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New tools and insights in physiology and chromosome dynamics of Clostridioides difficile

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Acknowledgements

English Summary

Clostridioides difficile is an anaerobic bacterium, that is, it is an organism that needs an environment that lacks oxygen in order to develop, like the environment found in the human intestine. *C. difficile* can colonize the colon of the humans but it can also be found in the environment, as dormant cells called spores. Spores are a type of daughter cell that is capable of withstanding adverse conditions, such as antibiotics or cleaning products, but also oxygen levels that are normally toxic to *C. difficile*. Due to the ability to produce spores, it can remain in the environment for long periods of time.

C. difficile infection was identified in 1978 in patients with pseudomembranous colitis and is a major cause of diarrhoea acquired in a hospital setting. It can manifest itself with diverse symptoms, ranging from relatively mild diarrhoea to severe inflammation of the colon, which can lead to the patient's death. Patients acquire *C. difficile* by ingesting spores from their environment, and when coming into contact with contaminated surfaces. The infection can easily pass from patient to patient. *C. difficile* can also be found in people that do not demonstrate symptoms of the disease, so-called asymptomatic carriers. Up to 15% of people who are hospitalized carry *C. difficile* without any development of the disease.

After spores are ingested, they can grow out to regular bacterial cells again in the intestinal tract when they find favourable conditions. The intestinal tract contains a complex community of bacteria (microbiome) that not only help us to metabolize food but also protects us from pathogenic organisms. But, for instance, when we take antibiotics, part of our microbiome is eliminated. Under these conditions, the growth of disease-causing microorganisms such as *C. difficile* is possible. Other factors such as age of the person or the presence of other diseases can also lead to a disturbed microbiome and/or promote the development of infections. When *C. difficile* colonizes the intestinal tract, it proliferates and is able to produce toxins that damage the intestinal walls, cause inflammation and lead to the symptoms of the disease.

The first step in the treatment of *C. difficile* infection is to discontinue the use of the antibiotics that may have triggered the disease. Additional treatment depends on the severity of the disease and the patient's comorbidities. Currently, there are three antibiotics recommended to fight *C. difficile* infection. However, on average, 15 to 30% of treated patients experience a recurrence of symptoms. Though the reasons for this are not very well understood, these patients have a less diverse microbiome than healthy people, which may prevent the recovering from infection. Recently, a new technique called faecal microbiota transplantation has been developed, in which a stable and diverse microbiome from healthy volunteers is

transferred to the patient. This successfully prevents development of the disease in up to 90% of cases, even when previous treatments have failed multiple times.

Since 2000, the incidence and severity of *C. difficile* infections have increased. Several outbreaks in healthcare facilities around the world have been reported, with higher mortality rates. As a result, the study of this organism and its life cycle has gained particular interest in recent years. The current knowledge relevant to the research described in this thesis is summarized in **Chapter 1**.

In this PhD project, we wanted to explore molecular details of processes that are important for *C. difficile* cells, such as how genetic information (DNA) is copied in a process called DNA replication and how it is organized within the cell. However, we quickly realized that in order to do so, it was also necessary to develop new tools that would allow us to answer the questions of this study.

One of the tools involves the production of a protein, luciferase, that emits light in the presence of a substrate. This protein can be used as an indicator for when and how the bacterium transcribes genes and, as a result, produces proteins. *C. difficile* contains mechanisms that allow the cell to direct produced proteins to the outside of the cell. We took advantage of this ability to direct the secretion of luciferase to develop a new tool, which allowed us to evaluate the expression of cellular components by sampling the medium in which the cells were grown. The luciferase, which we called sLuc^{opt}, has the same amino acid sequence as a commercially available luciferase but has been engineered with the secretion signal of a *C. difficile* protein. Using the same strategy, we also made a secreted protein that can degrade starch, called AmyE^{opt}. Both these proteins are described in **Chapter 2**. In our other work, we have extended the luciferase toolkit by deriving systems from sLuc^{opt} for different *in vivo* assays; these systems make use of the fact that you can split the luciferase protein into pieces that when brought together again form the functional protein. The HiBit^{opt} system allowed us to determine whether one side of the protein is inside of a bacterial cell, or is exposed on the outside, described in **Chapter 3**; the other system, bitLuc^{opt}, was used to study whether proteins interact in bacterial cells and is described in **Chapter 5**.

We also used for the first time another tool in *C. difficile*, the HaloTag. This is a small protein that emits fluorescence when combined with a substrate (the substrates come in different colours). We were able to explore the limitations and advantages of the HaloTag for fluorescence microscopy in live *C. difficile* cells, but also of other fluorescent proteins that were previously used in *C. difficile* (CFP^{opt}, mCherry^{Opt}, phiLOV2.1 and SNAP^{Cd}). This data led to **Chapter 6**. We observed during this work that *C. difficile* itself also emits fluorescence, in

the green spectrum, which forms a limitation for the use of green fluorescent proteins for *C. difficile* studies. Our work elucidated that the green fluorescence of *C. difficile* becomes higher when cultures grow for a longer time and is also higher when cells are exposed to oxygen.

As indicated, we applied our tools in our investigations of the molecular mechanisms of important cellular processes. For instance, the HiBiT^{opt} system allowed us to investigate TcdC, a protein with a controversial role in the regulation of the toxins that cause disease. In **Chapter 3**, we characterize this enigmatic protein, which is present in the same genetic context as the toxins. This protein was believed to repress toxin gene expression, by interfering with the "on-switch" for toxin production, TcdR, that is located inside the cell. We found, however, that the most important part of TcdC is located outside the cell. This is not compatible with the function that was previously hypothesized, as the outside localization may suggest a role in the detection of stimuli present in the environment.

Two other experimental chapters deal with how the genetic information of *C. difficile* is organized and copied.

In **Chapter 4**, we identified the location where the replication of DNA is initiated, the origin of replication (*oriC*). The first step in this process is mediated by a protein called DnaA and consists of opening up of the double-stranded DNA (unwinding). Using bioinformatics analyses and biochemical experiments, we showed *in vitro* where DnaA induces this unwinding. We also showed that these first steps in copying of DNA are likely to be similar in other clostridia (these include for instance *Clostridium botulinum*, known for its ability to produce the neurotoxin botox or *Clostridium perfringens*, that can cause food poisoning) and possibly even more distantly related bacteria.

In **Chapter 5** we looked at the effects of the HupA protein, that belongs to a family of proteins that frequently protect DNA, or change its shape. We used biochemical experiments, including measurements on single molecules, and fluorescent microscopy to show this protein can change the shape of DNA and in cells causes DNA to be compacted. This is similar, in some aspects, to so-called histones, proteins that compact DNA in human cells, and therefore, proteins such as HupA are sometimes called bacterial histone-like proteins.

Finally, we summarize the results of all our studies in **Chapter 7**, with a discussion of some of the implications of our findings and the prospects for future research on *C. difficile* based on our work.