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Determination of the *in vitro* Sporulation Frequency of *Clostridium difficile*

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[Abstract] The anaerobic, gastrointestinal pathogen, *Clostridium difficile*, persists within the environment and spreads from host-to-host via its infectious form, the spore. To effectively study spore formation, the physical differentiation of vegetative cells from spores is required to determine the proportion of spores within a population of *C. difficile*. This protocol describes a method to accurately enumerate both viable vegetative cells and spores separately and subsequently calculate a sporulation frequency of a mixed *C. difficile* population from various *in vitro* growth conditions (Edwards *et al.*, 2016b).
Keywords: *Clostridium difficile*, *Clostridium difficile* infection (CDI), Anaerobe, Spores, Sporulation, Ethanol resistance

[Background] Sporulation is a complex developmental process that results in the formation of a metabolically dormant spore. The physical properties of the *C. difficile* spore form provide intrinsic resistance to many environmental stresses and disinfectants, permitting its long-term survival outside of the host (reviewed in: Paredes-Sabja *et al.*, 2014). To differentiate between the vegetative cells and spores of *C. difficile*, various techniques that take advantage of the physical and resistant properties of spores have been developed, including a short exposure to wet-heat or ethanol (Burns *et al.*, 2010; Lawley *et al.*, 2010; Edwards *et al.*, 2014). However, these techniques may inadvertently cause long-term damage to the spores, depending on the strain of *C. difficile* tested, resulting in inaccurate recovery rates. Here, we describe an optimized method using a lower concentration of ethanol than previously described (40% less ethanol) to eliminate all vegetative cells within a heterogeneous *C. difficile* population without reducing the viability of spores. This technique provides highly reproducible and less variable results for quantifying *C. difficile* spore formation.

Materials and Reagents

1. Sterile inoculating loops (Grenier Bio One, catalog number: 731170)
2. GeneMate 1.7 ml microcentrifuge tubes (BioExpress, catalog number: C-3260-1)
3. Petri dishes (94 x 16 mm) (Grenier Bio One, catalog number: 633161)
4. Glass test tubes with caps (18 x 150 mm) (Thermo Fisher Scientific, Fisher Scientific, catalog number: 14-961-32)

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5. 0.22 µm filter and syringe (for sterilization of taurocholate solutions) (CELLTREAT Scientific Products, catalog number: 229747)
6. 0.45 µm filter and syringe (for sterilization of D-fructose and L-cysteine solutions) (CELLTREAT Scientific Products, catalog number: 229749)
7. *Clostridium difficile* strains of interest, including the isogenic parent strain (*e.g.*, 630Δ*erm* or R20291) as a reference strain or positive control, test strains, and a negative control strain that is unable to sporulate, preferably in the same isogenic background as the parent (*e.g.*, a *spo0A* null mutant [Heap *et al.*, 2007; Dawson *et al.*, 2012; Deakin *et al.*, 2012; Fimlaid *et al.*, 2013; Mackin *et al.*, 2013; Edwards *et al.*, 2014; Edwards *et al.*, 2016a])
8. 95% ethanol (190 proof) (Decon Labs, catalog number: 2805SG)
9. Sterile water
10. Taurocholate (Sigma-Aldrich, catalog number: T4009)
11. Brain heart infusion (BHI) (BD, catalog number: 237300)
12. Yeast extract (BD, catalog number: 212730)
13. Agar for solid medium (BD, catalog number: 214010)
14. Bacto peptone (BD, catalog number: 211677)
15. Proteose peptone (BD, catalog number: 211684)
16. Tris base (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP152)
17. Ammonium sulfate [$(NH_4)SO_4$] (Sigma-Aldrich, catalog number: A5132)
18. L-cysteine (Sigma-Aldrich, catalog number: C7352)
19. Sodium chloride (NaCl) (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP358)
20. Potassium chloride (KCl) (Thermo Fisher Scientific, Fisher Scientific, catalog number: P217)
21. Sodium phosphate dibasic heptahydrate (Na_2HPO_4) (Thermo Fisher Scientific, Fisher Scientific, catalog number: S373)
22. Potassium phosphate monobasic (KH_2PO_4) (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP362)
23. D-fructose (Thermo Fisher Scientific, Fisher Scientific, catalog number: L96)
24. 10% (w/v) sodium taurocholate (see Recipes)
25. Pre-reduced BHIS agar (brain heart infusion supplemented with yeast extract; see Recipes)
    Note: All media needs to be reduced before use. This is achieved by bringing plates or liquid medium into the anaerobic chamber at least 2 h for plates or overnight for liquid medium before use (for additional details, see Edwards et al., 2013).
26. Pre-reduced BHIS agar supplemented with 0.1% taurocholate
27. BHIS liquid medium (see Recipes)
28. 70:30 sporulation agar (one plate per strain; see Recipes)
29. 1x PBS (see Recipes)
30. 20% D-fructose (see Recipes)
31. 10% L-cysteine (see Recipes)

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Equipment

1. Anaerobic chamber (Coy Type A or Type C Chamber)
   Note: All steps are performed within the anaerobic chamber unless otherwise noted. Details on C. difficile cultivation as well as use and maintenance of an anaerobic chamber are described in (Edwards et al., 2013).
2. Spectrophotometer (Biochrom, model: CO8000)
3. Autoclave

Procedure

1. Inoculate 10 ml BHIS medium supplemented with 0.1% taurocholate and 0.2% fructose with a single *C. difficile* colony from a plate and incubate overnight at 37 °C. Include selective antibiotics, if necessary for plasmid maintenance.
   Note: For additional details on basic C. difficile cultivation, including propagation from frozen glycerol stocks, see Edwards et al., 2013. Taurocholate is a germinant that will promote germination of any spores present in the C. difficile colony. Fructose is an additional carbon source that prevents sporulation and reduces the accumulation of spores in the overnight culture. To ensure that the cultures are in logarithmic growth phase in the morning, an additional overnight culture of each strain may be made by back diluting the original culture 1:200-1:1,000 after inoculation into the same medium conditions.
2. In the morning, ensure that cultures are actively growing in mid-exponential phase, *e.g.*, the OD_{600} is ≤ 0.9. If cultures are past an OD_{600} = 0.9, and thus, entering transition or stationary phase, it may be necessary to back dilute cultures into fresh BHIS to an OD_{600} < 0.5 to allow the cultures to outgrow and re-enter logarithmic growth before initiating the experiment (approximately 30-90 min, depending on the original density of the overnight culture). If cultures are below OD_{600} < 0.9, indicating that the cells are in logarithmic growth, continue with step 3.
3. Allow cultures to grow, or back dilute late exponential phase cultures (OD_{600} < 0.9) using fresh BHIS, to a final OD_{600} = 0.5. Apply 250 µl of culture onto the surface of fresh, pre-reduced 70:30 sporulation agar and gently spread evenly over the surface with a sterile inoculating loop. Mark the time as H_0 and incubate plates for 24 h at 37 °C.
4. Immediately perform H_0 ethanol resistance sporulation assays as a control to ensure no spores are present in the exponential phase cultures. Add 0.5 ml of the back diluted (OD_{600} = 0.5) culture to a microcentrifuge tube containing 0.3 ml 95% ethanol and 0.2 ml dH_2O. Vortex well and incubate for 15 min. Evenly spread 100 µl of each H_0 control onto pre-reduced BHIS supplemented with 0.1% taurocholate agar plates and incubate at 37 °C for at least 24 h.
   Note: This control ensures that there are not a significant number of spores carried over from the overnight culture or previous passages and confirms that sporulation is not already occurring

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at the beginning of the sporulation assay, potentially misrepresenting the final sporulation frequencies.

5. At H_{24}, perform ethanol resistance sporulation assays:
   a. Enumerate vegetative cells
      i. Scrape *C. difficile* from the surface of the plate (approximately 1/8 of the plate, depending on the density of the cells on the plate surface) with a sterile inoculating loop and suspend cells in approximately 5 ml BHIS to an OD_{600} = approximately 1.0.
      ii. Perform serial dilutions of suspended cells in pre-reduced BHIS liquid medium to enumerate the total number of vegetative cells. Evenly spread 100 µl onto pre-reduced BHIS agar. Typically, plating 100 µl each of 10^{-4} and 10^{-5} dilutions will yield countable colonies.
   b. Enumerate spores
      i. Aliquot 0.5 ml of culture from step 5a.i to a tube containing 0.2 ml dH_2O and 0.3 ml 95% ethanol (final concentration = 28.5% ethanol). Vortex well and incubate for 15 min.
      Note: This step may be performed anaerobically or aerobically as spores are fully resistant to oxygen exposure.
      ii. Serially dilute the ethanol and spore mixture from step 5b.i in 1x PBS + 0.1% taurocholate and spread 100 µl onto pre-reduced BHIS supplemented with 0.1% taurocholate plates (*e.g.*, 100 µl of a 10^{-4} dilution for 630Δ*erm* typically yields approximately 150-200 CFU). Plate as many serial dilutions as necessary to ensure countable colonies (approximately 30-400 colonies per plate).
      Note: As a control, plate 100 µl of the undiluted non-sporulating C. difficile strain after ethanol treatment (step 5b.i). This plate should yield no colony forming units (CFU), as all vegetative cells are eliminated in the presence of 28.5% ethanol. Additionally, 0.1% taurocholate must be included in the diluent and in the medium to efficiently recover all viable spores, as taurocholate serves as a germinant for C. difficile (Sorg and Sonenshein, 2008).
6. Calculate sporulation frequency
   a. Enumerate CFU on all plates after > 24 h incubation at 37 °C. Separately calculate the total number of vegetative cells and spores, accounting for both the serial dilution and the plate dilution in the calculation.
   Note: Remember to multiply the ethanol resistant spores by a factor of 2 to account for the one-half dilution that occurs during ethanol exposure. In addition, there should be few (> 100) to no CFU on the H_0 plates, and no CFU present on the spo0A mutant control plate. If more than 500 CFU are recovered at H_0, this may impact the final sporulation frequency, and caution is warranted when interpreting the results. Finally, this step can be performed aerobically and with a digital colony counter to facilitate enumeration.

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b. Calculate sporulation frequency according to equation below.

$$\frac{\text{Total spores (ethanol resistant)}}{\text{Total cells (vegetative cells on BHIS + spores [ethanol resistant])}} = \text{Sporulation frequency}$$

Data analysis

To calculate sporulation frequency as a percentage (%), multiply the final number by 100. Statistical analyses used will depend on the number of strains and conditions tested.

Notes

1. In addition to determining sporulation frequency using ethanol resistance, we encourage the use of a second method, such as phase contrast microscopy (Edwards *et al.*, 2014 and 2016b), to confirm the sporulation phenotype observed.
2. Alternatively, this protocol may be adapted to perform sporulation assays in liquid cultures (*e.g.*, 70:30 liquid medium, BHIS liquid medium or TY liquid medium). If these experimental conditions are desired, follow the details outlined in Edwards *et al.,* 2014. Briefly, dilute the exponential phase culture(s) (OD_{600} = 0.5; from step 3) 1:10 into pre-reduced medium (the initial OD_{600} of the liquid cultures will be approximately 0.05). Follow growth using a spectrophotometer until the cultures reach an OD_{600} = 1.00, which corresponds to the beginning of transition phase for most *C. difficile* strains. This time point is denoted as T_0. At T_2-T_4 (two to four hours after an OD_{600} = 1.00 is achieved), plate for total cells (vegetative cells and spores) present in the culture by performing serial dilutions and plating onto BHIS agar supplemented with 0.1% taurocholate. At T_{24}, use 0.5 ml culture to perform the ethanol resistance assay as described in step 5b.i and continue the protocol as described. This method provides for the comparison of ethanol resistant spores present in the culture at T_{24} versus the total number of cells (vegetative cells and spores) at peak growth at T_{24} to calculate the sporulation frequency in liquid culture. It is important to note that much higher and more consistent sporulation frequencies are observed on plates than in liquid medium (Fimlaid *et al.,* 2013; Edwards *et al.*, 2016b).

Recipes

1. 10% (w/v) sodium taurocholate
   Dissolve 1 g taurocholate in 10 ml deionized water. Sterilize with a 0.22 µm syringe filter and store at room temperature
2. BHIS medium
   37 g/L brain heart infusion
   5 g/L yeast extract
   15 g/L agar for solid medium

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Bring to 1 L with deionized water and autoclave for 20 min at 121 °C to sterilize

For BHIS agar supplemented with taurocholate, add 10 ml 10% taurocholate (final concentration, 0.1%) once medium cools to 60-65 °C after autoclaving

Notes:

a. *The use of an infrared thermometer is an easy way to monitor the medium temperature.*
b. *Allow medium to cool for an additional 20 min (to approximately 45-50 °C) before pouring plates. Adding the taurocholate to medium warmer than 60 °C eliminates any potential contamination from Mollicute species, which can grow on BHIS in the presence of taurocholate, a cholesterol derivative. These contaminants appear as pinpoint colonies on the surface of the agar.*

3. 70:30 sporulation medium

   63 g/L Bacto peptone

   3.5 g/L proteose peptone

   11.1 g/L brain heart infusion

   1.5 g/L yeast extract

   1.06 g/L Tris base

   0.7 g/L ammonium sulfate [$(NH_4)_2SO_4$]

   15 g/L agar for solid medium

   Bring to 1 L with deionized water and autoclave for 20 min at 121 °C to sterilize. After autoclaving, add 3 ml 10% (w/v) L-cysteine (final concentration, 0.03%)

   Notes:

   a. *When pouring plates, use 35 ml medium per Petri dish (94 x 16 mm) to ensure consistency between each plate.*
   b. *If using strains that harbor plasmids, use thiamphenicol at 2 µg/ml for maintenance. This concentration stably maintains plasmids that confer thiamphenicol resistance while not interfering with the sporulation frequency of the parent strain containing the control vector. The optimal concentration may need to be empirically determined for other antibiotics.*

4. 1x PBS

   8.01 g/L NaCl

   0.2 g/L KCl

   2.72 g/L $Na_2HPO_4 \cdot 7H_2O$

   0.27 g/L KH_2PO_4

   Bring to 1 L with deionized water and autoclave for 20 min at 121 °C or use a 0.45 µm filter to sterilize

5. 20% D-fructose

   Dissolve 10 g D-fructose in 50 ml deionized water. Sterilize with a 0.45 µm syringe filter and store at room temperature

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6. 10% L-cysteine
   Dissolve 0.5 g L-cysteine in 5 ml deionized water. Sterilize with a 0.45 µm syringe filter. This solution precipitates and needs to be made fresh before each use

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