A genome-wide analysis of adhesion in *Caulobacter crescentus* identifies new regulatory and biosynthetic components for holdfast assembly

David M. Hershey¹, Aretha Fiebig¹ and Sean Crosson¹,²*

¹ Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, USA.
² Department of Microbiology, University of Chicago, Chicago, IL 60637, USA.

* To whom correspondence should be addressed: scrosson@uchicago.edu; (773) 834-1926

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Abstract: Due to their intimate physical interactions with the environment, surface polysaccharides are critical determinants of fitness for bacteria. Caulobacter crescentus produces a specialized structure at one of its cell poles called the holdfast that enables attachment to surfaces. Previous studies have shown that the holdfast is a carbohydrate-based material and identified a number of genes required for holdfast development. However, incomplete information about its chemical structure, biosynthetic genes and regulatory principles has limited progress in understanding the mechanism of holdfast synthesis. We have leveraged the adhesive properties of the holdfast to perform a saturating screen for genes affecting attachment to cheesecloth over a multi-day time course. Using covariance in the temporal profiles of mutants in a transposon library, we defined discrete clusters of genes with related effects on cheesecloth colonization. Holdfast synthesis, flagellar motility, type IV pilus assembly and smooth lipopolysaccharide (SLPS) production represented key classes of adhesion determinants. Examining these clusters in detail allowed us to predict and experimentally define the functions of multiple uncharacterized genes in both the holdfast and SLPS pathways. In addition, we showed that the pilus and flagellum control holdfast synthesis separately by modulating the holdfast inhibitor hflA. This study defines a set of genes contributing to adhesion that includes newly discovered genes required for holdfast biosynthesis and attachment. Our data provide evidence that the holdfast contains a complex polysaccharide with at least four monosaccharides in the repeating unit and underscore the central role of cell polarity in mediating attachment of C. crescentus to surfaces.

Importance: Bacteria routinely encounter biotic and abiotic materials in their surrounding environments, and they often enlist specific behavioral programs to colonize these materials. Adhesion is an early step in colonizing a surface. Caulobacter crescentus produces a structure called the holdfast, which allows this organism to attach to and colonize surfaces. To understand
how the holdfast is produced, we performed a genome-wide search for genes that contribute to
adhesion by selecting for mutants that could not attach to cheesecloth. We discovered complex
interactions between genes that mediate surface contact and genes that contribute to holdfast
development. Our genetic selection identified what likely represents a comprehensive set of genes
required to generate a holdfast, laying the groundwork for a detailed characterization of the
enzymes that build this specialized adhesin.

Introduction: The bacterial cell envelope is a highly dynamic structure that is essential for growth
and division(1). Carbohydrate-based compounds often form the outermost layer of the envelope,
comprising a specialized surface that each cell displays to the surrounding environment(2). The
roles of surface polysaccharides such as capsules, exopolysaccharides and O-antigens in
promoting colonization of preferred niches are well established for both free-living and infectious
cacteria(3-5). However, the enzymes that synthesize and export these polysaccharides have been
difficult to characterize due to the chemical complexity of the metabolic intermediates(6).
Defining the molecular details of how extracellular carbohydrates are produced is critical to
understanding bacterial colonization and how it can be controlled.

The aquatic bacterium Caulobacter crescentus has a dimorphic lifestyle characterized by an
association with exogenous surfaces. Division in C. crescentus is asymmetric and produces two
distinct cell types, a chemotactic swarmer cell and a sessile, replication-competent stalked cell(7).
After replication initiation, a swarmer cell sheds its flagellum, disassembles its pili and undergoes
a transition into a stalked cell before dividing(8). Stalked cells are named for a specialized
envelope extension called the stalk that emerges from the new pole after disassembly of the
flagellum and pili. During the swarmer to stalked transition, cells often produce a polysaccharide
rich matrix called the holdfast at the site of stalk development(9). This highly adhesive material allows *C. crescentus* to form essentially permanent interactions with exogenous surfaces (Fig 1A).

Due to the irreversible nature of surface attachment in *C. crescentus*, the timing of holdfast production is tightly controlled. When grown in defined medium only a small proportion of cells produces a holdfast, as compared to nearly all cells when grown in complex medium(10). This effect is due to elevated expression of the *holdfast inhibitor A (hfiA)* gene in defined medium (Fig 1B). *hfiA* expression is also coordinated with the cell-cycle. Its transcript levels drop during the swarmer to stalked transition, which corresponds to the developmental stage at which holdfasts begin to appear. Numerous signaling pathways target the *hfiA* promoter, allowing the cell to integrate environmental, nutritional and developmental cues into a single output that regulates adhesion(10, 11). In yet another regulatory regime, holdfast synthesis can be induced when a swarmer cell encounters a surface(12). Physical disruption of flagellar rotation or pilus oscillation upon surface contact stimulates the production of a holdfast (13, 14). How the numerous regulatory pathways converge to control holdfast development remains unclear, but the complexity of these networks reflects the significance of committing to a surface-associated lifestyle.

Genetic analysis of non-adhesive mutants indicates that the holdfast is a polysaccharide-based material. The *holdfast synthesis (hfs)* genes include predicted glycosyltransferases, carbohydrate modification factors and components of a *wzy*-type polysaccharide assembly pathway(15-18). *wzy*-dependent carbohydrate assembly utilizes a lipid carrier known as undecaprenylpyrophosphate (UPP) on which glycosyltransferases assemble an oligosaccharide repeating unit(19). The resulting glycolipid is flipped from the cytoplasmic face of the inner membrane to the periplasmic face where the oligosaccharide is polymerized and exported to the cell surface(20). The *wzy* mechanism is used to produce an impressive diversity of
polysaccharides and is broadly conserved among bacteria (21). Thus, characterizing enzymes involved in the biosynthesis of the holdfast has the potential to uncover broadly applicable principles about how bacteria produce carbohydrate polymers.

The chemical nature of the holdfast matrix remains poorly characterized. The holdfast binds to the N-acetylglucosamine (GlcNAc) specific lectin wheat germ agglutinin (WGA) and is sensitive to the GlcNAc specific hydrolases cellulase and lysozyme, indicating that GlcNAc is a component of the matrix (22). Little other information about the carbohydrate content has been reported. Extracellular DNA and unidentified protein component(s) also contribute to the mechanical properties of the holdfast, but only mutations in polysaccharide biosynthesis genes or pleiotropic regulators of cell polarity abolish holdfast production (23). Currently, three glycosyltransferase steps are known to be required for holdfast synthesis. An initial reaction carried out by one of the genetically redundant HfsE, PssY or PssZ enzymes is thought to be followed by the actions of HfsG and HfsJ, suggesting that the polysaccharide may be composed of a triscaccharide repeat (10, 17). However, new hfs genes continue to be discovered, hinting that additional glycosyltransferases may remain unidentified (10, 24). Uncertainty about both the composition of the holdfast and the saturation of screens for hfs genes presents a major obstacle to characterizing enzymatic reactions in the pathway.

Here, we utilized saturating transposon mutagenesis to probe holdfast production at the genome scale. We developed a barcoded transposon library in C. crescentus and enriched for non-adhesive mutants by passing across multiple days in presence of cheesecloth. We discovered a surprising number of genes with distinct adhesion phenotypes that ranged from hyper-adhesive to non-adhesive. We found that disrupting the smooth lipopolysaccharide (SLPS) leads to ectopic adhesion throughout the cell surface, and we used the temporal adhesion profile of known SLPS mutants to identify and characterize new genes in the SLPS pathway. The same covariance
approach was used to place previously uncharacterized genes in the holdfast pathway. We further demonstrated that disrupting the assembly of polar surface appendages modulates the activity of the holdfast inhibitor, hfiA. In particular, individual mutations in the pilus machinery had a range of adhesion phenotypes suggesting that distinct intermediates in the pilus assembly pathway have opposing effects on hfiA. Based on our comprehensive analysis of holdfast regulation, biosynthesis and assembly, we propose a model that outlines the complete set of enzymatic steps required to produce the holdfast polysaccharide.

Results:

A screen for mutants with altered adhesion

The holdfast promotes adhesion of C. crescentus cells to a variety of surfaces (25). We reasoned that adhesive cells could be depleted from liquid cultures by adding an attachment substrate with a sufficiently large surface area. Cheesecloth has been used in this manner to enrich for holdfast mutants in both C. crescentus and Asticcaaulis biprosthecum, another stalked bacterium in the Caulobacteraceae clade (26, 27). Adding sterile cheesecloth to wild-type C. crescentus cultures decreased the turbidity of the medium by titrating adhesive cells from the broth. This effect was amplified in the hyper-adhesive ΔhfiA strain and not observed in the holdfast deficient ΔhfsJ strain demonstrating the effectiveness of cheesecloth at capturing cells with a holdfast (Fig 1B). We concluded that growth in the presence of cheesecloth could be used as the basis of a selection to identify mutants defective in adhesion.

Saturating transposon mutagenesis coupled with transposon insertion sequencing (TnSeq) offers the advantage of scoring phenotypes for all non-essential genes in the genome simultaneously (28). Thus, combining TnSeq-based mutant profiling with cheesecloth depletion seemed appropriate to perform a saturating screen for holdfast biosynthesis genes and identify
missing biosynthesis factors. We developed a randomly barcoded transposon library in *C. crescentus* to enable the use of BarSeq(29) for profiling mutant fitness. Adhesive cells were depleted by passaging the library in cheesecloth for five cycles. For each passage, the library was cultured for 24-hours in the presence of cheesecloth after which the unattached cells in the medium were used to re-inoculate a fresh culture containing cheesecloth. An aliquot of unattached cells in the medium was also harvested for BarSeq analysis. Each passaging experiment was performed in triplicate, and three parallel passaging experiments were also performed in medium without cheesecloth to control for effects of mutation on growth rate (Table S2).

The abundance of each mutant during the passaging steps was assessed using BarSeq, providing a temporal fitness profile for each gene over the course of the experiment(29). Genes with higher fitness scores reflect mutants with adhesion defects that were enriched in medium that had been depleted with cheesecloth. As expected most genes had inconsequential effects on adhesion. However there were a significant number of genes whose mutation caused strong cheesecloth dependent changes in abundance over the course of the multi-day experiment. The 250 genes with the highest fitness values, above or below the baseline level, are shown in Fig 1C.

The time resolved nature of the experiment allowed us to group mutants with similar fitness profiles into distinct classes. Mutants with similar temporal adhesion profiles often mapped to genes with similar or complementary annotations, indicating that they represented groups of functionally related genes. We predicted the functions of uncharacterized genes using known functions for genes with similar fitness profiles. For example, a cluster of genes whose mutants show a continuous increase in abundance after each passage contains many known *hfs* genes, and any uncharacterized genes that share this fitness profile would be predicted to contribute to holdfast synthesis as well. We identified a total of four clusters containing mutants that display
distinct fitness profiles for the cheesecloth passaging experiment. Each cluster is described in detail below.

**Mutants defective in smooth lipopolysaccharide display ectopic adhesion**

We identified a cluster of mutants with strong fitness decreases (i.e. increased adhesion to cheesecloth) in early passages that recovered to near neutral or even positive fitness values as passaging proceeded (Fig 2A). Many of the genes in this “recovery” cluster had annotations associated with polysaccharide biosynthesis but had no known cellular function. However, the *wbq* genes that are required for the production of smooth lipopolysaccharide (SLPS) comprised a subset of the recovery cluster (30). This suggested that mutants sharing this fitness profile might also be defective in the biosynthesis of SLPS.

We focused on three uncharacterized genes in the recovery cluster. *CCNA_00497* is annotated as a putative rhamnosyl transferase, *CCNA_02386* is annotated as an O-antigen ligase and *CCNA_03744* is homologous to *rfbB*, a gene required for the biosynthesis of dTDP-L-rhamnose (31). Mutants in *CCNA_03744* were previously shown to suppress the holdfast attachment defect observed in a ∆*hfaD* mutant, but SLPS was not examined in these cells (32). We created in-frame deletions of *CCNA_00497*, *CCNA_02386* and *rfbB* and analyzed SLPS production by immunoblotting. A deletion of *wbqP*, which is thought to encode the initial glycosyltransferase step in the O-polysaccharide biosynthesis pathway, was used as a positive control. Disruption of *CCNA_02386*, *rfbB* or *wbqP* led to the loss of detectable SLPS, while ∆*CCNA_00497* cells showed a reduction in SLPS levels (Fig 2C). Additionally, all four mutants released the S-layer protein RsaA into the spent medium, an additional hallmark of SLPS defects in *C. crescentus* (Fig 2C) (33). None of the mutants displayed observable changes in rough LPS, demonstrating that they were not defective in the production of lipid A or the core oligosaccharide (Fig S1). All of the defects could
be complemented by ectopic expression of the target gene, confirming their roles in the 
production of SLPS (Fig 2C and Table S4).

The fitness profiles for early stages of cheesecloth passaging suggested that disrupting 
SLPS led to hyper-adhesive cells that were rapidly depleted by cheesecloth. We chose to 
investigate adhesion in detail using a defined medium because adhesion of wild-type cells is lower 
in defined medium than in complex medium, making this condition better suited for detecting 
increases in adhesion. We found that the SLPS mutants were indeed hyper-adhesive, producing CV 
staining values ranging from 2 to 4 times that of wild type (Fig 2B). To understand the basis of 
hyper-adhesion, ∆wbqP cells were imaged after staining with fluorescently labeled wheat germ 
agglutinin (fWGA) to label holdfasts. Most wild-type cells displayed a fluorescent focus at the tip of 
the stalk, and stalks from multiple cells often aggregated around a single focus to form rosette 
structures that are characteristic of holdfast production. In the ∆wbqP background, a comparable 
number of cells produced a holdfast, but the structure of the rosettes was altered. Cells that 
assembled around a holdfast were more tightly packed, and not all of them adhered to the 
aggregates through the tip of the stalk (Fig 2D).

The unusual rosette structures in the ∆wbqP mutant suggested that cells with disrupted 
SLPS might have a second mode of adhesion that did not require holdfast. We compared fWGA 
staining in the holdfast deficient ∆hfsJ strain to a ∆hfsJ ∆wbqP double mutant that lacks both 
holdfast and SLPS. ∆hfsJ cells did not stain with fWGA and did not form aggregates. ∆hfsJ ∆wbqP 
cells did not stain with fWGA, but in contrast to the ∆hfsJ strain, the cells still formed aggregates 
(Fig 2D). These aggregates appeared not to be mediated by stalk-stalk interactions but rather 
through interactions within the cell body. This further supported the idea that a holdfast-
independent mode of adhesion operates in SLPS mutants. Consistent with this model, bulk 
adhesion in the ∆hfsJ ∆wbqP double mutant was not abolished, and was, in fact, higher than wild
type (Fig 2B). We conclude that disrupting SLPS production causes defects in the cell surface leading to a holdfast independent mode of adhesion that represents the dominant mode of adhesion for these mutants early in our experimental time course.

The flagellum and type IV pili regulate holdfast production

A second cluster of mutants displayed steady decreases in fitness throughout passaging in cheesecloth. This group primarily contained genes known to participate in chemotaxis and flagellar motility as well as components of the type IV pilus machinery. Thus, the data suggested that disrupting the assembly of polar appendages, either pili or the flagellum, leads to hyper-adhesion. To study the effects of polar appendages on adhesion, we deleted the genes for the flagellar basal body component FlgH and the pilus assembly protein CpaH (34, 35). We confirmed that ΔflgH showed the expected loss in motility and that ΔcpaH was resistant to the type IV pilus specific phage ΦCBK (Figs S2 and S3).

Both the ΔflgH and the ΔcpaH mutants showed adhesion defects in complex medium (Table S4). However, in defined medium, both mutants displayed increased adhesion relative to wild type, indicating that disrupting the pilus or the flagellum causes hyper-adhesion under these conditions (Fig 3B). To reconcile these differences we used fWGA staining to measure the proportion of cells that produced a holdfast. The ΔflgH and ΔcpaH mutants produced more holdfasts than wild-type in both complex and defined medium (Fig S3 and Table S6). We conclude that flagellum and pilus mutations increase holdfast production, but that loss of either appendage also leads to holdfast-independent defects in surface colonization. Pili and flagella often have similar effects on surface colonization in other systems (36, 37). Because the baseline level of holdfast production is low in defined medium, the enhanced holdfast production ΔflgH and ΔcpaH backgrounds appears to outweigh surface colonization defects under these conditions (Fig S3).
A recent report showed that flagellar hook mutants displayed decreased transcription from the \(hfiA\) promoter \((P_{hfiA})\) in defined medium and that this effect did not occur in the absence of the diguanylate cyclase PleD\((38-40)\). This led us to examine the relationships between our polar appendage mutants, \(hfiA\) and \(pleD\). We used a \(P_{hfiA}\)-\(lacZ\) reporter to measure transcription from the \(hfiA\) promoter. Because expression from \(P_{hfiA}\) is nearly undetectable in complex medium, we focused on transcriptional changes that occurred in defined medium. The \(\Delta flgH\) and \(\Delta cpaH\) mutants showed reduced \(hfiA\) transcription in the reporter assay (Fig 3D). These effects were abrogated in the \(\Delta flgH \Delta pleD\) and \(\Delta cpaH \Delta pleD\) double mutants (Fig 3D). Likewise, bulk adhesion in defined medium reverted to near wild-type levels in the \(\Delta flgH \Delta pleD\) and \(\Delta cpaH \Delta pleD\) mutants, confirming that \(pleD\) contributes to the modulation of \(P_{hfiA}\) caused by pilus and flagellar mutants (Fig 3C). We note, however, that a full reversion of the hyper-holdfast phenotype would be predicted to display bulk adhesion levels below that of wild type due to the holdfast-independent attachment defects seen in pilus and flagellar mutant backgrounds. Thus, while \(pleD\) does contribute to the enhanced adhesion seen in the \(\Delta flgH\) and \(\Delta cpaH\) mutants, the effect is not completely dependent on this gene.

To test whether the hyper-adhesive phenotypes in the polar appendage mutants could be explained by regulation of \(hfiA\), we created \(\Delta flgH \Delta hfiA\) and \(\Delta cpaH \Delta hfiA\) double mutants. Though \(\Delta flgH \Delta hfiA\) cells remained hyper-adhesive in defined medium, bulk attachment was not significantly increased relative to the \(\Delta flgH\) single deletion. This suggests that \(\Delta flgH\) minimizes the effects of \(hfiA\), and that holdfast-independent adhesion defects lower the maximum level of CV staining that can be achieved in a \(\Delta flgH\) background. The enhanced attachment seen in the \(\Delta cpaH\) background was further increased in a \(\Delta cpaH \Delta hfiA\) double mutant \((P < 0.0001; \text{Fig } 3C)\). Thus, \(\Delta cpaH\) has an intermediate effect on \(hfiA\) activity by dampening but not completely masking its activity. Consistent with this, the percentage of \(\Delta cpaH\) cells that produced a holdfast in defined
medium was intermediate between that of wild type and ΔhfiA, supporting the idea that ΔcpaH causes both intermediate enhancement of holdfast production and holdfast-independent surface-attachment defects (Fig S3 and Table S6). Finally, bulk adhesion in a ΔflgH ΔcpaH double mutant was indistinguishable from ΔflgH, and P_{hfiA} transcription was lower in ΔflgH ΔcpaH than either the ΔflgH (P < 0.0001) or ΔcpaH (P < 0.0001) single mutants (Fig 3B and C). This result indicates that holdfast production is likely maximized in the ΔflgH mutant and that the ΔflgH and ΔcpaH mutants modulate P_{hfiA} through separate pathways.

A complex role for the pilus in regulating adhesion

The ΔcpaH phenotype suggested that disrupting pilus assembly leads to increased holdfast production via the repression of hfiA. However, a closer examination of the fitness profiles for genes involved in type IV pilus assembly revealed a range of phenotypes for various components of the apparatus (Fig 4A). Mutants in most of the genes encoding components of the pilus secretion machinery, including cpaH, showed decreased fitness scores consistent with hyper-adhesion. However, mutations in the gene coding for the main pilin subunit, PilA, displayed the opposite trend. pilA mutants showed an increase in fitness during passaging in cheesecloth that would be expected for mutants with adhesion defects. We confirmed that, indeed, the ΔpilA strain was defective in surface attachment both in complex and defined medium (Fig 4B and Table S4).

To examine the relationship between pilA-dependent loss of adhesion and the activation of adhesion observed in mutants that disrupt pilus and flagellum assembly we created ΔflgH ΔpilA and ΔcpaH ΔpilA double mutants. The phenotypes for these mutants were similar in both complex and defined medium (Table S5). Surface attachment levels in the ΔflgH ΔpilA mutant were intermediate to those of ΔpilA (P < 0.0001) and ΔflgH (P < 0.0001), suggesting that flgH and pilA regulate adhesion through independent, additive pathways (Fig 4C and Table S5). In contrast,
adhesion in the ΔcpaH ΔpilA mutant was indistinguishable from ΔpilA demonstrating that the
effects of pilA on adhesion are epistatic to those of cpaH (Fig 4C and Table S5). We conclude that
the pilin subunit PilA is required for the holdfast promoting effect caused by disruption of the
pilus assembly apparatus.

We further explored the model that pilA contributed to the modulation of adhesion by cpaH
by examining the relationships between pilA, hfiA and pleD. The decreased adhesion observed in
the ΔpilA mutant was not reversed in a ΔpilA ΔpleD background indicating that the effect of pilA on
adhesion is pleD-independent. Adhesion in a ΔpilA ΔhfiA double mutant was elevated to a level
slightly below that of the ΔhfiA strain (P < 0.0001; Fig 4B). The difference in surface attachment
between the ΔpilA ΔhfiA and ΔhfiA mutants likely reflects the holdfast-independent surface
attachment defects caused by the loss of a functional pilus. Expression from P_{hfiA} was only slightly
elevated in the ΔpilA mutant (Fig 4D). Because hfiA is already highly expressed under these
conditions, it is difficult to determine if P_{hfiA} is activated further in the ΔpilA mutant. Finally, β-
glactosidase activity from the P_{hfiA} reporter in both the ΔflgH ΔpilA and ΔcpaH ΔpilA backgrounds
was restored to wild-type levels demonstrating that ΔpilA blocks hfiA activation in the ΔflgH and
ΔcpaH backgrounds (Fig 4D).

New factors in the holdfast biosynthesis pathway

The final two clusters of mutants presented in Fig 1C displayed a continuous increase in fitness
during growth in cheesecloth that would be predicted for mutants with adhesion defects. We used
the magnitude of the measured fitness increases to separate these genes into 1) a cluster with
higher fitness scores that contained all of the hfs genes known to be required for robust adhesion
and 2) a cluster displaying more modest fitness increases which contained the hfsK gene. hfsK
codes for an enzyme thought to modify the holdfast polysaccharide in order to produce a fully
adhesive holdfast (24). We chose three uncharacterized genes from these clusters of mutants to examine in detail for holdfast defects.

Disruption of CCNA_01242, which encodes a predicted amino acid permease, leads to the highest fitness increase of any gene in the cheesecloth passaging experiment (Fig 5A). However, the ΔCCNA_01242 strain had only a modest defect in surface attachment (Fig 5B). There were no obvious holdfast defects in the mutant and we could not detect a significant adhesion defect under any conditions tested (Fig 5C and Table S4). ΔCCNA_01242 had an unusual, biphasic growth profile. In complex medium, log phase was shorter than wild type, leading to a lower optical density as growth began to slow prematurely. Growth of this strain continued slowly over the next 24 hours and eventually plateaued at a similar optical density to wild type (Fig S4). The biphasic growth likely confounds fitness calculations for samples collected during the sequential passaging experiment. It is not clear why CCNA_01242 mutants were more enriched when cheesecloth was included in the medium, but we conclude nonetheless that the gene does not contribute to holdfast production.

CCNA_02360 is a predicted member of the GT2 family of glycosyltransferases. Its disruptions have a fitness profile closely resembling many of the known hfs genes (Fig 5A). A ΔCCNA_02360 mutant was non-adhesive in CV staining assays and did not stain with fWGA under any conditions tested (Figs 5B and 5C). Given the lack of holdfast production in ΔCCNA_02360 cells, we predict that CCNA_02360 encodes a glycosyltransferase that contributes one or more monosaccharides to repeating unit of the holdfast polysaccharide and have named this gene hfsL. Previous studies have mentioned mutants in CCNA_02360 as a holdfast-deficient control strain (24, 39), but, curiously, the identification of the gene and characterization of its phenotype have not been reported. Closely related genes could be identified in many stalked bacteria within the Caulobacterales clade suggesting that HfsL carries out a conserved step in holdfast biosynthesis.
Identifying close homologs in more distantly related Alphaproteobacteria was difficult due to the abundance of GT2 family glycosyltransferases in bacterial genomes. Nevertheless, HfsL represents a fourth glycosyltransferase that is required for holdfast biosynthesis in C. crescentus.

CCNA_02722 is a hypothetical protein that does not show homology to any known protein families. It has a predicted N-terminal signal peptide for export to the periplasm. The fitness profile is similar to hfsK, showing a modest increase in fitness during passaging in cheesecloth suggestive of intermediate defects in adhesion (Fig 5A). The ∆CCNA_02722 mutant was significantly in impaired surface-attachment (Fig 5B). When planktonic ∆CCNA_02722 cells were stained with fWGA, holdfast staining was apparent (Fig 5C). However, using adhesion assays in which cells are grown in the presence of a glass slide that is then washed, stained with fWGA and imaged we detected a holdfast attachment defect. Wild-type cells normally coat the surface of the slide and show fWGA foci at the site of attachment, but we observed very few attached ∆CCNA_02722 cells. Instead, the slide was coated with an abundance of fWGA reactive material (Fig 5C). This holdfast shedding phenotype is characteristic of mutants with defects in attaching the holdfast matrix to the surface of the stalk(27), and the gene has been named hfaE accordingly. Like hfsL, hfaE could be identified in the genomes of many other stalked bacteria suggesting a conserved role in holdfast attachment.

Discussion

Holdfast production in C. crescentus presents an attractive model system to interrogate the assembly of polysaccharides in bacteria. Lectin staining, enzymatic sensitivity and functional annotations for the hfs genes indicate that the holdfast contains a polysaccharide(16, 17, 22). However, despite decades of work, surprisingly little else is known about the chemical structure of the holdfast. As part of our efforts to characterize the biosynthetic pathway, we sought a complete
list of enzymes required for holdfast biosynthesis. In order to saturate the search for hfs genes, we
developed a TnSeq-based method to measure the adhesion phenotype conferred by each non-
essential gene in the genome.

The genome-wide approach provided a surprisingly rich set of insights into adhesion in C.
crescentus. Not only did the screen identify non-adhesive mutants representing missing
components of the holdfast pathway, but it also resolved mutants that displayed increased
adhesion. One class of such mutants was defective in the production of smooth-LPS. Disrupting
SLPS resulted in elevated adhesion levels in both holdfast producing and holdfast deficient
backgrounds. SLPS mutants no longer adhered exclusively at the stalks of holdfast producing cells,
but rather displayed a generalized form adhesion throughout the cell surface. Our analysis of these
mutants supports a model in which the C. crescentus envelope is structured to ensure that the cell
surface is non-adhesive, maximizing the opportunity for polar adhesion via the holdfast.

A holdfast independent form of adhesion could explain the unexpected fitness profiles seen
in SLPS mutants during passaging in cheesecloth. The unique mode of aggregation conferred by
having both a holdfast and a disrupted cell surface clearly leads to an initial depletion of these
mutants from the culture medium by cheesecloth. However, as non-adhesive hfs mutants begin to
dominate the culture, this adhesion strategy may lose its effectiveness, leading to a recovery
phase. Regardless of mechanism, our analysis allowed for the identification of new SLPS
biosynthesis genes in C. crescentus simply by defining a group of mutants that shared the
characteristic depletion and recovery profile over the course of the passaging experiment. Such
co-fitness approaches have been useful in other contexts(41) and allowed us to greatly expand the
number of genes in the C. crescentus SLPS pathway (Table S3).

Genes with predicted functions in motility, flagellar biosynthesis and type IV pilus
assembly displayed a hyper-adhesive profile that could be distinguished from SLPS mutants by the
lack of a recovery phase. Mutations in components of the flagellar basal body were recently shown to enhance holdfast production by inhibiting the expression of hfiA, a result that we confirmed here in our examination of flgH(40). Co-fitness analysis indicated that the cpa genes, which code for components of the type IV pilus, have a similar phenotype, and we showed that mutating the inner-membrane pilus assembly component cpaH also increased holdfast production by repressing hfiA. However, mutating pilA, which codes for the main subunit of the pilus filament, reduced adhesion. The adhesion defect in ΔpilA cells was partially restored in a ΔpilA ΔflgH background but remained unchanged in ΔpilA ΔcpaH cells. Thus, although the ΔflgH and ΔcpaH mutations both enhance holdfast production, the two pathways can be distinguished by their requirement for pilA. Disentangling the specific routes by which the various polar appendage mutants modulate hfiA activity will require identifying intermediate factors in the signaling pathways, but our results underscore the interconnectedness of the flagellum, pilus and holdfast.

Numerous reports have debated the roles of pili and the flagellum in surface attachment(12-14, 42, 43). Our unbiased, genome-wide analysis of adhesion unambiguously identified both appendages as determinants of attachment. Two recent studies, in particular, showed that mutating the flagellar basal body represses hfiA and that disruption of flagellar rotation upon surface contact stimulates holdfast production(14, 40). In our cheesecloth passaging experiment flagellar motor (mot), flagellin glycosylation (flm) and chemotaxis (che) genes shared the same hyper-adhesive fitness profile as components of the flagellar basal body (Table S3). Some of these mutants would be predicted to disrupt flagellar rotation without affecting assembly per se(44). We propose that disrupting flagellar function, either through physical interaction with a surface or mutation of motility genes, stimulates holdfast production. It will be interesting to test this model by determining whether the repression of hfiA seen in the flagellar mutants is required to activate holdfast production after surface contact. We also note
that future studies should take into account the finding that mutating \textit{flgH} reduces biofilm formation in a holdfast-independent manner (Fig S3), suggesting flagellar motility likely also promotes productive interactions with a surface that lead to permanent attachment.

Much like flagellar mutants, disrupting components of the type IV pilus also causes both increased holdfast production and holdfast-independent surface colonization defects. A recent report proposed that contact with a surface inhibits the retraction of PilA filaments leading to a stimulation of holdfast production\(^{(13)}\). One might initially conclude that pilus assembly defects in the \textit{cpa} mutants mimic the obstruction of pilus filament oscillation. However, the situation is more complex because \textit{pilA} was required for increased holdfast production in the \textit{\Delta cpAH} mutant. These findings can be reconciled in a model in which the disruption of filament oscillation caused either by surface contact or upon mutation of the \textit{cpa} genes leads to changes in the PilA pool that serve as a signal to stimulate holdfast production. A similar model was proposed for the regulation of biofilm formation by the \textit{Agrobacterium tumefaciens} pilus\(^{(37)}\). Again, determining if repression of \textit{hfiA} is required for the induction of holdfast production upon surface contact would help to clarify whether the \textit{hfiA}-dependent and surface stimulated pathways are one in the same.

Finally, the initial goal of this study was to saturate the search for holdfast production factors. We used the cheesecloth passages to define a temporal fitness profile that was shared by genes known to be required for holdfast biosynthesis and used this pattern to search for missing genes in the pathway. In addition to a new holdfast attachment factor (\textit{hfaE}), we identified a glycosyltransferase (\textit{CCNA\_02360}), which we have named \textit{hfsL}, and showed that it is required for holdfast production. Due to the saturating nature of our experiment, we believe that HfsE (along with the redundant PssY and PssZ), HfsJ, HfsG and HfsL carry out the only four glycosyltransferase steps involved in holdfast biosynthesis. Thus, we predict that a repeating unit of four or more monosaccharides makes up the holdfast polysaccharide because each of these enzymes likely
contributes at least one sugar to the glycolipid intermediate that serves as a substrate for polymerization.

Implicit in defining the complete set of genes in the holdfast pathway is knowledge of genes that do not contribute. Many bacterial polysaccharides contain specialized monosaccharide components (45, 46). Such intermediates are synthesized as nucleotide activated precursors that can be directly utilized by glycosyltransferases (47, 48). We did not identify any genes involved in nucleotide sugar metabolism that had significant adhesion defects when disrupted. This suggests that the holdfast does not contain specialized monosaccharide components but is instead composed of “housekeeping” sugars that are shared by other cellular processes. Such insights into the nature of the sugar components will help inform our ongoing efforts to characterize the chemical structure of the holdfast polysaccharide. Additionally, the ability to utilize standard nucleotide sugars as substrates without requiring specialized chemical syntheses will make the holdfast biosynthetic enzymes useful models for probing the catalytic mechanisms of bacterial polysaccharide biosynthesis.

More broadly, the findings reported here highlight the advantages to probing mutant phenotypes in parallel. Classical genetic selections in which mutants are enriched then isolated and analyzed separately inherently favor both the most extreme phenotypes and large genes that have the highest likelihood of acquiring mutations. Screening pools of mutants in parallel using TnSeq eliminates these biases, allowing detection of a range of phenotypes and analysis of mutations in short genes that represent a small fraction of the mutant pool. In this study, we discovered new regulatory networks that modulate holdfast synthesis by identifying mutants that display modest adhesion increases using a genetic background in which cells are already adhesive. Such mutants would be extremely difficult to isolate using a classical approach. Additionally, the saturating nature of these experiments makes them ideal for outlining complete biosynthetic
pathways. Having a reasonable measure of completeness allowed us to leverage the results of the genome-wide screen to propose a model for the enzymatic steps in the holdfast pathway that will inform efforts to reconstitute the biosynthetic reactions in vitro (Fig 6).

**Methods**

*Strains, growth conditions and genetic manipulation*

Strains and plasmids used in this study are listed in Table S1. *E. coli* strains were grown in LB at 37°C with diaminopimelic acid (60 mM), kanamycin (50 μg/mL) or tetracycline (12 μg/mL) included as needed. *C. crescentus* CB15 was grown either in PYE (complex) or M2 salts containing 0.15% xylose (M2X, defined) at 30°C (49). When necessary sucrose (3%), kanamycin (25 μg/mL) or tetracycline (2 μg/mL) was included in solid medium. Kanamycin (5 μg/mL) or tetracycline (1 μg/mL) was included in liquid medium when necessary. Standard techniques were used for cloning and sequencing plasmids. Plasmids were introduced by electroporation or, in the case of the pFC1948 reporter plasmid, by tri-parental mating. Unmarked mutations were created through two-step deletion using a SacB counter-selection. Mutants were complemented by inserting target genes into pMT585, which allows for the integration of the gene at the *xyl* locus under the transcriptional control of *P*<sub>xyl</sub>(50). When necessary, target genes were inserted into pMT585 in reverse orientation under the control of their native promoters.

*Library development and mapping*

The barcoded HiMar transposon pool APA_752 developed by Wetmore et al.(29) was used to create a barcoded Tn library in *C. crescentus*. Construction of the library has been reported previously along with its associated statistics(51). Briefly, the transposon pool was introduced into *C. crescentus* by conjugation. Transconjugants appearing on selective plates were pooled, used
to inoculate a liquid culture with Kanamycin and grown for 3 doublings. Glycerol was added to a final concentration of 15% and 1mL aliquots were frozen and stored at -80°C. TnSeq also followed the method of Wetmore et al(29). A 1mL library aliquot was centrifuged and genomic DNA was extracted from the pellet. The DNA was sheared, size selected for ~300bp fragments and end repaired. A custom Y-adapter (Mod2_TS_Univ annealed to Mod2_Truseq) was ligated and transposon junctions were amplified by PCR using the Nspacer_BarSeq_pHIMAR and P7_mod_TS_index1 primers. An Illumina HiSeq2500 was used to generate 150bp single-end reads of the library. The genomic positions of were determined with BLAT. The barcode corresponding to each insertion site was determined using MapTnSeq.pl. This information was used to develop a list of barcodes that mapped to unique insertion sites using DesignRandomPool.pl (available at https://bitbucket.org/berkeleylab/feba).

Passaging in cheesecloth

1 mL aliquots of the DH1 transposon library was thawed at room temperature and 300 μL of the library was inoculated into a well of a12-well microtiter plate containing 1.2 mL PYE and a stack of 5 ~10 mm x 10 mm squares of sterile cheesecloth was added. The culture was grown with 155 rpm shaking at 30°C for 24 hours after which 100 μL of the planktonic culture was used to inoculate a fresh well containing 1.4 mL PYE and a fresh piece of sterile cheesecloth. An additional 500 μL of the planktonic culture medium was centrifuged and the pellet was stored at -20°C for BarSeq analysis. The process was repeated for a total of five passages and each passaging experiment was performed in triplicate. The same procedure was used to perform passaging experiments in which no cheesecloth was added.

Fitness determination with BarSeq
Cell pellets from 0.5mL of planktonic culture medium that had been frozen after each passage were used as templates for PCR reactions that simultaneously amplified the barcode region of the transposons while adding TruSeq indexed Illumina adaptors (29). The PCR products were purified, pooled and multiplexed on a single Illumina 4000 lane for sequencing. Fitness values for each gene were determined using the pipeline described by Wetmore et al (29). Barcodes for each read were mapped using MultiCodes.pl and correlated with their associated insertion positions using combineBarSeq.pl. This data was used to calculate fitness using FEBA.R. This analysis determines strain fitness as the log₂ ratio of barcode counts in a sample to the barcode counts in the reference condition, for which we used the first passage of the library in PYE without cheesecloth. Gene fitness was calculated by determining the weighted average of the insertion mutants with a given gene, excluding the first and last 10% of the ORF. The scripts used for fitness determination can be found at https://bitbucket.org/berkeleylab/feba.

Data availability

The raw sequence data for each BarSeq sample along with a table describing the barcode abundances and their genomic insertion sites has been uploaded to the NCBI Gene Expression Omnibus (GEO) at https://www.ncbi.nlm.nih.gov/geo/ under the accession: GSE119738.

Analysis of fitness data

We focused on identifying genes with the highest absolute (positive or negative) fitness scores. For each passaging step (with and without cheesecloth), an average and a standard deviation for the three replicate samples were calculated. Genes for which the largest standard deviation across the ten passages was greater than the largest absolute fitness score were eliminated from further analysis. Fitness scores determined for each passage without cheesecloth were subtracted from
those of the corresponding passage with cheesecloth to normalize for growth defects. These normalized values were used to rank each gene according to its largest absolute fitness score at any stage of cheesecloth passaging. The top 250 genes were then sorted by hierarchical clustering\cite{52} to identify genes with related fitness patterns. Groups derived from the clustering analysis were curated manually to produce the genes in Tables S4-S8.

Crystal violet (CV) staining of adherent cells

Saturated cultures of \textit{C. crescentus} grown in PYE were diluted to OD$_{660}$=0.4 with PYE, 1\textmu L was inoculated into the wells of 48-well microtiter plates containing 450\textmu L medium and the cultures were shaken for 24 hours. For cultures used to measure the effects of motility mutants on adhesion, the incubation was shortened to 17 hours. The cultures were then discarded and the plates were washed thoroughly with a steady stream of tap water. The wells were stained for 5 min with 0.01\% crystal violet and washed again with tap water. Stain was dissolved for 5 min in 100\% EtOH and quantified by reading absorbance at 575nm.

Fluorescent wheat germ agglutinin (fWGA) staining

For staining of planktonic cells, 400 \textmu L of liquid culture was added to an Eppendorf tube and Alexa594 conjugated-WGA (ThermoFisher) was added to a final concentration 0.5 mg/mL. After incubating the cells for 5 min in the dark, 1\textmu L of sterile water was added and the cells were centrifuged at 6k x g for 2 min. The pellet was re-suspended in 5 \textmu L of medium, spotted on a glass slide and imaged. Cells were imaged with a Leica DM500 microscope equipped with a HCX PL APO 63X/1.4na Ph3 objective. fWGA staining was visualized using an RFP fluorescence filter (Chroma set 41043). For quantifying the number of holdfast-producing cells, a culture was inoculated to an
OD$_{660}$ of 0.002 and harvested when the OD$_{660}$ reached 0.05-0.1 to minimize the number of rosettes.

For staining attached cells, a glass coverslip was washed with ethanol and added to a 1mL PYE culture of the appropriate strain in a 12-well plate. The cultures were grown for 6-8hrs. Unattached cells were removed from the coverslip by washing under a stream of distilled water. One side of the glass was covered with a solution of 0.5 mg/mL fWGA in PYE, incubated for 5 min in the dark and washed under a stream of distilled water. The coverslip was placed stain side down on a glass slide and imaged with phase contrast and fluorescence as described above.

**Smooth lipopolysaccharide (SLPS) Immunoblotting**

Lysates for immunoblotting were prepared by the method of Walker et al(53). Pellets from saturated cultures were treated with DNase and lysozyme, mixed with SDS-running buffer and digested with proteinase K. Samples were separated on a Tris-glycine SDS-PAGE gel with 12% acrylamide and transferred to nitrocellulose. The amount of sample loaded was normalized to the final optical density of the culture at harvest. The membrane was blocked in 5% milk, probed with a 1 in 20,000 dilution of anti-SLPS serum raised in rabbit, washed, probed with HRP-conjugated goat-anti-rabbit antibody, washed again and visualized with peroxidase substrate(33). The top half of the membrane was removed before blocking and stained with Ponceau S as a loading control.

**Analysis of Rough LPS**

LPS was extracted by the method of Darveau and Hancock(54). Cells were isolated from saturated 50mL *C. crescentus* cultures grown in M2X by centrifugation, re-suspended in 2 mL of 10 mM Tris-HCl pH 8.0 containing 2 mM MgCl$_2$, and sonicated. DNaseI and RNaseA were added to 100 μg/mL
and 25 µg/mL, respectively and the lysate was incubated for 1 hour at 37°C. Additional DNaseI and RNAseA were added to 200 µg/mL and 50 µg/mL, respectively and the lysate was incubated for an additional 1 hour at 37°C. SDS and EDTA were added to 2% and 100 mM, respectively, and the lysate was incubated for 2 hours at 37°C. The solution was then centrifuged for 30 min at 50,000 x g. Proteinase K was added to 50 µg/mL in the supernatant and the solution was incubated for 2 hours at 60°C after which the LPS was precipitated with 2 volumes of ice-cold 0.375 M MgCl₂ in 95% EtOH and collected by centrifugation at 12,000 x g. The precipitate was re-suspended in 2% SDS containing 100mM EDTA, incubated overnight at 37°C, and re-precipitated with 0.375 M MgCl₂ in 95% EtOH. The precipitate was then suspended in 10 mM Tris-HCl pH 8.0 and centrifuged for 2 hours at 200,000 x g. The LPS pellets from each strain were suspended in SDS loading dye and separated on by Tris-Tricine SDS-PAGE on an 18% acrylamide gel containing 6M urea. LPS was stained using the periodate-silver method of Kittelberger and Hilbink(55).

Bacteriophage ΦCBK sensitivity

Saturated cultures of C. crescentus grown in PYE were diluted to OD₆₆₀=0.4 with PYE, serially diluted and spotted on PYE plates containing 0.15% xylose that had been spread with 1*10⁻⁷ PFU/mL ΦCBK or PYE plates with 0.15% xylose alone. The plates were incubated for 48hrs at 30°C and photographed.

Soft-agar swarming assay

Saturated cultures of C. crescentus grown in PYE were spotted in plates of PYE containing 0.3% agar and 0.15% xylose. The plates were incubated for 4 days at 30°C and photographed.

lacZ reporter assay
Cultures for measuring reporter activity were grown in M2X medium and the amount of culture required to achieve an $OD_{660}$ of 0.0005-0.00075 was added to fresh M2X medium. These cultures were grown to an $OD_{660}$ of 0.05-0.15 and β-galactosidase activity was measured as previously described(10).

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References

1. **Silhavy TJ, Kahne D, Walker S.** 2010. The Bacterial Cell Envelope. Cold Spring Harbor Perspectives in Biology 2:a000414–a000414.

2. **Whitfield C.** 2006. Biosynthesis and Assembly of Capsular Polysaccharides in Escherichia coli. Annu Rev Biochem 75:39–68.

3. **Joiner KA.** 1988. Complement evasion by bacteria and parasites. Annu Rev Microbiol 42:201–230.

4. **Roberson EB, Firestone MK.** 1992. Relationship between Desiccation and Exopolysaccharide Production in a Soil Pseudomonas sp. Appl Environ Microbiol 58:1284–1291.

5. **Geno KA, Gilbert GL, Song JY, Skovsted IC, Klugman KP, Jones C, Konradsen HB, Nahm MH.** 2015. Pneumococcal Capsules and Their Types: Past, Present, and Future. Clin Microbiol Rev 28:871–899.

6. **Whitfield C.** 2010. Polymerases: Glycan chain-length control. Nat Chem Biol 6:403–404.

7. **Poindexter JS.** 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol Rev 28:231–295.

8. **Degnen ST, Newton A.** 1972. Chromosome replication during development in Caulobacter crescentus. J Mol Biol 64:671–680.

9. **Henrici AT, Johnson DE.** 1935. Studies of Freshwater Bacteria: II. Stalked Bacteria, a New Order of Schizomycetes. J Bacteriol 30:61–93.

10. **Fiebig A, Herrou J, Fumeaux C, Radhakrishnan SK, Viollier PH, Crosson S.** 2014. A Cell Cycle and Nutritional Checkpoint Controlling Bacterial Surface Adhesion. PLoS Genet 10:e1004101–14.

11. **Purcell EB, Siegal-Gaskins D, Rawling DC, Fiebig A, Crosson S.** 2007. A photosensory two-component system regulates bacterial cell attachment. Proc Natl Acad Sci U S A 104:18241–18246.

12. **Li G, Brown PJB, Tang JX, Xu J, Quadrokus EM, Fuqua C, Brun YV.** 2011. Surface contact stimulates the just-in-time deployment of bacterial adhesins. Mol Microbiol 83:41–51.

13. **Ellison CK, Kan J, Dillard RS, Kysela DT, Ducret A, Berne C, Hampton CM, Ke Z, Wright ER, Biais N, Dalia AB, Brun YV.** 2017. Obstruction of pilus retraction stimulates bacterial surface sensing. Science 358:535–538.

14. **Hug I, Deshpande S, Sprecher KS, Pfohl T, Jenal U.** 2017. Second messenger-mediated tactile response by a bacterial rotary motor. Science 358:531–534.

15. **Kurtz HD Jr, Smith J.** 1992. Analysis of a Caulobacter crescentus gene cluster involved in attachment of the holdfast to the cell. J Bacteriol 174:687–694.
16. **Smith CS, Hinz A, Bodenmiller D, Larson DE, Brun YV.** 2003. Identification of Genes Required for Synthesis of the Adhesive Holdfast in Caulobacter crescentus. *J Bacteriol* 185:1432–1442.

17. **Toh E, Kurtz HD, Brun YV.** 2008. Characterization of the Caulobacter crescentus Holdfast Polysaccharide Biosynthesis Pathway Reveals Significant Redundancy in the Initiating Glycosyltransferase and Polymerase Steps. *J Bacteriol* 190:7219–7231.

18. **Wan Z, Brown PJB, Elliott EN, Brun YV.** 2013. The adhesive and cohesive properties of a bacterial polysaccharide adhesin are modulated by a deacetylase. *Mol Microbiol* 88:486–500.

19. **Samuel G, Reeves P.** 2003. Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. *Carbohydr Res* 338:2503–2519.

20. **Islam ST, Lam JS.** 2014. Synthesis of bacterial polysaccharides via the Wzx/Wzy-dependent pathway. *Can J Microbiol* 60:697–716.

21. **Hong Y, Reeves PR.** 2014. Diversity of O-Antigen Repeat Unit Structures Can Account for the Substantial Sequence Variation of Wzx Translocases. *J Bacteriol* 196:1713–1722.

22. **Merker RI, Smit J.** 1988. Characterization of the adhesive holdfast of marine and freshwater caulobacters. *Appl Environ Microbiol* 54:2078–2085.

23. **Hernando-Pérez M, Setayeshgar S, Hou Y, Temam R, Brun YV, Dragnea B, Berne C.** 2018. Layered Structure and Complex Mechanochemistry Underlie Strength and Versatility in a Bacterial Adhesive. *mBio* 9:e02359–17-19.

24. **Sprecher KS, Hug I, Nesper J, Potthoff E, Mahi M-A, Sangermani M, Kaever V, Schwede T, Vorholt J, Jenal U.** 2017. Cohesive Properties of the *Caulobacter crescentus* Holdfast Adhesin Are Regulated by a Novel c-di-GMP Effector Protein. *mBio* 8:e00294–17-15.

25. **Berne C, Ma X, Licata NA, Neves BRA, Setayeshgar S, Brun YV, Dragnea B.** 2013. Physiochemical Properties of Caulobacter crescentus Holdfast: A Localized Bacterial Adhesive. *J Phys Chem B* 117:10492–10503.

26. **Umbreit TH, Pate JL.** 1978. Characterization of the holdfast region of wild-type cells and holdfast mutants of Asticcacaulis biprosthecum. *Arch Microbiol* 118:157–168.

27. **Ong CJ, Wong ML, Smit J.** 1990. Attachment of the adhesive holdfast organelle to the cellular stalk of *Caulobacter crescentus*. *J Bacteriol* 172:1448–1456.

28. **van Opijnen T, Bodi KL, Camilli A.** 2009. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. *Nature Methods* 6:767–772.

29. **Wetmore KM, Price MN, Waters RJ, Lamson JS, He J, Hoover CA, Blow MJ, Bristow J, Butland G, Arkin AP, Deutschbauer A.** 2015. Rapid Quantification of Mutant Fitness in Diverse Bacteria by Sequencing Randomly Bar-Coded Transposons. *mBio* 6:e00306–15-15.
30. Awram P, Smit J. 2001. Identification of lipopolysaccharide O antigen synthesis genes required for attachment of the S-layer of Caulobacter crescentus. Microbiology 147:1451–1460.

31. Jiang XM, Neal B, Santiago F, Lee SJ, Romana LK, Reeves PR. 1991. Structure and sequence of the rfb (O antigen) gene cluster of Salmonella serovar typhimurium (strain LT2). Mol Microbiol 5:695–713.

32. Hardy GG, Toh E, Berne C, Brun YV. 2018. Mutations in Sugar-Nucleotide Synthesis Genes Restore Holdfast Polysaccharide Anchoring to Caulobacter crescentus Holdfast Anchor Mutants. J Bacteriol 200:e00597–17–24.

33. Walker SG, Karunaratne DN, Ravenscroft N, Smit J. 1994. Characterization of mutants of Caulobacter crescentus defective in surface attachment of the paracrystalline surface layer. J Bacteriol 176:6312–6323.

34. Dingwall A, Gober JW, Shapiro L. 1990. Identification of a Caulobacter basal body structural gene and a cis-acting site required for activation of transcription. J Bacteriol 172:6066–6076.

35. Christen M, Beusch C, Bösch Y, Cerletti D, Flores-Tinoco CE, Del Medico L, Tschan F, Christen B. 2016. Quantitative Selection Analysis of Bacteriophage φCbK Susceptibility in Caulobacter crescentus. J Mol Biol 428:419–430.

36. Haiko J, Westerlund-Wikström B. 2013. The Role of the Bacterial Flagellum in Adhesion and Virulence. Biology 2:1242–1267.

37. Wang Y, Haitjema CH, Fuqua C. 2014. The Ctp type IVb pilus locus of Agrobacterium tumefaciens directs formation of the common pili and contributes to reversible surface attachment. J Bacteriol, 2nd ed. 196:2979–2988.

38. Aldridge P, Paul R, Goymer P, Rainey P, Jenal U. 2003. Role of the GGDEF regulator PleD in polar development of Caulobacter crescentus. Mol Microbiol 47:1695–1708.

39. Levi A, Jenal U. 2006. Holdfast Formation in Motile Swarmer Cells Optimizes Surface Attachment during Caulobacter crescentus Development. J Bacteriol 188:5315–5318.

40. Berne C, Ellison CK, Agarwal R, Severin GB, Fiebig A, Morton RI III, Waters CM, Brun YV. 2018. Feedback regulation of Caulobacter crescentusholdfast synthesis by flagellum assembly via the holdfast inhibitor HfiA. Mol Microbiol 9:e1003744–20.

41. Price MN, Wetmore KM, Waters RJ, Callaghan M, Ray J, Liu H, Kuehl JV, Melnyk RA, Lamson JS, Suh Y, Carlson HK, Esquivel Z, Sadeeshkumar H, Chakraborty R, Zane GM, Rubin BE, Wall JD, Visel A, Bristow J, Blow MJ, Arkin AP, Deutschbauer AM. 2018. Mutant phenotypes for thousands of bacterial genes of unknown function. Nature 557:503–509.

42. Hoffman MD, Zucker LI, Brown PJB, Kysela DT, Brun YV, Jacobson SC. 2015. Timescales and Frequencies of Reversible and Irreversible Adhesion Events of Single Bacterial Cells. Anal Chem 87:12032–12039.
Nesper J, Hug I, Kato S, Hee C-S, Habazettl JM, Manfredi P, Grzesiek S, Schirmer T, Emonet T, Jenal U. 2017. Cyclic di-GMP differentially tunes a bacterial flagellar motor through a novel class of CheY-like regulators. eLife 6:1–25.

Leclerc G, Wang SP, Ely B. 1998. A new class of Caulobacter crescentus flagellar genes. J Bacteriol 180:5010–5019.

O'Riordan K, Lee JC. 2004. Staphylococcus aureus Capsular Polysaccharides. Clin Microbiol Rev 17:218–234.

Stenutz R, Weintraub A, Widmalm G. 2006. The structures of Escherichia coli O-polysaccharide antigens. FEMS Microbiology Reviews 30:382–403.

Olivier NB, Chen MM, Behr JR, Imperiali B. 2006. In Vitro Biosynthesis of UDP- N, N'-Diacetylbacillosamine by Enzymes of the Campylobacter jejuni General Protein Glycosylation System †. Biochemistry 45:13659–13669.

Mostafavi AZ, Troutman JM. 2013. Biosynthetic Assembly of the Bacteroides fragilis Capsular Polysaccharide A Precursor Bactoprenyl Diphosphate-Linked Acetamido-4-amino-6-deoxygalactopyranose. Biochemistry 52:1939–1949.

Ely B. 1991. Genetics of Caulobacter crescentus. Meth Enzymol 204:372–384.

Thanbichler M, Iniesta AA, Shapiro L. 2007. A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in Caulobacter crescentus. Nucleic Acids Research 35:e137–e137.

Hentchel KL, Ruiz LMR, Curtis PD, Fiebig A, Coleman ML, Crosson S. 2018. Genome-scale fitness profile of Caulobacter crescentus grown in natural freshwater. The ISME Journal 1–14.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868.

Walker SG, Smith SH, Smit J. 1992. Isolation and comparison of the paracrystalline surface layer proteins of freshwater caulobacters. J Bacteriol 174:1783–1792.

Darveau RP, Hancock RE. 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough Pseudomonas aeruginosa and Salmonella typhimurium strains. J Bacteriol 155:831–838.

Kittelberger R, Hilbink F. 1993. Sensitive silver-staining detection of bacterial lipopolysaccharides in polyacrylamide gels. J Biochem Biophys Methods 26:81–86.
Figure 1 A genome-wide screen for holdfast biosynthesis genes identifies multiple classes of mutants affecting adhesion

A) During the dimorphic C. crescentus life-cycle, each cell division produces a motile swarmer cell and a sessile stalked cell. Swarmer cells stop swimming, shed their flagellum and pili and develop into stalked cells before dividing. Stalked cells adhere strongly to exogenous surfaces using a specialized material called the holdfast. B) The holdfast can be visualized by staining with fluorescently labeled wheat germ agglutinin (fWGA) and biofilm formation can be quantified by crystal violet staining. ΔhfiA cells overproduce holdfast and are hyper-adhesive. ΔhfsJ cells do not produce holdfasts and are non-adhesive. Attachment is reduced in minimal medium due to high levels of hfiA expression. C) Fitness profiles for the 250 genes with the strongest adhesion phenotypes in the cheesecloth passaging experiment. Lines are drawn to connect mean fitness scores at days zero through five for each of the 250 genes. Genes in the four major fitness clusters are colored according to the legend, with a specific example listed. Genes shown in black do not fit into the any of the four clusters.
Figure 2 Disrupting SLPS production leads to ectopic adhesion
A) Fitness profiles for genes in the SLPS cluster. A full list of these genes and their annotations is provided in Table S3. wbqP was not characterized in our library due to low insertion density in this region. B) Surface attachment of SLPS mutants measured by CV staining. Cultures were grown for 24 hours in M2X medium before staining surface attached cells. Disrupting SLPS leads to increased adhesion in a holdfast-independent fashion. The graph shows the average (± standard deviation) of five biological replicates. Statistical significance was assessed by ANOVA with a pairwise Dunnett’s post test to determine which samples differed from wild type. ns – not significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. C) Smooth LPS production is disrupted in ΔCC_0465, ΔCC_2301 and ΔrfbB. Top: western blot to detect SLPS. Middle: Total protein stained with ponceau S as a loading control for SLPS blot. Bottom: Coomassie staining of spent medium from the cultures. Each mutant shows a loss of or a decrease in SLPS production by western blot and releases the S-layer protein RsaA into the spent medium. The cell-surface defects can be complemented by ectopic expression of the appropriate gene. “Empty” refers to plasmid control strains without inserts. D) FwGA staining (shown in red) shows ectopic adhesion in the ΔwbqP mutant. The rosettes in ΔwbqP cells are more compact than wild-type and have a different shape. ΔhfsJ ΔwbqP cells aggregate despite the lack of holdfasts and this aggregation occurs throughout the cell surface.
Figure 3 Disrupting polar appendages stimulates holdfast production

A) Fitness profiles for genes in the polar appendage cluster. A full list of these genes and their annotations is provided in Table S3. B) Surface attachment of motility mutants measured by CV staining. Cultures were grown for 17 hours in M2X medium before staining surface attached cells. Deletion of the genes for either the outer-membrane flagellar base protein FlgH or the inner-membrane type IV pilus component CpaH caused increased adhesion. In both mutant backgrounds, hfsJ is required for attachment. The graph shows the average (± standard deviation) of five biological replicates. C) Effect of hfiA and pleD deletions on surface attachment of cpaH and flgH mutants. ΔhfiA does not affect adhesion in the ΔflgH background and increases adhesion in the ΔcpaH background. The increased adhesion in both the ΔflgH and ΔcpaH mutants can be eliminated by deletion of pleD. The graph shows the average (± standard deviation) of four biological replicates. D) PflgH-lacZ reporter activity in motility mutants. The chart shows the average (± standard deviation) of four biological replicates. Statistical significance was assessed by ANOVA with a pairwise Dunnett’s post test to determine which samples differed from wild type. nd – not detected; ns – not significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. When necessary, P values for additional pairwise comparisons pertinent to interpretation are indicated in the text.
Figure 4 Opposing effects of pilus mutants on adhesion

A) Fitness profiles for genes at the pilus assembly locus. A full list of these genes and their annotations is provided in Table S3. B) Surface attachment of pilus mutants measured by CV staining. Cultures were grown for 17 hours in M2X medium before staining. Deletion of the gene for the main pilin subunit (PilA) reduces adhesion. ΔpilA is epistatic to ΔcpaH but not ΔflgH. The graph shows the average (± standard deviation) of seven biological replicates. C) Effect of hfiA and pleD deletions on surface attachment in the ΔpilA background. Staining is slightly lower in ΔhfiA ΔpilA than ΔhfiA reflecting the holdfast-independent defect in surface attachment when the pilus is disrupted. pleD has no effect on adhesion in the ΔpilA mutant. The graph shows the average (± standard deviation) of six biological replicates. D) P_hfiA-lacZ reporter activity in various pilA mutants. The chart shows the average (± standard deviation) of four biological replicates. Statistical significance was assessed by ANOVA with a pairwise Dunnett’s post test to determine which samples differed from wild type. ns = not significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. When necessary, P values for additional pairwise comparisons pertinent to interpretation are indicated in the text.
Figure 5 New holdfast biosynthesis factors

A) Fitness profiles for genes in the hfs (magenta) and holdfast modification (orange) clusters. Full lists of these genes and their annotations are provided in Table S3. B) Surface attachment of putative holdfast mutants measured by CV staining. Cultures were grown for 24 hours in PYE medium before being stained. ∆CCNA_01242 and ∆CCNA_02722 displayed reduced staining, and ∆CCNA_02360 was non-adhesive. The graph shows the average (± standard deviation) of five biological replicates. C) Analysis of holdfast phenotypes by fWGA staining. The top panels show overlays of phase contrast and fluorescence images after staining planktonic cells as described in materials and methods. Adherent cells from the slide attachment assay are shown as phase contrast images in the middle set of panel, and the fluorescence channel showing attached holdfast material from the same slides is represented in the bottom panels. ∆CCNA_01242 does not have an apparent holdfast defect, ∆CCNA_02722 has a holdfast attachment defect and ∆CCNA_02360 does not produce holdfasts. Statistical significance was assessed by ANOVA with a pairwise Dunnett’s
post test to determine which samples differed from wild type. nd – not detected; ns – not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. 

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Figure 6 Updated model for holdfast biosynthesis

The model shows a wzy-type polysaccharide biosynthesis pathway. Four glycosyltransferases, HfsE, HfsJ, HfsG, and the newly described HfsL add monosaccharides sequentially onto the UP carrier to produce a glycolipid repeating unit. This intermediate is flipped across the membrane by HfsF, polymerized by HfsC and exported by a putative HfsABD transevelope complex. Attachment of the holdfast matrix is mediated by the Hfa proteins, including the newly-identified HfaE, reported here. Disruptions to the flagellum or the pilus activate holdfast production by relieving the inhibition of HfsJ by HfiA.
Supplemental Materials

A genome-wide analysis of adhesion in *Caulobacter crescentus* identifies new regulatory and biosynthetic components for holdfast assembly

David M. Hershey, Aretha Fiebig and Sean Crosson

| Strains | Organism | Genotype | Description | Source |
|---------|----------|----------|-------------|--------|
| FC19    | *C. crescentus* CB15 | CB15     | Wild-type   | ATCC 19089 |
| FC1365  | *C. crescentus* CB15 | ΔhfiA    | In-frame deletion of CC_0817 | Ref. 10 |
| FC1974  | *C. crescentus* CB15 | ΔhfsJ    | In-frame deletion of CC_0095 | Ref. 10 |
| FC3105  | *C. crescentus* CB15 | ΔpleD    | In-frame deletion of CC_2462 | This work |
| FC3054  | *C. crescentus* CB15 | ΔCCNA_00497 | In-frame deletion of CC_0465 | This work |
| FC3055  | *C. crescentus* CB15 | ΔCCNA_02386 | In-frame deletion of CC_2301 | This work |
| FC3056  | *C. crescentus* CB15 | ΔrfbB    | In-frame deletion of CC_3629 | This work |
| FC3057  | *C. crescentus* CB15 | ΔwbqP    | In-frame deletion of CC_1486 | This work |
| FC3058  | *C. crescentus* CB15 | ΔhfsJ ΔwbqP | In-frame deletion of CC_1486 in FC1974 background | This work |
| FC1266  | *C. crescentus* CB15 | ΔflgH    | In-frame deletion of CC_2066 | This work |
| FC3013  | *C. crescentus* CB15 | ΔcpaH    | In-frame deletion of CC_2940 | This work |
| FC1265  | *C. crescentus* CB15 | ΔpilA    | In-frame deletion of CC_2948 | This work |
| FC3019  | *C. crescentus* CB15 | ΔCCNA_01242 | In-frame deletion of CC_1184 | This work |
| FC3021  | *C. crescentus* CB15 | ΔhfsL    | In-frame deletion of CC_2277 | This work |
| FC3020  | *C. crescentus* CB15 | ΔhfaE    | In-frame deletion of CC_2639 | This work |
| FC3015  | *C. crescentus* CB15 | ΔhfsJ ΔflgH | In-frame deletion of CC_2066 in FC1974 background | This work |
| FC3016  | *C. crescentus* CB15 | ΔhfsJ ΔcpaH | In-frame deletion of CC_2940 in FC1974 background | This work |
| FC3107  | *C. crescentus* CB15 | ΔflgH ΔcpaH | In-frame deletion of CC_2940 in FC1266 | This work |
| FC3085  | *C. crescentus* CB15 | ΔflgH ΔhfiA | In-frame deletion of CC_0817 in FC1266 background | This work |
| FC3083  | *C. crescentus* CB15 | ΔcpaH ΔhfiA | In-frame deletion of CC_0817 in FC3013 background | This work |
| FC3084  | *C. crescentus* CB15 | ΔpilA ΔhfiA | In-frame deletion of CC_0817 in FC1265 background | This work |
| FC3104  | *C. crescentus* CB15 | ΔflgH ΔpleD | In-frame deletion of CC_2462 in FC1266 background | This work |
| FC3103  | *C. crescentus* CB15 | ΔcpaH ΔpleD | In-frame deletion of CC_2462 in FC3013 background | This work |
| FC3108  | *C. crescentus* CB15 | ΔpilA ΔpleD | In-frame deletion of CC_2462 in FC1265 background | This work |
| FC3017  | *C. crescentus* CB15 | ΔflgH ΔpilA | In-frame deletion of CC_2948 in FC1266 background | This work |
| Strain | Organism | Insertion | Description | Reference |
|--------|----------|-----------|-------------|-----------|
| FC3018 | C. crescentus CB15 | ΔcpaH ΔpilA | In-frame deletion of CC_2948 in FC3013 background | This work |
| FC3097 | C. crescentus CB15 | ΔCCNA_00497 xyl::P<sub>xyl</sub>-empty | pMT585 integrated at xylose locus of FC3054 | This work |
| FC3098 | C. crescentus CB15 | ΔCCNA_00497 xyl::P<sub>xyl</sub>-CCNA_00497 | pFC3080 integrated at xylose locus of FC3054 | This work |
| FC3101 | C. crescentus CB15 | ΔCCNA_02386 xyl::P<sub>xyl</sub>-empty | pMT585 integrated at xylose locus of FC3055 | This work |
| FC3102 | C. crescentus CB15 | ΔCCNA_02386 xyl::P<sub>xyl</sub>-CCNA_02386 | pFC3082 integrated at xylose locus of FC3055 | This work |
| FC3095 | C. crescentus CB15 | ΔrfbB xyl::P<sub>xyl</sub>-empty | pMT585 integrated at xylose locus of FC3056 | This work |
| FC3096 | C. crescentus CB15 | ΔrfbB xyl::P<sub>xyl</sub>-rfbB | pFC3079 integrated at xylose locus of FC3056 | This work |
| FC3099 | C. crescentus CB15 | ΔwbqP xyl::P<sub>xyl</sub>-empty | pMT585 integrated at xylose locus of FC3057 | This work |
| FC3100 | C. crescentus CB15 | ΔwbqP xyl::P<sub>xyl</sub>-wbqP | pFC3081 integrated at xylose locus of FC3057 | This work |
| FC3075 | C. crescentus CB15 | ΔflgH xyl::P<sub>flgE</sub>-empty | pFC3094 integrated at xylose locus of FC1266 | This work |
| FC3074 | C. crescentus CB15 | ΔflgH xyl::P<sub>flgE</sub>-flgH | pFC3093 integrated at xylose locus of FC1266 | This work |
| FC3070 | C. crescentus CB15 | ΔcpaH xyl::P<sub>xyl</sub>-empty | pMT585 integrated at xylose locus of FC3013 | This work |
| FC3071 | C. crescentus CB15 | ΔcpaH xyl::P<sub>xyl</sub>-cpaH | pFC3090 integrated at xylose locus of FC3013 | This work |
| FC3073 | C. crescentus CB15 | ΔpilA xyl::P<sub>pilA</sub>-empty | pFC3092 integrated at xylose locus of FC1265 | This work |
| FC3072 | C. crescentus CB15 | ΔpilA xyl::P<sub>pilA</sub>-pilA | pFC3091 integrated at xylose locus of FC1265 | This work |
| FC3126 | C. crescentus CB15 | ΔCCNA_01242 xyl::P<sub>CCNA_01242</sub>-empty | pFC3124 integrated at xylose locus of FC3019 | This work |
| FC3127 | C. crescentus CB15 | ΔCCNA_01242 xyl::P<sub>CCNA_01242</sub>-CCNA_01242 | pFC3125 integrated at xylose locus of FC3019 | This work |
| FC3088 | C. crescentus CB15 | ΔhfsL xyl::P<sub>xyl</sub>-empty | pMT585 integrated at xylose locus of FC3021 | This work |
| FC3089 | C. crescentus CB15 | ΔhfsL xyl::P<sub>xyl</sub>-hfsL | pFC3078 integrated at xylose locus of FC3021 | This work |
| FC3086 | C. crescentus CB15 | ΔhfaE xyl::P<sub>xyl</sub>-empty | pMT585 integrated at xylose locus of FC3020 | This work |
| FC3087 | C. crescentus CB15 | ΔhfaE xyl::P<sub>xyl</sub>- | pFC3077 integrated at xylose | This work |
### hfaE locus of FC3020

| APA_752 | E. coli WM3064 | Tn-HiMar (Km<sup>R</sup>) | Bacoded transposon pool for created BarSeq libraries | Ref. 29 |

| **Plasmids** | **Description** | **Antibiotic** | **Reference** |
|---------------|-----------------|----------------|--------------|
| pNPTS138      | Suicide plasmid for making unmarked deletions in *C. crescentus*; carries sacB for counter-selection | Km | M. R Alley unpublished |
| pFC3059       | To delete CCNA_01242; contains fusion of CC_1184 flanking regions with first and last 12 nucleotides of CC_1184 ORF included | Km | This work |
| pFC3060       | To delete hfaE; contains fusion of CC_2639 flanking regions with first and last 12 nucleotides of CC_2639 ORF included | Km | This work |
| pFC3061       | To delete hfsL; contains fusion of CC_2277 flanking regions with first 12 and last 96 nucleotides of CC_2277 ORF included | Km | This work |
| pFC3063       | To delete CCNA_00497; contains fusion of CC_0465 flanking regions with first and last 12 nucleotides of CC_0465 ORF included | Km | This work |
| pFC3062       | To delete CCNA_02386; contains fusion of CC_2301 flanking regions with first and last 12 nucleotides of CC_2301 ORF included | Km | This work |
| pFC3068       | To delete rfbB; contains fusion of CC_3629 flanking regions with first and last 12 nucleotides of CC_3629 ORF included | Km | This work |
| pFC3069       | To delete wbqP; contains fusion of CC_1486 flanking regions with first and last 12 nucleotides of CC_1486 ORF included | Km | This work |
| pFC1267       | To delete pilA; contains fusion of CC_2948 flanking regions with first and last 12 nucleotides of CC_2948 ORF included | Km | This work |
| pFC1268       | To delete flgH; contains fusion of CC_2066 flanking regions with first and last 12 nucleotides of CC_2066 ORF included | Km | This work |
| pFC3065       | To delete cpaH; contains fusion of CC_2940 flanking regions with first and last 12 nucleotides of CC_2940 ORF included | Km | This work |
| pFC3067       | To delete pldD; contains fusion of CC_2462 flanking regions with first and last 12 nucleotides of CC_2462 ORF included | Km | This work |
| pMT585        | Contains multiple cloning site downstream of PxyI; integrates at upstream of *xylX*; used for complementations | Km | Ref. 50 |
| pFC3077       | pMT585 containing hfaE under the control of *P*<sub>xyI</sub> for | Km | This work |
Table S1 Strains and plasmids used in this study

| Plasmid     | Description                                                                 |
|-------------|-----------------------------------------------------------------------------|
| pFC3078     | pMT585 containing *hfsL* under the control of *P*\_xyl for integration at xylX locus | Km | This work |
| pFC3080     | pMT585 containing *CCNA_00497* under the control of *P*\_xyl for integration at xylX locus | Km | This work |
| pFC3082     | pMT585 containing *CCNA_02386* under the control of *P*\_xyl for integration at xylX locus | Km | This work |
| pFC3079     | pMT585 containing *rfbB* under the control of *P*\_xyl for integration at xylX locus | Km | This work |
| pFC3081     | pMT585 containing *wbqP* under the control of *P*\_xyl for integration at xylX locus | Km | This work |
| pFC3091     | pMT585 containing *pilA* under the control of *P*\_pilA for integration at xylX locus; 316bp upstream of *CC_2948* fused to the *CC_2948* ORF was inserted in reverse orientation into pMT585 | Km | This work |
| pFC3092     | pMT585 containing *pilA* without insert for integration at xylX locus; 316bp upstream of *CC_2948* was inserted in reverse orientation into pMT585 | Km | This work |
| pFC3093     | pMT585 containing *flgH* under the control of *P*\_flgF for integration at xylX locus; 226bp upstream of *CC_2063* fused to the *CC_2066* ORF was inserted in reverse orientation into pMT585 | Km | This work |
| pFC3094     | pMT585 containing *flgE* without insert for integration at xylX locus; 216bp upstream of *CC_2063* was inserted in reverse orientation into pMT585 | Km | This work |
| pFC3090     | pMT585 containing *cpaH* under the control of *P*\_xyl for integration at xylX locus | Km | This work |
| pFC3124     | pMT585 containing *CCNA_01242* without insert for integration at xylX locus; 99bp upstream of the *CC_1184* ORF was inserted in reverse orientation into pMT585 | Km | This work |
| pFC1948     | pRKlac290 containing the *hfiA* promoter fused to *lacZ*                      | Tet | Ref. 10 |

To analyze the sequence data used for fitness calculations, we used the *C. crescentus* NA1000 genome. NA1000 is directly derived from CB15, but its genome has more detailed annotations and is better curated. To facilitate interpretation of the BarSeq data, genotypes in the text and supplemental tables use the NA1000 locus numbers and nomenclature. We note the corresponding CB15 numbers in the “Description” column.
| Sample name | Index | Sample description | Total reads | Usable reads | Unique barcodes | Proportion of reads in top percentile |
|-------------|-------|--------------------|-------------|-------------|-----------------|--------------------------------------|
| SCD_50.31   | CACGAT| PYE 1A             | 4018890     | 3698106     | 233280          | 0.117                                |
| SCD_50.32   | CACTCA| PYE 1B             | 4492543     | 4130359     | 246118          | 0.1227                               |
| SCD_50.33   | CAGGCG| PYE 1C             | 4449306     | 4044087     | 150780          | 0.1236                               |
| SCD_50.34   | CATGGC| PYE 2A             | 4137845     | 3779486     | 226993          | 0.1249                               |
| SCD_50.35   | CATTTT| PYE 2B             | 4499135     | 4137664     | 228359          | 0.1274                               |
| SCD_50.36   | CCAACA| PYE 2C             | 4213003     | 3750243     | 228855          | 0.1316                               |
| SCD_50.37   | CCGAAT| PYE 3A             | 6735128     | 6171057     | 262618          | 0.1504                               |
| SCD_50.38   | CTAGCT| PYE 3B             | 6127591     | 5614129     | 249692          | 0.1471                               |
| SCD_50.39   | CTATAC| PYE 3C             | 5322215     | 4857843     | 235579          | 0.1571                               |
| SCD_50.40   | CTCAGA| PYE 4A             | 4562846     | 4108435     | 242916          | 0.1455                               |
| SCD_50.41   | GACGAC| PYE 4B             | 6394697     | 5855783     | 248801          | 0.1691                               |
| SCD_50.42   | TAATCG| PYE 4C             | 2975382     | 2755150     | 172812          | 0.1675                               |
| SCD_50.43   | TACGAC| PYE 5A             | 5799597     | 5301223     | 246151          | 0.2002                               |
| SCD_50.44   | TATAAT| PYE 5B             | 6016430     | 5569218     | 234947          | 0.1972                               |
| SCD_50.45   | TACTTC| PYE 5C             | 4811202     | 4428232     | 209890          | 0.2178                               |
| SCD_50.46   | TCCCCA| Cheese 1A          | 4562846     | 4108435     | 242916          | 0.1455                               |
| SCD_50.47   | TCGAAG| Cheese 1B          | 4313848     | 3922295     | 233599          | 0.1484                               |
| SCD_50.48   | TCGGCA| Cheese 1C          | 4773752     | 4335186     | 247032          | 0.1516                               |
| SCD_50.49   | AAACAT| Cheese 2A          | 6050903     | 5557666     | 232487          | 0.2488                               |
| SCD_50.50   | AAAGCA| Cheese 2B          | 7013967     | 6384659     | 253431          | 0.2793                               |
| SCD_50.51   | AAATGC| Cheese 2C          | 4421479     | 4054218     | 211068          | 0.2713                               |
| SCD_50.52   | AAGCGA| Cheese 3A          | 6616953     | 5904400     | 235379          | 0.4118                               |
| SCD_50.53   | AAATTC| Cheese 3B          | 5935535     | 5450038     | 209707          | 0.4585                               |
| SCD_50.54   | AAGACT| Cheese 3C          | 7750956     | 7158882     | 204209          | 0.5887                               |
| SCD_50.55   | AAGCGA| Cheese 4A          | 4002747     | 3664336     | 167909          | 0.6349                               |
| SCD_50.56   | AAGGAC| Cheese 4B          | 3156990     | 2902069     | 151371          | 0.605                                |
| SCD_50.57   | AATAGG| Cheese 4C          | 1882499     | 1730719     | 115437          | 0.6777                               |
| SCD_50.58   | ACAAGC| Cheese 5A          | 2475323     | 2197424     | 99108           | 0.787                                |
| SCD_50.59   | ACATTG| Cheese 5B          | 2419554     | 2241238     | 99833           | 0.7796                               |
| SCD_50.60   | ACCAGG| Cheese 5C          | 2865211     | 2583906     | 107021          | 0.8014                               |

**Table S2** Samples used for BarSeq analysis of gene fitness across cheesecloth passages

The "Index" column represents the TruSeq indices used to de-multiplex the samples after sequencing. Cheese and PYE represent passages with and without cheesecloth, respectively. The numbers indicate which of the five passages each sample represents, and the letters indicate which of the three replicates. The "Proportion of reads in top percentile" column is calculated by ranking the barcodes in each sample by their abundance and determining the proportion of reads that map to the top 1% of barcodes.
| Cluster 1: SLPS | locusid; annotation | 0  | 1  | 2  | 3  | 4  | 5  |
|----------------|----------------------|----|----|----|----|----|----|
| CCNA_00044; ribosome maturation protein RimP | 0.013 | -2.93 | -4.145 | -3.319 | -1.57 | 0.067 | |
| CCNA_00217; thiol:disulfide interchange protein DsbD | -0.07 | -2.488 | -2.869 | -1.883 | 0.313 | 1.215 | |
| CCNA_00290; autotransporter protein | 0.009 | -3.141 | -3.74 | -3.294 | 2.788 | 1.794 | |
| CCNA_00390; ADP-heptose--LPS heptosyltransferase | -0.008 | -2.321 | -4.402 | -2.913 | 2.413 | 1.408 | |
| CCNA_00497; putative rhamnosyl transferase | -0.074 | -3.219 | -3.706 | -2.835 | -1.19 | 0.371 | |
| CCNA_00502; glycosyl transferase family protein | 0.024 | -3.405 | -3.06 | -3.883 | 2.296 | 1.313 | |
| CCNA_00512; GTP-binding protein, probable translation factor | 0.082 | -3.203 | -3.936 | -3.087 | 1.455 | 0.104 | |
| CCNA_00519; conserved hypothetical protein | 0.009 | -4.406 | -4.912 | -4.798 | 3.134 | 1.521 | |
| CCNA_00667; lipopolysaccharide biosynthesis protein | -0.194 | -2.497 | -2.358 | -1.846 | 0.306 | 1.286 | |
| CCNA_00668; capsular polysaccharide biosynthesis protein | -0.001 | -2.031 | -3.171 | -3.547 | 1.836 | 1.285 | |
| CCNA_00669; glycosyltransferase family 99 protein WbsX | -0.022 | -3.396 | -3.205 | -2.343 | 0.788 | 0.792 | |
| CCNA_01055; GT1 family glycosyl transferase | -0.043 | -3.088 | -4.106 | -3.578 | -2.06 | 0.533 | |
| CCNA_01062; GDP-mannose 4,6 dehydratase | -0.117 | -3.685 | -4.072 | -3.613 | 1.995 | 0.595 | |
| CCNA_01063; UDP-perosamine 4-acetyl transferase | -0.038 | -3.41 | -2.969 | -2.665 | 0.986 | 0.2 | |
| CCNA_01064; perosamine synthetase | -0.112 | -3.481 | -4 | -3.214 | 1.594 | 0.064 | |
| CCNA_01065; glycosyltransferase | -0.076 | -3.761 | -3.547 | -3.178 | 1.581 | 0.043 | |
| CCNA_01066; glycosyltransferase | -0.038 | -2.802 | -3.277 | -2.57 | 1.632 | 0.394 | |
| CCNA_01068; glycosyltransferase | -0.15 | -4.496 | -3.787 | -3.662 | 1.324 | 0.508 | |
| CCNA_01086; GTP-binding protein lepA | 0.043 | -3.449 | -3.931 | -3.509 | 2.112 | -0.57 | |
| CCNA_01103; ADP-heptose--LPS heptosyltransferase | 0.034 | -2.691 | -2.823 | -3.069 | 1.778 | 0.077 | |
| CCNA_01199; glucose-1-phosphate thymidyltransferase | -0.008 | -4.037 | -3.851 | -3.099 | 1.424 | 0.062 | |
| CCNA_01375; lactoylglutathione lyase | 0.048 | -3.392 | -4.654 | -4.066 | - | - |
| Accession       | Protein Name                                      | Value 1 | Value 2 | Value 3 | Value 4 | Value 5 | Value 6 |
|-----------------|--------------------------------------------------|---------|---------|---------|---------|---------|---------|
| CCNA_01427;     | beta-barrel assembly machine (BAM) protein BamE | 0.052   | -2.633  | -4.028  | -4.39   | 2.727   | 1.235   |
| CCNA_01430;     | conserved hypothetical protein                   | -0.013  | -1.923  | -2.959  | -3.144  | -2.08   | 0.903   |
| CCNA_01447;     | homoserine dehydrogenase                         | 0.02    | -2.348  | -3.724  | -2.91   | 1.286   | 0.261   |
| CCNA_01497;     | ADP-L-glycero-D-manno-heptose-6-epimerase         | 0.022   | -2.19   | -2.593  | -2.199  | -1.98   | -0.42   |
| CCNA_01955;     | zinc metalloprotease                             | 0.005   | -2.151  | -2.316  | -2.232  | 1.865   | 1.314   |
| CCNA_01971;     | peptidyl-prolyl cis-trans isomerase               | 0.013   | -2.17   | -2.493  | -1.791  | 0.927   | 0.591   |
| CCNA_02219;     | hypothetical protein                              | 0.017   | -2.85   | -2.949  | -2.521  | 2.268   | 1.687   |
| CCNA_02326;     | acetylornithine aminotransferase/succinylidaminopimelate aminotransferase | 0.019   | -1.712  | -2.166  | -1.194  | 0.525   | 0.791   |
| CCNA_02347;     | phosphomannomutase/phosphoglucomutase            | -0.064  | -4.09   | -4.85   | -4.033  | 2.455   | 0.889   |
| CCNA_02386;     | O-antigen ligase related enzyme                   | -0.134  | -2.762  | -3.457  | -2.585  | 1.025   | 0.537   |
| CCNA_02463;     | UDP-N-acetylglucosamine 4-epimerase               | 0.029   | -2.627  | -3.781  | -3.758  | 2.374   | 0.882   |
| CCNA_02650;     | N-acetyl-anhydromuramyl-L-alanine amidase         | -0.018  | -1.617  | -3.035  | -2.194  | 0.678   | 0.911   |
| CCNA_02941;     | transcription elongation factor greA              | -0.119  | -1.233  | -2.748  | -2.498  | -0.92   | 0.629   |
| CCNA_03026;     | two-component response regulator petR            | -0.055  | -2.619  | -4.063  | -3.652  | 2.577   | 1.705   |
| CCNA_03195;     | RNA polymerase sigma factor RpoH                 | -0.001  | -2.089  | -3.735  | -3.307  | 1.951   | 1.294   |
| CCNA_03352;     | YebC/PmpR transcriptional regulator              | -0.009  | -3.942  | -4.169  | -3.705  | 2.805   | 1.706   |
| CCNA_03475;     | homoserine kinase                                 | -0.028  | -1.569  | -3.168  | -2.323  | 0.697   | 0.84    |
| CCNA_03609;     | outer membrane protein                           | -0.011  | -2.28   | -3.936  | -3.203  | 1.489   | 0.261   |
| CCNA_03705;     | conserved hypothetical protein                   | 0.053   | -1.304  | -3.383  | -1.826  | 0.864   | 0.665   |
| CCNA_03713;     | RNA polymerase sigma-54 factor rpoN              | 0.02    | -1.566  | -3.418  | -2.288  | 1.299   | 0.198   |
| CCNA_03733;     | mannose-1-phosphate guanylyltransferase          | -0.137  | -2.754  | -3.111  | -2.202  | 1.085   | 0.29    |
| Locus ID; Annotation | Cluster 2: Polar Appendages |
|----------------------|---------------------------|
| CCNA_03744; dTDP-glucose 4,6-dehydratase | -0.009 | -2.964 | -3.124 | -1.88 | 1.002 | 0.388 |
| CCNA_03748; dTDP-4-dehydrorhamnose 3,5-epimerase | -0.059 | -2.155 | -2.242 | -1.967 | 0.249 | 0.731 |
| CCNA_03859; two-component response regulator cenR | 0.01 | -1.898 | -3.244 | -2.126 | 0.879 | 0.579 |
| CCNA_03909; conserved hypothetical protein | 0.028 | -2.113 | -4.213 | -2.917 | 1.893 | 0.234 |
| CCNA_03984; hypothetical protein | 0.007 | -1.779 | -2.747 | -2.011 | 0.292 | 0.697 |
| Cluster 2: Polar Appendages | locUsID; Annotation | 0 | 1 | 2 | 3 | 4 | 5 |
| CCNA_00233; UDP-N-acetylglucosamine 4,6-dehydratase | 0.008 | -0.398 | -0.647 | -1.464 | 1.536 | 1.994 |
| CCNA_00234; WecE-family cell wall biogenesis enzyme | 0.005 | -0.587 | -1.119 | -1.518 | 1.539 | -2.71 |
| CCNA_00444; chemotaxis protein methyltransferase | -0.017 | -0.73 | -1.261 | -1.337 | -1.85 | -0.249 |
| CCNA_00447; chemotaxis protein cheD | -0.008 | -0.393 | -1.262 | -1.868 | 2.689 | 2.926 |
| CCNA_00449; cheYIII | -0.001 | -0.649 | -1.243 | -1.71 | 2.675 | 2.985 |
| CCNA_00542; hypothetical protein | 0.003 | -0.471 | -1.169 | -1.452 | 1.965 | 2.529 |
| CCNA_00787; chemotaxis motA protein | -0.012 | -0.721 | -0.806 | -1.894 | 2.588 | 3.936 |
| CCNA_00821; hypothetical protein | -0.027 | -0.613 | -1.472 | -0.837 | 1.245 | 2.046 |
| CCNA_00823; LuxR-like DNA-binding protein | -0.058 | -0.97 | -1.218 | -1.518 | 2.066 | 2.612 |
| CCNA_00942; flagellar hook-associated protein FlgL | 0.004 | -0.424 | -0.765 | -1.385 | 1.835 | 2.326 |
| CCNA_00943; flagellar hook-associated protein FlaN | -0.032 | 0.105 | -0.548 | -0.928 | 2.707 | 2.553 |
| CCNA_01004; flagellar basal-body rod protein FlgB | 0.004 | -0.37 | -0.935 | -1.357 | 2.617 | 3.031 |
| CCNA_01005; flagellar basal-body rod protein FlgC | 0.004 | -0.426 | -0.738 | -1.599 | 2.121 | 3.296 |
| CCNA_01094; hypothetical protein | -0.002 | 0.146 | -0.421 | -1.277 | 1.473 | -3.97 |
| CCNA_01117; conserved hypothetical protein | 0.006 | -0.472 | -0.715 | -1.256 | 1.147 | 1.777 |
| CCNA_01524; FlbA protein | 0.001 | -0.427 | -0.921 | -1.919 | 1.998 | 2.886 |
| Gene ID          | Description                              | Value 1   | Value 2   | Value 3   | Value 4   | Value 5   | Value 6   |
|-----------------|------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| CCNA_01527; fliL  | flagellin fljL                            | 0.011     | -0.601    | -1.477    | -1.912    | 2.791     | 2.989     |
| CCNA_01530; fliJ  | flagellin FljJ                            | -0.005    | -0.245    | -0.754    | -1.332    | 1.409     | 2.203     |
| CCNA_01532; regY  | regulatory protein flaY                   | 0.004     | -0.56     | -0.887    | -1.34     | 1.497     | 1.985     |
| CCNA_01562; aaldo  | 4-hydroxy-2-oxoglutarate/2-dehydro-3-deoxyphosphogluconate aldolase | -0.001   | 0.111     | -0.489    | -1.482    | 2.752     | 3.307     |
| CCNA_01644; motB  | chemotaxis motB protein                   | -0.002    | -0.479    | -0.629    | -1.493    | 2.599     | 2.458     |
| CCNA_01675; ompr  | outer membrane protein                    | -0.008    | -0.328    | -0.695    | -1.409    | 1.926     | 2.472     |
| CCNA_01676; protY  | conserved hypothetical protein            | 0.006     | -0.455    | -0.55     | -1.207    | -2.14     | 2.362     |
| CCNA_02142; fgbF  | flagellar basal-body rod protein flgF     | -0.011    | -0.368    | -1.402    | -1.304    | 2.054     | 3.858     |
| CCNA_02143; fgbG  | flagellar basal-body rod protein flgG     | -0.008    | -0.273    | -1.237    | -1.728    | 2.163     | 2.703     |
| CCNA_02144; flrP  | flagella basal body P ring formation protein flgA | 0.004 | -0.357 | -0.887 | -1.701 | 1.911 | 3.179 |
| CCNA_02145; frH  | flagellar L-ring protein flgH             | -0.005    | -0.473    | -1.193    | -1.891    | 2.175     | 3.406     |
| CCNA_02322; eflxP  | Co2+/Mg2+ efflux protein ApaG             | 0.01      | -0.146    | -0.529    | -0.943    | 1.572     | 2.072     |
| CCNA_02411; pty  | putative lytic transglycosylase PleA      | 0.008     | -0.039    | -0.349    | -0.84     | 1.079     | 1.064     |
| CCNA_02526; soP  | dihydroorotase                            | 0         | -0.85     | -0.636    | -1.913    | 2.573     | 3.177     |
| CCNA_02667; fgbF  | flagellar basal-body protein FlbY         | -0.011    | -0.328    | -0.781    | -1.413    | 1.898     | 3.416     |
| CCNA_02796; protY  | conserved hypothetical protein            | -0.019    | -0.623    | -1.316    | -2.031    | 2.836     | -4.51     |
| CCNA_02946; rsP  | spsF-related cytidylyltransferase         | 0.002     | -0.342    | -0.765    | -1.462    | 1.667     | 1.885     |
| CCNA_02947; rpsP  | spsG-related polysaccharide biosynthesis protein | 0.007 | -0.126 | -0.843 | -0.56 | -2.42 | 2.072 |
| CCNA_02950; protY  | hypothetical protein                      | 0.006     | -0.564    | -1.334    | -1.874    | 2.604     | 2.534     |
| CCNA_02951; wAmpC-like family protein | WbqC-like family protein                  | 0.011     | -0.302    | -0.744    | -1.461    | 1.905     | 2.271     |
| CCNA_02961; dncP  | NeuB-family N-acetylneuraminate synthase  | 0.005     | -0.404    | -0.841    | -1.145    | 2.067     | 2.738     |
| CCNA_03035; dcp  | TadC-related pilus assembly protein       | 0.013     | -0.501    | -0.716    | -1.382    | 1.531     | 1.851     |
| locusId; annotation | 0   | 1   | 2   | 3   | 4   | 5   |
|---------------------|-----|-----|-----|-----|-----|-----|
| CCNA_03036; TadB-related pilus assembly protein | 0.004 | -0.411 | -0.608 | -1.411 | 1.647 | 2.348 |
| CCNA_03041; pilus assembly protein CpaB | 0.004 | -0.193 | -0.661 | -0.824 | 0.946 | 1.653 |
| CCNA_03890; conserved hypothetical protein | 0.007 | 0.049 | -0.268 | -1.598 | 1.602 | 2.476 |

**Cluster 3: Pilus assembly**

| locusId; annotation | 0   | 1   | 2   | 3   | 4   | 5   |
|---------------------|-----|-----|-----|-----|-----|-----|
| CCNA_03033; Flp pilus assembly protein TadD | 0.008 | 0.009 | -0.62 | -0.629 | 0.758 | 1.594 |
| CCNA_03035; TadC-related pilus assembly protein | 0.013 | -0.501 | -0.716 | -1.382 | 1.531 | 1.851 |
| CCNA_03036; TadB-related pilus assembly protein | 0.004 | -0.411 | -0.608 | -1.411 | - | - |
| CCNA_03037; pilus assembly ATPase CpaF | 0.005 | 0.095 | -0.253 | -0.32 | 0.409 | 0.403 |
| CCNA_03038; pilus assembly ATPase CpaE | -0.004 | -0.237 | -0.211 | -0.606 | 0.457 | 1.147 |
| CCNA_03039; pilus assembly protein CpaD | 0.037 | -0.125 | -0.317 | -0.922 | -1.06 | 0.943 |
| CCNA_03040; outer membrane pilus secretion channel CpaC | 0.008 | -0.244 | -0.478 | -0.827 | -1.23 | 1.472 |
| CCNA_03041; pilus assembly protein CpaB | 0.004 | -0.193 | -0.661 | -0.824 | 0.946 | 1.653 |
| CCNA_03042; pilus assembly prepilin peptidase CpaA | -0.001 | -0.267 | 0.188 | 0.554 | 0.736 | 0.911 |
| CCNA_03043; type IV pilin protein pilA | 0.006 | 0.615 | 1.513 | 2.023 | 2.366 | 2.569 |
| CCNA_03044; CpaC-related secretion pathway protein | 0.008 | -0.301 | -0.433 | -1.517 | 1.481 | 1.527 |
| CCNA_03045; TadG-related pilus assembly protein | 0  | 0.045 | -0.047 | 0.016 | - | 0.154 |
| CCNA_03046; TadE-related pilus assembly protein | -0.004 | -0.33 | -0.304 | -0.389 | 0.182 | 0.06 |

**Cluster 4: Holdfast synthesis**

| locusId; annotation | 0   | 1   | 2   | 3   | 4   | 5   |
|---------------------|-----|-----|-----|-----|-----|-----|
| CCNA_00094; WecG/TagA-family glycosyltransferase HfsJ | -0.001 | 1.792 | 3.826 | 5.461 | 6.707 | 7.85 |
| CCNA_01241; Zn-dependent hydrolase, glyoxalase II family | -0.014 | 0.565 | 2.753 | 4.296 | 5.334 | 6.595 |
| CCNA_01242; amino acid permease | 0.008 | 2.034 | 5.865 | 8.127 | 8.789 | 9.649 |
| CCNA_02360; glycosyl transferase family 2 protein | 0.006 | 1.507 | 2.857 | 3.987 | 4.727 | 5.919 |
| CCNA_02436; hypothetical protein | -0.011 | 0.37 | 1.598 | 2.491 | 4.207 | 5.78 |
Cluster 5: Holdfast modification

| locusId; annotation | 0   | 1   | 2   | 3   | 4   | 5   |
|---------------------|-----|-----|-----|-----|-----|-----|
| CCNA_00006; enoyl-CoA hydratase   | 0.006 | 1.416 | 2.106 | 2.633 | 2.838 | 2.885 |
| CCNA_00011; chaperone protein DnaJ | -0.002 | 0.391 | 1.424 | 2.487 | 3.42 | 3.55 |
| CCNA_00134; surface protein | 0.012 | 1.004 | 1.223 | 2.175 | 2.703 | 3.104 |
| CCNA_00135; trypsin-like peptidase | 0.024 | 1.408 | 1.849 | 2.536 | 2.983 | 3.508 |
| CCNA_00247; two-component receiver protein SpdR | -0.022 | 0.746 | 1.113 | 2.001 | 1.936 | 2.122 |
| CCNA_00527; conserved hypothetical protein | 0.015 | 0.266 | 0.896 | 2.136 | 3.469 | 4.796 |
| CCNA_00543; methyl-accepting chemotaxis protein | 0.009 | 1.134 | 1.716 | 2.128 | 2.157 | 2.226 |
| CCNA_00551; hypothetical protein | -0.004 | 0.866 | 1.543 | 2.139 | 2.308 | 2.69 |
| CCNA_00554; methyltransferase | -0.001 | 0.062 | 0.822 | 2.006 | 2.545 | 2.978 |
| CCNA_00908; 3-oxoacyl-(acyl-carrier-protein) synthase III | 0.407 | 0.586 | 0.845 | 1.489 | 2.322 |
| CCNA_00948; CtrA inhibitory protein SciP | 0.007 | 1.162 | 1.949 | 2.067 | 2.008 | 2.039 |
| CCNA_01020; LacI-family transcriptional regulator | 0.007 | 0.551 | 1.386 | 2.301 | 2.185 | 2.402 |
| CCNA_01214; YjgP/YjgQ family membrane permease | 0.015 | 2.881 | 3.481 | 3.156 | 3.126 | 3.309 |
| CCNA_01215; histidine triad (HIT) hydrolase | 0.028 | 1.556 | 1.748 | 1.705 | 2.032 | 2.724 |
| CCNA_01345; short chain dehydrogenase | 0.005 | 0.092 | 0.3 | 0.598 | 1.441 | 2.418 |
| CCNA_01354; myo-inositol 2-dehydrogenase IdhA | 0.011 | 0.138 | 0.551 | 0.717 | 1.425 | 2.056 |
| CCNA_01893; SnoaL-like domain protein | -0.014 | 0.189 | 0.498 | 0.484 | 1.23 | 2.187 |
| CCNA_02087; deoxyguanosinetriphosphate | 0.002 | 0.592 | 1.448 | 1.871 | 2.344 | 2.614 |
| Protein Name                        | Passage 1 | Passage 2 | Passage 3 | Passage 4 | Passage 5 |
|------------------------------------|-----------|-----------|-----------|-----------|-----------|
| triphosphohydrolase                 | 0.008     | 1.149     | 2.18      | 2.796     | 3.456     | 4.419     |
| CCNA_02242; PHB granule-associated protein, phasin2 | -0.018    | 0.695     | 1.362     | 1.519     | 1.819     | 2.214     |
| CCNA_02415; Xre-family transcriptional regulator | 0.001     | -0.156    | 2.237     | 3.038     | 2.641     | 2.593     |
| CCNA_02619; stomatin/prohibitin-related protein | -0.013    | 0.45      | 1.896     | 3.271     | 3.38      | 3.866     |
| CCNA_02722; conserved hypothetical protein | 0.002     | 0.867     | 1.936     | 2.676     | 3.336     | 4.092     |
| CCNA_02846; DegP/HtrA-family serine protease | 0.018     | 0.267     | 1.041     | 1.554     | 1.965     | 2.587     |
| CCNA_02880; terminase-like family protein | -0.003    | 0.224     | 0.389     | 0.732     | 1.32      | 2.122     |
| CCNA_02934; conserved hypothetical protein | -0.003    | 0.633     | 1.144     | 2.146     | 2.127     | 2.572     |
| CCNA_03099; hypothetical protein | 0.002     | 0.378     | 0.678     | 1.271     | 1.525     | 2.473     |
| CCNA_03161; transcriptional regulator xylR | 0.017     | 1.201     | 1.769     | 2.792     | 2.9       | 2.983     |
| CCNA_03386; multimodular transpeptidase-transglycosylase PbpC | 0.002     | 0.775     | 1.546     | 2.13      | 2.477     | 2.602     |
| CCNA_03465; glycine cleavage system aminomethyltransferase T | 0.012     | 1.186     | 1.447     | 1.937     | 2.076     | 2.344     |
| CCNA_03611; glutathione-regulated potassium-efflux system protein kefC | 0.005     | -0.076    | 1.577     | 2.82      | 3.551     | 3.907     |
| CCNA_03803; acetyltransferase family holdfast biogenesis protein HfsK | -0.003    | 0.895     | 1.957     | 2.71      | 3.132     | 3.698     |
| CCNA_03902; conserved hypothetical protein | -0.009    | 0.681     | 1.566     | 2.037     | 2.339     | 3.165     |

**Table S3** Fitness scores across cheesecloth passages for mutant clusters shown in Fig 1C

Fitness values represent the average of the three replicates for each passage. The first passage without cheesecloth (PYE1) represents the time 0 sample.
| Genotype       | Deletion (PYE) | Empty vector (PYE) | Complement (PYE) | Deletion (M2X) | Empty Vector (M2X) | Complement (M2X) |
|----------------|---------------|-------------------|-----------------|---------------|-------------------|-----------------|
| Wild-type      | 1.00 ± 0.03   | n.m.              | n.m.            | 1.00 ± 0.06   | n.m.              | n.m.            |
| ΔhfiA          | 1.28 ± 0.16   | n.m.              | n.m.            | 4.34 ± 0.23   | n.m.              | n.m.            |
| ΔhfsJ          | 0.00 ± 0.00   | n.m.              | n.m.            | 0.00 ± 0.00   | n.m.              | n.m.            |
| ΔCCNA_01242    | 0.68 ± 0.06   | 0.98 ± 0.06       | 0.98 ± 0.13     | 0.81 ± 0.19   | 0.74 ± 0.09       | 1.09 ± 0.17     |
| ΔhfaE          | 0.34 ± 0.08   | 0.32 ± 0.12       | 1.02 ± 0.08     | 0.06 ± 0.03   | 0.05 ± 0.02       | 1.08 ± 0.19     |
| ΔhfsL          | 0.00 ± 0.00   | 0.00 ± 0.00       | 1.00 ± 0.12     | 0.00 ± 0.01   | 0.00 ± 0.01       | 1.10 ± 0.21     |
| ΔCCNA_00497    | 1.12 ± 0.07   | 1.02 ± 0.07       | 1.08 ± 0.04     | 2.10 ± 0.23   | 3.47 ± 0.35       | 0.84 ± 0.10     |
| ΔCCNA_02386    | 0.72 ± 0.07   | 0.68 ± 0.08       | 1.10 ± 0.14     | 1.97 ± 0.30   | 2.73 ± 0.19       | 0.72 ± 0.13     |
| ΔrfbB          | 1.34 ± 0.06   | 1.40 ± 0.03       | 1.08 ± 0.07     | 3.24 ± 0.71   | 2.14 ± 0.24       | 1.48 ± 0.31     |
| ΔwbqP          | 1.29 ± 0.11   | 1.09 ± 0.11       | 1.11 ± 0.12     | 3.87 ± 0.51   | 2.31 ± 0.40       | 1.10 ± 0.26     |
| ΔflgH          | 0.58 ± 0.05   | 0.62 ± 0.06       | 1.03 ± 0.04     | 2.91 ± 0.08   | 2.42 ± 0.26       | 1.23 ± 0.25     |
| ΔcpaH          | 0.71 ± 0.05   | 0.76 ± 0.08       | 1.03 ± 0.07     | 2.68 ± 0.07   | 1.85 ± 0.24       | 0.90 ± 0.31     |
| ΔpilA          | 0.34 ± 0.04   | 0.44 ± 0.04       | 1.01 ± 0.06     | 0.20 ± 0.16   | 0.08 ± 0.05       | 0.93 ± 0.36     |

**Table S4** Complementation of adhesion defects

Normalized crystal violet staining values are shown as the average ± standard deviation from at least 4 biological replicates. All values shown reflect trends that were consistent across at least five independent experiments. Cells were grown for 17 hours in M2X or 24 hours in PYE medium before staining. n.m. – not measured.
| Genotype | Deletion (PYE) | ΔhfiA (PYE) | ΔpleD (PYE) | Deletion (M2X) | ΔhfiA (M2X) | ΔpleD (M2X) |
|----------|---------------|-------------|-------------|----------------|-------------|-------------|
| Wild-type | 1.00 ± 0.03   | 1.28 ± 0.16 | 0.81 ± 0.01 | 1.00 ± 0.06    | 5.61 ± 0.27 | 1.38 ± 0.19 |
| ΔhfiA    | 1.28 ± 0.16   | n.m.        | 1.08 ± 0.07 | 4.34 ± 0.23    | n.m.        | 5.53 ± 0.16 |
| ΔhfsJ    | 0.00 ± 0.00   | n.m.        | n.m.        | 0.00 ± 0.00    | n.m.        | n.m.        |
| ΔwbqP ΔhfsJ | 0.48 ± 0.25 | n.m.        | n.m.        | 2.51 ± 0.64    | n.m.        | n.m.        |
| ΔflgH    | 0.58 ± 0.05   | 0.62 ± 0.07 | 0.31 ± 0.02 | 2.91 ± 0.08    | 2.78 ± 0.21 | 1.41 ± 0.33 |
| ΔcpaH    | 0.71 ± 0.05   | 0.91 ± 0.05 | 0.19 ± 0.01 | 2.68 ± 0.07    | 4.29 ± 0.16 | 1.22 ± 0.18 |
| ΔpilA    | 0.34 ± 0.04   | 0.73 ± 0.03 | 0.43 ± 0.01 | 0.20 ± 0.16    | 5.95 ± 0.18 | 0.25 ± 0.08 |
| ΔflgH ΔhfsJ | 0.00 ± 0.00 | n.m.        | n.m.        | 0.01 ± 0.01    | n.m.        | n.m.        |
| ΔcpaH ΔhfsJ | 0.00 ± 0.00 | n.m.        | n.m.        | 0.01 ± 0.02    | n.m.        | n.m.        |
| ΔflgH ΔcpaH | 0.40 ± 0.01 | n.m.        | n.m.        | 4.13 ± 0.18    | n.m.        | n.m.        |
| ΔflgH ΔpilA | 0.32 ± 0.02 | n.m.        | n.m.        | 2.00 ± 0.25    | n.m.        | n.m.        |
| ΔcpaH ΔpilA | 0.43 ± 0.03 | n.m.        | n.m.        | 0.12 ± 0.04    | n.m.        | n.m.        |

**Table S5 Additional adhesion phenotypes for motility mutants**

Normalized crystal violet staining values are shown as the average ± standard deviation from at least 4 biological replicates. All values shown reflect trends that were consistent across at least five independent experiments. Cells were grown for 17 hours in M2X or 24 hours in PYE medium before staining. n.m. – not measured.
Table S6 Holdfast counts for motility mutants

The values represent the fraction of cells (out of 1) that stained with a holdfast focus with the associated standard deviation from three biological replicates. The total number of cells counted is shown in parentheses. Cells were harvested from low-density cultures (Material and Methods) to minimize the formation of rosettes. However, in the event that rosettes were observed, all cells in the rosette were counted as holdfast producing.

| Strain      | PYE          | M2X          |
|-------------|--------------|--------------|
| Wild-type   | 0.686 ± 0.045 (739) | 0.081 ± 0.006 (743) |
| ΔhfiA       | 0.863 ± 0.021 (526) | 0.722 ± 0.039 (703) |
| ΔhfsJ       | 0.000 ± 0.000 (628) | 0.000 ± 0.000 (638) |
| ΔflgH       | 0.869 ± 0.037 (662) | 0.620 ± 0.013 (609) |
| ΔcpaH       | 0.874 ± 0.031 (531) | 0.296 ± 0.094 (689) |
| ΔpilA       | 0.673 ± 0.030 (464) | 0.092 ± 0.016 (367) |
| ΔpleD       | 0.496 ± 0.024 (457) | 0.084 ± 0.007 (500) |
Figure S1 Analysis of rough LPS in SLPS mutants Rough LPS was isolated and analyzed as described in materials and methods. None of the four SLPS mutants show apparent defects in rough LPS.
Figure S2 Validation of pilus and flagellum phenotypes in polar appendage mutants
Top: Swimming phenotypes for relevant mutants are shown. Note that ΔpilA has a slight but reproducible increase in swarm size that can be complemented by ectopic expression of pilA in trans. Mutants lacking flgH show the expected non-motile phenotype. Bottom: ΦCBK sensitivity of motility mutants. Mutants lacking pilA or cpaH show the expected ΦCBK resistance phenotype.
Figure S3 Comparison of crystal violet staining and holdfast counts for motility mutants

Surface attachment was assessed by CV staining (violet bars) and holdfast production by fWGA staining (orange bars) as described in Material and Methods. The values for growth in complex medium are shown on the left and defined medium on the right.
Figure S4 Complementation of growth defect in ΔCCNA_01242

The ΔCCNA_01242 mutant displays a biphasic growth curve indicating a defect. Normal growth is restored by ectopic complementation with CCNA_01242.