Vesicle Isolation from *Bacillus subtilis* Biofilm

Lisa Brown[1]*, Anne Kessler[2] and Arturo Casadevall[1, 3]

[1]Department of Microbiology and Immunology, [2]Department of Pathology and [3]Department of Medicine, Albert Einstein College of Medicine, New York, USA

*For correspondence: Lisa.Brown@phd.einstein.yu.edu

[Abstract] Bacterial biofilms are associated clinically with many bacterial infections including those caused by bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In recent years, extracellular vesicles produced by bacteria have been isolated from biofilm communities. Vesicles have been described in depth and can encapsulate various virulence factors including toxins and immunomodulatory compounds. Vesicles may be important for virulence and survival by serving as a vehicle for the secretion and concentrated delivery of these molecules. Studying extracellular vesicles is an important step towards understanding biofilm formation, structure, and disruption with the ultimate goal of preventing or treating hospital infections caused by bacterial pathogens residing in biofilms. Here we describe the protocol for isolating vesicles from biofilm produced by *Bacillus subtilis.*

Materials and Reagents

1. *Bacillus subtilis* (*B. subtilis*) 168 bacterial strain (available from ATCC, catalog number: 23857)
2. BHI broth/agar (BD, catalog number: 211059/211065)
3. Amicon Ultra centrifugal filter units Ultra-4 (MWCO 100 kDa) (Millipore, catalog number: UFC910024)
4. 1x phosphate buffered saline (PBS) (see Recipes)
5. MSgg medium (see Recipes)

Equipment

1. Petri dishes (100 x 55 mm) (Corning, catalog number: 351029) (dish size can be variable and ultimately depends on the amount of vesicles you wish to purify)
2. 0.22 µm syringe filters (Fisher, catalog number: 09-719C)
3. Tube (thickwall, polyallomer, 3.5 ml, 13 x 51 mm) (Beckman Coulter, catalog number: 349623)
4. Optima TL ultracentrifuge (Beckman Coulter)
5. TLA 100.3 rotor (Beckman Coulter)

6. Centrifuge capable of 15,000 *x g*

Procedure

A. Biofilm growth assay
   1. Inoculate a plate of *Bacillus subtilis* lab strain 168 (from -80 °C) on a BHI agar plate overnight (~18 h) at 37 °C.
   2. Inoculate 5 ml BHI broth from overnight agar plate (one colony) and grow for 4 h with shaking (~200 rpm) at 37 °C.
   3. Inoculate 1:1,000 of broth culture (12 µl) into 12 ml MSgg media in petri dishes.
   4. Let biofilm incubate (covered with dish lid without shaking) at 37 °C for desired time period (usually 3 and 7 days). Ensure that incubator has a water container to ensure the dishes do not dry out.

B. Purification of vesicles from biofilm
   1. After desired time period, remove pellicle and supernatant from plates by pipetting and centrifuge at 15,000 *x g* for 20 min at 4 °C to remove cells and large debris.
   2. To remove remaining cells and debris, filter the spun supernatant with 0.22 µm syringe filters.
   3. Centrifuge filtered, cell-free supernatant with centrifugal filter units to concentrate to less than 3 ml in volume.
   4. To pellet vesicles from supernatant, ultracentrifuge concentrated supernatant at 195,000 *x g* for 1 h at 4 °C in polyallomer tubes.
   5. Remove supernatant without disturbing the vesicle pellet by pipetting.
   6. Repeat spin after washing in 500 µl PBS.
   7. Remove PBS wash from tube without disturbing the vesicle pellet.
   8. Resuspend pellet in desired volume of PBS.

Notes

1. *Bacillus subtilis* grows as a biofilm pellicle on the surface of the media (Figure 1). It may need a few washings to remove all cells for vesicle collection. Figure 2 shows electron micrograph of negatively stained vesicles.
2. Biofilms can be grown in various plates to achieve desired volume of vesicles. Ideally you want to have a visible pellet after ultracentrifugation. If no pellet is visible, scale up the plate size or number of biofilms for vesicle purification.

3. Vesicle pellet after ultracentrifugation should be visible, but in cases of small culture volumes the pellet may be difficult to see.

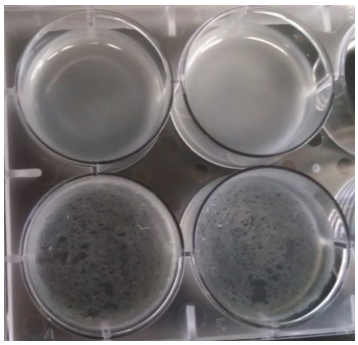

Figure 1. Representation of what biofilm pellicles may look like in a 6-well plate

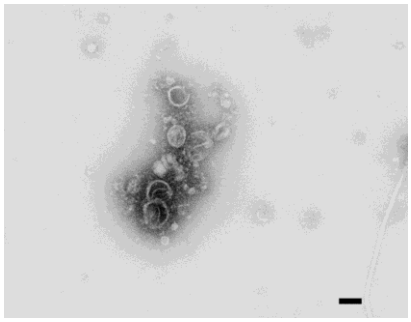

Figure 2. Negative stain TEM of vesicles isolated from *Bacillus subtilis* biofilm. Scale bar = 100 nm

Recipes

1. 1x phosphate buffered saline (PBS) (1 L, pH 7.4) (filter or autoclave sterilize)
   137 mM NaCl
   2.7 mM KCl
   10 mM Na_2HPO_4
   1.8 mM KH_2PO_4
   Bring to pH 7.4 with HCl
   Dissolve in H_2O up to 1 L
2. MSgg medium (pH 7) (filter sterilize)
   50 µM $MnCl_2$
   5 mM KH_2PO_4
   1 µM $ZnCl_2$

50 μM $FeCl_3$
2 mM $MgCl_2$
700 μM $CaCl_2$
50 μg/ml threonine, tryptophan, and phenylalanine
0.5% glutamate
0.5% glycerol
2 μM thiamine
100 mM morpholinepropanesulfonic acid (MOPS) (pH 7)

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Protocol was adapted from Brown *et al.* (2014) and McLoon *et al.* (2011).

References

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