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Note

High-density, homogeneous endospore monolayer deposition on test surfaces

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ABSTRACT

Bacillus subtilis spores were deposited in high-density single layers on metal, glass, and polymer substrates using vacuum filtration followed by a wetted filter transfer step. Quantitative analysis of spore transfer was performed using culture-based and germinability assays, and spore distributions were observed with electron microscopy.

The ability to create a high-density single layer of microparticles is useful in microbiology (Rogers et al., 2005; Rastogi et al., 2009), biochemical assays (Dugas et al., 2005), ink-jet printing (de Gans et al., 2004; Park and Moon, 2006), and astrobiology (Tauscher et al., 2006). Here we present a method for depositing *Bacillus subtilis* spores onto metal, glass, and polymer test surfaces to enable spore inactivation studies with relevance to spacecraft bio-burden reduction (i.e., planetary protection) (Tauscher et al., 2006; Schubert and Beaudet, 2011), decontamination after a bioterror attack (Rogers et al., 2005), and hospital sterility assurance (Rutala et al., 1993; Oie et al., 2011).

The most common deposition method for both spores and other microparticles is the droplet evaporation method where droplets of the suspension are allowed to evaporate on a surface leaving only the solute behind. This method leads to the common problem known as "the coffee-ring effect (named for the characteristic ring-like deposit left from a spill of coffee)," where the colloidal solute is preferentially deposited in a ring along the original droplet perimeter (Fig. 1a). The effect is primarily due to contact line pinning, which prevents a reduction in the droplet diameter during evaporation and results in edge-ward capillary flow (Deegan et al., 1997, 2000).

There are a number of approaches for combating the coffee-ring effect including changing the solvent–solute relationship (Park and Moon, 2006), electrowetting (Eral et al., 2011), changing the shape of the particles or adding a surfactant (Yunker et al., 2011; Still et al., 2012), and changing the drying conditions (Majumder et al., 2012). However, these methods are not suitable for use with spore applications as water is the preferred solvent and the shape of the spore cannot be altered; furthermore, the addition of a surfactant may cause spores to germinate (Setlow, 2003), or leave an undesired chemical film on the spores.

Aerosol deposition of spores and cells to create desired test surfaces has also been investigated (Brown et al., 2007; Baron et al., 2008; Heimbuch et al., 2009; Lee et al., 2011). These methods have the advantage of working on a variety of different surfaces, but generally require specialized equipment, cannot easily achieve densities greater than ~10^5 spore/cm^2 (Brown et al., 2007; Heimbuch et al., 2009), suffer from inconsistency and lengthy prep time (Baron et al., 2008), or result in stacked layers of spores (Lee et al., 2011).

A proven way to achieve high density layers, while avoiding spore stacking is through vacuum filtration of a spore suspension (Schiza et al., 2005; Brooke et al., 2008). In order to facilitate quantitative studies of spore survival and behavior, we developed two closely related methods to transfer the spore layer created by vacuum filtration to a variety of surfaces of interest (with the requirement that the surface of the substrate have a mirror polish or micron level smoothness).

For this work stock suspensions of *B. subtilis* spores (ATCC 27370) were prepared as described previously (Yung and Ponce, 2008). While *B. subtilis* spores were the only species tested, the results described should apply to most spores due to their similar properties; however, species with substantial amounts of extracellular polymeric material could behave differently. Total endospore concentrations of the stock were determined using a Petroff–Hausser hemocytometer. The methods were tested on 4 mm diameter stainless-steel tabs, 12.5 mm aluminum mirrors, borosilicate glass microscope slides, and ~3 × 1 cm rectangular pieces of polydimethylsiloxane (PDMS) (Rogers Corp. HT-6240-.010 Transparent). Transfer to the tabs was analyzed both qualitatively and quantitatively using an environmental scanning

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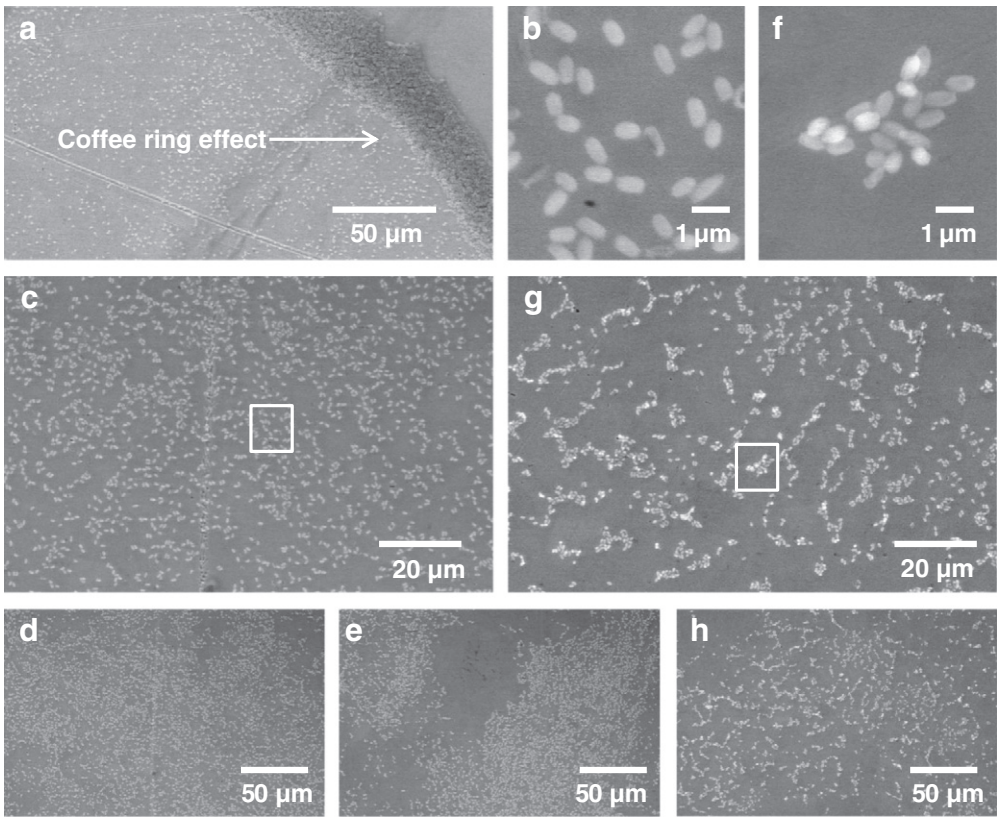

Fig. 1. ESEM images of *B. subtilis* spores on stainless steel tabs. a. An example of the coffee ring effect where spores are piling up at the contact line of an evaporated droplet. b–c. A typical distribution of spores for the wet–wet method at two different magnifications. d–e. An example of the inconsistency sometimes observed over larger distance scales with the wet–wet method. f–h. A typical distribution of spores for the wet–dry method at three different magnifications. The application is more consistent over larger distances than the wet–wet method, but the drying leads to a more clustered distribution on a smaller scale.

electron microscope (ESEM) (Philips, XL 30 ESEM-FEG) and culturing by TSA spread plating in triplicate. The ESEM was operated at 4 torr H_2O using a 20 keV beam with a spot size of 3, and a gaseous secondary electron (GSE) detector. Hi-Res, 1424 × 968 pixel images were acquired after manual adjustment of the contrast. Spores were recovered from the tabs for culturing by 2 min of sonication (40 kHz, Branson 3510) in water. Initially, using the ratio of filter size to tab size and assuming 100% transfer, a concentration range of 3.5×10^6–1.2×10^2 spores/tab was prepared to determine both transfer efficiency and linearity. This was compared against a droplet evaporation method of deposition where the exact number, but not the distribution, of spores can be controlled. The maximum density of spores that could be filtered and then transferred without spores stacking on top of each other was determined by visual inspection of ESEM images at the highest concentrations filtered. These concentrations, based on the filter-to-tab size ratios, were increased by intervals of 5×10^5 spores from 1.5×10^6–2.5×10^6 spores/tab. The true upper limit density, factoring in the transfer efficiency, was quantified by both culturing and enumerating spores in the images. After the maximum density was determined, transfer efficiency to both borosilicate glass microscope slides and PDMS was quantitated at that highest filter density. TSA spread plating was used to quantitate spore transfer to borosilicate glass microscope slides, and transfer efficiency to the PDMS was determined by enumerating germinable spores (Yung and Ponce, 2008).

The two methods developed were a "wet–wet" application and a "wet–dry" application; in both methods, the filter must be saturated prior to contact with the substrate, and then either removed while still wet ("wet–wet") or dried ("wet–dry") in place before removal. Transfer to metal surfaces worked with either technique, while transfer to glass only worked reproducibly with the wet–dry technique and transfer to polymer surfaces worked best with the wet–wet technique.

The initial steps of both methods are as follows: 1. Prepare a well-mixed solution of the total number of desired spores in 150 mL sterile water. 2. Setup a vacuum filtration apparatus (a 47 mm VWR coarse (40–60 μm pores) glass support frit was used in this work) with a 0.2 μm polycarbonate (PC) membrane filter (Nuclepore Track-Etch Membrane, Whatman # 111606) on top of a backing filter (Whatman #2 Qualitative filter paper). 3. Vacuum-filter the spore suspension to create a single layer. 4. Remove the PC membrane from the apparatus and place it onto a previously saturated backing filter paper (we used the Whatman #2 qualitative paper again) in a petri dish, spore side up. 5. To transfer the spores to a secondary substrate, move the saturated PC membrane from the backing filter to the substrate of choice and place the spore side in contact with the substrate in a smooth even fashion. It is critical that the PC membrane remains saturated while making contact between the surfaces in order to facilitate a full and reproducible transfer. The smoothness of the substrate surface is also critical here to allow for even contact everywhere with the filter.

Then for a. the wet–dry method:

6a. The back side of the membrane is then dried with gaseous nitrogen (or air) and the membrane is removed from the substrate after drying is complete, leaving the spore layer behind.

Or for b. the wet–wet method:

6b. The still wet membrane should be pulled carefully back up off of the substrate leaving the spore layer behind. This removal requires some practice in order to prevent droplets being left behind on the surface. When done carefully and consistently the removal should leave only one small droplet at the final point of contact between the filter and the substrate.