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Comparative analysis of quantitative methodologies for *Vibrionaceae* biofilms

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Abstract Multiple symbiotic and free-living *Vibrio* spp. grow as a form of microbial community known as a biofilm. In the laboratory, methods to quantify *Vibrio* biofilm mass include crystal violet staining, direct colony-forming unit (CFU) counting, dry biofilm cell mass measurement, and observation of development of wrinkled colonies. Another approach for bacterial biofilms also involves the use of tetrazolium (XTT) assays (used widely in studies of fungi) that are an appropriate measure of metabolic activity and vitality of cells within the biofilm matrix. This study systematically tested five techniques, among which the XTT assay and wrinkled colony measurement provided the most reproducible, accurate, and efficient methods for the quantitative estimation of *Vibrionaceae* biofilms.

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Introduction

Biofilms are communities of microbes that are composed of cells attached to a surface and encapsulated in an extracellular matrix (composed primarily of polysaccharides, proteins, and DNA; Watnick and Kotler 2000; Yildiz and Visick 2008). Biofilms develop when cells transition from a planktonic (free-living) lifestyle to surface-attached complex multicellular communities (Watnick and Kotler 2000). These microscopic communities can form unique microbiomes that are common in nature, and can range from a healthy consortium of beneficial bacteria to those that can be the primary source of dangerous chronic diseases (Watnick and Kotler 2000; Costerton et al. 1999).

Biofilms formed by symbiotic bacteria in the family *Vibrionaceae* (pathogenic and mutualistic) have been studied for over 20 years, and diverse methodologies for studying *Vibrio* biofilms under laboratory conditions have been proposed by multiple research groups (Yildiz and Visick 2008). However, this area of research is in constant change and is still under development. For example, a recent methodology developed to measure *Vibrio* biofilm mass included examining cell viability and identification of common biofilm phenotypes (such as formation of wrinkled or rugose bacterial colonies; Ray et al. 2011), while another popular semiquantitative method (that has been used extensively for multiple bacterial biofilms) includes the use of crystal violet in a colorimetric assay to stain biofilms attached to a surface (O'Toole 2011). In the case of fungal biofilms (such as those formed by *Candida* and *Cryptococcus*), there is a commonly used colorimetric assay that accurately shows cellular viability within the biofilm through the metabolic use of formazan salts (Kuhn et al. 2003). Interestingly, this method is not routinely used in *Vibrio* (and other bacterial) biofilms for its quantitative capability or detection limits. All of these proposed methods

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have been important tools to measure in vitro formation of biofilms. These procedures vary widely as to their time and cost requirements, and in variation reported in assay performance. An important element of these proposed methods is the necessity to accurately and reproducibly quantify viable cells in the biofilms as can be accomplished by a metabolism-based assay such as the XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide} reduction assay. Therefore, the goals of this current study were to test the efficacy of the XTT assay in *Vibrionaceae* biofilms as well as to make a comparative analysis of time, efficiency, and cost of different quantitative assays. Studies included the use of crystal violet staining, dry cell mass measurement, viable colony counting (direct enumeration of bacteria in biofilms), phenotype observation (wrinkled colony development), and the use of the XTT reduction assay.

Methods

Six wild-type *Vibrio* strains (biosafety level (BSL) 1) were selected for this study: *Vibrio rotiferianus* (Chowdhury et al. 2011), *Vibrio corallilyticus* (ATCC BAA450), *Vibrio parahaemolyticus* (ATCC 17802), two *Vibrio fischeri* strains isolated from *Euprymna* squid hosts: ES114 (*Euprymna scolopes* from Kaneohe Bay, O'ahu, HI, USA) and ETJB1H (*Euprymna tasmanica* from Jervis Bay, New South Wales, Australia), and one free-living (seawater) isolate (*V. fischeri* CB31 from Coogee Bay, New South Wales, Australia). We also selected mutant *V. fischeri* strains (from the ETJB1H isolate) that have been reported to be defective in biofilm formation (Ariyakumar and Nishiguchi 2009; Chavez-Dozal et al. 2012). Mutant strains had interruptions in genes responsible for (a) twitching motility and pilus assembly (Δ*pilT*, Δ*pilU*, Δ*mshA*; Ariyakumar and Nishiguchi 2009; Chavez-Dozal et al. 2012), (b) flagellum assembly and functionality (Δ*flgF*, Δ*motY*; Chavez-Dozal et al. 2012), and (c) stress responses such as heat shock (Δ*ibpA*) and magnesium-dependent induction (Δ*mifB*; Chavez-Dozal et al. 2012).

To evaluate biofilm formation, cultures were grown overnight at 28 °C and 250 rpm in Luria Bertani high-salt media (LBS; 10 g tryptone, 5 g yeast extract, 20 g sodium chloride, 50 mL 1 mol/L Tris pH 7.5, 3.75 mL 80 % glycerol, and 950 mL distilled water). Biofilm quantification was measured by five different methodologies, including crystal violet (CV) staining (O'Toole 2011), XTT assay (Pierce et al. 2010), dry cell mass measurement (Taff et al. 2012), colony counting (Merrit et al. 2005), and wrinkly colony development (Ray et al. 2011).

For the CV and XTT assays, all strains were subcultured and grown to a cell density of 1×10^8 colony-forming units (CFU)/mL. Aliquots of each *Vibrio* isolate (200 μL) were added to individual wells on a flat-bottom, polystyrene 96-well microtiter plate (Corning, Sigma-Aldrich CLS3628, St. Louis, MO) and incubated for 24 h under conditions previously described (Chavez-Dozal et al. 2012). After incubation, planktonic (those not forming biofilms) cells were removed by briskly shaking the plate and attached cells were washed three times with sterile media. For the CV assay, crystal violet (2 % aqueous solution) was added to each well and incubated at room temperature for 30 min. After incubation, CV was removed and the plate was washed five times with sterile media. CV was then quantified by solubilizing with 95 % ethanol, and optical density (A_{562}) readings were recorded at 562 nm for each biofilm in individual wells. For the XTT assay, planktonic cells were removed and plates washed as previously described (O'Toole 2011; Ariyakumar and Nishiguchi 2009; Chavez-Dozal et al. 2012). Metabolic activity was measured by the XTT reduction assay (Pierce et al. 2010). In brief, 0.010 mol/L menadione (Sigma-Aldrich, St. Louis, MO) stock solution (diluted in acetone) was mixed with XTT/Ringer's lactate solution (0.5 g of XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide} from Sigma diluted in 1 L of 1× PBS or Ringer's lactate solution) at a final concentration of 1 μmol/L. An aliquot of the XTT/Ringer's/menadione solution was then added to each prewashed well. The plates were covered in aluminum foil and incubated for 2 h at 28 °C. If the XTT is effectively reduced by metabolically active cells, the original clear solution is transformed into an orange solution that can be measured at A_{490}. For CV and XTT assays, experiments were performed three times independently (biological replicates), each in quadruplicate (technical replicates) including inoculated sterile LBS as a negative control.

For dry cell mass determination, biofilms were formed in 96-well microplates and planktonic bacteria were removed after 24 h of incubation (as described previously; Taff et al. 2012). Biofilms were dried for 30 min at room temperature and then were disrupted by scraping with a sterile spatula and diluted into 500 μL of sterile water. The biofilm suspension was filtered through a preweighted filter (0.45 μm) and dried in an incubator at 105 °C for 2 h, after which the filter was weighed again. The dry mass of the biofilm was calculated based on mass differences between the control and samples.

For enumeration of bacteria in biofilms, the biofilm assay plates were inoculated, incubated, and washed as described for the CV and XTT assays. Each individual well was cut with scissors and 100 μL of 1× PBS was added. The well (plus the PBS) was placed into a separate 10-mL tube containing 1.9 mL of 1× PBS. The sample was sonicated for 5 s at 30 % power (higher sonication times compromised cell viability of some strains). The sample was plated in triplicate onto LBS plates and incubated for 24 h at 28 °C. Viable counts of colony-forming units were performed. For each strain, the experiment was performed in triplicate.

We additionally performed a semiquantitative method to measure biofilm formation by observation of wrinkled colony

development as described previously (Merrit et al. 2005) with minor modifications. In brief, an aliquot of overnight cultures was subcultured in 5 mL of fresh LBS at a 1:100 dilution and grown to an A_{600} of 0.2. After incubation, 1 mL of culture was pelleted and washed twice with 1× PBS, and resuspended in 1 mL of 1× PBS. Ten-microliter subsamples were spotted onto a fresh LBS plate (three spots per plate) and incubated for 24 h at 28 °C. Morphology and spot size were observed, and light micrographs of colonies were acquired using an inverted microscope (Micromaster digital inverted microscope with infinity optics, Fisher Scientific, Waltham, MA). The diameter of the colony was recorded digitally using the data acquisition software Micron 2.0.0 (Westover Scientific, Milpitas, CA). This experiment was performed in triplicate for each strain.

Results were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc or Tukey comparison post-test. Differences between groups were considered to be significant at a P value of <0.05. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

Findings

In vitro studies of biofilms have been increasing in number over the last decade. *Vibrio* biofilms play an important role in the environment and have been studied in the laboratory for over a decade (Yildiz and Visick 2008). There are multiple assays that have been proposed for quantification of *Vibrio* biofilms; for example, crystal violet is one of the most commonly used methods (Ray et al. 2011; O'Toole 2011; Kuhn et al. 2003; Chowdhury et al. 2011), and consists of a colorimetric assay where crystal violet solution (water or ethanol based) is used to stain cells and their extracellular matrices. The amount of CV absorbed by the biofilm is quantified by optical density readings of dissolved crystal violet, which is directly proportional to the biofilm mass. An alternative method consists of weighing the dried biofilm. This is one of the techniques used to calculate the total amount of biofilm but does not account for cell viability within the biofilm. The colony-forming unit determination assay (CFU counts) is a labor-intensive method that is solely based on cell viability. Moreover, the recently proposed method of observation of wrinkled colonies provides a more reliable method of quantifying biofilm development, which also allows the evaluation of the tri-dimensional structure and patterning of a particular *Vibrio* biofilm former. Some limitations of CFU and wrinkled colony development assays include lengthy assay time and requirements for previous adjustments to ensure reproducibility. In addition, strains with growth defects are usually difficult to analyze and cells in the viable but non-culturable state will not be detected (McDougald et al. 1998). For those types of assays that require removal of adherent biofilms (dry cell measurement and CFU counting), removal of cells may be inconsistent between samples.

An alternative method that has been widely used and has been proven to be especially useful for the study of fungal biofilms (in particular *Candida albicans*) is a colorimetric assay based on cellular viability involving the use of tetrazolium salts (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide, or XTT) and measurement of its orange-colored formazan product (due to activity of succinoxidase and cytochrome P450 enzymes). Since this assay is easy to perform, we included this analysis in quantification of *Vibrio* biofilms to combine measurements of cell viability with biofilm mass.

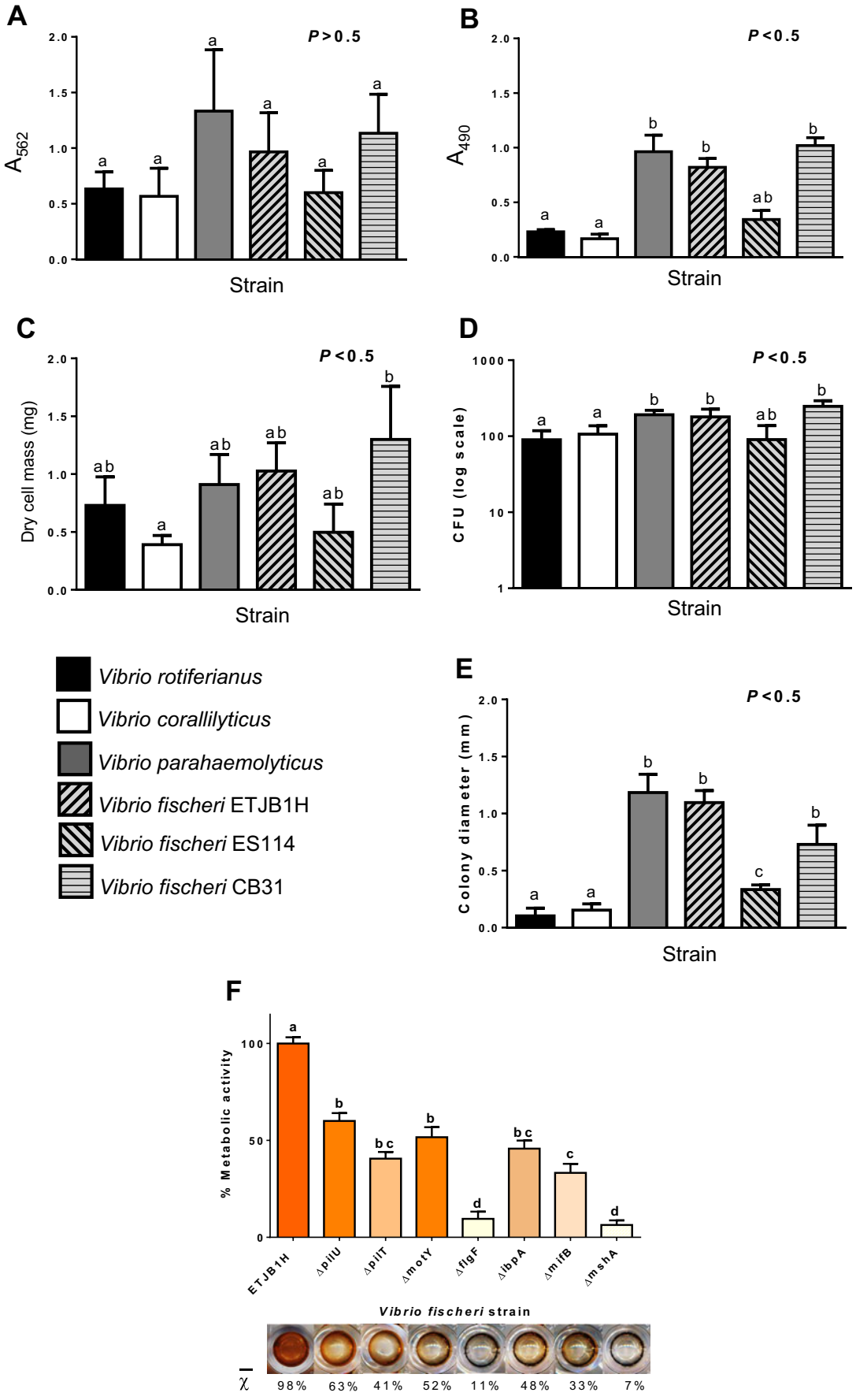
Since it is not advisable to conduct biofilm formation experiments on strains with growth defects, we performed growth curves on all the strains used in this study and none of them exhibited defects in growth. Additionally, for those biofilm formation experiments done in 96-well plates (including crystal violet and XTT), we measured the optical density (A_{600}) of the plate after incubation and prior to addition of either CV or menadione/Ringer's. All strains were between an A_{600} range of 5–6.

Among the assays tested, the most time consuming (but accurate and reproducible) were the CFU counting and the wrinkled colony development, whereas the crystal violet and dry cell mass assays were the most inaccurate and least reproducible (Table 1). Additionally, the XTT assay was the most reliable, the least time consuming, and the least costly. Figure 1 shows a more detailed comparison of the assays tested and their variability according to statistical differences (P values). An additional advantage of the use of metabolism-based assays (XTT assay) was that it allowed comparison of biofilm formation efficacy of mutant cells with the parental strain. This is illustrated in Fig. 1f, where different *V. fischeri* mutants in genes that have been reported to be important for

Table 1 Summary of the different methods used to quantify biofilms

Assay	Accuracy	Reproducibility	Time
XTT assay	++++	++++	+
Crystal violet	+	+	+
Dry cell mass	++	++	++
CFU count	+++	+++	++++
Wrinkled colony development	++++	++++	+++

Accuracy represents whether the data was consistent among technical replicates, as well as whether differences between the strains were significant ($P < 0.05$). Reproducibility was determined by the coefficient of variation (CV) for each set of data between biological replicates; ++++ = CV <0.1, +++ = CV of 0.1–0.15, ++ = CV of 0.15–0.2, and + = CV of >0.2. Time accounts for both the total length of the protocols and the amount of labor required for each (++++ representing the most time-consuming protocols)


A
P > 0.5
A562
Strain
B
P < 0.5
A490
Strain
C
P < 0.5
Dry cell mass (mg)
Strain
D
P < 0.5
CFU (log scale)
Strain
Vibrio rotiferianus
Vibrio corallilyticus
Vibrio parahaemolyticus
Vibrio fischeri ETJB1H
Vibrio fischeri ES114
Vibrio fischeri CB31
E
P < 0.5
Colony diameter (mm)
Strain
F
% Metabolic activity
ETJB1H
ΔpilU
ΔpilT
ΔmotY
ΔflgF
ΔibpA
ΔmifB
ΔmshA
Vibrio fischeri strain
χ
98%
63%
41%
52%
11%
48%
33%
7%

◀ **Fig. 1** Comparison of five in vitro biofilm quantification methods for *Vibrionaceae* biofilms. Each graph represents the data of the average (with *error bars* indicating standard deviations) of three independent experiments (biological replicates). *Different letters* on the *abscissa* denote significant differences between groups according to the Tukey post hoc comparison. *P* values indicate significant ($P < 0.5$) or non-significant ($P > 0.5$) overall differences according to the one-way ANOVA test. Absorbancies (A_{562} and A_{490}) of biofilms using **a** crystal violet (CV) or **b** XTT assay, respectively. **c** Biofilm quantification via dry cell mass measurement. **d** Colony-forming unit (CFU) determination of cells in biofilms formed in 96-well microplates. **e** Diameter of wrinkled colonies measured after 24 h of incubation. **f** Metabolic activity of wild-type (ETJB1H) and mutant *Vibrio fischeri* strains. Δ*pilT*, Δ*pilU*, and Δ*mshA* are type IV pilus mutants; Δ*flgF* and Δ*motY* are mutants in flagellum assembly and functionality; Δ*ibpA* is a mutant of a chaperonin responsible for the heat stress response, and Δ*mifB* is a mutant of the magnesium-dependent induction response. Metabolic activity is calculated as percentage in relation to *A* readings (A_{490}) of the wild-type parental strain. *Different letters* indicate significant differences according to the Tukey post hoc comparison test. *Wells* indicate the representative image of the intensity of the orange product as a result of formazan production by each biofilm. $\overline{\chi}$ represents the median value of the metabolic activity (in percentage)

biofilm development (but that are not defective in planktonic growth) were compared based on metabolic activity.

For the *Vibrionaceae* strains tested, we found that the XTT assay is the most reproducible and efficient method for measurement of biofilm biomass. The observation of development of wrinkled colonies could be used as a complementary test as it allows observation of the tri-dimensional structure of the biofilm and complements the colorimetric approach.

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