Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Caulobacter crescentus holdfast: A bio-based and water-resistant adhesive

B.1.2 Abstract

Caulobacter crescentus is an aquatic bacterium, which produces an adhesive called holdfast. With an adhesive strength of 68 N/mm^2, the holdfast is the strongest natural adhesive encountered thus far. The exact chemical structure and composition of holdfast are not yet known due to the small amount of holdfast secreted by the bacterium and the challenge of purifying it. However, it is known that a polysaccharide with N-acetylglucosamine (GLcNAc) as the basic unit is one of the main components of holdfast, together with 3-O-methylglucose, glucose, mannose and xylose. Proteins and DNA have also been encountered in the holdfast matrix. The holdfast has a lot of potential as a natural and biobased wet-adhesive for medical and industrial applications due to its incredible strength. Synthetic adhesives for underwater/wet application often lack strength. The aim of this proposal is to determine the exact structure and chemical components of holdfast to better understand its adhesive properties. A second aim is the determination of the whole biosynthetic pathway and related genes of holdfast production, which will also help in determining the chemical make-up of holdfast. Then, before any feasible future application, the yield of the holdfast production needs to be increased. The third aim of this proposal is to increase biosynthesis in the bacterium itself or by using recombinant DNA techniques to express holdfast synthesis in the model organism *E. coli*.

B.1.3 Layman’s summary

Due to climate change, society demands more environmentally friendly products. An excellent way to approach this is to develop more circular, waste-free, zero/negative emission or biodegradable products. For something to be biodegradable it has to be completely biobased. A few examples of biobased materials are hemp isolation panels and furniture created out of mycelium bio-composites.

Something that does not have a biobased alternative yet is a good performing underwater glue. There is a lot to gain here because even synthetic glues lack strength under wet or underwater conditions. This hypothetical biobased glue could be incredibly useful in the industrial field but also in the medical field to quickly repair bleeding wounds. In order to create a new biobased and environmentally friendly product of high quality, it is often a great start to look at the solutions provided by nature.

Organisms first developed in the sea and animals like sand-castle worms, mussels and barnacles have already found ways to make excellent underwater glue. They mainly use a glue that is made of proteins to adhere to any underwater surface. Scientists have tried to mimic these glues but due to their complexity they have not yet succeeded in making an equally strong and marketable biobased underwater glue.

Another type of organisms that produce underwater glue are bacteria, especially the bacterium *Caulobacter crescentus*, which creates a holdfast that is currently the record holder of the strongest natural underwater glue ever measured. According to a few experiments it could hold a weight of 680 kg per cm^2, the equivalent of holding a large horse with a glue surface area the size of a small coin.

However, because the bacterium *C. crescentus* produces so little of the holdfast, it has been difficult to study and we currently do not know where its adhesive strength comes from. A few advancements have been made recently that might enable to investigate this holdfast further. Once the exact structure and chemical components of holdfast have been discovered we might be able to understand why it is so strong. This might help us with developing a new type of glue inspired by this holdfast.

Other than its exact chemical composition we also want to know how the holdfast is produced by studying its biosynthetic pathway. This will not only give us more insights in how holdfast is created but might also allow us to mimic it ourselves.

To produce the glue ourselves, it might be possible to genetically modify *C. crescentus* so that it produces a lot more of the holdfast. Alternatively, we could try to insert the biosynthetic pathway of holdfast in another more well-known and easy to culture bacterium like *Escherichia coli.* Then this bacterium will produce the holdfast for us in large quantities, something that is already done with certain medicine like hormones and vaccines. When we produce enough holdfast, we can do more mechanical testing and experiments on the properties of holdfast to find out if it would be suitable as a new underwater/wet adhesive.

B.1.4 Keywords

Biobased adhesive, Water-resistant glue, *C. crescentus* holdfast, biosynthesis

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic

Introduction

Climate change, pollution and depletion of essential materials have caused an increased demand for healthy, renewable and environmentally friendly products. Increasing the use of biobased materials is an intuitive response to this demand. By directly using the materials as provided by nature, pollution and emissions can be avoided. Furthermore, these natural materials can be included in the circular economy, are renewable and are often less harmful to the environment. The main challenge and bottleneck for these materials is to compete with mass-produced and cheaper synthetic materials (Vinod et al. 2020). Furthermore, due to lack of knowledge and technology most of the biobased materials are difficult to market or produce on a large scale. However, with the right direction of research and incentive, creating marketable biobased materials is definitely possible.

One example is the increased use of flax and hemp as natural insulation in houses. The thermal properties, biodegradability and carbon negative production emissions of flax insulation mats make them a good replacement for the commonly used synthetic glass wool (Kymäläinen and Sjöberg 2008; “Bouwisolatie - Isovlas” n.d.). Another example is the use of mycelium to create strong and versatile bio-composites. The roots of the fungus will bind an organic substrate together and after drying the material can be used as wall panels and interior furniture with excellent properties (Vandelook et al. 2021). Creating a biobased and biodegradable product without using a synthetic binder or material.

Recently, multiple biobased underwater glues inspired by mussels are developed with the potential to replace and outperform synthetic glues (Hiraishi et al. 2015). However, because these are quite complex to make, none of them are currently available on the market for a competitive price.

Wet adhesion is highly desired in a wide field of applications such as underwater building, wound dressing and leakage repairing. Since water molecules weaken the contact adhesion of current synthetic glues, the potential of new glues that mimic the underwater adhesives found in nature is high (Nyarko, Barton, and Dhinojwala 2016).

Aquatic animals like mussels, barnacles and sandcastle worms, secrete a natural glue that hardens due to a change in the environmental conditions (pH, ionic strength). In this way, they are able to adhere to various underwater substrate surfaces such as rocks, ship hulls and cables while simultaneously withstanding large forces like waves and erosion of seawater (Ma et al. 2021). However, a large part of the strength of the attachment is caused by post translational modifications and structure of the polymers which is difficult to synthetically mimic (Budisa and Schneider 2019). Natural adhesives follow a complicated path of protein expression, transportation, extrusion and curing inside or outside the organisms’ body. Mussels can attach themselves to almost any substrate using a system called the byssus which is composed of collagenous threads secreted by the mussel foot. A process that undertakes less than 30s in juvenile mussels (Waite 2017). Each distal part of the byssus threads has an adhesive plaque, which connects the thread to any chosen substrate. Within the plaque, a mixture of 6 mussel foot proteins (Mfp-1 to Mfp-6) are mainly responsible for the adhesive strength of the mussel (Ma et al. 2021). Each type of Mfp plays a different but equally

important role in the adhesion mechanism. Mfp-1 provides the byssal cuticle with high stiffness and extensibility (Zeng et al. 2010). Mfp-2 is the most abundant protein in the plaque and enhances cohesion of the byssus (Hwang et al. 2010). Mfp-3 and Mfp-5 are mainly responsible for the strong adhesion of mussels, they contain 10-30 mol% DOPA, which is the major constituent of the amino acid sequences of mussel foot proteins and causes the adhesive qualities of the proteins (Lee et al. 2011). Mfp-4 is speculated to connect the byssal thread with the plaque (Zhao and Waite 2006) and Mfp-6 is believed to stimulate crosslinking between the different Mfps and to maintain the redox balance during the formation of the byssus threads (Zhang et al. 2020).

The adhesion strength of mussels varies by their living conditions. Mytilus californianus lives in relatively harsh conditions with strong currents, and exhibits stronger attachment than other mussels of the Mytilus genus (Bell and Gosline 1996). The attachment apparatus of the approximately 150 g weighing mussel can withstand a 40 kg gravitational force (Witman and Suchanek 1984). However, since bivalve glues consist of multiple complicated protein networks, thus far no method that exactly mimics the adhesive in composition and strength has been found (Cui et al. 2017).

Another group of organisms that also produce underwater adhesives are bacteria. They use adhesives for attachment but also as components in biofilm matrices (Fritts et al. 2017). Bacteria from the *Alphaproteobacterial* class are unusual as they are able to secrete adhesins very locally and with exceptional adhesive capabilities (Hershey et al. 2019). In fact, the strongest natural underwater adhesive measured thus far comes from the bacterium *Caulobacter crescentus* (Nyarko, Barton, and Dhinojwala 2016). Nevertheless, despite the apparent strength, this bacterium and adhesive have received comparably little attention. The holdfast from *C. crescentus* differs from bivalve adhesives since it is not protein based but consists mainly of mono- and polysaccharides. Since the detailed biosynthetic pathway and structure of holdfast is still not clear (Liu, Zhang, and Xu 2021), there is a possibility that understanding it might lead to the development of a new type of underwater adhesive.
In this research proposal, I suggest to study the chemical structure and biosynthetic pathway of the *C. crescentus* holdfast in such a way that mimicry or biological manufacture as a marketable biobased adhesive might be possible in the future.

State of the art

The bacterium *Caulobacter crescentus* is more properly known as *Caulobacter vibrioides* (Henrici and Johnson 1935) but both names are used. *C. vibrioides* was first named and classified in 1935, but was possibly discovered by Omeliansky in 1914 (Omeliansky 1914). It was the most commonly found bacteria in Lake Alexander, a fresh-water lake in Minnesota, and were found to be ubiquitously observed in stagnant and tap water as well (Henrici and Johnson 1935). The bacterium was described as a stalked vibrio with distinctly curved cells and rounded polar ends. Its multiplication occurred by transverse binary fission. The outermost cell is set free after cell division and would find a new substrate and proceed to secrete a new stalk. At the end of the slender stalk they observed a distinct button-like expansion which they considered to be the holdfast (Henrici and Johnson 1935). *C. vibrioides* was named to be the type species of the *Caulobacter* genus. The full taxonomy is as follows: Bacteria > Proteobacteria > Alphaproteobacteria > Caulobacteriales > Caulobacteraceae > *Caulobacter*.

C. crescentus was first classified in 1964 (Poindexter 1964) but was actually found to be synonymous to *C. virbioides* in 1999 after a DNA study (Abraham et al. 1999). Now, the name *C. crescentus* is more commonly used and will therefore also be used throughout this proposal.

C. crescentus is an aquatic Gram-negative bacterium that thrives in nutrient-poor environments. It is commonly used as a model organism to study bacterial cell biology, asymmetric cell division, and cellular differentiation. *C. crescentus* has two different states, first it persists as a mobile swarmer cell with a single flagellum. The bacterium will then differentiate to a cell with a tubular stalk structure with an adhesive holdfast material at its end to permanently adhere to any surface. Only in the stalked state will a new cell division start, forming a swarmer cell (Barrows and Goley 2023). (**Error! Reference source not found.**, (Hughes, Jiang, and Brun 2012))

Little is known thus far about the holdfast, with a diameter of around 400 nm and a thickness of 40-50 nm (Liu, Zhang, and Xu 2021), partially because the low amount of biomass per holdfast makes it difficult to study. However, with an adhesive strength of 68 N/mm^2 (680 kg/cm^2) it is arguably the strongest natural adhesive measured thus far (Nyarko et al. 2020). The holdfast is gelatinous in nature and has elastic characteristics. In its 'wet' stage it is three times the size of its 'dry' state. Holdfast can adhere to substrates of a wide variety in physical and chemical properties, which makes it an attractive model for industrial and medical adhesives (Hershey et al. 2019).

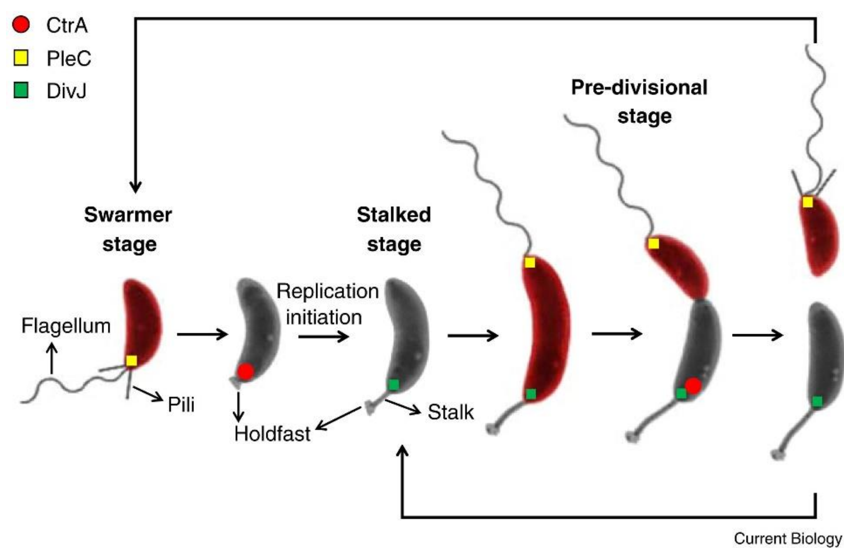

Figure 1: Cell cycle of *Caulobacter crescentus*, derived from Hughes et al. (2012)

Composition

The main component of holdfast has been identified with target specific enzymes and fluorescein as a polymer polysaccharide with N-acetylglucosamine (GLcNAc) as the basic unit (Berne et al. 2013). Other monosaccharide components of the holdfast matrix include 3-O-methylglucose, glucose, mannose and xylose (Hershey et al. 2019).

After lysosome digestion, of which the common substrates include oligomers of GLcNAc, viscosity of holdfast was reduced to 10% of its original strength. This indicates that GLcNAc polymers also play an important role for the viscosity of holdfast (Li et al. 2005). This was confirmed in a different study where atomic force microscopy (AFM) and enzymatic assays were used to study the structure of holdfast. The presence of DNA and polypeptides were also found in holdfast and had an effect on the viscous strength as well (Hernando-Pérez et al. 2018).

The current model of holdfast suggests that it is a heterogeneous material that consists of two layers: a flexible and far reaching surface layer and a rigid core layer (figure 2). A 1,4-linked backbone of glucose, GLcNAc, mannose and xylose with glucose and mannose branches at the C-6 position plays an important structural role within the core ((Hershey et al. 2019) and DNA and peptides are probable constituents of the surface layer (Hernando-Pérez et al. 2018). Mechanical characterization suggests that the core becomes more homogeneous as it shifts from its fluid secreted state to a solid cured state, similar to the hardening of epoxy glues. This will distribute applied force on the bacterium equally among the surface bonds, increasing its strength. The polymers in the surface layer can extend over a distance that is multiple times the radius of the core. The surface layer, also called the brush, is mainly thought to explore the surface and initiate a weak adhesion through the use of DNA. Scientists haven't succeeded in completely removing the brush layer yet so this remains speculation. Progressive compaction of the surface-bound brush layer will then close the gap between the surface and the holdfast core (Hernando-Pérez et al. 2018). Other bacteria related to C. *crescentus* have been found to create similar nanoscopic adhesive structures (Fritts et al. 2017; Merker and Smit 1988).

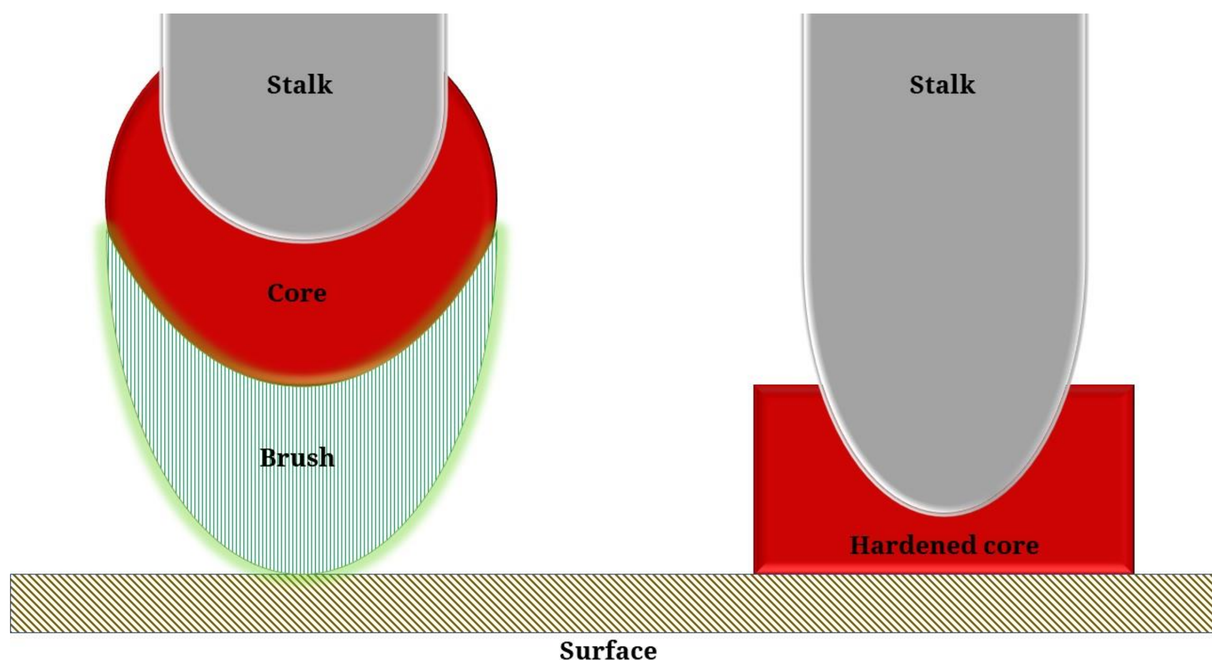

Figure 2: Current 2-layer model of the structure of *C. crescentus* holdfast. The holdfast consists of a brush and a stiff core. The brush is composed of DNA and peptides and makes an initial weak adherence to the surface. Subsequent compaction leads to the hardening of the core in a way similar to an epoxy glue, where molecules are aligned in a more homogeneous way. The core is mainly composed of a polysaccharide with N-acetylglucosamine (GLcNAc) as the basic unit but also contains 3-O-methylglucose, glucose, mannose and xylose. With a proposed structure of a 1,4-linked backbone of glucose, GLcNAc, mannose and xylose with glucose and mannose branches at the C-6 position. But there might be other chemical compounds involved in both the core and brush.

Holdfast is only once secreted in the life cycle of *C. crescentus*, partly for this reason the amount of holdfast per cell is very low, furthermore it also has a relatively difficult purification process due to its adhesive nature. The available experimental techniques for these small amounts of holdfast have been insufficient to fully resolve its chemical components, and even less to know about its precise structural features. To this day, a large part of the composition and structure of holdfast remains a mystery.

Related genes

Genetic determinants of holdfast biosynthesis provide invaluable insight into its chemical makeup. Multiple genes have been identified as important for holdfast production and predicting their encoded machinery has helped with understanding the structure of holdfast (Hershey et al. 2019).

Adhesion-deficient mutants of *C. crescentus* were originally grouped into three different classes based on phenotypic characteristics. One class is for the holdfast synthesis (*hfs*) related genes. Mutants with inserted defects in these genes fail to produce holdfast and are unable to adhere to any surface, indicating that these *hfs* genes are essential for holdfast synthesis. There are also the holdfast anchoring (*hfa)* genes, these mutants showed defects in the surface adhesion. Notably, mutants were the holdfast had a reduced ability to attach to the tip of the stalk, causing the whole adhesive holdfast polysaccharide to be released from the cell, also belong to these group. The last class has no discerning nomenclature and is for the genes were mutation has multiple developmental effects (Toh, Kurtz, and Brun 2008).
An overview of the identified genes related to holdfast synthesis and anchoring are shown in Table 1 (Hershey et al. 2019). They represent the major factors required for holdfast production.

Table 1: Overview of identified genes for holdfast synthesis and anchoring, adapted from Hershey et al. (2019).

Gene	**Locus**	**Mutant phenotype**	**Annotation**	**Reference(s)**
hfsA	CC_2431	No holdfast production (HF^-)	Polysaccharide copolymerase Wzz	(Smith et al. 2003)
hfsB	CC_2430	Few cells with holdfasts; small holdfasts; adhesion defect	Polysaccharide secretion autokinase	(Smith et al. 2003; Javens et al. 2013)
hfsC	CC_2429	Redundant with *hfsI*; Δ*hfsC* Δ*hfsI* mutant is HF^-	Polysaccharide polymerase Wzy	(Toh, Kurtz, and Brun 2008)
hfsD	CC_2432	No holdfast production (HF^-)	Polysaccharide secretin Wza	(Smith et al. 2003)
hfsE	CC_2425	Redundant with *pssY* and *pssZ*; Δ*hfsE* Δ*pssY* Δ*pssZ* mutant is HF^-	Hexose phosphate transferase (PHPT)	(Toh, Kurtz, and Brun 2008)
hfsF	CC_2426	Smaller holdfasts; adhesion defect	Polysaccharide flippase Wzx	(Toh, Kurtz, and Brun 2008; Hardy et al. 2018)
hfsG	CC_2427	No holdfast production (HF^-)	GT2 family glycosyltransferase	(Toh, Kurtz, and Brun 2008)
hfsH	CC_2428	Loss of holdfast cohesiveness; adhesion defect	Polysaccharide deacetylase	(Toh, Kurtz, and Brun 2008)
hfsI	CC_0165	Redundant with *hfsC*;	Polysaccharide	(Toh, Kurtz, and

		Δ*hfsC* Δ*hfsI* mutant is HF⁻	polymerase Wzy	Brun 2008)
hfsJ	CC_0095	No holdfast production (HF⁻)	WecG/TagA family glycosyltransferase	(Fiebig et al. 2014)
hfsK	CC_3689	Loss of holdfast cohesiveness; adhesion defect	CelD family acyltransferase	(Sprecher et al. 2017)
hfsL	CC_2277	No holdfast production (HF⁻)	GT2 family glycosyltransferase	(Hershey, Fiebig, and Crosson 2019)
hfaA	CC_2628	Holdfast anchoring defect; holdfast shedding	CsgA-like curlin protein	(Ong, Wong, and Smit 1990; Hardy et al. 2010)
hfaB	CC_2629	Holdfast anchoring defect; holdfast shedding	CsgG family curlin secretion protein	(Ong, Wong, and Smit 1990; Hardy et al. 2010)
hfaD	CC_2630	Holdfast anchoring defect; holdfast shedding	Hypothetical protein; signal peptide	(Ong, Wong, and Smit 1990; Hardy et al. 2010)
hfaE	CC_2639	Holdfast anchoring defect; holdfast shedding	Hypothetical protein; signal peptide	(Hershey, Fiebig, and Crosson 2019)
pssY	CC_0166	Redundant with *hfsE* and *pssZ*; Δ*hfsE* Δ*pssY* Δ*pssZ* mutant is HF⁻	Hexose phosphate transferase (PHPT)	(Toh, Kurtz, and Brun 2008)
pssZ	CC_2384	Redundant with hfsE and *pssY*; Δ*hfsE* Δ*pssY* Δ*pssZ* mutant is HF⁻	Hexose phosphate transferase (PHPT)	(Toh, Kurtz, and Brun 2008)

Among these genes, there were many annotated to encoding machinery for the production of an extracellular polysaccharide (Smith et al. 2003). Four of them encode glycosyltransferases (GTs), implying that the holdfast polysaccharide has a repeating unit of at least four monosaccharides (Hershey et al. 2019).

The mechanism of holdfast production (figure 3) seems to be very similar to the Wzx/Wzy-dependent group I capsular polysaccharide synthesis pathway in *Escherichia coli* (Cuthbertson et al. 2009). HfsE, PssY and PssZ, all annotated glucosyltransferases, initiate holdfast polysaccharide synthesis by transferring activated sugar phosphates from UDP to a lipid carrier in the cytoplasm (Toh, Kurtz, and Brun 2008). Then, three other annotated glycosyltransferases HfsG, HfsJ and HfsL, add monosaccharide substituents to form a repeat unit on the lipid carrier (Chepkwony, Hardy, and Brun 2022). The deacetylase HfsH and an annotated acetyltransferase HfsK will subsequently modify some of the sugar residues on the repeat units (Chepkwony, Hardy, and Brun 2022). Thereafter, the lipid carrier with the polysaccharide repeat unit is translocated into the periplasm by HfsF, an annotated flippase (Toh, Kurtz, and Brun 2008). It is thought that the two polymerases HfsC and HfsI then assemble the repeat units into the holdfast polysaccharide (Toh, Kurtz, and Brun 2008). Finally, the holdfast polysaccharide would be secreted through an export protein complex composed of HfsA, HfsB and HfsD (Smith et al. 2003).

The HfaA, HfaB, HfaD and HfaE anchoring proteins are responsible for the anchoring of the holdfast polysaccharide. Removing these proteins causes (partial) shedding of the holdfast, where removing HfaB causes the most shedding (Hardy et al. 2010). It is therefore thought that

HfaB has an additional function, such as the formation of a complex with the holdfast polysaccharide or the secretion of other unidentified holdfast proteins (Hardy et al. 2010). The anchoring mechanism is still very poorly understood and it is only known that deacetylation of the holdfast polysaccharide is required for its connection to the cell surface (Wan et al. 2013).

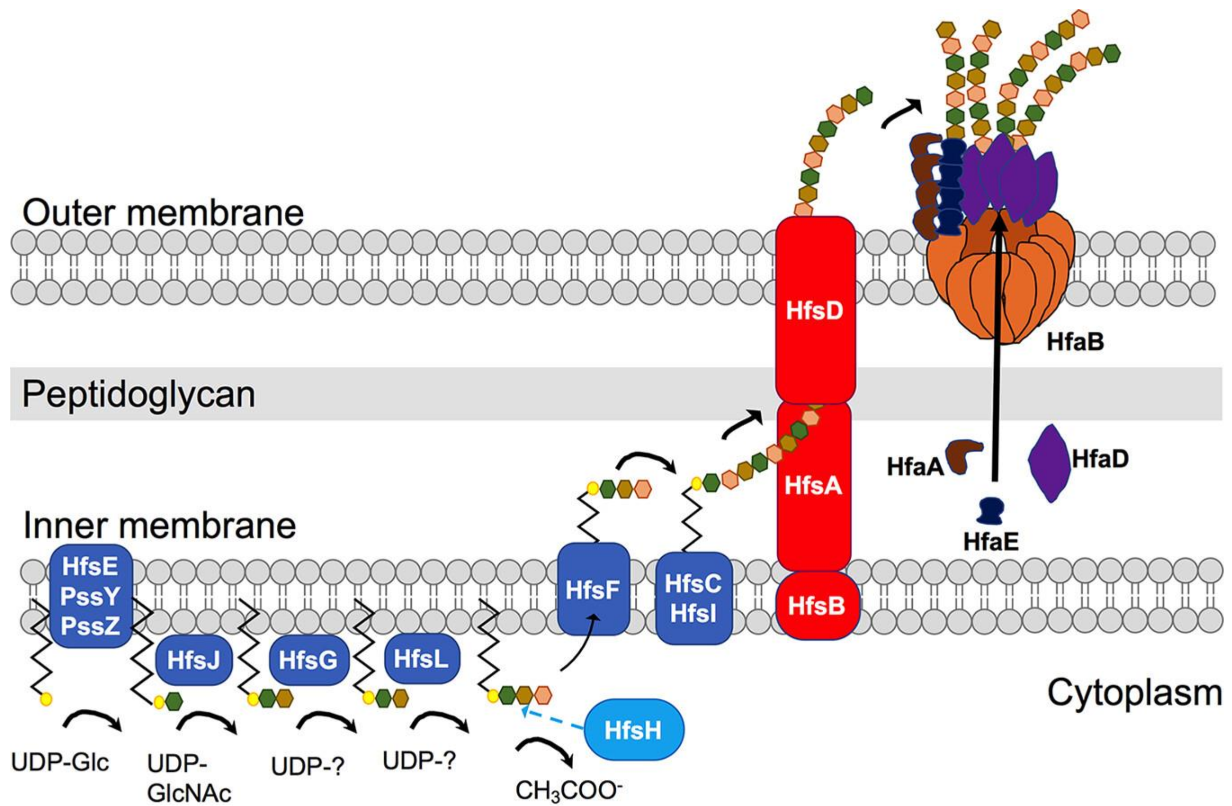

Figure 3: Current model of the production of the *C. crescentus* holdfast. This schematic overview shows the related synthesis, modification, secretion and anchoring machinery of the holdfast polysaccharide. The glycosyltransferases HfsE, PssY, PssZ, HfsJ, HfsG and HfsL add different monosaccharide components to a lipid-linked repeating unit in the cytoplasm. HfsH deacetylates some of the sugar residues before HfsF transport the structure into the periplasm. Repeat units are then assembled by the polymerases HfsC and HfsI and secreted by an export protein complex composed of HfsA, HfsB and HfsD. An anchoring complex composed of HfaA, HfaB, HfaD and HfaE tethers the holdfast polysaccharide to the cell wall. The different colors of the hexagons that represent the holdfast polysaccharide indicate different sugars. Adapted from Chepkwony, Hardy and Brun (2022)

Aim

The structure and production of *C. crescentus* holdfast is still very poorly understood. Despite being one of the strongest natural glues measured so far, it was not until recently that more research has been performed on holdfast. Its potential as a biobased underwater adhesive could have great future impact on wound repair in medical surgeries or other wet applications. However, before any applications will be possible, the holdfast needs to be understood thoroughly. I therefore propose, in order to understand *C. crescentus* holdfast in such a way that biomimicry or biosynthesis of the glue might be possible in the future, to research the following:

1. *How does the chemical structure of* Caulobacter crescentus *holdfast correlate with its adhesive strength?*

My first aim is to study the chemical composition of holdfast using highly specialized machinery like nuclear magnetic resonance (NMR) spectrometry and single-molecule force spectrometry (SMFS) to identify all molecules that make-up the holdfast. Singling out all the components of holdfast and studying their interactions will lead to new insights of what exactly causes the adhesive strength in holdfast. Subsequent comparing of these findings with the holdfast of different strains and species might also clarify differences in adhesive strength or characteristics.

2. *The whole biosynthetic pathway of* Caulobacter crescentus *holdfast has to be identified.*

My second aim focuses on studying the biosynthetic pathway and related genes of holdfast. Using whole genome sequencing and function analysis of all related genes the complete pathway of holdfast should be mapped out. Once the whole pathway is known, a deeper understanding of the exact build-up of holdfast will be gained. This will be extremely valuable for endeavours in mimicking the holdfast or increasing its production by deleting inhibitors or other production limiting factors.
Comparative genomics studies with different species or strains that produce holdfast might also identify holdfast characteristics related genes, which will give a deeper understanding of certain behavioural differences in the holdfasts.

3. *Can the yield of* Caulobacter crescentus *holdfast be increased?*

In my third aim I explicitly focus on the potential of holdfast as an useable underwater adhesive. Here, I want to dramatically increase the amount of holdfast production. This will be achieved by either increasing production in *C. crescentus* itself through genetic modification or by translocating the biosynthetic production apparatus of holdfast to a more well known organism like *Escherichia coli* to create recombinant holdfast. Once enough holdfast is produced, more mechanical experiments can be performed to identify all the properties of holdfast and thus gain a deeper understanding of its potential as a future natural adhesive.

B.2.2 Approach

The aims of this research as described in the previous section are here supplemented with suggestions for their approach. Before any experiment can be performed. a few strains of *C. crescentus/C. vibrioides* need to be ordered. Luckily, these are readily available at the ATCC and DSMZ micro-organism and cell culture collections. Obtaining *C. crescentus* strains will therefore not pose any problems. The approaches are divided in the three distinct packages related to their aims, this means that at least three different papers could be produced by this research, as is mandatory for a PhD research. The time to perform this research would therefore consist of 4 years, a time-frame which seems pretty accurate to perform these experiments.

1. **How does the chemical structure of *Caulobacter crescentus* holdfast correlate with its adhesive strength?**

Most performed experiments thus far on holdfast include electron microscopy (EM) studies and atomic force microscopy (AFM), since they could be used on single cells. AFM has been

used to create three-dimensional images of the holdfast at nanoscale resolution to discover new structural and local chemical characteristics of holdfast (Hernando-Pérez et al. 2018).

However, the composition and precise structure of *Caulobacter crescentus* holdfast has not been fully elucidated. Techniques from structural biology and crystal analysis can be used to further understand the chemical composition and structural features of holdfast. Once enough of the holdfast is obtained nuclear magnetic resonance analysis (NMR) and mass spectrometry can be performed to determine the order of the monosaccharides in the holdfast polysaccharide. A new technique called single-molecule force spectroscopy (SMFS) can unravel material properties of purified bio-adhesives at a single-molecule level and could be very valuable here as well (Hernando-Pérez et al. 2018).

Besides the holdfast polysaccharide there might still be some other chemical components that together form the holdfast. NMR and mass spectrometry can also identify these molecules, especially NMR is capable of forming two dimensional or three dimensional structures of a targeted area. This will therefore be a set of very important experiments once enough holdfast is gathered.

Once the complete structure of holdfast is known, it will be possible to determine what exactly causes the adhesive strength of holdfast. This could be made visual with computer modelling of the structure and chemical interactions of holdfast and the surface.

After the exact structure of the *C. crescentus* holdfast polysaccharide has been discovered, the same experiments could be performed on similar species containing holdfast such as *Hischia baltica.* The differences in holdfast composition and/or structures could then explain differences in the adhesive characteristics.

2. **The whole biosynthetic pathway of *Caulobacter crescentus* holdfast has to be identified.**

A lot of genes related to the holdfast synthesis have been identified already. However, not all functions are completely understood. To better understand their role and function in holdfast production a few different experiments involving genomics and bioinformatics techniques can be performed. Whole genome sequencing and a functional analysis of all encountered genes might find more holdfast related genes which have not been discovered yet. In that case, a series of gene knock-out or deletion experiments will have to be performed to better understand the function of these genes in relation to holdfast production. *In vitro* studies should help in clarifying the function of different holdfast synthesis related enzymes and proteins. Isolating the associated enzymes and proteins will help to study their behaviour in a more controlled environment.

Once the whole biosynthetic pathway is understood, it will also help in understanding the composition and structure of holdfast better. Furthermore, key important genes would then be identified that ensure holdfast production. Understanding which genes these are will help with future studies related to the yield or mimicry of holdfast.

Additionally, comparative genomics experiments with different strains and species might also clarify functions and characteristics of some holdfast specific genes. For example, some genes encoding a different enzyme might explain a distinct phenotype in the differing type of holdfasts. Understanding these characteristics associated genes could potentially lead to adaptions of holdfast, resulting in an adhesive that suits each individual environment. In a

high-ionic environments such as the sea, the biosynthesis pathway of *Hirschia baltica* holdfast might produce the best adhesive for this environment (Chepkwony, Hardy, and Brun 2022), whereas the holdfast of *C. crescentus* might be more suitable for applications in fresh water or medicine (Nyarko, Barton, and Dhinojwala 2016). Furthermore, understanding the differences in the production related genes of holdfast could also expose the reason for other differing characteristics observed in holdfast from different species, such as the adhesive strength (Hernando-Pérez et al. 2018).

3. Can the yield of *Caulobacter crescentus* holdfast be increased?

Before the *C. crescentus* holdfast has any potential as a future adhesive, it is of paramount importance that the yield is increased. There are different approaches to this problem. The first is to increase production by the bacteria itself, this was done by Hershey *et al.* through a double mutant of *C. crescentus* (Hershey et al. 2019). These mutants had deletions of the HfiA, a holdfast inhibitor gene, and the HfaB and HfaD loci, which are essential for holdfast anchoring. The result was overproduction and release of holdfast in the culture medium, allowing easier extraction for subsequent analysis (Hershey, Fiebig, and Crosson 2019). However, they themselves stated already that their method is still not good enough to create large enough quantities of holdfast for specific experiments (Hershey et al. 2019). Their extraction method does therefore provides a framework for optimizing and scaling up the production of holdfast in *C. crescentus.*

First of all, after a more thorough investigation of the holdfast synthesis related genes, more inhibitors could be discovered that could be deleted as well. It might even be possible to create a mutant that has multiple stalks from where to create holdfast, or to remove the stalk and make holdfast synthesis something which occurs over the whole cell envelope. These suggestions would however translate to a lot of different experiments with high risks of being unsuccessful, especially since not all related factors of *C. crescentus* holdfast production are currently known. Therefore, a different approach would be to translocate all genes related to the holdfast production apparatus to a more understood and studies species such as *Escherichia coli* through vector placement. In this case, *E. coli* would then produce recombinant holdfast polysaccharides. This might make it easier to adjust the holdfast production to specific experimental needs since it occurs in a slightly more controlled environment. Furthermore, *E. coli* does not possess a stalk which might make production of holdfast easier and faster. This method is already widely used in the pharmacy industry for the production of certain medicine such as vaccines, insulin, hormones and antibodies and has therefore been very successful before (Kay et al. 2015).

Both of these suggestions have the advantage that the whole composition and structure does not have to be understood exactly to perform these experiments. However, once these are understood it might also be possible to artificially produce larger quantities of holdfast by mimicking its chemical structure. Techniques such as polycondensation or ring-opening polymerization (ROP) are well suited for artificially producing a desired polysaccharide. These techniques do however produce a more heterogeneous solution of polysaccharides and for more pure synthesis with the exact desired monomer order there are techniques such as automated glycan assembly (AGA) which performs a total synthesis of polysaccharides. AGA does however also involve large machinery and significantly more effort. For a more sustainable production process this might therefore not be the most favourable option, but it will eliminate the need of using bacteria. Steps like bacteria culture maintenance and holdfast polysaccharide isolation would then not be needed anymore.

The drawback of artificially producing holdfast is that the structure of holdfast might proof to be too complex to synthetically create with the currently available techniques. However, it could potentially mimic the most important features of holdfast that are responsible for its adhesive strength. Thus creating a more simplified, but also strong, underwater adhesive inspired by the holdfast polysaccharides.

B.2.3 Feasibility / Risk assessment

What has impeded the research on *C. crescentus* holdfast so far is the small quantity of holdfast that the organism biosynthesizes per cell and the difficulty of isolating the holdfast because of its adhesive nature. This is the reason that many available experiments could not be performed and it will undoubtedly effect this research as well. However, a few methods have been discovered to make the process of purification easier and the quantity of the holdfast polysaccharide in the medium larger (Hershey et al. 2019). These methods enable the execution of new experiments, such as the ones described previously.

In this proposal, we mainly suggest research techniques that have worked on other organisms, adhesives or polysaccharides before. By implementing these techniques in our own experiments with *C. crescentus* we minimize the risk of failure of these experiments. When we perform the experiments on a different *C. crescentus* strain or species such as *Hirscha baltica*, we will only do that after the experiments on the experimental strain of *C. crescentus* have succeeded. Repeating proven methods with these different organisms will be essential before new experiments could be performed.

The risk mainly lies in the optimization of these methods. It is uncertain if scaling up of holdfast production might result in a loss of adhesive strength, it is possible that it only works as an adhesive at a nanoscopic level. Especially because of the unanticipated complexity of both the structure and composition of holdfast. This would mean that it could potentially never function as a commercial adhesive. However, it could still be useful in other (nanoscopic) applications and all newly acquired knowledge will be valuable.

B.2.4 Scientific and societal impact

The impact of this research could potentially be enormous. On a scientific level, there will be a lot more understanding of the *C. crescentus* holdfast chemistry and biosynthesis pathway. This could lead to different kinds of experiments where the proposed methods could form as an example to investigating other species or where new functions and applications of holdfast could be discovered.

Societally speaking, this research could lead to the eventual manufacture of a new biobased and more environmentally friendly underwater adhesive, something that is currently non-existent. The prospect is that the developed adhesive could even outperform current synthetic adhesives, considering synthetic adhesives have difficulty with good underwater performance (Nyarko, Barton, and Dhinojwala 2016). This would mean that there is a huge market for these type of adhesives, increasing its societal impact. But not only in underwater conditions could this adhesive be useful, there is also a need for a healthy wound repairing adhesive in the medical industry and adhesives in humid environment, for which this new adhesive might be

suitable. This means that the impact of this adhesive on an industrial and medical level could be considerate.

However, maybe equally important, the impact on the way of thinking and practice in society could be tremendous as well. Creating a good-performing adhesive, inspired from nature and manufactured by nature, shows the ingenuity and genius of nature. Stimulating the movement towards more bio-inspired and bio-based environmentally friendly products. This adhesive would help indicate that the time of a fossil- and synthetic-based economy is over and the time for a bio-based and environmentally friendly economy is starting, something that should reflect in the everyday life of everyone in the near future.

B.2.5 Ethical considerations

In a few of the proposed experiments it would be necessary to create genetically modified organisms (GMOs). The creation of GMOs has always been a controversial topic with ethical concerns. These range from more philosophical considerations, such as the morality of tinkering with the DNA of organisms, to more practical considerations, such as the danger of the new-species outperforming the old species in survivability and resources (Burkhardt 2001). Religion often disagrees with genetic modification as well since, according to their beliefs, mankind should not possess such power.

The practical form of concerns we can modulate by only producing the GMOs in ML1 certified laboratories and minimizing the possibility of spreading by autoclaving every utensil that has been in contact with this genetically modified species. The more philosophical concerns are harder to address, here it can only be said that it is done with the best intentions to eventually create a more healthy and liveable planet that would preserve and restore the habitat for a lot of different species. Although there is opposition against GMOs, the advancements it has brought are indisputable. Many vaccines are nowadays produced by GMOs so that they are also affordable for third world countries. Fruit and vegetables that can be bought at the supermarket came from genetically modified plants. They are modified to ensure increased quality production and pathogen resistance. Crossbreeding, which is arguable also genetic engineering, cannot keep up to everyday production demands due to the fast spreading of diseases and the increased world population.

If through genetic modification an environmentally friendly adhesive could be created from the *C. crescentus* holdfast, the benefits to the planet and environment might outweigh the impediments. Furthermore, it would also prove that biobased products can be of high quality as well and might inspire others to produce more environmentally friendly and circular products as well.

B.2.6 Literature/references

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