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Isolating and Purifying *Clostridium difficile* Spores

Adrianne N. Edwards and Shonna M. McBride*

Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA 30322

Summary

The ability for the obligate anaerobe, *Clostridium difficile*, to form a metabolically dormant spore is critical for the survival of this organism outside of the host. This spore form is resistant to a myriad of environmental stresses, including heat, desiccation and exposure to disinfectants and antimicrobials. These intrinsic properties of spores allow *C. difficile* to survive long-term in an oxygenated environment, to be easily transmitted from host-to-host and to persist within the host following antibiotic treatment. Because of the importance of the spore form to the *C. difficile* lifecycle and treatment and prevention of *C. difficile* infection (CDI), the isolation and purification of spores are necessary to study the mechanisms of sporulation and germination, investigate spore properties and resistances, and for use in animal models of CDI. This chapter provides basic protocols, *in vitro* growth conditions and additional considerations for purifying *C. difficile* spores for a variety of downstream applications.

Keywords

Clostridium difficile; sporulation; endospore; anaerobe; anaerobic chamber; antibiotic-associated diarrhea

1. Introduction

Clostridium difficile is a significant gastrointestinal pathogen of humans and other animals and is the primary agent of antibiotic-associated diarrhea. *C. difficile* infection (CDI) is responsible for billions of dollars in healthcare costs and causes greater than 14,000 deaths annually in the United States alone [1, 2]. *C. difficile* was recently named an urgent public health threat by the United States Centers for Disease Control and Prevention [1]. This anaerobic organism survives outside of its host by forming a metabolically inactive spore. Spore formation is a complex process in which a vegetative cell forms a dormant structure that protects *C. difficile* from exposure to oxygen, heat, disinfectants and desiccation and provides inherent resistance to antibiotics and other antimicrobials. Once ingested by the host, the spore germinates in the presence of bile salts [3], producing the vegetative form of the bacterium. The vegetative cell reproduces and secretes toxins, which cause disease symptoms. As the vegetative cells transit through the gastrointestinal tract, unknown host and intracellular signals spur sporulation. Once shed from the host in feces, *C. difficile*

*Corresponding author: shonna.mcbride@emory.edu.

spores can survive in the environment for long periods of time and are an enormous obstacle in preventing CDI in short-term and long-term healthcare facilities.

Because *C. difficile* spores are the infectious form of the organism, they are used to study infection in animal models of CDI [4-6] and to perform molecular biological experiments to discover sporulation and germination characteristics, resistances and susceptibilities [7-9]. The emergence of the epidemic 027 ribotype strain [10] has underscored the need for further studies on spore properties and the development of antimicrobials that directly target the *C. difficile* spore.

Here, we describe how to elucidate the best conditions to optimize the production of *C. difficile* spores *in vitro*. We provide protocols that allow for the isolation of *C. difficile* spores and for further spore preparation purification, necessary for some downstream applications, of spore preparations. These protocols rely on the intrinsic heat, cold and chemical resistances of *C. difficile* spores to kill vegetative cells and separate these and other debris from the spores. Finally, conditions and additional considerations for the use and long-term storage of *C. difficile* spores are discussed. Propagation and purification of *C. difficile* spores is relatively simple as long as a strict anaerobic environment is provided during *C. difficile* growth and spore formation.

2. Materials

For reliable growth of *C. difficile*, is it critical to pre-reduce all liquid and solid media in the anaerobic chamber for at least two hours before use. Handle all materials with appropriate aseptic technique to prevent contamination from other prevalent spore forming species (e.g. *Clostridium perfringens*).

2.1. Culture Media

1. Brain Heart Infusion Supplement (BHIS) Medium: Dissolve 37 g brain heart infusion extract (BD Difco) and 5 g yeast extract (BD Difco) in 800 ml dH₂O. Bring to 1 L with dH₂O. Autoclave for 30 min and store at room temperature. Add 15 g agar per liter before autoclaving for 1.5% (w/v) agar plates.
2. BHIS medium supplemented with 0.1% taurocholate: Prepare medium as described above and autoclave. To prevent the growth of contaminating *Mycoplasma* species on the surface of plates containing taurocholate (which is a cholesterol derivative [11]), add 10 ml of 10% taurocholate to 1 L BHIS when the autoclaved medium reaches ~60-65°C. Incubate medium with 0.1% taurocholate for 20 min as the medium cools, before pouring plates.
3. 70:30 Sporulation Medium [12; Note 1]: Dissolve 63 g Bacto peptone (BD Difco), 3.5 g proteose peptone (BD Difco), 0.7 g ammonium sulfate

¹ 70:30 sporulation medium is a mixture of 70% SMC medium [14, 25] and 30% BHIS medium and was specifically developed to enhance and reliably determine the sporulation frequency of *C. difficile* [12].

((NH₄)₂SO₄), 1.06 g Tris base, 11.1 g brain heart infusion extract (BD Difco) and 1.5 g yeast extract (BD Difco) in 800 ml dH₂O. Bring to 1 L with dH₂O. Autoclave for 30 min; once medium is cool to touch, add 3 ml 10% (w/v) cysteine (dissolve 0.4 g cysteine in 4 ml dH₂O and filter sterilize with a 0.45 µm filter; see Note 2). Add 15 g agar per liter before autoclaving for 1.5% (w/v) agar plates; use 35 ml per plate for consistent sporulation results. Important: 70:30 sporulation medium provides the best conditions for efficient *C. difficile* sporulation if used fresh (within a week; see Note 3).

2.2. Media Supplements

1. Taurocholate: To induce germination of *C. difficile* spores, 0.1% (w/v) taurocholate must be supplemented in BHIS medium [13, 14]. Prepare a stock solution of 10% (w/v) taurocholate (1 g taurocholate dissolved in 10 ml dH₂O) and filter sterilize with a 0.22 µm filter. Store at room temperature.
2. Fructose: To prevent high rates of sporulation of *C. difficile* in overnight cultures (unpublished data, Edwards and McBride), 0.2% (w/v) fructose may be added to BHIS medium. Prepare a stock solution of 20% (w/v) D-fructose (2 g fructose dissolved in 10 ml dH₂O) and filter sterilize with a 0.45 µm filter. Store at room temperature.
3. Antibiotic supplements: The use of antibiotics can influence spore formation in *C. difficile* [15]; however, antibiotic supplements are necessary for maintaining plasmids. Using the lowest concentration of antibiotic necessary for stable plasmid maintenance is strongly recommended (e.g. use a final concentration of 2 µg/mL thiamphenicol [15] instead of the commonly used 10-15 µg/mL thiamphenicol [16, 17]).

2.3. Solutions for *C. difficile* spore stocks

1. 10X Phosphate Buffered Saline (PBS) Stock Solution: Dissolve 25.6 g Na₂HPO₄ · 7H₂O, 80 g NaCl, 2 g KCl and 2 g KH₂PO₄ in 800 ml dH₂O. Bring to 1 L. Autoclave for 30 min and store at room temperature.
2. 1X PBS: Dilute 10X PBS Stock Solution 1:10 (100 ml 10X PBS into 900 ml dH₂O). Sterilize by autoclaving for 30 min or using a 0.45 µm vacuum filter. Store at room temperature.
3. 1X PBS + 1% (w/v) BSA: Dissolve 10 g bovine serum albumin (BSA) into 1 L 1X PBS. Sterilize only by using a 0.45 µm vacuum filter as BSA

²A stock solution of 10% cysteine must be prepared fresh as cysteine will precipitate when stored at room temperature or cooler.

³At H₂₄ (24 h after plates are inoculated with an actively growing *C. difficile* culture), sporulation efficiency is ~30% for most strains tested when grown on 70:30 sporulation medium agar plates (Figure 1; unpublished data, Edwards and McBride), which is similar to previously observed sporulation frequencies on 70:30 sporulation plates for similar strains [12]. For unknown reasons, the sporulation efficiency significantly decreases after 70:30 sporulation medium is a week old. To obtain consistent results, we suggest using plates that are no more than three days old.

denatures and precipitates in high heat and pressure. Store at room temperature.

2.4. Additional reagents

1. 95% ethanol
2. 0.7% (w/v) agarose: Dissolve 0.7 g agarose in 100 ml of dH₂O and heat by stir plate or microwave to fully solubilize agarose. The agarose solution can be stored as a solid at room temperature and be reheated for subsequent use or as a liquid at 60°C for long-term storage.
3. 50% (w/v) sucrose: Dissolve 50 g in 100 ml dH₂O. Filter sterilize.

3. Methods

One of the more vital considerations to remember when working with *C. difficile* spores is that their hydrophobic and anionic properties allow them to adhere to many surfaces, including pipet tips and polypropylene. When working with spore stocks, it is important to pipet up and down several times, especially when making dilutions, to ensure as few spores as possible are left behind. The use of polypropylene materials, such as conical tubes, is sufficient when working with high concentrations of spore stocks (greater than 10⁷ colony forming units (CFU)/ml) for a short period of time; however, for long-term storage of high concentration spore stocks and when diluting spores to a concentration of 10⁶ CFU/ml or less, the use of glass [15] or Teflon-coated tubes [18] is highly recommended to prevent loss and obtain reproducible spore counts. Finally, the use of 1% BSA in final spore preparations decreases clumping of spores, prevents loss and facilitates accurate enumeration [15].

While it is not necessary to use phase contrast microscopy to track sporulation, as described in Section 3.1, the use of this method is a reliable and efficient way to determine the progress of sporulation without removing the plates from the anaerobic chamber and interrupting an experiment. This technique allows for the accurate enumeration of sporulation efficiency in various conditions and at different time points before embarking on the more time-consuming spore isolation and purification protocol.

3.1 Growth and sporulation efficiency of *C. difficile* on various media

This protocol describes the general methods needed for determining the best sporulation conditions in preparation for isolating and purifying spore stocks. Methods for general cultivation of *C. difficile* and anaerobic chamber maintenance can be found elsewhere [19, 20].

1. Streak strain(s) from glycerol stock(s) onto BHIS medium agar plates and incubate overnight at 37°C.
2. Inoculate 10 ml BHIS supplemented with 0.1% taurocholate and 0.2% fructose from a single colony using a sterile inoculating loop (see Note 4). Incubate overnight at 37°C.

3. In the morning, while the overnight culture is still in active growth ($OD_{600} < 1.0$; again, see Note 4), backdilute the cells to an OD_{600} of 0.5 with BHIS. Spread 250 μ l of culture onto a fresh, pre-reduced 70:30 agar plate and incubate anaerobically at 37°C for a minimum of 24 h.
4. After a minimum of 24 h, take a small sample from the plate with a sterile inoculating loop and resuspend in 500 μ l BHIS in a microcentrifuge tube. Remove sample(s) from the anaerobic chamber (see Note 5).
5. Centrifuge the cells at 13000 rpm for 30 sec at room temperature and decant the supernatant. Resuspend the cells in 10 – 50 μ l BHIS.
6. Prepare a microscope slide by applying a small amount (< 0.5 ml) of 0.7% agarose in the center of slide (see Note 6). Allow to solidify for ~2 min.
7. Apply 5 μ l of resuspended culture to the agarose pad and carefully place a coverslip over the sample.
8. Observe cells by phase contrast microscopy (see Note 7). Mature spores will appear phase bright while vegetative cells and prespores will appear phase dark and phase gray (partially refractile), respectively (Figure 1). Ensure that the field of view contains only a single layer of cells, as overlapping vegetative cells may falsely appear phase bright.
9. To determine the sporulation efficiency, enumerate the number of phase bright spores and phase dark vegetative cells and use the equation detailed below:

$$\text{Sporulation efficiency (\%)} = \frac{\text{Spores}}{\text{Vegetative cells} + \text{Spores}} * 100$$

Count at least 1000 cells to obtain an accurate representation of the population.

10. This protocol can be performed and repeated on different types of medium and at multiple time points to determine the best conditions for sporulation for the strain or mutant of interest (Figure 1; see Note 8).

⁴We achieve the most reliable and reproducible results in a variety of molecular biological assays by starting with *C. difficile* cultures that are in exponential phase. Oftentimes, overnight cultures of *C. difficile* will have already reached stationary phase after 12-16 h of growth. To combat this, we immediately dilute our initial, already-inoculated overnight culture 1:500 into another test tube with 10 ml BHIS supplemented with 0.1% taurocholate and 0.2% fructose. This results in two overnight cultures: the original culture and a 1:500 dilution of the original culture.

⁵Because the protocol from this point is performed outside of the chamber, the use of pre-reduced BHIS medium is not important in this step. However, if the final time point has been reached or the plates will no longer be needed, the plates can be removed from the chamber, and this step may be performed in aerobic conditions.

⁶The use of a thin agarose pad (0.7-1.0% agarose) immobilizes the bacteria for microscopy. We caution against the use of higher percentages of agarose as this decreases the amount of visible light that can be seen through the sample.

⁷Phase contrast microscopy allows the observation of the later stages of sporulation (Stage IV+) in endospore-forming bacteria [26, 27] and is a frequently used technique to observe and quantitate sporulation frequencies in *C. difficile* [12, 15, 28-31]. Although this technique requires special equipment, it is easy to perform, does not require special dyes, sample fixation or preparation and is relatively inexpensive beyond the initial cost of the equipment. For the most accurate results, 100X magnification with oil immersion is needed.

3.2 Isolation of *C. difficile* spores from solid medium

This method relies on the intrinsic alcohol and heat resistance of *C. difficile* spores to kill vegetative cells present in the spore stock. Importantly, this protocol does not remove lysed or killed vegetative cells or debris; however, this is not necessary for many downstream applications, including most animal studies [15]

1. Once the optimal sporulation conditions are determined for the *C. difficile* strain(s) of interest (Section 3.1), inoculate 3-10 fresh agar plates with an actively growing overnight culture, as outlined in Section 3.1, and incubate for the appropriate amount of time.
2. At the appropriate time point(s), verify spore formation via phase contrast microscopy as detailed above.
3. Remove plates from the anaerobic chamber. Scrape off the entire bacterial lawn using a sterile inoculating loop and resuspend in 10 ml 1X PBS. Vortex well to thoroughly resuspend the cells.
4. Centrifuge the cells at 3000 rpm for 10 min at room temperature.
5. Decant the supernatant and thoroughly resuspend the cell pellet in 10 ml 1X PBS.
6. Add 10 ml of 95% ethanol to the resuspended cell pellet, vortex well and incubate at room temperature for 1 h.
7. Centrifuge the cells at 3000 rpm for 10 min at room temperature.
8. Decant the supernatant and resuspend in 20 ml 1X PBS. Wash twice more in 20 ml 1X PBS to ensure complete removal of residual ethanol.
9. Resuspend the cell pellet in 10 ml 1X PBS + 1% BSA after final wash.
10. Heat spore stock to 70°C for 20 min (see Note 9). Allow spore stock to cool to room temperature. Time permitting, immediately enumerate the spore stock (see Section 3.4).

3.3 Purification of *C. difficile* spores

Some downstream applications, including spore germination and outgrowth studies [7, 9], require pure spore stocks in which the spores are physically separated from vegetative cells, lysed mother cells and other debris. This protocol describes a slightly different purification method than described in Section 3.2, and utilizes a density gradient centrifugation step to obtain free *C. difficile* spores [7, 9, 21].

⁸Some strains do not always grow or sporulate well on 70:30 sporulation medium. Spores for these strains can instead be propagated on BHIS or TY agar plates. To increase the production and recovery of spores on these richer media, incubate plates for 4-5 days before harvesting.

⁹To ensure that the 10 ml volume of spores is thoroughly heated to 70°C, we have found it easier to divide the spores into 10 – 1 ml aliquots in microcentrifuge tubes and heat the spore preparation using a benchtop heat block. Once this step is completed, the aliquots are then recombined into a fresh conical tube.

1. Propagate spores as described in Section 3.1 to achieve the highest sporulation efficiency possible. At the appropriate time point(s), verify spore formation via phase contrast microscopy as detailed above.
2. Remove plates from the chamber and resuspend cells from one-half of a plate in 1 ml sterile, ice cold dH₂O. Repeat for the remaining half.
3. Centrifuge at 13000 rpm for 2 min at 4°C. Carefully remove supernatant and resuspend in 1 ml sterile, ice cold dH₂O. Wash with sterile, ice cold dH₂O two more times.
4. Incubate the spore preparation at -20°C for 48 h to facilitate the lysis of mother cells and subsequent release of mature endospores.
5. After incubation, centrifuge at 13000 rpm for 2 min at 4°C, decant the supernatant and resuspend the spore preparation with 1 ml sterile, ice cold dH₂O. Repeat for a total of five washes in sterile, ice-cold dH₂O as described in Step 3.
6. After the final wash, combine both cell pellets in 3 ml sterile, ice cold dH₂O.
7. Apply the entire 3 ml spore preparation slowly to the top of a 10 ml 50% sucrose gradient in a 15 ml polypropylene conical tube.
8. Centrifuge in a swinging bucket rotor at 4000 × g for 20 min at 4°C. The vegetative cells and debris will collect at the interface and throughout the gradient, while the spores move through the gradient and form a pellet at the bottom of the centrifuge.
9. Remove the cell debris at the interface, then remove the rest of the solution, leaving the spore pellet.
10. Resuspend the spore pellet in 1 ml of sterile dH₂O. Centrifuge at 13000 rpm for 2 min at room temperature, decant the supernatant and resuspend the spore preparation in 1 ml sterile dH₂O.
11. Repeat step 10 for a total of five washes in sterile dH₂O and three washes in 1X PBS + 1% BSA.
12. Resuspend the final spore preparation in 1 ml 1X PBS + 1% BSA and quantify as described in Section 3.4.

3.4 Enumeration of initial spore stock and serial dilutions

To determine the number of viable spores per ml for the spore preparation, a range of serial dilutions of the primary, highly concentrated spore stock are plated and enumerated. The range of serial dilutions required to achieve accurate enumeration of a spore stock depends on the sporulation efficiency of the strain, the type and number of agar plates used for spore propagation, and the length of time the plates are incubated before beginning spore isolation and purification. Remember: when making serial dilutions of spores, vortex each dilution

well and pipet up and down multiple times to reduce the retention of spores on tube walls and in pipet tips.

1. Pre-reduce the same number of BHIS supplemented with 0.1% taurocholate agar plates as the number of serial dilutions performed.
2. Vortex the spore stock vigorously for 15 s.
3. Prepare a series of 10-fold dilutions in 1X PBS + 1% BSA. Dilute 100 µl of the spore stock into 900 µl 1X PBS + 1% BSA (which results in the 10^{-1} dilution). Dilute 100 µl of the 10^{-1} dilution into 900 µl 1X PBS + 1% BSA (which results in the 10^{-2} dilution). Continue until the appropriate dilutions have been made (see Note 10 for an example).
4. Heat the dilutions to 55°C for 20 min. Important: If you are performing these dilutions immediately after Step 10 in Section 3.2, the dilutions do not have to be reheated. However, a small proportion of spores become “inactivated” over time, and reheating the spore stocks immediately before plating assists in *in vitro* germination and provides more accurate representation of spores.
5. Cycle the reheated, serial dilutions into the anaerobic chamber. Apply 100 µl of each serial dilution onto individual pre-reduced BHIS supplemented with 0.1% taurocholate agar plates and evenly spread the dilution onto the surface of the plate using a sterile inoculating loop.
6. Incubate plates at 37°C for at least 24 hours to allow spore germination and sufficient growth of a colony.
7. Remove plates from chamber. For most accurate enumeration, use a plate that has between 30-300 colonies with few to no overlapping or clustered colonies (see Note 11).
8. Calculate the number of spores per ml of spore stock using the following equation:

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{Number of colonies (CFU)}}{\text{Dilution factor} * \text{Volume plated (ml)}}$$

9. Finally, one last concern with the high concentration spore stocks is the presence of contaminants. Optionally, a small amount of the spore stock (50-100 µl) may be plated onto i) a BHIS agar plate and incubated aerobically and ii) a pre-reduced BHIS agar plate and incubated anaerobically to ensure no growth of non-*C. difficile* bacteria occurs. It is

¹⁰When initially enumerating a new spore stock, we typically plate 100 µl of 10^{-4} , 10^{-5} and 10^{-6} dilutions of the original spore stock. With most strains, we easily obtain spore stocks of 1×10^8 CFU/ml from three to four 70:30 sporulation medium agar plates incubated for 48-72 h. For example, with a spore stock of this concentration, we obtain approximately 100 CFU on a BHIS supplemented with 0.1% taurocholate agar plate inoculated with 100 µl of a 10^{-5} dilution.

¹¹Before removing plates from the chamber, ensure that all of the colonies are large enough to be accurately counted. If not, allow colonies to grow longer in the anaerobic chamber. While counting, we find placing the plate on top of a light source and using a digital colony counter allows the accurate enumeration of smaller and clustered colonies and reduces user error.

important to note here that a small number of *C. difficile* spores will germinate in the absence of taurocholate, so it is possible to see some *C. difficile* colonies on the BHIS agar plate incubated anaerobically. This will vary based on the strain of *C. difficile* used.

3.5 Preparation of final working spore stocks

To create a working spore stock, the original spore preparation needs to be diluted to the desired concentration. The working spore stock concentration will vary depending upon the downstream application (e.g. germination studies, spore outgrowth inhibition concentration (OIC) assays and animal studies all use a wide range of spore concentrations [3, 7, 9, 15, 22-24]).

1. Calculate the dilution required to achieve the needed working spore stock concentration using the following equation (solve for X):

$$\frac{\text{Original stock concentration (CFU/ml)} \times X \text{ ml original stock}}{\text{Desired working stock concentration (CFU/ml)} \times \text{Total volume of working stock required}}$$

Of note: it will likely be necessary to perform a few dilutions before making the final dilution of the final working spore stock.

2. Perform all dilutions using 1X PBS + 1% BSA as the diluent. Store the final working spore stock in glass or Teflon-coated tubes, as mentioned above, at room temperature. The shelf-life of spores varies by strain and stock, and should be retested if more than two weeks old. Remember to always reheat the spore stock to 55°C for 20 min before replating.

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Figure 1.

Phase contrast micrographs of *C. difficile* strains 630 [32, 33], R20291 [34], 5325 (ATCC BAA-1875) and VPI 10463 (ATCC 43255) grown on 70:30 sporulation medium agar plates or BHIS agar plates for the indicated amount of time (h). All strains were obtained from the ATCC except R20291, which was a gift from Linc Sonenshein. Images were corrected for exposure differences, which was applied uniformly across each image.