Research Advances in Biosynthesis Mechanism of Ultra-High Adhesive Material - Holdfast

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ABSTRACT
As a unique secretary product of Caulobacter crescentus, holdfast is the most powerful natural viscous substance known so far, and its detailed synthetic mechanism is still not clear. The biosynthetic process of holdfast is complex, including the synthesis, transportation and secretion of monosaccharides and polysaccharides, as well as the various modifications in each above-mentioned step. Many genes related to the sugar metabolism, the synthesis and modification of proteins, the modification of polysaccharides, the transportation and the secretion of polymer are involved in this process. In addition, the production of holdfast is also affected by cell cycle and stress. This review focuses on the recent advances in the study on the physicochemical property, transportation, secretion and modification of holdfast, as well as its synthetic pathways. The complex associations of genes involved in holdfast biosynthesis are also summarized, which helps deepen the understanding of its biosynthetic mechanism, thus gives the guidance on the future research and development of holdfast.

KEYWORDS: Holdfast; Caulobacter crescentus; Biosynthesis; Deacetylation; Transportation and Secretion

INTRODUCTION
In the microbial world, holdfast, the natural substance with the strongest adhesion, presents in Caulobacter crescentus [1,2], and is not easily observed. As a natural adhesive material with high viscosity, high environmental adaptability, high biocompatibility and no chemical pollution, the application of holdfast is extensive, especially in précis ion instrument manufacturing and underwater engineering. Holdfast is an ultra-viscous polysaccharide, which is different from the natural adhesive substances commonly found in nature. For example, mussels secrete the foot filament proteins Mfp-3 and Mfp-5 interacted with the interface through their DOPA structure to produce viscosity [3-5]. Barnacles secrete the adhesive protein cp19k, which is a self-assembling fibrin resulted in the viscosity through the aggregation of its amyloid fibrils [6,7]. In general,
polysaccharide substances such as chitosan, hyaluronic acid and dermatan sulfate are not highly viscous, which are usually developed into health care or medical products [8-11]. On the other hand, the discovery of holdfast breaks the perception that polysaccharides are not so sticky. Current researches on underwater adhesive materials mainly focused on natural adhesive proteins secreted by mussels and barnacles, but holdfast adhesive polysaccharide is more characteristic, which possesses a greater potential of development [12,13]. Its ultra-high viscosity can be applied to the field of safety devices with stringent requirements for adhesion force, its high environmental adaptability can be applied to the environment (such as underwater or special interface) where adhesives cannot be used for bonding or the bonding effect is poor, its high biocompatibility can be applied to the medical field, especially the implantable medical devices, and its property of non-chemical pollution meets the requirements to the development of adhesive industry towards green environmental protection.

This review attempted to briefly summarize the recent progress in holdfast, to comb the findings related to its biosynthesis, and to provide assistance for further studies and artificial manufacture of holdfast. The components of holdfast are mainly N-acetylglucosamine. The mechanism of holdfast biosynthesis is very complex, including the synthesis, transport and secretion of polysaccharides, as well as various modifications. Many genes are involved in the biosynthesis of holdfast; therefore, its regulation mode and pathways are extremely complex. In addition, the production of holdfast is also regulated by cell cycle and stress. Defining the biosynthetic mechanism of holdfast will help to understand why it is ultra-viscous and develop new viscous materials based on its properties.

COMPOSITION AND PHYSICOCHEMICAL CHARACTERISTICS OF HOLDFAST

The composition and chemical structure of holdfast are not yet completely expounded. A research provides a method to extract the holdfast from *C. crescentus* cultures and analyzes the monosaccharide components of the adhesive matrix, which includes Glucose, 3-O-methylglucose, mannose, N-acetylglucosamine, and xylose[14]. The treatment of holdfast with multiple enzymes reveals that the proteolytic enzymes can’t change its integrity and viscosity, while chitinase and lysozyme can destroy the viscosity of holdfast. It is known that the common substrates of chitinases and lysozymes include oligomers of N-acetylglucosamine (GlcNAc). After incubation with holdfast using a variety of fluorescein-conjugated lectins, respectively, only wheat germ agglutinin (WGA) can bind to holdfast.

The specific binding substrate of WGA is also GlcNAc, thus demonstrating that the main component of holdfast is not a protein but a polymer of polysaccharide with N-acetylglucosamine as the basic unit [15]. Meanwhile, when testing holdfast viscosity, the elastic strength of holdfast reduced to 10% of its original value after lysozyme treatment, demonstrating that GlcNAc is the main component of holdfast that produces viscosity [13]. Since holdfast retains a portion of its viscosity after lysozyme treatment, but the low viscosity of PGA is observed by comparison with the GlcNAc polymer poly-β-1, 6-N-acetylglucosamine (PGA), demonstrating that there may be other components affecting the high viscosity of holdfast [16]. In order to further clarify the chemical composition of holdfast, some researchers have used atomic force microscopy (AFM) to perform nanoindentation measurements on holdfast treated with different enzymes. It is found that the holdfast viscosity strength decreases about one-third after DNase I and lysozyme treatment at the same time, by referring to the viscosity change of holdfast after treatment, indicating that DNA and GlcNAc may play an important role in its viscosity. However, the holdfast core stiffness is significantly decreased after treatment with proteinase K, demonstrating the presence of polypeptides in holdfast and their effect on the viscous strength [17]. In another study, researchers employed spectroscopic techniques to probe holdfast. Results indicate the similarity of the holdfast to peptidoglycan from other bacterial species; surface-sensitive sum frequency generation show that aromatic and hydroxyl groups related to this protein content at the adhesive interface could be playing a crucial role in adhesion [18]. In order to determine the size of holdfast, atomic force microscopy (AFM) and fluorescence microscopy are used to determine the final state of solidification after secretion of holdfast, which is disk-shaped, with a diameter of about 400 nm and a thickness of about 40 ~ 50 nm [19,20]. The holdfast polysaccharide has some gel-like properties, and the determination of the size of its two states of dryness and wetness by AFM has revealed that the air-dried holdfast is one-third of its original thickness [21]. Due to the less secretion of holdfast and its difficulty in purification, the available experimental techniques are still insufficient to fully resolve its chemical components, and even less to know about its precise structural features.
The means to determine the viscous strength of trace substances include techniques such as laser tweezers, atomic force microscopy, flow chamber shear pressure and micropipetting [22-25]. Because of the low molecular weight and high viscous strength of holdfast, all the means above can't measure the viscosity of holdfast directly. Tsang PH et al. designed an AFM-based holdfast viscosity test protocol, which first allowed *Caulobacter crescentus* to adhere to a micropipette tip, and then uses a micromanipulator to pull the bacteria vertically to separate the holdfast from stalk or micropipette tip. The magnitude of tensile force was calculated by the deformation distance of the micropipette tip at the time of separation, and the maximum adhesion force of holdfast was up to 68N/mm2 (Figure 1). Their results showed that the location of the fracture was at the interface between holdfast and the bacteria, so it was presumed that its actual adhesive force might be higher than that of the result [26].

**Figure 1:** A single *C. crescentus* cell is attached to a thin flexible pipette. The micromanipulation holding suction pipette pulls the cell until detached. The force of adhesion can be calculated from the amount of bending required to break the cell–pipette contact.

**BIOSYNTHESIS OF HOLDFAST**

Through the combinatorial analysis of genes involved in the synthesis of holdfast, a biosynthetic pathway of holdfast can be roughly established. As a polysaccharide, the synthesis of holdfast starts with the transfer of glucose-1-phosphate from an uracil diphosphate (UDP) nuclear carrier to an undecaprenyl phosphate (Und-P) lipid carrier by the glycosyltransferase HfsE (holdfast synthesis E) to form monosaccharide glycolipids [27,28]. Then the glycosyltransferase HfsJ can synthesize monosaccharide lipids into disaccharide lipids, and the glycosyltransferase HfsG, which continues the work of HfsJ, further transfers N-acetylglucosamine to disaccharide lipids and further increasing the length of oligosaccharides. The deacetylase HfsH, partially deacetylates oligosaccharides, and both the synthesis and modification of these oligosaccharides are completed on the intracellular membrane. Subsequently, oligosaccharides cross the intracellular membrane into the periplasm by the flipase HfsF [29-31]. Oligosaccharides entering the cell periplasm are polymerized to form polysaccharides by the polymerase complex HfsC/I. After the polymerization of the polysaccharide, it is required to transport the polysaccharide to the outer membrane of the cell via the transmembrane transport complex HfsDAB [32,33]. Finally, the holdfast polysaccharide will localize to the anchoring protein complex HfaABD and undergo surface adhesion after secretion into the extracellular space through the transport complex. Subsequent secretion and adhesion processes are regulated by genes such as cell cycle-related factors and signal transduction [34,35,36]. A diagram of the holdfast biosynthetic pathway is shown in figure 2.
Figure 2: Diagram of Holdfast Biosynthesis Pathway. The synthesis of holdfast starts with the transfer of glucose-1-phosphate by the glycosyltransferase HfsE. Then the length of oligosaccharide is increased by HfsJ and HfsG. Oligosaccharide is partially deacetylated by the esterase HfsH. Oligosaccharide is transferred to the periplasm by the flippase HfsF and polymerized by the polymerase HfsC/I. Via the transmembrane transport complex HfsDAB, the holdfast polysaccharide is exported and localized to the anchoring protein complex HfaABD. The synthesis of holdfast is inhibited by HfiA which is regulated by c-di-GMP. The c-di-GMP also effects the N-acetyltransferase-like enzyme HfsK. PleD, the regulator of c-di-GMP contributes to holdfast induction along with flagellar assembly.

At present, some progress has been made in the study of its synthesis mechanism at the gene level, and more is known about the genes involved in the synthetic pathway of holdfast through whole-genome sequencing and analysis of Caulobacter Crescentus [41,42]. Genes directly involved in the synthesis and secretion of holdfast are divided into two gene clusters, named as holdfast synthesis (hfs) and holdfast anchoring (hfa) gene clusters [43,44]. On the other hand, the subsequent gene found to inhibit holdfast synthesis is named holdfast inhibitor (hfi) gene [45]. Functional analysis of these genes and their encoded proteins will help to further define the components, chemical modifications, and their detailed synthetic pathways of holdfast.

HOLDFAST POLYSACCHARIDE SYNTHESIS RELATED GENES

First, the initial step of holdfast synthesis is the transfer of glucose-1-phosphate from an uracil diphosphate (UDP) nuclear carrier to an undecaprenyl phosphate (Und-P) lipid carrier molecule by glycosyltransferase (HfsE) to form monosaccharide glycolipids. As the initial enzyme in the synthesis of holdfast, HfsE is replaceable. Synthesis of holdfast is not affected in the hfsE knockout strain. By genome sequence analysis, two hfsE homologs are found. Gene pssZ and pssY can express glycosyltransferases in strains of ΔhfsE [46].

The key gene in the synthesis of oligosaccharides from monosaccharide lipids is hfsJ, which encodes a glycosyltransferase that synthesizes monosaccharide lipids into disaccharide lipids. It has been shown that strains with a deletion mutation in hfsJ are unable to form holdfast, illustrating the irreplaceability of this gene [47]. Further studies have revealed that the gene hfaA, which has an
inhibitory effect on holdfast synthesis, can specifically suppress the expression of hfsJ. At the same time, hfsA is regulated by multiple developmental regulatory proteins during cell cycle progression. Thus, hfsA is the connection of the holdfast synthesis pathway to the cell cycle regulatory network in *Caulobacter Crescentus* [48,49]. The hfsG gene, which encodes a glycosyltransferase, is responsible for transferring N-acetylglucosamine onto disaccharide lipids and further increasing the sugar chain length of oligosaccharides, while deletion of the hfsG gene results in failure of holdfast synthesis, demonstrating its critical role in holdfast synthesis [50].

**Holdfast Modification and Transport Related Genes**

After the oligosaccharide chains reach a certain length, the deacetylase encoded by the hfsH gene can partially deacetylate oligosaccharides. Studies have shown that strains with hfsH knockout can secrete holdfast normally, but it has almost no adhesive function. At the same time, strains overexpressing the hfsH gene have higher adhesion efficiency than that of wild-type strains [50,51]. Studies of polysaccharide deacetylation have been proved its effect on viscosity enhancement, although the degree of deacetylation of holdfast polysaccharides is positively correlated with their viscosity, the upper limit of deacetylation cannot be determined. For example, galactosamine galactan outside the fungal cell mediates adhesion and biofilm formation by deacetylation, acetylated chitosan material mixed with chitin nanofibers improves the bond strength. Chitosan with a relatively high degree of deacetylation has higher bond strength and tensile strength compared with other adhesives [53-55]. Both the synthesis and modification of the oligosaccharides described above are completed on the inner membrane in the cytoplasm, and the oligosaccharides subsequently modified by deacetylation cross the intracellular membrane into the periplasm via the flippase encoded by the hfsF gene [56]. Oligosaccharides entering the periplasm of cells undergo polymerization to form polysaccharide chains by the polysaccharide copolymerase protein complex encoded by the hfsC/I gene, which is commonly found in the copolymerization transport system of exopolysaccharides and capsular polysaccharides of Gram-negative bacteria [57,58].

After completing the polymerization of polysaccharide chains, it is required to transport polysaccharides to the outer membrane of the cell through the transmembrane transport complex encoded by the holdfast transport-related gene cluster hfsDAB, which is unique in *Caulobacter Crescentus*. All the deletion mutants of hfsA, hfsB and hfsD show defects in adhesion function [59,60]. The protein encoded by hfsA has sequence similarity with the polysaccharide transporter GumC from *X. Campestris*, which has been demonstrated that GumC is involved in the transport of xanthan from the cytoplasm to the cell exterior [61]. HfsB and HfsD have no significant similarities to available protein sequences.

**Holdfast Anchoring Related Genes**

Synthetic holdfast polysaccharides will localize to the anchoring protein complex HfaABD and go to surface adhesion after secretion into the extracellular space through the transport complex. The occurrence and elongation of the organelle stalk for secretion of holdfast is associated with the cell cycle factor Pod J. In the swarmer cell the Pod J protein is located at the flagellar terminal in a short sequence. When the swarmer cell switches to the stalk cell, Pod J begins to synthesize the full-length form and localizes to terminal of stalk. The full-length Pod J can promote the elongation of stalk. Eventually the Pod J protein is processed into a short sequence in the late stalk cell and relocates to the dividing swarmer cell flagellar terminal after cell division [62,63]. During stalk formation for holdfast secretion, HfaABD protein complex begins to be synthesized and localized to the front of stalk extension. Gene hfaA, hfaB and hfaD are mutated and deleted, respectively, showing that holdfast would be released into the surrounding medium and unable to form adhesion. The hfaB mutant has the strongest holdfast shedding phenotype. HfaB and HfaD are shown to be membrane proteins by protein sequence analysis, while HfaA may have the ability to anchor holdfast to stalk tips [64,65]. Further studies on HfaABD protein complex show that their localization is mediated by both Pod J and Hfs (D, A, B) proteins, suggesting that the processes of holdfast synthesis, secretion, and anchoring are performed by a series of proteins in cooperation with each other [66].

**EFFECT OF CELL CYCLE AND STRESS ON HOLDFAST**

The process of secretion and adhesion of holdfast is closely related to cell cycle. There are two cell states in the growth process of *Caulobacter Crescentus*: swarmer cell and stalk cell. When the bacterium swims in water, it is in the swarmer cell state. When it contacts the surface of the medium to prepare for colonization, it elongates through the protrusions of the cell wall to form a structure called stalk, and permanently adheres to the surface of the medium after secretion of holdfast from the front end of stalk extension, and the cells in this state are called stalk cells [38,39]. After the stalk cell colonizes the
surface of the medium and enters the next cell cycle to start dividing, the dividing cell leaves as a swarmer cell morphology. The transformation of these two cellular states is regulated by cell cycle-related signaling molecules and accompanied by the regulation of holdfast synthesis and secretion [40,41], the different states of Caulobacter Crescentus during the cell cycle are as follows (Figure 3). The synthesis and secretion of holdfast is not carried out during the whole cell cycle, and its initiation signal comes from the contact stimulation of the swarmer cell flagellum with the medium surface [67,68]. Studies on flagella have shown that in contrast to complete media, Caulobacter Crescentus does not stimulate holdfast synthesis due to its inability to synthesize flagella in defined media. Moreover, mutant strains lacking flagella adhere more efficiently to media surfaces than wild-type strains over time in defined media [69]. For this phenomenon, the current explanation is that the synthesis of c-di-GMP is downregulated due to decrease of PleD, a regulator of diguanylate cyclase, in the flagellar mutant, while transcription of the holdfast repressed gene hfiA is dependent on c-di-GMP, and eventually the synthesis of holdfast is initiated prematurely due to reduced expression of hfiA (Figure 4) [70]. Another research also discovers that PleD contributes to holdfast induction along with flagellar assembly as a developmental regulator, and proposes a model through which the flagellum integrates mechanical stimuli into the C. crescentus developmental program to coordinate adhesion [74]. During the study of the effect of c-di-GMP on holdfast synthesis, a new gene is identified named hfsK. The hfsK gene encodes an N-acetyltransferase-like enzyme which is also an effector of c-di-GMP. HfsK is localized in the cytoplasm by c-di-GMP mediation and inactivated at high concentrations of c-di-GMP, but the specific function of hfsK in the pathway of holdfast synthesis has not been clearly defined [71].

Figure 3: The cell cycle of Caulobacter crescentus. Surface contact stimulates the development of holdfast and stalk.
Figure 4: Stimulation contact of Flagellum and regulation of holdfast (HF) synthesis. Surface attachment results in a decrease of Pled and an increase of c-di-GMP, which reduces transcription of the holdfast synthesis inhibitor gene hfiA, thereby resulting in an increase in holdfast synthesis.

CONCLUSION AND PROSPECT

In conclusion, although the composition and precise structure of holdfast have not been fully elucidated, and its biosynthetic pathway has not been clear yet, the synthetic mechanism of holdfast in *Caulobacter crescentus* can be deeply studied at multiple levels with multi-disciplinary technologies in the future. Microbial engineering techniques can be used to solve the enrichment problem of holdfast, and obtaining a certain amount of this substance is the basis for conducting subsequent related studies. The synthetic pathway of holdfast can be regulated and simulated by means of genetic engineering as well as bio-manufacturing techniques, which can increase the yield of holdfast. New techniques such as structural biology and crystal analysis can be used to further understand the structural features of holdfast, mass spectrometry and nuclear magnetic resonance techniques can be applied in analyzing the complete chemical composition and structure of holdfast. Using genomics and bioinformatics techniques, the whole genome sequencing and functional analysis of *Caulobacter crescentus* can be performed to identify potential holdfast synthesis and modification-related genes, and further refine the understanding of its biosynthetic pathway. In the future, biomimetic adhesives will focus on the research and development of multi-material mixing substances. Adhesives inspired by mussel, barnacle, sandcastle worm and bacteria can combine the advantages of protein, polysaccharides and other adhesive components. With the rapid development of structural biology, material science and synthetic biology, the composition, structure and physicochemical characteristics of holdfast will be completely elucidated, and the clarification of its biosynthetic pathway and regulatory mechanism will lay the foundation for holdfast application and provide a new strategy for the development of novel biomimetic viscous materials.

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