



Comparative Analysis of Ionic Strength Tolerance between Freshwater and Marine *Caulobacterales* Adhesins

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ABSTRACT Bacterial adhesion is affected by environmental factors, such as ionic strength, pH, temperature, and shear forces. Therefore, marine bacteria must have developed adhesins with different compositions and structures than those of their freshwater counterparts to adapt to their natural environment. The dimorphic alpha-proteobacterium *Hirschia baltica* is a marine budding bacterium in the clade *Caulobacterales*. *H. baltica* uses a polar adhesin, the holdfast, located at the cell pole opposite the reproductive stalk, for surface attachment and cell-cell adhesion. The holdfast adhesin has been best characterized in *Caulobacter crescentus*, a freshwater member of the *Caulobacterales*, and little is known about holdfast compositions and properties in marine *Caulobacterales*. Here, we use *H. baltica* as a model to characterize holdfast properties in marine *Caulobacterales*. We show that freshwater and marine *Caulobacterales* use similar genes in holdfast biogenesis and that these genes are highly conserved among the species in the two genera. We determine that *H. baltica* produces a larger holdfast than *C. crescentus* and that the holdfasts have different chemical compositions, as they contain *N*-acetylglucosamine and galactose monosaccharide residues and proteins but lack DNA. Finally, we show that *H. baltica* holdfasts tolerate higher ionic strength than those of *C. crescentus*. We conclude that marine *Caulobacterales* holdfasts have physicochemical properties that maximize binding in high-ionic-strength environments.

IMPORTANCE Most bacteria spend a large part of their life spans attached to surfaces, forming complex multicellular communities called biofilms. Bacteria can colonize virtually any surface, and therefore, they have adapted to bind efficiently in very different environments. In this study, we compare the adhesive holdfasts produced by the freshwater bacterium *C. crescentus* and a relative, the marine bacterium *H. baltica*. We show that *H. baltica* holdfasts have a different morphology and chemical composition and tolerate high ionic strength. Our results show that the *H. baltica* holdfast is an excellent model to study the effect of ionic strength on adhesion and provides insights into the physicochemical properties required for adhesion in the marine environment.

KEYWORDS adhesion, bacterial adhesin, *Hirschia baltica*, holdfast, ionic strength, marine *Caulobacterales*, polysaccharides

In their natural environments, bacteria preferentially form surface-associated communities known as biofilms (1). To irreversibly adhere to surfaces and form these complex multicellular communities, bacteria produce strong adhesins, mainly composed of proteins or polysaccharides (2, 3). Bacterial adhesion is affected by different environmental conditions, such as pH, temperature, shear forces, and ionic strength (2, 4–6). In marine environments, bacteria face 500-times-higher ionic strength than in freshwater (7). Therefore, marine bacteria have evolved ways to overcome the effect of ionic strength and bind permanently to surfaces in high-salt environments, such as seas

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and oceans. Higher ionic strength might affect the structures of adhesins or neutralize their charges, impairing their interaction with surfaces (2).

Caulobacterales are *Alphaproteobacteria* found in various habitats from oligotrophic aquatic and nutrient-rich soil environments (8, 9). The aquatic *Caulobacterales* species live in a wide range of environments with different salinity levels, such as pristine fresh river and lake waters, brackish ponds, and marine waters, making them a good model for studying bacterial adhesion in different ionic environments. *Caulobacterales* species use a polar adhesin structure called a holdfast to adhere permanently to surfaces and form biofilms (8, 10, 11). The holdfast has been primarily studied in *Caulobacter crescentus*, a freshwater member of the *Caulobacterales* (2, 3, 12). The *C. crescentus* holdfast uses both electrostatic and hydrophobic interactions to attach to different surfaces (6). The binding affinity of the *C. crescentus* holdfast is dramatically impaired in the presence of NaCl (6), yet marine *Caulobacterales* adhere to surfaces at considerably higher ionic strength, suggesting that their holdfasts have different properties. However, little is known about holdfasts from marine *Caulobacterales*, and the molecular mechanism used to adhere successfully to surfaces in saline environments is currently unknown.

The *C. crescentus* holdfast is the strongest characterized bioadhesive, with an adhesion force of 70 N/mm² (13). Despite being identified almost 85 years ago (14), the exact composition and structure of the *C. crescentus* holdfast remain elusive. Wheat germ agglutinin (WGA) lectin-binding assays show that the holdfast contains *N*-acetylglucosamine (GlcNAc) residues (10), while other studies suggest that the holdfast is also composed of unidentified peptide and DNA residues (15). The *C. crescentus* holdfast polysaccharide is produced via a polysaccharide synthesis and export pathway similar to the group I capsular polysaccharide synthesis Wzy/Wzx-dependent pathway in *Escherichia coli* (16, 17), leading to a model for the holdfast synthesis pathway (Fig. 1A). Holdfast polysaccharide synthesis is hypothesized to be initiated in the cytoplasm by the putative glycosyltransferase HfsE, which is thought to transfer activated sugar phosphate from UDP (UDP-glucose) to an undecaprenyl-phosphate (Und-P) lipid carrier (18). Additional sugar residues, including GlcNAc, are then added to form a repeat unit on the lipid carrier with three glycosyltransferases, HfsG, HfsJ (17), and HfsL (19). The acetyltransferase HfsK (20) and the polysaccharide deacetylase HfsH (30) modify one or more sugar residues. The lipid carrier with the repeat units is transported across the inner membrane into the periplasm by a flippase (HfsF) (17, 22). In the periplasm, the repeat units are polymerized by two polysaccharide polymerases HfsC and HfsI (17). The holdfast polysaccharide chain is then secreted through the export protein complex, composed of HfsA, HfsB, and HfsD (21, 23, 24). Once outside the cell, holdfast polysaccharides are anchored to the cell envelope by the action of holdfast anchor (*hfa*) proteins: HfaA, HfaB, HfaD, and HfaE (19, 25–27).

Hirschia baltica is a marine member of the *Caulobacterales* isolated from surface water taken from a boat landing in the Kiel Fjord inlet of the Baltic Sea (Germany) (28). *H. baltica* has a dimorphic life cycle similar to that of *C. crescentus* (28) but reproduces by budding from the tip of the stalk (Fig. 1B). Newborn swarmer cells are motile by means of a polar flagellum and differentiate into sessile stalked cells after flagellum ejection. The sessile cells produce a holdfast at the same pole as the flagellum and synthesize a stalk at the opposite pole (29). *H. baltica* cells have been shown to produce holdfasts containing GlcNAc residues, using fluorescent WGA lectin (29, 30). The vast majority of studies on holdfasts have been done using *C. crescentus*, and therefore, the *H. baltica* holdfast is poorly understood.

As bacteria have to develop different strategies to adhere to surfaces in a given environment, we hypothesized that *H. baltica* produces holdfasts with different physicochemical properties because *H. baltica*'s natural habitat is high-ionic-strength seawater (28), while the freshwater *C. crescentus* holdfast is highly sensitive to salt (6). Here, we study *H. baltica* holdfast composition and properties. Using both genetics and bioinformatics analyses, we show that freshwater and marine *Caulobacterales* use orthologous genes in holdfast biogenesis and that these genes are highly conserved in

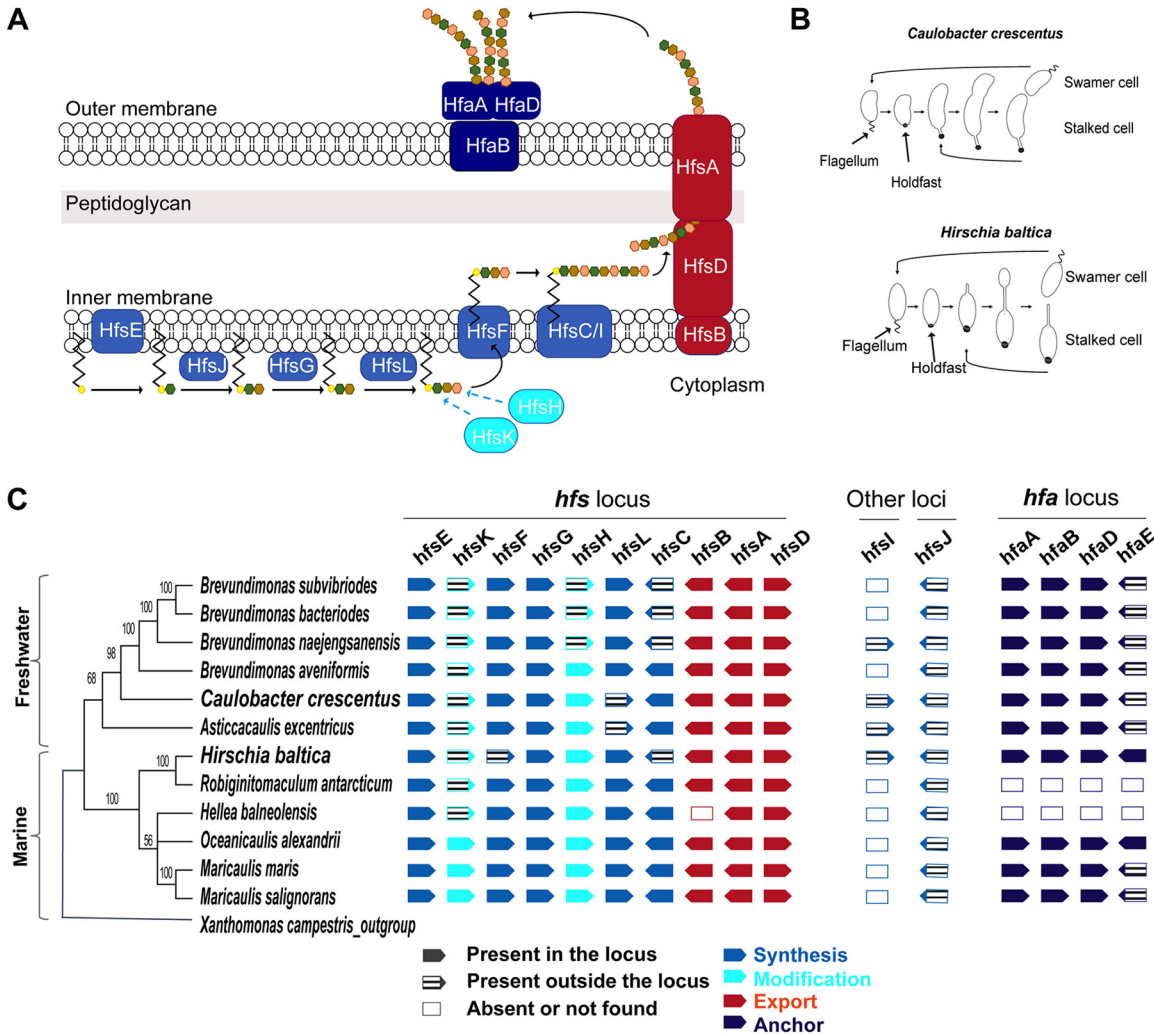


FIG 1 Organization of the holdfast gene cluster in *H. baltica*. (A) Schematic of holdfast synthesis, modification, secretion, and anchor machineries. Holdfast polysaccharide synthesis is initiated by the glycosyltransferase HfsE, which transfers activated sugar precursors in the cytoplasm to a lipid carrier. Three glycosyltransferases, HfsJ, HfsG, and HfsL, add different sugars to the growing polysaccharide. The acetyltransferase HfsK and the deacetylase HfsH modify one or more sugar residues, and then a flippase, HfsF, transports the lipid carrier into the periplasm. Repeat units are polymerized by polymerases HfsC and HfsI. The polysaccharide is exported outside the cell through the HfsA-HfsB-HfsD complex. The exported polysaccharide is then anchored to the cell body by the secreted proteins HfaA, HfaB, and HfaD. The different colored hexagons represent different sugars. (B) Diagrams of *C. crescentus* and *H. baltica* dimorphic cell cycles. A motile swarmer cell differentiates into a stalked cell by shedding its flagellum and synthesizing a holdfast at the same cell pole. *C. crescentus* stalked cells divide asymmetrically to produce a motile swarmer and a stalked cell (top), and *H. baltica* reproduces by budding a motile swarmer off the stalk (bottom). (C) Maximum-likelihood phylogeny inferred from 16S rRNA sequences of selected freshwater and marine members of *Caulobacterales*. The node values represent clade frequencies of 1,000 bootstraps. The genes were identified using reciprocal best-hit analysis on fully sequenced *Caulobacterales* genomes. Solid gene symbols represent genes within the *hfs* or *hfa* loci, while hatched symbols indicate the genes translocated from these loci to a different location in the genome. Empty boxes indicate absent or missing genes.

the two genera. We show that *H. baltica* produces more holdfast material than *C. crescentus* and that the holdfasts of the two genera have different chemical compositions and behave differently. In addition to GlcNAc monosaccharides, we show that *H. baltica* holdfasts contain galactose residues and uncharacterized peptides different than the ones found in *C. crescentus* holdfasts. Finally, we demonstrate that the *H. baltica* holdfast tolerates higher ionic strength than that of *C. crescentus*.

RESULTS

Organization of the holdfast genes in *H. baltica*. The genes essential for holdfast synthesis and export in the *C. crescentus* *hfs* locus (*hfsG*, *hfsB*, *hfsA*, and *hfsD*) are conserved in *H. baltica* (29, 30). To determine if the genomic organization of all the

TABLE 1 Genes involved in holdfast synthesis, modification, and anchoring

Gene name	Locus tag name		
	<i>C. crescentus</i>		<i>H. baltica</i>
	CB15	NA1000	IFAM 1418 ^T
Export apparatus			
<i>hfsA</i>	CC2431	CCNA_02513	Hbal_1968
<i>hfsB</i>	CC2430	CCNA_02512	Hbal_1967
<i>hfsD</i>	CC2432	CCNA_02514	Hbal_1969
Synthesis genes			
<i>hfsC</i>	CC2429	CCNA_02511	Hbal_1972
<i>hfsE</i>	CC2425	CCNA_02507	Hbal_1963
<i>hfsJ</i>	CC0095	CCNA_00094	Hbal_1784
<i>hfsG</i>	CC2427	CCNA_02509	Hbal_1964
<i>hfsL</i>	CC2277	CCNA_02361	Hbal_1966
<i>hfsI</i>	CC0500	CCNA_00533	Hbal_2115
<i>hfsF</i>	CC2426	CCNA_02508	Hbal_0100
Modification genes (nonessential)			
<i>hfsH</i>	CC2428	CCNA_02510	Hbal_1965
<i>hfsK</i>	CC3689	CCNA_03803	Hbal_0069
Anchor genes (nonessential)			
<i>hfaA</i>	CC2628	CCNA_02711	Hbal_0652
<i>hfaB</i>	CC2630	CCNA_02712	Hbal_0651
<i>hfaD</i>	CC2629	CCNA_02713	Hbal_0650
<i>hfaE</i>	CC2639	CCNA_02722	Hbal_0649

known holdfast-related genes is conserved in both species, we performed reciprocal best-hit analyses using the *C. crescentus* *hfs* (holdfast synthesis, modification, and export) and *hfa* (holdfast anchoring) genes (Fig. 1C). We also extended our analysis to other fully sequenced available *Caulobacteriales* genomes for a more global overview of the organization of these genes in the clade (Fig. 1C). Table 1 gives the locus tag names of all the holdfast-related genes used in this study for *C. crescentus* CB15 (31), *C. crescentus* NA1000 (32), and *H. baltica* IFAM 1418^T (29) type strains.

All the genes reported to be involved in holdfast synthesis in *C. crescentus* are present in the analyzed genomes, with a few rearrangements (Fig. 1C). The general organization of the *hfs* locus is conserved in all the *Caulobacteriales* genomes analyzed, with the genes encoding proteins essential for holdfast synthesis (the glycosyltransferase gene *hfsG* and the export genes *hfsA*, *hfsB*, and *hfsD*) and the initiating glycosyltransferase gene *hfsE* in an organization similar to that in *C. crescentus*. Some of the genes involved in holdfast synthesis and modification in *C. crescentus* are not part of the *hfs* gene cluster (genes encoding the polymerase HfsI [17], the glycotransferases HfsJ [33] and HfsL [19], and *N*-acetyltransferase HfsK [20]); these genes are also present in *H. baltica*. Interestingly, in the genomes of the marine *Caulobacteriales* *Oceanicaulis alexandrii*, *Maricaulis maris*, and *Maricaulis salignorans*, all the *hfs* genes except *hfsJ* are found in one locus (Fig. 1C). This suggests that the ancestral *hfs* locus might have contained most of the *hfs* genes. Most of the genomes analyzed had only one polysaccharide polymerase gene, *hfsC*, while others had a paralogous polysaccharide polymerase gene, *hfsI* (Fig. 1C) (17).

Once exported outside the cell by the HfsDAB complex, the holdfast is anchored to the cell envelope by the actions of anchor proteins that have been identified and characterized in *C. crescentus* HfaA, HfaB, and HfaD (19, 25–27). The organization of the three anchor genes *hfaA*, *hfaB*, and *hfaD* in the *hfa* locus is conserved in all the analyzed *Caulobacteriales* genomes (Fig. 1C). In *C. crescentus* and most of the tested *Caulobacteriales*, the recently identified holdfast anchor gene *hfaE* (19) is not part of the *hfaABD* operon, while it is present in the *hfa* locus in both *H. baltica* and *O. alexandrii* (Fig. 1C). We could not find orthologs of the *hfa* genes in the genomes of *Robiginitomaculum antarcticum* and *Hellea balneolensis*, but this may be due to the incomplete nature of

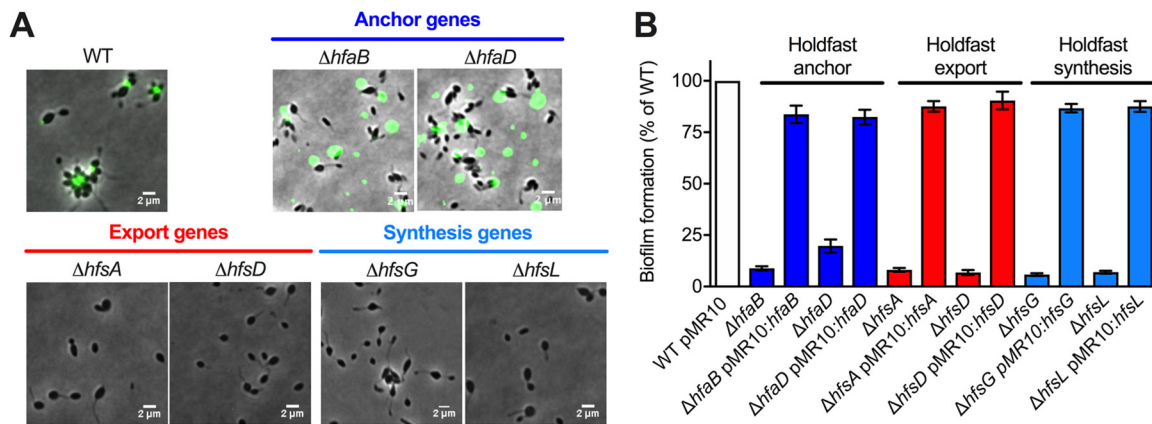


FIG 2 Role of the *hfs* and *hfa* genes in *H. baltica* holdfast production. (A) Representative images showing merged phase and fluorescence channels of different *H. baltica* WT and mutant strains with holdfasts labeled with WGA-AF488 (green): *H. baltica* holdfast anchor mutants ($\Delta hfaB$ and $\Delta hfaD$), export mutants ($\Delta hfsA$ and $\Delta hfsD$), and synthesis mutants ($\Delta hfsG$ and $\Delta hfsL$). (B) Quantification of biofilm formation using crystal violet assay after 12 h for *H. baltica* *hfs* and *hfa* mutants. The data are expressed as averages from 5 independent replicates, and the error bars represent the standard errors.

their genome sequences. Alternatively, these species may have a different mechanism to anchor the holdfast to the surface of the cell, as is the case for several other *Alphaproteobacteria* (34, 35).

Roles of the *hfs* and *hfa* genes in *H. baltica*. To determine if the genes identified in Fig. 1C are involved in holdfast production and anchoring in *H. baltica*, we created in-frame deletion mutants of the *hfa* genes encoding the anchor proteins and the *hfs* genes shown to be essential for holdfast synthesis in *C. crescentus* (12). We first monitored the presence of holdfasts in these mutants using fluorescence microscopy with fluorescently labeled WGA lectin (10) (Fig. 2A). We also quantified biofilm formation after 12 h of incubation at room temperature on a plastic surface by normalizing crystal violet staining to the optical density of the cells using 24-well PVC plates (Fig. 2B). All the mutants could be complemented *in trans* by a replicating plasmid carrying a copy of the deleted gene (Fig. 2B).

We first deleted the holdfast anchor genes encoding the HfaB and HfaD proteins. Both *H. baltica* $\Delta hfaB$ and $\Delta hfaD$ mutants produced holdfasts, but they failed to anchor them to the cell envelope, resulting in the holdfasts being shed in the medium (Fig. 2A). *H. baltica* $\Delta hfaB$ was not able to permanently attach to surfaces and could not form a biofilm (Fig. 2B). In contrast, *H. baltica* $\Delta hfaD$ mutants were not completely deficient for permanent adhesion, with around 20% biofilm formation compared to the wild type (WT) (Fig. 2B). These results are in agreement with what has been reported for *C. crescentus* $\Delta hfaB$ and $\Delta hfaD$ mutants (26), suggesting that the Hfa proteins have similar functions in both organisms.

We then made in-frame deletions of the genes encoding the export proteins HfsA and HfsD. These genes are essential for holdfast production in *C. crescentus* (23). Deletion of the genes in *H. baltica* similarly completely abolished holdfast production (Fig. 2A) and surface attachment (Fig. 2B). These results show that deletion of the export genes is sufficient for complete loss of holdfast production and that a holdfast is crucial for surface attachment in *H. baltica*.

Finally, we made in-frame deletions of the genes encoding the glycosyltransferases HfsG and HfsL, which are essential for holdfast formation in *C. crescentus* (17, 19). Similarly, *H. baltica* $\Delta hfsG$ and $\Delta hfsL$ mutants did not produce holdfasts or form biofilms (Fig. 2A and B).

Effects of modulating *hfsL* and *hfsG* expression on *H. baltica* holdfast properties. We investigated if varying the expression of the *hfsL* and *hfsG* genes could change holdfast synthesis and properties. To achieve this goal, we first engineered a replicating plasmid harboring an inducible promoter suitable for *H. baltica*. We adapted the system

developed for a tightly controlled heavy metal (copper) promoter-inducible system in *Hyphomonas neptunium*, a marine member of the *Caulobacteriales* closely related to *H. baltica* (36). Similarly, we used the promoter for the copper-resistant protein operon *copAB* (*Pcu*) in *H. baltica* (*copA*, *hbal_0699*, and *copB*, *hbal_0698*) (see Fig. S1A, top, in the supplemental material). We first showed that *H. baltica* can tolerate up to 500 μM CuSO_4 without a significant effect on growth (see Fig. S1B and C). We then fused 500 bp upstream of the *copAB* operon (*Pcu*) to the *lacZ* gene and assembled the construct onto the pMR10 replicating plasmid (see Fig. S1A, bottom) to assess *Pcu* promoter activity, using β -galactosidase as a reporter. We showed that *Pcu* is a tightly controlled promoter, with a working inducible range of CuSO_4 from 10 to 250 μM (see Fig. S1D), concentrations that do not impact *H. baltica* growth (see Fig. S1B and C).

We expressed *hfsL* or *hfsG* under the control of the *Pcu* inducible promoter in *H. baltica* $\Delta hfsL$ and $\Delta hfsG$ mutants. In both cases, when gene expression was highly induced (250 μM CuSO_4), holdfast size and adhesion were restored to WT levels (Fig. 3A and B). At lower levels of induction (10 μM CuSO_4), both complemented strains produced small holdfasts (Fig. 3A) but failed to form biofilms after 12 h (Fig. 3B). To test if these results were due to altered adhesive properties of the smaller holdfasts or if their smaller size did not enable the cells to be retained on the surface, we combined the $\Delta hfsL$ and $\Delta hfsG$ mutations with an in-frame deletion of the holdfast anchor gene *hfaB*, resulting in mutants that produced holdfasts shed in the medium upon CuSO_4 induction (Fig. 3C). We grew exponential-phase cultures of the double mutants on glass coverslips for 4 h to allow them to attach to the surface. After incubation, the slides were rinsed with distilled H_2O (dH_2O) to remove all the cells that were unable to anchor their holdfasts to their cell bodies, resulting in coverslips displaying attached holdfasts and no cells (Fig. 3C). At low levels of induction of *hfsL* or *hfsG*, shed holdfasts from *H. baltica* $\Delta hfaB$ $\Delta hfsL$ and *H. baltica* $\Delta hfaB$ $\Delta hfsG$, though smaller than those from *H. baltica* $\Delta hfaB$, were still able to efficiently bind to glass slides (Fig. 3C). We determined the numbers of holdfasts attached at different levels of induction of *hfsL* and *hfsG* (Fig. 3D). At low induction, the mutants produced 50% of the number of WT holdfasts (Fig. 3D). To visualize how cells with small holdfasts interact with the glass surface, we performed time-lapse microscopy in a microfluidic device, starting with static conditions and adding flow after 2 min to allow the cells to bind to the surface (Fig. 3E). We observed that at low induction of *hfsL* (10 μM CuSO_4), cells efficiently bound to the surface, despite their small holdfasts. However, when the flow was adjusted to 1.4 $\mu\text{l}/\text{min}$ in the microfluidic device, generating a drag force of 4 nN, the *hfsL* mutant cells detached, whereas wild-type cells remained attached. Attached *C. crescentus* cells have been shown to withstand high drag forces, up to 1 μN (13). These results show that the small holdfasts are not sufficient to withstand high shear forces. At high induction of *hfsL* (250 μM CuSO_4), cells produced bigger holdfasts and were able to bind to the surface and resist the drag force. This result confirms that the smaller holdfasts are still adhesive, but their size is probably not sufficient to allow cells to resist larger drag forces.

***H. baltica* produces large holdfasts by developmental and surface contact stimulation pathways.** It was previously shown that WGA binds to *C. crescentus* and *H. baltica* holdfasts (29, 30). However, side-by-side microscopy imaging using fluorescent WGA suggested that *H. baltica* holdfasts might be larger than *C. crescentus* holdfasts (Fig. 4A). To quantify relative holdfast size, we imaged mixed cultures of *H. baltica* and *C. crescentus* simultaneously labeled with fluorescent WGA lectin. We measured the area of fluorescent WGA staining on single cells for each strain (Fig. 4A) and determined that, on average, the fluorescence area was 5 times larger for *H. baltica* holdfasts than for those of *C. crescentus* (Fig. 4B), whereas the fluorescence intensities were not statistically different. Since WGA binds to GlcNAc residues in the holdfast, either *H. baltica* holdfasts are larger than those of *C. crescentus* or *H. baltica* and *C. crescentus* holdfasts are similar in size but *H. baltica* holdfasts contain more GlcNAc residues, yielding an increased fluorescence area from bound WGA. To reliably measure the sizes of holdfasts, we used atomic-force microscopy (AFM) and imaged dry holdfasts depos-

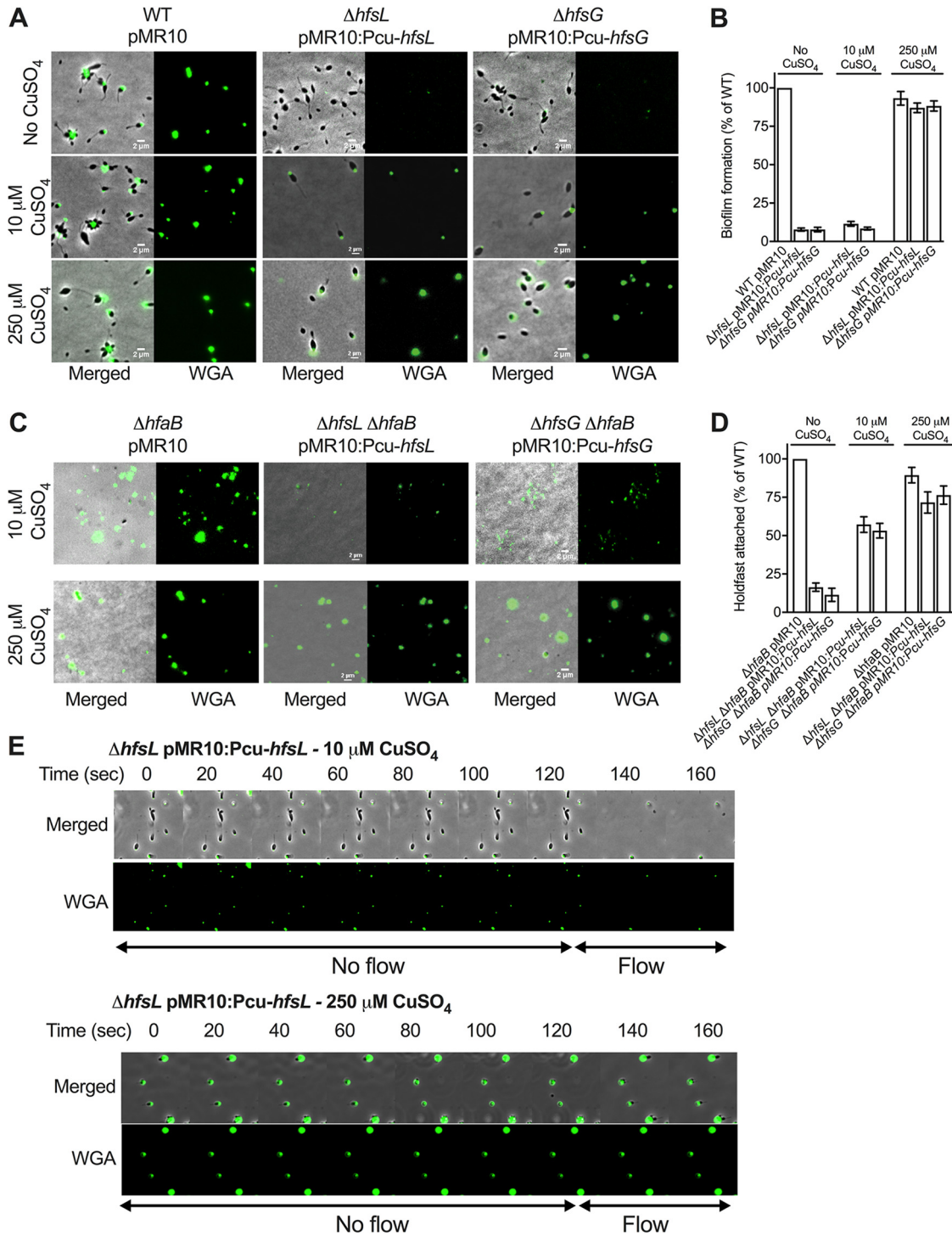


FIG 3 Effect of modulating *hfsL* and *hfsG* expression on *H. baltica* holdfast properties. (A) Representative images showing merged phase and fluorescence channels of *H. baltica* WT and $\Delta hfsL$ and $\Delta hfsG$ mutants complemented with copper-inducible promoter constructs and grown in marine broth with 0 μM, 10 μM, and 250 μM CuSO₄. The holdfasts were labeled with WGA-AF488. (B) Biofilm quantification after 12 h using crystal violet assay of $\Delta hfsL$ and $\Delta hfsG$ mutants and complementations under copper-inducible promoters in marine broth supplemented with 0 μM, 10 μM, and 250 μM CuSO₄. The data are expressed as averages from 6 independent replicates, and the error bars represent the standard errors. (C) Images of WGA-AF488-labeled *H. baltica* $\Delta hfaB$, *H. baltica* $\Delta hfaB \Delta hfsL$ pMR10:P_{cu}-hfsL, and *H. baltica* $\Delta hfaB \Delta hfsG$ pMR10:P_{cu}-hfsG shed holdfasts bound to glass slides. Cells were grown in marine broth with 0 μM, 10 μM, and 250 μM CuSO₄ induction for 4 h. (D) Percentages of holdfasts bound to glass slides per field of view at different CuSO₄ induction levels shown in panel C. The data are expressed as averages from 5 independent replicates, and the error bars represent the standard errors. (E) Time-lapse montage of *H. baltica* $\Delta hfsL$ pMR10:P_{cu}-hfsL induced with 10 μM (top) and 250 μM (bottom) CuSO₄ in a microfluidic device, initially with no flow and then with a flow of 1.4 μl/min, generating a drag force of 4 nN, introduced into the microfluidic device. The arrows indicate the times when no flow (first 120 s) and flow (later times) were applied to the device.

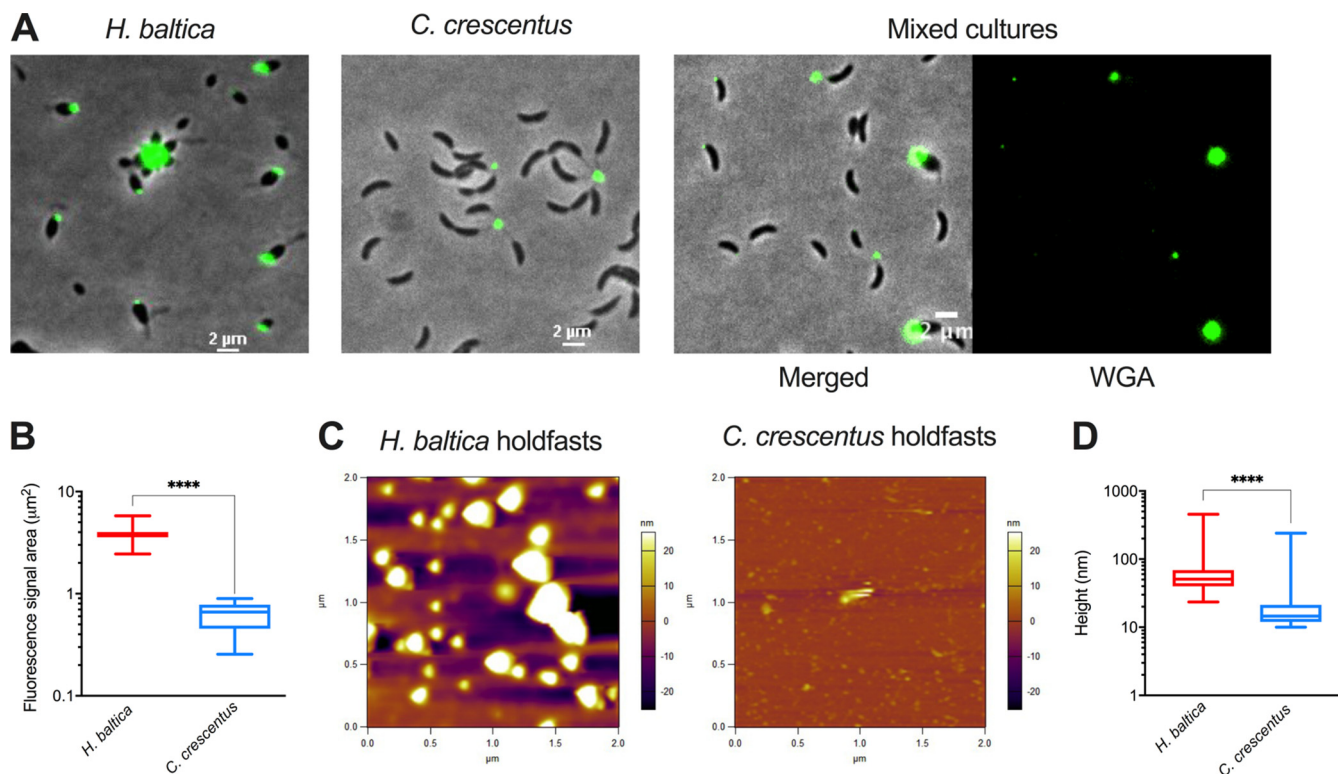


FIG 4 *H. baltica* produces large holdfasts. (A) Images of *H. baltica*, *C. crescentus*, and mixed culture with holdfasts labeled with WGA-AF488 (green). (B) Quantification of holdfast size based on WGA-AF488 fluorescence area. The data in the box-and-whisker plots represent 5 independent replicates of 200 holdfasts from each strain. The variance between *H. baltica* and *C. crescentus* holdfast fluorescent areas was analyzed using a *t* test. ****, $P < 0.0001$. (C) AFM images of dry shed holdfasts from *H. baltica* Δ*hfaB* and *C. crescentus* Δ*hfaB* deposited on a mica surface. The colors on the scale represent the height of the holdfast relative to the surface. (D) Box-and-whisker plots of holdfast height distribution from AFM images. More than 500 holdfasts were measured in 10 independent images. The variance between *H. baltica* and *C. crescentus* holdfast heights was analyzed using a *t* test. ****, $P < 0.0001$.

ited on a clean mica surface, free of any stain. The results confirmed that *H. baltica* produces larger holdfasts than *C. crescentus*. *H. baltica* holdfasts had a median height of 68 nm, while *C. crescentus* produced holdfasts with a median height of 19 nm (Fig. 4C and D), in agreement with previous reports (6, 37).

C. crescentus can regulate holdfast synthesis by two distinct pathways, a complex developmental program in a cell cycle-regulated manner or activation upon cell contact with a surface, independent of the cell cycle (38–41). Some *Alphaproteobacteria*, such as *Asticcacaulis biprosthecum* (39) and *Prosthecomicrobium hirschii* (42), are also able to produce holdfasts via developmental and surface-contact-stimulated pathways, while others, like *Agrobacterium tumefaciens*, produce holdfasts only upon contact with a surface (39, 43). To determine how holdfast production is regulated in *H. baltica*, we measured the timing of holdfast synthesis in the presence or absence of a hard surface. To test whether *H. baltica* holdfast production can be stimulated upon contact with a surface, we performed time-lapse microscopy in a microfluidic device where cells were in close proximity to a glass surface, and we tracked single cells as they reached the surface. We observed holdfast production by including fluorescently labeled WGA in the medium, and we recorded the difference between the time when a cell first reached the surface and the time when a holdfast was synthesized (Fig. 5A, top). We observed that *H. baltica* produces holdfasts within approximately 3 min of surface contact (Fig. 5A and B), showing that surface contact stimulates holdfast synthesis in the species. To assess cell cycle progression and the timing of holdfast synthesis independent of a hard surface, we tracked single cells and monitored cell differentiation and holdfast synthesis by time-lapse microscopy on soft agarose pads containing fluorescent WGA (Fig. 5A, bottom, and Fig. 5B). *H. baltica* newborn swarmer cells produced holdfasts within 15 to 25 min after budding on an agarose pad (Fig. 5A and

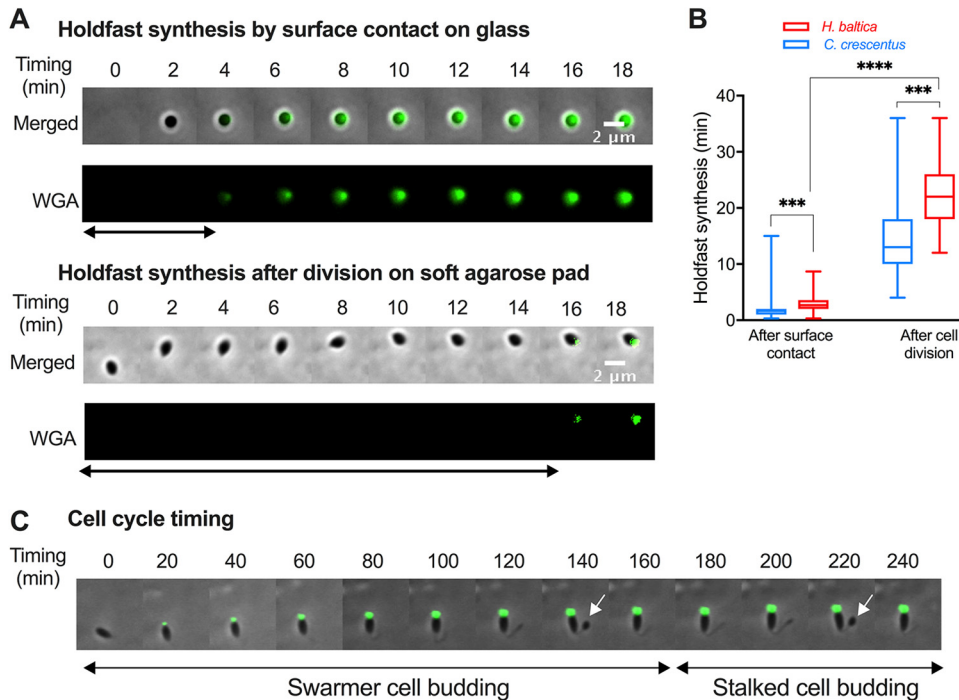


FIG 5 *H. baltica* holdfast synthesis is regulated by a developmental pathway and in response to surface contact. (A) Montages of *H. baltica* holdfast synthesis by a newly budded swarmer cell on a glass surface on a microfluidic device (top) and on soft agarose pads (bottom). The holdfasts were labeled with WGA-AF488 (green). Images shown were acquired every 2 min, and holdfast synthesis timing was processed using MicrobeJ. The arrows indicate the time it took for holdfasts to be detected after surface contact. (B) Box-and-whisker plots representing the quantification of *H. baltica* holdfast timing via surface contact stimulation and developmental pathways. The data for *C. crescentus* holdfast synthesis timing were extracted from reference 44. The total number of cells analyzed was 100 for each setup. The variance between *H. baltica* and *C. crescentus* holdfast synthesis times was analyzed using a *t* test. ***, *P* < 0.001; ****, *P* < 0.0001. (C) Time-lapse montage of an *H. baltica* swarmer cell differentiating into a budding stalked cell on an agarose pad containing WGA-AF488 to label the holdfast. Images were collected every 5 min for 3 h. The arrows point to incipient swarmer cells in predivisional cells.

B), showing that *H. baltica* can produce holdfasts through progression of the cell cycle, as part of a developmental pathway. To determine the timing of holdfast production relative to the cell cycle length, we measured the time required for a newborn swarmer cell to complete its first and second budding divisions on agarose pads (Fig. 5C). *H. baltica* swarmer cells completed their first budding within 160 to 200 min (Fig. 5C). Thus, the holdfast is synthesized within ~1/10 of the cell cycle, similar to *C. crescentus*, which synthesizes holdfasts within 8 to 12 min of a 90- to 120-min cell cycle under these conditions (44).

***H. baltica* holdfasts contain GlcNAc and galactose monosaccharides and proteins.** Holdfasts in diverse *Alphaproteobacteria* bind to WGA, showing that they contain GlcNAc residues (3). Previous studies using lectin labeling showed that GlcNAc polymers are the main polysaccharides present in *C. crescentus* holdfasts, while other *Caulobacterales* strains may have additional monosaccharides in their holdfasts (10). Indeed, WGA lectin (specific to GlcNAc) and *Dolichos biflorus* agglutinin (specific to *N*-acetylgalactosamine) both bind *Caulobacter henricii* holdfasts (10), while *Caulobacter subvibrioides* holdfasts were shown to interact with *D. biflorus* agglutinin (specific to *N*-acetylgalactosamine), concanavalin A (specific to α -mannose), and *Ulex europaeus* agglutinin (specific to α -fucose) but not WGA (10).

To identify the types of saccharides present in *H. baltica* holdfasts, we screened a variety of fluorescent lectins to attempt to label *H. baltica* holdfasts (Table 2; see Table S3 in the supplemental material). Our results indicate that, in addition to binding to WGA, *H. baltica* holdfasts also bind to *Solanum tuberosum* potato lectin (STL), *Lycopersicon esculentum* tomato lectin (LEL), and *Datura stramonium* lectin 1 (DSL1), all lectins

TABLE 2 Lectin-binding assay results

Lectin	Specificity	Presence in holdfast ^a	
		<i>H. baltica</i>	<i>C. crescentus</i>
WGA	GlcNAc	+	+
<i>L. esculentum</i> tomato lectin	GlcNAc 1-4	+	+ ^b
<i>D. stramonium</i> lectin	GlcNAc 1-4	+ ^b	–
<i>S. tuberosum</i> potato lectin	GlcNAc; prefers trimers and tetramers	+	+ ^b
<i>R. communis</i> agglutinin	Galactose	+	–
<i>G. simplicifolia</i> lectin 1	α-GalNAc, α-galactose	+	–
Soybean agglutinin	α-GalNAc	–	–

^a+, fluorescent signal detected; –, no fluorescent signal detected.

^bBinding was enhanced on rosettes, with weaker signals on single cells.

specific to GlcNAc residues (Table 2), confirming that *H. baltica* holdfasts contain GlcNAc residues. In addition, lectins that specifically recognize α-galactose residues, *Griffonia simplicifolia* agglutinin 1 (GSL1) and *Ricinus communis* agglutinin 120 (RCA120) also bind to *H. baltica* holdfasts (Table 2), while they do not bind to *C. crescentus* holdfasts (Fig. 6A). Interestingly, soybean agglutinin lectin (45) did not bind to *H. baltica* holdfasts, showing that these holdfasts contain only galactose and no *N*-acetylgalactosamine (GalNAc) residues (Table 2). These results show that *H. baltica* holdfasts have a different sugar composition than *Caulobacter* holdfasts and contain both GlcNAc and galactose residues. To confirm that the observed galactose-specific binding was holdfast dependent, we labeled *H. baltica* $\Delta hfsA$ and *H. baltica* $\Delta hfsG$ (holdfast-negative strains) and $\Delta hfaB$ (a holdfast-shedding strain) mutants with both WGA and GSL1 lectins. None of the lectins labeled the holdfast-deficient $\Delta hfsA$ and $\Delta hfsG$ mutants, but they labeled shed holdfasts produced by the $\Delta hfaB$ mutant (Fig. 6A), confirming that *H. baltica* holdfasts contain galactose residues.

C. crescentus holdfasts have been recently shown to contain peptides and DNA residues (15). To test whether *H. baltica* holdfasts contain proteins, we attempted to label putative cysteines in the holdfasts using a fluorescent maleimide dye (Alexa Fluor 488 maleimide [AF488mal]). As for *C. crescentus* holdfasts, *H. baltica* holdfasts could be stained with AF488mal, showing that these holdfasts possess molecules with free, accessible thiols, suggesting the presence of peptides containing cysteines (Fig. 6B). The staining was holdfast specific, as AF488mal did not label the holdfast-deficient $\Delta hfsA$ and $\Delta hfsG$ mutants (Fig. 6B). It has been shown that in *C. crescentus*, holdfast labeling by AF488mal was specific to holdfasts attached to cells, as shed holdfasts from a holdfast anchor mutant were not labeled, suggesting that the cysteine-containing HfaD in cell-anchored holdfasts is responsible for the labeling of those holdfasts with AF488mal (15). In *H. baltica*, both the anchor proteins HfaB and HfaD contain cysteines. In order to test whether AF488mal interacts with HfaB or HfaD, we stained shed holdfasts produced by an *H. baltica* $\Delta hfaB \Delta hfaD$ double mutant and could detect staining (Fig. 6B). This is in stark contrast with *C. crescentus* holdfasts, which react with AF488mal only when attached to WT cells (15, 44). This result shows that the holdfast compositions in the two microorganisms are different.

To probe for the presence of DNA in *H. baltica* holdfasts, we labeled holdfasts with the fluorescent DNA dye YOYO-1, which binds to double-stranded DNA molecules. As previously reported, *C. crescentus* holdfasts were labeled with YOYO-1 (15). However, YOYO-1 failed to label *H. baltica* holdfasts (Fig. 6C), suggesting that *H. baltica* holdfasts do not contain DNA. It has been previously shown that, in *C. crescentus*, extracellular DNA (eDNA) released during *C. crescentus* cell lysis binds specifically to *C. crescentus* holdfasts, preventing adhesion to surfaces and biofilm formation (46), and it has been hypothesized that this could be due to a specific interaction between the DNA present in the holdfast and eDNA (15). We showed above that *H. baltica* holdfasts were devoid of DNA, so we tested whether eDNA could inhibit *H. baltica* binding. We performed short-term adhesion assays in the presence of *H. baltica* and *C. crescentus* eDNAs (see Fig. S2A in the supplemental material). When *C. crescentus* eDNA was present, the

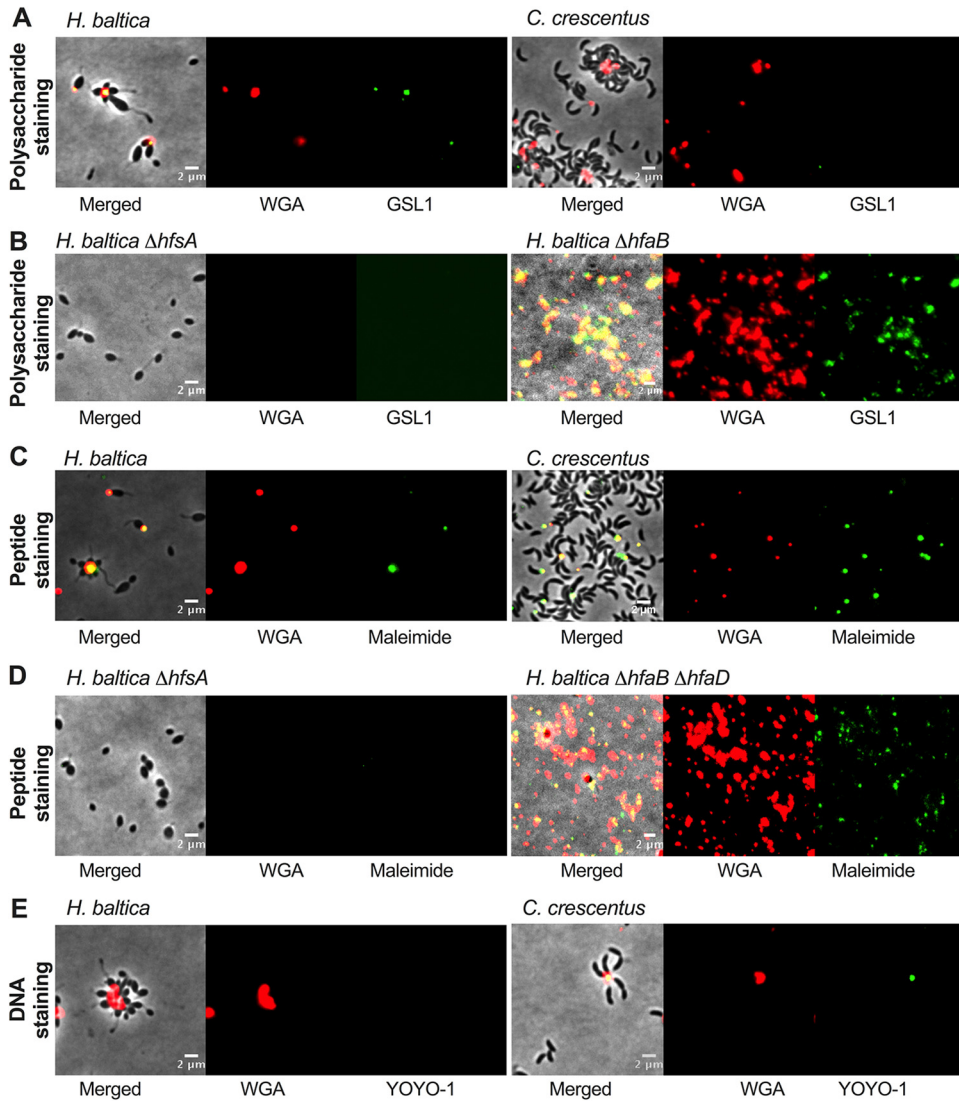


FIG 6 *H. baltica* holdfasts contain GlcNAc and galactose monosaccharides and proteins. (A to C) Representative images showing merged phase and fluorescence channels on the left and fluorescence channels alone in the middle and on the right. (A) *H. baltica* and *C. crescentus* holdfasts were colabeled with WGA-AF594 (red, GlcNAc) and GSL1-AF488 (green, galactose) lectins to stain polysaccharides. (B) *H. baltica* and *C. crescentus* holdfasts were colabeled with WGA-AF594 (GlcNAc) lectin and AF488mal to stain peptides. (C) *H. baltica* and *C. crescentus* holdfasts were colabeled with WGA-AF594 (GlcNAc) lectin and YOYO-1-AF488 to stain DNA.

number of *C. crescentus* cells attached to the glass slide after 60 min was dramatically decreased compared to when *H. baltica* eDNA was added and to the control (no DNA addition) (see Fig. S2A), confirming previous studies that showed that, in *C. crescentus*, eDNA inhibition was specific for *C. crescentus* eDNA (46). However, *H. baltica* adhesion was not impaired by the presence of eDNA, from itself or from *C. crescentus* (see Fig. S2A). We also performed long-term biofilm assays in the presence of eDNA and showed that *H. baltica* biofilm formation is not impaired by the presence of eDNA in the medium after 24 h of incubation (see Fig. S2B).

Taking these results together, we showed that the *H. baltica* holdfast is different than that of *C. crescentus*: it is larger and contains GlcNAc, galactose, and peptide residues but is devoid of DNA.

***H. baltica* holdfasts tolerate high ionic strength.** It has been shown that *C. crescentus* holdfasts are very sensitive to ionic strength, as the efficiency of purified-holdfast binding to glass decreased by 50% with the addition of 10 mM NaCl (6). *C.*

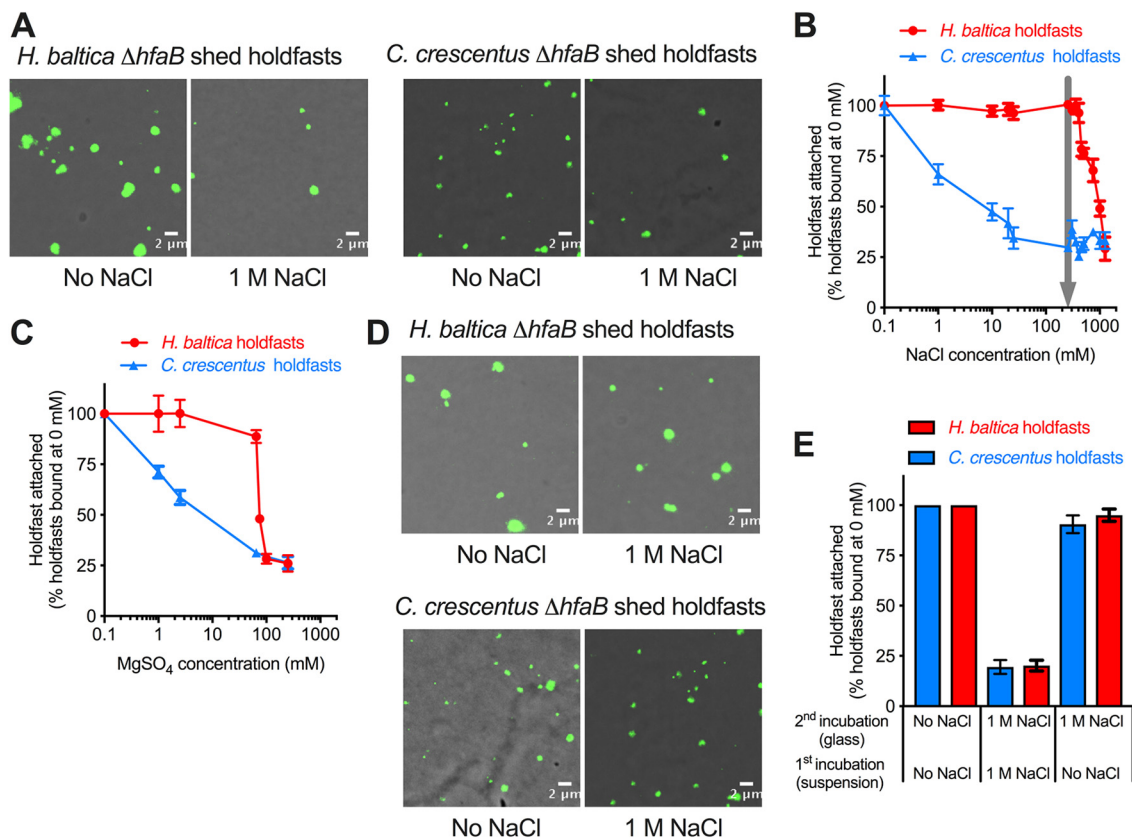


FIG 7 *H. baltica* holdfasts tolerate higher ionic strength than *C. crescentus* holdfasts. (A) Images of WGA-AF488-labeled *H. baltica* $\Delta hfaB$ and *C. crescentus* $\Delta hfaB$ shed holdfasts bound to glass slides and incubated in different concentration of NaCl for 4 h. (B) Percentages of holdfasts bound per field of view at different concentrations of NaCl. The number of holdfasts bound per field of view at 0 M NaCl was standardized to 100%. The arrow indicates the ionic strength of marine broth and the Baltic Sea (250 mM), from which *H. baltica* was isolated. The data are expressed as averages from 6 independent replicates, and the error bars represent the standard errors. (C) Percentages of holdfasts bound per field of view at different concentrations of MgSO₄. The number of holdfasts bound per field of view at 0 M MgSO₄ was standardized to 100%. The data are expressed as averages from 4 independent replicates, and the error bars represent the standard errors. (D) Images of WGA-AF488-labeled holdfasts already bound to a glass surface and incubated in 0 mM NaCl and 1 M NaCl for 12 h. (E) Percentages of holdfasts bound per field of view at 0 M or 1 M NaCl. The first incubation was done by adding 0 M or 1 M NaCl to a holdfast suspension spotted on a glass slide. After a 12 h of incubation, the second incubation was done, after washing off unbound holdfasts, by adding 0 M or 1 M NaCl directly to the holdfasts attached to the glass slide and incubating them for another 12 h. The number of holdfasts bound per field of view at 0 M NaCl was standardized to 100%. The data are expressed as averages from 5 independent replicates, and the error bars represent the standard errors.

crescentus is a freshwater bacterium and has probably evolved without selective pressure to bind under high ionic strength. This compelled us to investigate how purified holdfasts from *H. baltica* are affected by ionic strength. We first used NaCl to study the effects of ionic strength on holdfast binding, since it is the most abundant ionic element in marine water and it has been used in many studies to assess the effect of ionic strength on bacterial adhesins (6, 47–49). Holdfasts from both species were purified from a holdfast-shedding mutant during 2 h of growth in peptone yeast extract (PYE) following growth in optimal medium. We quantified purified holdfast binding to glass at different NaCl concentrations, using fluorescent WGA, and plotted the relative numbers of holdfasts per field of view bound to glass at different concentrations of NaCl (Fig. 7A and B). Our results confirmed that the *C. crescentus* holdfast is very sensitive to NaCl, as only 50% of holdfasts could bind to glass when 10 mM NaCl was added (Fig. 7B). However, *H. baltica* holdfasts tolerated up to 500 mM NaCl without any effect on surface binding (Fig. 7B). There was a 50% decrease in *H. baltica* holdfast binding at 600 mM (Fig. 7B), showing that *H. baltica* holdfasts are more than 50 times more resistant to NaCl than those of *C. crescentus*. *H. baltica* was originally isolated from the Baltic Sea, which has 250 mM NaCl (Fig. 7B, arrow) (28), and at that NaCl concen-

tration, the binding efficiency of *H. baltica* holdfasts was still at its maximum. Interestingly, *H. baltica* holdfasts still bound efficiently at low ionic strength. We observed similar results using different concentrations of MgSO_4 (Fig. 7C): *H. baltica* holdfasts were 50 times more resistant to MgSO_4 than those of *C. crescentus*, showing that the binding inhibition is not specific to NaCl but is rather dependent on ionic strength. The salt dose-response curves are noticeably different for the two types of holdfasts: while *C. crescentus* holdfast binding slowly decreases as the salt concentration increases, the binding efficiency of *H. baltica* holdfasts remains steady up to 500 mM NaCl or 250 mM MgSO_4 and sharply decreases at high salt concentrations (Fig. 7B and C). This shows that the two holdfasts behave differently under increased ionic strength, suggesting different properties.

Our results show that, in *H. baltica*, initial holdfast binding to glass did not change for NaCl concentrations up to 500 mM and then drastically decreased to reach around 25% of holdfasts attached at 1 M NaCl (Fig. 7B). To test whether high ionic strength could remove holdfasts previously attached to the glass surface, we first incubated purified holdfasts for 4 h without any salt added and then added 1 M NaCl for 12 h to the bound holdfasts (Fig. 7D). Bound holdfasts from *H. baltica* and *C. crescentus* were not dislodged from the glass surface (Fig. 7D and E), indicating that while high ionic strength inhibits holdfasts from binding to a surface, it cannot dislodge bound holdfasts from a glass surface (Fig. 7E).

DISCUSSION

Different bacterial species harbor an adhesive holdfast and use it to attach to surfaces (2, 3, 9, 50, 51). They represent an extremely diverse group in terms of their physiologies and the natural environments they inhabit (soil, freshwater, and marine environments). They have evolved the ability to adhere to surfaces with vastly different compositions under varying environmental conditions (salinity, pH, temperature, etc.). Holdfast chemical properties have been mainly studied in the model organism *C. crescentus* CB15, a freshwater member of the *Caulobacteriales* (6, 10, 13, 15, 19, 20, 30, 37, 52). Little is known about holdfast properties and composition in *Caulobacteriales* isolated from habitats other than oligotrophic freshwater environments. In this study, we used *H. baltica* as a model species living in a marine environment and found that it has a holdfast tailored for adhesion under high-salinity conditions. We show that holdfasts in *H. baltica* are different than those of *C. crescentus*: they are larger, have a different chemical composition, and have a high tolerance for ionic strength.

The bioinformatics analysis of holdfast genes indicated that the *hfs* and *hfa* loci are highly conserved among *Caulobacteriales*, with some reshuffling of the genes (Fig. 1C). The arrangement of the holdfast genes in the *hfs* and *hfa* loci appears to be ancestral, while the relocation of some of the genes is a recent event that could affect their levels of expression (53). Through deletion and complementation of important *hfs* and *hfa* genes, we confirmed that holdfast biogenesis and anchoring to the cell body in *H. baltica* use genes similar to those identified in *C. crescentus* (2, 19) (Fig. 2).

We showed that the two glycosyltransferase genes *hfsL* and *hfsG* are essential for holdfast production and that their expression level modulates the amount of sugar monosaccharides added to holdfast polysaccharides. Small holdfasts with fewer polysaccharides bind to glass, but not strongly enough to support cell adhesion to glass in flow (Fig. 3). This phenomenon could be due to the smaller surface contact area of the small holdfasts being insufficient to resist drag and shear forces during the washing steps of our assays or to a change in holdfast structure or composition due to the lower expression of the glycosyltransferases HfsL and HfsG. More studies on the roles of HfsL and HfsG will help us to determine if these enzymes play important roles in specific physicochemical properties of *H. baltica* holdfasts.

In *C. crescentus*, the growing holdfast polysaccharide repeat units are thought to be modified by the acetyltransferase HfsK (20) and the polysaccharide deacetylase HfsH (21) (Fig. 1A). These two enzymes are not essential for holdfast production in *C. crescentus*, but they modify the adhesiveness and cohesiveness of the holdfasts. *C.*

crenscentus $\Delta hfsH$ and $\Delta hfsK$ mutants produced thread-like holdfasts with weaker adhesion (20, 30). In addition, fully acetylated purified holdfasts from the *C. crescentus* $\Delta hfsH$ mutant were not affected by ionic strength (6), suggesting that holdfast modification can modulate salt tolerance. Our future work will determine how holdfast modification impacts *H. baltica* holdfast tolerance for high ionic strength and the possible roles of HfsH and HfsK.

The exact composition and structure of the holdfast in the model organism *C. crescentus* are still unknown. Lectin-binding assays and lysozyme treatment support GlcNAc as one of the important components in holdfasts (10, 37). Treating *C. crescentus* holdfasts with proteinase K and DNase I affects their structure and force of adhesion, suggesting that they contain peptide and DNA residues (15). In this work, we identified different components present in *H. baltica* holdfasts: these holdfasts contain galactose monosaccharides in addition to GlcNAc (Fig. 6A). In the different *hfs* mutants generated in this study, galactose monosaccharides were not detected on the cell pole (Fig. 6A), suggesting that GlcNAc and galactose are parts of the same polysaccharide or secreted by the same proteins. Shed holdfasts from *H. baltica* $\Delta hfaB$ contain both GlcNAc and galactose (Fig. 6A), implying that they are both anchored to the cell envelope with the same anchor proteins. *H. baltica* holdfasts are void of DNA, a stark contrast to those of *C. crescentus* (Fig. 6C). In addition, *H. baltica* holdfasts could be successfully stained with a fluorescent maleimide dye, which suggests the presence of a protein or peptide with a cysteine residue (54). The maleimide dye stains only cells with a holdfast and interacts with holdfasts without the presence of cells, indicating that the reactive molecules are an intrinsic part of *H. baltica* holdfasts (Fig. 6B), another notable difference from *C. crescentus* holdfasts, where maleimide dye interacts only with holdfasts attached to cells (15). In aggregate, our results suggest that the two holdfasts from *H. baltica* and *C. crescentus* have different compositions.

Bacterial adhesins have been shown to use electrostatic and hydrophobic interactions to attach to surfaces (6). Electrostatic interactions are impaired in high-ionic-strength environments, like seawater, with 600 mM NaCl (7). The *C. crescentus* holdfast uses both ionic and hydrophobic interactions, and its binding is impaired in the presence of NaCl in the medium (6). We have shown that *H. baltica* holdfasts tolerate high ionic strength compared to *C. crescentus* (Fig. 7A to C). Marine *Caulobacteriales* face a higher-ionic-strength environment than the freshwater bacteria; therefore, it is vital that marine *Caulobacteriales* produce holdfasts that are more tolerant of ionic strength and strongly adhere in saline environments. Holdfasts do not efficiently bind at 1 M NaCl, but holdfasts already attached to a surface cannot be removed by adding 1 M NaCl (Fig. 7D), suggesting that the binding inhibition at 1 M NaCl takes place during the initial stage of surface interaction. These results imply that holdfasts interact with surfaces initially by using electrostatic interactions before a permanent molecular bond is formed (6, 55). The differences in ionic tolerance between freshwater and marine *Caulobacteriales* indicate that there are significant differences in physicochemical properties between the two types of holdfasts. Holdfast structure and binding properties could depend on the types and the amounts of sugars polymerized in the holdfast polysaccharides that are specialized to interact with different surfaces (56).

In conclusion, we have shown that *H. baltica* produces holdfasts with different binding and physicochemical properties than *C. crescentus* holdfasts. This suggests that there are additional holdfast-related genes or regulators that have not been identified. A careful genetic screen of *H. baltica* will provide more insights into holdfast production and the underlying mechanisms yielding enhanced adhesion at high ionic strength.

MATERIALS AND METHODS

Identification of orthologous holdfast genes and phylogenetic analysis. *C. crescentus* holdfast genes were used to find bidirectional best hits (BBH) on *Caulobacteriales* genomes. The putative genes were selected for an E value of $>10^{-4}$ and a sequence identity of $>30\%$. The phylogenetic tree was built using 16S rRNA sequences of the selected *Caulobacteriales*. Sequences were aligned using MUSCLE software (47). The aligned sequences were used to construct a maximum-likelihood phylogeny using

MEGA6 software (57). The LG+G+I models and analysis of 1,000 bootstraps were used to generate the node values for each clade.

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table S1 in the supplemental material. *H. baltica* strains were grown in marine medium (Difco marine broth/agar; reference 2216), except when studying the effect of ionic strength on holdfast binding, when they were grown in PYE medium (8) supplemented with 0 or 1.5% NaCl or MgSO_4 . *C. crescentus* was grown in PYE medium. Both *H. baltica* and *C. crescentus* strains were grown at 30°C. When appropriate, kanamycin (Kan) was added at 5 $\mu\text{g}/\text{ml}$ in liquid and 20 $\mu\text{g}/\text{ml}$ on agarose plates. *H. baltica* strains with copper-inducible promoters were grown in marine broth supplemented with 0 to 250 μM CuSO_4 . *E. coli* strains were grown in Luria-Bertani medium (31) at 37°C with no antibiotics or with 30 $\mu\text{g}/\text{ml}$ Kan in liquid or 25 $\mu\text{g}/\text{ml}$ on agarose plates when needed.

Strain construction. All the plasmids and primers used in this study are listed in Tables S1 and S2 in the supplemental material, respectively. In-frame deletion mutants were obtained by double homologous recombination as previously described (58), using suicide plasmids transformed into the *H. baltica* host strains by mating or electroporation (59). Briefly, genomic DNA was used as the template to PCR amplify 500-bp fragments from upstream and downstream regions of the gene to be deleted. The pNPTS139 plasmid was cut using EcoRV-HF endonuclease from New England Biolabs (NEB). The primers used to amplify 500 bp upstream and downstream of the gene were designed to have 25 bp overlapping for isothermal assembly (60) using the New England Biolabs NEBuilder tools for Gibson assembly into plasmid pNPTS139. Then, pNPTS139-based constructs were transformed into an α -select *E. coli* strain and introduced into the host *H. baltica* by mating or electroporation (59). The two-step selection for homologous recombination was carried out using sucrose resistance and kanamycin sensitivity (61).

For gene complementation, the pMR10 plasmid was cut with EcoRV-HF, and 500 bp of the promoter and the gene was ligated into plasmid pMR10 using NEBuilder tools. The pMR10-based constructs were transformed into an α -select *E. coli* strain and introduced into the *H. baltica* host by mating or electroporation, followed by Kan selection. The plasmid constructs and mutants were confirmed by sequencing.

Holdfast labeling using fluorescently labeled lectins. Alexa Fluor (AF)-conjugated lectins (Vector Laboratories) (Table 2; see Table S3) were added to 100 μl of exponential-phase culture to a final concentration of 0.5 $\mu\text{g}/\text{ml}$ and incubated at room temperature for 5 min; 3 μl of the labeled culture was spotted on a glass cover slide and covered with a 1.5% (wt/vol) SeaKem LE agarose (Lonza) pad in water and visualized by epifluorescence microscopy. Holdfasts were imaged by epifluorescence microscopy using an inverted Nikon Ti-E microscope with a Plan Apo 60 \times objective, a green fluorescent protein (GFP)/DsRed filter cube, an Andor iXon3 DU885 EM charge-coupled device (CCD) camera, and Nikon NIS Elements imaging software with a 200-ms exposure time. Images were processed in ImageJ (45).

Short-term and biofilm binding assays. Short-term and biofilm binding assays were performed as previously described (30) with the following modifications. For short-term binding, exponential cultures (optical density at 600 nm [OD_{600}] = 0.6 to 0.8) were diluted to an OD_{600} of 0.4 in fresh marine broth, added to 24-well plates (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 0.10, added to 24-well plates (1 ml per well), and incubated at room temperature for 12 h with shaking (100 rpm). In both setups, OD_{600} values were measured before the wells were rinsed with dH_2O to remove nonattached bacteria, stained using 0.1% crystal violet (CV), and rinsed again with dH_2O to remove excess CV. The CV was dissolved in 10% (vol/vol) acetic acid and quantified by measuring the absorbance at 600 nm (A_{600}). The biofilm formation was normalized to the A_{600}/OD_{600} and expressed as a percentage of the WT value.

hfsL and hfsG expression using copper-inducible promoters. Strains bearing copper-inducible plasmids were inoculated from freshly grown colonies into 5 ml marine broth containing 5 $\mu\text{g}/\text{ml}$ Kan and incubated with shaking (200 rpm) at 30°C overnight. The overnight cultures were diluted in the same culture medium to an OD_{600} of 0.10 and incubated until an OD_{600} of 0.4 was reached. When needed, copper sulfate dissolved in marine broth was added to a final concentration of 0 to 250 μM . The induced cultures and controls were added to a 24-well plate (1 ml per well) and incubated with shaking (100 rpm) at room temperature for 4 to 8 h. Then, OD_{600} values were measured before the wells were rinsed with dH_2O to remove nonattached bacteria, stained using 0.1% CV, and rinsed again with dH_2O to remove excess CV. The CV was dissolved in 10% (vol/vol) acetic acid and quantified by measuring the A_{600} . The biofilm formation was normalized to the A_{600}/OD_{600} and expressed as a percentage of the WT value.

Visualization of holdfasts attached to a glass surface. Visualization of holdfast binding to glass surfaces was performed as described previously (30) with the following modifications. *H. baltica* and *C. crescentus* strains grown to exponential phase (OD_{600} = 0.2 to 0.6) were incubated on washed glass coverslips at room temperature in a saturated humidity chamber for 4 to 8 h. After incubation, the slides were rinsed with dH_2O to remove unbound cells, and the holdfasts were labeled using 50 μl of fluorescent Alexa Fluor 488 (AF488)- or AF594-conjugated lectins (Molecular Probes or Vector Laboratories) (Table 2) at a final concentration of 0.5 $\mu\text{g}/\text{ml}$. Then, the slides were rinsed with dH_2O and topped with a glass coverslip. The holdfasts were imaged by epifluorescence microscopy using an inverted Nikon Ti-E microscope with a Plan Apo 60 \times objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera, and Nikon NIS Elements imaging software with a 200-ms exposure time. Images were processed in ImageJ (45).

Atomic-force microscopy. AFM imaging was performed using the tapping mode on a Cypher AFM (Asylum Research) at 20°C, as described previously (6, 22), with the following modifications. *H. baltica* $\Delta hfaB$ and *C. crescentus* $\Delta hfaB$ grown to exponential phase were diluted and spotted on freshly cleaved mica. Samples were grown overnight at room temperature in a humid chamber. The samples were then

rinsed with sterile dH₂O to remove unbound cells and debris and air dried. AFM topographic images of dried holdfasts attached to the mica surface were obtained using a silicon Olympus AC160TS cantilever (resonance frequency = 300 kHz; spring constant = 26 N/m). Forty images of 4 independent replicates were obtained. Holdfast height was determined using the built-in image analysis function of the Igor Pro/Asylum Research AFM software.

Holdfast synthesis timing by time-lapse microscopy on agarose pads. *H. baltica* holdfast synthesis timing was observed in live cells on agarose pads by time-lapse microscopy, as described previously (40), with some modifications. A 1- μ l aliquot of exponential-phase cells ($OD_{600} = 0.4$ to 0.8) was placed on top of a pad containing 0.8% agarose in marine broth with 0.5 μ g/ml WGA-AF488. A coverslip was placed on top of the agarose pad and sealed with VALAP (petrolatum, lanolin, and paraffin wax). Time-lapse microscopy images were taken every 2 min for 4 h using an inverted Nikon Ti-E microscope and a Plan Apo 60 \times objective, a GFP/DsRed filter cube, and an Andor iXon3 DU885 EM CCD camera. Time-lapse movies were visualized in ImageJ (45) to manually assess the timing of a swarmer cell producing a holdfast (lectin detection) after budding. The time difference between holdfast synthesis and budding was determined using MicrobeJ (62).

Holdfast synthesis timing by time-lapse microscopy in microfluidic devices. The holdfast synthesis timing experiment was performed as previously described (41) with the following modifications. Cell cultures were grown to mid-exponential phase ($OD_{600} = 0.4$ to 0.6), and 200 μ l of culture was diluted in 800 μ l fresh marine broth in the presence of 0.5 μ g/ml WGA-AF488 for holdfast labeling. One milliliter of the cell culture was flushed into a microfluidic device containing a 10- μ m-high linear chamber fabricated in polydimethylsiloxane (PDMS) as described previously (40). After injection of the cells into the microfluidic chamber, the flow rate was adjusted so that attachment could be observed under static conditions or at a low flow rate of 1.4 μ l/min. The drag force generated by the flow in the microfluidic device was calculated as previously described by Persat and colleagues (63).

Time-lapse microscopy was performed using an inverted Nikon Ti-E microscope and a Plan Apo 60 \times objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera, and Nikon NIS Elements imaging software. Time-lapse videos were collected for strains over a period of 3 h at 20-s intervals. Cell attachment was detected at the glass-liquid interface within the microfluidic chamber using phase-contrast microscopy, while holdfast synthesis was detected using fluorescence microscopy. Cells that hit the surface and attached permanently via their holdfasts during this 3-h period were analyzed for the timing of holdfast synthesis. The time difference between holdfast synthesis and cell surface contact was determined using MicrobeJ (62) and define as holdfast delay. Cells that were present on the surface at the start of the time-lapse experiment were not analyzed.

Holdfast labeling using fluorescently labeled maleimide and YOYO-1. Alexa Fluor (AF488mal)-conjugated maleimide C₅ (ThermoFisher Scientific) was added to 100 μ l of exponential-phase culture to a final concentration of 0.5 μ g/ml and incubated at room temperature for 5 min. Similarly, YOYO-1 (a fluorescent DNA stain; Molecular Probes) was added to 100 μ l of exponential-phase culture to a final concentration of 0.5 μ g/ml and incubated at room temperature for 5 min; 3 μ l of the labeled culture was spotted on a glass cover slide, covered with a 1.5% (wt/vol) agarose pad in water, and visualized by epifluorescence microscopy. Holdfasts were imaged by epifluorescence microscopy using an inverted Nikon Ti-E microscope with a Plan Apo 60 \times objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera, and Nikon NIS Elements imaging software with a 200-ms exposure time. Images were processed in ImageJ (45).

Effect of ionic strength on holdfast binding. Purified holdfasts attached to a surface at different ionic strengths were visualized as described previously (6) with a few modifications. Briefly, *H. baltica* $\Delta hfaB$ and *C. crescentus* $\Delta hfaB$ cells were grown to late exponential phase ($OD_{600} = 0.6$ to 0.8) in PYE plus 1.5% NaCl and plain PYE, respectively. The cells were pelleted by centrifugation for 30 min at $4,000 \times g$, resuspended in PYE, and incubated for 2 h at 30°C to produce shed holdfasts. Then, the cells were again pelleted by centrifugation, and 100 μ l of supernatant containing free holdfasts shed by the cells was mixed with PYE-NaCl to make a final concentration of 0 to 1,000 mM NaCl. Fifty microliters of the mixture was incubated on washed glass coverslips at room temperature in a saturated humidity chamber for 4 to 12 h. After incubation, the slides were rinsed with dH₂O to remove unbound material, and labeled holdfasts were visualized with Alexa Fluor lectins (Vector Laboratories). The holdfasts were imaged by epifluorescence microscopy using an inverted Nikon Ti-E microscope with a Plan Apo 60 \times objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera, and Nikon NIS Elements imaging software with a 200-ms exposure time. Images were processed in ImageJ (45). The number of holdfasts bound per field of view was determined using MicrobeJ (62).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00061-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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