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Production and analysis of a *Bacillus subtilis* biofilm comprised of vegetative cells and spores using a modified colony biofilm model

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ABSTRACT

Bacillus subtilis is a spore-forming soil bacterium that is capable of producing robust biofilms. Sporulation can occur in *B. subtilis* biofilms and it is possible that the spores embedded in the protective matrix could present a significant challenge to disinfecting agents or processes. This article describes a method for the growth and quantification of a reproducible *B. subtilis* ATCC 35021 biofilm comprised of vegetative cells and spores using a modified colony biofilm model. In this method, membranes were inoculated and incubated for a total of 8 days to promote biofilm formation and subsequent sporulation within the biofilm. Representative samples were taken over the course of the incubation period to evaluate the biofilms using enumerative, microscopic, and spectrometric methods. At various time points, the total numbers of cells and spores were quantified. A Congo red agar (CRA) method was utilized to detect the TasA matrix protein, a primary component of the *B. subtilis* biofilm matrix. The presence of TasA was also confirmed using mass spectrometry. The biofilm morphologies were correlated to the enumeration data with a variety of correlative imaging techniques: confocal microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). At the end of the incubation period, the biofilm contained > 7 logs total colony forming units with spores comprising approximately 10% of the biofilm. The biofilm generated using this method allows researchers to use a new, more robust challenge for efficacy testing of chemical and physical antimicrobial treatments such as antibiotics, disinfectants, or heat.

1. Introduction

Bacillus subtilis is a Gram positive, spore-forming soil bacterium that is frequently isolated from processing equipment in the food and pharmaceutical industries (Wu and Liu, 2007; Soni et al., 2016). As such, United States Pharmacopeia (USP) < 1072 > 'Disinfectants and Antiseptics' recommends using the spores of *B. subtilis* as a challenge organism for sporicide efficacy testing due to their resistant nature (United States Pharmacopeia, 2017). Additionally, the spores of strain ATCC 35021 are used as a biological indicator to monitor and validate steam sterilization processes (Odlaug et al., 1981).

B. subtilis forms biofilms, which are defined as communities of microbes adhering to and growing on surfaces, encased in an extracellular polysaccharide (EPS) matrix of microbial origin (Parkar et al., 2004). Research has indicated that biofilms are typically more resistant to antimicrobial treatments than their planktonic counterparts, partly due to the protective nature of the EPS matrix (Olson et al., 2002; Stewart, 2015). It is also known that sporulation can occur within biofilms, including those formed by *B. subtilis* (Branda et al., 2001). Therefore, it is possible that the spores embedded in the protective EPS matrix could exhibit even higher resistance to disinfecting agents.

Standard methods exist to grow *Pseudomonas aeruginosa* biofilms in reactor-based systems, such as the CDC, drip flow, and rotating disk reactors, as well as a high throughput system called the MBEC assay (ASTM International, 2013a, b, 2017a, b, c). Each of these methods produces biofilms with particular characteristics that are dependent on the environment in which they grew. However, not all organisms will form biofilms under the conditions outlined in these methods. In particular, *B. subtilis* prefers to form biofilms at air interfaces, either on the surface of agar plates (colonies) or floating biofilms at the air-liquid interface of liquid (pellicles) (Branda et al., 2001). For this reason, reactor-based systems with submerged biofilm growth surfaces have not typically been effective for the formation of robust *B. subtilis* biofilms. The colony and pellicle biofilms formed by *B. subtilis* exhibit typical biofilm characteristics such as matrix formation and spatiotemporal organization of phenotypically differentiated subpopulations (Branda

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et al., 2001; Romero et al., 2010). It has been shown that the *B. subtilis* matrix is primarily composed of an exopolysaccharide component and a protein component, TasA (Branda et al., 2006). TasA forms amyloid fibers that are critical to the structural integrity of *B. subtilis* biofilms (Romero et al., 2010).

This paper describes a method to grow and quantify a reproducible *B. subtilis* biofilm as well as the analyses performed to determine the properties of the colony biofilms created. The colony biofilm model (CBM) first described by Anderl et al., 2000 was modified to stress the organisms and promote sporulation. The CBM uses an inoculated semipermeable membrane, placed on the surface of a nutrient agar plate, as the biofilm growth substrate. In the original method, the inoculated filters are transferred to fresh agar plates regularly (usually every 24–48 h) to provide new nutrients to the membrane-grown cells (Merritt et al., 2005). In the method described herein, membranes were inoculated and incubated for 24 h before transferring the membrane to a new media plate. This initial growth step allowed the colony biofilm to grow and spread across the filter. After the transfer, the colony biofilms were incubated for an additional 7 days without transfer to new media, inducing sporulation to occur within the biofilm.

This method has advantages over reactor-based methods. For example, reactors require cleaning and sterilization of equipment and creation and delivery of nutrient broth, while the CBM requires no equipment or delivery of nutrient broth over time and is scalable to much greater degrees by culturing multiple filters per agar plate and as many total plates as desired without significant additional effort. The biofilms produced using this method contain higher populations of both vegetative cells and spores than in previously reported studies using the CDC reactor to grow a *Bacillus* spp. biofilm with spores (Deal et al., 2016). It has been shown that *Bacillus* spp. form thicker biofilms at the air interface than in the submerged state, giving the CBM the advantage of providing a greater challenge when used to analyze the effect of various treatments such as antibiotics, disinfectants, or heat (Wijman et al., 2007).

The purpose of this study was to produce a *B. subtilis* biofilm using the CBM and evaluate the biofilm over the course of the 8-day incubation period using multiple enumerative, microscopic, and spectrometric methods. Quantification of the total number of cells, including vegetative and spore populations, occurred over various time points. Additionally, a spore count was determined by heat shocking the cells. The biofilm morphologies and locations of the spores were examined with a variety of correlative imaging techniques: confocal microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Finally, the Congo red agar method and mass spectrometry were utilized to detect the presence of TasA matrix protein (de Castro Melo et al., 2013; Kaiser et al., 2013; Romero et al., 2010).

2. Materials and methods

2.1. Production of a B. subtilis biofilm

2.1.1. Bacterial broth culture growth conditions

A *Bacillus subtilis* ATCC 35021 spore suspension was used to prepare a streak plate on trypticase soy agar (TSA; Becton, Dickinson, and Co., Sparks, MD). The plate was incubated in a calibrated and monitored 30–35 °C incubator (Caron Inc., Marietta, OH, USA) for 18–24 h. After incubation, a representative isolated colony was used to inoculate a 50 mL conical tube containing 20 mL of trypticase soy broth (TSB; Becton, Dickinson, and Co., Sparks, MD). The broth culture was incubated on a shaker (IKA Works, Inc., Wilmington, NC) in a 30–35 °C incubator for 18–24 h at 100 RPMs.

2.1.2. Inoculation and incubation of membranes

Prior to inoculation, 0.2 μm, nitrocellulose, 13 mm, membrane filters (EMD Millipore, Billerica, MA) were decontaminated in a laminar flow hood (NuAire, Plymouth, MN) using an ultraviolet lamp (UVP, Upland, CA) for at least 30 min per side. The membranes were placed on TSA or Congo red agar (CRA) using flame-sterilized forceps, 5 per plate. CRA was prepared by adding Congo red (Sigma-Aldrich, St. Louis, MO, USA) to molten TSA at a concentration of 0.8 g/L. The center of each membrane was inoculated with 5 μL of the bacterial broth culture, ensuring that only one droplet was delivered to each membrane and that the broth did not leak off the membrane. This was repeated to prepare the appropriate amount of membranes for the study. After the inoculum dried, the plates were inverted and incubated at 30–35 °C for approximately 24 h. After the first 24 h of incubation, the membranes were transferred to a fresh TSA or CRA plate using sterile forceps, taking care not to touch the growth on the membrane. The membranes were incubated at 30–35 °C for an additional 7 days (for a total of 8 days of incubation after inoculation). To prevent desiccation of the agar, the plates were placed in an unsealed zipper storage bag.

2.1.3. Biofilm sampling

The biofilms on the membranes were sampled in duplicate at various time points during the incubation period - approximately 4 h after inoculation and at 1 d, 2 d, 3 d, 4 d, 7 d, and 8 d. Using sterile forceps, randomly selected membranes were removed from the agar and gently immersed in approximately 10 mL sterile water (Baxter Healthcare Corporation, Deerfield, IL) to remove any planktonic or loosely attached cells.

2.2. Biofilm analysis

2.2.1. Bacterial enumeration

The biofilms grown on TSA were enumerated as follows. The membranes were aseptically transferred to individual glass tubes containing 10 mL sterile 1% Tween 80 (Sigma-Aldrich, St. Louis, MO) and 3–4 sterile 6 mm glass borosilicate beads (Kimble Chase, Rockwood, TN). Biofilm removal and disaggregation was performed in a series of five 30 s steps, alternating vortexing at the highest setting with sonication at 40 kHz for 30 s (Branson, Danbury, CT), i.e. tubes are vortexed, sonicated, vortexed, sonicated, and vortexed.

The 10 mL disaggregated cell suspension was split into two new test tubes, with 5 mL fractions transferred to each. One tube was heat shocked at 98 ± 2 °C for 15 min, a process that is lethal to vegetative cells, but not spores. To do so, a recirculating water bath (Cole Parmer, Vernon Hills, IL) was heated to 100 °C as measured by a calibrated thermometer. The tubes undergoing heat shock were placed in the bath along with a tube containing 5 mL 1% Tween 80 and a thermocouple to monitor the temperature. A timer was started when the thermocouple reading reached 96 °C. After heat shocking for 15 min, the tubes were placed on ice for at least 5 min. The heat shocked tubes provided the concentration of spores per membrane. The non-heat shocked tubes were enumerated to determine the total population, reported as total CFU per membrane. The total population includes vegetative cells and spores.

All samples were serially diluted in sterile water and plated on TSA in duplicate. The plates were incubated at 30–35 °C for a minimum of 24 h. After incubation, the colonies on each plate were counted and duplicate plate counts averaged. The Log_{10} total CFU or spores per membrane, here after referred to as log total CFU or log spores respectively, was calculated using the following equation:

$$\mathrm{LOG}_{10}(\text{total CFU/membrane) or (spores/membrane)} = \mathrm{LOG}_{10}[(X/V)*(10\ \text{mL}/D)]$$

where:

X = mean plate count
V = volume plated
D = dilution.