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Use of the Soft-Agar Overlay Technique to Screen for Bacterially-Produced Inhibitory Compounds

--Manuscript Draft--

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Abstract:	The soft-agar overlay technique was originally developed over 70 years ago and has been widely used in several areas of microbiological research, including work with bacteriophages and bacteriocins, proteinaceous antibacterial compounds. This approach is relatively inexpensive, with minimal resource requirements. This technique consists of spotting supernatant from a donor strain (potentially harboring a toxic compound(s)) onto a solidified soft agar overlay that is seeded with a bacterial test strain (potentially sensitive to the toxic compound(s)). We utilized this technique to screen a library of Pseudomonas syringae strains for intraspecific killing. By combining this approach with a precipitation step and targeted gene deletions, multiple toxic compounds produced by the same strain can be differentiated. The two commonly recovered antagonistic agents recovered using this technique are bacteriophages and bacteriocins. These two agents can be differentiated using two simple additional tests. Performing a serial dilution on a supernatant containing bacteriophage will result in individual plaques becoming less in number with greater dilution, whereas serial dilution of a supernatant containing bacteriocin will result a clearing zone that becomes uniformly more turbid with greater dilution. Additionally, a bacteriophage will produce a clearing zone when spotted onto a fresh soft agar overlay seeded with the same strain, whereas a bacteriocin will not produce a clearing zone when transferred to a fresh soft agar lawn, owing to the dilution of the bacteriocin.
Author Comments:	Dear Dr. Zaman, Thank you, Dr. Mehta, and the other editorial members and outside reviewers for you efforts in evaluating this manuscript. I believe that we have addressed all comments regarding the manuscript, with all modification to the manuscript being indicated with 'track changes'. I have uploaded a word document with a point by point response.

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May 30, 2016

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Editors of JoVE,

We wish to have the manuscript titled “Use of the Soft-Agar Overlay Technique to Screen for Bacterially-Produced Inhibitory Compounds” considered for publication in *JoVE*. The work presented in this manuscript describes a simple, cheap, and reliable technique for screening bacterial cultures for the production of antibacterial agents, predominantly induced temperate bacteriophage and bacteriocins (proteinaceous antibacterial compounds). Although this technique (or variations of it), have been widely utilized by many research laboratories, the unique format of *JoVE* will help facilitate adoption of this method by scientists who have not trained in a bacteriophage- or bacteriocin-oriented laboratory, but are interested in pursuing such research. As we have elected to make this article open-access (should it be accepted for publication), this article will greatly benefit scientists in regions or institutions with limited access to scientific journal subscriptions.

David Baltrus and I designed the experimental approach for our system and interpreted the data, whereas I conducted the experiments and wrote the manuscript. Teena Mehta assisted with the submission process.

Both of the authors of this manuscript have read and approved of its content and confirm that all of the work is original, that all prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected, and that consent is given for publication in *JoVE*, if accepted. I will serve as corresponding author. My contact information is:

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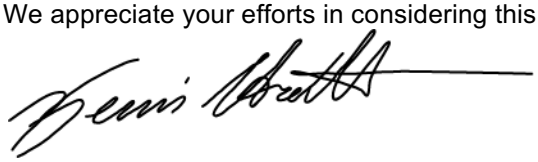
We appreciate your efforts in considering this submission.



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TITLE:
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KEYWORDS:
Bacteria; bacteriophage; bacteriocin; tailocin; antimicrobial agent; bacterial antagonism; PEG precipitation

SHORT ABSTRACT:
We describe a simple method for screening bacterial cultures for the production of compounds inhibitory towards other bacteria.

LONG ABSTRACT:
The soft-agar overlay technique was originally developed over 70 years ago and has been widely used in several areas of microbiological research, including work with bacteriophages and bacteriocins, proteinaceous antibacterial agents. This approach is relatively inexpensive, with minimal resource requirements. This technique consists of spotting supernatant from a donor strain (potentially harboring a toxic compound(s)) onto a solidified soft agar overlay that is seeded with a bacterial test strain (potentially sensitive to the toxic compound(s)). We utilized this technique to screen a library of *Pseudomonas syringae* strains for intraspecific killing. By combining this approach with a precipitation step and targeted gene deletions, multiple toxic compounds produced by the same strain can be differentiated. The two commonly recovered antagonistic agents recovered using this technique are bacteriophages and bacteriocins. These two agents can be differentiated using two simple additional tests. Performing a serial dilution on a supernatant containing bacteriophage will result in individual plaques becoming less in number with greater dilution, whereas serial dilution of a supernatant containing bacteriocin will result a clearing zone that becomes uniformly more turbid with greater

dilution. Additionally, a bacteriophage will produce a clearing zone when spotted onto a fresh soft agar overlay seeded with the same strain, whereas a bacteriocin will not produce a clearing zone when transferred to a fresh soft agar lawn, owing to the dilution of the bacteriocin.

INTRODUCTION:

Recently, there has been significant interest in deepening our understanding of microbial ecology (particularly the microbiomes of various environments), as well as new antibacterial compounds to use in combating antibiotic resistant pathogens[1,2]. An interconnecting theme between these interests is understanding antagonistic interactions among bacterial strains in their natural environment. There are numerous ways in which bacteria antagonize competitors[3]. Bacteriocins, a diverse group of proteinaceous, antibacterial compounds, have long been studied for their role in mediating interbacterial antagonism, with two of the most studied species being *Pseudomonas aeruginosa*[4,5] and *Escherichia coli*[6], important human pathogens. In addition to bacteriocins, induced prophages can also act as anticompetitor agents, allowing a strain to invade into a niche that is already colonized[7]. *Pseudomonas syringae* is a plant pathogen known to produce an array of antimicrobial agents, including single protein bacteriocins[8], bacteriophage tail-derived bacteriocins[9] (termed tailocins), as well as non-proteinaceous secondary metabolites[10]. Recently there has been interest in understanding how these antimicrobials influence the ecology of this organism, as well as how they can be harnessed to control plant disease [11].

A widely utilized method for studying both bacteriocins and bacteriophage is the soft-agar overlay technique. This method was first described by Gratia in 1936 to aid in enumerating bacteriophage[12,13].

Here we describe the application of the soft-agar overlay method, in combination with targeted genetic manipulation and polyethylene glycol (PEG) precipitation, in distinguishing among three different antimicrobial agents (a bacteriophage, a high molecular weight bacteriocin, and a low molecular weight bacteriocin) produced by a single bacterial strain. The benefit of this approach is that it is relatively simple and cheap, which is why, despite it being decidedly 'low-tech', it is still widely utilized.

PROTOCOL:

1. Preparation of supernatant to be tested for activity.

1.1) Prepare a 0.5 mg/ml stock solution of mitomycin C by dissolving the appropriate amount into sterile 0.1 M $MgSO_4$ buffer (e.g. 1 mg mitomycin C/ 2 ml buffer). Store stock at 4 °C in a light protected container (mitomycin C is light sensitive).

1.2) Inoculate a single colony of *P. syringae* into 3 ml of King's medium B (KB) broth[14]. Incubate over night with shaking (200 rpm) at room temperature (21-25 °C).

1.3) The following morning, dilute the broth culture 1/100 into 3 ml of fresh KB broth.

Incubate for 3-4 h with shaking at room temperature. Add mitomycin C (0.5 µg/ml, final concentration). Incubate the culture over night with shaking at room temperature.

Note: Mitomycin C causes double stranded DNA breaks, thus stimulating the cell's SOS response, leading to the production of both bacteriophage and bacteriocins.

1.4) Pellet 1-2 ml mitomycin C induced cultures by centrifugation at 20,000 x *g* for 5 min in a bench top microcentrifuge.

1.5) Remove and sterilize culture supernatants either by passing the supernatant through a 0.22 µm pore size filter or by treating the supernatant with chloroform (100 µl chloroform per 1 ml supernatant).

1.5.1) If using chloroform, vortex the mixture for 15 s and let incubate at room temperature for 1 h. Following incubation, centrifuge the mixture at 20,000 x *g* for 5 min.

Note: Chloroform efficiently kills cells by solubilizing their membranes. The benefit of using chloroform over filter sterilization is that it is cheaper and easier to scale-up if processing many (>20) samples at a time. There may be a possibility that a given killing activity is lost following chloroform treatment as a result of partitioning into the organic phase.

1.5.2) Remove the upper, aqueous phase using a 1 ml pipet to a fresh, sterile 1.5 or 2.0 ml microfuge tube. Be careful not to transfer any of the lower chloroform layer (it is better to remove less of the aqueous phase to avoid carry-over).

1.5.3) Incubate the transferred supernatant uncapped in a fume hood to allow the residual chloroform to evaporate (several hours). Store supernatants at 4 °C.

2. Separation and concentration of high molecular weight bactericidal compounds by polyethylene glycol (PEG) precipitation.

2.1) To the sterile supernatant, add NaCl and PEG 8000 to 1 M and 10% final concentrations, respectively. Repeatedly invert the sample until both the NaCl and PEG are completely dissolved. Incubate samples in an ice bath for 1 h or overnight at 4 °C.

2.2) Centrifuge samples at 16,000 x *g* for 30 min at 4 °C. A pellet should form at the bottom of the centrifuge tube. Decant the supernatant and resuspend the pellet in desired volume (such as 1/10 or 1/100 of the original supernatant volume) of buffer (10 mM Tris, 10 mM $MgSO_4$, pH 7.0) by repeated pipetting.

2.3) Remove residual PEG by two sequential extractions with an equal volume of chloroform.

2.3.1) Combine chloroform with the resuspended pellet and vortex for 10 to 15 s, then centrifuge the mixture at 20,000 x *g* for 5 min. Transfer the upper, aqueous phase to a

fresh microfuge tube. Repeat this extraction until no white interface between the aqueous and organic phase is visible (usually 2 extractions total). Allow residual chloroform to evaporate from extracted supernatants in a fume hood.

3. Preparation of overlay and testing supernatants for activity.

3.1) Inoculate a single colony of a *P. syringae* strain to be tested for sensitivity into 3 ml of KB, incubate over night with shaking at room temperature.

3.2) The following morning, back dilute the culture 1/100 into fresh KB. Incubate 3-4 h with shaking at room temperature.

3.3) Prepare sterilized water agar by autoclaving a suspension 0.35-0.7% (w/v) agar in ultrapure water.

Note: A stock of soft water agar can be melted in a microwave and reused repeatedly, fresh overlay does not need to be prepared for each experiment. If water agar is reused, it is critical to ensure it is completely melted following microwaving. If not, the overlay will have a grainy texture upon solidifying that will make interpretation difficult. If this occurs, melt the agar for several minutes longer in the microwave than done previously.

3.4) Maintain the molten soft agar in a 60 °C water bath prior to use. Using a sterile serological pipet, transfer 3 ml of soft agar to a sterile culture tube. Return the culture tube to water bath to maintain in a molten state.

3.5) To pour the overlay, first allow the molten agar to cool (it should feel warm but not hot to touch), but do not allow to solidify.

3.6) In a sterile hood, inoculate 100 µl of the tester strain culture into the soft agar and vortex to mix. Rotate culture by hand for 10-15 s, then pour onto a bottom agar (KB agar). Tilt the plate in all directions to ensure the soft agar evenly covers the bottom agar.

Note: The bottom agar can be any solidified (1.5% agar) medium on which the test strain grows vigorously. For a standard 100 mm diameter Petri dish, use ~20 ml melted medium. The bottom agar can be prepared several weeks ahead of time (if maintained at 4 °C without drying) or can be prepared the same day. If prepared the same day as performing the overlay, it is best to do so prior to step 3.4.

3.7) Cover the plate and allow it 20-30 min to solidify. Be careful not to disturb the plate while solidifying.

3.8) Once solidified, spot 2-5 µl of supernatant (generated in sections 1 and 2, above) onto the overlay. Allow the plates to incubate over night at room temperature. Observe and record results the following morning.

Note: It may be useful to perform and spot from a serial dilution of the supernatant. This will allow researchers to distinguish between bacteriophage and bacteriocin clearing activity. In this case, it is recommended to perform 1:5 or 1:10 dilutions.

REPRESENTATIVE RESULTS:

The combination of the soft agar overlay and PEG precipitation can be used to identify and characterize different antimicrobial agents produced by the same strain. **Figure 1** shows two strains of *P. syringae* (A and B) that are inhibited by a third strain of the same species. The two strains, however, are inhibited by different bacteriocins. The clearing zone on strain A exhibits a sharp edge, whereas the clearing zone on strain B is larger, and exhibits a non-sharp border. Genetic manipulations within the producing strain that specifically inactivate either the tailocin (tailocin deficient) or low molecular weight bacteriocin (bacteriocin deficient) confirm that strains A and B are sensitive to distinct bacteriocins. **Figure 2** shows that bacteriophage-mediated killing can be distinguished from other non-replicative antimicrobials by comparing a dilution series. Because both bacteriocins and prophage can be induced by mitomycin C treatment, activities of these two agents can closely resemble each other (particularly if the activated phage is abundant). In the case of bacteriophage-mediated clearing, dilution of the supernatant will resolve into individual plaques (clearing zones) while bacteriocin-mediated clearing will not resolve into such plaques.

Figure 1: Phenotypic distinction of multiple antimicrobial agents produced by the same strain. Strain A is sensitive to a high molecular weight bacteriocin (tailocin) but not an alternative low molecular weight bacteriocin, as evidenced by lack of clearing in the tailocin deficient strain, but not the bacteriocin deficient strain. Conversely, strain B is insensitive to the tailocin, but is sensitive to the low molecular weight bacteriocin. The tailocin is efficiently recovered following PEG precipitation, while lower molecular weight bacteriocins are not. WT: wild type.

Figure 2: Distinguishing between non-replicative antimicrobials and bacteriophages. A) Dilution of a high titer of bacteriophages results in individual plaques that become less numerous (high titer top, low titer bottom). **B)** Dilution of bacteriocins results in uniformly less clearing that does not resolve into individual plaques.

DISCUSSION:

The soft-agar overlay technique described here has been widely applied for many decades by researchers interested in bacteriophages or bacteriocins. The main benefits of this approach are that it is simple, cheap and relatively easy to interpret. By combining the soft-agar overlay with PEG precipitation and serial dilution, antimicrobial agents can be separated into high vs. low molecular weight agents, and replicative vs. non-replicative agents (i.e. bacteriophage vs. tailocin, respectively). Finally, incorporation of targeted genetic manipulation (deletion and complementation) of putative bacteriocin or prophage loci can firmly establish the identity of a given antagonistic compound. We have recently used this method to describe a new bacteriophage-derived bacteriocin prevalent among *Pseudomonas syringae* strains[9].

The growth media and conditions indicated in this protocol work well for *Pseudomonas syrignae* and would likely suffice for other Gram-negative bacteria that grow vigorously under these conditions. However, researchers using this method will want to optimize the timing, culture medium, induction method, and incubation temperature for their system. The critical parameters include inducing the producing culture while in the logarithmic growth phase, as well as seeding the soft-agar overlay with sufficient logarithmic phase culture to ensure a uniform bacterial distribution, but not excessive culture, which will obscure potential clearing zones. There are many bacteriocins that are not induced as part of the SOS response, but rather are induced through peptide-based quorum sensing systems (particularly Gram-positive bacteriocins[15]), thus, a researcher may want to screen several different induction methods (such as modifying nutrient content of inducing medium, or allowing for extended incubation of the producing strain).

When using this technique, the soft-agar overlay must be thoroughly melted and maintained at 55-60 °C prior to the addition of the seeding strain. We have had several experiments where the soft-agar was not thoroughly melted (though visually it appeared to be) and resulted in an overlay with a grainy appearance. Results from a grainy overlay can still be generally interpretable, however, this is dependent on the strength of the inhibition (weaker inhibition will be more difficult to observe). A final, confounding variable to consider when using this technique is that the temperature of the molten agar is allowed sufficient time to cool prior to inoculation with the seeding strain, so as to avoid killing the seeding strain. To avoid this issue, we ensure the soft-agar is warm, but not hot, to touch. Along these lines, we have also observed that if we give inadequate time for a seeding culture to acclimate to the molten agar, prior to pouring the overlay, we recover generally poor bacterial growth, which makes interpretation of specific inhibition difficult or impossible to interpret. This is why we allow 10-15 additional seconds of incubation between vortexing and pouring the overlay. The trade-off between maintaining the agar in a completely molten state (hotter is better) but not lethal to the seeding strain (hotter is not better) is one that will likely need to be determined by a researcher through trial and error.

If a PEG precipitation step is used, it would be beneficial to include a negative control where a sterile broth medium is processed identically to the culture supernatant. This will ensure that killing activity is not the result of residual PEG or chloroform within the sample supernatant.

As both bacteriophages and bacteriocins tend to be highly specific, it is not uncommon to encounter no apparent killing activity with a given combination of strains. This can result from a variety of strain-specific resistance mechanisms, one being that the tester strain either lacks or has an unrecognized version of the receptor needed for targeting by a given bacteriocin or bacteriophage.

An alternative method, bacteriocin screening method has been described by Kawai et al., but suffers from drawbacks and has not been widely adopted[16]. Specifically, this

technique requires observing broth samples at time intervals that can be burdensome, and does not allow for the discrimination between bacteriophage-derived and bacteriocin-derived killing activities.

The main limitations of this technique is that it is not well suited for organisms that do not grow robustly (and thus will lack easily discernable clearing zones) or that harbor prophages or bacteriocins that are not robustly produced under laboratory conditions. Adaption of this technique to detect bacteriocins produced in environmental samples would both expand the utility of this technique and facilitate greater insight into the role of bacteriocins within natural settings.

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DISCLOSURES:
The authors have nothing to disclose.

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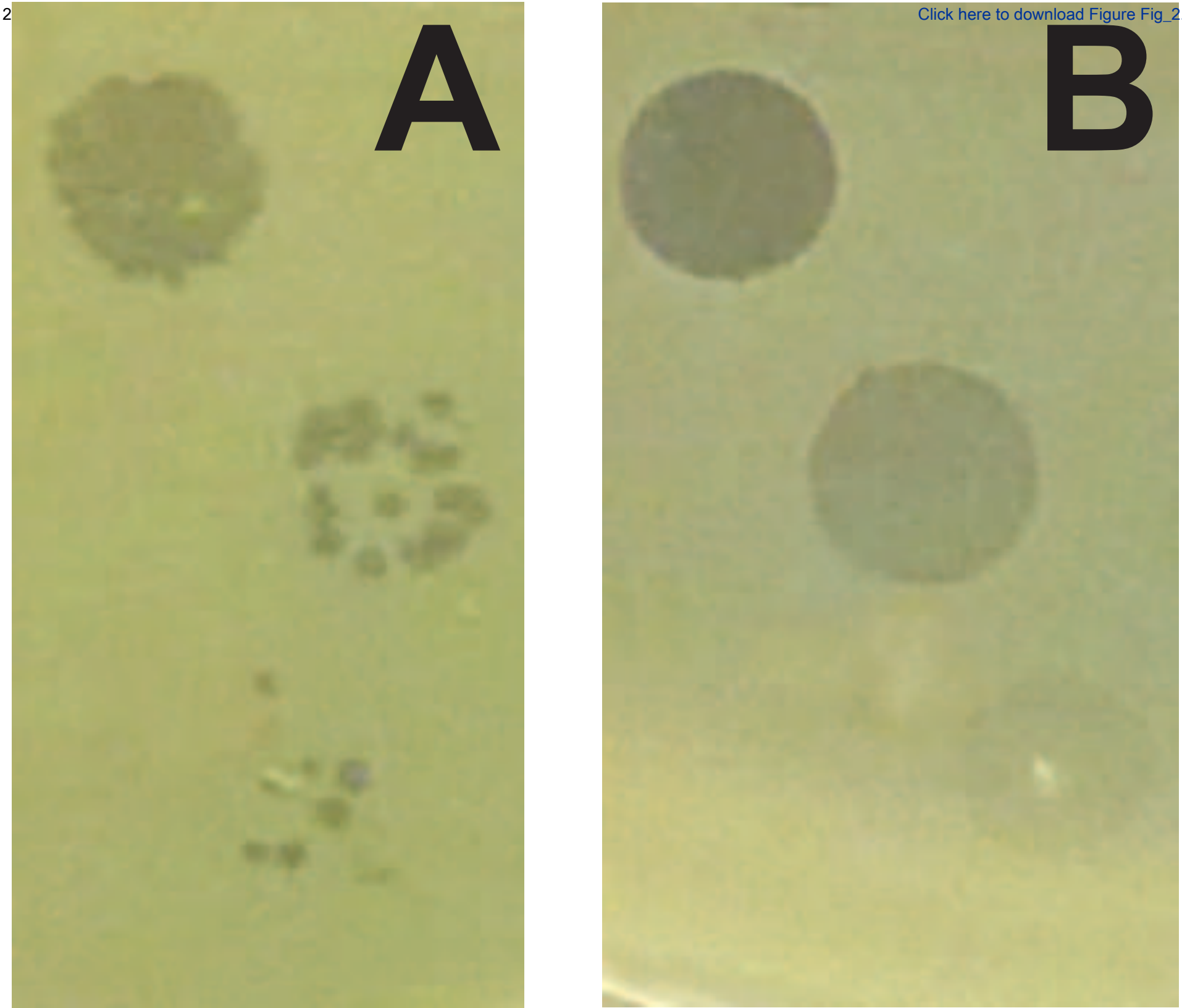
Figure 1

Figure 2



Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/Equipment	Company	Catalogue Number
Mitomycin C	Santa Cruz Biotechnology	sc-3514
Chloroform	Sigma-Aldrich	34854
Polyethylene Glycol (PEG)	Amresco	0159
Bacteriological grade agar	Genesee Scientific	20-274

Comments/Description

Carcinogenic. use appropriate PPE (i.e. gloves, eye protection, and face mask), particularly whe
Acutely toxic, respiratory hazard. Use appropriate PPE (glove and eye shield), handle open cont

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CORRESPONDING AUTHOR:

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Institution:	University of Arizona
Article Title:	Use of the Soft-Agar Overlay Technique to Screen for Bacterially-Produced Inhibitory Compounds
Signature:	Kevin Hockett
Date:	8/30/16

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Editorial comments:

Please sign the Article and Video Licence Agreement, either digitally or by hand. Please scan and upload it with your manuscript files.

Unfortunately, I am currently unable to scan and upload a signed version of the Article and Video License Agreement today as I am currently traveling. I have uploaded an electronically signed version, and will be able to upload a scanned version later in the evening tomorrow (9/1/16).

•Formatting:

-2.1 - "To sterilized supernatant, add" Unless the supernatant was sterilized prior to this step (as part of the protocol), please change to "To the sterile supernatant, add".

Modified as indicated

-References – Please abbreviate journal titles.

The journal titles have been abbreviated.

•Discussion: Please include alternative methods in discussion of significance and include independent citations. Please also discuss the limitations and future applications of the method.

Discussion of a recently published technique has been included, as well as discussion of limitations and future applications of the agar overlay technique (lines 299-313).

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript Hockett and Baltrus describe a strategy for the screening of culture supernatants on the presence of antagonism-mediating compounds. This method allows to distinguish between bacteriophage, high and low molecular weight bacteriocins. The experimental set-up of the experiment is clear and potential errors that may occur during the experiment are described in sufficient detail. Interaction phenotypes that a researcher may be looking for are clearly mentioned as well, which I appreciate.

Major Concerns:

There are no major concerns

Minor Concerns:

I have some small textual comments:

- Sentence L56 reads difficult? Word missing?

Sentence has been revised to read more smoothly.

- L61: Pseudomonas
Typo has been corrected.

- 2.1 L110: I would expect that stirring is needed to dissolve the NaCl and the PEG? Or not?
A sentence was included indicating that the sample should be repeatedly inverted until both the NaCl and PEG are dissolved.

- Figure 1: I would have expected strain A on the left side of the panel? This doesn't pose any dfficulty towards interpretation though…
- L229: of the producing?
The sentence was modified to include 'the', which was originally omitted (line 235).

- L269: ref 4 is a bit outdated if it concerns work on Pseudomonas bacteriocins. The authors may want to replace the ref by Ghequire and De Mot. 2014. FEMS Microbiol Rev. 38:523?
We agree that inclusion of the Ghequire and De Mot reference benefits the manuscript. We think inclusion of the Michel-Briand and Baysse reference, dated as it may be, is still beneficial as it includes more biochemical detail not included in the more contemporary review.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The authors describe the use of a soft-agar overlay to screen for inhibitory compounds. Through targeted gene deletion and PEG precipitation, one can distinguish between a phage, high molecular weight bacteriocin and low molecular weight bacteriocin. It represents a relatively inexpensive way to screen supernatants collected from a number of strains.

Major Concerns:
N/A

Minor Concerns:
1. In line 33, the wording of the sentence is a bit awkward "bacteriophages and bacteriocins, proteinaceous antibacterial compounds.
This sentence has been slightly modified, with *agents* replacing *compounds*.

2. The introduction describes Pseudomonas aeruginosa and E. coli as being known to produce bacteriocins but doesn't explain the reason why P. syringae was used. It would be helpful to add in a bit more information about known

bacteriocins or inhibitory compounds that are secreted by P. syringae.
Two sentences have included into the introduction both indicating briefly the different sorts of antimicrobial agents produced by *P. syringae*, as well as the current interest in antimicrobials from this organism (lines 67-72).

3. It would be useful to explain the reason for mitomycin C addition earlier.
A note has been added regarding the role of mitomycin C within the protocol (lines 104-105).

4. Can a negative control be added to the protocol? PEG can be difficult to get rid of and can kill bacterial strains.
This is an excellent suggestion, a note on inclusion of a negative control has been included within the discussion (lines 269-272).

5. Is there an advantage to adding chloroform to supernatants in step 1.5.1? It would be helpful if the authors included the benefits to adding chloroform to supernatants.
A note has been included indicating that chloroform is cheaper and easier to scale-up if processing many samples at a time (lines 118-119).

6. In Figure 1, would one expect to see a larger zone of inhibition for PEG precipitated tailocin as compared to untreated supernatants? PEG treatments removes low molecular weight which would enrich for tailocins so would that increase activity? What is known about the effective concentration of tailocins and relative amounts produced by P. syringae strains?
Tailocins (because of their size) are limited in their mobility within the agar matrix and do not diffuse away from the spot of inoculation (as noted within the representative results section). The best way to distinguish between samples with differing tailocin concentrations would be through comparing extinction points for serial dilution of each sample.

7. It would helpful if the authors included a potential result of a strain being immune to the supernatants collected from another strain. Would that mean the strain also contains the same bacteriocin locus? What are other possible explanantions?
A short note has been added at the end of the discussion regarding this comment (lines 277-281).

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
This is a very clearly written protocol, describing a simple but effective and very

useful technique for identification of producer of either replicative or non-replicative antimicrobial agents. I have used the technique in my own lab for screening both phage host ranges and antimicrobial activity of macro algae frond sections for antimicrobial activity. As such I believe a visual presentation and written protocol of this technique would be exceptionally useful and widely accessed. It appears that the authors have made every attempt to fully address the original reviewers comments. I have a few points of a minor nature which the authors should address prior to final acceptance

Major Concerns:
None

Minor Concerns:
Line 55-56 "is an understanding antagonistic" should read "is an understanding of the antagonistic"
This sentence has been revised in an alternative way to what the reviewer has indicated, but in a way that is grammatically correct.

Protocol 1.1 - although the authors mention the reason for using mitomycin C in lines 185-6, it might be useful to the viewer/reader to describe why mitomycin C is added to the culture at this stage, rather than (or in addition to) later on in the manuscript.
See response to reviewer #2, comment 3 above.

Line 93-94 - perhaps add a few words on what chloroform does here - is there a worry that perhaps active agents might partition into the chloroform as in a crude solvent extract? I think this should be considered and may be worth mentioning.
These concerns have been addressed within the note describing the difference between filter sterilization and chloroform treatment (lines 118-122).

Line 180-1 - the manuscript should contain details of the strains used - what are the genetic manipulations?
While we agree with the reviewer that inclusion of details regarding the genetic manipulations within manuscripts should normally be included, we believe such description would be beyond the scope of this manuscript, which focuses on the agar-overlay protocol and only uses the mutant strain for illustrative purposes.

Line 215 "We've" to "We have"
This change has been made.

Line 227 - is circuits the appropriate term here? QS regulated pathways?
Circuits has been modified to *systems*.

Line 233-5 - this sentence might be confusing for the reader - how will the individual know if it is properly melted, especially as you state that it may visually appear melted? I would be included to remove "(though it visually appeared to

be)”
We agree that our description is seemingly contradictory, however, this coming from our experience with this protocol. We believe that that the final sentence of this paragraph indicating that trial and error will be necessary will help resolve this issue for researchers.

Line 246 - "between maintain the agar" should read "between maintaining the agar”
Good catch, this mistake has been corrected.

Additional Comments to Authors:
N/A

[**Editorial recommendation**: Please keep JoVE’s protocol requirements in mind as you address the above comments - the protocol must contain sufficient detail in order to enable users to accurately replicate your technique. We recommend NOT removing any details from the protocol text.]