Chapter 9

A Role for Secreted Immune Effectors in Microbial Biofilm Formation Revealed by Simple In Vitro Assays

Assunta Liberti, Brittany A. Leigh, Zachary Graham, Ojas Natarajan, and Larry J. Dishaw

Abstract

The formation of biofilms is critical for the successful and stable colonization of mucosal surfaces by microbes, which often build three-dimensional environments by exuding exopolysaccharides and other macromolecules such as proteins, lipids, and even DNA. It is not just bacteria, but fungi such as yeast, that form these adherent interacting communities. Historically, biofilms have been studied in the context of pathogenesis, but only recently it has been recognized that important relationships among members of host-associated microbiomes are maintained within the context of biofilms. Host immune responses impact biofilm formation in various ways; for example, it is likely that formation of stable biofilms by non-pathogens improves barrier defenses by not just filling available niche spaces but also by helping to ward off pathogens directly. Recently, it was found that soluble immune effector molecules such as immunoglobulin A (IgA) in mammals serve essential roles in modulating complex biofilm communities in ways that benefit the host. Additional lines of evidence from other secreted immune effectors, such as the variable region-containing chitin-binding proteins (VCBPs) in protochordates, now suggest that this phenomenon is much more widespread than previously recognized. The activity of these immune molecules also likely serves roles beyond those of simple defense strategies; rather, they may be improving the outcome of symbiotic interactions benefiting the host. Thus, traditional immune assays that are aimed at studying the function of secreted immune effectors, such as agglutination assays, should take into account the possibility that the first observation may not be the last if the microbes under study are not directly killed. Here, we describe a series of simple approaches to characterize biofilm formation when bacteria (or yeast) are cultured in the presence of a secreted immune effector. To model this approach, we use microbes isolated from the gut of *Ciona robusta*, each grown in the presence or absence of VCBPs. The approaches defined here are amenable to diverse model systems and their microbes.

Key words Bacteria, Fungi, Biofilm, VCBP, Secreted immune effectors

1 Introduction

Studies of host-microbiome interactions have become a central focus of biomedical research. An important area of study focuses on how the host immune system manages these microbes. Mucosal immunity manages microbial ecosystems in luminal spaces and is

Jonathan Rast and Katherine Buckley (eds.), *Immune Receptors: Methods and Protocols*, Methods in Molecular Biology, vol. 2421, https://doi.org/10.1007/978-1-0716-1944-5_9,

fundamentally different than how immune defense strategies protect the host when a pathogen breaches epithelial barrier. The mucosal immune system can manage settlement, and support the expansion of adherent microbial communities into complex, interacting biofilms. While virulence of some pathogens is known to depend on the formation of biofilms, a variety of mechanisms involving nonspecific innate effectors can shape these adherent communities (e.g., [1]).

Secretory immunoglobulin A (SIgA) in mammals is known to associate with mucosal barriers [2, 3], modulating settlement and adherence of diverse microbiota [2, 4–6]. Several studies spanning almost 15 years [2, 5, 7–9], including a recent high-profile description [10], revealed that the presence of SIgA may, in some cases, actually support the formation of commensal biofilms that help to shape barrier defenses. It is likely that this phenomenon is more widespread than previously anticipated [11–13].

We have shown previously that an unrelated immunoglobulin (Ig)-containing immune effector, variable region-containing chitin-binding protein C, or VCBP-C, shapes in vitro biofilm formation among bacteria isolated from the gut of *Ciona robusta* [14], an invertebrate chordate that relies exclusively on innate immune mechanisms to modulate mucosal barriers and other interfaces with the microbial-rich seawater environment. Unique to protochordates, the VCBP gene family has been identified in the cephalochordate, *Branchiostoma floridae,* and the urochordate, *Ciona robusta* [15–17]. VCBPs are characterized by the presence of two Ig variable domains (IgV) at the N-terminus and a chitin-binding domain (CBD) at the C-terminus. These molecules, and some of their functions, have been more thoroughly studied in *Ciona*. These efforts have revealed their spatial-temporal expression patterns within the developing gut, i.e., during metamorphosis [18], and localized them specifically to the digestive tract and various blood cells [17]. VCBP-C, one of four VCBPs in *Ciona*, is secreted into the gut lumen where it can become tethered to chitin-rich mucus and bind bacteria [14]; untethered VCBP-C can bind fungi [19], suggesting that VCBPs are likely influencing bacterial and fungal communities in the gut. In vitro, VCBP-C can bind and opsonize bacteria and shape biofilm formation [14, 17]. It is likely that secreted immune effectors of various types across phylogenetically distinct organisms shape how microbes interact with mucosal surfaces and regulate the formation of biofilms, a predominant lifestyle of microbes colonizing mucosal surfaces. Here we highlight simple yet distinct approaches that can be used to determine if a secreted immune effector protein influences biofilm formation (*see* **Note 1**). Recombinant VCBP-C [14, 17] is used for reference only, but these methods are applicable to a diverse range of native and recombinant proteins.

2 Materials

2.1 Bacteria and Fungus Isolation from Ciona *Gut*

1. *C. robusta* guts, dissected from 5 animals.
2. Dounce homogenizer.
3. Centrifuge and microcentrifuge.
4. 50 mL conical tubes.
5. 2 mL microcentrifuge tubes.
6. 40 μm sterile cell strainer baskets.
7. 70% ethanol.
8. Artificial Sea Water (ASW), prepared as reported in [20].
9. Marine bacteria are most often isolated using some variant of marine broth (MB) agar plates (2% agar). Media variations can be used to increase yield of diverse species and we, like others [21], find it useful to implement sea salts into the recipes.
10. Fungi are often cultured using media/agar plates such as yeast-peptone-dextrose (YPD) agar plates (2% BactoPeptone, 1% Yeast extract, 2% dextrose, and 2% Agar); for marine species, the media is made with ASW, with or without penicillin/streptomycin (200 U/mL and 0.2 mg/mL, respectively) antibiotics.

2.2 Characterization of the Bacterial or Fungal Isolates

2.2.1 Identification of Bacteria or Fungi

1. Bacterial 16S rRNA PCR primers (27F: 5′- AGAGTTT GATCMTGGCTCAG -3′ and 1492R: 5′- GGTTACCTTGT TACGACTT-3′) [22, 23].
2. Ribosomal internal transcribed spacer (ITS) 18S primers (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5′-TCCTCC GCTTATTGATATGC-3′) [24].
3. PCR master mix (e.g., Promega PCR Master Mix).
4. PCR tubes and thermal cycler.
5. Genomic DNA extraction optimized for microbes (e.g., Dneasy PowerSoil kit, Qiagen).

2.2.2 Quantification of Bacteria or Fungi

1. Media, both MB and YPB, agar plates without antibiotics.
2. Liquid media, such as MB and YPD.
3. 15 mL conical tubes or 14 mL round-bottom tubes.
4. 50 mL conical tubes.
5. 1.5 or 2 mL microcentrifuge tubes.
6. 96-well microtiter dishes (sterile).
7. UV spectrophotometer.
8. Hemocytometer.

9. Incubator with shaker at 20 °C.
10. Multi-channel pipette.

2.3 Biofilm Assay Setup for Crystal Violet or for TOTO-1 and SYTO 60 Staining

1. Overnight culture of bacterial or yeast strains grown to log phase.
2. Liquid media, such as MB and YPD.
3. 12-well dishes.
4. 35 mm glass bottom dishes (No. 1.5, uncoated).
5. Humid chamber or any dark box with moistened paper towels.
6. Immune effector protein, appropriately purified.

2.4 Preparation of Biofilms for XTT Assay

1. Overnight culture of bacterial or yeast strains grown to log phase.
2. Liquid media, such as MB and YPD.
3. 1.5 or 2 mL microcentrifuge tubes.
4. 96-well microtiter dishes (sterile).
5. Humid chamber or dark box with moistened paper towels.
6. Immune effector protein, appropriately purified.

2.5 Biofilm Detection and Quantification

2.5.1 Crystal Violet Staining

1. 1× Phosphate-buffered saline (PBS) solution.
2. 1% Crystal violet aqueous solution.
3. Two bowls with running water.
4. Paper towels.
5. 30% acetic acid.
6. 96-well optical bottom dish for ELISA reader.
7. Multiwell reader set to read optical density at 560 nm.

2.5.2 TOTO-1 and SYTO 60 Staining (See **Note 2**)

1. 1× PBS.
2. TOTO-1 Iodide 514/533 (Invitrogen).
3. SYTO 60 Red (Invitrogen).
4. Inverted microscope.

2.5.3 XTT Assay

1. 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2*H*-tetrazolium hydroxide (XTT; Invitrogen) 0.5 g/L in 1× PBS.
2. 10 mM Menadione (Sigma), solubilized in 100% acetone.
3. 1× PBS.
4. 500 mL bottle-top (vacuum) filters, 0.22 μm pore size.
5. Aluminum foil.
6. 96-well optical bottom dish for ELISA reader.

7. Multiwell reader set to read optical density at 490 nm.
8. Reagent preparation for XTT assay (*see* **Note 3**).

3 Methods

3.1 Isolation, Culture, and Identification of Bacteria and Fungi from the Ciona Gut

Ciona robusta were collected from Mission Bay near San Diego, CA (M-REP, Carlsbad, CA, USA) (*see* **Note 4**).

1. Intestinal contents are cleared from animals by maintaining them in 0.22 μm-filtered sea water (FSW) for at least 24 h, unfed, and with routine water changes (*see* **Note 5**).
2. The *Ciona* tunic should be wiped with 70% ethanol and washed with sterile ASW [20]. Excise the gut aseptically from five animals and disrupt using a dounce homogenizer to liberate bacteria and fungi from the mucosal surface.
3. Separate host tissue by processing the homogenate through a 40 μm filter (sterile cell strainer/basket) and into a 50 mL conical tube. Centrifuge at 3000 rpm (or 1000 × *g*) for 10 min. The flow-through is retained, as it contains the microorganisms of interest.
4. Collect the microorganisms by pelleting in 2 mL microcentrifuge tubes for 10 min at 8000 rpm (or 6700 × *g*) (bacteria) or 5000 rpm (or 2700 × *g*) (fungi), respectively, washing and resuspending in 1 mL ASW at least twice.
5. Use MB to culture and isolate bacterial species (*see* **Note 6**).
6. Use YPD to culture and isolate fungal species (*see* **Note 7**).
7. To identify bacteria and fungi (*see* **Note 8**).

3.2 Characterization of Bacterial and Fungal Strains

Before setting up the biofilm assays, it is important to know a bit about the microorganisms under study and establish some basic growth parameters. Growth curves should be performed so that appropriate concentrations for the assays (i.e., reflecting exponential growth) can be determined. Growth curves follow standard protocols, and are summarized below.

3.2.1 Determination of Bacterial Growth Curve and Concentration

1. Pick a bacterial colony from a freshly streaked MB agar plate and inoculate in 3.5 mL MB liquid medium and incubate overnight in an orbital shaker (180–220 rpm) at 20 °C.
2. Dilute overnight culture 1:5 and 1:10 in fresh MB liquid medium (final volume 10 mL).
3. Follow growth of each dilution over time (by checking optical density at 600 nm).
4. Plot bacterial growth (via increased OD) and look for OD range that corresponds to log-phase growth.

5. At each time point, in parallel to the OD measurement, colony-forming units (CFUs, representing the number of viable bacterial cells present and used for calculating bacterial concentration) are determined (*see* **Note 9**).
6. The bacterial concentration used in the assays below should correspond to the OD at log phase of growth.

3.2.2 Determination of Yeast Growth Curve and Concentration

1. Pick a yeast colony from a freshly streaked YPD agar plate (about 3 days old), inoculate into 5 mL YPD liquid medium, and incubate overnight in an orbital shaker (180–220 rpm) at 20 °C.
2. Dilute yeast cultures 1:5 and 1:10 in fresh YPD liquid medium (final volume 10 mL), and growth is monitored and measured each hour as above at OD 600. Likewise, the measured ODs are graphed to help determine log phase of growth. Yeast cells can be counted using a hemocytometer.
3. The yeast concentration used in the following assays should correspond to the OD at log phase of growth.

3.3 Setup and Detection of the Biofilm Assay

Bacterial or fungal biofilms, by definition, are complex (often polymicrobial) communities of cells embedded in a self-produced "sticky" matrix rich in extracellular polymeric substances (EPS), which facilitate adherence to surfaces [25]. The EPS matrix, which provides structure and protection to the community, is composed of proteins, nucleic acids, diverse polysaccharides, and lipids. Several assays have been described that aid in the observation and quantification of biofilms, such as the biomass assays that include crystal violet (CV) or the matrix quantification assays that often utilize fluorescent imaging. In the former, staining can help quantify the EPS matrix but will stain living, dying, and dead cells equally; in the latter, specific staining of components is observed, such as eDNA with TOTO-1 or living cells, with SYTO-60. With viability assays, such as the XTT assay, metabolic activity of viable cells is monitored and quantified, allowing one to estimate the proliferative rate of cells forming the biofilm.

Below, we describe the setup and execution of simple assays with or without the addition of a secreted immune effector protein. In each case, appropriate protein controls should be used and can include heat-denatured versions. Generally, all steps of the assays are carried out within a cleanroom cabinet using aseptic conditions and sterile materials to avoid external contamination that can influence assay outcomes.

3.3.1 Setup of Biofilms Assays for Crystal Violet Staining

The protocol reported below is adapted and modified from previously established methods [26, 27].

1. Single colonies of bacteria or yeast are grown in MB or YPD medium, respectively, overnight at 20 °C, in an incubator with orbital shaking at appropriate speeds (e.g., 180–220 rpm).

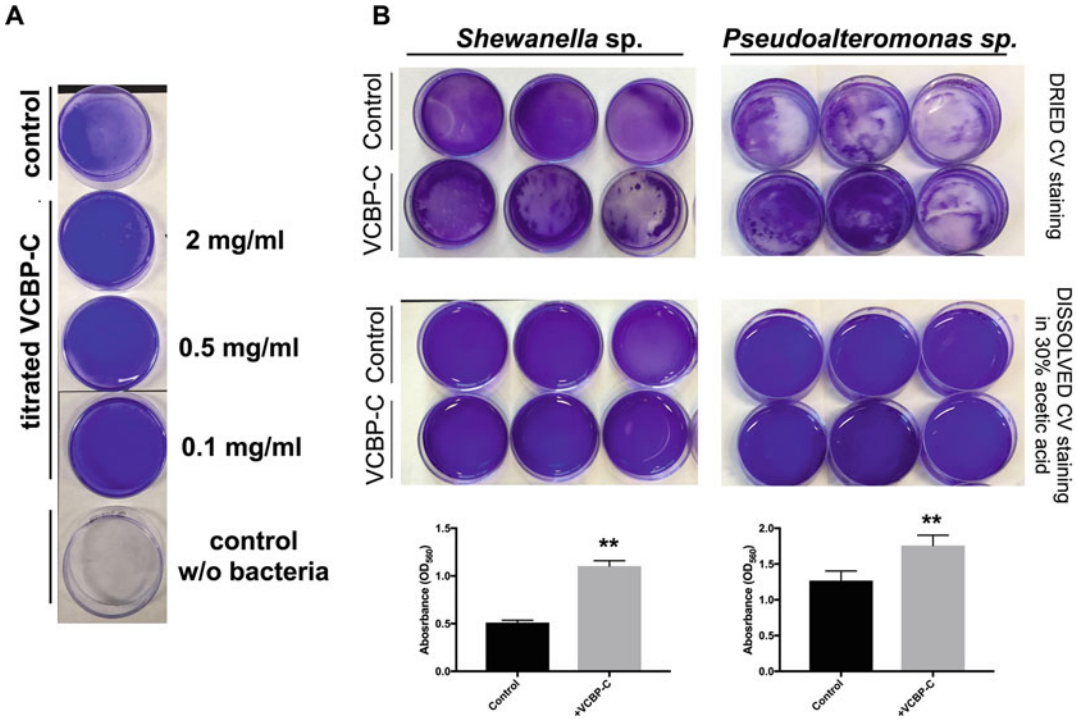

Fig. 1 Staining and quantification of biofilms with crystal violet. (**a**) Titration experiment of (recombinant) VCBP-C purified protein on the formation of *Shewanella* sp. biofilm cultured for 48 h, stained with CV, and imaged as dried/stained plates. Lower concentrations of VCBP-C protein (0.1 mg/mL) were equally effective in shaping biofilm formation compared to the higher concentration (2 mg/mL); hence a lower amount of protein, as 0.05 mg/mL, was used in the assays. (**b**) *Shewanella* sp. and *Pseudoalteromonas* sp. biofilms are grown for 4–5 days in the presence/absence of VCBP-C (0.05 mg/mL) and stained with CV. Plates are shown as dried and stained (upper images) or dissolved staining in 30% acetic acid (lower images). The lowest graphic shows quantification of the dissolved CV staining, measured with a microplate reader at OD_{560}. Biofilm formation in both bacterial strains was increased in the presence of VCBP-C. Asterisks indicate statistical significance calculated using two tailed *t*-tests ($P < 0.01$)

2. The concentration of the overnight culture is estimated by OD_{600}, and diluted in MB or YPD to a final volume suitable for the experiment and at the OD/mL corresponding to log phase, as calculated in Subheading 3.2 above. In some cases, further dilution may be desired prior to plating.
3. One milliliter of the diluted culture is added to each well of a 12-well dish. Each strain is plated in duplicate for all conditions, including the control samples. The protein being tested should be titrated (Fig. 1a). Immediately after plating, the appropriate volume of the purified immune protein is added and mixed well. Buffer controls should also be plated to measure background staining that may result from media components.

4. The dishes are incubated as stationary cultures at 20 °C in a humid chamber to reduce evaporation. Biofilm formation (and outcomes) can be observed after 24–72 h; however, these cultures should be carefully monitored daily under a stereoscope to visualize tertiary structures whose formation can change in the presence or absence of the immune protein. Many biofilm assays are allowed to develop for up to 5 days, but can be visualized and/or stained at 24-h intervals as long as sufficient dishes were prepared at the onset.
5. Biofilm growth and development is stopped by gently removing (by aspiration) the excess culture liquid that includes any remaining planktonic bacteria or yeasts. Adherent biofilms at the bottom of the dishes (and as rings that can form at the upper liquid/air or liquid/plastic interface) are washed at least twice with sterile PBS to remove un-adhered cells and media components. Finally, the remaining liquid is removed, and the plate is allowed to dry completely by overturning the plates onto paper towels and left overnight.
6. The amount of biofilm can be qualitatively observed and then stained with CV. Briefly, at least 1 mL of a 0.1% solution of CV diluted in deionized water is added to each well and incubated at room temperature for 10 min.
7. Dishes are rinsed twice with deionized water by submerging them in a bowl filled with running deionized water. The dishes are then firmly held and overturned over the sink to remove all the water and then rigorously tapped onto a stack of paper towels and left on the towels to dry overnight.
8. For qualitative observations, the dried 12-well dishes are photographed to record gross differences among the biofilms, with and without culturing in the presence of the immune molecule (Fig. 1b).
9. To quantify the biofilm, 1 mL of 30% acetic acid is added to each well and incubated for 10 min; the acetic acid re-solubilizes the CV trapped in the biofilm (Fig. 1b).
10. To complete the quantification, OD readings are acquired with an ELISA reader. Briefly, 100 μL of the solution (**step 9**) is transferred to 96-well optically clear dishes, in triplicate, for each biofilm well and the absorbance determined at OD_{560}. The wells in which sterile media was incubated in the original 12-well dish are used as a blank control, and this value is subtracted from the value of the biofilm samples. The final biofilm measurement for each condition is the mean of the biological duplicates, and the value of each biological duplicate is the arithmetic mean of the absorbance of the technical triplicates (Fig. 1b). The assay should be repeated at least three times for each microbial strain.

11. The statistical significance is determined with two tailed *t*-tests to compare each time point in the samples with and without the immune molecules, and a one-way ANOVA test can be used to compare biofilms among the different time points.

3.3.2 Setup of Biofilm Assay for TOTO-1 and SYTO 60 Staining and Observation

1. One milliliter of bacterial culture, prepared as reported above (Subheading 3.3.1, **steps 1–4**), is plated onto 35 mm dishes with glass bottom coverslips (in duplicate) for each condition defined above, and the assay followed as before.
2. Remove (by aspiration) the excess liquid, which includes planktonic bacteria or yeast, to stop biofilm growth and development. Wash the biofilms twice with sterile PBS, and stain with TOTO-1 (diluted in PBS as reported in manufacturer's instructions) for 10 min. Excess dye is then removed, and the biofilm is washed again twice with PBS and counterstained with SYTO 60 (diluted in PBS as reported in manufacturer's instructions) for 10 min. After excess dye is removed, biofilms are washed with PBS and then held in PBS for observation on an inverted fluorescent microscope to visualize and estimate cell viability and the amount of the eDNA present in the biofilms.
3. Images are captured (Fig. 2) and semi-quantification of the signal intensity can be made using ImageJ, or related software, to compare overall fluorescence intensity and/or pixel densities per field of view.

3.3.3 Biofilm Setup for XTT Assay

1. To initiate the XTT assay on the biofilms, the same procedures are followed as in Subheading 3.3.1, **steps 1–4**, with the exception that the microbial culture is plated in 96-well microtiter dishes, in triplicate for each condition, adding 100 μL of the diluted culture per well.
2. Aspirate the excess liquid, which includes the planktonic bacteria or yeast, to stop biofilm growth and development. Wash the adhered biofilm three times with sterile PBS.
3. After thawing the XTT solution, menadione is added at a final concentration of 5 μM and mixed well. Then, 100 μL of the XTT-menadione solution is added to each prewashed well. The plates are covered with foil and incubated for 2 h at room temperature. The reaction develops an orange color and is then quantified using an ELISA plate at OD_{490} by first transferring 80 μL of the reaction to a new 96-well optically clear dish. The final value is the arithmetic mean of the biological triplicates. The assay must be repeated at least three times for each microbial strain.
4. The statistical analysis applied is the same reported for the CV assay (Subheading 3.3.1).

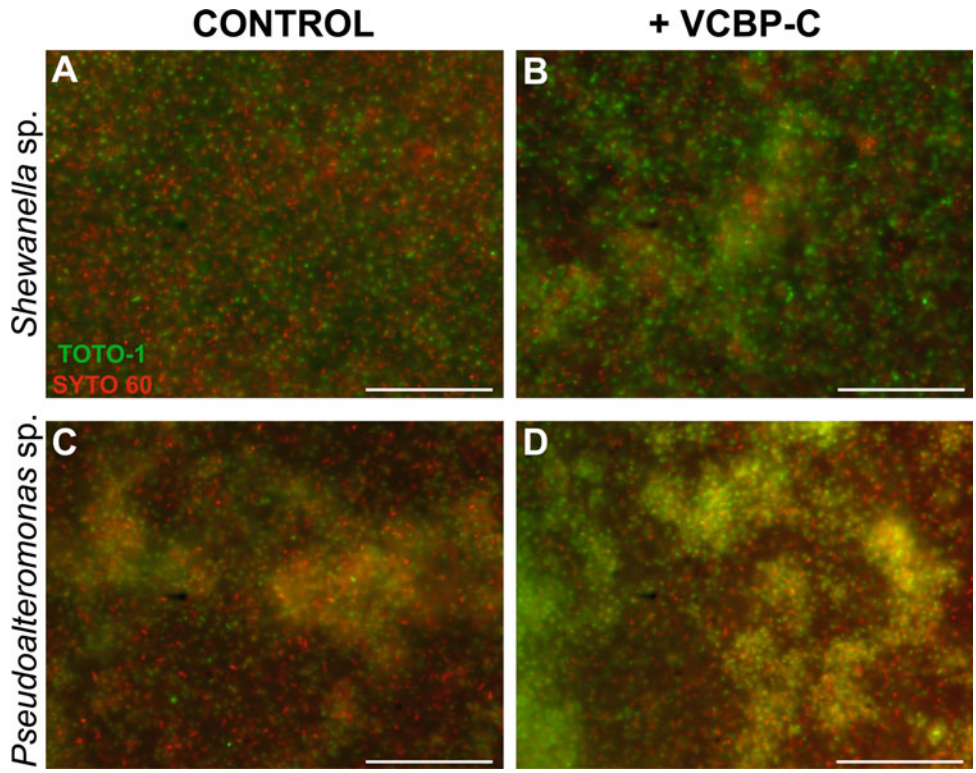

Fig. 2 Staining of biofilms with TOTO-1 and SYTO 60. *Shewanella* sp. (**a**/**b**) and *Pseudoalteromonas* sp. (**c**/**d**) biofilms are cultured for 48 h in presence (**b** and **d**) or absence (**a** and **c**) of VCBP-C protein (0.05 μg/mL). Live bacteria are stained in red with SYTO 60 and eDNA is stained in green with TOTO-1. The assay reveals an increase of eDNA in bacterial biofilm samples matured in the presence of VCBP-C (**b** and **d**). Scale bars: 50 μm

3.4 Conclusions or Anticipated Results

Secreted immune effector molecules have the capacity to transform the ecology of microbial communities associating with mucosal surfaces. These adherent communities most often thrive as complex, interacting, biofilms. Microbial biofilm formation is an intricate, multistep process that involves various genes and regulatory mechanisms [26]. The assays described here serve as straightforward and simple approaches to determine if secreted immune effector molecules impact the in vitro formation of biofilms among cultured microbes that are known to colonize host mucosal surfaces. Diverse microorganisms can be assessed, and various properties can be simultaneously examined, such as formation and quantification of the EPS matrix (using CV assay), cellular metabolic activity/viability (using XTT assay), and the presence of specific matrix components such as eDNA (using TOTO-1 Iodide 514/533 dye), most often resulting from cell turnover. In prior published examples, we have shown that the binding of VCBP-C to bacteria often induces an increase in biofilm formation among some bacterial strains isolated from the *C. robusta* gut, such as *Shewanella*

sp. and *Pseudoalteromonas* sp., while showing little impact on others [14]. Similarly, a role for VCBP-C in modulating yeast biofilm formation in terms of both EPS secretion and cell viability is currently under investigation (Liberti and Dishaw, unpublished data).

These aforementioned assays represent an excellent "first approach" to study, in vitro, the role that candidate immune effectors (which are not cytotoxic) could have in modulating specific microbial behavior, such as biofilm assembly. The simplicity of these assays affords the opportunity to test various microbial strains and experimental conditions (e.g., protein titration and other substrates) to rapidly assess a role for immune effectors in modulating microbial ecology.

4 Notes

1. Biofilm formation should be monitored (under a stereo microscope) to compare and record changes in gross morphology over time. In addition to the methods described here, where appropriate, the user may choose to supplement their approaches for visualizing or quantifying biofilms, such as with methylene blue staining [28, 29] or comparing "dry weight" (DW) measurements [30, 31]. Changes to motility or swarming behaviors, for example, can be estimated and/or visualized with simple motility assays on agarose dishes [32–34].
2. TOTO-1 (Iodide 514/533) and SYTO 60 red are fluorescent stains that can be used to study biofilms. For example, TOTO-1 can detect extracellular DNA (eDNA), which can be incorporated as scaffolding material into biofilms. SYTO 60 is a dye that can distinguish living and dead cells, helping to reveal the proportion of viable cells in the biofilm.
3. XTT powder is diluted in PBS at a final concentration of 0.5 g/L; the solution is then filtered with a 0.22 μm 500 mL bottle-top filter, covered with foil, and stored at −80 °C in 10 mL aliquots. The menadione solution is prepared by diluting the powder in 100% acetone at a final concentration of 10 mM and stored at −80 °C in aliquots of 200 μL.
4. *Ciona robusta* is a cosmopolitan species distributed in all seas from the tropics to the poles (Millar, 1953) and is considered an invasive species. The animals were harvested with an appropriate collector's license (M-Rep, Carlsbad, CA, USA), transported overnight to the lab, and allowed to acclimate in seawater.

5. As a filter-feeder, the gut of *Ciona* at any given time will reflect the surrounding seawater and dietary contents. To culture and identify microbes specific to the gut microbiome, it is important to clear the gut lumen of dietary material by maintaining the animals in FSW without any food source. All subsequent steps to isolate microorganisms are carried out in a benchtop cleanroom or a laminar-flow, HEPA-filtered cabinet, using aseptic conditions and sterilized materials.
6. To isolate bacteria, plate 10–15 μL of the suspension on MB agar plates and culture overnight, or until bacterial colonies are observed at 20 °C. Each colony is isolated by replica-streaking, and then selected colonies are used for PCR and grown overnight in 3 mL MB liquid medium in orbital shaker at 20 °C. Overnight cultures are then stored in 20% glycerol at −80 °C.
7. To isolate fungi, 300 μL of the suspension is plated onto YPD agar plates with antibiotics. Fungi are grown for 7–10 days before clonal growth is established and maintained by replica-streaking on YPD plates without antibiotics. Each fungal isolate is then inoculated in 5 mL YPD liquid medium, cultured overnight (or up to 1 week for filamentous fungi) in an orbital shaker at 20 °C, and then mixed with glycerol (final concentration 10% for filamentous fungi and 15% for yeasts) for long-term storage at −80 °C.
8. Isolating genomic DNA and sequencing individually cloned rRNA amplification products are essential for the identification of bacteria and fungi. More specifically, sequencing amplicon products of the 16S rRNA genes, using 27F and 1492R primers, can identify bacteria, and sequencing of amplification products from the ITS region of the 18S rRNA gene, using ITS1 and ITS4 primers, can identify fungi.
9. To determine colony-forming units, serial dilutions in MB liquid medium (10 μL of bacterial growth and 90 μL of bacterial medium) are made in triplicate until the 10^{-8} dilution is achieved. Ten microliters of each dilution is plated on MB agar and grown overnight at 20 °C. On the following day, the dilution at which single colonies are distinguishable is used for calculating the number of bacteria per milliliter.

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