The Effects of Redox Potential on the Gas Production in *Clostridioides difficile*

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Introduction

Prior experiments indicated that *C. difficile* can be influenced by sulfur sources. Nutrients, including the concentration of cysteine, influence toxin production. This needs to be explored to understand the impact of redox potential on bioenergetics and toxin production.

In a study by Gu et. al. 2018, the *C. difficile* R20291 response to cysteine was studied (5). When *C. difficile* R20291 was supplemented with 5 mM cysteine, it grew rapidly to the exponential phase. The growth rate of R20291 with 5 mM or 10 mM cysteine were the most efficient, and growth inhibition occurred with cysteine concentration above 10 mM (1).

In this report, *C. difficile* was grown with varying amounts of cysteine to observe how gas production was influenced; growth was observed over two days and optical density was measured at the end of each study. Cysteine is necessary for growth, but it still remains in question how much is necessary for efficient gas production. The objective of this investigation is to determine if the redox potential of routine growth media for *C. difficile* affects gas production.

Methods and Materials

Two *C. difficile* strains of differing ribotype (JIR8094, ribotype 012; R20291, ribotype 027) were designated as wild-type depending on use of mutants with differing genetic backgrounds: LB-CD7 (*selD::ermB*, [JIR8094]), KNM6 (Δ*selD*, [R20291]), and KNM9 (*selD::selD*$^{+}$, [R20291]). *C. difficile* was grown in supplemented brain heart infusion (BHIS; 37 g/L BHI, 5 g/L yeast extract). L-cysteine was included in BHIS, but was excluded for this experiment because it was a thiol reductant that was going to be tested on the *C. difficile* to observe gas production.

12x75 mm glass culture screw cap tubes with inverted Durham tubes, and 6 milliliters were dispensed in each tube with a serological pipette. The tubes were sterilized with an autoclave and subsequently cooled to room temperature. The cysteine was filter sterilized and added aseptically to the medium at concentrations of 0 mM, 1 mM, 5 mM, 10 mM, 15 mM, 20 mM, and 25 mM with triplicates for each; the cysteine was not added prior to autoclaving as it is a heat-labile reagent. The loosely-capped tubes were placed in an anaerobic chamber overnight to allow the media to reduce. Overnight cultures were started by inoculating several BHIS broths with the indicated *C. difficile* strains and were incubated at 37 °C for 12-16 hours.

The next day, overnight cultures were diluted 50-fold into each tube. The gas results were read over the next week, and pictures of tubes were taken to monitor growth of *C. difficile* and any gas production that was visible from the formation of bubbles.

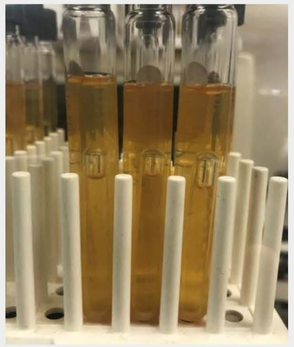

Figure 2: JIR8094: 10 mM Cysteine, Day 2

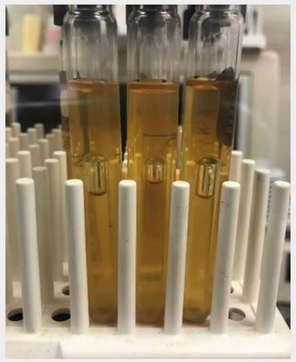

Figure 3: JIR8094: 15 mM Cysteine, Day 2

Figure 4: JIR8094: 20 mM Cysteine, Day 2

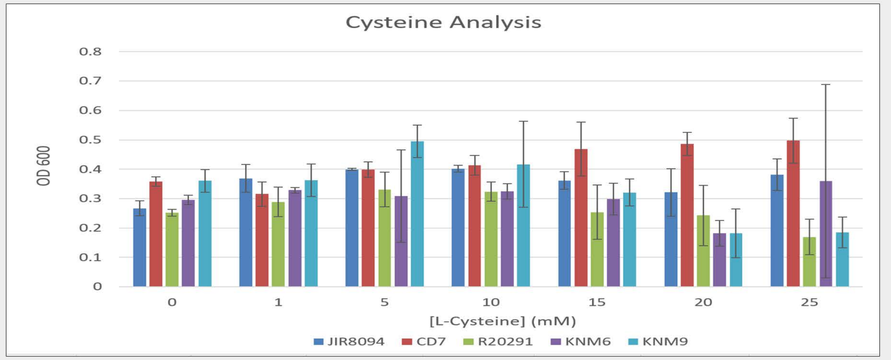

Figure 1: Optical Density Reading Averages for all Strains

	JIR8094			LD-CD7		
mM Cysteine	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
0	++	++	++	++	++	++
1	++	++	++	++	++	++
5	++	++	++	++	++	++
10	++	++	++	++	++	++
15	++	++	++	++	++	+
20	0	++	++	++	0	+
25	0	0	+	+	0	0

Table 1: Gas Production Bubble Rating System for JIR8094 and LD-CD7

	R20291			KNM6			KNM9		
mM Cysteine	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
0	++	++	++	++	++	++	++	++	+
1	++	++	++	++	++	++	++	++	+
5	++	++	++	0	+	+	+	+	+
10	++	++	++	+	++	++	++	++	+
15	++	++	++	++	++	++	++	++	++
20	++	++	++	+	++	+	0	0	0
25	+	0	0	0	0	0	+	0	0

Table 2: Gas Production Bubble Rating System for R20291, KNM6, and KNM9

Results

- Optical density measurements were taken for all of the samples, and had varying results. Gu et al., 2018
- For JIR8094, there was a peak reading for optical density for two of the replicates around the 10 mM, which decreased slightly at concentrations from 15-25 mM (Table 1).
- For LD-CD7, two of the replicates showed the peak of optical density reading at the 15 mM cysteine concentration (Table 1).
- R20291 had a less significant peak, however the optical density readings were typically at a maximum at the 10 mM cysteine concentration and slightly decreased in the higher concentrations (Table 2).
- KNM6 and KNM9 both had maximum optical density readings at 10 mM as well (Table 2).

Discussion

Optimal *C. difficile* growth occurred at 10 mM of cysteine concentration and decreased at concentrations above 20 mM. A rating system was formulated to categorize the gas bubbles in each tube (Tables 1 and 2). One plus sign was used to indicate minimal gas production, two plus signs for moderate gas production, and three plus signs for a bubble which took up a majority of the height of the solution in the tube. Although some tubes had no gas production and none had three plus signs, there was variability. In future cases, it would be important to require other individuals to also analyze the gas production using this scale, as it is a subjective method of observation and could be more efficient with multiple analyses.

These findings are similar to those of the study by Gu et al., 2018 - experiments demonstrated how cysteine at 5 mM or 10 mM provided the best conditions for growth of mutant R20291, and growth inhibition at concentrations above 10 mM (1). With this information, the hypothesis that *C. difficile* produces or uses gas in response to redox potential can be further tested with the use of defined a medium, and different thiol reductants instead of cysteine.

References

1. . Huawei Gu, Kan Shi, Zhengping Liao, Haonan Qi, Shuyi Chen, Haiying Wang, Shan Li, Yi Ma, Jufang Wang. (2018). Time-resolved transcriptome analysis of Clostridium difficile R20291 response to cysteine. Microbiological Research, (215), 114-125