPLC vials.

## 3 Methods

Carry out all experiments at room temperature unless otherwise noted.

### 3.1 Prepare Chemical Matter for Testing

1. Carefully weigh the dry test compound or natural product extract, collectively referred to as "drug" below. Create a stock solution at 10 mg/ml of extract (mixture of compounds) in vehicle (DMSO or water, depending on solubility), or 1 mg/ml suggested for individual compounds.
2. Vortex the stock solution for 1 min. Sonicate in a water bath for 15 min or more. Monitor to ensure that no solids are floating in the stock. Store at 4 °C until needed for assays.

### 3.2 Measure Growth Inhibitory Effects

1. Determine the Minimum Inhibitory Concentration (MIC) of your drug by broth microtiter dilution, following established Clinical & Laboratory Standards Institute (CLSI) guidelines (M100-S23) [9]. Briefly, overnight cultures of *S. aureus* should be standardized to reach a final inoculum density of $5 \times 10^5$ colony forming units per ml (CFU/ml) in Cation-adjusted Mueller-Hinton Broth (CAMHB), verified by colony plate counts. Your drug should be added to the top well on a 96-well plate and serial diluted down to the desired concentration test range. Following an 18 h period of incubation at 37 °C in a humidified chamber, the MIC can be determined by identifying the lowest concentration well with no visible growth (determined by eye or by a plate reader at an optical density of 600 nm).
2. Select a sub-MIC concentration for the starting point in serial dilutions of the chemical matter for õ-hemolysin quantification. In general, we recommend starting at concentrations at less than 50% growth inhibitory levels.

### 3.3 Grow Cultures and Harvest Supernatant

1. *Day 1*: Make a fresh 3-way streak plate of *S. aureus* on tryptic soy agar (TSA). Incubate at 37 °C overnight (18–24 h).
2. *Day 2*: Add 6 ml of tryptic soy broth to a 14 ml snap cap test tube. Using a sterile inoculating loop, inoculate the tube with a single colony from the overnight plate. Place in the incubator at a 45° angle; incubate at 37 °C with shaking (200 rpm) overnight (18–24 h).

3. *Day 3*: Standardize the inoculum to a final starting density of $5 \times 10^5$ colony forming units (CFU) per ml by diluting into fresh TSB. The final starting inoculum density (CFU/ml) should be checked by dilution plating and colony counts.
4. The freshly diluted inoculum should be placed in a test tube or flask at a volume-to-flask ratio of ~1:10 for optimal aeration with shaking. For example, 1.5 ml of inoculum is added to 14 ml snap cap test tubes. Use three untreated growth controls (no drug added) for establishing the baseline toxin levels for that particular strain. Vehicle controls should also be included in the study for comparison.
5. To test a single concentration of the drug, add an appropriate amount of the drug stock to achieve a sub-MIC concentration, preferably at a level eliciting less than 50% inhibition. Then, add the appropriate amount of inoculum created in **step 4**. Always vortex the stock solution well prior to use. If the drug exhibits issues with solubility, additional sonication prior to use may be helpful. Perform all tests with four replicates. To determine the volume of drug stock to add, use the following formula:

$$\frac{x\,\mu g}{z\,\mathrm{ml}} = \frac{y\,\mu g}{\mathrm{ml}}$$

   where $x$ = amount of drug required to achieve $y$ desired final test concentration in $z$ volume (*see* **Note 1**).
6. To investigate dose-response activity, test serial dilutions of the drug following the methods described in **step 5**, with the exception that you start with double the desired final volume in tube 1. Twofold serial dilutions can be made by vortexing tubes between dilution steps (*see* **Note 2**). Perform all tests with four replicates. To achieve twofold serial dilution with four final test concentrations, add 1.5 ml of inoculum from **step 3** to tubes 2–4. Place cap on tube 1 and vortex to mix the drug and inoculum. Transfer 1.5 ml of tube 1 to tube 2 and vortex with cap. Continue this process of transfer and vortexing from tube 2 to 3 and tube 3 to 4. Dispose of the last 1.5 ml from tube 4. The final concentration per tube in this example will be 32 μg/ml in tube 1, and then 16, 8, and 4 μg/ml in tubes 2–4, respectively.
7. Incubate the cultures at 37 °C for 15 h at 275 rpm for optimal growth. If using test tubes for this step, it is critical to place the tubes in the incubator rack at a 45°angle to achieve optimal aeration and toxin production.
8. Label microcentrifuge tubes and HPLC vials with the strain and drug information in preparation for Day 4 steps.

9. *Day 4*: Remove cultures from the incubator and immediately place tubes on ice. Take a 100 μl aliquot of each culture and transfer to a 96-well plate for a plate read at an optical density (OD) of 600 nm. This information will be used to check for potential growth inhibitory activity of the drug and will also be used to normalize toxin production by OD.
10. Vortex test tubes and transfer the remaining volume into pre-labeled microcentrifuge tubes. Use a benchtop microcentrifuge to spin down the cell pellets at 8000 × *g* for 5 min.
11. Using a 1 ml pipette, carefully transfer at least 0.7 ml of the supernatant to an HPLC vial without disturbing the cell pellet. Seal vials with lids and store at −20 °C until ready for testing by HPLC. Do not thaw and refreeze prior to testing.

### 3.4 HPLC Quantification of δ-Hemolysin

1. Prepare mobile phases A and B, mixing each solvent well, and if necessary filtering with 0.2 μm filter. Attach the Resource PHE 1-ml column to the HPLC. Flush the HPLC system, check the system for leaks, and flush the column with at least 10 column volumes (10 ml) of mobile phase at initial conditions until a stable system pressure is obtained. *See* **Note 3** for instrumentation details.
2. Thaw the supernatant samples in their HPLC vials at room temperature. Vortex each vial prior to loading in the HPLC autosampler.
3. Program the HPLC to the following parameters: 500 μl injection volume with one injection per vial; flow rate of 2 ml/min; column temperature at 25 °C; UV/Vis monitored at 214 nm. Use mobile phases (a) 0.1% (vol/vol) TFA in $H_2O$ and (b) 0.1% (vol/vol) TFA in ACN with the linear gradient profile for HPLC analysis reported in Table 1.

**Table 1**
**Linear gradient profile for HPLC analysis**

| Time (min) | % A | % B |
|---|---|---|
| 0 | 90 | 10 |
| 3.00 | 90 | 10 |
| 10.50 | 10 | 90 |
| 10.51 | 0 | 100 |
| 12.00 | 0 | 100 |
| 12.01 | 90 | 10 |
| 15.50 | 90 | 10 |

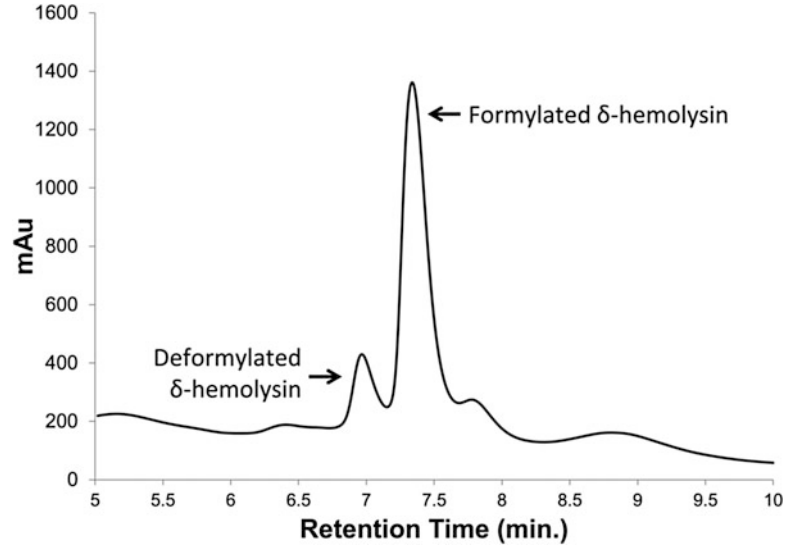


**Fig. 1** HPLC chromatogram of õ-hemolysin, with deformylated and formylated peaks highlighted

4. The chromatogram will show two prominent peaks, one corresponding to the deformylated õ-hemolysin at a retention time of ~7.2 min, and the second corresponding to the formylated õ-hemolysin at ~7.5 min (Fig. 1). There can be a large strain to strain variation in total levels of õ-hemolysin produced (Fig. 2). For this reason, we recommend that high level producers be used for drug screening initiatives in order to better detect potential inhibitors.
5. Integrate the peaks corresponding to the deformylated (~7.2 min) and formylated (~7.5 min) forms of õ-hemolysin at 214 nm. Adjust the LC software to use a "perpendicular drop method" of peak detection. The parameters need to accurately detect the baseline under the peaks and drop vertical lines from the valley between the peaks to the extended baseline. For samples with moderate to high levels of õ-hemolysin production the software defined integration parameters usually do not require manipulation. Care needs to be taken with samples that have no or low levels of toxin production, verify that the correct peak is being identified and integrated.
6. Calculate the standard deviation of the deformylated and formylated õ-hemolysin peak integrations separately for each set of replicates.
7. In order to normalize the õ-hemolysin production, the area corresponding to the deformylated and formylated õ-

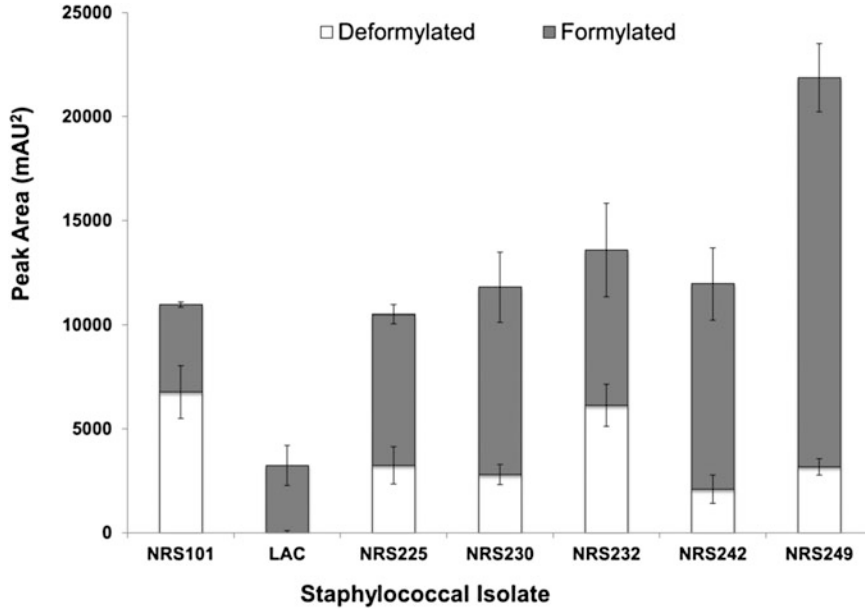


**Fig. 2** Staphylococcal strains exhibit different capacities for õ-hemolysin production, illustrated here by total peak area of the deformylated and formylated peaks. All isolates are *S. aureus,* with exception of NRS101 (*S. epidermidis*)

hemolysin must be divided by the OD reading determined in Subheading 3.2, **step 9**

$$A_{\text{norm}} = \frac{A_{\text{raw}}}{\text{OD}_{600}}$$

8. Once the integration data has been normalized, then it may be presented as separate deformylated and formylated õ-hemolysin values including standard deviation using a stacked histogram chart (Fig. 2).

## 4 Notes

1. Example: To achieve a final test concentration of 32 μg/ml, using a drug stock concentration of 10 mg/ml and a final volume of 1.5 ml in the test tube:
$\frac{x\ \mu g}{1.5\ \text{ml}} = \frac{32\ \mu g}{\text{ml}}$; $=1.5 \times 32$; then $x = 48$ μg need to be added.

   If working with a drug stock concentration of 10 mg/ml, or 10 μg/μl, then $48\ \mu g \times \frac{\mu l}{10\ \mu g} = 4.8\ \mu l$ should be added to achieve the desired final concentration in a total volume of 1.5 ml (1.4952 ml of inoculum + 4.8 μl drug stock).

2. Example: To achieve a starting concentration of 32 μg/ml, using a drug stock concentration of 10 mg/ml and a starting volume of 3 ml in the first test tube:

   $\frac{x\ \mu g}{3\ \text{ml}} = \frac{32\ \mu g}{\text{ml}}$; $= 3 \times 32$; then $x = 96$ μg need to be added.

   If working with a drug stock concentration of 10 mg/ml, then $96\ \mu g \times \frac{\mu l}{10\ \mu g} = 9.6\ \mu l$ should be added to achieve the desired final concentration in a total volume of 3 ml (2.9904 ml of inoculum + 9.6 μl drug stock).

3. The method was performed on an Agilent 1260 Infinity system equipped with a quaternary pump, automatic liquid sampler, thermostatted column compartment, and diode array detector. The system was controlled and data processed using OpenLab CDS ChemStation (Agilent Technologies, Santa Clara, CA, USA).

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