Comparative analysis of ionic strength tolerance between freshwater and marine Caulobacterales adhesins

Nelson K. Chepkwony, Cécile Berne and Yves V. Brun*

Département de microbiologie, infectiologie et immunologie, Université de Montréal, C.P. 6128, succ. Centre-ville, Montréal (Québec) H3C 3J7, Canada.

Department of Biology, Indiana University, 1001 E. 3rd St. Bloomington, Indiana 47405, USA

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*Address correspondence to Yves V. Brun, ybrun@indiana.edu.

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ABSTRACT

Bacterial adhesion is affected by environmental factors, such as ionic strength, pH, temperature, and shear forces, and therefore marine bacteria must have developed holdfasts with different composition and structures than their freshwater counterparts to adapt to their natural environment. The dimorphic α-proteobacterium *Hirschia baltica* is a marine budding bacterium in the Caulobacterales clade. *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 composition 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 two genera. We also determine that *H. baltica* produces larger holdfast than *C. crescentus* and that those holdfasts have a different chemical composition, 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 amount of their lifespan attached to surfaces, forming complex multicellular communities called biofilms. Bacteria can colonize virtually any surface, 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

42 morphology and chemical composition, and tolerate high ionic strength. Our results show that *H.*
43 *baltica* holdfast is an excellent model to study the effect of ionic strength on adhesion and
44 providing insights on the physicochemical properties required for adhesion in the marine
45 environment.

46

47 **INTRODUCTION**

48 In their natural environments, bacteria preferentially form surface-associated
49 communities, known as biofilms (1). To irreversibly adhere to surfaces and form these complex
50 mutil-cellular communities, bacteria produce strong adhesins, mainly composed of proteins or
51 polysaccharides (2, 3). Bacterial adhesion is affected by different environmental conditions such
52 as pH, temperature, shear forces, and ionic strength (2, 4-6). In marine environments, bacteria
53 face 500 times higher ionic strength than in freshwater (7), therefore, marine bacteria have
54 evolved ways to overcome the effect of ionic strength and bind permanently to surfaces in high
55 salt environments such as seas and oceans.
56 Caulobacterales are Alphaproteobacteria found in various habitats, from oligotrophic
57 aquatic and nutrient-rich soil environments (8, 9). The aquatic Caulobacterales species live in a
58 wide range of environments with different salinity levels, such as pristine fresh river and lake
59 waters, brackish ponds, and marine waters, making them a good model for studying bacterial
60 adhesion in different ionic environments. Caulobacterales species use a polar adhesin structure
61 called the holdfast to adhere permanently to surfaces and form biofilms (8, 10, 11). The holdfast
62 has been primarily studied in *Caulobacter crescentus*, a freshwater member of the
63 Caulobacterales (2, 3, 12). The *C. crescentus* holdfast uses both electrostatic and hydrophobic
64 interactions to attach to different surfaces (6). The binding affinity of the *C. crescentus* holdfast
65 is dramatically impaired in the presence of NaCl (6), yet marine Caulobacterales adhere to
66 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 salinity environments is currently unknown.

C. crescentus holdfast is the strongest characterized bioadhesive, with an adhesion force of 70N / mm^2 (13). Despite being first identified almost 85 years ago (14), the exact composition and structure of the *C. crescentus* holdfast remains 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 *E. coli* (16, 17) (Fig. 1A). Holdfast polysaccharide synthesis is thought to be initiated in the cytoplasm by a glycosyltransferase HfsE, which transfers activated sugar phosphate from uridine diphosphate (UDP-GlcNAc) to undecaprenyl-phosphate (Und-P) lipid carrier. Additional sugar residues are then added to form a repeat unit on the lipid carrier by three glycosyltransferases HfsG, HfsJ (17) and HfsL (18). The acetyltransferase HfsK (32) and the polysaccharide deacetylase HfsH (21) modify one or more sugar residue. The lipid carrier with the repeat units is transported across the inner membrane into the periplasm by a flippase (HfsF) (17, 19). 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 proteins complex, composed of HfsA, HfsB and HfsD (20-22). Once outside the cell, holdfast polysaccharides are anchored unto the cell envelop by the action of holdfast anchor (*hfa)* proteins HfaA, HfaB, HfaD and HfaE (18, 23-25).

Hirschia baltica is a marine Caulobacterale isolated from surface water taken from a boat landing in the Kiel Fjord inlet of the Baltic sea (Germany) (26). *H. baltica* has a dimorphic life-cycle similarly to *C. crescentus* (26), 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 (27). *H. baltica* have been shown to produce holdfast containing GlcNAc residues, using fluorescent WGA lectin (27, 28). The vast majority of studies on holdfast have been done using *C. crescentus,* therefore *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* natural habitat is high ionic strength sea water (26), 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 these genes are highly conserved among these genera. We show that *H. baltica* produces more holdfast material than *C. crescentus* and that the holdfasts of the two genera have a different chemical composition and behave differently. In addition to GlcNAc monosaccharides, we show that *H. baltica* holdfasts also contain galactose residues, and uncharacterized peptides different than the ones found in *C. crescentus* holdfasts. Finally, we demonstrate that *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* (27, 28). To determine if the genomic organization of all the 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 Caulobacterales genomes for a more global overview of the organization of these genes in this clade (Fig. 1C). Table 1 gives the locus tag names of all the holdfast related genes used in this study for *C. crescentus* CB15 (29), *C. crescentus* NA1000 (30), and *H. baltica* IFAM 1418^{T} (27) type strains.

All the genes reported 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 Caulobacterales genomes analyzed, with the genes encoding proteins essential for holdfast synthesis (glycosyltransferase gene *hfsG* and export genes *hfsA*, *hfsB*, and *hfsD*) and the initiating glycosyltransferase gene *hfsE* in a similar organization as 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 (31) and HfsL (18), and N-acetyltransferase HfsK (32)); these genes are also present in *H. baltica.* Interestingly, in the genomes of the marine Caulobacterales *Oceanicaulis alexandrii, Maricaulis maris and Maricaulis salignorans,* all the *hfs* genes are found in one locus except *hfsJ* (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 other have 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 action of anchor proteins that have been identified and characterized in *C. crescentus* HfaA, HfaB, and HfaD (18, 23-25). The organization of the three anchor genes *hfaA, hfaB* and *hfaD* in the *hfa* is conserved in all the analyzed Caulobacterales genomes (Fig. 1C). In *C. crescentus* and most of the tested Caulobacterales, the recently identified holdfast anchor gene *hfaE* (18) is not part of the *hfaABD* operon, while it is present in the *hfa* locus in both *H. baltica* and *Oceanicaulis alexandrii* (Fig. 1C). We could not find orthologs of the *hfa* genes in the genomes of *Robiginitomaculum antarticum* and *Hellea balneolensis*, but this may be due to the incomplete nature of the genome. Alternatively these species have a different mechanism to anchor holdfast to the surface of the cell, as is the case for several other Alphaproteobacteria (33, 34).

Role 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 labelled WGA lectin (10) (Fig. 2A). We also quantified biofilm formation after 12 h of incubation at room temperature on a plastic surface, using 24-well PVC plates (Fig. 2B). All mutants could be complemented *in trans* by a replicating plasmid encoding a copy of the deleted gene (Fig. 2B).

We first deleted the holdfast anchor genes encoding the HfaB and HfaD proteins. Both *H. baltica* Δ*hfaB* and Δ*hfaD* mutants produced holdfast, but they failed to anchor it to the cell body, resulting in holdfast being shed in the medium (Fig. 2A). *H. baltica* Δ*hfaB* was not able to permanently attach to surfaces, and could not form a biofilm (Fig. 2B). In contrast, *H. baltica*

162 Δ*hfaD* mutants were not completely deficient for permanent adhesion, with around 20% of
163 biofilm formed compared to WT (Fig. 2B). These results are in agreement with what was
164 reported for *C. crescentus* Δ*hfaB* and Δ*hfaD* mutants (24), suggesting that the Hfa proteins have
165 a similar function in both organisms.

166 We then made in-frame deletions of the genes encoding export proteins HfsA and HfsD.
167 These genes are essential for holdfast production in *C. crescentus* (20). Deletion of these genes
168 in *H. baltica* similarly completely abolished holdfast production (Fig. 2A) and surface attachment
169 (Fig. 2B). These results show that deletion of the export genes is sufficient for a complete loss
170 of holdfast production, and that a holdfast is crucial for surface attachment in *H. baltica*.

171 Finally, we made in-frame deletions of the genes encoding glycosyltransferases HfsG
172 and HfsL, which are essential for holdfast formation in *C. crescentus* (17, 18). Similarly, *H.*
173 *baltica* Δ*hfsG* and Δ*hfsL* mutants did not produce holdfasts nor form biofilms (Fig. 2A and 2B).
174

175 **Effect of modulating *hfsL* and *hfsG* expression of *H. baltica* holdfast properties.**

176 We investigated if tunable expression of the *hfsL* and *hfsG* genes, are essential for
177 holdfast production, could change holdfast synthesis and properties. To achieve this goal, we
178 first engineered a replicating plasmid harboring an inducible promoter suitable for *H. baltica*. We
179 adapted the system developed for a tightly controlled heavy metal (copper) promoter inducible
180 system in *Hyphomonas neptunium,* a marine Caulobacterale closely related to *H. baltica* (35).
181 Similarly, we used the promoter for copper resistant protein operon *copAB* (P*cu*) in *H. baltica*
182 *(copA*, *hbal_0699,* and *copB, hbal_0698)* (Fig. S1A top panel). We first showed that *H. baltica*
183 can tolerate up to 500 µM of $CuSO_4$ without significant effect on growth (Fig. S1B-C). We then
184 fused 500 pb upstream of the *copAB* operon (P*cu*) to *lacZ* gene and assembled the construct
185 onto the pMR10 replicating plasmid (Fig. S1A, bottom panel), to assess P*cu* promoter activity
186 using ß-galactosiade as a reporter. We showed that P*cu* is a tightly controlled promoter, with a

187 working inducible range of $CuSO_4$ from 10 to 250 µM (Fig. S1D), concentrations that do not
188 impact *H. baltica* growth (Fig S1 B-C).
189 We expressed *hfsL* or *hfsG* under control of the P*cu* inducible promoter in *H. baltica*
190 Δ*hfsL* and Δ*hfsG* mutants. In both cases, when the gene expression is highly induced (250 µM
191 $CuSO_4$), holdfast size and adhesion are restored to WT levels (Fig. 3A-B). At lower level of
192 induction (10 µM $CuSO_4$), both complemented strains produced small holdfasts (Fig. 3A), but
193 failed to form biofilms after 12 h (Fig. 3B). To test if these results were due to altered adhesive
194 properties of these smaller holdfasts, or if their smaller size was not enabling cells to be retained
195 on the surface, we combined the Δ*hfsL* and Δ*hfsG* mutations with an in-frame deletion of the
196 holdfast anchor gene *hfaB*, resulting in mutants that produce holdfasts shed in the medium upon
197 $CuSO_4$ induction (Fig. 3C). We grew exponential cultures of the double-mutants on glass
198 coverslips for 4 h to allow them to attach to the surface. After incubation, the slides were rinsed
199 with dH_2O to remove all cells that are unable to anchor their holdfast to their cell body, resulting
200 in coverslips displaying attached holdfasts and no cells (Fig. 3C). At low level of induction of
201 *hfsL* or *hfsG,* shed holdfasts from *H. baltica* Δ*hfaB* Δ*hfsL* and Δ*hfaB* Δ*hfsG*, though smaller than
202 those from the *H. baltica* Δ*hfaB,* were still able to efficiently bind to glass slide (Fig. 3C). We
203 quantified the number of holdfasts attached at different levels of induction of *hfsL* and *hfsG* (Fig.
204 3D). At low induction, the mutants produced 50% the amount of holdfast compared to WT
205 holdfasts (Fig. 3D). To visualize how cells with small holdfasts interact with glass surface, we
206 performed time-lapse microscopy in a microfluidic device, starting with static conditions, and
207 adding flow after 2 minutes, to allow cells to bind to the surface (Fig. 3E). We observed that at
208 low induction of *hfsL* (10 µM $CuSO_4$), cells efficiently bind to the surface, despite their small
209 holdfasts. However, once flow was added to the microfluidic device, cells detached, showing
210 that the small holdfasts are not sufficient to withstand high shear forces. At high induction of
211 *hfsL* (250 µM $CuSO_4$), cells produced bigger holdfasts and are able to bind to the surface
212 permanently. This result confirms that the smaller holdfasts are still adhesive, but their size is

213 probably not sufficient to allow the cells to efficiently permanently bind to surfaces and form
214 biofilms.
215
216 ***H. baltica* produces large holdfasts by developmental and surface contact stimulation**
217 **pathways.**

218 It was previously shown that WGA interacts with *C. crescentus* and *H. baltica* holdfasts
219 (27, 28). However, side-by-side microscopy imaging using fluorescent WGA suggested that *H.*
220 *baltica* holdfasts might be larger than *C. crescentus* holdfasts (Fig. 4A). To quantify relative
221 holdfast size, we imaged mixed cultures of *H. baltica* and *C. crescentus* simultaneously labeled
222 with fluorescent WGA lectin. We measured the area of fluorescent WGA staining on single cells
223 for each strain (Fig. 4A) and determined that, on average, the fluorescence area is 5 times
224 larger for *H. baltica* holdfasts compared to those of *C. crescentus* (Fig. 4B). Since WGA binds to
225 GlcNAc residues in the holdfast, either *H. baltica* holdfasts are larger than those of *C.*
226 *crescentus* or *H. baltica* and *C. crescentus* holdfasts are similar in size, but *H. baltica* holdfasts
227 contain more GlcNAc residues, yielding an increased fluorescence area from bound WGA. To
228 reliably measure the size of holdfasts, we used Atomic Force Microscopy (AFM) and imaged dry
229 holdfasts deposited on a clean mica surface, free of any straining. Results confirmed that *H.*
230 *baltica* produces larger holdfasts than *C. crescentus*. *H. baltica* holdfasts have a median height
231 of 68 nm, while *C. crescentus* produces holdfasts with median height of 19 nm (Fig. 4C-D), in
232 agreement with previous reports (6, 36).

233 *C. crescentus* can produce holdfast by two distinct pathways, as part of a complex
234 developmental program in a cell cycle regulated manner or upon contact with a surface,
235 independent of the cell cycle (37-40). Some Alphaproteobacteria, such as *Asticaccaulis*
236 *biprosthecum* (38) or *Prosthecomicrobium hirschii* (41) are also able to produce holdfasts via
237 developmental and surface-contacted stimulated pathways, while others, like *Agrobacterium*

238 *tumefaciens*, only produce holdfasts upon contact with a surface (38, 42). To determine how
239 holdfast production is regulated in *H. baltica,* we measured the timing of holdfasts synthesis in
240 the presence or absence of a hard surface. To test whether *H. baltica* holdfast production can
241 be stimulated upon contact with a surface, we performed time-lapse microscopy in a microfluidic
242 device where cells are in close proximity with a glass surface, and we tracked single cells as
243 they reached the surface. We observed holdfast production by including fluorescently labeled
244 WGA in the medium, and we recorded the difference between the time when a cell first reaches
245 the surface and the time when a holdfast is synthesized (Fig. 5A, top panels). We observed that
246 *H. baltica* produces holdfasts within approximately 3 min upon surface contact, (Fig. 5A-B),
247 showing that this species is able to trigger holdfast synthesis upon contact with a surface. To
248 assess cell cycle progression and timing of holdfast synthesis independent of a surface, we
249 tracked single cells and monitored cell differentiation and holdfast synthesis by time-lapse
250 microscopy on soft agarose pads containing fluorescent WGA (Fig 5A, lower panel and 5B). *H.*
251 *baltica* newborn swarmer cells produced holdfast within 15-25 minutes after budding on an
252 agarose pad (Fig. 5A-B), showing that *H. baltica* can produce holdfasts through progression of
253 the cell cycle, as part of a developmental pathway. To determine the timing of holdfast
254 production relative to the cell cycle length, we measured the time required for a newborn
255 swarmer cell to complete its first and second budding divisions on agarose pads (Fig. 5C). *H.*
256 *baltica* swarmer cells complete their first budding within 160 - 200 mins (Fig. 5C), similarly to *C.*
257 *crescentus* production of swarmer cells in PYE complex medium (43), meaning that the holdfast
258 is synthetized within ~1/10 of the cell cycle, similarly to *C. crescentus* (37).

259

260 **_H. baltica_ holdfast contains GlcNAc and galactose monosaccharides and proteins**

261 Holdfasts in diverse Alphaproteobacteria bind to WGA, showing that they contain
262 GlcNAc residues (3). Previous studies using lectin labelling showed that GlcNAc polymers are

263 the main polysaccharide present in *C. crescentus* holdfast, while other Caulobacterales strains
264 may have additional monosaccharides in their holdfasts (10). Indeed, WGA lectin (specific to
265 GlcNAc) and Dolichos Biflorus agglutinin (specific to N- acetylgalactosamine) both bind
266 *Caulobacter henricii* holdfasts (10), while *Caulobacter subvibriodes* holdfasts was shown to
267 interact with Dolichos Biflorus Agglutinin (specific to N- acetylgalactosamine), Concanavalin A
268 (specific to α-mannose) and Ulex Europaeus agglutinin (specific to α-fucose), but not WGA
269 (10).

270 To identify the type of saccharides present in *H. baltica* holdfast, we screened a variety
271 of fluorescent lectins to attempt to label *H. baltica* holdfast (Table 2 and Table S3). Our results
272 indicate that, in addition to binding to WGA, *H. baltica* holdfast also binds to Solanum
273 Tuberosum potato lectin (STL), Lycopersicon Esculentum tomato lectin (LEL), and Datura
274 Stramonium Lectin (DSL1), all lectins specific to GlcNAc residues (Table 2), confirming that *H.*
275 *baltica* holdfasts contain GlcNAc residues. In addition, lectins that specifically recognize α-
276 galactose residues, Griffonia Simplicifolia (GSL1), and Ricinus Communis Agglutinin 1
277 (RCA120), also interact with *H. baltica* holdfasts (Table 2), while not binding to *C. crescentus*
278 holdfasts (Fig. 6A). Interestingly, Soybean Agglutinin lectin (SBA) did not bind to *H. baltica*
279 holdfasts, showing that these holdfasts only contain galactose and no N-acetylgalactosamine
280 residues (GalNAc) (Table 2). These results show that *H. baltica* holdfasts have a different sugar
281 composition than Caulobacter and contain both GlcNAc and galactose residues. To confirm that
282 observed galactose-specific binding was holdfast dependent, we labeled *H. baltica* Δ*hfsA,*
283 Δ*hfsG* (holdfast minus strains) and Δ*hfaB* (holdfast shedding strain) mutants with both WGA and
284 GSL1 lectins. None of the lectins labelled the holdfast deficient Δ*hfsA* and Δ*hfsG* mutants, but
285 they labelled shed holdfast produced by the Δ*hfaB* mutant (Fig. 6A), confirming that *H. baltica*
286 holdfasts contain galactose residues.

287 *C. crescentus* holdfasts have been recently shown to contain peptides and DNA
288 residues (15). To test whether *H. baltica* holdfast contains proteins, we attempted to label
289 putative cysteines in the holdfast using a fluorescent maleimide dye (AF488mal). As for *C.*
290 *crescentus* holdfasts, *H. baltica* holdfasts could be stained with AF488mal, showing that these
291 holdfasts possess molecules with free accessible thiols, suggesting the presence of peptides
292 containing cysteines (Fig. 6B). The staining was holdfast-specific, as AF488mal did not label the
293 holdfast-deficient Δ*hfsA* and Δ*hfsG* mutants (Fig. 6B). It has been shown that in *C. crescentus*,
294 holdfast labeling by AF488mal was specific to holdfasts attached to cells, as shed holdfasts
295 from a holdfast anchor mutant were not labeled, suggesting that the cysteine-containing HfaD in
296 cell-anchored holdfasts is responsible for the labeling of those holdfasts with AF488mal (15). In
297 *H. baltica*, both the anchor proteins HfaB and HfaD contain cysteines. In order to test whether
298 AF488mal interacts with HfaB or HfaD, we stained shed holdfasts produced by a *H. baltica*
299 Δ*hfaB* Δ*hfaD* double mutant and could detect staining (Fig. 6B). This is in stark contrast with *C.*
300 *crescentus* holdfasts that react with AF488mal only when attached to WT cells (15, 44). This
301 result show that the holdfast composition in these two microorganisms is different.

302 To probe for the presence of DNA in *H. baltica* holdfasts, we labeled holdfasts with the
303 fluorescent DNA dye YOYO-1 that binds to double-stranded DNA molecules. As previously
304 reported, *C. crescentus* holdfasts was labeled with YOYO-1 (15). However, YOYO-1 failed to
305 label *H. baltica* holdfasts (Fig. 6C), suggesting that *H. baltica* holdfasts do not contain DNA. It
306 has been previously shown that, in *C. crescentus,* extracellular DNA (eDNA) released during *C.*
307 *crescentus* cell lysis binds specifically to *C. crescentus* holdfasts, preventing adhesion to
308 surfaces and biofilm formation (45), and it has been hypothesized that it could be due to a
309 specific interaction between the DNA present in the holdfast and eDNA (15). We showed above
310 that *H. baltica* holdfasts were devoid of DNA, so we tested whether eDNA could inhibit *H. baltica*
311 binding. We performed short term adhesion assays in the presence of *H. baltica* and *C.*

312 *crescentus* eDNA (Fig. S2A). When *C. crescentus* eDNA is present, the number of *C.*
313 *crescentus* attached to the glass slide after 60 minutes is dramatically decreased, compared to
314 when *H. baltica* eDNA is added and to the no DNA addition control (Fig S2A), confirming
315 previous studies that showed that, in *C. crescentus,* eDNA inhibition was specific for *C.*
316 *crescentus* eDNA (45). However, *H. baltica* adhesion is not impaired by the presence of eDNA,
317 from itself or from *C. crescentus* (Fig. S2A). We also performed long term biofilm assays in the
318 presence of eDNA and showed that *H. baltica* biofilm formation is not impaired by the presence
319 of eDNA in the medium after 24 h of incubation (Fig. S2B).

320 Taken together, we show that *H. baltica* holdfasts are different from *C. crescentus* ones:
321 they are larger, contain GlcNAc, galactose, and peptide residues, but are void of DNA.
322
323 **_H. baltica_ holdfast tolerates high ionic strength**

324 It has been shown that *C. crescentus* holdfasts are very sensitive to ionic strength, as
325 purified holdfast binding efficiency to glass decreased by 50% with addition of 10 mM NaCl (6).
326 *C. crescentus* is a freshwater bacterium and has probably evolved without selective pressure to
327 bind under high ionic strength. This compelled us to investigate how the holdfasts from *H.*
328 *baltica* are affected by ionic strength. We first used NaCl to study the effects of ionic strength on
329 holdfast binding, since it is the most abundant ionic elements in marine water and it has been
330 used in many studies to assess the effect of ionic strength on bacterial adhesins (6, 46-48). We
331 quantified purified holdfast binding to glass at different NaCl concentrations, using fluorescent
332 WGA, and plotted the relative number of holdfasts per field of view bound to glass at different
333 concentrations of NaCl (Fig. 7A-B). Our results confirmed that *C. crescentus* holdfast is very
334 sensitive to NaCl, as only 50% of holdfasts can bind to glass when 10 mM NaCl is added (Fig.
335 7B). However, *H. baltica* holdfast tolerated up to 500 mM NaCl without any effect on surface
336 binding (Fig. 7B). There was a 50% decrease in *H. baltica* holdfast binding at 600 mM (Fig. 7B),

337 showing that *H. baltica* holdfasts are more than 50 times more resistant to NaCl than those of *C.*
338 *crescentus*. *H. baltica* was originally isolated from the Baltic Sea, which has 250 mM NaCl (Fig
339 7B, gray arrow) (26), and at that NaCl concentration, the binding efficiency of *H. baltica*
340 holdfasts is maximal. Interestingly, *H. baltica* holdfasts still bound efficiently at low ionic
341 strength. We observed similar results using different concentrations of $MgSO_4$ (Fig. 7C): *H.*
342 *baltica* holdfasts were 50 times more resistant to $MgSO_4$ than those of *C. crescentus*, showing
343 that the binding inhibition is not specific to NaCl but is rather dependent on ionic strength.
344 Our results show that, in *H. baltica*, initial holdfast binding to glass is not changed for
345 NaCl concentrations up to 500 mM, then drastically decreased to reach around 25% of holdfasts
346 attached at 1 M NaCl (Fig. 7B). To test whether high ionic strength could remove holdfasts
347 previously attached to the glass surface, we first incubated purified holdfasts for 4 h without any
348 salt added, and then added 1M of NaCl for 12 hours to the bound holdfasts (Fig. 7D). Bound
349 holdfasts from *H. baltica* and *C. crescentus* were not dislodged from the glass surface (Fig. 7D
350 and E), indicating that while high ionic strength inhibits holdfast from binding to the surface, it
351 cannot dislodge bound holdfast from a glass surface (Fig. 7E).
352
353 **DISSCUSION**
354 Different bacterial species harbor an adhesive holdfast and use it to attach to surfaces
355 (2, 3, 9, 49, 50). They represent an extremely diverse group in terms of their physiology and the
356 natural environments they inhabit (soil, freshwater, and marine environments). They have
357 evolved the ability to adhere to surfaces with vastly different composition in varying
358 environmental conditions (salinity, pH, temperature, etc.). Holdfast chemical properties have
359 been mainly studied in the model organism *C. crescentus,* a freshwater Caulobacterale (6, 10,
360 13, 15, 18, 28, 32, 36, 51), and little is known about holdfast properties and composition in
361 marine Caulobacterales. In this study, we used *H. baltica* as a model species living in a marine

362 environment and found that this bacterium has a holdfast tailored for adhesion in high salinity
363 conditions. We show that holdfasts in *H. baltica* are different than those of *C. crescentus*: they
364 are larger, have a different chemical composition, and have a high tolerance to ionic strength.
365 The bioinformatics analysis of holdfast genes indicated that the *hfs* and *hfa* loci are
366 highly conserved among Caulobacterales, with few reshufflings of these genes (Fig. 1C). The
367 arrangements of the holdfast genes in the *hfs* and *hfa* loci appears to be ancestral while the
368 relocation of some of the genes is a recent event that could affect their level of expression (52).
369 Through deletion and complementation of important *hfs* and *hfa* genes, we confirmed that
370 holdfast biogenesis and anchoring to the cell body in *H. baltica* use similar genes to those
371 identified in *C. crescentus* (2);(18) (Fig 2).
372 We showed that the two glycosyltransferases *hfsL* and *hfsG,* are essential for holdfast
373 production and regulate the amount of sugar monosaccharides added to holdfast
374 polysaccharides, as cells expressing low levels of these proteins produce smaller holdfasts (Fig.
375 2, 3). Small holdfasts with less polysaccharides binds to glass but not strongly enough to
376 support cells (Fig. 3). This phenomenon could be due to the smaller surface contact area
377 between the small holdfasts being insufficient to resist drag and shear forces during the washing
378 steps of our assays or to a change in holdfast structure or composition due to the lower
379 expression of the glycosyltransferases HfsL and HfsG. More studies on the role of HfsL and
380 HfsG will help us to determine if these enzymes play an important role in specific
381 physicochemical properties of *H. baltica* holdfasts.
382 In *C. crescentus*, the growing holdfast polysaccharide repeat units are modified by the
383 acetyltransferase HfsK (32) and the polysaccharide deacetylase HfsH (21) (Fig. 1A). These two
384 enzymes are not essential for holdfast production in *C. crescentus*, but modify adhesiveness
385 and cohesiveness of the holdfast. Holdfasts produced by Δ*hfsH* or Δ*hfsK* mutants produced
386 thread-like holdfasts with weaker adhesion strength (28, 32). In addition, fully acetylated purified
387 holdfasts from the *C. crescentus* Δ*hfsH* mutant holdfasts were not affected by ionic strength (6),

388 suggesting that holdfast modification can modulate salt tolerance. Our future work will determine
389 how holdfast modification impacts *H. baltica* holdfasts tolerance to high ionic, and the possible
390 role of HfsH and HfsK.

391 The exact composition and structure of holdfast is still unknown in the model organism
392 *C. crescentus*. Lectin binding assays and lysozyme treatment support GlcNAc as one of the
393 important components in holdfasts (10, 36). Treating holdfast with proteinase K and DNase I
394 affects *C. crescentus* holdfast structure and force of adhesion, suggesting that it contains
395 peptide and DNA residues (15). In this work, we identified different components present in *H.*
396 *baltica* holdfasts: these holdfasts contain galactose monosaccharides in addition to GlcNAc (Fig.
397 6A). In the different *hfs* mutants generated in this study, galactose monosaccharides were not
398 detected on the cell pole (Fig. 6A), suggesting that GlcNAc and galactose are produced
399 together or secreted by the same proteins. Shed holdfasts from *H. baltica* Δ*hfaB* contain both
400 GlcNAc and galactose (Fig. 6A), implying that they are both anchored to the cell envelope with
401 the same anchor proteins. *H. baltica* holdfasts are void of DNA, a stark contrast to *C. crescentus*
402 (Fig. 6C). In addition, *H. baltica* holdfasts could be successfully stained with a fluorescent
403 maleimide dye, which suggest the presence of a protein or peptide with a cysteine residue (53).
404 The maleimide dye stains only cells with a holdfast, and interacts with holdfasts without the
405 presence of cells, indicating that the reactive molecules are intrinsic part of *H. baltica* holdfast
406 (Fig. 6B), another notable difference with *C. crescentus* holdfasts where maleimide dye only
407 interacts with holdfasts attached to cells (15). Our results suggest that the two holdfasts from *H.*
408 *baltica* and *C. crescentus* have different composition.
409 Bacterial adhesins have been shown to use electrostatic and hydrophobic interactions to
410 attach to surfaces (6). Electrostatic interactions are impaired in high ionic environment like
411 seawater with 600 mM of NaCl (7). *C. crescentus* holdfast uses both ionic and hydrophobic
412 interactions and its binding is impaired in presence of NaCl in the media (6). We have shown

that *H. baltica* holdfasts tolerate high ionic strength compared to *C. crescentus* (Fig. 7A-C). Marine Caulobacterales face a higher ionic strength environment than the freshwater bacteria, therefore, it is vital that marine Caulobacterales produce holdfasts that are more tolerant to 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 when adding 1 M NaCl (Fig. 7D), suggesting that the binding inhibition at 1 M NaCl takes place during the initial stage of surface interaction, because it has no effect on surface bound holdfasts (Fig. 7D). These results imply that holdfast interacts with surfaces initially using electrostatic interactions, before a permanent molecular bond is formed (6, 54). The differences in ionic tolerance between fresh and marine Caulobacterales indicates that there are significant differences in physicochemical properties between the two types of holdfasts. Holdfast structure and binding properties could depend on the type and the amount of sugars polymerized in the holdfast polysaccharide that are specialized to interact with different surfaces (55).

In conclusion, we have shown that *H. baltica* produces holdfasts with different binding and physicochemical properties compared to *C. crescentus* holdfasts. This could suggest that there are additional holdfast related genes or regulators that have not been identified. A careful genetic screen in *H. baltica* will provide more insights about holdfast production and the underlying mechanisms yielding to an enhanced adhesion at high ionic strength. The molecular mechanism by which *H. baltica* and other marine Caulobacterales overcome the effect of ionic strength on holdfast binding will be our next focus.

MATERIALS AND METHODS

Identification of orthologous holdfast genes and phylogenetic analysis.

C. crescentus holdfast genes were used to find bi-directional best hits (BBH) on Caulobacterales genomes. The putative genes were selected for E^{-} value > 10^{-4} and sequence identify > 30%. The phylogenic tree was built using 16S rRNA sequences of the selected Caulobacterales. Sequences were aligned using MUSCLE software (46). The aligned sequences were used to construct the maximum likelihood phylogeny using the MEGA6 software (58). The LG+G+I models and analysis of 1000 bootstraps were used to generate the nodes values for each clade.

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 reference 2216), except when studying the effect of ionic strength on holdfast binding where they were grown in Peptone Yeast Extract (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, antibiotics were added to the following concentrations: kanamycin (Kan) 5 µg/ml in liquid and 20 µg/ml on agarose plates. *H. baltica* strains with copper inducible promoter were grown in marine broth supplemented with 0-250 µM of $CuSO_4$. *E. coli* strains were grown in Luria-Bertani medium (LB) at 37 ˚C with no antibiotics or with 30 µg/ml of Kan in liquid or 25 µg/ml on agarose plate when needed.

Strains construction.

All the plasmids and primers used in this study are listed on Table S1 and S2 respectively. In-frame deletion mutants were obtained by double homologous recombination as

461 previously described (59), using suicide plasmids transformed into the *H. baltica* host strains by
462 mating or electroporation (60). Briefly, genomic DNA was used as the template to PCR-amplify
463 500 bp fragments from upstream and downstream regions of the gene to be deleted. pNPTS139
464 plasmid was cut using EcoRV-HF endonuclease from New England Biolabs. The primers used
465 to amplify 500 bp upstream and downstream of the gene were designed to have overlapping 25
466 bp for isothermal assembly (61) using the New England Biolabs (NEB) NEBuilder tools for
467 Gibson assembly into plasmid pNPTS139. Then pNPTS139-based constructs were transformed
468 into α-select *E. coli* strain and introduced in the host *H. baltica* by mating or electroporation (62).
469 The two-step selection for homologous recombination was carried out using sucrose resistance
470 and kanamycin sensitivity (63).
471 For gene complementation, the pMR10 plasmid was cut with EcoRV-HF and 500 bp of
472 the promoter and the gene were ligated into plasmid pMR10 using NEBuilder tools. The pMR10-
473 based constructs were transformed into α-select *E. coli* strain and introduced in the host *H.*
474 *baltica* by mating or electroporation, followed by Kan selection. The plasmid constructs and
475 mutants were confirmed by sequencing.
476
477 **Holdfast labeling using fluorescently labeled lectins**
478 Alexa Fluor (AF) conjugated lectins (Vector Labs, Table 2 and Table S3) were added to
479 100 µl of exponential culture to a final concentration of 0.5 µg/ml and incubated at room
480 temperature for 5 min. 3 µl of the labeled culture was spotted on glass cover slide and covered
481 with 1.5 % (w/v) SeaKem LE agarose (Lonza) pad in water and visualized by epifluorescence
482 microscopy. Holdfasts were imaged by epifluorescence microscopy using an inverted Nikon Ti-
483 E microscope with a Plan Apo 60X objective, a GFP/DsRed filter cube, an Andor iXon3 DU885
484 EM CCD camera and Nikon NIS Elements imaging software with 200 ms exposure time.
485 Images were processed in ImageJ (64).

Short-term and biofilm binding assays

This assay was performed as previously described (28) with the following modification. For short-term binding, exponential cultures (OD_{600} = 0.6 - 0.8) were diluted to OD_{600} = 0.4 in fresh marine broth, added into 24-well plate (1 ml per well), and incubated shaking (100 rpm) at room temperature for 4 h. For biofilm assays, overnight cultures were diluted to OD_{600} = 0.10, added to 24-well plate (1 ml per well), and incubated at room temperature for 12 hours with shaking (100 rpm). In both set-ups, OD_{600} were measured before the wells were rinsed with distilled H_2O to remove non-attached bacteria, stained using 0.1% crystal violet (CV) and rinsed again with dH_2O to remove excess CV. The CV was dissolved into 10% (v/v) acetic acid and quantified by measuring the absorbance at 600 nm (A_{600}). The biofilm formation was normalized to A_{600} / OD_{600} and expressed as a percentage of WT.

***hfsL* and *hfsG* expression using copper inducible promoter.**

Strains bearing copper inducible plasmids were inoculated from freshly grown colonies into 5 ml marine broth containing 5 µg/ml Kan and incubated shaking (200 rpm) at 30˚C overnight. Overnight cultures were diluted in the same culture medium to OD_{600} = 0.10 and incubated until OD_{600} = 0.4 was reached. When needed, copper sulfate dissolved in marine broth was added to a final concentration of 0-250 µM. The induced cultures and controls were added to 24-well plate (1 ml per well) and incubated shaking (100rpm) at room temperature for 4-8 h. Then, OD_{600} were measured before the wells were rinsed with distilled H_2O to remove non-attached bacteria, stained using 0.1% crystal violet (CV) and rinsed again with dH_2O to remove excess CV. The CV was dissolved into 10% (v/v) acetic acid and quantified by measuring the absorbance at 600 nm (A_{600}). The biofilm formation was normalized to A_{600} / OD_{600} and expressed as a percentage of WT.

511

512 **Visualization of holdfasts attached on a glass surface**

513 Visualization of holdfast binding to glass surfaces were performed as described
514 previously in (28) with the following modification. *H. baltica and C. crescentus* strains grown to
515 exponential phase (OD_{600} = 0.2 – 0.6) were incubated on washed glass coverslips at room
516 temperature in a saturated humidity chamber for 4 - 8 h. After incubation, the slides were rinsed
517 with dH_2O to remove unbound cells, and holdfasts were labelled using 50 µl of fluorescent Alexa
518 Fluor (AF488 or AF594) conjugated lectins (Molecular Probes or Vector Labs, Table 2) at a final
519 concentration of 0.5 µg/ml. Then, slides were rinsed with dH_2O and topped with a glass
520 coverslip. Holdfasts were imaged by epifluorescence microscopy using an inverted Nikon Ti-E
521 microscope with a Plan Apo 60X objective, a GFP/DsRed filter cube, an Andor iXon3 DU885
522 EM CCD camera and Nikon NIS Elements imaging software with 200 ms exposure time.
523 Images were processed in ImageJ (64).

524

525 **Atomic Force Microscopy (AFM)**

526 AFM imaging was performed using the tapping mode on a Cypher AFM (Asylum
527 Research) at 20°C, as described previously (6, 19) with the following modifications. Exponential
528 phase grown *H. baltica* Δ*hfaB* and *C. crescentus* Δ*hfaB* were diluted and spotted on a freshly
529 cleaved mica. Samples were grown overnight at room temperature in a humid chamber. The
530 samples were then rinsed with sterile dH_2O to remove unbound cells and debris, and air-dried.
531 AFM topographic images of dried holdfasts attached to the mica surface were obtained using a
532 silicon Olympus AC160TS cantilever (Resonance frequency = 300 kHz, Spring constant = 26
533 N/m). 40 images of 4 independent replicates were obtained. Holdfast height was determined
534 using the built-in image analysis function of the Igor Pro/Asylum Research AFM software.

535

536 **Holdfast synthesis timing by time-lapse microscopy on agarose pads.**

537 *H. baltica* holdfast synthesis timing were observed in live cells on agarose pads by time-
538 lapse microscopy as described previously (39) with some modifications. A 1 µl aliquot of
539 exponential-phase cells (OD_{600} = 0.4 – 0.8) was placed on top of a pad containing 0.8% agarose
540 in marine broth with 0.5 µg/ml AF-WGA 488. A coverslip was placed on top of the agarose pad
541 and sealed with VALAP (Vaseline, lanolin and paraffin wax). Time-lapse microscopy images
542 were taken every 2 min for 4 h using an inverted Nikon Ti-E microscope and a Plan Apo 60X
543 objective, a GFP/DsRed filter cube, and an Andor iXon3 DU885 EM CCD camera. Time-lapse
544 movies were visualized in ImageJ (64) to manually assess the timing of a swarmer cell
545 producing holdfast (lectin detection) after budding. The time difference between holdfast
546 synthesis and budding was determined using MicrobeJ (65).
547
548 **Holdfast synthesis timing by time-lapse microscopy on microfluidic device.**
549 This experiment was performed as previously described (40) with the following
550 modifications. Cell cultures were grown to mid-exponential phase (OD_{600} = 0.4 - 0.6) and 200 µl
551 of culture was diluted into 800 µl fresh marine broth in the presence of 0.5 µg/ml AF-WGA 488
552 for holdfast labeling. One ml of the cell culture was flushed into a microfluidic device containing
553 a 10 µm high linear chamber fabricated in PDMS (Polydimethylsiloxane) as described
554 previously (39). After injection of the cells into the microfluidic chamber, the flow rate was
555 adjusted so that attachment could be observed under static conditions or low flow rate.
556 Time-lapse microscopy was performed using an inverted Nikon Ti-E microscope and a
557 Plan Apo 60X objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera and
558 Nikon NIS Elements imaging software. Time-lapse videos were collected for strains over a
559 period of 3 h at 20-second intervals. Cell attachment was detected at the glass-liquid interface
560 within the microfluidic chamber using phase contrast microscopy while holdfast synthesis was
561 detected using fluorescence microscopy. Cells that hit the surface and attached permanently via
562 their holdfast during this 3 h period were analyzed for the timing of holdfast synthesis. The time

563 difference between holdfast synthesis and cell-surface contact was determined using MicrobeJ
564 (65) and define as holdfast delay. Cells that were present on the surface at the start of the time-
565 lapse experiment were not analyzed.
566
567 **Holdfast labeling using fluorescently labeled Maleimide and YOYO-1**
568 Alexa Fluor (AF-mal488) conjugated Maleimide C_5 (ThermoFisher Scientific) were added
569 to 100 µl of exponential culture to a final concentration of 0.5 µg/ml and incubated at room
570 temperature for 5 mins. Similarly, YOYO-1 (fluorescent DNA stain, Molecular Probes) was
571 added to 100ul of exponential culture to a final concentration of 0.5 µg/ml and incubated at room
572 temperature for 5 mins. 3 µl of the labeled culture was spotted on glass cover slide and covered
573 with 1.5 % (w/v) agarose pad in water and visualized by epifluorescence microscopy. Holdfasts
574 were imaged by epifluorescence microscopy using an inverted Nikon Ti-E microscope with a
575 Plan Apo 60X objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD camera and
576 Nikon NIS Elements imaging software with 200 ms exposure time. Images were processed in
577 ImageJ (64).
578
579 **Effect of ionic strength on holdfast binding.**
580 Purified holdfasts attached to a surface in different ionic strength were visualized as
581 described previously (Berne *et al*., 2013), with few modifications. Briefly, *H. baltica* Δ*hfaB* and *C.*
582 *crescentus* Δ*hfaB* cells were grown to late exponential phase (OD_{600} = 0.6 – 0.8) in PYE + 1.5%
583 NaCl, and plain PYE respectively. The cells were pelleted by centrifugation for 30 min at 4,000 x
584 *g* and resuspended in PYE and incubated for 2 h at 30 °C. Then, the cells were again pelleted
585 by centrifugation and 100 µl of supernatant, containing free holdfasts shed by the cells, were
586 mixed with 100 µl of NaCl in PYE to make a final concentration of 0 - 1000 mM of NaCl. 50 µl of
587 the mixture was incubated on washed glass coverslips at room temperature in a saturated
588 humidity chamber for 4 - 12 h. After incubation, the slides were rinsed with dH_2O to remove

589 unbound material, and labelled holdfast were visualized with Alexa Fluor lectins (Vector labs).
590 Holdfasts were imaged by epifluorescence microscopy using an inverted Nikon Ti-E microscope
591 with a Plan Apo 60X objective, a GFP/DsRed filter cube, an Andor iXon3 DU885 EM CCD
592 camera and Nikon NIS Elements imaging software with 200 ms exposure time. Images were
593 processed in ImageJ (64). The number of holdfasts bound per field of view was quantified using
594 MicrobeJ (65).
595
596 **β-galactosidase assays to assess copper inducible *copA* promoter activity**.
597 This assay was performed as previously described with few modification (44). Strains
598 bearing plasmids with *lacZ* gene controlled by copper inducible promoter *copAB* were
599 inoculated from freshly grown colonies into 5 ml marine broth containing 5 µg/ml Kan and
600 incubated at 30˚C overnight. Overnight cultures were diluted in the same culture medium to
601 OD_{600} = 0.10 and incubated until an OD_{600} = 0.4 was reached, where copper sulfate dissolved in
602 marine broth was added to a final concentration of 0 - 250 µM. The induced cultures and
603 controls were incubated for 2 - 4 h at 30 ˚C. β-galactosidase activity was measured
604 colorimetrically as described previously (66). Briefly, 200 µl of culture was mixed with 600 µl Z
605 buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-
606 mercaptoethanol). Cells were then permeabilized using 50 µl chloroform and 25 µl 0.1% SDS.
607 200 µl of substrate *o*-nitrophenyl- β-D-galactoside (4 mg/ml) was added to the permeabilized
608 cells. Upon development of a yellow color, the reaction was stopped by raising the pH to 11 with
609 addition of 400µl of 1M Na_2CO_3. Absorbance at 420 nm (A_{420}) was determined and the Miller
610 Units of β-galactosidase activity were calculated as $(A_{420})(1000)/(OD_{600})(t)(v)$ where t is the time
611 in minutes and v is the volume of culture used in the assay in mL.
612
613 **Growth measurements**.

614 Impact of $CuSO_4$ on *H. baltica* growth was measured using 24-well plates. 1 ml of cultures
615 (Starting OD_{600} = 0.05) were incubated for 12 h at 30˚C, using marine Broth and various $CuSO_4$
616 concentrations. OD_{600} were recorded after overnight incubation, to determine the growth yield for
617 the different $CuSO_4$ concentrations. Growth curves using 0 or 500 µM $CuSO_4$ were recorded
618 every 30 minutes for 20 h. All OD_{600} were recorded using a Biotek Synergy HT.
619

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786 Biology.

787 **Table 1: Genes involved in holdfast synthesis, modification and anchoring**

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

788

789

790 **Table 2: Lectin binding assays**

Lectin	Specificity	*H. baltica* holdfast	*C. crescentus* holdfast
Wheat Germ Agglutinin	GlcNAc	√	√
Lycopersicon Esculentum Tomato Lectin	GlcNAc 1-4	√	√*
Datura Stramonium Lectin	GlcNAc 1-4	√*	-
Solanum Tuberosum Potato Lectin	GlcNAc , prefers trimers and tetramers	√	√*
Ricinus Communis Agglutinin	Galactose	√	-
Griffonia Simplicifolia Lectin 1	α-GalNAc, α-galactose	√	-
Soybean Agglutinin	α-GalNAc	-	-

791

792 √ Fluorescent signal detected

793 - No fluorescent signal detected

794 * Binding is enhanced on rosettes but weaker signals on single cells.

795

796 **Table S1: Strains and Plasmids used in this study**

Strain or Plasmid	Description and or genotype	Reference or source
E. coli		
α select	*deoR endA1 relA1 gyrA96 hsdR17($r_K^-m_K^+$) supE44 thi-1 Δ(lacZYA-argFV169) Φ80δlacZ*ΔM15 F^-	Bioline
YB8430	α select /pNPTS139Δ*hfsA*	This study
YB8431	α select /pNPTS139Δ*hfsL*	This study
YB8432	α select /pNPTS139Δ*hfaB*	This study
YB8439	α select /pNPTS139Δ*hfaD*	This study
YB8440	α select /pNPTS139Δ*hfsD*	This study
YB172	α select /pNPTS139Δ*hfsG*	This study
YB8441	α select/ pMR10:*hfsA*	This study
YB8442	α select/ pMR10:P*hfa-hfaB*	This study
YB8443	α select/ pMR10:P*hfsE-hfsL*	This study
YB8429	α select/ pMR10:P*cu-hfsL*	This study
YB8433	α select/pMR10:P*hfaA-hfaD*	This study
YB8436	α select/ pMR10:*hfsD*	This study
YB8437	α select/ pMR10:Pcu-*lacZ*	This study
YB173	α select/ pMR10:Pcu-*hfsG*	This study
C. Crescentus		
YB135	Wild-type strain CB15	(8)
YB4251	CB15 Δ*hfaB*	(24)
H. baltica		
YB5842	IFAM 1418^T Wild-type strain	(26)
YB8404	YB5842 Δ*hfsA*	This study
YB8405	YB5842 Δ*hfL*	This study
YB8406	YB5842 Δ*hfaB*	This study
YB210	YB5842 Δ*hfaB* Δ*hfaD*	This study
YB8409	YB5842 Δ*hfsA* /pMR10:*hfsA*	This study
YB8410	YB5842 Δ*hfaB* /pMR10:P*hfa-hfaB*	This study
YB8414	YB5842 Δ*hfsL* /pMR10:P*hfsE-hfsL*	This study
YB8417	YB5842 Δ*hfsG* Δ*hfaB* /pMR10:P*cu-hfsG*	This study
YB8418	YB5842 Δ*hfsL* Δ*hfaB* /pMR10:P*cu-hfsL*	This study
YB8424	YB5842 Δ*hfsL* /pMR10:P*cu-hfsL*	This study
YB8425	YB5842 Δ*hfaD*	This study
YB8426	YB5842 Δ*hfaD* /pMR10:P*hfaA-hfaD*	This study
YB8427	YB5842 ΔhfsD	This study
YB8434	YB5842 ΔhfsD /pMR10:*hfsD*	This study
YB8438	YB5842 pMR10:Pcu-*lacZ*	This study
YB173	YB5842 Δ*hfsG*	This study
YB174	YB5842 pMR10:Pcu-*hfsG*	This study
Plasmids		
pNPTS139	pLitmus 39 derivative, *ori*T, *sac*B, Kan^r	M.R.K Alley
pNPTS139Δ*hfsA*	pNPTS139 containing 500 bp fragments upstream and downstream of *hfsA*	This study
pNPTS139Δ*hfsL*	pNPTS139 containing 500 bp fragments upstream and downstream of *hfsL*	This study
pNPTS139Δ*hfaB*	pNPTS139 containing 500 bp fragments upstream and downstream of *hfaB*	This study
pNPTS139Δ*hfaD*	pNPTS139 containing 500 bp fragments upstream and downstream of *hfaD*	This study
pNPTS139Δ*hfsD*	pNPTS139 containing 500 bp fragments upstream and downstream of *hfsD*	This study
pNPTS139Δ*hfsG*	pNPTS139 containing 500 bp fragments upstream and downstream of *hfsG*	This study
pMR10	Mini-RK2 cloning vector; RK2 replication and stabilization functions	R. Roberts and C. Mohr
pMR10:*hfsA*	pMR10 containing *hfsA* gene with its native promoter	This study
pMR10:P*hfa-hfaB*	pMR10 containing native *hfaA* promoter and the *hfaB* gene	This study
pMR10:P*hfsE-hfsL*	pMR10 containing native *hfsE* promoter and the *hfsL* gene	This study
pMR10:P*cu-hfsL*	pMR10 containing copper inducible promoter of *CopA* and the *hfsL* gene	This study
pMR10:P*hfaA-hfaD*	pMR10 containing native *hfaA* promoter and the *hfaD* gene	This study
pMR10:*hfsD*	pMR10 containing *hfsD* gene with its native promoter	This study
pMR10:Pcu-*lacZ*	pMR10 containing copper inducible promoter of *CopA* and the *lacZ* gene	This study
pMR10:Pcu-*hfsG*	pMR10 containing copper inducible promoter of *CopA* and the *hfsG* gene	This study

Table S2: Primers used in this study

Primers	Sequence (5'→3')	Description
Hb*hfsA*UpF	GCGAATTCTGGATCCACGATGAAATACGCCCGGATTATTG	5' region for deletion of *hfsA*
Hb*hfsA*FR	ATACTTAGTCATTCTGATTCTGCTTTATCTAAAC	
Hb*hfsA*UpR	CAGAAAGCTTCCTGCAGGATTAATTTAGTATCCGCCACAC	3' region for deletion of *hfsA*
Hb*hfsA*RR	GAATCAGAATGACTAAGTATTTGTTATTTAATTAAAAAATATACTTTTC	
Hb*hfsGT*_pUpF	GCCAAGCTTCTCTGCAGGATCAGTATTGTTATTCCAACATTTCG	5' region for deletion of *hfsL*
Hb*hfsGT*_UpR	GTGAGTTTGGGTTGAATGCGATCCAAATC	
Hb*hfsGT*_DwF	CGCATTCAACCCAAACTCACAAACTGAG	3' region for deletion of *hfsL*
Hb*hfsGT*_pDwR	GCGAATTCGTGGATCCAGATTTGGTCCAGCTCATAACG	
Hb*hfaB*UpF	CGCGTTCGGCCGTGCTAGCGGATCATTGCTTATTCCCG	5' region for deletion of *hfaB*
Hb*hfaB*FRev	TCGCCAATTATTGCGAATTGGGCTAGTC	
Hb*hfaB*UpR	GCAGGATATCGTGGATCCAGGAAATATCGTTGACACTGG	3' region for deletion of *hfaB*
Hb*hfaB*RRev	CAATTCGCAATAATTGGCGATAAACTTCGC	
p*hfaD*upF	GTGCTAGCGAATTCTGGATCCACGATGTCTTGTCGAAACAGAATCTCTGGAAG	5' region for deletion of *hfaD*
*hfaD*upR	CTAAGTTTCTATATGTATATTGAGAACTTGGTGTCTGAGACCTTTTAGATAGGC	
*hfaD*dwF	GCCTATCTAAAAGGTCTCAGACACCAAGTTCTCAATATACATATAGAAACTTAG	3' region for deletion of *hfaD*
p*hfaD*dwR	GGCGCCAGAAAGCTTCCTGCAGGATATAGTGATGCAATGTTCGATGGTGG	
*hfsD*upF	GTGCTAGCGAATTCTGGATCCACGATTTTCTGCTATCTCTTGGGCAATTTTAG	5' region for deletion of *hfsD*
*hfsD*upR	CTAGTGTTTAGTTCAGCAATCTGAGGGTGCTTTCTTAATGCATCCGTTTTG	
*hfsD*dwF	CCAAAACGGATGCATTAAGAAAGCACCCTCAGATTGCTGAACTAAACACTAG	3' region for deletion of *hfsD*
*hfsD*dwR	GGCGCCAGAAAGCTTCCTGCAGGATACAGTAAAAGAAAATTCATGTACAAC	
*hfsA*_upF	ACGCCAAGCTTCCATGGGATGAAATACGCCCGGATTATTG	Complementation of *hfsA*
*hfsA*_DwpR	GCTCTGCAGGAGATCTCGATTAATTTAGTATCCGCCACAC	
*hfaB*_upF	ACGCCAAGCTTCCATGGGATAATTGCGCCATTGTG	Complementation of *hfaB*
*hfaB*_DwpR	GCTCTGCAGGAGATCTCGATGAAATATCGTTGACACTGGC	
P*hfsE_hfsL*upF	CCATGATTACGCCAAGCTTCCATGGGATGGCCATACAAATATAAGCGGTGCTC	Complementation of *hfsL*
P*hfsE_hfsL*upR	CAATACTGACTTTTACGGATTGGTTCATTCACGAAGAACACAGAGTGTCTCC	using *hfsE* promoter
P*hfsE_hfsL*dwF	GGAGACACTCTGTGTTCTTCGTGAATGAACCAATCCGTAAAAGTCAGTATTG	
P*hfsE_hfsL*dwR	CTAGAGCTCTGCAGGAGATCTCGATTTAAGTTGCGCTTTTGATAACTTTTTTG	
Pcu_*hfsL*upF	CTAGAGCTCTGCAGGAGATCTCGATTATACACGGATCGCACGCC	Complementation of *hfsL*
Pcu_*hfsL*upR	GGGTGTGTAATGCCAATCAATCATGATGTTCTCCTTCTTGCGTTGGAC	using copper promoter
Pcu_*hfsL*dwF	GTCCAACGCAAGAAGGAGAACATCATGATTGATTGGCATTACACACCC	
Pcu_*hfsL*dwR	CCATGATTACGCCAAGCTTCCATGGGATTTAAGTTGCGCTTTTGATAACTTTTTTG	
p*hfaABD*F	CCATGATTACGCCAAGCTTCCATGGGATCGAGACGAAAACATGAACAGTTTCAC	Complementation of *hfaB*
p*hfaABD*F	CTAGAGCTCTGCAGGAGATCTCGATCAGACAAACAGTTAGAAGAATTTAGAAATC	
comp*hfsD*upF	CCATGATTACGCCAAGCTTCCATGGGATTTTCTGCTATCTCTTGGGCAATTTTAG	Complementation of *hfsD*
comp*hfsD*dwR	CTAGAGCTCTGCAGGAGATCTCGATTTAGAAGGCGTTGTCTTTTAGGTTG	
Cu*lacZ*upF	CTAGAGCTCTGCAGGAGATCTCGATTATACACGGATCGCACGCC	Expression of *lacZ* under
Cu*lacZ*upR	CCCAGTCACGACGTTGTAAAACGACCATGATGTTCTCCTTCTTGCGTTGGACG	copper inducible promoter
Cu*lacZ*dwF	CGTCCAACGCAAGAAGGAGAACATCATGGTCGTTTTACAACGTCGTGACTGGG	
Cu*lacZ*dwR	GATTACGCCAAGCTTCCATGGGATCGGTGGCGGCCGCTCTAGAAC	
*hfsG*upF	GTGCTAGCGAATTCTGGATCCACGATGGTTTTAACAATCAGATTATTCGTGTC	5' region for deletion of *hfsG*
*hfsG*upR	CGTTTTAATTTGGCGGGAAGGGTACATTGGATGCCTAGCGCTGTGTTTTTG	
*hfsG*dwF	CAAAAACACAGCGCTAGGCATCCAATGTACCCTTCCCGCCAAATTAAAACG	3' region for deletion of *hfsG*
*hfsG*dwR	GGCGCCAGAAAGCTTCCTGCAGGATCAACATTAATTCCGGGAAGAATACC	
Pcu_*hfsG*upF	GATTACGCCAAGCTTCCATGGGATATCAATCATGAAGAGCCTCCGCATATATG	Complementation of *hfsG*
Pcu_*hfsG*upR	CGTCCAACGCAAGAAGGAGAACATCATGAACACAACGCCCCAACTTAGCG	using copper promoter
Pcu_*hfsG*dwF	CGCTAAGTTGGGGCGTTGTGTTCATGATGTTCTCCTTCTTGCGTTGGACG	
Pcu_*hfsG*dwR	CTAGAGCTCTGCAGGAGATCTCGATTATACACGGATCGCACGCCTGACAATG	

Table S3: Lectin binding assays for all the lectins used.

Lectin	Specificity	*H. baltica holdfast*	*C. crescentus holdfast*
Wheat Germ Agglutinin	GlcNAc, sialic acid	√	√
Succinylated Wheat Germ Agglutinin	GlcNAc	√	√
Lycopersicon Esculentum Tomato	GlcNAc 1-4	√	√*
Datura Stramonium Lectin	GlcNAc 1-4	√*	-
Solanum Tuberosum Potato Lectin	GlcNAc, prefers trimers and tetramers	√	√*
Ricinus Communis Agglutinin	Galactose	√	-
Griffonia Simplicifolia Lectin 1	α-GalNAc, α-galactose	√	-
Soybean Agglutinin	α-GalNAc	-	-
Concanavalin A	α-linked mannose	-	-
Dolichos Biflorus Agglutinin	α-linked acetylgalactosamine	-	-
Peanut Agglutinin	Galactosyl β-1,3 N-acetylgalactosamine	-	-
Soybean Agglutinin	α or β acetylgalactosamine	-	-
Ulex Europaeus Agglutinin 1	N- acetylgalactosamine, sialic acid or chitobiose	-	-
Len Culinaris Agglutinin	α-linked mannose	-	-
Pisum Sativum Agglutinin	α-linked mannose, fucose or N-acetylchitobiose	-	-
Erythrina Cristagalli Lectin	Galactose, prefers Galactosyl β-1,4 N- acetylgalactosamine	-	-
Jacalin	Galactosyl β-1,3 N-acetylgalactosamine	-	-
Griffinia Simplicifolia Lectin 2	α or β acetylgalactosamine	-	-
Vicia Villosa Lectin	α or β terminal N-acetylgalactosamine	-	-

√ Fluorescent signal detected

- No fluorescent signal detected

* Binding is enhanced on rosettes but weaker signals on single cells.

803 **FIGURE LEGEND.**

804 **Figure 1: Organization of the holdfast gene cluster in *H. baltica***

805 **A.** Schematic of holdfast synthesis, modification, secretion, and anchor machineries. Holdfast
806 polysaccharide synthesis is initiated by glycosyltransferase HfsE, which transfers activated
807 sugar precursors in the cytoplasm to a lipid carrier. Three glycosyltransferases HfsJ, HfsG and
808 HfsL add different sugars to the growing polysaccharide. The acetyltransferase HfsK and the
809 deacetylase HfsH modify one or more sugar residue and then a flippase HfsF transports the
810 lipid carrier into periplasm. Repeat units are polymerized by polymerases HfsC and HfsI. The
811 polysaccharide is exported to the outside of the cell through the HfsA, HfsB, HfsD complex. The
812 exported polysaccharide is then anchored to the cell body by secreted protein HfaA, HfaB and
813 HfaD. The different colors of hexagons represent different sugars. **B.** Diagrams of *C. crescentus*
814 and *H. baltica* dimorphic cell cycles. A motile swarmer cell differentiates into stalked cell by
815 shedding its flagellum and synthesizing holdfast at the same cell pole. *C. crescentus* stalked cell
816 divide asymmetrical to produce a motile swarmer and a stalked cell (top panel) and *H. baltica*
817 reproduces by budding a motile swarmer off the stalk (bottom panel). **C.** Maximum likelihood
818 phylogeny inferred from 16S rRNA sequences of selected freshwater and marine members of
819 Caulobacterales. Node values represent clade frequency of 1000 bootstraps. The genes were
820 identified using reciprocal best hit analysis on fully sequenced Caulobacterales genomes. Solid
821 gene symbols indicate genes within the *hfs* or *hfa* loci while striped symbols indicate the genes
822 translocated from these loci to a different location in the genome. Empty boxes indicate absent
823 or missing genes. Blue represents synthesis genes, turquoise represents modification genes,
824 red represents polysaccharide export genes and navy blue represents anchor genes.
825
826 **Figure 2: Role of the *hfs* and *hfa* genes in *H. baltica* holdfast production.**
827 **A.** Representative images showing merged phase and fluorescence channels of different *H.*
828 *baltica* WT and mutant strains with holdfast labeled with WGA-AF488 (green): *H. baltica* holdfast

anchor mutants Δ*hfaB* and Δ*hfaD,* export mutants Δ*hfsA* and Δ*hfsD,* and synthesis mutants Δ*hfsG* and Δ*hfsL.* **B.** Quantification of biofilm using crystal violet assay after 12 hours for *hfs* and *hfa H. baltica* mutants. Data are expressed as an average of 5 independent replicates and the error bars represent the standard error.

Figure 3: Effect of modulating *hfsL* and *hfsG* expression in *H. baltica* holdfast properties. **A.** Representative images showing merged phase and fluorescence channels of *H. baltica* WT, Δ*hfsL* and Δ*hfsG* mutants complemented with copper inducible promoter constructs and grown in marine broth with 0 μM,10 μM and 250 μM $CuSO_4$. Holdfast is labeled with WGA-AF488. **B.** Biofilm quantification after 12 h using crystal violet assay of Δ*hfsL* and Δ*hfsG* mutants and complementations under copper inducible promoter in marine broth supplemented with 0 μM, 10 μM and 250 μM $CuSO_4$. Data are expressed as an average of 6 independent replicates and the error bars represent the standard error. **C.** Images of WGA-AF488 labeled *H. baltica* Δ*hfaB, H. baltica* Δ*hfaB* Δ*hfsL* pMR10:P_{cu}*hfsL*, *H. baltica* Δ*hfaB* Δ*hfsG* pMR10:P_{cu}*hfsG* shed holdfasts bound to glass slide. Cells were grown in marine broth with 0 μM, 10 μM and 250 μM $CuSO_4$ induction for 4 hrs. **D.** Percentage of holdfasts bound to glass slide per field of view at different $CuSO_4$ induction measured in (C). Data are expressed as an average of 5 independent replicates and the error bars represent the standard error. **E.** Time-lapse montage of a *H. baltica* Δ*hfsL* pMR10:P_{cu}*hfsL* induced with 10 μM (upper panels) and 250 μM (lower panels) $CuSO_4$ in a microfluidic device with initially no flow and then low flow introduced to the microfluidic device. Arrows represent time when no flow (first 120 seconds) and flow (later times) was applied to the device.

Figure 4: *H. baltica* produces large holdfasts

853 **A.** Images of *H. baltica, C. crescentus* and mixed culture with holdfasts labeled with WGA-
854 AF488 (green). **B.** Quantification of holdfast size based on fluorescent area covered by WGA-
855 AF488 collected in (A). Data on the box and whiskers plots represent 5 independent replicates
856 of 200 holdfasts from each strain. **C** AFM images of dry shed holdfasts from *H. baltica* Δ*hfaB*
857 and *C. crescentus* Δ*hfaB* deposited on a mica surface. The colors on the scale represent the
858 height of the holdfast relative to the surface. **D.** Box and whiskers plots of holdfast height
859 distribution from the AFM images collected in (C). More than 500 holdfasts were measured in 10
860 independent images.
861
862 **Figure 5: *H. baltica* produces holdfasts via developmental pathway and upon contact**
863 **with a surface.**
864 **A.** Montages of *H. baltica* holdfast synthesis by a newly budded swarmer cell on a glass surface
865 on a microfluidic device (holdfast production after surface contact, top panel), and on soft
866 agarose pads (holdfast production after cell division, bottom panel). Holdfasts are labeled with
867 WGA-AF488 (green). Images were acquired every 20 sec (top panel) and 2 min (bottom panel),
868 and holdfast synthesis timing was processed using MicrobeJ. The arrow indicates the time it
869 takes for holdfast to be detected after surface contact. **B.** Box and whisker plots representing
870 the quantification of *H. baltica* holdfast timing via surface contact stimulation and developmental
871 pathway. Data for *C. crescentus* holdfast synthesis timing were extracted from (2). Total number
872 of cells analyzed is 100 for each set up. **C.** Time-lapse montage of a *H. baltica* swarmer cell
873 differentiating into a budding stalked cell on agarose pad containing WGA-AF488 to
874 label holdfast. Images were collected every 5 min for 3 h. The arrow indicates the time it
875 takes for holdfast to be detected after cell division.
876

877 **Figure 6: *H. baltica* holdfast contains GlcNAc and galactose monosaccharides, and**
878 **proteins.**
879 **(A-C)** Representative images showing merged phase and fluorescence channels on the left and
880 fluorescence channels alone on the middle and right. **A.** *H. baltica* and *C. crescentus* holdfasts
881 were co-labeled with WGA-AF594 (GlcNAc) and GSL1-AF488 (galactose) lectins, to stain
882 polysaccharides. **B** *H. baltica* and *C. crescentus* holdfasts were co-labeled with WGA-AF594
883 (GlcNAc) lectin and AF488mal, to stain peptides. **C.** *H. baltica* and *C. crescentus* holdfasts were
884 co-labeled with WGA-AF594 (GlcNAc) lectin and YOYO-1-AF488, to stain DNA.
885
886 **Figure 7: *H. baltica* holdfast tolerates higher ionic strength.**
887 **A.** Images of WGA-AF488 labeled *H. baltica* Δ*hfaB* and *C. crescentus* Δ*hfaB* shed holdfasts
888 bound to glass slide, incubated in different concentration of NaCl for 4 hours. **B.** Percentage of
889 holdfasts bound per field of view at different concentrations of NaCl. Gray arrow indicates ionic
890 strength of marine broth and Baltic sea (250 mM), natural habitat for *H. baltica*. Data are
891 expressed as an average of 6 independent replicates and the error bars represent the standard
892 error. **C.** Percentage of holdfasts bound per field of view at different concentrations of $MgSO_4$.
893 Data are expressed as an average of 4 independent replicates and the error bars represent the
894 standard error **D.** Images of WGA-AF488 labeled holdfasts already bound to a glass surface
895 and incubated in 0 mM NaCl (left) and 1 M NaCl (right) for 12hrs. **E.** Percentage of holdfasts
896 bound per field of view at 0 M or 1 M NaCl. The first incubation was done by adding 0 M or 1 M
897 of NaCl to a holdfast suspension spotted on a glass slide. After a 12 h incubation, the second
898 incubation was done after washing off unbound holdfasts, by adding 0 M or 1 M NaCl directly to
899 the holdfasts attached to the glass slide, and by incubating for another 12 h. Data are expressed
900 as an average of 5 independent replicates and the error bars represent the standard error.
901
902 **Figure S1: Design of a copper inducible promoter system in *H. baltica.***

903 **A.** Chromosomal arrangement of one of the copper sensitive operon (*Cso*) genes in *H. baltica*
904 genome, showing copper operon repressor *csoR* and copper binding proteins *copA* and *copB*
905 (top panel). The bottom diagram shows the fusion of copper-inducible promoter (P_{cu}) to *lacZ*
906 reporter gene. **B.** Effect of different concentration of $CuSO_4$ added into marine broth on *H.*
907 *baltica* growth. Growth yield (OD_{600}) was measured on overnight cultures with different
908 concentration of $CuSO_4$. Data represent mean of four independent replicates and the error bars
909 represent standard error. **C**. Representative growth curves of *H. baltica* growing in marine broth
910 without or with 500 µM $CuSO_4$. OD_{600} representing bacterial growth in a 24 well plate was
911 measured every 30 min. **D.** β–galactosidase activity representing the P_{cu} activity when induced
912 with different concentrations of $CuSO_4$. Exponential cultures were induced for 4 hrs. Data shown
913 is representative of three independent replicates.
914
915 **Figure S2: DNA inhibition of holdfast binding and biofilm formation.**
916 **A.** *C. crescentus* (upper panel) and *H. baltica* (lower panel) cells bound to a glass surface in
917 presence of eDNA from each strain. Holdfasts labeled with WGA-AF488 lectins after
918 exponentially grown cells were bound to a glass slide for 45 min. **B.** Biofilm quantification after
919 24 h for *C. crescentus and H. baltica* in presence of eDNA. Data are expressed as an average
920 of 4 independent replicates and the error bars represent the standard error.

Figure 1

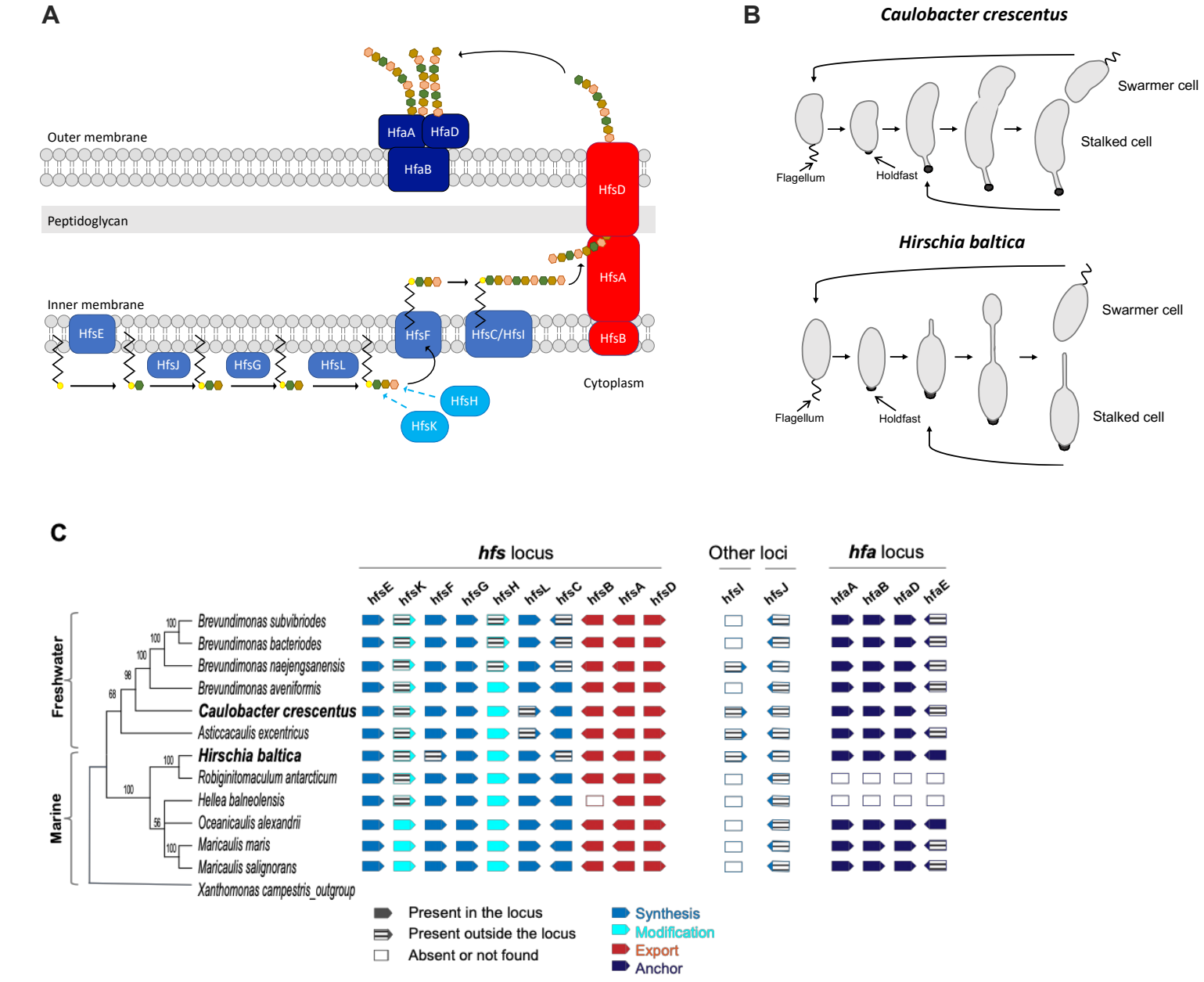
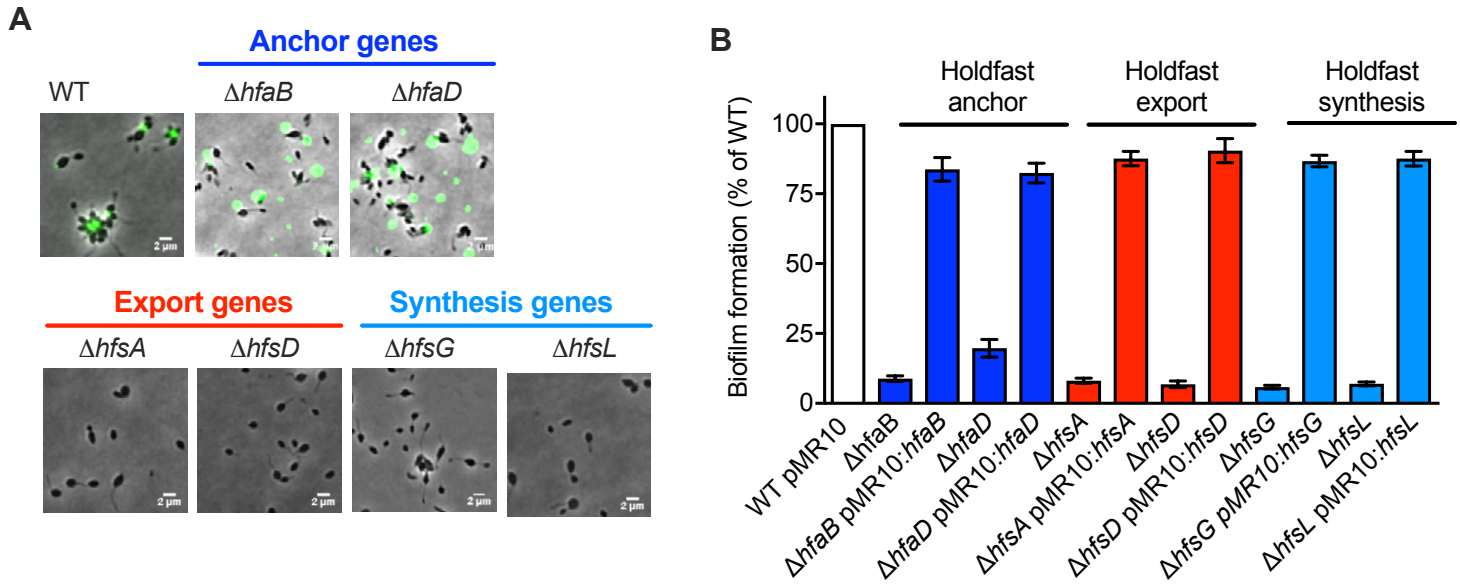


Figure 2



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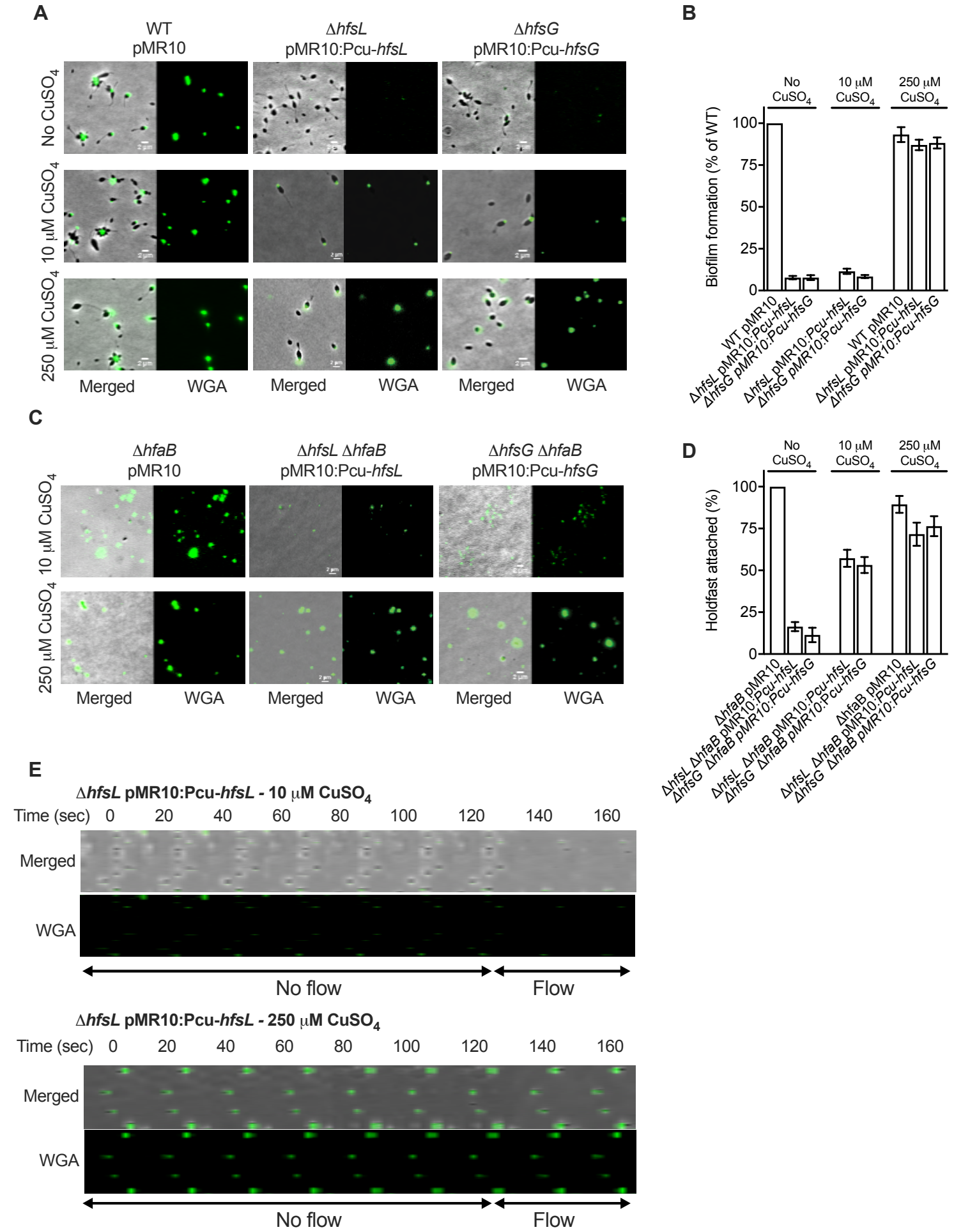

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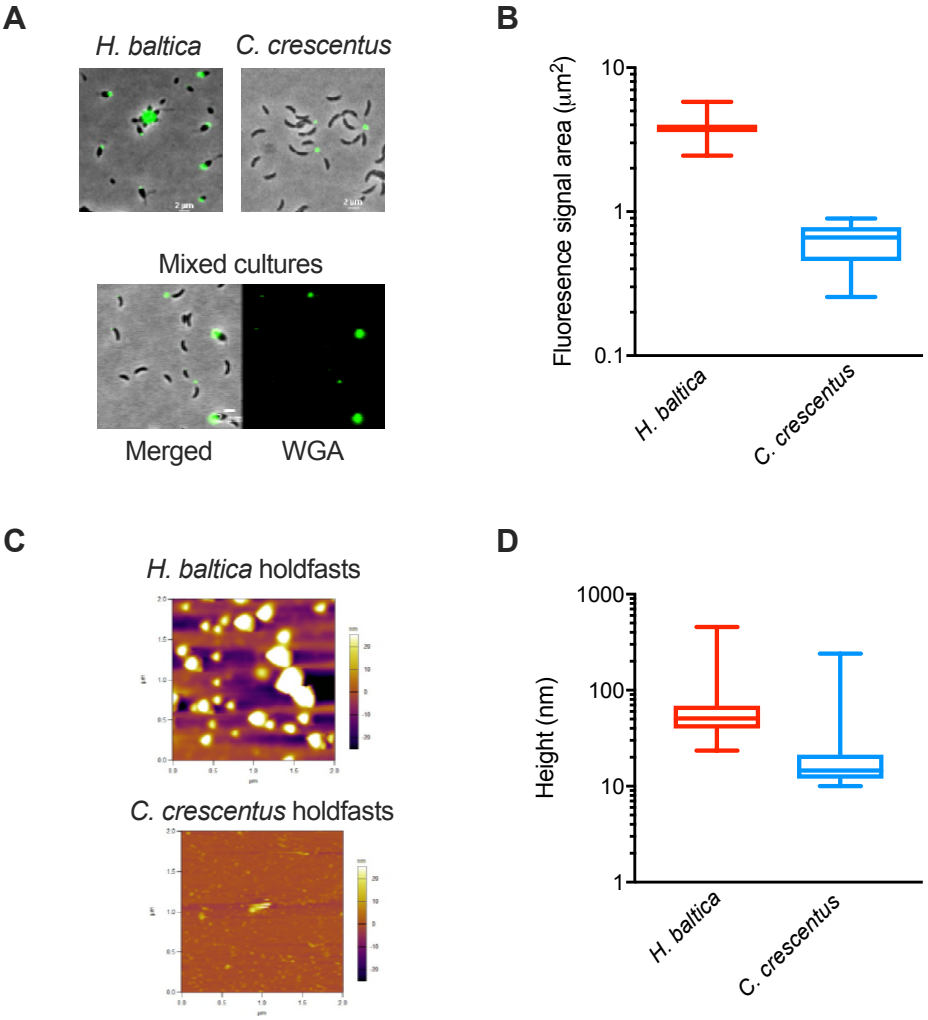

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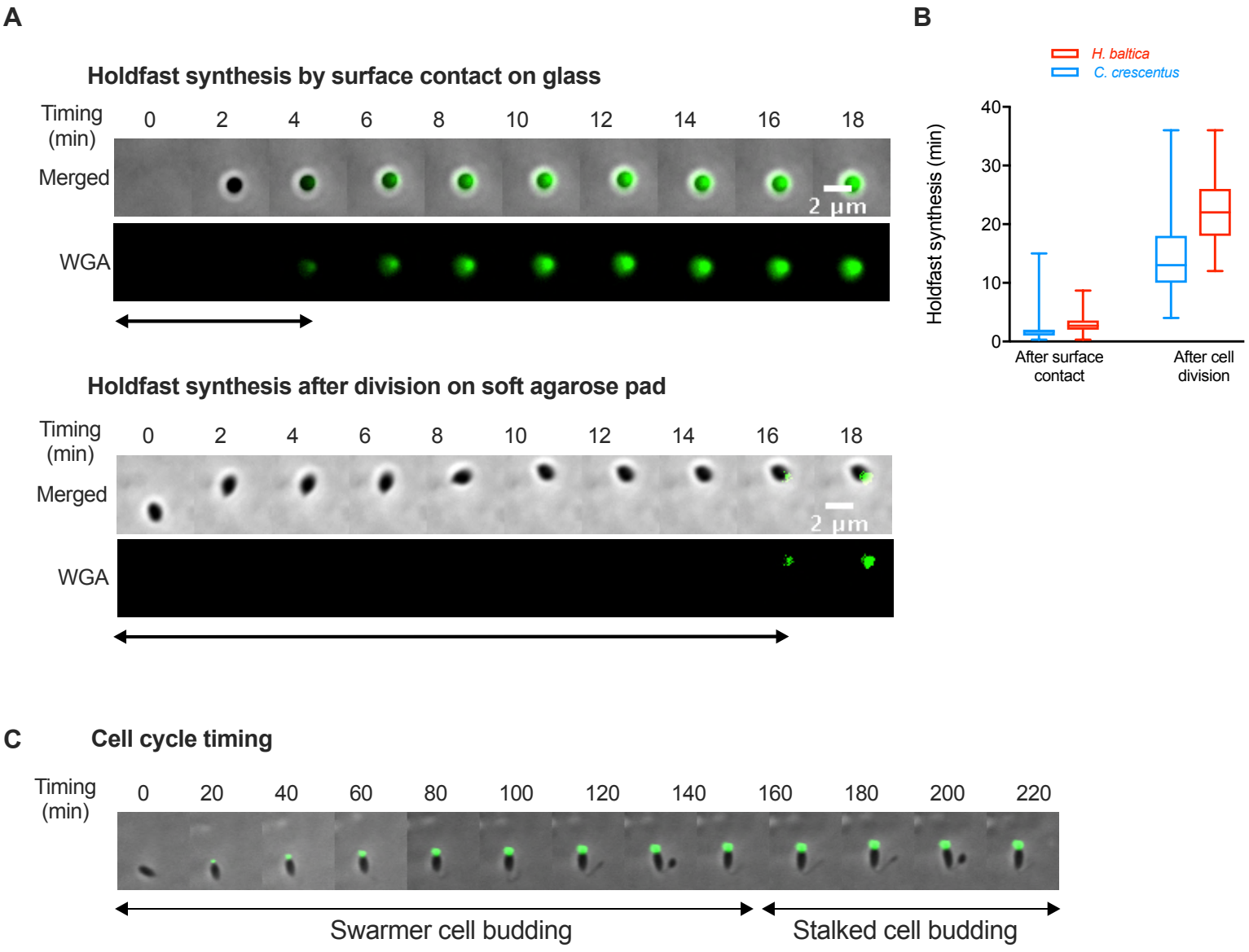
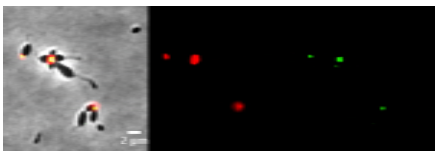


Figure 6

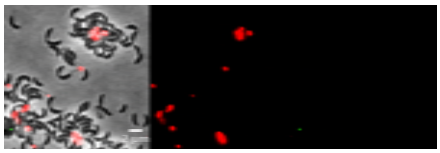
A **Polysaccharide staining**

H. baltica



Merged WGA GSL1

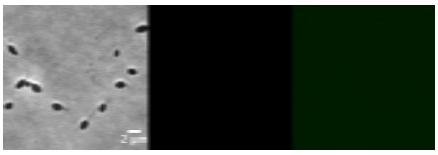
C. crescentus



Merged WGA GSL1

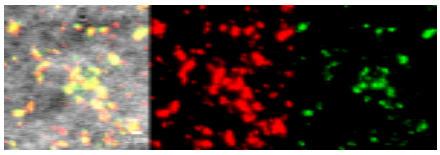
B **Polysaccharide staining**

H. baltica ΔhfsA



Merged WGA GSL1

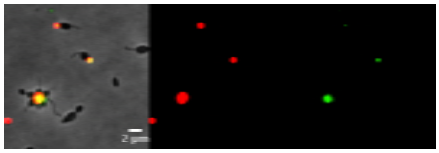
H. baltica ΔhfaB



Merged WGA GSL1

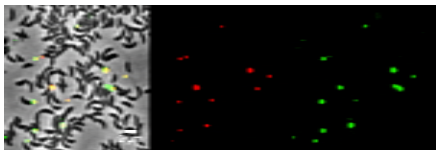
C **Peptide staining**

H. baltica



Merged WGA Maleimide

C. crescentus



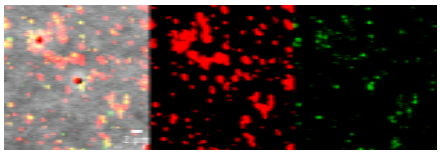
Merged WGA Maleimide

D **Peptide staining**

H. baltica ΔhfsA

Merged WGA Maleimide

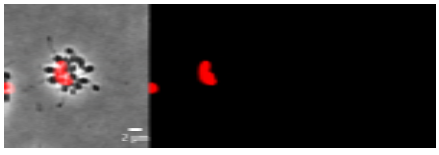
H. baltica ΔhfaB ΔhfaD



Merged WGA Maleimide

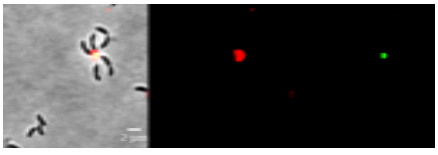
E **DNA staining**

H. baltica



Merged WGA YOYO-1

C. crescentus



Merged WGA YOYO-1

Figure 7

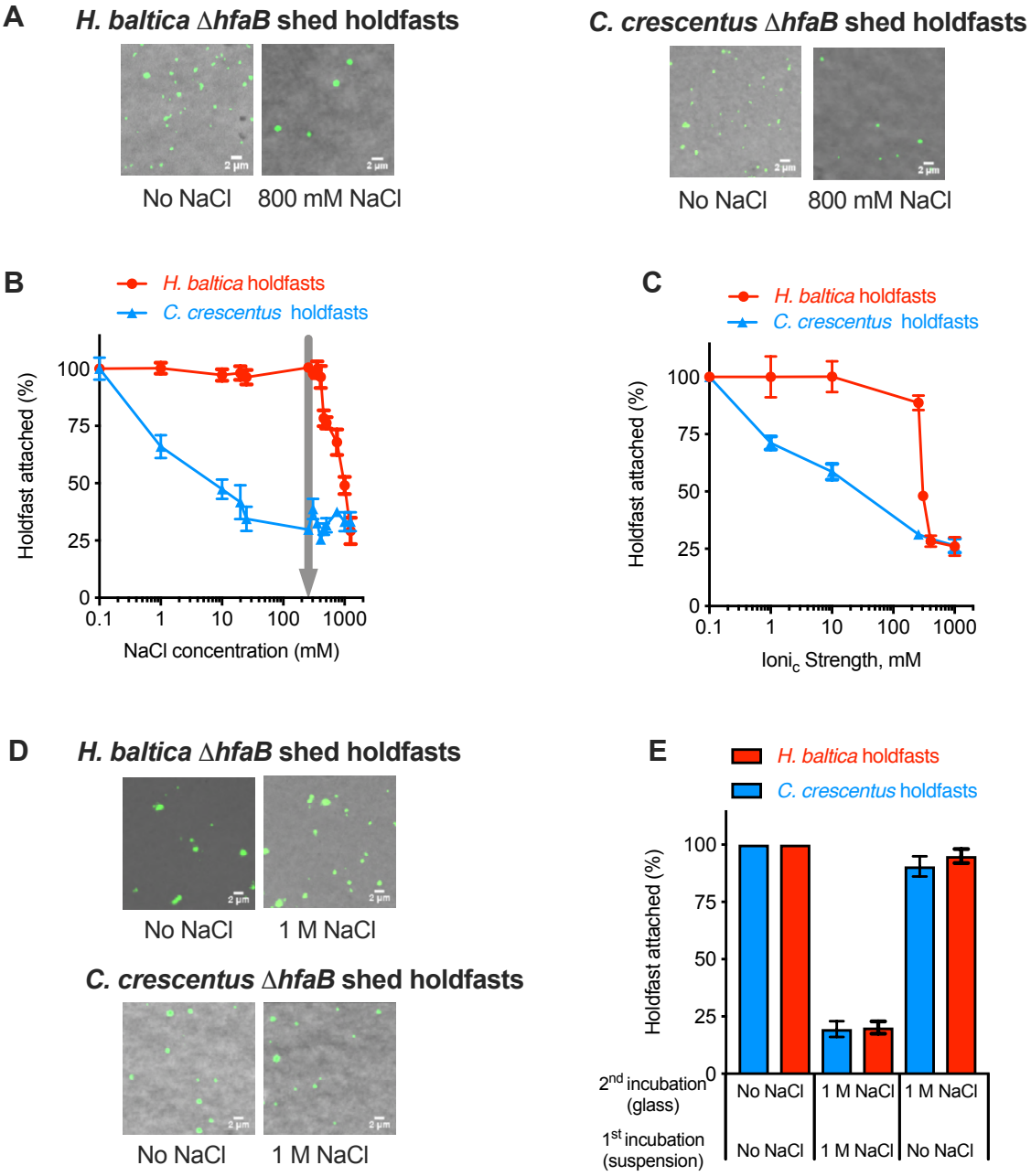


Figure S1

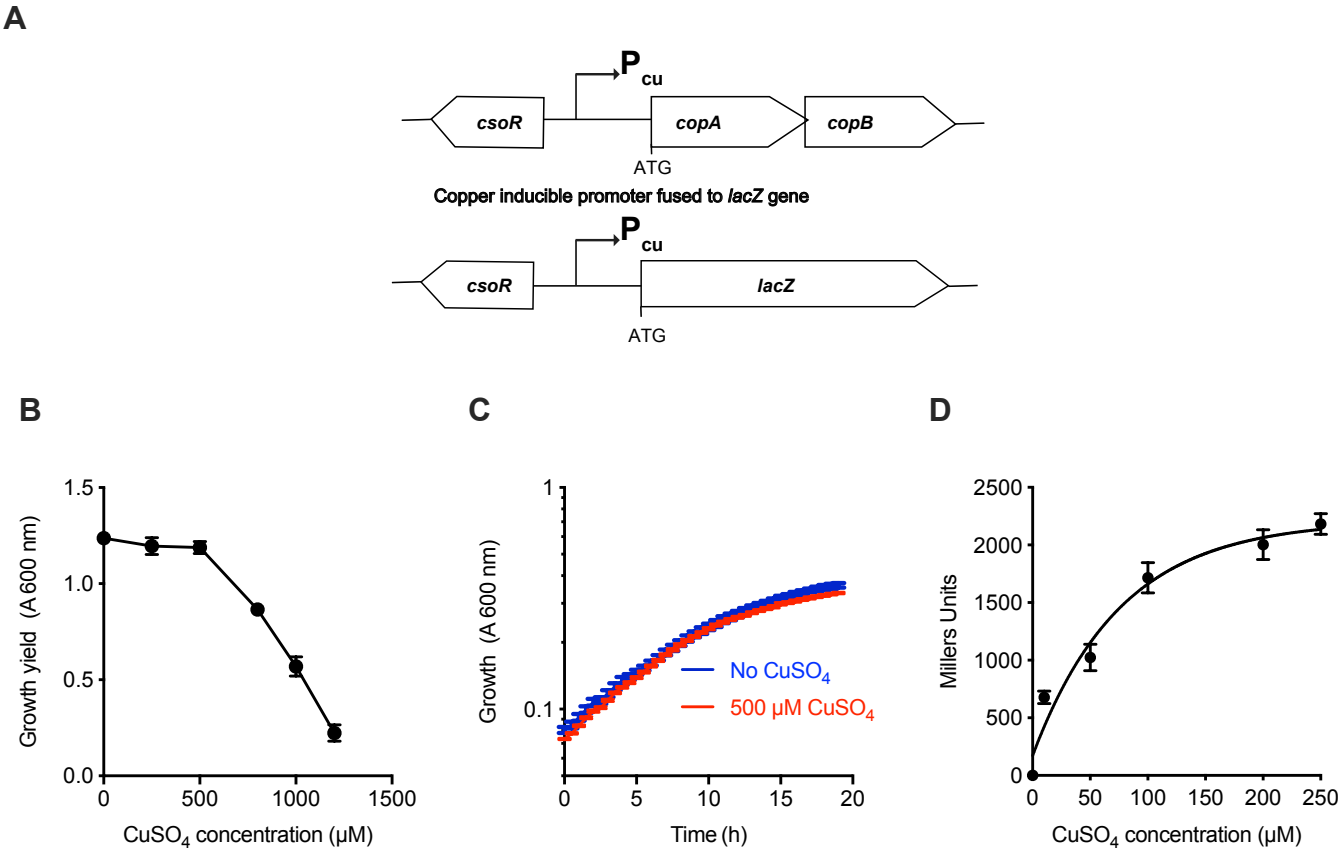


Figure S2