HfaE is a component of the holdfast anchor complex that tethers the holdfast adhesin to the cell envelope

Nelson K. Chepkwony\textsuperscript{a}, Gail G. Hardy\textsuperscript{b} and Yves V. Brun\textsuperscript{a}\textsuperscript{*}

\textsuperscript{a}Département de microbiologie, infectiologie et immunologie, Université de Montréal, Montréal, Québec, Canada

\textsuperscript{b}Department of Biology, Indiana University, Bloomington, Indiana, USA

Running title: HfaE strengthens holdfast-cell envelope association

\textsuperscript{*}Address correspondence to Yves V. Brun, yves.brun@umontreal.ca

Keywords: Holdfast, Bacterial adhesin, Adhesion, Biofilm, Extracellular Polysaccharides, Caulobacterales.
ABSTRACT

Bacteria use adhesins to colonize different surfaces and form biofilms. The species of the Caulobacterales order use a polar adhesin called holdfast, composed of polysaccharides, proteins, and DNA to irreversibly adhere to surfaces. In *C. crescentus*, a freshwater Caulobacterales, the holdfast is anchored at the cell pole via the holdfast anchor (Hfa) proteins HfaA, HfaB, and HfaD. HfaA and HfaD co-localize with holdfast and are thought to form amyloid-like fibers that anchor holdfast to the cell envelope. HfaB, a lipoprotein, is required for translocation of HfaA and HfaD to the cell surface. Deletion of the anchor proteins leads to a severe defect in adherence resulting from holdfast not properly attached to the cell and shed into the medium. This phenotype is greater in a ΔhfaB than a double ΔhfaA hfaD mutant, suggesting that HfaB has other functions besides the translocation of HfaA and HfaD. Here, we identify an additional HfaB-dependent holdfast anchoring protein, HfaE, which is predicted to be a secreted protein. HfaE is highly conserved among Caulobacterales species with no predicted function. In planktonic culture, *hfaE* mutants produce holdfasts and rosettes similar to wild type. However, holdfasts from *hfaE* mutants bind to the surface but are unable to anchor cells, similar to other anchor mutants. We showed that fluorescently-tagged HfaE co-localizes with holdfast, and HfaE forms an SDS-resistant high molecular weight species consistent with amyloid fiber formation. We propose that HfaE is a novel holdfast anchor protein, and that HfaE functions to link holdfast material to the cell envelope.
IMPORTANCE

For surface attachment and biofilm formation, bacteria produce adhesins that are composed of polysaccharides, proteins and DNA. Species in the Caulobacterales produce a specialized polar adhesin, holdfast, which is required for permanent attachment to surfaces. In this study, we evaluate the role of a newly identified holdfast anchor protein HfaE in holdfast anchoring to the cell surface in two different Caulobacterales with drastically different environments. We show that HfaE plays an important role in adhesion and biofilm formation in Caulobacterales. Our results provide insight into bacterial adhesins and how they interact with the cell envelope and surfaces.

INTRODUCTION

Many bacteria spend their lives attached to or associated with surfaces, forming a complex community called a biofilm. Bacteria attach to surfaces using a variety of adhesins, which are mainly composed of polysaccharides, proteins, and DNA (1, 2). Most polysaccharide adhesins are synthesized in the cytoplasm and secreted by either an ABC-transporter dependent, a synthase-dependent, or a Wzx/Wzy-dependent translocation pathway, and can be associated with or anchored in the cell envelope (2-4). Protein adhesins are secreted via a Sec-dependent pathway involving an N-terminal leader peptide, and can be subsequently anchored to the cell surface by covalent linkage to the peptidoglycan in Gram-positive bacteria or the Gram-negative outer membrane, for example curli in Escherichia coli (5), protein F1 of Streptococcus pyogenes (6), or the fibronectin binding domain of Staphylococcus aureus (7). However, there are many other polysaccharide and protein adhesins whose anchoring systems are poorly understood.

Species of the order Caulobacterales use a polar adhesin called holdfast to adhere permanently to surfaces and form biofilms (2). The best characterized holdfast adhesin is from C. crescentus, a freshwater Caulobacterales. Hirschia baltica, a marine Caulobacterales, produces holdfast adhesin that binds better to surfaces in high ionic strength environments than the holdfast produced by C. crescentus (8). Holdfast is capable of binding to a variety of chemically distinct surfaces with impressive force (9-11). Although the
complete composition of *C. crescentus* holdfast is unknown, it has been shown to contain *N*-acetylglucosamine (GlcNAc), glucose, 3-*O*-methylglucose, mannose, and xylose monosaccharides (12, 13), as well as proteins and DNA (14). *H. baltica* holdfast contains GlcNAc and galactose monosaccharides, and proteins (8).

The holdfast polysaccharide is produced via a mechanism similar to the Wzx/Wzy-dependent group I capsular polysaccharide synthesis pathway in *Escherichia coli* (15, 16). A putative glycosyltransferase HfsE initiates the synthesis of holdfast polysaccharide in the cytoplasm by transferring an activated sugar phosphate from uridine diphosphate (UDP) to an undecaprenyl-phosphate (Und-P) lipid carrier (16). Additional monosaccharide substituents are then added to form a repeat unit on the lipid carrier by three putative glycosyltransferases HfsG (16), HfsJ (17), and HfsL (18). Some of the sugar residues on the repeat units are enzymatically modified by a deacetylase, HfsH (19, 20), and a putative acetyltransferase, HfsK (21). The lipid carrier with the polysaccharide repeat unit is subsequently translocated across the inner membrane into the periplasm by the putative flippase HfsF (16). The repeat units are then predicted to be assembled into the mature polysaccharide in the periplasm by two polymerases, HfsC and HfsI (16). The assembled holdfast polysaccharide is then believed to be secreted through an export protein complex, composed of HfsA, HfsB, and HfsD (22-24).

Holdfast polysaccharides are anchored to the cell envelope by the action of the holdfast anchor (Hfa) proteins HfaA, HfaB, HfaD, and HfaE (18, 25-27). Deletion of anchor genes leads to holdfast shedding into the medium and a decrease in adhesion and subsequent biofilm formation (18, 25-27). HfaA, HfaB, and HfaD are colocalized at the tip of the stalk with the holdfast (26, 28). HfaA shares similarity with the curli monomer CsgA and forms a high molecular weight, SDS-resistant complex, a property of amyloid proteins (26). HfaA polymerization depends on HfaD, which shares limited sequence similarity to surface layer proteins and other adhesins (25, 26, 29). HfaB, a lipoprotein with sequence similarity to the curli secretin CsgG, is required for the stability of HfaA and HfaD and their localization to the cell surface (26). Loss of HfaA, HfaD, or both leads to partial shedding of holdfast into the medium, while loss of HfaB
leads to severe holdfast shedding and abolishes biofilm formation (25, 26). It has been postulated that HfaB has a separate function in addition to facilitating the secretion of HfaA and HfaD, such as the formation of a complex with holdfast polysaccharides or secretion of other unidentified holdfast proteins (25, 26, 29). The mechanism by which holdfast anchor proteins and holdfast polysaccharides interact is poorly understood. However, deacetylation of holdfast polysaccharides is required for their tethering to the cell surface and may affect their association with the anchor proteins (20). We hypothesize that the amines generated as a result of deacetylation are important for the formation of bonds with anchor proteins, either covalently or electrostatically (20).

In this study, we characterize the HfaE protein in both *C. crescentus* and *H. baltica*. We show that HfaE is a secreted protein involved in holdfast anchoring. Deletion of *hfaE* in both *C. crescentus* and *H. baltica* reduces cell adhesion and biofilm formation; however, these mutants produce holdfasts and form rosettes similar to wild-type (WT) during planktonic growth. When cells are attached to a glass surface, even though the holdfasts produced by ΔhfaE mutants are able to adhere to the surface, the cells are easily washed away, indicating a holdfast anchoring defect. Finally, we show that HfaE colocalizes with holdfast and forms a high molecular weight, SDS resistant complex, a property of amyloids. We postulate that HfaE is a secreted holdfast protein that is required for holdfast to associate with the cell envelope, in addition to HfaA, HfaB, and HfaD.

**RESULTS**

**HfaE is involved in cell adhesion**

The mechanism by which holdfast polysaccharides are tethered to the cell envelope remains unknown. Three conserved *hfa* genes, *hfaA*, *hfaB*, and *hfaD* are found in the *hfa* operon in both *C. crescentus* and *H. baltica* (Fig. 1A). Shed holdfasts from double ΔhfaA ΔhfaD mutants contain peptides or proteins, suggesting that there are other unidentified holdfast proteins in addition to the known anchor
proteins (8, 30). To identify uncharacterized holdfast genes, we explored genes adjacent to the hfa locus to test whether they may have a role in bacterial attachment or biofilm formation. *hbal* _0649_, a predicted protein with unknown function, is immediately downstream of the _H. baltica_ hfa locus (Fig. 1A). We performed reciprocal best-hit analysis and identified a homolog of *hbal* _0649_ in the _C. crescentus_ genome, *CC* _2639_ (*CCNA* _02722_), which is eight genes downstream of the _C. crescentus_ hfa locus (Fig. 1A). *Hbal* _0649_ and *CC* _2639_ are predicted to be secreted. Cells lacking *CC* _2639_ were previously shown to be defective in surface attachment and holdfast anchoring in _C. crescentus_ and the gene was named *hfaE_ (18).

We extended the reciprocal best-hit analysis to other Caulobacterales genomes and found that HfaE is highly conserved among the Caulobacterales. We identified *hfaE_ gene in all fully sequenced Caulobacterales genomes (25 fully sequenced and annotated genomes) and in 204 out of 238 partially or drafted Caulobacterales genomes. Interestingly, HfaE and HfaA are two of the eleven proteins that are only found in Caulobacterales and not in any other sequenced genomes (31). Basic sequence analyses of HfaE proteins from different Caulobacterales species showed that HfaE has a highly conserved C-terminal region (half of the protein, Fig. 1B). HfaE from _C. crescentus_ and _H. baltica_ were 33% identical (ID) and 46% similar across their entire protein sequence. Although HfaE, HfaA, and HfaD all have similar overall sequence conservation between _C. crescentus_ and _H. baltica_ (~33% ID), the C-terminus of HfaE is more conserved than average (~43% ID), while the N-terminal region is more divergent (~23% ID, 32). In particular, there are three C-terminal motifs that are highly conserved amongst all HfaE sequences analyzed: PHPASQV, CNERSLLR, and DGGVG (Fig. 1B, red asterisks). We hypothesized that these conserved motifs could be important for multimerization, protein-protein interactions, or protein-polysaccharide binding. We analyzed HfaE amino acid sequences using AGGRESCAN and PASTA2.0 to identify amino acid sequences important for promoting amyloid fiber formation (33, 34). We identified two common aggregation domains that are within the conserved regions of HfaE (Fig. 1B, black lines), suggesting HfaE might aggregate to form high molecular weight complexes similar to HfaA and HfaD.
We generated clean, in-frame deletions of \textit{CC\_2639} (\textit{hfaE}CC) and \textit{hbal\_0649} (\textit{hfaE}HB) in \textit{C. crescentus} and \textit{H. baltica} respectively, to determine whether they are involved in adhesion and/or biofilm formation. We found that deletion of \textit{hfaE}CC in \textit{C. crescentus} resulted in a 60\% decrease in biofilm formation, similar to a \textit{\Delta hfaA} or \textit{\Delta hfaD} mutant (Fig. 1C). Deletion of \textit{hfaE}HB in \textit{H. baltica} led to a 40\% reduction in biofilm formation compared to wildtype (WT), while a 60\% reduction was observed for the \textit{hfaA} and \textit{hfaD} mutants (Fig. 1D). We complemented both the \textit{\Delta hfaE}CC and \textit{\Delta hfaE}HB mutants with their native copy of \textit{hfaE} in \textit{trans}, which restored biofilm formation to WT levels (Fig. 1C-D), confirming that \textit{HfaE} is involved in biofilm formation in both \textit{C. crescentus} and \textit{H. baltica}. In \textit{C. crescentus}, the triple \textit{\Delta hfaA} \textit{\Delta hfaD} \textit{\Delta hfaE} mutant phenocopies the double \textit{\Delta hfaA} \textit{\Delta hfaD} or each of the single mutants (Fig. 1C). In \textit{H. baltica}, we observed that the triple \textit{\Delta hfaA} \textit{\Delta hfaD} \textit{\Delta hfaE} mutant phenocopies the double \textit{\Delta hfaA} \textit{\Delta hfaD}, \textit{\Delta hfaA}, and \textit{\Delta hfaD} mutants but not the \textit{\Delta hfaE} mutant, which has higher biofilm formation (Fig. 1D). These results suggest that \textit{HfaE} does not contribute to biofilm formation independently of \textit{HfaA} and \textit{HfaD} and thus may function alongside or downstream of \textit{HfaA} and \textit{HfaD}. Importantly, the \textit{\Delta hfaA} \textit{\Delta hfaD} \textit{\Delta hfaE} triple mutant did not phenocopy the \textit{\Delta hfaB} mutant (Fig. 1C-D), suggesting that the additional hypothesized function of \textit{HfaB} mentioned above is not just the secretion of \textit{HfaE}. The \textit{\Delta hfaB} mutants were completely deficient in biofilm formation in both \textit{C. crescentus} and \textit{H. baltica}, which suggests that \textit{HfaE} functions downstream of \textit{HfaB} in holdfast anchoring, and may be involved in the same processes as \textit{HfaA} and \textit{HfaD} (Fig. 1C-D).

\textbf{HfaE contributes to holdfast anchoring}

In order to study the role of \textit{HfaE} in \textit{C. crescentus} and \textit{H. baltica} holdfast anchoring, we examined holdfasts using fluorescence microscopy with AF488 conjugated to the wheat germ agglutinin (WGA) lectin (AF488-WGA), which binds to GlcNAc moieties in the holdfast polysaccharide (12). In planktonic culture, \textit{C. crescentus} and \textit{H. baltica} WT cells formed organized clusters of cells attached together by their
holdfast called rosettes. In addition, all holdfasts stained by AF488-WGA were attached to cells (Fig. 2A). The *C. crescentus* Δ*hfaE* and *H. baltica* Δ*hfaE* mutants were both labeled by AF488-WGA, indicating the presence of holdfast polysaccharide attached to cells (Fig. 2A). However, some holdfasts could also be detected away from any cell, suggesting that they were shed from cells in both mutant backgrounds (Fig. 2A, white arrows). Interestingly, when we quantified rosette formation in the Δ*hfaE* mutant, we observed that it was similar to WT (Fig. 2A, blue arrows and Fig. 2B), implying that holdfast anchoring in cells lacking HfaE was not severely impaired compared to the Δ*hfaA*, Δ*hfaD*, and Δ*hfaB* mutants which have a more reduced number of rosettes (Fig. 2B).

The reduction in biofilm formation in the Δ*hfaE* mutant was significant for both *C. crescentus* and *H. baltica* (Fig. 1C-D), however, we observed few shed holdfasts in the medium (Fig. 2A). This suggests that holdfasts from the Δ*hfaE* mutant may be loosely tethered to the cell surface. To test this hypothesis, we spotted exponentially growing cell cultures onto a glass coverslip and allowed cells to bind for 1 h. The coverslip was then washed to remove unbound cells and holdfast were labelled with AF488-WGA. As expected, WT cells were bound to coverslips via their holdfasts (Fig. 2C). However, for both *C. crescentus* and *H. baltica* Δ*hfaE* mutants, most cells were washed off the coverslip surface, leaving only their holdfasts attached (Fig. 2C). The strains complemented with *hfaE* in *trans* restored holdfast anchoring (Fig. 2C). These results indicate that HfaE plays an important role in holdfast anchoring for both *C. crescentus* and *H. baltica*.

We observed that both *C. crescentus* Δ*hfaE* and *H. baltica* Δ*hfaE* were strongly deficient in holdfast anchoring when cells were incubated with a surface for a short while (1 h, Fig. 2C), but *H. baltica* Δ*hfaE* showed increased cell adhesion after incubation with a surface for a longer period (4 h – 12 h, Fig. 2D). We hypothesized that over longer periods of time, the Δ*hfaE* cells are able to attach more efficiently to surfaces. To test this hypothesis, we quantified biofilm formation after 12 h and 24 h. We observed no significant increase in biofilm formed by *C. crescentus* Δ*hfaE* (Fig. 4E). However, *H. baltica* Δ*hfaE* showed an...
increase in biofilm formation to almost WT levels after 24 h (Fig. 4E). These results suggest that the role of HfaE varies between the two species, or that the contribution of HfaE to holdfast anchoring in both species differ due to differences in holdfast composition or holdfast anchoring mechanisms.

**Epistasis analysis of hfaE and other hfa genes**

In the current model of holdfast anchoring in *C. crescentus*, HfaB forms a secretion pore through the outer membrane for the translocation of HfaA and HfaD to the cell surface, where they polymerize into high molecular weight fibers or complexes (26, 28). Recent studies on holdfast anchor mutants suggest that HfaB may have an additional function beyond the secretion of HfaA and HfaD (29). This was based on the observation that the loss of adhesion of a double *C. crescentus ΔhfaA ΔhfaD* mutant could be suppressed by mutations in sugar-nucleotide synthesis genes, whereas a *C. crescentus ΔhfaB* mutant could not be suppressed by similar mutations (29). The additional HfaB function is thought to be stabilization of the holdfast anchoring machinery, directly interacting with the polysaccharides or secretion of other unidentified anchor proteins.

We hypothesized that HfaB could be interacting with or transporting HfaE, thus deletion of *hfaE* in a ΔhfaA ΔhfaD background could increase holdfast shedding to levels observed in the ΔhfaB mutant. We monitored for the presence of holdfast in anchor mutants using AF488-WGA lectin. In planktonic culture for both *C. crescentus* and *H. baltica*, we observed shed holdfasts in all the anchor mutants (Fig. 3A). We quantified the number of shed holdfasts in planktonic cultures (Fig. 3B). In WT, it is very rare to observe shed holdfasts, while the ΔhfaB mutant has the highest shedding phenotype (Fig. 3B). We observed the lowest number of shed holdfasts in the ΔhfaE mutant compared to all the anchor mutants. The triple ΔhfaA ΔhfaD ΔhfaE mutant had a similar amount of shed holdfasts compared to the double ΔhfaA ΔhfaD mutant for both *C. crescentus* and *H. baltica*. These results suggest that HfaE may not be the only unidentified holdfast component that is secreted by HfaB, and further emphasize that HfaB may have additional unknown roles in holdfast anchoring.
Holdfasts from both *C. crescentus* and *H. baltica* are stained by maleimide, which indicates the presence of thiol components in the holdfast (8). To test whether the cysteines in HfaE are the thiol component of holdfasts, we allowed exponential-phase cultures to bind to coverslips for 1 h, washed to remove unbound cells, and co-labeled with both AF488-WGA (GlcNAc-specific, green) and the maleimide dye AF594-Mal (thiol-specific, red). As expected, the WT cells from *H. baltica* and *C. crescentus* were labeled with both AF488-WGA and AF594-Mal (Fig. 3C), indicating the presence of thiols in the holdfast. The shed holdfasts from the ΔhfaB mutants were also labelled with both AF488-WGA lectin and AF594-Mal (Fig. 3C). The single anchor mutants and the triple mutants ΔhfaA ΔhfaD ΔhfaE also showed similar labelling (Fig. 3C, Fig. S1). These results suggest that there are thiol components in the holdfast in addition to HfaA, HfaD, and HfaE cysteines, and that they are not secreted by HfaB.

**HfaE co-localizes with the holdfast and forms high molecular weight complexes.**

The holdfast anchor proteins HfaA, HfaD, and HfaB have been mainly studied in *C. crescentus* and they all colocalize with the holdfast polysaccharides (26). We decided to pursue our studies of HfaE in *C. crescentus* because its anchor proteins have been well characterized, and because *H. baltica* ΔhfaE has a less severe adhesion phenotype, complicating certain types of analyses. To determine the localization of HfaE in *C. crescentus*, HfaE was fused to mCherry at the C-terminus and fluorescent microscopy was used to examine HfaE::mCherry localization. We first confirmed that HfaE::mCherry is functional as it restores biofilm formation in *C. crescentus* ΔhfaE (Fig. S2). Next, an exponentially growing culture expressing HfaE::mCherry was labelled with AF488-WGA. We observed that HfaE::mCherry localized at the cell pole or at the tip of the stalk in stalked cells (Fig. 4A). In cells that have holdfasts (35 % of WT produce holdfasts under our growth conditions), HfaE::mCherry colocalized with holdfast (Fig. 4A). To test whether HfaE requires HfaA, HfaB, or HfaD to localize at the tip of the stalk, we generated in-frame deletions of *hfaA*, *hfaB* and *hfaD* in the *hfaE::mCherry* background. We added WGA to label holdfast and use fluorescent microscopy to observe HfaE::mCherry localization in these anchor mutants. In all the anchor mutants,
HfaE::mCherry was mislocalized (Fig. 4A). These results indicate that HfaE requires HfaA, HfaD, and HfaB for correct localization at the tip of the stalk.

HfaA, HfaD and HfaB localization at the tip of the stalk is independent of the presence of holdfast polysaccharides but depends on the holdfast secretion machinery (26). To test whether HfaE localization depends on the presence of holdfast polysaccharide or its secretion machinery, we generated a clean deletion of the holdfast export genes hfsDAB (no holdfast polysaccharide export protein complex and no holdfast produced) and a glycosyltransferase hfsG (presence of holdfast polysaccharide export protein complex but no holdfast polysaccharides) in the hfaE::mCherry background. We observed mislocalization of HfaE in ΔhfsDAB and correct localization in ΔhfsG (Fig. 4B), similarly to what has been observed with HfaA, HfaD, and HfaB localization (26). These results indicate that HfaE, like the other anchor proteins, requires the holdfast polysaccharide export complex but not the presence of the polysaccharide itself to localize at the tip of the stalk. In order to determine when HfaE localizes to the cell pole relative to when holdfast is synthesized, we performed time-lapse microscopy on soft agarose pads with AF488-WGA to label holdfast. We observed that HfaE::mCherry first localizes at cell pole in the pre-divisional cell (Fig. 4C, blue arrow). After cell division, HfaE::mCherry remains polarly localized in the swarmer cell where holdfast is synthesized (Fig. 4C, white arrows). This localization pattern is similar to what has been observed for the other anchor proteins (26).

HfaD and HfaA depend on each other for stability and localization (26). In order to test whether HfaA, HfaD, and/or HfaB localization depends on HfaE, we generated a clean deletion of hfaE in strains with C-terminally FLAG-tagged HfaA or HfaD (hfaA::M2 and hfaD::M2). We performed immunofluorescence microscopy with an anti-FLAG antibody (IR-680), and observed that HfaA and HfaD correctly localized in ΔhfaE mutants (Fig. 5A). We also deleted hfaE in a strain expressing HfaB tagged with mCherry at its C-terminus (hfaB::mCherry). We observed that HfaB localization is not affected in the ΔhfaE mutant (Fig. 5B). These results indicate that HfaA, HfaD, and HfaB localization do not depend on HfaE.
HfaA and HfaD have been shown to form high molecular weight complexes that are resistant to heat and SDS denaturation, a characteristic of amyloid proteins (26). To determine if HfaE has similar properties, we performed western blot analysis of *C. crescentus hfaE::mCherry* whole cell lysates. We observed a high molecular weight species in the wells (Fig. 6A), similar to what was observed with HfaA and HfaD (26). To test whether the formation of this high molecular weight species depends on other anchor proteins, we deleted hfaA, hfaB and hfaD in the *C. crescentus hfaE::mCherry* background. We observed that HfaE multimerization was not affected in any of the anchor mutants (Fig. 6A, Fig.S3). These results suggest that HfaE assembles into a high molecular weight complex that is resistant to heat and SDS treatment, suggestive of an amyloid-forming protein (Fig. 1B). HfaA and HfaD depend on each other for stability and the formation of high molecular weight multimers (26). In order to test whether HfaE contributes to the stability of HfaA and HfaD, we performed western blots on strains with hfaA::M2 and hfaD::M2. As expected, HfaA stability was affected in the hfaD mutant (Fig. 6B, upper panels). However, we observed that HfaA stability was not affected in the hfaE mutant (Fig. 6B, upper panels). We also observed similar results for HfaD (Fig. 6B, lower panels). These results indicate that HfaA and HfaD do not depend on HfaE for multimerization and stability.

**Mutations in sugar-nucleotide synthesis pathways suppress the hfaE mutation.**

Little is known about how the holdfast anchor complex interacts with holdfast polysaccharides. Deacetylation of holdfast polysaccharides is required for strong interactions between the holdfast polysaccharides, anchor complex, and the thiol-containing component(s) of the holdfast (19, 20). Mutations in sugar-nucleotide synthesis genes have been shown to suppress holdfast shedding in hfaA and hfaD mutants, but not a hfaB mutant (29). These mutations are hypothesized to alter lipopolysaccharide (LPS) properties on the cell envelope, permitting an alternative mechanism for holdfast polysaccharide to interact with the cell envelope (18, 29). To test whether similar mutations can suppress the holdfast shedding phenotype of the hfaE mutant, we generated clean deletions of *wbqV* (dehydratase that catalyzes the conversion pf UDP-GlcNAC to UDP-Qui2NAc) and *rfbB* (dTDP-glucose 4,6-dehydratase that converts
dTDP-D-glucose to dTDP-6-deoxy-D-glucose) in the C. crescentus ΔhfaE background. We allowed cells to bind to a glass surface for 1 h, added AF488-WGA, and washed away unattached cells. We observed that both wbqV and rfbB mutations suppressed holdfast shedding in the hfaE mutant (Fig. 7A). We observed similar restoration of adhesion and biofilm formation to WT levels in both ΔwbqV ΔhfaE and ΔrfbB ΔhfaE mutants (Fig. 7B).

**DISCUSSION**

Reversible attachment of bacteria to surfaces is often facilitated by pili and flagella, while permanent adhesion is mediated by synthesized adhesins composed of exopolysaccharides and/or proteins (1, 35). Members of the Caulobacterales use a polar adhesin composed of polysaccharides and proteins called the holdfast to permanently attach to surfaces and form biofilms (1, 2, 8, 12, 36). Holdfast polysaccharides are tethered to the cell surface by the anchor proteins HfaA, HfaB, and HfaD (25, 26). HfaB is predicted to be an outer membrane localized pore-forming lipoprotein which facilitates secretion of HfaA and HfaD (22, 26). Because the holdfast anchoring defect of a hfaB mutant is much more severe than that of a double ΔhfaA ΔhfaD mutant (25, 26), we hypothesized that there were still unidentified holdfast anchor genes. By analyzing the synteny of holdfast anchor genes, we identified CC_2639 from C. crescentus and hbal_0649 from H. baltica as putative additional anchor genes. While this work was in progress, Hershey et al. identified CC_2639 (hfaE) in C. crescentus as a gene whose deletion causes holdfast shedding (18).

HfaE has a signal sequence that is predicted to target it to the periplasm or outer membrane. Deletion of hfaE in C. crescentus decreases biofilm formation to half that of WT, similar to deletion of hfaA or hfaD. In H. baltica, deletion of hfaE decreases biofilm formation, but not to the same levels as the ΔhfaA or ΔhfaD mutants. These results indicate that HfaE is involved in bacterial adhesion, although its contribution appears to differ between species. This could be due to differences in anchoring mechanisms, since holdfasts in H. baltica are anchored to the cell pole and cover a larger area compared to C. crescentus.
holdfasts, which are anchored at the tip of the thin stalk. Alternatively, the observed differences could be due to the differences in holdfast composition and structure. *H. baltica* holdfasts contain galactose monosaccharides, which are absent in *C. crescentus* (8).

We showed that cells lacking HfaE shed few holdfasts into the medium during planktonic growth, and that cells bearing a holdfast form rosettes similarly to WT. However, when cells are attached to a glass coverslip surface, most ΔhfaE cells detached from the coverslip after washing, leaving only their holdfasts. Over longer periods of growth, *H. baltica* ΔhfaE was able to increase the proportion of cells attached to coverslips, while *C. crescentus* ΔhfaE remained poorly attached. These results imply that the role of HfaE is to strengthen the association between other holdfast matrix components and the cell envelope, and that this requirement is variable between these species. HfaE::mCherry colocalizes with holdfast in *C. crescentus*, similar to HfaA and HfaD (26), suggesting that it may be part of the anchor complex. Deletion of *hfaB* in both *C. crescentus* and *H. baltica* leads to severe holdfast shedding and abolishes biofilm formation (25, 26). It was intriguing that a ΔhfaA ΔhfaD ΔhfaE triple mutant did not phenocopy a ΔhfaB mutant, suggesting that there are likely other unidentified anchor proteins or that HfaB interacts directly with holdfast polysaccharides. Holdfast has been shown to contain thiols other than the cysteines of holdfast anchor proteins HfaA and HfaD based on labelling with a thiol-reactive maleimide dye (8, 9). We hypothesized that HfaE could be the main thiol-containing component of holdfast since it has 10 cysteine residues, however our results showed that this is not the case.

Using western blot analysis, we showed that HfaE forms a high molecular weight complex that is resistant to heat and SDS denaturation, similar to HfaA, HfaD, and other amyloid-forming proteins (26). HfaA and HfaD depend on each other for stability and multimerization (26). Our results show that HfaE depends on HfaA, HfaB, and HfaD for localization to the cell pole, however, HfaE forms a high molecular weight complex without these anchor proteins. This suggests that either HfaE self-polymerizes, or that there is an unidentified protein involved in HfaE assembly, similarly to the way HfaA and HfaD depend on each other for stability and polymerization.
How holdfast polysaccharides are attached to the cell envelope via the anchor complex is still unknown. Polysaccharide deacetylase mutants shed holdfast similarly to anchor mutants, suggesting that deacetylation of holdfast polysaccharides is required for strong interactions between the holdfast polysaccharides and the anchor complex (19, 20). Mutations in the sugar-nucleotide synthesis genes, \textit{wbqV} and \textit{rfbB}, suppress holdfast shedding and restore biofilm formation in the \textit{hfaE} mutant, similar to what has been observed in the \textit{hfaA} and \textit{hfaD} mutants (29). These mutations in the sugar-nucleotide synthesis pathway are hypothesized to (1) alter LPS properties on the cell envelope, permitting an alternative mechanism for holdfast polysaccharide to interact with the cell envelope or (2) alter holdfast polysaccharide composition and increases its interaction with some other components of the cell envelope (18, 29). However, these sugar-nucleotide biosynthetic mutations do not suppress the \textit{hfaB} mutation (29). This result suggests that HfaE is a part of the anchor complex along with HfaA and HfaD, and that HfaB is directly interacting with the polysaccharides or that there are other unidentified anchor proteins that play a role in suppression of the \textit{hfaA}, \textit{hfaD}, and \textit{hfaE} mutations.

Even though HfaE is functionally similar to HfaA and HfaD, there are some differences in the phenotypes of mutants in each of these genes which suggest that they play slightly different roles, despite all co-localizing with the holdfast polysaccharides and forming multimeric complexes. We propose that HfaE strengthens the anchor complex, and that in the absence of HfaE, the anchor complex (HfaA-HfaB-HfaD) is still able to partially tether the holdfast polysaccharides to the cell envelope, but that this anchoring is unable to withstand shear forces. Whether these proteins are all part of the same anchoring complex, and how they contribute to holdfast anchoring individually and collectively, will be the focus of future studies.
**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.**

The bacterial strains used in this study are listed in Table S1. *H. baltica* strains were grown in marine medium (Difco™ Marine Broth/Agar 2216). *C. crescentus* was grown in PYE medium. Both *H. baltica* and *C. crescentus* strains were grown at 30 °C. When appropriate, antibiotics were added at the following concentrations: kanamycin, 5 µg/ml in liquid medium and 20 µg/ml in agar plates; streptomycin, 5 µg/ml in liquid medium and in plates. *E. coli* strains were grown in LB medium (37) at 37 °C and supplemented with 30 µg/ml of kanamycin in liquid medium or 25 µg/ml in agar plates; 100 µg/ml of spectinomycin in liquid medium or 50 µg/ml in agar plates; and 12 µg/ml of chloramphenicol in liquid medium and in agar plates.

**Strain construction.**

All the plasmids and primers used in this study are listed in Table S1 and S2, respectively. In-frame deletion mutants were generated by double homologous recombination as previously described (38) using suicide plasmids transformed into the *C. crescentus* or *H. baltica* host strains by electroporation (39). Briefly, genomic DNA was used as the template to PCR-amplify 500 bp fragments upstream and downstream of the gene to be deleted. The primers were designed with 25 bp of overlapping sequence for isothermal assembly (40) into pNPTS139 plasmid digested using EcoRV-HF (New England Biolabs, Ipswich, MA) using the NEBuilder HiFi Assembly Master mix (NEB). Assembled pNPTS139-based constructs were transformed into α-select *E. coli* for screening and sequence confirmation before introduction into the host *C. crescentus* or *H. baltica* strains by electroporation. Introduction of the desired mutation onto the *C. crescentus* or *H. baltica* genome was verified by sequencing.

For gene complementation, pMR10 was digested with EcoRV-HF and 500 bp upstream of *hfaE* containing the promoter elements, and the *hfaE* gene itself, were ligated into pMR10 using NEBuilder HiFi Master mix (NEB). The pMR10-based constructs were transformed into α-select *E. coli* for screening and
sequence confirmation before introduction into either *C. crescentus* or *H. baltica* by electroporation, followed by selection for kanamycin resistance.

For generation of the HfaE::mCherry construct, pCHYC-1 and a 500 bp 'hfaE C-terminal PCR product were digested with HindIII-HF and KpnI (NEB) and ligated with T4 DNA ligase (NEB). The pCHYC-1-hfaE::mCherry construct was transformed into α-select *E. coli* for screening and sequence confirmation before conjugation into *C. crescentus* using *E. coli* SM10, a S17-1 derivative. Transconjugants were identified via streptomycin resistance.

**Holdfast labeling using fluorescent lectins**

Holdfast labeling with AF488-WGA (ThermoFisher, Waltham, MA) was performed as previously described (8) with the following modifications. Overnight cultures were diluted in fresh medium to an OD₆₀₀ of 0.2 and incubated for 4 h to an OD₆₀₀ of 0.6 – 0.8. AF488-WGA was added to 100 µl of the resultant exponential culture to a final concentration of 0.5 µg/ml and incubated at room temperature for 5 min. Five microliters of the labeled culture was then spotted onto a glass cover slide, covered with a 1.5 % (w/v) agarose (Sigma-Aldrich) pad in water, and visualized by epifluorescence microscopy. Imaging was performed using an inverted Nikon Ti-E microscope with a Plan Apo 60X objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera, and Nikon NIS Elements imaging software with 200 ms exposure time. Images were processed in ImageJ (41).

**Biofilm formation assays**

Biofilm formation assays were performed as previously described (8) with the following modifications. For short-term binding assays, exponential cultures (OD₆₀₀ of 0.6 - 0.8) were diluted to an OD₆₀₀ of 0.4 in fresh marine broth (*H. baltica*) or PYE (*C. crescentus*), added to a 24-well plate (1 mL per
well), and incubated with shaking (100 rpm) at room temperature for 4 h. For biofilm assays, overnight cultures were diluted to an OD$_{600}$ of 0.1, added to a 24-well plate (1 mL per well), and incubated at room temperature for 12 h with shaking (100 rpm). In both set-ups, OD$_{600}$ was measured before the wells were rinsed with distilled H$_2$O to remove non-attached bacteria, stained using 0.1% crystal violet (CV), and rinsed again with dH$_2$O to remove excess CV. The CV was dissolved with 10% (v/v) acetic acid and quantified by measuring the absorbance at 600 nm (A$_{600}$). Biofilm formation was normalized to A$_{600}$/OD$_{600}$ and expressed as a percentage of WT.

**Visualization of holdfasts attached to glass coverslips**

Visualization of holdfast binding to coverslips was performed as described previously (8) with the following modifications. *H. baltica* and *C. crescentus* strains grown to exponential phase (OD$_{600}$ of 0.4 – 0.6) were incubated on washed glass coverslips at room temperature in a saturated humidity chamber for 4 - 8 h. After incubation, the slides were rinsed with dH$_2$O to remove unbound cells and holdfasts were labelled using 50 µL of AF488-WGA at a concentration of 0.5 µg/ml for 5 min at room temperature. Excess lectin was washed off and the slides were topped with a glass coverslip. Holdfasts were imaged by epifluorescence microscopy using an inverted Nikon Ti-E microscope with a Plan Apo 60X objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera, and Nikon NIS Elements imaging software with 200 ms exposure time. Images were processed in ImageJ (41). Dual labelling with HfaE::mCherry and AF488-WGA was performed as previously described in Hardy et al 2010.

**Holdfast labeling using fluorescent maleimide**

Alexa Flour conjugated Maleimide C$_5$ (AF594-mal, ThermoFisher Scientific) was added to 100 µl of exponential culture to a final concentration of 0.5 µg/ml and incubated at room temperature for 5 min.
A 5 µl aliquot of the labeled culture was spotted onto a glass coverslide, covered with a 1.5 % (w/v) agarose (Sigma-Aldrich) pad in water, and visualized by epifluorescence microscopy. Imaging was performed using an inverted Nikon Ti-E microscope with a Plan Apo 60X objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera, and Nikon NIS Elements imaging software with 200 ms exposure time. Images were processed in ImageJ (41).

**Holdfast synthesis and HfaE localization by time-lapse microscopy on soft agarose pads**

*C. crescentus* holdfast synthesis was observed in live cells on agarose pads by time-lapse microscopy as described previously (8) with some modifications. A 1 µl aliquot of exponential-phase cells (OD₆₀₀ of 0.4 – 0.8) was placed on top of a 0.8% agarose pad in PYE with 0.5 µg/ml of AF488-WGA. The pad was overlaid with a coverslip and sealed with VALAP (Vaseline, lanolin and paraffin wax). Time-lapse microscopy images were taken every 10 min for 6 h using an inverted Nikon Ti-E microscope and a Plan Apo 60X objective, a GFP/DsRed filter cube, and an Andor iXon3 DU885 EM CCD camera. Time-lapse movies were processed using ImageJ (41).

**Sample preparation, SDS-PAGE, and Western blot analysis**

Cell lysates were prepared from exponentially growing cultures (OD₆₀₀ 0.6-0.8) as previously described in (26), with the following modifications. The equivalent of 1.0 ml of culture at OD₆₀₀ 0.6-0.8 was centrifuged at 16,000 × g for 5 min at 4 °C. The supernatant was removed, and cell pellets were resuspended in 50 µl of 10mM Tris, pH 8.0. 50 µl of 2x SDS sample buffer was then added to the cell suspension. Samples were boiled for 5 min at 100 °C before separation on a 12% (w/v) polyacrylamide gel and transfer to a nitrocellulose membrane. Membranes were blocked for 30 min in 5% (w/v) non-fat dry milk in TBST (20 mM Tris, pH 8, 137 mM NaCl, and 0.05% (w/v) Tween 20), and incubated at 4 °C overnight with primary antibodies. Anti-FLAG tag or anti-mCherry primary antibodies were used at a
concentration of 1:1,250 (Sigma, St. Louis, MO). Then, a 1:20,000 dilution of HRP-conjugated goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA), was incubated with the membranes at room temperature for 1 h. Membranes were developed with SuperSignal West Dura Substrate (Thermo Scientific, Rockford, IL) and imaged with a Bio-Rad Chemidoc MP imager (Bio-Rad).

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described (42) for unpermeabilized cells with some modification. Briefly, cells were grown to an OD_{600} of 0.3–0.8 and fixed in 2.5% formaldehyde (Ted Pella, Redding, CA) for 15 min. Cells were spun at 8,000 g for 5 min at 4°C and washed thrice with phosphate-buffered saline (PBS), pH 7.2 and resuspended in 0.5% (w/v) blocking reagent from Roche Molecular Biochemicals in 1× PBS for 30 min at 37°C. Primary antibody (anti-FLAG) was added (1:100 dilution) and incubated for 2 h. at 37°C. Cells were washed three times in 1× PBS. Secondary antibody [fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit; Jackson Immunological Research, West Grove, PA] was added (1:100) in blocking buffer and incubated for 1 h. at 37°C. Cells were washed three times in PBS–0.05% Tween 20. Imaging was performed using an inverted Nikon Ti-E microscope with a Plan Apo 60X objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera, and Nikon NIS Elements imaging software with 200 ms exposure time. Images were processed in ImageJ (41).

ACKNOWLEDGEMENTS

We thank the members of the Brun laboratory for the discussion and providing critical comments on the manuscript. This study was supported by grant R35GM122556 from the National Institutes of Health to YVB. YVB is supported by a Canada 150 Research Chair in Bacterial Cell Biology from the Canadian Institutes of Health Research.
REFERENCES:


<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Description and/or genotype</th>
<th>Reference / source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α select</td>
<td>F'deoR endA1 relA1 gyrA96  hsdR17(π, μ +) supE44 thi-1 ΔlacZYA-argF169 980lacZAM15 λ</td>
<td>Bioline</td>
</tr>
<tr>
<td>S17-1</td>
<td>E. coli 294::RP4-2(Tc::Mu)(Km::Tn7)</td>
<td>(43)</td>
</tr>
<tr>
<td>SM10</td>
<td>thi-1 thr leu tonA supE44 recA::RP4-2 Tc::Mu, KmR</td>
<td>(43)</td>
</tr>
<tr>
<td><strong>C. crescentus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YB135</td>
<td>Wild-type strain CB15</td>
<td>(44)</td>
</tr>
<tr>
<td>YB4250</td>
<td>CB15 ΔhfaA</td>
<td>(26)</td>
</tr>
<tr>
<td>YB4251</td>
<td>CB15 ΔhfaB</td>
<td>(26)</td>
</tr>
<tr>
<td>YB4252</td>
<td>CB15 ΔhfaD</td>
<td>(26)</td>
</tr>
<tr>
<td>YB4288</td>
<td>CB15 ΔhfaA ΔhfaD</td>
<td>(26)</td>
</tr>
<tr>
<td>YB</td>
<td>CB15 pU142</td>
<td>(26)</td>
</tr>
<tr>
<td>YB5618</td>
<td>CB15 ΔhfaA pU142::hfaAM2</td>
<td>(26)</td>
</tr>
<tr>
<td>YB5620</td>
<td>CB15 ΔhfaD pU142::hfaDM2</td>
<td>(26)</td>
</tr>
<tr>
<td>YB5637</td>
<td>CB15 ΔhfaB pCHYC-1::hfaABmCherry</td>
<td>(26)</td>
</tr>
<tr>
<td>YB188</td>
<td>CB15 ΔhfaE</td>
<td>This study</td>
</tr>
<tr>
<td>YB197</td>
<td>CB15 ΔhfaE pMR10::hfaE</td>
<td>This study</td>
</tr>
<tr>
<td>YB204</td>
<td>CB15 ΔhfaA ΔhfaD ΔhfaE</td>
<td>This study</td>
</tr>
<tr>
<td>YB8734</td>
<td>CB15 hfaE::pCHYC-1::hfaEmCherry</td>
<td>This study</td>
</tr>
<tr>
<td>YB8735</td>
<td>CB15 hfaE::pJM21::hfaEM2</td>
<td>This study</td>
</tr>
<tr>
<td>YB9557</td>
<td>CB15 ΔhfaE ΔhfaA pU142::hfaAM2</td>
<td>This study</td>
</tr>
<tr>
<td>YB9558</td>
<td>CB15 ΔhfaE ΔhfaD pU142::hfaDM2</td>
<td>This study</td>
</tr>
<tr>
<td>YB9614</td>
<td>CB15 ΔhfaA hfaE::pJM21::hfaEM2</td>
<td>This study</td>
</tr>
<tr>
<td>YB9615</td>
<td>CB15 ΔhfaD hfaE::pJM21::hfaEM2</td>
<td>This study</td>
</tr>
<tr>
<td>YB9617</td>
<td>CB15 ΔhfaA ΔhfaD hfaE::pJM21::hfaEM2</td>
<td>This study</td>
</tr>
<tr>
<td>YB9618</td>
<td>CB15 ΔhfaB hfaE::pJM21::hfaEM2</td>
<td>This study</td>
</tr>
<tr>
<td>YB9619</td>
<td>CB15 ΔhfaE ΔhfaB pCHYC-1::hfaBmCherry</td>
<td>This study</td>
</tr>
<tr>
<td>YB9627</td>
<td>CB15 ΔhfaE Δfbb</td>
<td>This study</td>
</tr>
<tr>
<td>YB9628</td>
<td>CB15 ΔhfaA ΔwbqV</td>
<td>This study</td>
</tr>
<tr>
<td><strong>H. baltica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YB5842</td>
<td>IFAM 1418 Wild-type strain</td>
<td>(45)</td>
</tr>
<tr>
<td>YB8404</td>
<td>YB5842 ΔhfaB</td>
<td>(8)</td>
</tr>
<tr>
<td>YB8425</td>
<td>YB5842 ΔhfaD</td>
<td>(8)</td>
</tr>
<tr>
<td>YB176</td>
<td>YB5842 ΔhfaE</td>
<td>This study</td>
</tr>
<tr>
<td>YB191</td>
<td>YB5842 ΔhfaE pMR10::hfaE</td>
<td>This study</td>
</tr>
<tr>
<td>YB186</td>
<td>YB5842 ΔhfaA</td>
<td>(8)</td>
</tr>
<tr>
<td>YB208</td>
<td>YB5842 ΔhfaA ΔhfaD</td>
<td>(8)</td>
</tr>
<tr>
<td>YB209</td>
<td>YB5842 ΔhfaA ΔhfaD ΔhfaE</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference / source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMR10</td>
<td>Mini-RK2 cloning vector; RK2 replication and stabilization functions, KmR</td>
<td>R. Roberts and C. Mohr</td>
</tr>
<tr>
<td>pCHYC-1</td>
<td>integrating plasmid with C-terminal mCherry fusion, StrR/SpR</td>
<td>(46)</td>
</tr>
<tr>
<td>pJM21</td>
<td>integrating plasmid with C-terminal M2 tag; KmR</td>
<td>(47)</td>
</tr>
<tr>
<td>pLV9</td>
<td>conjugation helper plasmid carrying a CoIE1 mob, CmR</td>
<td>G. Warren, unpublished</td>
</tr>
<tr>
<td>pMR10::hfaE_CC</td>
<td>pMR10 containing hfaE gene from C. crescentus for complementation</td>
<td>This study</td>
</tr>
<tr>
<td>pMR10::hfaE_HB</td>
<td>pMR10 containing hfaE gene from H. baltica for complementation</td>
<td>This study</td>
</tr>
<tr>
<td>pCHYC-1::hfaE</td>
<td>integrating plasmid containing a 500 bp C-terminal hfaE fragment with a C-terminal mCherry tag</td>
<td>This study</td>
</tr>
<tr>
<td>pJM21::hfaEM2</td>
<td>integrating plasmid containing a 500 bp C-terminal hfaE fragment with a C-terminal M2 tag</td>
<td>This study</td>
</tr>
<tr>
<td>pNPTS139ΔhfaE_CC</td>
<td>pNPTS139 containing 500 bp fragments upstream and downstream of C. crescentus hfaE</td>
<td>This study</td>
</tr>
<tr>
<td>pNPTS139ΔhfaE_HB</td>
<td>pNPTS139 containing 500 bp fragments upstream and downstream of <em>H. baltica</em> hfaE</td>
<td>This study</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>pNPTS139ΔrfbB</td>
<td>pNPTS139 containing 500 bp fragments upstream and downstream of <em>C. crescentus</em> rfbB</td>
<td>(29)</td>
</tr>
<tr>
<td>pNPTS139ΔwbqV</td>
<td>pNPTS139 containing 500 bp fragments upstream and downstream of <em>C. crescentus</em> rfbB</td>
<td>(29)</td>
</tr>
<tr>
<td>Primers</td>
<td>Sequence (5’→3’)</td>
<td>Description</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>C2639upF</td>
<td>GTGCTAGCGAATTCTCTGATCCACAGATGCGGTGGGCCGGAATATC</td>
<td>5’ region for deletion of hfaE in <em>C. crescentus</em></td>
</tr>
<tr>
<td>CC2639upR</td>
<td>GCTGCGGGCGCCAGACAGACAGCGCCTGGTTCTCTTCACCTCAGG</td>
<td></td>
</tr>
<tr>
<td>CC2639dwF</td>
<td>CCCGAGGTTAAGGCGGACTGCTGCTGTGGCTGCCGCCGAGG</td>
<td>3’ region for deletion of hfaE in <em>C. crescentus</em></td>
</tr>
<tr>
<td>CC2639dwR</td>
<td>GCCGCCAGAAGCTTCTTCGAGGTGCGGGGCCAAGAAGCAGCCGAGCCATC</td>
<td></td>
</tr>
<tr>
<td>hbal0469upF</td>
<td>CTAGCGAATTCTCGTATCCACAGATGCGGACACTTTCTATCATTTGTTGATAAC</td>
<td>5’ region for deletion of hfaE in <em>H. baltica</em></td>
</tr>
<tr>
<td>hbal0469upR</td>
<td>GCCTCTTGATGGGTCAATTCTATAATAATCTACCGACCAGCTCACCAGCTGAG</td>
<td></td>
</tr>
<tr>
<td>hbal0469dwF</td>
<td>GCCGGGAGCTGAGTCTGAGTATATTATAGAATTTGACCTAAGGAGCCTTTC</td>
<td>3’ region for deletion of hfaE in <em>H. baltica</em></td>
</tr>
<tr>
<td>hbal0469dwR</td>
<td>GCCCACTGTAAGGTCCTTGAGGCATATCTTGTGATTGATCGCAATAAGGACCTG</td>
<td></td>
</tr>
<tr>
<td>Compcc2639F</td>
<td>CCATGATTCCAAGCTTCCATGCGATCGCGCGCCGGAATATCGCGG</td>
<td>Complementation of hfaE in <em>C. crescentus</em></td>
</tr>
<tr>
<td>Compcc2639R</td>
<td>GAGCTCTGCGGAGATTTGCGCCGCGTCACTGCACTGACGAGC</td>
<td></td>
</tr>
<tr>
<td>Comphb06649F</td>
<td>CTAGAGCTCTCAGGAGATCTCGATTTAATAAACGATTTGACCAACGCCACC</td>
<td>Complementation of hfaE in <em>H. baltica</em></td>
</tr>
<tr>
<td>Comphb06649R</td>
<td>GATTACGCGCAAGCTTCCATGCGATCGCGCATCAATCGCTGGTTCCACAG</td>
<td></td>
</tr>
<tr>
<td>2639PstEndM2R</td>
<td>AATGGGGCTCGACTGCAAGTGGCGAGTGCGG</td>
<td>3’ hfaE fragment for M2 tag construct <em>C. crescentus</em></td>
</tr>
<tr>
<td>2639Eco3252F</td>
<td>CCATGAGAATTCGACTGCGACGCGGC</td>
<td></td>
</tr>
<tr>
<td>2639Hind3252F</td>
<td>TTCAGCGCGCCTGAAATCCGAGGCGCGCGAAGG</td>
<td>3’ hfaE fragment for mCherry construct <em>C. crescentus</em></td>
</tr>
<tr>
<td>2639KpnendmChRnew</td>
<td>TGGGACCAGTGCCGTAACCAGACGCC</td>
<td></td>
</tr>
</tbody>
</table>
FIGURES AND FIGURE LEGENDS

Figure 1: HfaE is involved in cell adhesion

A. C. crescentus

B. H. baltica

C. C. crescentus biofilm assay (12 h)

D. H. baltica biofilm assay (12 h)
**Figure 1: HfaE is involved in cell adhesion**

**A.** Genomic organization of the holdfast anchor genes (*hfa*) in *C. crescentus* and *H. baltica*. Genes were identified using reciprocal best hit analysis with *C. crescentus* and *H. baltica* genomes. In the *C. crescentus* genome, *hfaE* is found outside the *hfa* locus, while in the *H. baltica* genome, *hfaE* is found downstream of *hfaD*. **B.** Multiple sequence alignment of HfaE from selected Caulobacterales. Residues are color coded based on their physicochemical properties using ClustalW (48). Consensus residues are indicated by an asterisk (*), and amyloid domains by black lines. **C-D.** Quantification of biofilm formation by the crystal violet assay after incubation for 12 h, expressed as a mean percent of WT crystal violet staining normalized to OD$_{600}$. Error is expressed as the standard error of the mean of three independent biological replicates, each with four technical replicates.
Figure 2: HfaE contributes to holdfast anchoring

A. Planktonic cells with holdfasts on agarose pads

- **C. crescentus**
  - WT
  - ΔhfaE
  - ΔhfaE pMR10::hfaE

- **H. baltica**
  - WT
  - ΔhfaE
  - ΔhfaE pMR10::hfaE

B. Rosette formation in planktonic cultures

- **C. crescentus**

- **H. baltica**

C. Cells and holdfasts attached to coverslips after 1 h

- **C. crescentus**
  - WT
  - ΔhfaE
  - ΔhfaE pMR10::hfaE

- **H. baltica**

D. Cells and holdfasts attached to coverslips after 4 or 12 h

- **C. crescentus**
  - 4 h
  - 12 h

- **H. baltica**

E. Biofilm assay

- **C. crescentus**
- **H. baltica**
Figure 2: HfaE contributes to holdfast anchoring

A. Representative images showing merged phase and fluorescence channels of exponentially growing planktonic cultures of *C. crescentus* (left) and *H. baltica* (right) strains. Holdfast polysaccharides are labeled with AF488-WGA (green). White arrows indicate shed holdfast, while blue arrows indicate rosettes. B. Quantification of rosettes formed in *C. crescentus* (left) and *H. baltica* (right) strains grown to an OD$_{600}$ of 0.8. Data are expressed as the mean number of rosettes formed. Error is represented as the standard error of the mean of two biological replicates with five technical replicates each. Scale bar, 2 µm. C-D. Representative images showing merged phase and fluorescence channels of *C. crescentus* and *H. baltica* strains bound to a glass slide. Holdfast is labeled with AF488-WGA (green). Exponentially growing cultures were incubated on the glass slides for 1 h (C), or 4 h to 12 h (D), and washed to remove unbound cells before AF488-WGA labeling. Scale bar, 2 µm. E. Quantification of biofilm formation by the crystal violet assay after incubation for 12 h and 24 h, expressed as a mean percentage of WT crystal violet staining normalized to OD$_{600}$. Error is expressed as the standard error of the mean of three independent biological replicates, each with four technical replicates.
Figure 3: Epistasis analysis of \textit{hfaE} and other \textit{hfa} genes

\textbf{A} \hspace{1cm} Holdfasts and cells on agarose pads (planktonic cultures)

\begin{itemize}
  \item \textit{C. crescentus}
  \item \textit{H. baltica}
\end{itemize}

\textbf{B} \hspace{1cm} Shed holdfasts in planktonic cultures

\textbf{C} \hspace{1cm} Merged \hspace{1cm} WGA \hspace{1cm} Mal

\textit{C. crescentus}

\textit{H. baltica}
Figure 3: Epistasis analysis of hfaE and other hfa genes

A. Representative images showing merged phase and fluorescence channels of exponentially growing, planktonic C. crescentus (left) and H. baltica (right) strains on agarose pads. Holdfast polysaccharides are labeled with AF488-WGA (green). Scale bar, 2 µm. B. Quantification of shed holdfasts in C. crescentus (left) and H. baltica (right) strains grown to an OD<sub>600</sub> of 0.8. Data are expressed as the mean number of shed holdfasts formed. Error is represented as the standard error of the mean of two biological replicates with five technical replicates each. C. Representative images showing merged phase and fluorescence channels of C. crescentus (left) and H. baltica (right) strains bound to a glass slide. Holdfast polysaccharides are labeled with AF488-WGA (GlcNAc, green) and holdfast thiols are labeled with the maleimide dye AF594-Mal (thiols, red). Exponential cultures were incubated on glass slides for 1 h, and washed to remove unbound cells before co-labelling with AF488-WGA and AF594-Mal. Scale bar, 2 µm.
Figure 4: HfaE co-localizes with the holdfast in *C. crescentus*

A  HfaE localization in holdfast anchor mutants

![Images of localization for hfaE::mCherry, ΔhfaA hfaE::mCherry, ΔhfaB hfaE::mCherry, ΔhfaD hfaE::mCherry, and ΔhfaA ΔhfaD hfaE::mCherry](image)

Merged | 97±3% | <1% | <1% | <1% | <1%

WGA | mCherry

B  HfaE localization in holdfast synthesis mutants

![Images of localization for ΔhfsDAB hfaE::mCherry and ΔhfsG hfaE::mCherry](image)

Merged | WGA | mCherry

ΔhfsDAB hfaE::mCherry: 97±3%<br>ΔhfsG hfaE::mCherry: 2 μm

C  Time-lapse for HfaE::mCherry localization in *C. crescentus*

![Images of time-lapse localization for Merged, mCherry, WGA, and Merged](image)

Time (min) 0 10 20 30 40 50 60 70 80 90
**Figure 4: HfaE co-localizes with the holdfast in C. crescentus**

A-B. Representative images showing merged phase and fluorescence channels of the indicated *C. crescentus* strains expressing HfaE::mCherry. Holdfasts were labeled with AF488-WGA (green), specific for GlcNAc in holdfast polysaccharides, and the mCherry channel (red) was used to visualize the localization of HfaE::mCherry. Exponential planktonic cultures were used to quantify the percentage of pre-divisional cells with HfaE::mCherry foci at the cell pole, which is indicated numerically at the bottom of each set of representative images. Data are expressed as the mean of two independent biological replicates, along with the standard error of the mean. A total of 5,000 cells were quantified per replicate using MicrobeJ. Scale bar, 2 µm.

C. Time-lapse montages of *C. crescentus hfaE::mCherry* on soft agarose pads (0.8 % agarose). Exponential cultures were placed on soft agarose pads containing holdfast specific AF488-WGA (green) and covered with a glass coverslip. The blue arrow indicates a pre-divisional cell and white arrows indicate polar localization of HfaE::mCherry as well as the newly synthesized holdfast in the swarmer cell. Images were collected every 10 minutes for 6 hr. Scale bar, 2 µm.
Figure 5: Localization of anchor proteins in hfaE mutants

A  HfaA and HfaD localization in C. crescentus

<table>
<thead>
<tr>
<th></th>
<th>WT EV</th>
<th>hfaA::M2</th>
<th>ΔhfaE hfaA::M2</th>
<th>hfaD::M2</th>
<th>ΔhfaE hfaD::M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merged</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR-680</td>
<td>0%</td>
<td>77±5%</td>
<td>83±4%</td>
<td>78±5%</td>
<td>82±5%</td>
</tr>
</tbody>
</table>

B  HfaB localization in C. crescentus

<table>
<thead>
<tr>
<th></th>
<th>hfaB::mCherry</th>
<th>ΔhfaE hfaB::mCherry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merged</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mCherry</td>
<td>93±5%</td>
<td>91±5%</td>
</tr>
</tbody>
</table>
Figure 5: Localization of anchor proteins in hfaE mutants

A. Representative immunofluorescence images showing merged phase and fluorescence channels of *C. crescentus* strains expressing HfaA-M2 or HfaD-M2. Exponentially growing cells were fixed with formaldehyde. Anti-FLAG (M2) primary antibody and goat anti-rabbit secondary antibody conjugated to IRDye 680 red fluorescence was used to visualize localization of the anchor protein. B. Representative images showing phase and fluorescence channels of exponentially growing *C. crescentus hfaB::mCherry* strains. The mCherry fluorescence channel was used to visualize HfaB-mCherry (red). For panels A-B, exponential planktonic cultures were used to quantify the percentage of pre-divisional cells with fluorescent foci at the cell pole, which is indicated numerically at the bottom of each set of representative images. Data are expressed as the mean of two independent biological replicates, along with the standard error of the mean. A total of 1,000 cells were quantified per replicate using MicrobeJ. Scale bar, 2 μm.
Figure 6: HfaE forms high molecular weight complexes

A Multimerization of HfaE

<table>
<thead>
<tr>
<th>HfaE::mCherry</th>
<th>HfaE::mCherry</th>
</tr>
</thead>
<tbody>
<tr>
<td>hfaE::mCherry</td>
<td>hfaE::mCherry</td>
</tr>
<tr>
<td>ΔhfaE</td>
<td>ΔhfaA</td>
</tr>
<tr>
<td>ΔhfaD</td>
<td>ΔhfaA</td>
</tr>
<tr>
<td>ΔhfaD</td>
<td>ΔhfaB</td>
</tr>
<tr>
<td>ΔhfaD</td>
<td>ΔmsDAB</td>
</tr>
</tbody>
</table>

α-mCherry | Wells |
α-McpA | 100 kD |

B Multimerization of HfaA and HfaD

<table>
<thead>
<tr>
<th>HfaA::M2</th>
<th>HfaD::M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>hfaA::M2</td>
<td>hfaD::M2</td>
</tr>
<tr>
<td>ΔhfaA</td>
<td>ΔhfaA</td>
</tr>
<tr>
<td>ΔhfaA</td>
<td>ΔhfaA</td>
</tr>
<tr>
<td>ΔhfaD</td>
<td>ΔhfaA</td>
</tr>
<tr>
<td>ΔhfaD</td>
<td>ΔhfaA</td>
</tr>
<tr>
<td>ΔhfaE</td>
<td>ΔhfaA</td>
</tr>
<tr>
<td>ΔhfaE</td>
<td>ΔhfaA</td>
</tr>
<tr>
<td>ΔhfaE</td>
<td>ΔhfaA</td>
</tr>
</tbody>
</table>

α-M2 | Wells |
α-M2 | 50 kD |
α-M2 | 37 kD |
α-McpA | 100 kD |
α-McpA | 75 kD |
Figure 6: HfaE forms high molecular weight complexes

A-B. Western blots of whole cell lysates of *C. crescentus hfaE::mCherry* (A), *C. crescentus hfaA::FLAG* and *C. crescentus hfaD::FLAG* (B) using anti-FLAG tag (α-M2) antibody with goat anti-rabbit HRP conjugated secondary antibody. Exponentially growing cells grown to an OD$_{600}$ of 0.6-0.8 were normalized to the equivalent of 1 mL of culture at an OD$_{600}$ of 1.0, and 10 ul of the cell lysate was loaded into each lane. Anti-McpA was used as a loading control.
Figure 7: Mutations in sugar-nucleotide synthesis genes suppress the hfaE mutation

A  Cells and holdfasts attached to coverslips after 1 h

WT  ΔwbqV  ΔrfbB

ΔhfaE  ΔhfaEΔwbqV  ΔhfaEΔrfbB

B  Biofilm assay (12 h)

Relative adherence (% of WT)

WT  ΔwbqV  ΔrfbB  ΔhfaE  ΔhfaEΔwbqV  ΔhfaEΔrfbB
Figure 7: Mutations in sugar-nucleotide synthesis genes suppress the hfaE mutation

A. Representative images showing merged phase and fluorescence channels of exponentially growing, planktonic C. crescentus strains. Exponentially growing cultures were incubated on glass slides for 1 h, and washed to remove unbound cells before labelling with AF488-WGA (GlcNAc, green). Scale bar, 2 µm. B. Quantification of biofilm formation by the crystal violet assay after incubation for 12 h, expressed as a mean percent of WT crystal violet staining normalized to OD_{600}. Error is expressed as the standard error of the mean of three independent biological replicates, each with four technical replicates.
Figure S1: Thiol labeling in holdfasts, related to figure 3

**C. crescentus**

- **WT**
- **ΔhfaA**
- **ΔhfaD**
- **ΔhfaB**
- **ΔhfaE**
- **ΔhfaE ΔhfaA ΔhfaD**

**H. baltica**

- **Merged**
- **WGA**
- **Mal**

*Image details*

- Scale bar: 2 μm

*Legend for colors*

- Red: Thiol labeling
- Green: Other labels

*Note*

This figure illustrates thiol labeling in holdfasts of *C. crescentus* and *H. baltica*. The results are compared across different genotypes, highlighting variations in thiol labeling intensity and distribution.
Figure S1: Thiol labeling in holdfasts, related to figure 3

Representative images showing merged phase and fluorescence channels of exponentially growing, planktonic *C. crescentus* (left) and *H. baltica* (right) strains. Holdfast polysaccharides are labeled with AF488-WGA (GlcNAc, green) and holdfast thiols are labeled with the maleimide dye AF594-Mal (thiols, red). For surface attachment, exponential cultures were incubated on glass slides for 1 h, and washed to remove unbound cells before co-labelling with AF488-WGA and AF594-Mal. Scale bar, 2 µm.
Figure S2: Complementation of *C. crescentus* Δ*hfaE* with *hfaE::mCherry*, related to figure 4.
Figure S2: Complementation of C. crescentus ΔhfaE with hfaE::mCherry, related to figure 4

Quantification of biofilm formation by the crystal violet assay after incubation for 12 h, expressed as a mean percent of WT crystal violet staining normalized to OD_{600}. Error is expressed as the standard error of the mean of three independent biological replicates, each with four technical replicates.
Figure S3: HfaE forms high molecular weight complexes, related to figure 6

A  HfaE Multimerization

C. crescentus hfaE::mCherry

HfaE::mCherry

McpA for loading control

MW (kD)

Wells

250
100
50
25
10

B  Hfa multimerization C. crescentus ΔhfaE

HfaA::M2

HfaD::M2

McpA for loading control

MW kD

Wells

250
75
52
25
Figure S3: HfaE forms high molecular weight complexes, related to figure 6

A-B. Western blots of whole cell lysates of *C. crescentus hfaE::mCherry* (A), *C. crescentus hfaA::FLAG* and *C. crescentus hfaD::FLAG* (B) using anti-FLAG tag (α-M2) antibody with goat anti-rabbit HRP conjugated secondary antibody. Exponentially growing cells grown to an OD$_{600}$ of 0.6-0.8 were normalized to an equivalent of 1 mL of culture at an OD$_{600}$ of 1.0, and 10 μL of the cell lysate was loaded into each lane. Anti-McpA was used as a loading control.
Figure 1

**A. C. crescentus**

```
\[
\begin{array}{c}
\text{hfaA} \rightarrow \text{hfaB} \rightarrow \text{hfaD} \\
\text{ORFs} \quad \text{(8 genes)} \quad \text{CC_2639} \quad \text{(hfaE)}
\end{array}
\]
```

**B. H. baltica**

```
\[
\begin{array}{c}
\text{hfaA} \rightarrow \text{hfaB} \rightarrow \text{hfaD} \\
\text{Hbal_0649} \quad \text{(hfaE)}
\end{array}
\]
```

**B. HfaE sequence alignment**

```
<table>
<thead>
<tr>
<th>H. baltica_hbal649/1-294</th>
<th>C. crescentus_cc2639/1-304</th>
<th>B. subriviodes/1-304</th>
<th>M. maris/1-298</th>
<th>A. biprosthecum/1-281</th>
<th>A. excentricus/1-272</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTTAAGTTTACAGATGTTTACAG</td>
<td>TTAACTTACAGATGTTTACAG</td>
<td>TTAACTTACAGATGTTTACAG</td>
<td>TAACTTACAGATGTTTACAG</td>
<td>TAACTTACAGATGTTTACAG</td>
<td>TAACTTACAGATGTTTACAG</td>
</tr>
<tr>
<td>CCGGTTTACAGATGTTTACAG</td>
<td>CCGGTTTACAGATGTTTACAG</td>
<td>CCGGTTTACAGATGTTTACAG</td>
<td>CCGGTTTACAGATGTTTACAG</td>
<td>CCGGTTTACAGATGTTTACAG</td>
<td>CCGGTTTACAGATGTTTACAG</td>
</tr>
<tr>
<td>GGGGTTTACAGATGTTTACAG</td>
<td>GGGGTTTACAGATGTTTACAG</td>
<td>GGGGTTTACAGATGTTTACAG</td>
<td>GGGGTTTACAGATGTTTACAG</td>
<td>GGGGTTTACAGATGTTTACAG</td>
<td>GGGGTTTACAGATGTTTACAG</td>
</tr>
<tr>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
</tr>
<tr>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
<td>TTTTACAGATGTTTACAG</td>
</tr>
</tbody>
</table>
```

**C. C. crescentus biofilm assay (12 h)**

```
```

**D. H. baltica biofilm assay (12 h)**

```
```

The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-NC-ND 4.0 International license.
Figure 1

A. Planktonic cells with holdfasts on agarose pads

C. crescentus

H. baltica

WT ΔhfaE ΔhfaE

pMR10::hfaE ΔhfaA ΔhfaB ΔhfaD

B. Rosette formation in planktonic cultures

C. crescentus

H. baltica

Number of rosettes per field of view

D. Cells and holdfasts attached to coverslips after 4 or 12 h

C. crescentus

H. baltica

WT ΔhfaE ΔhfaE

pMR10::hfaE ΔhfaA ΔhfaB ΔhfaD

E. Biofilm assay

Relative adherence (% of WT)

C. crescentus H. baltica

WT ΔhfaE ΔhfaE

ΔhfaE (24 hr) ΔhfaE (24 hr)

ns ***
A

Holdfasts and cells on agarose pads (planktonic cultures)

WT  \(\Delta hfaB\)  \(\Delta hfaE\)  \(\Delta hfaA\)  \(\Delta hfaD\)  \(\Delta hfaA \Delta hfaD\)

\(C. \ crescentus\)

\(H. \ baltica\)

B

Shed holdfasts in planktonic cultures

\(C. \ crescentus\)

\(H. \ baltica\)

C

\(C. \ crescentus\)

\(H. \ baltica\)

Merged  WGA  Mal
A  HfaE localization in holdfast anchor mutants

HfaE localization in holdfast anchor mutants

B  HfaE localization in holdfast synthesis mutants

C  Time-lapse for HfaE::mCherry localization in C. crescentus

Figure 4
Figure 5

A  HfaA and HfaD localization in *C. crescentus*

<table>
<thead>
<tr>
<th></th>
<th>WT EV</th>
<th>hfaA::M2</th>
<th>ΔhfaE hfaA::M2</th>
<th>hfaD::M2</th>
<th>ΔhfaE hfaD::M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merged</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR-680</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td>77±5%</td>
<td>83±4%</td>
<td>78±5%</td>
<td>82±5%</td>
</tr>
</tbody>
</table>

B  HfaB localization in *C. crescentus*

*hfaB::mCherry*  

Merged  
mCherry  

93±5%

*ΔhfaE hfaB::mCherry*  

Merged  
mCherry  

91±5%
**Figure 6**

**A** Multimerization of HfaE

<table>
<thead>
<tr>
<th></th>
<th>hfaE::mCherry</th>
<th>HfaE::mCherry</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔhfaE</td>
<td>α-mCherry</td>
<td>Wells</td>
</tr>
<tr>
<td>ΔhfaA</td>
<td>α-McpA</td>
<td>100 kD</td>
</tr>
<tr>
<td>ΔhfaD</td>
<td></td>
<td>75 kD</td>
</tr>
<tr>
<td>ΔhfaDAD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔhfaB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔhfsDAB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B** Multimerization of HfaA and HfaD

<table>
<thead>
<tr>
<th></th>
<th>HfaA::M2</th>
<th>HfaD::M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔhfaA</td>
<td>α-M2</td>
<td>α-M2</td>
</tr>
<tr>
<td>ΔhfaA</td>
<td>α-McpA</td>
<td>α-McpA</td>
</tr>
<tr>
<td>ΔhfaD</td>
<td>100 kD</td>
<td>50 kD</td>
</tr>
<tr>
<td>ΔhfaD</td>
<td>75 kD</td>
<td>37 kD</td>
</tr>
<tr>
<td>ΔhfaE</td>
<td></td>
<td>100 kD</td>
</tr>
<tr>
<td>ΔhfaE</td>
<td></td>
<td>75 kD</td>
</tr>
</tbody>
</table>
A  Cells and holdfasts attached to coverslips after 1 h

![Images of cells and holdfasts](image)

B  Biofilm assay (12 h)

![Bar chart showing relative adherence](image)
Figure S1: Thiols labeling in holdfasts, related to figure 3

**C. crescentus**

- **WT**
- **ΔhfaA ΔhfaD**
- **ΔhfaB**
- **ΔhfaE**
- **ΔhfaE ΔhfaA ΔhfaD**

**H. baltica**

- **Merged**
- **WGA**
- **Mal**

Scale bar: 2 μm
Figure S2: Complementation of C. crescentus with hfaE::mCherry, related to figure 4.
Figure S3: Western blot images, related to figure 6

A. HfaE multimerization

C. crescentus hfaE::mCherry

McpA for loading control

<table>
<thead>
<tr>
<th>MW (kD)</th>
<th>hfaE::mCherry</th>
<th>HfaE::mCherry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. Hfa multimerization C. crescentus ΔhfaE

McpA for loading control

<table>
<thead>
<tr>
<th>MW (kD)</th>
<th>HfaA::M2</th>
<th>HfaD::M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-NC-ND 4.0 International license. This version posted July 22, 2022. doi: bioRxiv preprint