Routes of Administration – Lab book

Sporulation assay

We would perform sporulation assays to determine the optimal conditions for spore production. Several sporulation media have been tested in *C. sporogenes* and other *Clostridium* species, so we would compare 5 different media described in the literature (table 1).

Table 1 - Compositions of the sporulation media we would test using sporulation assays:

Name	Components	Reference
Sporulation medium 1	3% Trypticase 10% Peptone 10% Ammonium sulfate	Yang, W.W., Crow-Willard, E. & Ponce, A., Production and characterization of pure *Clostridium* spore suspensions. Journal of Applied Microbiology. 2009, 106: 27-33.
Sporulation medium 2	39.5 g/L Blood agar base no.2 10 g/L Desiccated ox bile 5 g/L Sodium bicarbonate 0.5 mL/L Quinoline 50 mL/L Defibrinated horse blood	Phillips, K.D., A sporulation medium for *Clostridium perfringens*. Letters in Applied Microbiology. 1986, 3: 77-79.
Sporulation medium 3	0.4% Yeast extract 1.5% Proteose peptone 0.4% Soluble starch 0.1% Sodium thioglycolate 1% Sodium phosphate	Duncan, C.L. & Strong, D.H., Improved Medium for Sporulation of *Clostridium perfringens*. Applied Microbiology. 1968, 16 (1): 82-89.
Sporulation medium 4	1% Polypeptone 0.3% Yeast extract 0.3% Starch 0.01% Magnesium sulfate 0.15% Potassium phosphate 5% Sodium phosphate	Ellner, P.D., A medium promoting rapid quantitative sporulation in *Clostridium perfringens.* Journal of Bacteriology. 1956 Apr;71(4):495-496.
Sporulation medium 5	Per 100 mL of medium: 20 mg Magnesium sulfate 1 mg Manganese sulfate 1 mg Iron(II) sulfate 0.1 mg P-aminobenzoic acid 0.1 mg Thiamine hydrochloride 5 µg Biotin	Long, S., Jones, D.T., Woods, D,R,. Sporulation of *Clostridium acetobutylicum* P262 in a Defined Medium. Appl Environ Microbiol. 1983;45(4):1389-1393. doi:10.1128/AEM.45.4.1389-1393.1983

The sporulation assay results are typically presented by plotting the CFU/mL obtained for each time point, as shown below.

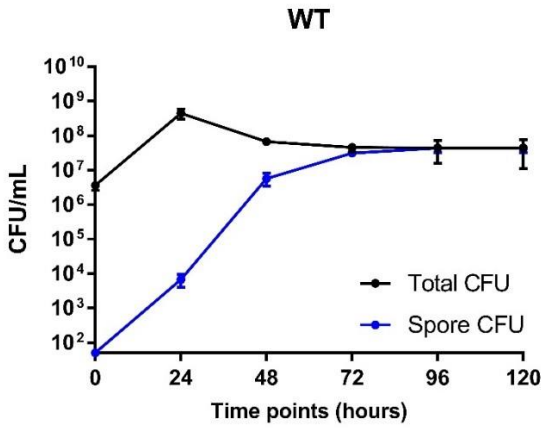

Figure 1: ***Sporulation profile of*** **C. sporogenes.** *Examples of results from sporulation assays performed with C. sporogenes wild type (WT). All the cultures would be synchronised at the beginning of the assay, and samples would be collected at 24 hour intervals during a total period of 120 hours. For the total CFU count, the cultures would be diluted and spotted into TYG agar. For spore CFU count, the cultures would be heat-treated at 80°C for 20 minutes prior to the dilutions. Data presented would show the mean and error bars represent the standard deviation of three biological and three technical replicates. The Y axis starts at the limit of detection (50 spores/mL).*

In figure 1, the blue lines (spore CFU) show the formation of spores (heat treated samples) and the black lines (total CFU) are used as controls, to evaluate the growth of the cultures (poor growth would affect the formation of spores). We would choose the medium that resulted in the highest CFU/mL (high number of spores produced), in the shortest amount of time.

Germination assay

The purpose of this assay would be to determine whether any of the components of the capsule could trigger germination. Ideally, we would want the cells to remain as spores in the capsule and only germinate upon reaching the gut.

Testable components:

- sodium alginate
- cellulose
- hydroxypropyl methylcellulose
- microcrystalline cellulose
- magnesium stearate

During germination, Ca^{2+} dipicolinic acid is released from the spore causing a drop in the OD_{600}. Then, as the germinated cells start to grow, the OD_{600} increases. If the spores don't germinate, the OD_{600} should remain relatively constant throughout.

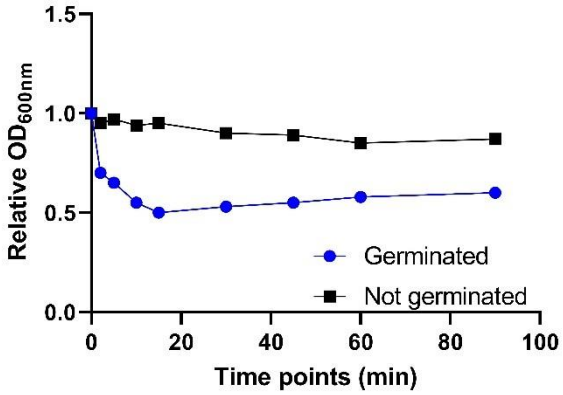

Figure 2: ***Germination profile of*** **C. sporogenes.** *Examples of results from a germination assay performed with C. sporogenes WT. Spores would be produced, purified, and re-suspended in the germinant being tested. The OD_{600} would be monitored over a period of 90 min, with measurements made at several time points. Data presented would show the mean and error bars represent the standard deviation of three biological and three technical replicates.*

The graph shown in figure 2 displays this, with the blue line showing a decrease in OD_{600} from 1.0 to 0.5 within 20 minutes, and then gradually increasing as the germinated cells start to grow. On the other hand, the black line represents spores that haven't germinated – evidenced by the relative lack of change in OD_{600} compared to blue line. If we see a result that mimics the blue line, this would suggest that our component is a recognised germination factor and triggers early germination of the spores. We want to avoid this as much as possible within the capsule as early germination has three effects:

- reduces shelf life of NeuroTone
- loss of viable cells through early exposure to extreme stomach conditions
- decreases likelihood of successful colony growth in the gut

How this would be implemented in the wet lab:

If we had access to the wet lab, we would perform the experiments described below. We would start by optimising the production of spores.

Sporulation assays (Routes of administration subgroup - protocol 4) to select sporulation medium

Step 1
Set up *C. sporogenes* overnight cultures (TYG broth). We would test *C. sporogenes* wild type, as well as the final strain (engineered to produce DBHB and with controlled sporulation). We would also include *C. sporogenes* Δspo0A as a negative control (this strain is available in the SBRC culture collection and is not able to produce heat-resistant spores).

Step 2
Sub-culture into fresh TYG broth (1:100) and incubate for 3 hrs.

Step 3
Sub-culture again into appropriate medium (1:100). For each strain, we would test TYG (control) and each of the 5 sporulation media described in table 1.

Steps 4 to 10 as described in protocol 4
Immediately take 0 hrs sample and divide into 2 aliquots.

We would produce graphs similar to the one presented in figure 1 (one graph for each strain/medium combination) to represent our results and use them to choose the optimal medium. We would then test the protocol for spore purification.

Purification of *Clostridium sporogenes* spores using a sucrose gradient (Routes of administration subgroup - protocol 1)

Step 1
Set up *C. sporogenes* cultures (wild type and final strain) in the selected sporulation medium.

Steps 2 to 14 as described in protocol 1

We would observe the samples using phase-contrast imaging. We would expect to see many round, phase-bright spores and only a few vegetative cell debris (phase dark).

Germination assay (Routes of administration subgroup - protocol 5)

Step 1
Set up *C. sporogenes* cultures (wild type and final strain) in selected sporulation medium.

Step 2 to 4 as described in protocol 5

Step 5
At time point 0 hrs, re-suspend the spores in the germinant being tested to an OD_{600} of 1.0. We would test 5 components: sodium alginate, cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose and magnesium stearate. We would use TYG as a positive control and dH_2O as a negative control.

Step 6
Monitor OD_{600} at several time points: 0 hrs, 2 mins, 5 mins, 10 mins, 15 mins, 30 mins, 45 mins, 60 mins and 90 mins. This can be done in the CLARIOstar plate reader.

We would plot the results as shown in figure 2, and select the components that did not trigger germination for the formulation of our capsule.

Note: we would not be able to test the industrial scale protocols even if we had access to the wet lab. Those are part of the protocol list just as examples.