

Surface contact stimulates the just-in-time deployment of bacterial adhesins

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Summary

The attachment of bacteria to surfaces provides advantages such as increasing nutrient access and resistance to environmental stress. Attachment begins with a reversible phase, often mediated by surface structures such as flagella and pili, followed by a transition to irreversible attachment, typically mediated by polysaccharides. Here we show that the interplay between pili and flagellum rotation stimulates the rapid transition between reversible and polysaccharide-mediated irreversible attachment. We found that reversible attachment of *Caulobacter crescentus* cells is mediated by motile cells bearing pili and that their contact with a surface results in the rapid pili-dependent arrest of flagellum rotation and concurrent stimulation of polar holdfast adhesive polysaccharide. Similar stimulation of polar adhesin production by surface contact occurs in *Asticcacaulis biprosthecum* and *Agrobacterium tumefaciens*. Therefore, single bacterial cells respond to their initial contact with surfaces by triggering just-in-time adhesin production. This mechanism restricts stable attachment to intimate surface interactions, thereby maximizing surface attachment, discouraging non-productive self-adherence, and preventing curing of the adhesive.

Introduction

Many organisms derive important competitive advantages from their attachment to surfaces and the mechanisms by which they settle on a surface are subject to important regulation. For example, in sessile marine and aquatic

invertebrates such as mussels, barnacles and oysters, it is well established that during the transition from the free swimming larval stage to the sessile adult, the larva reversibly contact the surface, testing and responding to the physical and chemical composition of its environment, prior to cementation and an irreversible commitment to stationary life (Morse, 1990; Tamburri *et al.*, 1992). Surface attachment is also advantageous for bacteria because it increases nutrient access and resistance to environmental stress, and they have evolved many strategies that improve the efficiency of the attachment process.

Decades of research have clearly determined that bacterial attachment proceeds in two stages, a reversible stage that involves transient surface interactions and an irreversible stage that depends on adhesins responsible for mediating permanent attachment (van Loosdrecht *et al.*, 1990a,b; Vigeant *et al.*, 2002; Hinsa *et al.*, 2003; Agladze *et al.*, 2005; Beloin *et al.*, 2008; Otto, 2008; Van Dellen *et al.*, 2008). Flagellar motility and thin surface structures such as pili often mediate the reversible adhesion phase and allow cells to overcome the electrostatic repulsion as they approach a surface. In addition, bacteria produce exopolymers, such as adhesive polysaccharides, to mediate the transition from reversible to irreversible binding and increase their strength of adhesion to surfaces. The temporal mechanism by which this transition occurs however has remained mysterious (Karatan and Watnick, 2009).

In this study, *Caulobacter crescentus* is used as a model bacterium to study the dynamics of cell-to-surface binding. *C. crescentus* exhibits a dimorphic life cycle in which each cell division produces a motile swarmer cell and a sessile stalked cell (Fig. 1). Swarmer cells (SW) harbour pili and a flagellum at the same pole. These cells are unable to initiate DNA replication and are motile for approximately 25–30 min under the conditions used in these studies, unless they attach to a surface. After the motile period, swarmer cells differentiate into stalked cells (ST) by shedding their flagellum, retracting their pili and synthesizing an adhesive polysaccharide holdfast and a stalk at the same pole. The holdfast is composed at least in part of a polysaccharide of *N*-acetylglucosamine that is required for irreversible adhesion to surfaces (Merker and Smit, 1988). Stalked cells initiate DNA replication, elongate and

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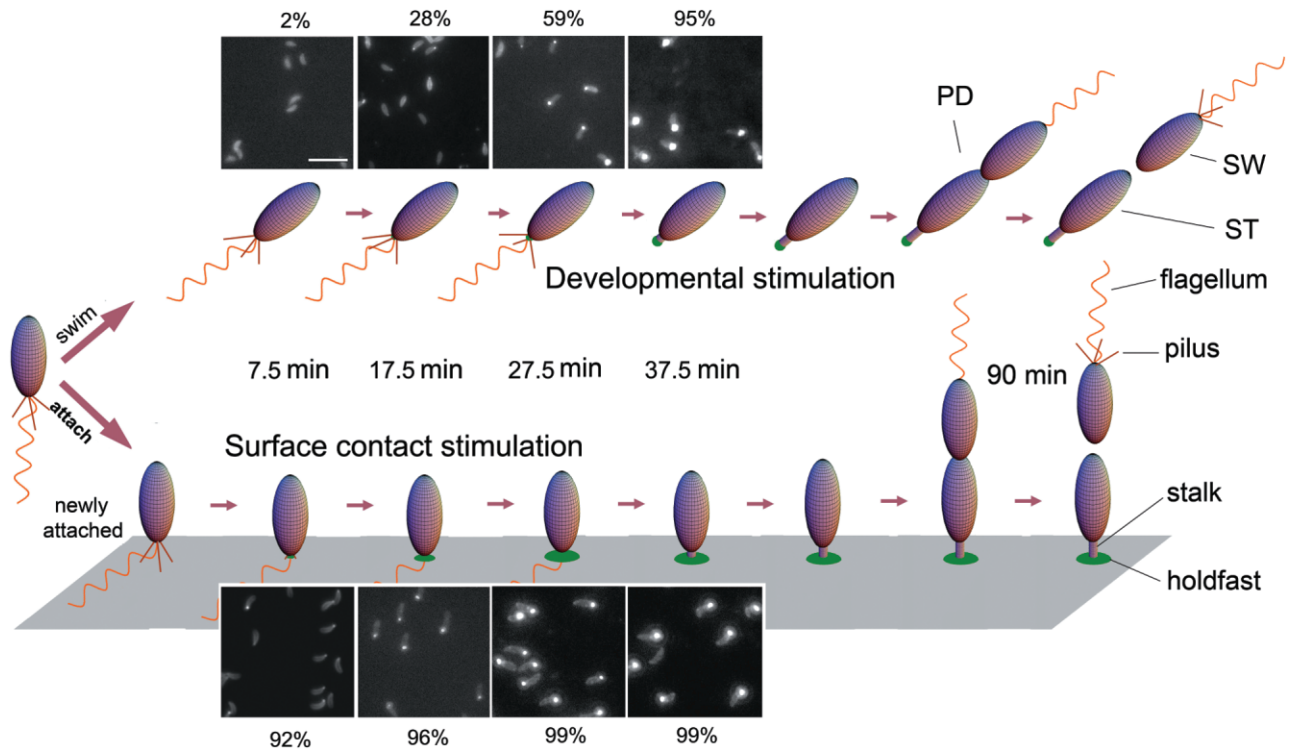


Fig. 1. Cell cycle diagram and holdfast production of unattached or attached *C. crescentus* cells. Fluorescence images show unattached (top) and attached (bottom) synchronized *C. crescentus* wild-type cells labelled with fluorescein-WGA and imaged at ages 7.5 ± 2.5 min, 17.5 ± 2.5 min, 27.5 ± 2.5 min and 37.5 ± 2.5 min. The contrast of the images was scaled up to show the dim cell body due to non-specific labelling. The bright spot at the pole of a cell body is the holdfast. The percentage of cells with a holdfast at each time point is indicated. Attached cells rapidly produce a holdfast after surface contact, whereas unattached swarmer cells produce a holdfast just prior to differentiating into stalked cells.

eventually synthesize a flagellum at the pole opposite the stalk, forming the pre-divisional cell (PD). Flagellum rotation is initiated just prior to the completion of cell division, which always produces a swarmer cell and a stalked cell. Initial surface adhesion occurs during the swarmer phase (Bodenmiller *et al.*, 2004; Levi and Jenal, 2006) and permanent adhesion is cemented by the holdfast, which binds to surfaces with an impressive strength in the μN range (Tsang *et al.*, 2006). Therefore, the availability of a portion of the life cycle in which the adhesive polysaccharide is absent allows the investigation of factors that regulate its synthesis and its correlation with surface adhesion.

Here, we take advantage of the fact that *C. crescentus* and several species of alphaproteobacteria synthesize polar adhesive polysaccharides (Laus *et al.*, 2006; Brown *et al.*, 2009; Tomlinson and Fuqua, 2009) to show that surface contact triggers the rapid production of adhesins and to investigate the mechanism that drives the transition between reversible and irreversible attachment. We find that the interplay between pili and the flagellum during initial surface interactions causes the rapid, just-in-time production of the *C. crescentus* adhesin, the holdfast polysaccharide, thereby driving the transition from reversible to irreversible attachment. We show that the surface

contact stimulation of adhesive polysaccharides is a general phenomenon as it also occurs in two other bacterial genera, the stalked bacterium *Asticcacaulis biprosthecum* and the plant pathogen *Agrobacterium tumefaciens*.

Results and discussion

Surface contact stimulates the rapid, protein synthesis independent deployment of the holdfast adhesin

In order to determine if surface contact influences the biosynthesis of the holdfast polysaccharide adhesin, *C. crescentus* swarmer cells, which do not harbour a holdfast, were collected following synchronization and divided into two treatments. To monitor holdfast production in attached cells, an aliquot of swarmer cells was placed on a coverslip for 5 min to allow cells to attach. Unattached cells were washed away and the attached cells were grown, followed by labelling for holdfasts. To observe holdfast production in unattached cells, synchronized swarmer cells were diluted 100-fold in peptone-yeast extract (PYE) medium such that few cells could contact the surfaces until they were labelled for holdfasts. The holdfasts of both populations were labelled at specific

time points with fluorescein-conjugated WGA lectin (fluorescein-WGA), which specifically binds to holdfast polysaccharide (Umbreit and Pate, 1978; Merker and Smit, 1988). Fluorescence microscopy analysis indicated that attached cells produce a holdfast much earlier than unattached cells; more than 90% of attached cells had a holdfast within 7.5 min in contrast to only 2% of unattached cells. Unattached cells took ~37.5 min to reach a level similar to that of attached cells at 2 min (Fig. 1).

To improve the time resolution of the study of holdfast synthesis in single cells, total internal reflection fluorescence (TIRF) microscopy was used to observe and quantify holdfast production in real time by measuring the integrated fluorescence intensity of the labelled holdfast of individual attached cells in the presence of fluorescein-WGA (Fig. 2A and Movie S1). During the first ~20 min after the synchrony, virtually all cells lacked holdfast prior to their contact with the surface (Figs 2B–D and S1A). Throughout this time interval, the timing of holdfast production depended on the time of attachment to the surface, rather than the age of the cell, and it occurred within 1–2 min of contact (Figs 2D and S1A). Furthermore, stimulation of holdfast production did not require *de novo* protein synthesis since holdfast synthesis was still triggered by surface contact in the presence of a growth inhibiting concentration of kanamycin (Fig. 3A). This observation is consistent with previous work showing that addition of kanamycin, chloramphenicol or tetracycline had no effect on attachment or holdfast biogenesis during the cell cycle of planktonic *C. crescentus* cells (Levi and Jenal, 2006). Thus, surface contact stimulates rapid holdfast production by a post-translational mechanism that would involve activating the holdfast polysaccharide biosynthesis or export machinery (Smith *et al.*, 2003; Toh *et al.*, 2008). In contrast, 25 min after synchronization, nearly all *C. crescentus* cells, including the unattached cells in suspension, had detectable holdfast (Figs 1, 2C and D and S1A). This observation is readily explained by cell cycle-dependent holdfast production, which occurs slightly prior to swarmer-to-stalk cell differentiation (Levi and Jenal, 2006).

The results presented above imply that the initial attachment of newborn swarmer cells to surfaces is not mediated by the holdfast, whose synthesis or export must be stimulated in order to transit from reversible attachment to holdfast-mediated permanent attachment. In order to test this model, we observed attachment of an $\Delta hfsA$ mutant, which is deficient in holdfast synthesis (Smith *et al.*, 2003), using a quantitative dark-field microscopy assay. After processing overlays of sequential images (see *Experimental procedures*) attached cells appear as stationary foci and are readily distinguished from swimming cells, which generate trajectories (Fig. S2). Swarmer cells of the $\Delta hfsA$ mutant initially attached to the glass surface nearly as efficiently as wild type (Fig. 4B). However, they

did not permanently adhere and progressively detached from the surface (Fig. 4C). In contrast, once attached, the number of attached wild-type cells remained the same for at least 90 min (data not shown). Thus, the holdfast is not required for the initial reversible weak attachment, suggesting that the stimulation of holdfast production drives the transition to the permanent adhesion stage.

Pili-mediated flagellar rotational arrest stimulates holdfast synthesis

How is reversible attachment mediated when cells encounter a surface? We used video microscopy to observe the dynamics of single cell attachment in real time. As *C. crescentus* wild-type cells attached to the surface, they rapidly became tethered (Movie S2). Immediately after attachment, the cell body continued to rotate due to the rotation of the flagellar motor. The majority of attached cells stopped rotation within a few seconds and formed a stable association with the surface, whereas a low proportion of cells continued rotation for a short period, but then detached and swam away. In order to quantify the efficiency of surface attachment in a population, synchronized cells were placed between two glass surfaces separated by 12 μm and were observed with dark-field microscopy with a 10 \times objective, allowing cells at any depth in the field of view to be imaged. Surface attachment was extremely rapid; 68% of the swarmer cells had attached to the surface within 1 min, and virtually all the cells had attached within 5 min (Fig. 4A).

Caulobacter crescentus pili are colocalized with the flagellum at the future pole of holdfast synthesis and are required for efficient surface attachment (Bodenmiller *et al.*, 2004; Entcheva-Dimitrov and Spormann, 2004; Levi and Jenal, 2006). In order to assess the role of pili in the dynamics of single cell attachment, we synchronized swarmer cells of a mutant lacking pili, $\Delta pilA$, and observed the cells by video microscopy. $\Delta pilA$ cells were rarely found attached to the surface. Occasionally, a $\Delta pilA$ cell became tethered to the surface by its flagellum, but the motor continued to rotate until the cell detached and swam away (Movie S3). Notably, the flagellum motor of tethered $\Delta pilA$ cells rotates considerably slower (60 Hz) than the motor of free-swimming $\Delta pilA$ cells (~330 Hz) (Li and Tang, 2006). Thus, the decrease in flagellum motor rotation upon tethering of $\Delta pilA$ cells does not lead to permanent attachment. In contrast, the complete stopping of motor rotation following tethering of wild-type cells results in permanent adhesion (Movie S2). Thus, we infer that pili are required to arrest flagellum rotation when cells are bound to a surface.

Quantitative cell attachment assays using dark-field microscopy indicated that, in stark contrast to wild-type cells, the effective attachment of $\Delta pilA$ swarmer cells only began to occur more than 20 min after cell division and

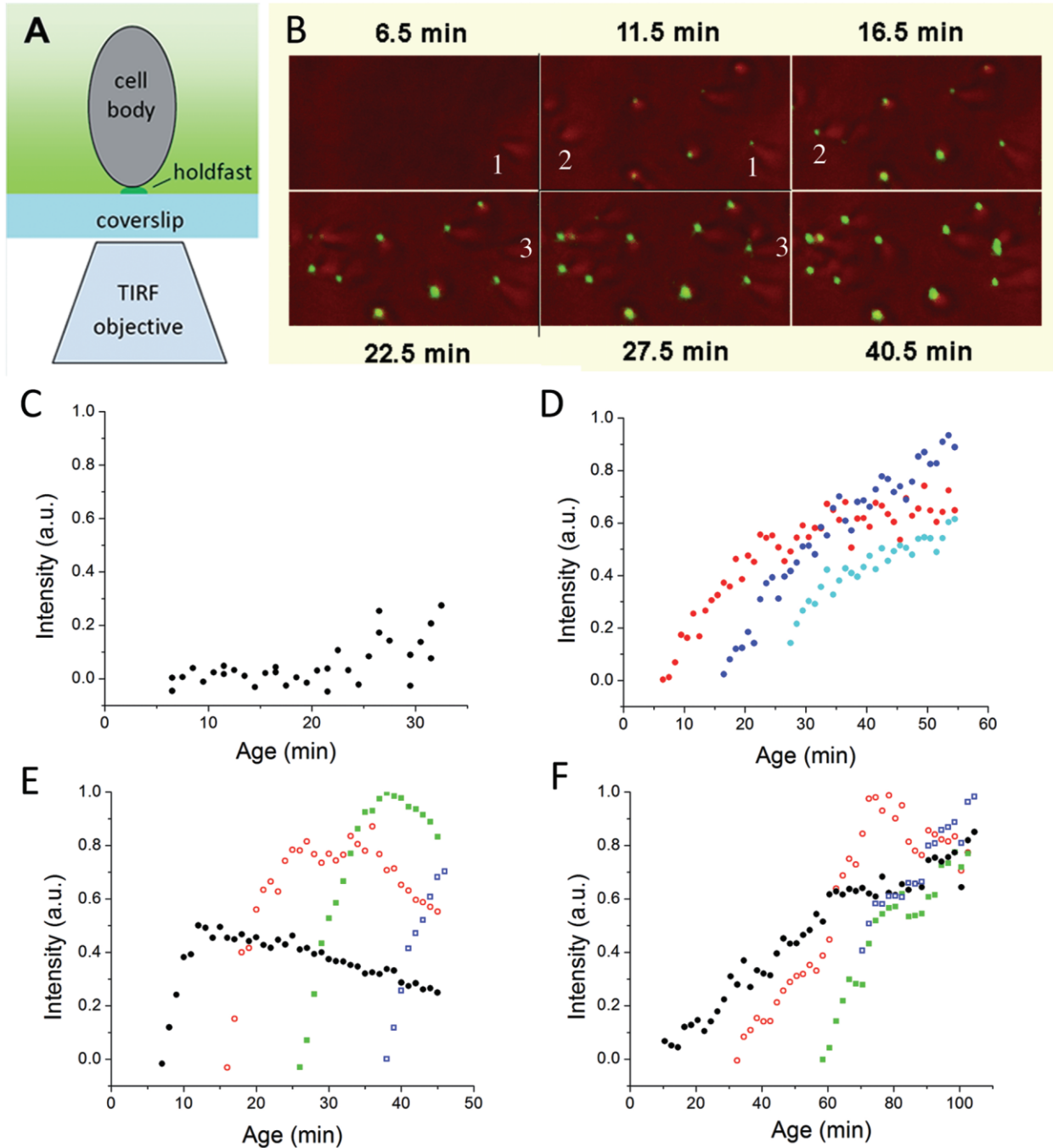


Fig. 2. Holdfast production observed for individual cells with TIRF microscopy.

A. Schematic drawing showing the configuration of the TIRF microscopy and a cell attached to the coverslip. Only the fluorescent dye (shown in green) within a couple hundred nanometres from the coverslip is visible.

B. Overlay of light and TIRF images obtained during a time-course with wild-type *C. crescentus*. Cells arrive at the surface without a holdfast but the holdfast is detected in the next image, which was taken five min later (see numbered examples).

C. Integrated intensity in arbitrary unit (a.u.) of the TIRF image of the holdfast of newly attached wild-type *C. crescentus* cells. Each black dot represents the data from a newly attached individual cell.

D. Integrated intensity of the TIRF images of the holdfasts of three wild-type *C. crescentus* CB15 cells after attachment at an age of 6.5 min (red), 16.5 min (blue) and 26.5 min (cyan). Each colour shows the fluorescence intensity for a single cell as it varies through time.

E. Integrated intensity of the TIRF images of the holdfasts of four *A. biprosthhecum* cells after attachment at an age of 7.5 min (black), 16.5 min (red), 26.5 min (green) and 38.5 min (blue). Each colour shows the fluorescence intensity for a single cell as it varies through time. The fluorescence intensity eventually declines due to photobleaching.

F. Integrated intensity of TIRF image of the holdfasts of four *A. tumefaciens* cells after attachment at an age of 10.5 min (black), 32.5 min (red), 58.5 min (green) and 70.5 min (blue). Each colour shows the fluorescence intensity for a single cell as it varies through time.

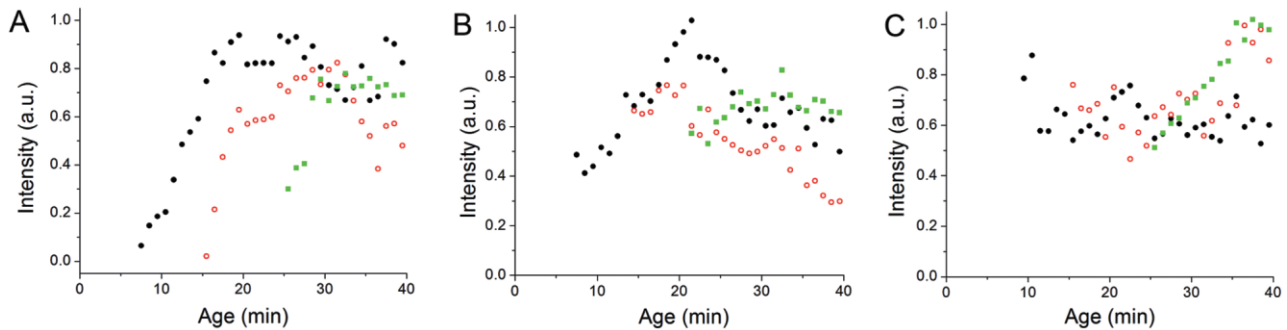


Fig. 3. Integrated intensity of the TIRF images of the holdfasts of (A) three *C. crescentus* wild-type cells in medium with $10 \mu\text{g ml}^{-1}$ kanamycin at ages of 7.5 min (black), 15.5 min (red) and 26.5 min (green); (B) three *C. crescentus* wild-type cells in medium with 10% (w/v) Ficoll 400 attached at an age of 7.5 min (black), 14.5 min (red) and 21.5 min (green); (C) three *C. crescentus* $\Delta pilA$ cells in medium with 10% (w/v) Ficoll 400 attached at an age of 9.5 min (black), 15.5 min (red) and 25.5 min (green).

occurred at a 24-fold lower rate than wild type (Fig. 4A). The timing of attachment of $\Delta pilA$ swarmer cells was consistent with the timing of holdfast synthesis of unattached cells (Figs 1, 2C and 5), suggesting that contact between a preexisting holdfast, whose synthesis was triggered by the developmental pathway, and the surface is responsible for the attachment. This hypothesis is supported by the fact that a mutant synthesizing neither pili nor holdfasts ($\Delta pilA \Delta hfsDAB$) does not bind to surfaces since both initial reversible attachment and permanent attachment have been abolished (data not shown). Thus, we conclude that pili-mediated initial attachment is followed by rapid holdfast production that drives the transition from reversible to permanent adhesion.

How does the initial reversible attachment stimulate holdfast production and the transition to permanent attachment? Video microscopy of the $\Delta pilA$ mutant binding to surfaces revealed that attachment of the flagellum filament enables transient attachment of the cell to the surface (Movie S3). Since the objective is focused on the surface and the cell pole remains out of focus, we infer that the flagellum-mediated attachment is not sufficient to bring the cell body into close proximity with the surface. Notably, tethering by the filament, even though it slows down flagellum rotation, appears insufficient to stimulate holdfast production since attachment of the $\Delta pilA$ mutant only becomes efficient once developmental regulation has triggered holdfast synthesis ~ 20 min after cell division (Figs 4 and 5). Like the $\Delta pilA$ mutant, cells from a double $\Delta pilA \Delta flgE$ mutant attached at a slow rate (Fig. 4D). In contrast to wild-type and $\Delta pilA$ cells, which swam to the surface and whose behaviour is consistent with tethering (Movies S2 and S3), cells from the $\Delta pilA \Delta flgE$ mutant simply diffused to the surface. We hypothesize that adhesion by the pili brings the flagellum filament in close proximity to the surface, thereby increasing the probability of filament adhesion. Indeed, the aggregate interaction of the filament and the pili with the surface causes the rapid inhibition of

motor rotation (Movie S2). This pure mechanical force may be sufficient to inhibit flagellum rotation, although it is possible that the mechanism involves a clutch or a brake type mechanism (Blair *et al.*, 2008; Paul *et al.*, 2010) regulated at the post-translational level. However, the rapidity by which the cell body is immobilized to the surface by the pili precludes the distinction between these mechanisms by video microscopy. Irrespective of the mechanism, inhibition of flagellum rotation is immediately followed by stimulation of holdfast synthesis, independent of the stage of the cell cycle.

Polymer crowding agents that arrest flagellum rotation stimulate holdfast synthesis

Inhibition of the flagellar motor has been reported to trigger various changes in bacteria (McCarter *et al.*, 1988; Anderson *et al.*, 2010). To test if stopping flagellar rotation could stimulate holdfast production independent of surface attachment, we used the observation that polymer crowding agents can cause bacteria to aggregate in solution (Eboigbodin *et al.*, 2005; Schwarz-Linek *et al.*, 2010), due to osmotic pressure caused by the crowding polymers being excluded from the space between neighbouring cells, an effect called the depletion effect (Tang *et al.*, 1997; Hosek and Tang, 2004). We found that *C. crescentus* cells formed aggregates in 10% Ficoll within 1 min after the addition of the polymer. Single cells and some aggregates continued to move (Movie S4), propelled by the rotating flagellar filaments in the aggregates. Almost all the cells and aggregates stopped moving at 4 min (Movie S5), indicating that all flagella in the aggregates stopped rotation. The cells at age 17.5 min were labelled with fluorescein-WGA for fluorescence microscopy. The majority of the cells (78%) showed clear holdfasts in contrast to free swimming cells in medium without crowding agent of which only 29% had holdfast (Table 1 and Fig. 6).

The stimulation of holdfast production was also observed with polymers of different chemical composition

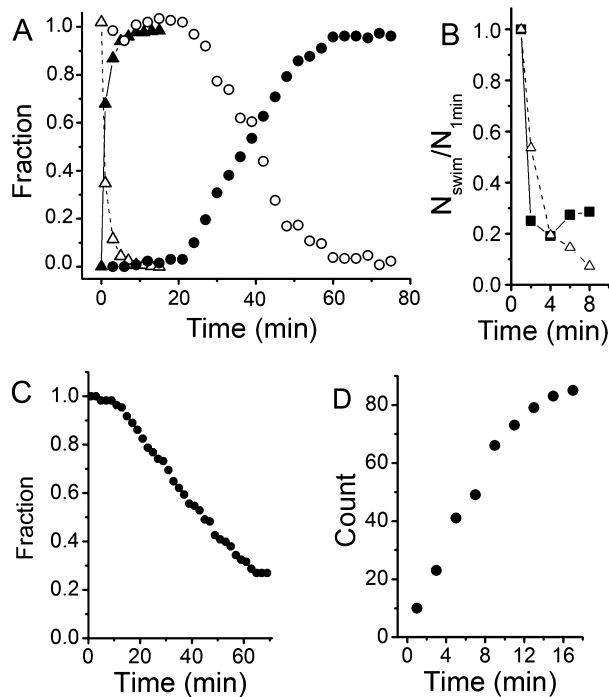


Fig. 4. Attachment of *C. crescentus* strains CB15 (wild type), $\Delta pilA$, $\Delta hfsA$, and $\Delta pilA \Delta flgE$ double mutant to glass surfaces. A. The fraction of attached (solid symbol) and swimming cells (open symbol) in the field of view at various times after the synchronized cells were placed between two glass surfaces separated by 12 μm . Syn synchronized wild-type cells (triangle) attached at a rate of 68% of cells per min immediately after birth and almost all of them had attached to the glass surface within 5 min. Synchronized $\Delta pilA$ cells (circle) only began to attach after 20 min and their attachment occurred at a rate of 2.8% of cells per min.

B. Attachment of *C. crescentus* wild-type (triangle) and $\Delta hfsA$ (square) cells. Since the $\Delta hfsA$ strain cannot be synchronized the number of swimming cells in mixed populations (containing swarmer, stalked and pre-divisional cells) of both strains was normalized to that at 1 min as a function of time after the culture at mid-exponential phase was placed between two glass surfaces separated by 12 μm .

C. Fraction of *C. crescentus* $\Delta hfsA$ cells attached to a surface decreases over time as observed under light microscopy. In a similar experiment, wild-type cells remained attached to the surface for at least 90 min (data not shown).

D. Number of cells from mixed culture of a $\Delta pilA \Delta flgE$ double mutant attaching to a surface. Cells were grown to mid-exponential phase, placed between two coverslips, and a 40 \times objective was focused on the bottom surface to take videos. Because these cells do not swim, most cells settled on the bottom surface. Unattached cells were distinguished from attached cells because unattached cells are subject to Brownian motion while attached cells are not.

that stopped flagellar motor rotation (Table 1). In contrast, polymers that did not stop flagellum rotation despite being added to a similar viscosity did not stimulate holdfast production (Table 1). The dependence of holdfast production on the inhibition of flagellum rotation triggered by viscous solution supports the notion that arrest of flagellum rotation is required for contact-dependent holdfast production. However, it is possible that the osmotic shock

induced by the introduction of the viscous solution may trigger membrane stress (or another physiological change) responsible for stimulating holdfast production, although not all polymer crowding agents had this effect.

These results were confirmed with TIRF microscopy experiments in the presence of Ficoll. In contrast to what was observed in complex medium, cells in the presence of 10% Ficoll arrived at the surface with a holdfast (Figs 3B and S3A), indicating that holdfast synthesis was stimulated prior to surface contact. Ficoll had a similar effect on $\Delta pilA$ cells, which also arrived at the surface with holdfasts already formed and adhered permanently to the surface (Figs 3C and S3B). Therefore, pili are not required for the stimulation of holdfast synthesis by viscous agents as they are for the surface contact stimulation. This result also supports our earlier inference that, prior to the developmental trigger of holdfast synthesis, $\Delta pilA$ cells do not adhere productively upon surface contact because they are unable to stimulate holdfast synthesis.

We conclude that the loss of flagellum rotation upon surface contact triggers holdfast synthesis. In addition, our results suggest that on surfaces pili are mediating the arrest of flagellum rotation, but with viscosity agents the flagellum is intrinsically impeded and the need for the pili in stimulating holdfast synthesis is bypassed. Future studies will utilize *fla* and *mot* mutants to directly determine the contribution of the flagellum filament and motor in initial binding and the transition to permanent adhesion. At present, these experiments are complicated by the fact that these mutants have a delay in cell separation and cannot be synchronized via either the plate synchrony or density centrifugation methods. Furthermore, these mutants have altered timing of cell cycle-dependent holdfast production (data not shown). We are currently exploring alternative methods of cell synchrony to enable these studies.

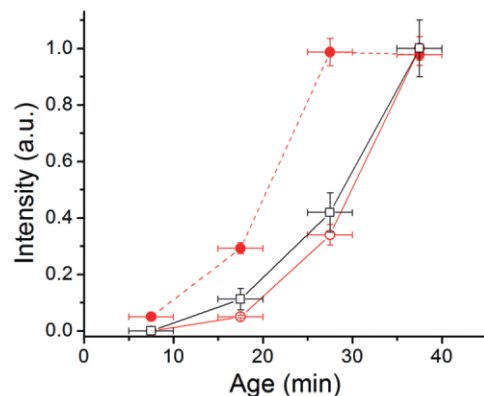


Fig. 5. Integrated fluorescence intensity of holdfast of unattached wild-type cells (open circle), wild-type attached cells (solid circle) and unattached $\Delta pilA$ cells (open square) at different ages. The error bar is the standard error in measurement of 2–4 cells for each data point. Age refers to the time after cell division.

Table 1. Effect of various polymers on holdfast synthesis.

Test matrix	Viscosity (cp)	Holdfasts/cells	% with holdfast	Aggregation	Motility at age 17.5 min
PYE (control)	1	152/529	29%	No	Swimming
Percoll, 67%	2.2	29/95	31%	No	Swimming
PEG (8000 Da), 3.5%	2.2	286/817	35%	Yes	Some moving aggregates and cells
PEG (35 000 Da), 5%	5.4	214/328	65%	Yes	No moving aggregates or cells
Dextran (100 000–200 000 Da), 5%	2.2	228/314	73%	Yes	No moving aggregates or cells
Ficoll (400 000 Da), 5%	2.2	151/179	84%	Yes	No moving aggregates or cells
Ficoll (400 000 Da), 10%	5.2	174/223	78%	Yes	No moving aggregates or cells

Surface-contact stimulation probably activates previously assembled holdfast synthesis machinery since it does not require *de novo* protein synthesis. Indeed, at least the holdfast anchoring machinery (Hardy *et al.*, 2010) and the outer membrane export protein HfsD (J. Javens, Z. Wan and Y.V. Brun, in preparation) are already localized at the flagellar pole in newborn swarmer cells, in close proximity to the flagellum and the pili.

Surface contact stimulation of adhesin synthesis in other bacterial genera

To determine if surface contact stimulated adhesin synthesis is a general phenomenon, we observed polar polysaccharide formation in *A. biprosthecum* and *A. tumefaciens*. *A. biprosthecum* has a dimorphic life cycle similar to that of *C. crescentus*; however, this bacterium produces two lateral stalks and the holdfast is made at the pole of the cell body (Pate *et al.*, 1973). The unipolar polysaccharide (UPP) of the plant pathogen *A. tumefaciens* is rarely detected in single planktonic cells, but is frequently detected in cells attached to each other in rosettes or single cells attached to a surface (Tomlinson and Fuqua, 2009). Stimulation of holdfast and UPP production following contact with a surface was readily observed in both species

using TIRF microscopy. For *A. biprosthecum*, nearly every cell arrived at the surface without a holdfast, but these were detected as early as 2 min after contact, irrespective of cell age (Figs 2E and S1B; Movie S6). For *A. tumefaciens*, most cells produced UPP within 3 min of attaching to a glass surface (Figs 2F and S1C; Movie S7). We also tested whether the production of UPP was stimulated by contact with *Arabidopsis* roots, a more natural surface for *A. tumefaciens* attachment. UPP production occurred preferentially among cells attached to an *Arabidopsis* root when compared with unattached cells from the same population (Fig. 7A; Movie S8) and UPP production occurred after initial attachment of *A. tumefaciens* cells to the *Arabidopsis* root (Fig. 7B; Movie S9). These data indicate that surface contact-dependent stimulation of polar polysaccharide production is conserved in multiple bacterial species.

Advantages of surface contact stimulation of adhesin synthesis

We have shown that the ability to rapidly produce a permanent adhesin is shared among at least three members of the Alphaproteobacteria, in which motile cells differentiate to become sessile cells. For many motile bacteria, initial attachment is reversible, allowing cells to detach

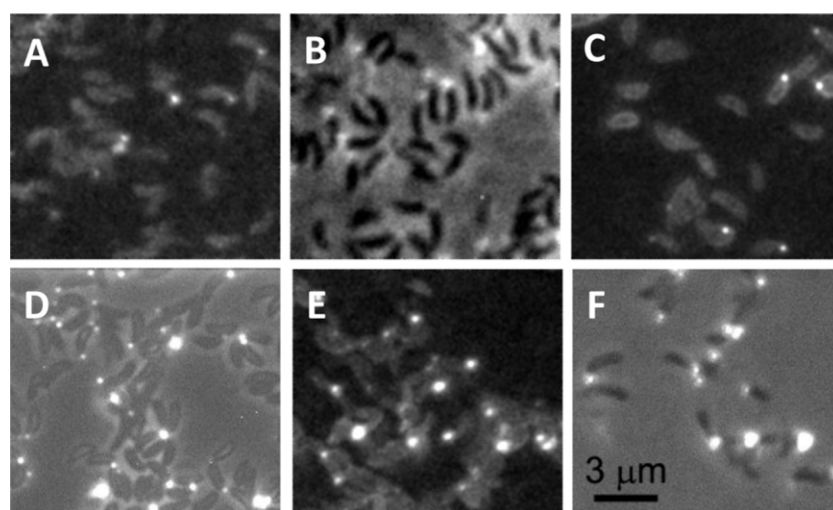


Fig. 6. Holdfast of cells at age 17.5 min grown in (A) PYE medium, (B) PYE medium with 67% Percoll, (C) PYE medium with 3.5% PEG 8000, (D) PYE medium with 5% PEG 35000, (E) PYE medium with 5% Dextran and (F) PYE medium with 10% Ficoll. Panels (A), (C) and (E) are fluorescence images, that clearly show the cell bodies in addition to the holdfasts. Panels (B), (D) and (F) are overlays of fluorescence and phase-contrast images.

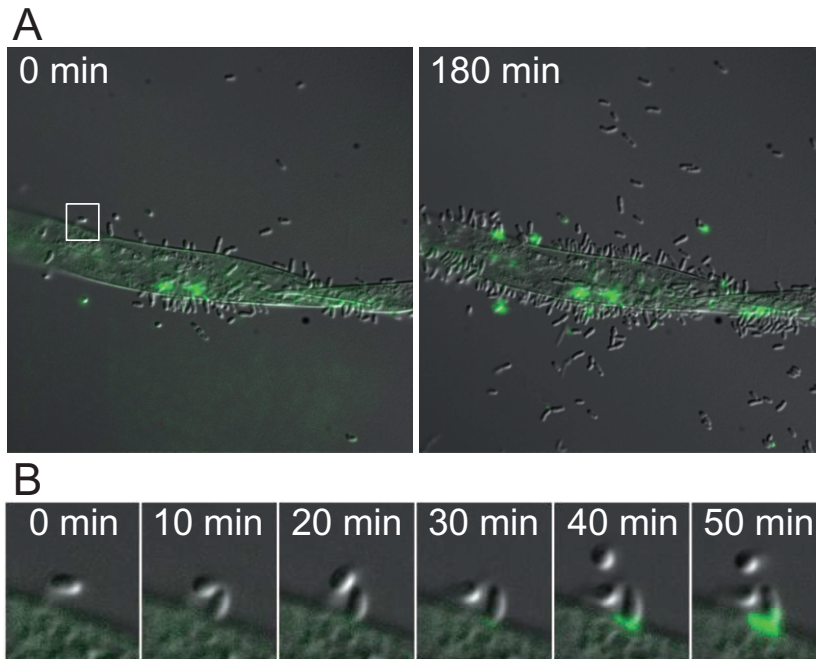


Fig. 7. Time-lapse microscopy of *Agrobacterium tumefaciens* cells attaching to *Arabidopsis* roots. Production of UPP was monitored using WGA-Alexa Fluor 488.

A. At the initial time point, few cells are attached to the root and little UPP is detected. After 180 min, many more cells are attached to the root and UPP is frequently detected along the junction between the bacterial cells and the root. UPP is rarely observed in the unattached cells.

B. Close-up of the region boxed in (A). A single *A. tumefaciens* cell attaches to the root and subsequent production of UPP is observed.

and sample other surfaces (Korber *et al.*, 1994; Hinsä *et al.*, 2003). The ability to rapidly deploy a permanent adhesin may be advantageous for swimming cells that have encountered a favourable environment, enabling colonization.

Another advantage of just-in-time adhesin deployment upon surface contact is that it reduces the chances that the adhesive will be inactivated. Once exported from the cell, two main factors can reduce the adhesiveness of an adhesin: curing, as observed in the mussel byssal thread (Kamino, 2008) and also with man-made glues, and occlusion of the adhesin with microscopic particles after random collision events. Either mechanism would be expected to decrease adhesiveness over time, limiting productive interactions with a surface. Indeed, previous experiments showed that permanent surface adhesion is highest during the swarmer phase of the *C. crescentus* developmental cycle and drops dramatically during the stalked cell stage despite the presence of a holdfast on stalked cells (Bodenmiller *et al.*, 2004; Levi and Jenal, 2006). These results support the hypothesis that holdfast adhesiveness decreases with time after export and suggest that the ability to rapidly stimulate adhesin formation upon initial contact with a surface dramatically improves the efficiency of surface interaction and colonization.

Polar attachment of single bacterial cells as a prelude to the transition from reversible to irreversible attachment has also been seen in other proteobacteria such as *Escherichia coli* (Agladze *et al.*, 2005) and *Pseudomonas aeruginosa* (Caiazza and O'Toole, 2004). Therefore, contact-dependent adhesin deployment may be a com-

monly utilized strategy to improve adhesion. As surface-associated adhesins may also act to stimulate host defence responses, this timing may also come into play during pathogenesis to ensure that potential antigens are not prematurely exposed to the host immune system.

Experimental procedures

Bacterial strains and synchronization

Caulobacter crescentus strains were grown at 30°C in PYE medium (Poindexter, 1964) with 10 µg ml⁻¹ kanamycin when indicated. The following *C. crescentus* strains were used in this study: YB135 (CB15 wild type), YB4038 (CB15 $\Delta pilA$), YB5086 (CB15 $\Delta pilA \Delta flgE$) and YB2833 (CB15 $\Delta hfsA$). *A. biprosthhecum* strain JH2 (YB1113) was grown at 26°C in PYE. *A. tumefaciens* strain C58 (YB2951) was grown at 26°C in LB. Synchronized newborn cells were obtained using the plate release technique (Degnen and Newton, 1972). Briefly, cultures were grown for ~10 h with shaking at 100 r.p.m. Three millilitres of the culture was added to 20 ml of fresh growth medium in a 9 cm diameter plastic Petri dish and grown at room temperature overnight (or 48 h for *A. tumefaciens* with medium changes every 12 h) with shaking at 60 r.p.m. After growth, a monolayer of cells was attached to the surface of the Petri dish. The Petri dish was washed thoroughly with water to remove loosely attached and unattached cells. Twenty millilitres of fresh medium was added to the Petri dish and the cells were grown further at room temperature for ~4 h with shaking at 60 r.p.m. The Petri dish was then grown for 1 more hour at 30°C for *C. crescentus* and 26°C for *A. biprosthhecum* and *A. tumefaciens*. The Petri dish was washed repeatedly with oxygenated fresh medium to remove unattached cells and 1 ml fresh medium was added to the plate, which was incu-

bated for 5 min without shaking. The synchronized cells were harvested from the 1 ml of fresh medium. The resulting newborn cells differ in age by a maximum 5 min after cell division.

Fluorescence labelling and fluorescence microscopy

Labelling holdfasts of attached cells. The holdfast is composed of a polysaccharide containing *N*-acetylglucosamine (GlcNac), which can be labelled with fluorescein-conjugated wheat germ agglutinin lectin (fluorescein-WGA) (Merker and Smit, 1988; Ong *et al.*, 1990). A drop of culture containing synchronized swarmer cells was placed on a coverslip for 5 min to allow some cells to attach to the glass surface. The unattached cells were rinsed with PYE. The cells attached to the coverslips were then grown in oxygenated PYE at 30°C for various amounts of time. After growth, the coverslips were rinsed with water and the cells were labelled with 0.02 mg ml⁻¹ fluorescein-WGA (Molecular Probes) and 0.05% w/v of the bactericide sodium azide on ice for 15 min. After labelling, the coverslips were rinsed with 0.05% w/v sodium azide three times and antiphotobleaching solution (20 µg ml⁻¹ catalase, 0.5 mg ml⁻¹ glucose, 0.1 mg ml⁻¹ glucose oxidase and 0.25% v/v mercaptoethanol) was added to the slide for fluorescence microscopy.

Labelling holdfasts of unattached cells. One millilitre of synchronized swarmer cells was added to 100 ml of oxygenated PYE and grown at 30°C without agitation for various amounts of time. In this large volume, very few cells get sufficiently close to the surface to attach. After growth, the unattached cells were supplemented with 0.05% w/v sodium azide and transferred to a pre-cooled glass beaker. Fluorescein-WGA was added to a final concentration of 20 µg ml⁻¹ and the cells were labelled for 15 min on ice. The solution was then passed through an Isopore membrane of 0.4 µm pore size (Millipore) to collect the cells. The collected cells on the membrane were further washed with cold 0.05% w/v sodium azide solution to remove excess dyes. Antiphotobleaching solution was added for fluorescence microscopy.

Attachment of *C. crescentus* on a glass surface

Immediately after synchronization, an aliquot of synchronized cells was placed between a coverslip and a glass slide that had been cleaned with a solution of 6.5 g of Nochromix (Godax Laboratories) in 100 ml of sulphuric acid for 30 min, rinsed thoroughly with water and dried in air before use. The slide sample was quickly transferred to a warm stage kept at 30°C and observed with dark-field microscopy with a 10× objective, allowing cells at any depth in the field of view to be imaged. To monitor the attachment process, a 1-s-long video was taken at 10 frames per second every 2–3 min. To identify attached cells, an average image was obtained by overlaying the 10 frames of a video and dividing the intensity of the overlay by 10. Since the swimming cells are moving, the brightness of swimming cells is 10 times weaker than that of attached cells in the average image, and therefore the attached cells can be identified by appropriately scaling the image to show only the bright spots (Fig. S2A and B). The

attached cells were counted from such images. Attached cells can be distinguished from unattached non-swimming cells because the later drift randomly due to Brownian motion, while attached cells do not. To image the swimming cells, we removed the attached cell from the video by subtracting the average image from each frame. Three consecutive frames were then overlaid and the number of trajectories was counted as the number of swimming cells (Fig. S2C and D). The thickness of the sample was measured using phase-contrast microscopy with a 100× objective by focusing on the top and bottom surfaces respectively (Li and Tang, 2004).

The strain YB2833 ($\Delta hfsA$) lacks a holdfast, is unable to form a stable monolayer and therefore cannot be synchronized by the plate release method. To quantify its attachment, YB2833 cells were grown to exponential phase in a mixed culture, centrifuged and resuspended in fresh PYE. An aliquot was confined between a coverslip and a glass slide and the sample was observed by dark-field microscopy at 30°C. The culture at mid-exponential phase is a mixture of swarmer cells, stalked cells and pre-divisional cells. We measured the decrease in the number of swimming cells as a measure of attachment by counting the number of cell trajectories as described above. The attached cells could easily be washed off the surface, unlike similarly treated wild-type cells, which are tightly attached through their holdfast. Although this strain is able to attach to glass surface through pili, the attachment is not permanent. To quantify the detachment, we added a drop of cell culture at mid-exponential phase to a coverslip, allowed it to sit for 2 min and rinsed gently to remove unattached cells. The number of attached cells was recorded under the light microscope over time.

Labelling holdfast in viscous media

The test viscosity agents used included Percoll (GE), PEGs of molecular weight 8000 Da and 35 000 Da (Fluka), dextran of molecular weight 100 000–200 000 Da (Sigma) and Ficoll of molecular weight 400 000 Da (Fluka). The viscosity was measured with the falling ball method (Tang *et al.*, 1999), except for PEG 8000 (Gunduz, 1996) and Ficoll (Chen and Berg, 2000), which are obtained from the literature. Synchronized *C. crescentus* wild-type cells were mixed with these agents at the designated concentration and 10 µg ml⁻¹ fluorescein-WGA in PYE in a 1 ml tube. A portion of the mixture was taken out to make slide samples at different times to observe cell swimming and aggregation. To observe holdfast labelling 15 min after mixing, the mixture was passed through a 0.44 µm microfilter (Millipore), followed by passing 60 ml of water to wash the cells trapped by the filter. About 100 µl of water was left in the filter to collect the cells, which became dispersed by the procedure. The cells were then dried on a coverslip for microscopic observation.

Fluorescence microscopy

A Nikon Eclipse E800 epifluorescence microscope with a 100× oil immersion objective lens (Plan Apo) was used to image the fluorescently labelled holdfast. A highly sensitive and linear CoolSnap camera controlled by MetaMorph software (Universal Imaging, PA) was used to record labelled

holdfast fluorescence. The fluorescence images were taken at 0.1 s exposure time. To keep the same photobleaching time for each sample, we focused on the holdfast by taking successive pictures under fluorescence microscopy after readjusting the sample stage height between pictures. If the holdfast was in focus prior to the 11th exposure, subsequent exposures were taken and the picture taken at the 11th exposure was used to measure the fluorescence intensity.

TIRF microscopy

TIRF microscopy was performed with a Nikon E2000 inverted microscope with a TIRF objective (NA = 1.49). Two hundred microlitres of synchronized swarmer cells (2.5 ± 2.5 min old) in $20 \mu\text{g ml}^{-1}$ fluorescein-WGA lectin solution in PYE was added to a cleaned coverslip sitting on top of the TIRF objective. Some of the dye adsorbed to the coverslip, facilitating focusing of the microscope. After focusing, the TIRF illumination was kept on for 2 min to photobleach the adsorbed dye. Since the TIRF setting varies from experiment to experiment, it is hard to control the light intensity on the holdfast and inconsistent levels of photobleaching are observed. Light microscopy images and TIRF images were taken alternately with 30 s interval to get a time series. The integrated intensity of the TIRF image of individual holdfasts was measured over time. For experiments in the presence of polymers, cells were mixed with the polymers as indicated and $20 \mu\text{g ml}^{-1}$ fluorescein-WGA lectin prior to TIRF observation. All the experiments were performed at approximately 22°C .

A. tumefaciens UPP production during attachment to an *Arabidopsis* root

Sterilized *Arabidopsis thaliana* seeds were placed on $1/2$ MS salts plates with 1% agar and 1% sucrose and allowed to germinate and grow until the roots were approximately 3 cm long (Ramey *et al.*, 2004). Wild-type *A. tumefaciens* cells were grown in LB medium at 26°C until reaching exponential phase. Two hundred microlitres of cell culture was washed and resuspended in a solution containing 1 mM calcium chloride and 0.4% sucrose. The bacterial cell suspension was spotted into a Petri dish and a root segment was floated in the bacterial cell suspension. The root segments were incubated in the bacterial culture overnight in the dark at room temperature. The root segments were rinsed in 1 mM calcium chloride and 0.4% sucrose to remove unattached cells and placed on an agarose pad containing Alexa Fluor 488 conjugated-WGA. The attachment of *A. tumefaciens* cells to the root segments was observed using time-lapse microscopy. *A. tumefaciens* attachment to the root occurs prior to the formation of UPP and this structure is rarely observed in the unattached cells (Fig. 7).

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References

- Agladze, K., Wang, X., and Romeo, T. (2005) Spatial periodicity of *Escherichia coli* K-12 biofilm microstructure initiates during a reversible, polar attachment phase of development and requires the polysaccharide adhesin PGA. *J Bacteriol* **187**: 8237–8246.
- Anderson, J.K., Smith, T.G., and Hoover, T.R. (2010) Sense and sensibility: flagellum-mediated gene regulation. *Trends Microbiol* **18**: 30–37.
- Beloin, C., Houry, A., Froment, M., Ghigo, J.M., and Henry, N. (2008) A short-time scale colloidal system reveals early bacterial adhesion dynamics. *PLoS Biol* **6**: e167.
- Blair, K.M., Turner, L., Winkelman, J.T., Berg, H.C., and Kearns, D.B. (2008) A molecular clutch disables flagella in the *Bacillus subtilis* biofilm. *Science* **320**: 1636–1638.
- Bodenmiller, D., Toh, E., and Brun, Y.V. (2004) Development of surface adhesion in *Caulobacter crescentus*. *J Bacteriol* **186**: 1438–1447.
- Brown, P.J., Hardy, G.G., Trimble, M.J., and Brun, Y.V. (2009) Complex regulatory pathways coordinate cell-cycle progression and development in *Caulobacter crescentus*. *Adv Microb Physiol* **54**: 1–101.
- Caiazza, N.C., and O'Toole, G.A. (2004) SadB is required for the transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas aeruginosa* PA14. *J Bacteriol* **186**: 4476–4485.
- Chen, X., and Berg, H.C. (2000) Torque-speed relationship of the flagellar rotary motor of *Escherichia coli*. *Biophys J* **78**: 1036–1041.
- Degnen, S.T., and Newton, A. (1972) Chromosome replication during development in *Caulobacter crescentus*. *J Mol Biol* **129**: 671–680.
- Eboigbodin, K.E., Newton, J.R.A., Routh, A.F., and Biggs, C.A. (2005) Role of nonadsorbing polymers in bacterial aggregation. *Langmuir* **21**: 12315–12319.
- Entcheva-Dimitrov, P., and Spormann, A.M. (2004) Dynamics and control of biofilms of the oligotrophic bacterium *Caulobacter crescentus*. *J Bacteriol* **186**: 8254–8266.
- Gunduz, U. (1996) Evaluation of viscosities of polymer-water solutions used in aqueous two-phase systems. *J Chromatogr B* **680**: 263–266.
- Hardy, G.G., Allen, R.C., Toh, E., Long, M., Brown, P.J., Cole-Tobian, J.L., and Brun, Y.V. (2010) A localized multimeric anchor attaches the *Caulobacter* holdfast to the cell pole. *Mol Microbiol* **76**: 409–427.
- Hinsa, S.M., Espinosa-Urgel, M., Ramos, J.L., and O'Toole, G.A. (2003) Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Mol Microbiol* **49**: 905–918.

- Hosek, M., and Tang, J.X. (2004) Polymer-induced bundling of F-actin and the depletion force. *Phys Rev E Stat Nonlin Soft Matter Phys* **69**: 0519071–0519079.
- Kamino, K. (2008) Underwater adhesive of marine organisms as the vital link between biological science and material science. *Mar Biotechnol (NY)* **10**: 111–121.
- Karatan, E., and Watnick, P. (2009) Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev* **73**: 310–347.
- Korber, D.R., Lawrence, J.R., and Caldwell, D.E. (1994) Effect of motility on surface colonization and reproductive success of *Pseudomonas fluorescens* in dual-dilution continuous culture and batch culture systems. *Appl Environ Microbiol* **60**: 1421–1429.
- Laus, M.C., Logman, T.J., Lamers, G.E., Van Brussel, A.A., Carlson, R.W., and Kijne, J.W. (2006) A novel polar surface polysaccharide from *Rhizobium leguminosarum* binds host plant lectin. *Mol Microbiol* **59**: 1704–1713.
- Levi, A., and Jenal, U. (2006) Holdfast formation in motile swarmer cells optimizes surface attachment during *Caulobacter crescentus* development. *J Bacteriol* **188**: 5315–5318.
- Li, G., and Tang, J. (2004) Diffusion of actin filaments within a thin layer between two walls. *Phys Rev E Stat Nonlin Soft Matter Phys* **69**: 061921.
- Li, G., and Tang, J.X. (2006) Low flagellar motor torque and high swimming efficiency of *Caulobacter crescentus* swarmer cells. *Biophys J* **91**: 2726–2734.
- van Loosdrecht, M.C., Lyklema, J., Norde, W., and Zehnder, A.J. (1990a) Influence of interfaces on microbial activity. *Microbiol Rev* **54**: 75–87.
- van Loosdrecht, M.C., Norde, W., and Zehnder, A.J. (1990b) Physical chemical description of bacterial adhesion. *J Biomater Appl* **5**: 91–106.
- McCarter, L., Hilmen, M., and Silverman, M. (1988) Flagellar dynamometer controls swarmer cell differentiation of *V. parahaemolyticus*. *Cell* **54**: 345–351.
- Merker, R.I., and Smit, J. (1988) Characterization of the adhesive holdfast of marine and freshwater *Caulobacters*. *Appl Environ Microbiol* **54**: 2078–2085.
- Morse, D.E. (1990) Recent progress in larval settlement and metamorphosis: closing the gaps between molecular biology and ecology. *Bull Mar Sci* **46**: 465–483.
- Ong, C.J., Wong, M.L.Y., and Smit, J. (1990) Attachment of the adhesive holdfast organelle to the cellular stalk of *Caulobacter crescentus*. *J Bacteriol* **172**: 1448–1456.
- Otto, K. (2008) Considering the first steps toward a stable and orderly way of bacterial life. *PLoS Biol* **6**: e180.
- Pate, J.L., Porter, J.S., and Jordan, T.L. (1973) *Asticcacaulis biprosthecum* sp.nov.: life cycle, morphology, and cultural characteristics. *Antonie Van Leeuwenhoek* **39**: 569–583.
- Paul, K., Nieto, V., Carlquist, W.C., Blair, D.F., and Harshey, R.M. (2010) The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a 'backstop brake' mechanism. *Mol Cell* **38**: 128–139.
- Poindexter, J.S. (1964) Biological properties and classification of the *Caulobacter crescentus* group. *Bacteriol Rev* **28**: 231–295.
- Ramey, B.E., Matthyse, A.G., and Fuqua, C. (2004) The FNR-type transcriptional regulator SinR controls maturation of *Agrobacterium tumefaciens* biofilms. *Mol Microbiol* **52**: 1495–1511.
- Schwarz-Linek, J., Dorken, G., Winkler, A., Wilson, L.G., Pham, N.T., French, C.E., et al. (2010) Polymer-induced phase separation in suspensions of bacteria. *Europhys Lett* **89**: 68003.
- Smith, C.S., Hinz, A., Bodenmiller, D., Larson, D.E., and Brun, Y.V. (2003) Identification of genes required for synthesis of the adhesive holdfast in *Caulobacter crescentus*. *J Bacteriol* **185**: 1342–1442.
- Tamburri, M.N., Zimmer-Faust, R.K., and Tamplin, M.L. (1992) Natural sources and properties of chemical inducers mediating settlement of oyster larvae: a re-examination. *Biol Bull* **183**: 327–338.
- Tang, J.X., Ito, T., Tao, T., Traub, P., and Janmey, P.A. (1997) Opposite effects of electrostatics and steric exclusion on bundle formation by F-actin and other filamentous polyelectrolytes. *Biochemistry* **36**: 12600–12607.
- Tang, J.X., Janmey, P.A., Stossel, T.P., and Ito, T. (1999) Thiol oxidation of actin produces dimers that enhance the elasticity of the F-actin network. *Biophys J* **76**: 2208–2215.
- Toh, E., Kurtz, H.D., and Brun, Y.V. (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.
- Tomlinson, A.D., and Fuqua, C. (2009) Mechanisms and regulation of polar surface attachment in *Agrobacterium tumefaciens*. *Curr Opin Microbiol* **12**: 708–714.
- Tsang, P.H., Li, G., Brun, Y.V., Freund, L.B., and Tang, J.X. (2006) Adhesion of single bacterial cells in the micronewton range. *Proc Natl Acad Sci USA* **103**: 5764–5768.
- Umbreit, T.H., and Pate, J.L. (1978) Characterization of the holdfast region of wild-type cells and holdfast mutants of *asticcacaulis biprosthecum*. *Arch Microbiol* **118**: 157–168.
- Van Dellen, K.L., Houot, L., and Watnick, P.I. (2008) Genetic analysis of *Vibrio cholerae* monolayer formation reveals a key role for DeltaPsi in the transition to permanent attachment. *J Bacteriol* **190**: 8185–8196.
- Vigeant, M.A.S., Ford, R.M., Wagner, M., and Tamm, L.K. (2002) Reversible and irreversible adhesion of motile *Escherichia coli* cells analyzed by total internal reflection aqueous fluorescence microscopy. *Appl Environ Microbiol* **68**: 2794–2801.

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