Effects of surface interaction on c-di-GMP regulation in *Clostridioides difficile*

Khalid Tunau-Spencer, Dr. Leila Reyes Ruiz, Dr. Rita Tamayo

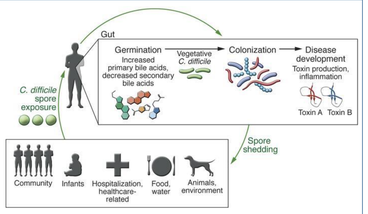
THE UNIVERSITY of NORTH CAROLINA at CHAPEL HILL

Abstract

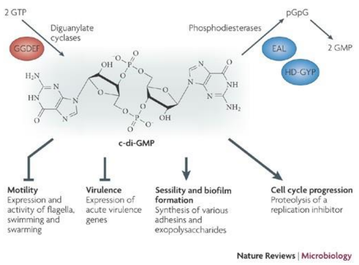
Clostridioides difficile is a bacterial pathogen that causes antibiotic-associated intestinal disease.[2] The signaling molecule cyclic diguanylate monophosphate (c-di-GMP) regulates various physiological changes such as biofilm formation, motility, and cell differentiation in many bacterial species.[1] In *C. difficile*, c-di-GMP regulates motility, colonization and virulence.[5] In many bacteria, an increase in c-di-GMP levels is associated with the transition from a motile to a sessile lifestyle.[1] In the presence of an abiotic surface, bacteria more likely organize themselves in a protective manner through biofilm formation and become sessile.[4] With these relationships in mind, we wanted to test if there was a difference in c-di-GMP levels between *C. difficile* growing in a liquid culture and cells on a solid surface. We hypothesize that being on a surface will lead to higher intracellular c-di-GMP levels. We used a plasmid where transcription of a red fluorescent reporter, *mCherry*, is regulated by an upstream riboswitch that allows for transcription of *mCherry* under high c-di-GMP levels. With this plasmid, we quantified the percentage of the bacterial population that fluoresced red, indicative of an increase in c-di-GMP levels, at different time points. In this study, we found that the percentage of fluorescent cells on a solid surface closely resembles those in a liquid medium within a 24-hour period, suggesting that the levels of c-di-GMP remained similar in both conditions. Further work is needed to understand at which timepoint the percentage of the population exhibiting a difference in c-di-GMP dependent regulation would differ following movement to a surface.

Introduction

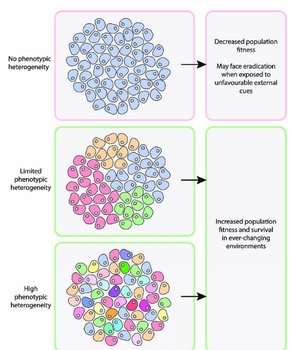
Clostridioides difficile is a gram-positive, spore-forming, obligate anaerobic bacterium. In humans, *C. difficile* colonizes the large intestines and is a leading cause of fatal nosocomial infections in the United States.[7] Transmission is via the fecal-oral route, and disease is mediated by the release of two main exotoxins. These are toxin A, the enterotoxin, and toxin B, the cytotoxin. The first causes increased intestinal penetrability and fluid discharge while the second leads to colonic inflammation.[8] Despite this knowledge, much of the basic biology of *C. difficile* is not known.



Cyclic diguanylate monophosphate (c-di-GMP) is a bacterial second-messenger molecule that is involved in the switch between planktonic, motile and non-motile bacterial forms.[9] An increase in the intracellular c-di-GMP concentration has been shown to induce exopolysaccharide synthesis and adhesion while inhibiting flagellar motility. C-di-GMP functions by binding to and influencing the activity of proteins and RNA riboswitches. A riboswitch is an RNA structure located in the 5′ untranslated regions of some mRNA that binds a specific metabolite; binding of the metabolite modifies the RNA structure in a way that alters expression of the downstream gene.

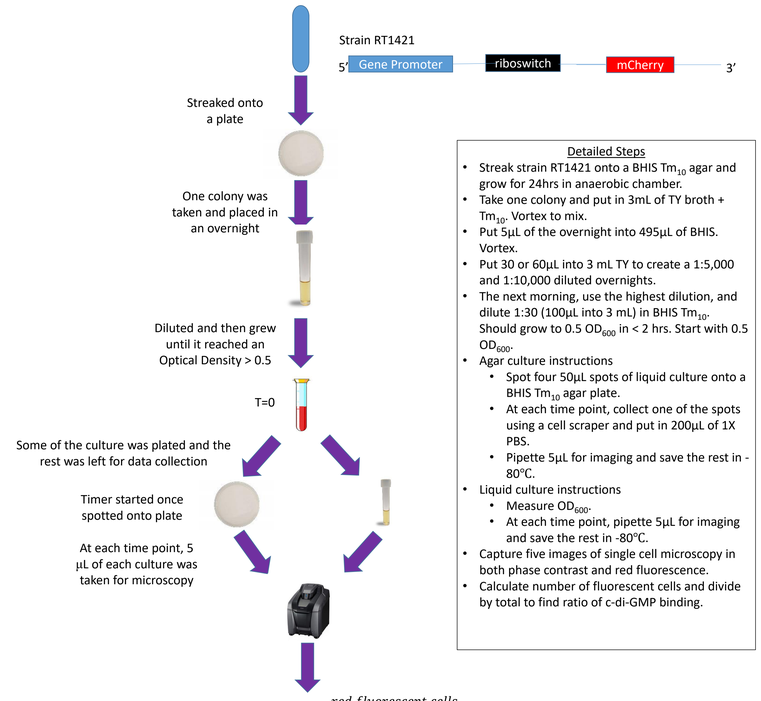


Phenotypic heterogeneity describes the presence of cells with distinct properties within an isogenic population. Phenotypic heterogeneity affects many aspects of the bacterial response to stimuli, and it is thought to increase bacterial fitness and the chances for survival of the population as a whole. Some survival strategies that are predicted to be affected by phenotypic heterogeneity include spore formation, biofilm formation, altered motility, and a differing response to antibiotics. As a whole, these strategies are evoked by environmental or bacterially produced signals. Depending on the stimuli, a change in the bacterial phenotype and response can occur.



In this study, we attempt to observe the effects of surface interaction on c-di-GMP binding, as well as identify if C. *difficile* exhibits a population heterogeneity in the activation of the c-di-GMP riboswitch.

Methods



Detailed Steps

- Streak strain RT1421 onto a BHIS Tm_{10} agar and grow for 24hrs in anaerobic chamber.
- Take one colony and put in 3mL of TY broth + Tm_{10}. Vortex to mix.
- Put 5µL of the overnight into 495µL of BHIS. Vortex.
- Put 30 or 60µL into 3 mL TY to create a 1:5,000 and 1:10,000 diluted overnights.
- The next morning, use the highest dilution, and dilute 1:30 (100µL into 3 mL) in BHIS Tm_{10}. Should grow to 0.5 OD_{600} in < 2 hrs. Start with 0.5 OD_{600}.
- Agar culture instructions
  - Spot four 50µL spots of liquid culture onto a BHIS Tm_{10} agar plate.
  - At each time point, collect one of the spots using a cell scraper and put in 200µL of 1X PBS.
  - Pipette 5µL for imaging and save the rest in -80°C.
- Liquid culture instructions
  - Measure OD_{600}.
  - At each time point, pipette 5µL for imaging and save the rest in -80°C.
- Capture five images of single cell microscopy in both phase contrast and red fluorescence.
- Calculate number of fluorescent cells and divide by total to find ratio of c-di-GMP binding.

$$\%\ of\ red\ fluorescent\ cells = \frac{red\ fluorescent\ cells}{total\ cells} * 100$$

Results

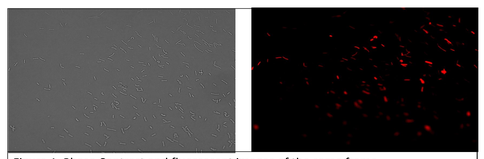


Figure 1. Phase Contrast and fluorescent images of the same frame.

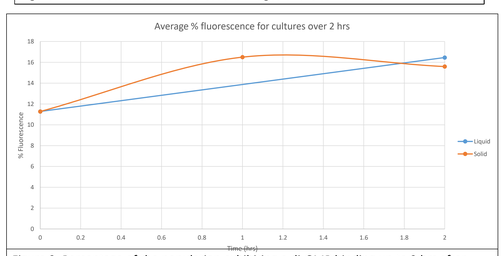


Figure 2. Percentage of the population exhibiting c-di-GMP binding up to 2 hrs after plating.

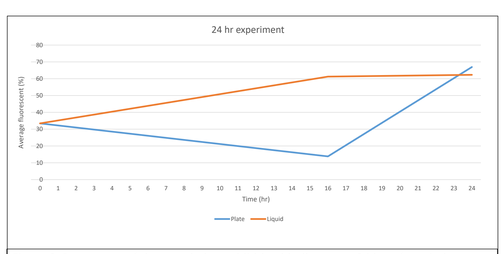


Figure 3. Percentage of the population exhibiting binding up to 24 hrs after plating.

Conclusion and Future Study

In this experiment, we used a fluorescent reporter to compare the percentage of *Clostridioides difficile* cells exhibiting high c-di-GMP when grown in liquid media or an agar plate. In the 2 hour experiment, the difference between the two cultures was minimal (<10% roughly). However, in the 24 hour experiment, there was a noticeable drop in the percentage of fluorescent cells at time 16 hours, suggesting a change in c-di-GMP levels. Since this was only performed once, more replicates are needed to test this time point. We also observed population heterogeneity: some cells showed higher red fluorescence than others, suggesting a difference in c-di-GMP levels. Mechanical errors, such as poor microscopy resolution, made it difficult to record the total number of cells within a frame. For future studies, additional experiment trials will need to be performed to provide more accurate data, as well as an experiment to test why some cells exhibit more c-di-GMP riboswitch binding than others.

References

1. Jenal, Urs, et al. "Cyclic Di-GMP: Second Messenger Extraordinaire." *Nature News*, Nature Publishing Group, 6 Feb. 2017, www.nature.com/articles/nrmicro.2016.190.
2. Schäffler, Holger, and Anne Breitrück. "*Clostridium Difficile* - From Colonization to Infection." *Frontiers in Microbiology*, Frontiers Media S.A., 10 Apr. 2018, www.ncbi.nlm.nih.gov/pmc/articles/PMC5902504/.
3. Sisti, Federico, et al. "Cyclic-Di-GMP Signalling Regulates Motility and Biofilm Formation in Bordetella Bronchiseptica." *Microbiology (Reading, England)*, Society for General Microbiology, May 2013, www.ncbi.nlm.nih.gov/pmc/articles/PMC4085988/.
4. Even, Catherine, et al. "Recent Advances in Studying Single Bacteria and Biofilm Mechanics." *Advances in Colloid and Interface Science*, Elsevier, 21 July 2017, www.sciencedirect.com/science/article/pii/S0001868617301872?via=ihub.
5. McKee, Robert W, et al. "Cyclic Diguanylate Regulates Virulence Factor Genes via Multiple Riboswitches in *Clostridium Difficile*." *MSphere*, American Society for Microbiology, 24 Oct. 2018, www.ncbi.nlm.nih.gov/pubmed/30355665.
6. Weigel, W.A., and P. Dersch. "Phenotypic Heterogeneity: a Bacterial Virulence Strategy." *Microbes and Infection*, Elsevier Masson, 1 Feb. 2018, https://www.sciencedirect.com/science/article/pii/S1286457918300418?via=ihub.https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4805733/
7. Garrett, Elizabeth M., et al. "Phase Variation of a Signal Transduction System Controls Clostridioides Difficile Colony Morphology, Motility, and Virulence." *BioRxiv*, Cold Spring Harbor Laboratory, 1 Jan. 2019, https://www.biorxiv.org/content/10.1101/690230v1.full.
8. Voth, Daniel E, and Jimmy D Ballard. "Clostridium Difficile Toxins: Mechanism of Action and Role in Disease." *Clinical Microbiology Reviews*, American Society for Microbiology, Apr. 2005, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1082799/.
9. Bordeleau, Eric, et al. "Cyclic Di-GMP Riboswitch-Regulated Type IV Pili Contribute to Aggregation of Clostridium Difficile." *Journal of Bacteriology*, American Society for Microbiology Journals, 1 Mar. 2015, https://jb.asm.org/content/197/5/819.long.
10. Seekatz, Anna M., and Vincent B. Young. "*Clostridium Difficile* and the Microbiota." *The Journal of Clinical Investigation*, American Society for Clinical Investigation, 1 Oct. 2014, www.jci.org/articles/view/72336.
11. Shlyakhtina, Yelyzaveta, et al. "Asymmetric Inheritance of Cell Fate Determinants: Focus on RNA." *Non-Coding RNA*, MDPI, 9 May 2019, www.ncbi.nlm.nih.gov/pubmed/31075989.
12. Hengge, Regine. "Principles of c-Di-GMP Signalling in Bacteria." *Nature News*, Nature Publishing Group, www.nature.com/articles/nrmicro2109.