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High-throughput assessment of bacterial growth inhibition by optical density measurements

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Abstract

The increasing incidence of antibiotic-resistant bacterial infections both in hospitals and in the community intensifies the need for new antibacterial strategies and targets. Although high-throughput screening against live bacteria allows rapid discovery of compounds with growth-inhibitory activities, these efforts have failed to fill the pipeline with the anticipated antibacterial compounds because target identification is often onerous. Recently, a strategy was reported that employs a bacteria growth inhibition assay readout using optical density measurements on paired strains – both a wildtype strain and a pathway-null mutant – to find inhibitors of wildtype bacterial growth that specifically target conditionally essential enzymes in the pathway of interest. Protocols are provided here for determining the robustness of an assay, screening in a high-throughput format and setting up dose-response curves in paired *Staphylococcus aureus* strains. However, the protocols can be used to screen for growth-inhibitory compounds in any bacterial strain of interest.

Keywords

high-throughput screening; *Staphylococcus aureus*; optical density; cell-based assay; pathway specific screen; bacterial growth

Introduction

The increasing incidence of antibiotic-resistant bacterial infections in both hospitals and the community has intensified the need for new antibacterial strategies and targets (Walsh, 2003; Grundmann et al., 2006). A relatively new idea for targeting bacteria is to inhibit virulence factors (Marra, 2004; Clatworthy et al., 2007). Broadly defined, virulence factors are required for a pathogenic bacterium to establish an infection in a host but are not required for its survival *in vitro*. Although inhibiting virulence factors is a promising antibacterial strategy, discovery of inhibitors that block their synthesis has been difficult. Several challenges include reconstituting the enzymatic machinery, obtaining lipid-linked substrates, and developing appropriate high-throughput assays (Inglese et al., 2007). The protocols in this article describe a newly reported screening strategy that circumvents these challenges and allows for the discovery of small molecules that specifically target downstream enzymes within certain virulence factor biosynthetic pathways in a cellular context.

Many pathogenic bacteria synthesize surface-displayed, polysaccharide-based polymers on bactoprenol carrier lipids that function as virulence factors, including exopolysaccharides,

O-antigens, capsular polysaccharides and wall teichoic acids. Interestingly, many of these virulence factor biosynthetic pathways share a common feature that lends itself to a general screening strategy: they contain conditionally essential genes. That is to say, once flux into the biosynthetic pathway has started, blocking it is detrimental to the organism. The fact that the polymers are non-essential to the bacteria means that the initiating genes are expendable. The downstream genes, however, are essential *unless* polymer initiation is prevented (see Figure 1). Hence, stably maintained deletions of conditionally essential genes are typically accompanied by suppressor mutations that prevent committed flux into these biosynthetic pathways (Yuasa et al., 1969; Katzen et al., 1998; Burrows & Lam, 1999; D'Elia et al., 2006; Xayarath & Yother, 2007).

The mixed gene dispensability pattern in these bactoprenol-dependent biosynthetic pathways can be exploited in a high-throughput screen to discover inhibitors of the downstream, conditionally essential enzymes. Chemicals are tested for growth-inhibitory activities against paired strains – the wildtype strain and a mutant in which flux through the pathway has been prevented (and the downstream enzymes are no longer essential). Those molecules that stop wildtype growth but do not inhibit the growth of the pathway-null mutant should be selective for the conditionally essential enzymes in the pathway of interest (see Table 1). Screening against paired strains combines advantages of both *in vitro* and *in vivo* screening by limiting the targets to the conditionally essential enzymes within the desired pathway and by identifying active compounds in a cellular context. The utility of this screening strategy has been demonstrated by the discovery of an inhibitor of wall teichoic acid biosynthesis in the clinically relevant pathogen *S. aureus* (see Background Information) (Swoboda et al., 2009; Swoboda et al., 2010).

The protocols outlined here allow one to design, optimize and carry out a high-throughput screen against paired bacterial strains to find growth-inhibitory compounds that target certain enzymes within the virulence factor pathway of interest. A simple assay using an optical density readout to measure the extent of bacterial growth is used. Calculation of Z' values for the assay (Basic Protocol 1) allow a screener to determine whether their incubation conditions give adequate and reproducible growth. The more robust the assay, the easier it becomes to identify screening positives with confidence. Basic Protocol 2, "Screening for growth-inhibitory compounds", describes the steps involved in setting up a high-throughput screen. It is assumed that the screener has access to a chemical library and a pin transfer robot. Once the data have been analyzed and screening positives have been identified, Basic Protocol 3 outlines the setup of 6-point dose-response curves for secondary screening. Using this protocol, it is possible to determine the minimum inhibitory concentrations (MICs) of 48 compounds in a single 384-well plate.

Strategic Planning

Acquiring bacterial strains

Mixed gene dispensability is common in biosynthetic pathways that have two things in common: (1) they make cell surface polymers and (2) they assemble them on a bactoprenol carrier. Many of these cell surface polymers function as virulence factors, and they all share key similarities in their biosynthetic pathways: you can knock out the first two genes but you cannot knock out downstream genes unless suppressor mutations that inactivate one of the first two enzymes are present (Figure 1). Theoretically, the screening strategy described here should be applicable to all of these biosynthetic pathways.

In order to screen paired bacterial strains for inhibitors of downstream enzymes within virulence factor biosynthetic pathways, it is first necessary to confirm that these enzymes are conditionally essential. Researchers can place the first gene in the pathway under an

inducible promoter and then, in the absence of inducer, make knock-outs of the downstream genes. One should then acquire growth curves of the resulting strains in the absence and presence of inducer. Knockout strains of conditionally essential enzymes will be able to grow in the absence of inducer, but not in its presence (D'Elia, et al., 2006; Swoboda, et al., 2009). Screening is then performed against a wildtype strain and a mutant in which the first enzyme in the targeted biosynthetic pathway is inactive.

NOTE: If one wishes to screen small molecules for growth-inhibitory activities against only a single bacterial strain, the protocols outlined below can easily be adapted for this purpose.

Screening in duplicate

The majority of high-throughput screens show a high degree of intrinsic variability and error. It is, therefore, recommended that these assays be run in duplicate in a second set of assay plates. This assay duplication is built into the high-throughput screening protocol described here. Acquiring duplicate data points for each library compound can reduce the rates of false positives by as much as 50%.

Biosafety considerations

If possible, it is best to screen against bacterial strains that have a biosafety level one (BSL1) rating. These strains can be handled on the bench-top and no extraneous safety measures need to be taken. However, if screening against a BSL2-rated pathogen, one must handle this strain in a biosafety cabinet at all times unless it is in a sealed container. To carry out a screen using a bacterial strain that requires bio-containment, initial media dispensing and pin transfer of compounds can happen on the bench-top, but once the plates have been inoculated with the bacteria, they must be transported and incubated in leak-proof containers (e.g., Nalgene® 7135). Because these containers are airtight, only ~10 assay plates can be incubated in each one. Otherwise, because of limited oxygen, the negative controls would not grow sufficiently for optimal assay conditions. To obtain assay data when growing a BSL2 strain, a plate reader would need to be used inside a biosafety cabinet and decontaminated before removal. Assay plates then need to be disposed of properly. These extra precautions can greatly reduce the throughput of an assay.

Selective antibiotics

Contamination can be introduced into an assay when liquid dispensing and pin transfer steps occur on the bench-top. If the contaminating bacteria are sensitive to the positive control compound, it is almost impossible to detect their presence through standard optical density measurements. Hence, it is suggested that selective antibiotics be used throughout the screen. They should be present in the media at all times (during both pin transfer and bacterial inoculation). Plasmid-borne selective markers have been successfully used to avoid contamination during screening (see Background Information) (Swoboda, et al., 2009).

Basic Protocol 1: Z' determination to assess screening assay robustness

INTRO

For high-throughput screening, it is necessary to ensure that the readout window be sufficiently large and reproducible to enable the identification of screening positives with confidence. A standard parameter used to assess the quality and robustness of an assay is the Z' value (Zhang et al., 1999).

$$Z' = 1 - \left[\frac{(3\sigma_{neg} + 3\sigma_{pos})}{|\mu_{neg} - \mu_{pos}|} \right]$$

Equation 1

This parameter requires a mean (μ) and standard deviation (σ) for both the positive and negative controls. Increasing the sample size improves the statistical significance, so half of a 384-well plate should be used for each control condition. An assay should have a Z' of at least 0.5 to be considered robust enough for high-throughput screening, while an assay with a Z' greater than 0.7 is considered excellent.

MATERIALS

Plated bacterial strain(s) of interest (e.g., *S. aureus*)

Sterile tryptic soy broth (TSB; 30 g/L; sterile filtered or autoclaved; BD® ref#211823)

Positive control compound that inhibits growth of bacterial strain (e.g., for *S. aureus*, erythromycin at 10 mg/mL in ethanol)

384-well clear-bottom plate (Corning® 3702)

Low evaporate lid (Corning® 3009)

Sterile solvent reservoirs (VWR® 89094-680)

Multi-channel pipet (volume range from 20–100 μ L)

Aerosol barrier tips that fit the multi-channel pipet

Incubator (30 °C)

Plate reader capable of measuring optical density at 600 nm (OD_{600}) in a 384-well format

STEPS

1. Inoculate 2 mL of TSB media with a single colony of bacteria and grow to saturation by shaking at 30 °C overnight.

For paired strain screening, it is necessary to establish a Z' for each strain (wildtype and mutant). Make sure to include selective antibiotics if necessary.
2. Dilute the bacteria to *ca.* 1×10^6 CFU/mL in 40 mL of TSB media.

For *S. aureus*, this is typically a 1/1000 dilution from a saturated culture; however, the proper dilution for the bacteria of interest can be calculated by following the Support Protocol.
3. Split the 40 mL culture in half and add the appropriate concentration of positive control compound to one of the 20 mL cultures. Retain the other 20 mL for the negative control wells.

For *S. aureus*, the positive control erythromycin at 10 μ g/mL final concentration can be used (1/1000 dilution of stock).
4. Dispense 80 μ L of positive control culture into all of the wells in the top half of a 384-well plate using a multi-channel pipet (see Figure 2).

Use one 384-well plate per strain. Be careful to avoid dispensing air bubbles at this point. They will not pop overnight, and will affect the optical density and, ultimately, the Z' value of the assay. Alternatively, use the liquid handling system (plate filler) that will be employed during screening, as this will equalize volumes and minimize the likelihood of bubbles forming in the bottoms of the wells. If a plate

filler is used, scale up the media to account for the dead volume in the tubing (~10 mL).

5. Dispense 80 μ L of negative control culture into all of the wells in the bottom half of the 384-well plate.
6. Cover the plate with a low evaporation lid and incubate overnight (typically 16–24 h) at 30 °C.

The incubation time needs to be determined experimentally. Take OD₆₀₀ readings of the plate each hour starting at 16 h (continue incubating at 30 °C between each reading) and calculate the Z' for each time point. Choose an incubation length that gives a maximal Z' value (minimal growth of the positive controls and maximal growth of the negative controls).

7. Using a plate reader, measure the optical density of each well at 600 nm and calculate the Z' value using Equation 1.

It is recommended that a screener repeat this protocol on three separate occasions to confirm that the growth conditions and controls yield a robust and reproducible assay.

Basic Protocol 2: Screening for growth-inhibitory compounds

INTRO

To find growth-inhibitory compounds of a wildtype strain, one should screen compound library plates in duplicate (see Strategic Planning). To discover inhibitors of conditionally essential enzymes in a non-essential biosynthetic pathway, one should also screen the compound library plates in duplicate against a mutant strain that lacks the first enzyme in the pathway of interest. Those compounds that inhibit growth of the wildtype strain but not the mutant should target the conditionally essential enzymes within the desired pathway (see Table 1). This protocol is designed for one screening session of 40 compound library plates in duplicate against a single bacterial strain of interest, or for 20 compound library plates in duplicate against both a wildtype and a mutant strain (four daughter plates per library plate). For this protocol, it is assumed that columns 23 and 24 of each compound library plate are empty and thus those columns can be used for negative (column 23) and positive (column 24) controls for the assay (Figure 3). The location of control wells can be changed as appropriate for screens using compound library plates that are formatted differently.

MATERIALS

Plated bacterial strain(s) of interest (e.g., *S. aureus*)

2.5 L sterile TSB (+ selective antibiotic, if appropriate)

20 mL media containing positive control compound (at 2 $\times$ final concentration; typically this is erythromycin at 20 μ g/mL)

Compound library plates, with compounds diluted in DMSO at 5 mg/mL or 10 mM stock concentration

384-well clear-bottom plates (80 per screening session; Corning® 3702)

Low evaporation lids (16–20; Corning® 3009)

Permanent marker

Sterile solvent reservoir (VWR® 89094-680)

Multi-channel pipet and tips (volume range from 20–100 μ L)

Microplate dispenser (Matrix *WellMate*® or comparable liquid handler)

Incubator (30 °C)

Plate reader capable of measuring optical density at 600 nm (OD₆₀₀) in a 384-well format (e.g., *Envision*® from Perkin Elmer)

Pin transfer robot

STEPS

1. Inoculate 2 mL of TSB media with a single colony of the bacteria and grow to saturation by shaking at 30 °C overnight.
Make sure to include selective antibiotics if necessary.
2. Dilute both the wildtype and mutant bacteria to *ca.* 2×10^6 CFU/mL each in 600 mL of TSB media.
For *S. aureus*, this is typically a 1/500 dilution from a saturated culture; however, the proper dilution for the bacterial strain of interest can be calculated by following the Support Protocol.

Screening the Library

3. Label the sides of the 384-well plates with numbers that correspond to compound library plates and with letters that designate replicate plates (e.g., wildtype: A and B; mutant: C and D).
4. Using a plate filler, dispense 40 μ L of TSB into columns 1–23 of each 384-well plate.
Wells in column 23 will serve as negative controls (media + bacteria only) for each plate.
5. Using a multi-channel pipet and a solvent reservoir, manually add 40 μ L of media containing 2 $\times$ positive control compound to the wells of column 24 (see Figure 3).
6. Stack the plates in order according to their labels and load them into the pin transfer robot.
7. Pin transfer 300 nL per well from each compound library plate into four daughter plates (A–D if screening against paired strains; if only one strain is being screened, pin transfer the compound plates into duplicate plates).
Assuming the library compounds are stored as 10 mM stocks, a 300 nL pin transfer into a final volume of 80 μ L will give a screening concentration of 38 μ M.
8. Using a plate filler, add 40 μ L of 2×10^6 CFU/mL wildtype bacteria to all wells in plates A and B, and 40 μ L of 2×10^6 CFU/mL mutant bacteria to the wells of plates C and D.
If paired strains are not being screened, simply make 1.2 L of 2×10^6 CFU/mL culture and inoculate all plates with the single bacterial strain.
9. Stack the plates no more than five high and cover the top plate with a low evaporation lid.

10. Place the plates in a 30 °C incubator overnight (16–24 h).
Optimize this time parameter during Z' determination (Basic Protocol 1).
11. Using a plate reader, measure the optical density of each well at 600 nm.
The use of plate stackers during this stage of the protocol will greatly increase the throughput of the assay and will minimize human error.

Analyzing the Data

12. Calculate the Z' value for each plate using Equation 1.
13. Normalize the wells of each plate for percent survival by scaling them to their respective negative and positive controls using Equation 2.

It is important to normalize the optical densities of the sample wells to the controls on that plate, especially if the pathway-null mutant has different growth characteristics than the wildtype. See Critical Parameters for more details.

$$\text{Percent survival} = \left(\text{OD}_{\text{sample}} - \text{OD}_{\text{pos}} \right) / \left(\text{OD}_{\text{neg}} - \text{OD}_{\text{pos}} \right) \times 100$$

Equation 2

14. Graph replicate data with sample wells from plate A as the x-coordinates and sample wells from plate B as the y-coordinates.
Those data points that do not fall near a trendline with a slope equal to one are considered outliers (i.e., they did not show equal growth-inhibitory activity in duplicate), and caution should be taken in following up on these compounds.
15. Define criteria for a screening “positive” (a typical HTS “hit rate” should be less than 0.5%) and request compounds for secondary screening.
For paired screening, “hits” could be defined as any compound that gives <10% survival of the wildtype while giving >50% survival of the pathway-null mutant.

Basic Protocol 3: Dose-response assays for secondary screening of potential bacterial growth inhibitors

INTRO

Once screening “positives” have been identified, it is necessary to confirm their activity through secondary screening. Not all active compounds from the primary screen will “re-hit” at this stage. This procedure allows one to determine the minimum inhibitory concentrations (MICs) of 48 compounds between 3 and 100 μM in a single 384-well plate and rank them in terms of activity for future follow up. If paired strains are being screened, it is advised that the MICs of all cherry picked compounds be determined against the wildtype strain first. The compounds that reproducibly inhibit growth of the wildtype strain can then be screened against the pathway-null mutant using the same procedure to test for target specificity.

MATERIALS

Plated bacterial strain(s) of interest (e.g., *S. aureus*)

20 mL sterile TSB (+ selective antibiotic, if appropriate)
15 mL sterile TSB containing 2% (v/v) DMSO (+ selective antibiotic, if appropriate)
5 mL media containing positive control compound at 2× final concentration
384-well clear-bottom plates (one per 48 compounds; Corning® 3702)
Low evaporation lids (one per plate; Corning® 3009)
Permanent marker
Sterile solvent reservoir (VWR® 89094-680)
Single channel pipet (volume range of 0.5–2.0 µL)
Multi-channel pipet (volume range of 15–30 µL)
Aerosol barrier tips that fit the multi-channel pipet
Incubator (30 °C)
Plate reader capable of measuring optical density at 600 nm (OD₆₀₀) in a 384-well format

STEPS: 1

1. Inoculate 2 mL of TSB media with a single colony of the bacteria and grow to saturation by shaking at 30 °C overnight.
Make sure to include selective antibiotics if necessary.
2. Dilute the bacteria to *ca.* 2×10^6 CFU/mL in 15 mL of TSB media and set aside.
For *S. aureus*, this is typically a 1/500 dilution from a saturated culture; however, the proper dilution for the bacterial strain of interest can be calculated by following the Support Protocol.
3. Add 30 µL of TSB to the wells in rows A and I of a labeled 384-well plate. Using a single channel pipet, mix a different test compound into each well.
Typically 0.6 µL of a 10 mM solution of the compound of interest is mixed into 30 µL of media to give a 200 µM stock in each well of rows A and I.
4. Using a multi-channel pipet, aliquot 15 µL of TSB containing 2% DMSO (v/v) to the wells of rows B–G and J–O.
5. Make a series of six two-fold dilutions down the plate using the multi-channel pipet by sequentially mixing 15 µL of each compound solution into the well directly beneath it (see Figure 4).
For example, 15 µL of the 200 µM stock in well A1 will be mixed with the 15 µL of TSB in well B1 to give 30 µL of a 100 µM solution, 15 µL of which is then mixed with the 15 µL of TSB in well C1 to give 30 µL of a 50 µM solution, and so forth. Tips need not be changed as dilutions are being made down the plate. The extra 15 µL in well F1 will need to be discarded (along with the tips). This dilution procedure is repeated to dilute all of the wells of row A and row I.
6. Using a multi-channel pipet, add 15 µL media containing 2× the final concentration of the positive control compound to the wells of rows H and P.

Typically 20 µg/mL erythromycin is used for this step when screening against *S. aureus*.

7. Using a multi-channel pipet or a plate filler, add 15 µL of the bacterial culture made in step 2 to all of the wells in the 384-well plate. Final compound concentrations are shown in Figure 4.
8. Cover the plate with a low evaporation lid and incubate it at 30 °C for 16–24 h.
9. Read the optical densities of each well, normalize them to the positive and negative controls using Equation 2, and determine the overall Z' value using Equation 1.
10. Plot MIC curves for the active compounds as percent survival vs. compound concentration.

Open source software is available for batch processing.

<http://ncgc.nih.gov/pub/openhts/curvefit/>

Support Protocol: Determining correct bacterial concentration for a screening assay

INTRO

Antibacterial assays are typically run at bacterial concentrations of 10^5 or 10^6 colony forming units (CFU) per milliliter. To achieve the proper dilution, it is necessary to generate a curve that relates bacterial density (OD_{600}) to bacteria concentration (CFU/mL). To do this, a bacterial culture is grown until it reaches $OD_{600} \sim 1$. It is then diluted to four or five different concentrations (to be accurate, they must absorb UV light above baseline), and the bacteria are enumerated on non-selective agar plates. Once calculated, CFU/mL is graphed versus OD_{600} (see Figure 5). Once this curve is generated, it is possible to determine the dilution of an overnight culture that will provide the correct bacterial concentration for a screening assay.

MATERIALS

Plated bacterial strain(s) of interest (e.g., *S. aureus*)

TSB or equivalent media

Non-selective agar plates (~20)

Permanent marker

Spreaders

2 mL eppendorf tubes

Vortex

UV-VIS spectrophotometer

Cuvettes

Graphing software

STEPS

1. Inoculate 2 mL of media with a single colony of bacteria and shake at 30 °C overnight.

Make sure to include selective antibiotics if necessary.

2. Dilute the overnight culture of bacteria 1/200 into fresh TSB and shake at 30 °C for about 3 h (until OD₆₀₀ ~1.0)

Do not overgrow the bacteria. The turbidity of the culture should be within the linear range of the UV-VIS instrument.

3. Dilute the freshly grown culture 4:1, 3:2, 2:3, 1:4, 1:9 (Culture:TSB) into fresh TSB and measure the OD₆₀₀ of each sample (including the original sample where OD~1).
4. Dilute each of the six samples from step 3 to 10⁻² by vortexing 10 µL of sample with 990 µL of TSB in a 2 mL tube; dilute the samples to 10⁻⁴ by vortexing 10 µL of the 10⁻² dilutions into 990 µL of TSB; plate 100 µL of the 10⁻⁴ dilution of each sample onto a labeled non-selective agar plate.
5. Dilute the solutions from step 4 further by vortexing 100 µL of the 10⁻⁴ dilutions into 900 µL TSB (to give 10⁻⁵); repeat this procedure using 100 µL of the 10⁻⁵ dilutions into 900 µL TSB (to give 10⁻⁶); plate 100 µL of the resulting 10⁻⁵ and 10⁻⁶ dilutions onto non-selective agar plates.
6. Incubate the plates overnight at 30 °C and count the resulting colonies.

Assuming six samples are plated at three different dilutions (10⁻⁴, 10⁻⁵, and 10⁻⁶), there should be 18 plates total.
7. Calculate the CFU/mL for each of the original six samples using Equation 3.

$$\text{CFU/ML} = \# \text{ colonies on plate} / \text{dilution} / \text{volume plated}$$

Equation 3

For example, 44 colonies from 100 µL of a 10⁻⁴ dilution would correspond to 4.4 × 10⁶ CFU/mL. Colony counts are significant if there are between 30 and 300 colonies on a plate.

8. Graph data with CFU/mL on the y-axis and OD₆₀₀ on the x-axis, setting the intercept equal to zero; add a linear trendline and record the resulting equation (see Figure 5).

Determining the Concentration of a Bacterial Culture

9. Dilute an overnight culture 1/10 into fresh TSB (to bring it into the linear range of the UV-VIS), and measure its OD₆₀₀. Multiply this value by the slope of the trendline to calculate CFU/mL present in the 1/10 dilution. Multiply this value by 10 to obtain the cellular density of the overnight culture. Dilute the overnight culture appropriately to obtain 1×10⁶ CFU/mL as the starting inoculum in each assay well for the bacterial growth screening assay.

For *S. aureus* RN4220, diluting saturated overnight cultures 1/1000 typically gives a starting inoculum in the right range.

COMMENTARY

Background Information

The protocols described in this article have been successfully employed to identify inhibitors of the wall teichoic acid (WTA) biosynthetic pathway in *Staphylococcus aureus* (Swoboda, et al., 2009). WTAs are phosphate-rich, carbohydrate-based polymers displayed on the surfaces of Gram-positive bacteria that affect both their physiology and pathogenicity. These anionic polymers are considered virulence factors because they are not essential for growth *in vitro* but they are required for the establishment of a robust infection. Strains lacking the

first enzyme in the pathway have been made and studied. It has been found that WTA-null *S. aureus* mutants are unable to cause infections in certain animal models (Weidenmaier et al., 2004; Weidenmaier et al., 2005; Weidenmaier & Peschel, 2008). WTAs are synthesized intracellularly on a bactoprenol carrier lipid and are then transported to the outside of the cell where they are covalently attached to the cell wall.

Similar to other virulence factor biosynthetic pathways, WTAs contain conditionally essential enzymes. The mixed gene dispensability of this pathway has been described previously (D'Elia, et al., 2006). Researchers were unable to knockout the downstream genes in a wildtype background; however, when the first gene of the pathway, *tarO*, was inactive the downstream genes were easily removed. These genetic experiments inferred that once flux through the WTA biosynthetic pathway had started, blocking it was detrimental to the organism. A screening strategy was devised to find growth-inhibitory compounds against wildtype *S. aureus* that would specifically target the conditionally essential enzymes in the WTA biosynthetic pathway. This was done by running a counter selection in tandem with the wildtype growth-inhibitory screen. A WTA-null ($\Delta tarO$) mutant was screened alongside the wildtype strain to distinguish between compounds that blocked processes unrelated to WTA biosynthesis and those that inhibited conditionally essential enzymes within the WTA biosynthetic pathway (see Table 1). Because the essentiality of the downstream enzymes is relieved in the $\Delta tarO$ mutant, compounds that selectively hit one of these targets will not affect the growth of the mutant strain.

Z' values were determined for both the wildtype and mutant strains using erythromycin at 10 $\mu\text{g}/\text{mL}$ as a positive control and incubating the bacteria at 30 °C for 18 hours ($Z' = 0.85$). The screen was then performed according to Basic Protocol 2 with a wildtype strain of *S. aureus* RN4220 that contained a plasmid encoding constitutively expressed green fluorescent protein (GFP) and a $\Delta tarO$ mutant that carried a plasmid encoding constitutively expressed mCherry fluorescent protein. Throughout the screen, both plasmids were maintained with 10 $\mu\text{g}/\text{mL}$ chloramphenicol, and library compounds were screened at 38 μM . Following overnight incubation, plates were read for both optical density and fluorescence at the appropriate wavelengths. Although each strain was marked with a unique fluorescent marker to provide an alternative readout of bacterial growth, optical density proved to be a more robust measure of growth and was used for hit determination. 55,000 compounds were screened in duplicate against the paired strains. A screening positive was defined as a compound that resulted in less than 10% survival of the wildtype with greater than 50% survival of the $\Delta tarO$ mutant. Forty compounds were cherry picked for dose-response determination (see Basic Protocol 3) and three of these compounds “re-hit” as being both active and selective (Swoboda, et al., 2009).

Critical Parameters

Z' values – assessing the success of an assay—Obtaining a Z' value for each plate during a high-throughput screen can help the screener identify plates that encountered sub-optimal conditions. The data obtained from plates that do not have an acceptable Z' (e.g., it is less than 0.5) should be scrutinized further. It may be that some error occurred during screening and the data need to be discarded. However, oftentimes one or two bad control wells can be enough to greatly reduce the Z'. If these improper controls happen at random, no action need be taken. Ideally, greater confidence can be placed in data obtained from plates with higher Z' values.

Normalizing wells to the plate median—If sufficient positive and negative controls cannot be accommodated on the assay plates because of the format of the library compounds to be screened, the screener would be unable to normalize the data according to Equation 2.

In this case it is possible to normalize the samples to themselves. It is assumed that most of the compounds are inactive and essentially act as vehicle controls. Because of this, the sample wells can be normalized to the median of the plate (the middle value when the samples are ranked in order). The median is used in place of the average because high-throughput screening attempts to identify the outliers on a plate. Normalizing to the plate average would thus diminish the apparent activity of the outlying data points or screening “positives” (Brideau et al., 2003).

Z scores – determining the validity of a “hit”—Up to this point, screening actives have been described as those wells that have the greatest relative activity. However, in some cases it may be useful to also rank “hits” using a statistical scoring method. Use of Z scores can help a screener detect data points that look different from the bulk of the samples (Malo et al., 2006). Identifying a “hit” with both potent activity and a high Z score can increase confidence that it is genuine. Z scores are calculated according to Equation 4 where μ_{plate} is the average of the sample wells (no controls) and σ_{plate} is the standard deviation of these values (Brideau, et al., 2003). This statistical measure calculates the number of standard deviations away from the mean for each well, and can be used to determine the probability that a screening positive is not due to background noise.

$$Z\ score = (OD_{sample} - \mu_{plate}) / \sigma_{plate} \quad \text{Equation 4}$$

More rigorous statistical methods that can salvage data from an assay with positional effects and other systematic variations have been described (Brideau, et al., 2003). However, it is not anticipated that these B scores would need to be calculated for a simple bacterial growth assay.

Troubleshooting

Optimal growth conditions—Basic Protocol 1 is used to determine whether the assay conditions are suitable for high-throughput screening. With bacterial growth assays, the parameters that can be altered are incubation time and temperature. Too short of an incubation time could affect the assay window by not allowing the negative control to fully grow. On the other hand, too long of an incubation time could lead to evaporation or to growth of resistant mutants. Incubation time should be experimentally optimized to maximize the assay window. That being said, the dynamic range of optical density measurements is relatively small (i.e., *S. aureus* cultures that give optical densities above background while not saturating the detector range from 10^6 – 10^7 CFU/mL). If a bacterial strain does not grow to a sufficient density (e.g., greater than $OD_{600}=0.6$), the assay window will likely be too narrow and the Z' value will be negatively affected. One way to avoid this problem is to utilize a more sensitive readout. The advantages and disadvantages of two alternative readouts are briefly described below.

Fluorescent proteins—Fluorescence can be used to quantify the number of bacteria present in a microtiter well because the amount of fluorescent protein expressed in properly engineered bacteria should be proportional to the concentration of bacteria. Plasmids containing constitutively expressed fluorescent protein genes can easily be introduced into the bacterial strain of interest (see above in Background Information). Fluorescent proteins form internal chromophores without the need of additional cofactors, enzymes or substrates other than molecular oxygen, making them amenable to high-throughput screening. However, because chemical libraries are most often composed of flat aromatic molecules, many of the compounds autofluoresce, causing interference with fluorescence output. This

interference can increase the number of false negatives encountered in a high-throughput screen.

Luminescence—Luminescence is produced by a chemical reaction in which light is generated by an enzyme, luciferase, acting on its substrate (luciferin). Plasmid-borne genes encoding both the enzyme and the proteins required for substrate synthesis can be introduced into bacterial strains. However, if an appropriate plasmid is not available, the reagent BacTiter Glo™ (Promega G8230) can be used. This reagent measures the levels of ATP in each well, which should be proportional to the number of bacteria present. The reagent contains the purified enzyme and necessary substrates to produce the reaction. Its use would add a dispensing step to the assay protocol (following overnight incubation) and would also significantly increase costs. Although luminescence gives a weaker signal than fluorescent proteins, its background is negligible, making it as much as 1000 more sensitive than fluorescent readouts (Inglese, et al., 2007). Also, because no excitation energy is needed to generate this signal, compound interference is greatly reduced. However, if present in the chemical library, luciferase inhibitors and luciferin analogs can interfere with the reaction.

Anticipated Results

Basic Protocol 1 allows the screener to determine whether the incubation conditions are suitable to give adequate growth and minimal variability. Following optimization, this protocol should be repeated on three independent occasions to confirm reproducibility. The assay conditions can then be applied to a high-throughput screen as outlined in Basic Protocol 2. Depending in part on the chemical diversity of the screened library and the bacterial strains used, a “hit” rate of approximately 0.5% should be obtained. **NOTE:** The outer membranes of Gram-negative bacteria act as a barrier to decrease the permeability of small molecules into the cells. Because of this added defense, the “hit” rates for Gram-negative screens tend to be much lower than for Gram-positive screens. Basic Protocol 3 describes a method to determine the minimal inhibitory concentrations of screening “positives” identified in Basic Protocol 2. Those compounds that retest as both active and selective (they inhibit the growth of the wildtype strain but not the pathway-null mutant) can be advanced for further evaluation.

Time Considerations

Z' determination can take as little as 24 hours, but should be repeated three times on at least two separate days. If compound library plates are tested against a single strain, 40 compound library plates (14,000 small molecules) can be screened in duplicate in one day. If paired strains are screened in duplicate (four assay plates/library plate), 20 compound library plates can be screened in a 24-hour period (7,000 small molecules). Data analysis can take several days. Once cherry picked compounds are received, dose-response setup for one 384-well plate (48 compounds) takes two to three hours. MICs can then be determined within 24 hours.

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Figure 1.

Schematic showing the mixed dispensability present in the biosynthetic pathways of many cell wall surface polymers that are assembled on a bactoprenol carrier lipid and act as virulence factors. The first two non-essential genes are typically glycosyltransferases (Gtfs), which are depicted here as light grey arrows; downstream genes shown as dark grey arrows are conditionally essential, that is, they cannot be deleted except in strains that lack one of the initiating Gtfs.

Figure 2.

Schematic showing a 384-well plate layout for Z' determination. In a growth-inhibitory assay, the positive control wells will most likely contain bacteria in media containing erythromycin (10 $\mu\text{g}/\text{mL}$) or another appropriate antibiotic, while the negative control wells will be filled with media and bacteria only.

Figure 3.

Schematic showing a 384-well plate layout for high-throughput screening. This format assumes that the library compounds are arrayed in columns 1–22, allowing for a column each of negative and positive controls. Columns 1–23 are filled with 40 μL of media by the plate filler while positive control media (40 μL media + erythromycin for a 10 $\mu\text{g}/\text{mL}$ final screening concentration) is added manually to column 24 using a multi-channel pipet. Following the pin transfer of small molecules from compound library plates, all wells are filled with 40 μL of media containing the bacteria of interest.

Figure 4.

Schematic showing a 384-well plate layout for dose-response screening of cherry-picked compounds. This procedure allows one to determine the MICs of 48 compounds between 3 μ M and 100 μ M in a single 384-well plate. Final screening concentrations for the compounds are shown in the schematic to the right of rows I through M. NC and PC are negative control and positive control rows, respectively.

Figure 5. Graph depicting bacterial concentration (CFU/mL) vs. optical density (OD₆₀₀). Once determined, the slope of the trendline can be used to determine the cellular density of a bacterial culture through a simple optical density measurement.

Table 1

Concept behind paired strain screening. Small molecules that inhibit the growth of the wildtype but not the pathway-null mutant should target the conditionally essential enzymes in the non-essential biosynthetic pathway of interest.

Compound	Bacterial strain	
	Wildtype	Mutant ^a
does not block any cellular process	Growth	Growth
blocks an essential cellular process	No Growth	No Growth
blocks a conditionally essential enzyme ^b	No Growth	No Growth

^aThe first enzyme in a non-essential pathway of interest is inactive

^bGrowth will be uninhibited in the mutant strain if the compound hits a conditionally essential enzyme within the deleted pathway