High-Throughput Co-Culture Assays for the Investigation of Microbial Interactions

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Abstract

The study of interactions between microorganisms has led to numerous discoveries, from novel antimicrobials to insights in microbial ecology. Many approaches used for the study of microbial interactions require specialized equipment, are expensive, and/or are time intensive. This paper presents a protocol for co-culture interaction assays that are inexpensive, scalable to large sample numbers, and easily adaptable to numerous experimental designs. Microorganisms are cultured together on 12-well plates, with each well representing one pairwise combination of microorganisms. A test organism is cultured on one side of each individual well, and first incubated in monoculture. Subsequently, target organisms are simultaneously inoculated onto the opposite side of each of the wells using a 3D printed inoculation stamp. After co-culture, the completed assays are scored for visual phenotypes, such as growth or inhibition. These assays can be used to confirm phenotypes or identify patterns among isolates of interest. Using this simple and effective method, users can analyze many combinations of microorganisms rapidly and efficiently. This co-culture approach is applicable to antibiotic discovery, as well as, culture-based microbiome research and has already been successfully applied to both applications.

SUMMARY

The co-culture interaction assays presented herein are inexpensive, high-throughput, and simple. These assays can be used to observe microbial interactions in co-culture, identify interaction patterns, and characterize the inhibitory potential of a microbial strain of interest against human and environmental pathogens.

Keywords

Antibiotics; antimicrobials; co-culture; inhibition assays; microbial interactions; phenotypic screen

INTRODUCTION

In nature, microorganisms rarely exist in isolation and consequently they are constantly interacting with other organisms in their environment. Therefore, studying how microorganisms interact with each other is essential to understanding a multitude of microbial behaviors[1]. Microbial interactions can be mutualistic, commensal, or antagonistic, and can critically affect not only the microorganisms themselves, but also the environments and hosts that the microorganisms colonize[1,2].

Many scientists study microbial interactions to identify new antimicrobial molecules. One of the first clinically important antimicrobial molecules was found through the study of microbial interactions. Sir Alexander Fleming observed a contaminating *Penicillium* spp. isolate that inhibited the growth of a *Staphylococcus* strain, which led to the discovery of the commonly used antibiotic penicillin[3]. Characterization of the mechanisms that microorganisms use to antagonize their competitors remains a fruitful resource for the discovery of antimicrobial molecules. For example, it was recently shown that *Streptomyces* sp. strain Mg1 produces antibiotic linearmycins, which have a lytic and degradative activity against *Bacillus subtilis*[4]. Further, a non-ribosomally synthesized peptide named lugdunin, was recently discovered after the observation that nasal commensal *Staphylococcus lugdunensis* inhibits *Staphylococcus aureus*[5]. Studies have, also, shown that mutualistic interactions between microorganisms are equally, as powerful as, antagonistic interactions for the discovery of antimicrobial molecules. For example, many fungus-farming ants in the tribe Attini harbor symbiotic bacteria called *Pseudonocardia* on their exoskeleton that produce antifungal molecules to inhibit an obligate pathogen of their fungal crop[6]. As the study of microbial interactions has been beneficial for discovering antimicrobial molecules, the use of high-throughput screens may result in the discovery of new antimicrobial molecules.

With respect to the cost and ease of performance, the methodologies used to study microbial interactions range from simple to complex. For instance, an agar plug assay is an inexpensive and simple method that can be used to investigate antagonism between multiple microorganisms[7]. However, an agar plug assay is not an efficient procedure and can be labor-intensive for many pairwise combinations. To assess the effects of microbially produced products on target isolates of interest in a high throughput manner, many laboratories use disk diffusion assays[8]. These assays are easy, inexpensive, and can be scalable to higher numbers of samples[7]. However, this assay requires the generation of microbial extracts and may produce misleading results for certain combinations of target organisms and antibiotics, such as *Salmonella* and cephalosporins[9].

The preceding approaches rely on isolated components to elicit a response in a target organism, instead of allowing microorganisms to interact with each other. This is of note because interactions between microbes may elicit the production of "cryptic" antimicrobial molecules that are not produced in monoculture. For instance, it was recently shown that the antimicrobial keyicin is only produced by a *Micromonospora* sp. when it is co-cultured with a *Rhodococcus* sp. that was isolated from the same sponge microbiome[10]. More complex interaction methodologies circumvent this potential monoculture hindrance. For instance,

the iChip is useful for isolating rare and difficult to cultivate bacteria from environmental samples, and allows for the observation of microbial interactions through growth in situ[11]. To investigate interactions in detail, matrix assisted laser desorption/ionization time-of-flight imaging mass spectrometry (MALDI-TOF-IMS) can be used. This approach provides detailed information on the composition and distribution of small molecules and peptides produced by interacting microbial colonies with high spatial resolution, and has been used in multiple studies of bacterial interactions to characterize the mechanisms of competition[12–15]. However, MALDI-TOF-IMS often requires laborious sample preparation, specialized expertise to operate the equipment, and expensive, specialized mass spectrometers. For these reasons, it is a difficult technique to use for high throughput studies. Thus, a simple, scalable, and high-throughput co-culture assay for microbial interactions that overcomes many limitations of the above approaches would be advantageous.

Here, a protocol for high-throughput microbial co-culture is presented. This assay is simple and easily incorporated into preexisting studies of microbial interactions. In contrast to many commonly used methods for the study of microbial interactions, our method is simple, inexpensive, and is amenable to investigating large numbers of interactions. These assays are not only easy to perform, but the materials are widely available from most laboratory suppliers or public resources (e.g., libraries and makerspaces). Consequently, this assay is advantageous as a first line of investigation to identify and parse interesting patterns among many pairwise combinations of microorganisms, and which may be especially useful for the investigation of microbial ecology.

PROTOCOL

Informed consent was obtained from the donor's parents, and the Human Subjects Committee at the University of Wisconsin—Madison approved the study (institutional review board [IRB] approval number H-2013–1044)

1.   Sample Culture

NOTE: This procedure was used for the study of interactions among bacteria isolates from the human nasal cavity. In principle, the following methods are applicable to any culture condition. Brain-heart-infusion Broth (BHI) was used for general propagation of nasal bacteria. All plates were solidified using 1.5% agar. For this study, samples were taken from saline solution flushed into donors' noses (nasal lavage), transferred into microcentrifuge tubes and frozen at −80 °C.

1.1 Use standard culture techniques to plate 100 μL of each of the thawed lavage samples onto BHI plates.

1.2 Incubate the plates aerobically at 37 °C for 1 week.

1.3 After incubation, select   2 colonies of each distinct morphotype per plate and passage the isolates aerobically on BHI plates by streaking a colony from the initial plate onto a new BHI plate and incubating the plate at 37 °C. Repeat until the bacterial cultures are pure.

NOTE: Bacterial isolates can be identified through colony morphology, Gram-staining, 16S rRNA gene sequencing, or another method. However, knowing the isolate identity is not necessary for continuing the protocol.

1.4 Cryopreserve all bacterial isolates at −80 °C after combining 1 mL of 50% glycerol with 1 mL of bacterial overnight culture (see step 3) in cryotubes.

2. 3D Printing Stamps

NOTE: Polycarbonate was selected as the stamping material due to its high glass transition temperature (147 °C) that exceeds standard autoclave temperatures (121 °C), which minimizes the potential for deformation after repeated uses.

2.1 Load the 3D printer with polycarbonate filament.

2.2 Apply white school glue (polyvinyl acetate) to the print bed to aid in adhesion and minimize warping of the inoculation stamp during the print.

2.3 Load the .STL model file (Supplementary Data File) for the inoculation stamp (Figure 1) into the 3D printer software.

2.4 Print the inoculation stamp at 290 °C nozzle temperature, 60 °C bed temperature, and using a layer height of 0.38 mm.

2.5 Wrap the stamp in the aluminum foil and sterilize by autoclaving for 1.5 h on a gravity cycle with 15 min of drying.

NOTE: Though polycarbonate is hydroscopic, the stamps only retain approximately 0.5% water weight after autoclaving.

3. Preparation of Overnight Cultures

3.1 Using a serological pipette, pipette 3 mL of sterile BHI broth into 14 mL culture tubes.

3.2 Using a sterile 1 μL inoculating loop, inoculate a bacterial colony into the broth. Swirl the loop to ensure the clump disperses into the broth. Vortex the culture tubes briefly before incubation.

3.3 Incubate the culture tubes at 37 °C overnight (~16 h) on a shaker at 250 rpm.

3.4 Vortex to break up the clumps of cells once the bacterial cultures reach enough turbidity (OD_{600} 1).

4. Preparation of Bioassay plates

NOTE: Bioassay plates are prepared in a laminar flow hood to maintain sterility.

4.1 Prepare BHI media with 1.5% agar and sterilize by autoclaving according to manufacturer instructions.

4.2 After autoclaving, cool the BHI media to 55 °C in a temperature-controlled water bath.

4.3 Using a serological pipette, pipette 3 mL of molten BHI media into each of the 12-wells on a 12-well plate. Ensure that the wells are as exact and even as possible. One liter of media will yield ~27 bioassay plates.

4.4 Allow the agar to set overnight.

5. Inoculating Bioassay Plates with the Test Organism

NOTE: A test organism refers to the organism for which the production of inhibitory activity (e.g., antibiotic production) is determined using the co-culture interaction assay. For this experiment, the test organisms were Actinobacteria isolated from nasal lavages samples.

5.1 Inoculate the test organism on a bioassay plate by inserting a sterile 10 μL inoculating loop into overnight culture and streaking a culture droplet over the left third of a plate well.

NOTE: It is recommended to only streak one third of the well, as overgrowth of the well prohibits later inoculation of target organisms.

5.2 Repeat until all 12-wells on the plate have been inoculated with the test organism.

5.3 Incubate plates upside down at the appropriate temperature for 7 days. At higher temperatures (37 °C) or in drier climates, store the plates in a humid container to prevent the plates from drying out.

6. Preparation of Target Organisms

NOTE: A target organism refers to the organism whose inhibition status is determined using the co-culture interaction assay. For this experiment, the target organisms were *Staphylococcus* spp. isolated from nasal lavages samples

6.1 After incubating the bioassay plates for 6 days, prepare overnight cultures of the specified target organisms, as above (see step 3).

7. Target Organism Inoculation

NOTE: After overnight incubation, ensure that the cultures are turbid (OD_{600} 1). Some bacterial cultures may flocculate at the bottom of the culture tube. Vortex the culture tubes to disperse clumps and assess the culture turbidity.

7.1 Prepare the target plate by filling each well of an empty 12-well plate with 1.8 mL of BHI and 200 μL of the target overnight culture.

7.2 Unwrap and place a sterile inoculation stamp into the target plate. Gently swirl the cultures around in the wells, taking care to ensure that the cultures do not cross contaminate neighboring wells.

7.3 Lift the inoculation stamp and ensure that there is a droplet of diluted target culture on each stamp tip.

7.4 Prepare a monoculture control plate by placing the inoculation stamp on an uninoculated bioassay plate and gently rock the stamp so that a culture drop inoculates each well. Once the inoculation stamp is removed, a droplet of culture should be visible in the wells of the bioassay plate.

7.4.1 If any wells are not inoculated with the inoculation stamp due to uneven levels of media, spot 3 μL of diluted overnight culture onto the right side of the wells using a pipette.

7.5 Inoculate the bioassay plates as above (see substep 7.4) but align the stamp so that the tips align with the right side of the 12-well plate. Ensure that the stamp does not contact the existing bacterial colony when inoculating the wells.

7.6 Carefully remove the stamp and place back into the target plate.

7.7 Repeat the inoculation for each bioassay plate until all the plates are inoculated.

7.8 Incubate bioassay plates upside down at the appropriate temperature for 7 days.

8. Scoring

8.1 After co-culturing the test and target organisms for 1 week, score the interactions based on the following visual assessment:

8.1.1 Score the wells with target organism growth that is exhibits growth that is indistinguishable from the monoculture control as 0 (no inhibition) (Figure 2A–B).

8.1.2 Score the wells with target organism organism that exhibits diminished growth compared to the control as 1 (weak inhibition) (Figure 2C).

8.1.3 Score the wells where the target organism did not grow as 2 (strong inhibition) (Figure 2D).

REPRESENTATIVE RESULTS

Co-culture interaction assays can be used to understand microbial interactions, identify patterns of interest, and uncover microbial isolates with intriguing activities. In these assays, a test organism is monocultured on one side of a 12-well agar plate and incubated for 7 days. Subsequently, a target organism is spotted next to the test organism and the two microbes were co-cultured for 7 days before scoring for the growth phenotype of the target organism. The assays are scored based on a visual analysis of the growth or inhibition of the target organism.

These co-culture assays were recently used to assess the inhibitory activity of Actinobacteria (test organisms) toward *Staphylococcus* spp. (target organisms) isolated from the human nasal cavity (Figure 2)[16]. The co-culture assays were used to identify specific inhibition patterns between Actinobacteria (n=21) and *Staphylococcus* isolates (n=39) and showed that Actinobacteria isolates showed variation in their ability to inhibit coagulase-negative staphylococci (CoNS). A total of 812 pairwise combinations were tested. In particular, *Corynebacterium propinquum* strongly inhibited CoNS (Figure 2D), especially when

compared to other *Corynebacterium* that weakly inhibited CoNS (Figure 2C) or had no effect on CoNS (Figure 2B), when compared to the monoculture control (Figure 2A)[16]. Using comparative genomics, a biosynthetic gene cluster for siderophore production was identified in *C. propinquum* genomes that was absent in the genomes of other *Corynebacterium* isolates[16]. Siderophores are chelators produced by microorganisms to scavenge iron from the environment[17]. Siderophore production by *C. propinquum* was confirmed and the siderophore was identified as dehydroxynocardamine[16]. This result led to the hypothesis that inhibition of CoNS was due to siderophore-mediated iron depletion. Subsequently, by performing co-culture interaction assays between *C. propinquum* and CoNS on both standard and iron-supplemented BHI medium, it was determined that the inhibition phenotype was iron-dependent (Figure 2E). Together, these results suggested that siderophore-mediated iron depletion was responsible for the strong inhibition of CoNS by *C. propinquum*[16].

DISCUSSION

Antibiotics and other secondary metabolites that mediate microbial interactions are useful for a multitude of applications, including drug discovery. Herein, a protocol for co-culture assays to assess large numbers of microbial interactions is presented. These co-culture interaction assays are a simple, affordable, scalable, and a high-throughput means to investigate many pairwise combinations of microorganisms in tandem. Target organisms are spotted next to test organisms in a well of a 12-well plate using an inoculation stamp, and inhibition of the target organisms is scored based on visual inspection of the target organism's phenotype. The majority of materials for these assays are readily available through most laboratory suppliers or through public resources. Therefore, these assays can be easily tailored to many laboratory environments. In the laboratory, these co-culture assays have been successful in investigating the interactions of microorganisms associated with many hosts from a variety of environments.

While there are many benefits to this technique, there are also a few limitations. The first notable limitation is that patterns from the co-culture assays are difficult to interpret without other metadata. In two recent papers that employed these co-culture assays[16,18], inhibition patterns of interest were only identified in conjunction with other metadata, such as taxonomic identity of the test organisms. Nevertheless, even without accompanying metadata, the methods outlined in this paper are amenable to identifying bacterial isolates with inhibitory activity toward specific pathogens or target microbes of interest. An additional limitation is that these assays are not commercially available, and the bioassay plates must be hand prepared, which can limit the efficiency of the assays. Plate preparation is critical to the experiment, and care should be taken while preparing the plates. If the wells are uneven, then the inoculation stamp may not be able to inoculate all wells. However, missed wells can simply be inoculated by directly pipetting the target organism culture onto the right side of each uninoculated well. Indeed, even if a few wells on each plate are missed by the stamp, the procedure is still more efficient than directly pipetting the target organism into every single well. Alternatively, users can forgo using the inoculation stamp and pipette the target organism into each well, but this process is more time consuming, especially when compared to stamping 12 wells simultaneously. Finally, the test organism may consume the

available nutrients in the well during monoculture before the target organism is inoculated. Though nutrient depletion may affect the observed inhibition patterns, it appears to be uncommon among the pairwise combinations that have been tested thus far. Notably, depletion of iron in the wells by *C. propinquum* allowed for the discovery of siderophore-mediated competition from members of the human nasal microbiota[16].

These co-culture interaction assays are customizable by modifying the media composition, timing, or even including multiple organisms or microbial consortia as the test organism. Furthermore, different scoring systems can be used depending on the desired level of detail required to describe the interaction phenotype. Examples scoring scales include those from 0–2 used to describe competitive interactions among bacteria isolated from the human nasal cavity[16] (Figure 2) and from 0–3 used to assess the antimicrobial potential of *Streptomyces* isolated from insect microbiomes[18]. However, with more nuanced scales, scoring becomes increasingly difficult. Thus, inhibition is most easily recognized using a binary scoring system (e.g., 0 is defined as no inhibition and 1 is defined as inhibition), which can eliminate any confusion and standardize scoring across multiple individuals. Moreover, in addition to scoring for inhibition phenotypes, these assays can also be scored for other phenotypes, including pigment production, sporulation, or any other phenotype which can be assessed visually. As these assays are highly scalable with analysis based upon visual inspection of the target organism, training sets for machine learning algorithms can be generated to facilitate phenotype scoring to further increase assay throughput.

A major strength of these co-culture assays is their ability to facilitate screening many combinations of pairwise interactions inexpensively and rapidly to uncover activities or inhibition patterns of interest. Subsequently, more complex and intensive methods, including genomic characterization, MALDI-TOF-IMS, or natural product isolation and characterization, may be used for deeper characterization of the microbes and interactions of interest identified by co-culture assays. As a recent example, these co-culture inhibition assays were used to show that insect-derived *Streptomyces* can inhibit Gram-negative bacteria and fungi better than their soil-derived counterparts. The inhibition assays allowed for quick and efficient visualization of inhibition patterns among 2003 *Streptomyces* isolates and led to the discovery of a new antifungal called cyphomycin, which is active against drug-resistant fungal pathogens[18]. Thus, these co-culture interaction assays are a powerful tool for microbiome research, antimicrobial discovery, and for gaining deeper insight into patterns of microbial interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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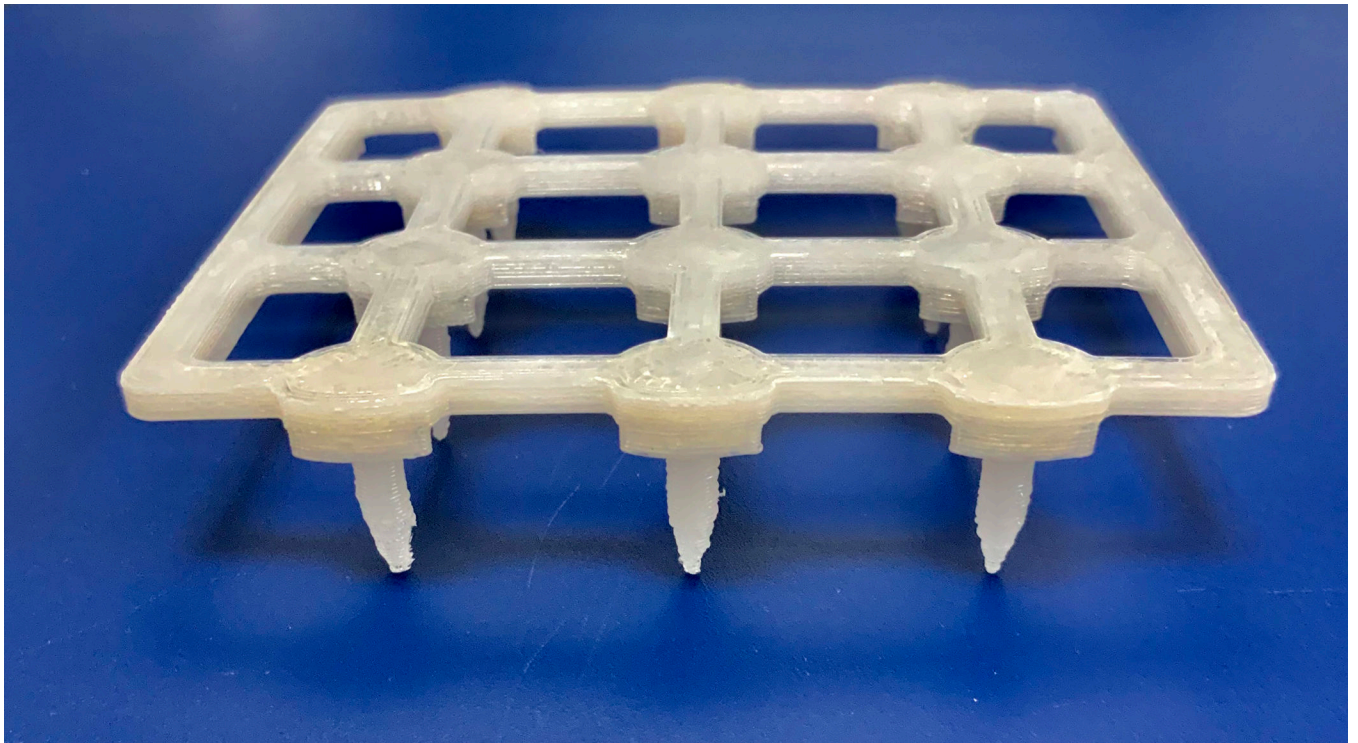
Figure 1:
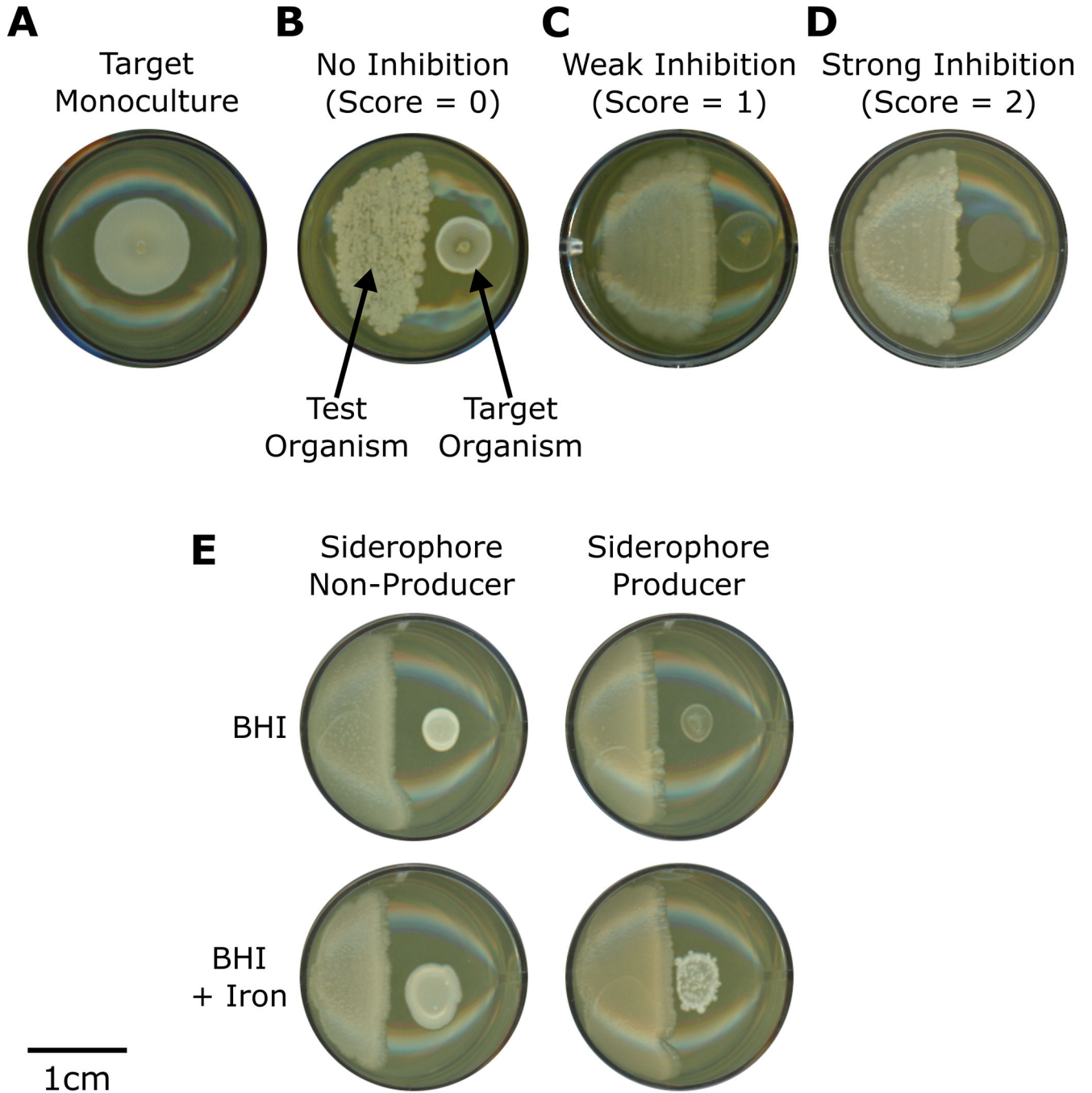Photograph of the bioassay inoculation stamp.

Figure 2: Co-culture interaction assays uncover siderophore-mediated inhibition of CoNS by *Corynebacterium* **propinquum.**

(**A**) Monoculture of CoNS (target organism) inoculated on a BHI bioassay plate. (**B-D**) Co-cultures between different strains of *Corynebacterium* spp. (test organisms, left) and the same strain of CoNS (target organism, right) inoculated on BHI bioassay plates. Each panel is a representative image showing interactions with (**B**) no inhibition (score = 0), (**C**) weak inhibition (score = 1), or (**D**) strong inhibition (score = 2). (**E**) Comparison of interactions between *Corynebacterium pseudodiphtheriticum* (siderophore non-producer) or

Corynebacterium propinquum (siderophore producer) with the same strain of CoNS on BHI media (BHI) and BHI media supplemented with 200 μM $FeCl_3$ (BHI + Iron).