Genetic Dissection of *Caulobacter crescentus* Surface Colonization

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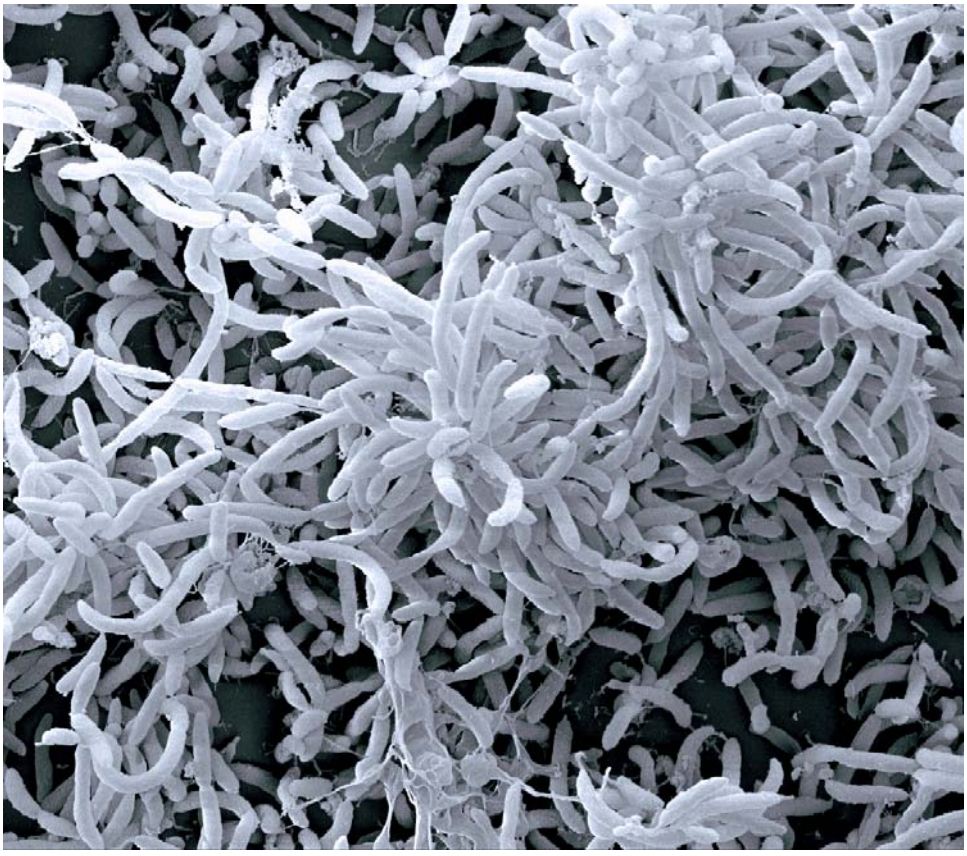
Genetic Dissection of *Caulobacter crescentus* Surface Colonization

"... It is quite evident that for the most part, water bacteria are not free floating organisms, but grow upon submerged surfaces"

Arthur T. Henrics
Journal of Bacteriology
1933, 25: 277-287



SEM image of *Caulobacter crescentus* CB15 microcolony grown on borosilicate surface

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SUMMARY

During its biphasic life cycle *Caulobacter crescentus* switches from a planktonic to surface attached life style. This transition requires the continuous remodeling of the cell poles through the temporally and spatially coordinated assembly and disassembly of polar organelles like the flagellum, pili, and an adhesive holdfast. A genetic screen for mutants affected in surface binding and colonization led to the identification of various genes required for motility, pili, and holdfast biogenesis, suggesting a specific role for all three organelles in *C. crescentus* surface colonization. Several novel holdfast genes were identified, which are potentially involved in the synthesis and regulation of the polysaccharidic component of the holdfast. Quantitative surface binding studies during the *C. crescentus* cell cycle revealed that optimal attachment coincides with the presence of flagellum, pili, and holdfast at the same pole. This indicated that accurate temporal control of polar appendices is critical for surface colonization of *C. crescentus* and represents the first example for developmentally controlled bacterial surface adhesion.

We have used genetic and biochemical analyzes to demonstrate that di-cyclic guanosine monophosphate (c-di-GMP) is a central regulatory compound involved in the timing of *C. crescentus* pole development. Mutants lacking the diguanylatecyclase PleD show a dramatic delay of holdfast formation during swarmer cell differentiation. In contrast, cells lacking the GGDEF-EAL composite protein CC0091 show premature holdfast formation, while overexpression of CC0091 also leads to a delayed appearance of holdfast. The observation that CC0091 is a c-di-GMP specific phosphodiesterase indicated that the antagonistic activities of PleD and CC0091 could be responsible for the correct timing of holdfast formation and flagellum ejection. Finally, our genetic screen identified a candidate for the c-di-GMP effector protein, which mediates holdfast synthesis in response to fluctuating levels of c-di-GMP. The glycosyltransferase CC0095 is strictly required for holdfast formation and its overexpression leads to premature holdfast synthesis. This and the observation that CC0095 is able to bind c-di-GMP lead to the hypothesis that holdfast synthesis is

regulated via allosteric control of the CC0095 glycosyltransferase. These data provide the first example of a developmental process being regulated by the bacterial second messenger, c-di-GMP.

OVERVIEW

What is a biofilm?

For most of the history of microbiology, microorganisms have primarily been characterized as planktonic, freely suspended cells and described based on their growth characteristics in nutritionally rich culture media. However, in the majority of natural environments, bacteria are rarely found in the planktonic, free-swimming phase. Rather, they are found in association with a biotic or abiotic surfaces in a structure known as a biofilm (22). It is believed that biofilms are the predominant microbial lifestyle. Surface association seems to be means for bacteria persisting in biological or pathogenic microenvironments. For aquatic or soil microorganisms, surface attachment and biofilm formation may provide an adaptive advantage. For example, high-density communities of attached bacteria could metabolize insoluble polymeric organic compounds, hemicellulose, or the exoskeletons of crustaceans and insects. Large negatively charged microbial cell aggregates found in biofilms may constitute a substratum to concentrate and chelate different limiting nutrients such as iron. Finally, biofilms are believed to provide protection from toxic compounds, antibiotics, stress factor and predators (104, 106, 193). It has been speculated that surface attachment and biofilm formation has evolved as a protective mechanism against grazing protozoan predators (105, 106, 193). The persistence stage of bacterial infections is often associated with biofilm formation, and as a result of increased resistance to antimicrobial and the scavenging forces of the immune system, is very difficult to eradicate (39). Persistence of *Vibrio cholerae* in aquatic environments is thought to be the main factor for seasonal occurrence of cholera epidemics (106). Biofilm-like colonization of the lungs of cystic fibrosis (CF) patients by *Pseudomonas aeruginosa* is considered as the principal cause of mortality in CF patients (40). In *Yersinia pestis*, the biological transmission of plague depends on blockage of the flea foregut by a biofilm-like cell mass. This blockage is dependent on the hemin storage (*hms*) locus. *Y. pestis hms* mutants, although established long-term infection of the flea's

midgut, failed to colonize the proventriculus. Thus, the *hms* dependent biofilm formation affects the course of *Y. pestis* infection in its insect vector, leading to a change in blood-feeding behavior and to efficient transmission of plague (28, 63). Another example of biofilm formation role in pathogenicity comes from *Staphylococcus aureus.* Recently, Kropec *et al*. (89) found that in three mouse models of infection (bacteremia, renal abscess formation, and lethality following high-dose intraperitoneal infection), using three divergent *S. aureus* strains, the loss of PNAG by deletion of the intracellular adhesion (*ica*) locus had a profound effect on virulence of this microorganism, which was more susceptible to innate host immune killing (88). Mutant strains showed significantly reduced abilities to maintain bacterial levels in blood, to spread systemically to the kidneys, or to induce a moribund/lethal state following intraperitoneal infection (89). Fluckiger *et al.* (46) have used a device-related infection model to show that PIA is detectable early in the infection course of *S. epidermidis,* and that its production in *S. aureus* is induced during the course of a device-related infection. They have shown that PIA production and biofilm formation of both species exist late in infection, and that the *ica* genes and biofilm formation are essential for staphylococcal colonization and endurance on implants (46). Persistence of uropathogenic *Escherichia coli* as biofilm-like communities was proposed to be the source for recurrent urinary tract infections (81).

Biofilm-associated cells can be distinguished from suspended cells by the formation of an extracellular polymeric substance (EPS) that acts as a matrix for the embedded cells. Biofilm associated cells often display reduced growth rates and a completely different genetic program compared with their planktonic counterpart (reviewed in (37)). Attachment of cells to each other and to surfaces is a complex process regulated by a diverse range of environmental and possibly host signals, which are still poorly understood. Attached bacteria may take the form of a dispersed monolayer of surface-bound cells, they can aggregate on the surface to form microcolonies, or they may be organized into a well structured three-dimensional biofilm (112).

Exopolysaccharides in biofilms

EPS may account for 50% to 90% of the total organic carbon of biofilms and is considered as the main matrix material of the biofilm (37). EPS consists of various biopolymers with different chemical and physical properties; however, it is primarily composed of polysaccharides. Some of these polysaccharides are polyanionic (175), which allow the association of divalent cations such as calcium and magnesium that could strengthen the matrix structure by cross linkage. In the case of some gram-positive bacteria, such as the *staphylococci*, the chemical composition of EPS may be quite different and may be primarily cationic (37). EPS is also highly hydrated and thus large amounts of water can become incorporated into its structure by hydrogen bonding. Sutherland (176) noted two important properties of EPS for its role in microbial biofilms. First, the chemical composition and structure of EPS might determine the biofilm conformation (175). For example, many bacterial EPS possess backbone structures that contain 1,3- or 1,4-β-linked hexose residues and tend to be relatively rigid and poorly soluble. Second, the EPS of biofilms is not generally homogeneous but may vary spatially and temporally (175). Leriche *et al.* exploited the binding specificity of different lectins to sugars in order to assess the polysaccharide properties during bacterial biofilm formation of different organisms (99), the results of this study indicated that distinct organisms produce different amounts of EPS which increases with biofilm development. EPS production is affected by the nutrient status of the cells, when an excess in available carbon with limiting nitrogen, potassium, or phosphate were shown to promote EPS synthesis (175). EPS may also contribute to the antimicrobial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm, probably by binding directly to these agents (38, 104) .

In many bacteria, EPS biosynthesis is underlain the regulation of various systems. In *V. cholerae* the expression of *Vibrio* polysaccharide synthesis genes (*vps*) was shown to be regulated by VpsR and VpsT, homologous to response regulators of two-component regulatory system (17). The *vps* genes expression in this microorganism was shown to be controlled also by absence of the

flagellar structure (191), and also by quorum sensing mediated signals (205). In *P. aeruginosa* alginate biosynthesis gene, *algC*, was shown to be upregulated within 15 minutes following contact with the surface (29); in addition to alginate synthesis genes, recent studies of the *P. aeruginosa* autoaggregative phenotype led to the identification of two genetic loci, *psl* and *pel*, that are involved in the production of two distinct carbohydrate-rich biofilm matrix components. The *pel* gene cluster is involved in the production of a glucose-rich matrix material, while the *psl* gene cluster is involved in the production of a mannose-rich matrix material (48). Hickman *et al.* demonstrated that the expression level of these gene clusters is increased in a *wspF* mutant, probably due to elevation in the cellular levels of c-di-GMP which is probably caused by the constitutive activation by phosphorylation of WspR (62). *S. aureus* biofilm formation seems to be mediated primarily by the production of the extracellular polysaccharide PIA/PNAG, which is composed of linear beta-1,6-linked glucosaminylglycans. The synthesis of PIA/PNAG depends on the expression of the intercellular adhesion genes *icaADBC* (55). While most of the *S. aureus* strains analyzed so far contain the entire *ica* gene cluster (23), these genes are only expressed in a few, probably due to the regulatory nature and the complex control of these genes. *ica* genes expression was shown to be subjected to environmental stimuli such as high osmolarity, anaerobic conditions, high temperature and certain antibiotics (140). Recent evidence indicates that SarA, a key regulator of *S. aureus* virulence factors, is required for the expression of *ica* genes and the synthesis of PIA/PNAG (184).

Biofilm as a developmental process

The term microbial development was defined as "…changes in form and function that play a prominent role in the life cycle of the organism…" (120). Recent genetic and molecular approaches used to study bacterial biofilms, have uncovered various genes and regulatory circuits important for initial cell-surface interactions and biofilm development. Studies to date suggest that the planktonic-biofilm transition is, like any other bacterial developmental process, complex and highly regulated. Biofilm development consists of a series of well-regulated discrete steps: i) reversible attachment, ii)

irreversible attachment, iii) maturation, and iv) dispersion (Figure 1) (156). Reversible attachment was shown in many organisms to be mediated by flagellar based motility and fimbrial adhesins (64). Active motility is thought to assist surface binding by helping the cell to overcome the charge barrier that prevents the negatively charged bacterial cell from reaching certain surfaces. It has been also postulated that an active flagellar motor could play a part in the regulation switch that upon surface binding of bacteria, leads to an up-regulation of exopolysaccharide synthesis (97). Irreversible attachment is mediated mainly by self-made polymeric substances, usually exopolysaccharides, which not only promote cell-cell and cell-surface contacts, but also construct part of the encapsulating matrix (33, 109). Flagella-independent motility (gliding or twitching) allows some bacteria to move on the surface and to form cell-aggregates known as microcolonies (60, 123). Clonal growth within these microcolonies together with EPS encapsulation results with the maturation of a biofilm. Finally, an active dissociation or stream shear forces trigger the dispersal of sub populations of the biofilm (52, 71, 188). Bacteria within each of these four stages of biofilm development are physiologically distinct (26). It is obvious that biofilm formation resembles other adaptive processes of bacterial development like fruiting body formation in *Myxococcus xanthus*, and although the molecular and regulatory mechanisms may differ from organism to organism, the stages of biofilm development seem to be similar in a wide range of microbes (121).

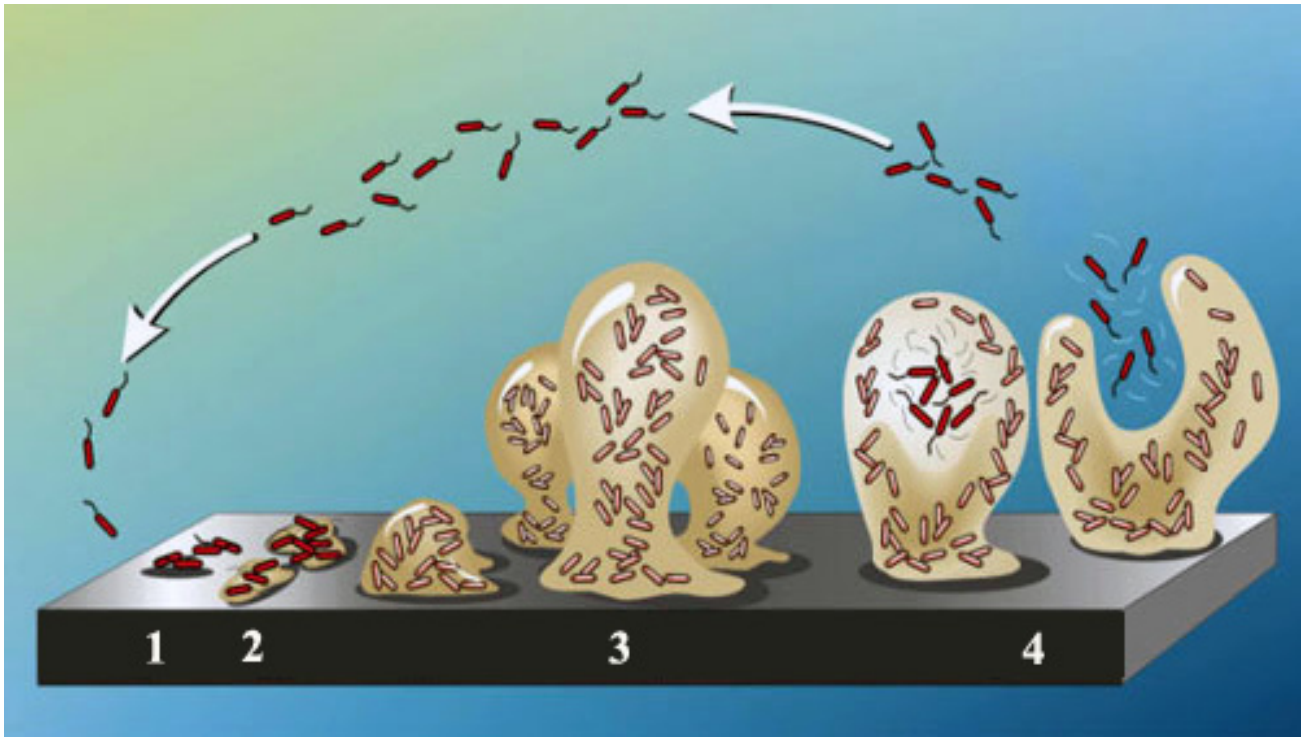

Figure 1) Illustration of the four main stages of biofilm development. Stage i) reversible attachment of cells to the surface mediated by flagellar motility and adhesive pili. Stage ii) irreversible cementing of the cells on the surface is a result of EPS production. Stage iii) maturation of biofilm architecture including water channels and pillars. Stage iv) dispersion of single cells from the biofilm. This figure was adapted from (174).

One of the hallmarks of a developmental process is near-complete changes of gene expression profile of the different stages. In accordance with this, differential stage-specific gene expression has been reported during biofilm formation. Genes required for the initial stage of biofilm formation, e.g. those coding for components of the flagellar motor and adherence pili, are usually repressed in the mature biofilms of many Gram-negative bacteria simply because although these structures required during the initial stages of biofilm development, they might destabilize the mature biofilm (155, 157, 196). In contrast, exopolysaccharides synthesis genes, which are critical for the adherence and for the maintenance of the biofilm structure, exhibit increased expression in biofilm-embedded cells. Thus, progression from the planktonic to the biofilm state requires a change of the cell's genetic program. Several studies have reported on this program change using global analysis of gene expression or protein synthesis.

A protein collection of all four stages during *P. aeruginosa* biofilm formation was established using 2-D gel electrophoresis (156). On average, consecutive stages differed by 35% of the detectable proteins. 29% of the protein spots changed upon reversible attachment, and 40% upon biofilm maturation (156). Escape from biofilm reduced the protein pool by 35% and re-established a protein profile similar to the one observed for planktonic cells (156). When comparing steady-state levels of proteins from planktonic and biofilm cells, more than 800 proteins showed a six-fold or higher change in abundance (156). The identified proteins fall into four main classes: metabolism, phospholipid and LPS-biosynthesis, protein transport and secretion, as well as adaptation and protective mechanisms (156). In another study performed with *P. aeruginosa*, genes responsible for alginate biosynthesis were shown to be upregulated within 15 minutes after cells adherence to surfaces, arguing that surface binding might initiate this genetic switch that leads to biofilm formation (29). A study by Sauer *et al.* showed that the expression of more than 30 operons was altered within 6 hours following *P. putida* surface attachment (155).

The comparison of global gene expression profiles of planktonic *vs.* biofilm cells was performed with several model organisms. When gene expression in *E. coli* biofilms grown in a flow chamber was compared with planktonic cells in stationary or exponential phase, an overall alteration of more than 600 genes was observed between stationary phase and biofilm cells (157). Only 230 genes were found to be differentially expressed in exponentially growing cells and biofilm cells (157). Among the genes that showed increased expression in biofilms, several were shown to be involved in adhesion and autoaggregation. In a parallel study, 38% of a random *E. coli lacZ* fusion library showed biofilm-specific expression (138); sessile bacteria showed specific up-regulation of genes involved in colanic acid biosynthesis (*wca* locus), while *fliC* (flagellin) was reduced in biofilms. Moorthy and Watnick used microarrays to study the transcriptome of *V. cholerae* during each stage of biofilm development (114). The transitions from planktonic to monolayer and mature biofilm identified up to 383 differentially regulated genes. Most of these genes were specific for only one of the three experimental stages analyzed. These results demonstrated that monolayer and mature biofilm stages of *V. cholerae* biofilm development are transcriptionally distinct. A similar analysis with a clinical isolate of *Staphylococcus aureus* (UAMS-1) reveled a total of 580 differentially expressed genes (10). In this study, the largest difference of total numbers of differentially expressed genes was observed between the biofilms and the exponentially grown planktonic cells (10). Taken together, these studies make it apparent that biofilms have gene-expression patterns that differ from those of planktonic bacteria, and telling something about the extensive physiological changes that occur during biofilm formation. These global gene expression analyzes facilitated the uncovering of the stage-specific cell physiology and morphology during biofilm development and demonstrated the complexity of this process.

Structural requirements for biofilm formation

Initial attachment and microcolony formation

Pseudomonas aeruginosa- In a pioneering work by O'Toole and Kolter (123), a screen for the isolation of *P. aeruginosa* Tn*5* insertion mutants defective in the initial steps of biofilm formation was undertaken, based on the ability of this bacterium to adhere to plastic surface of a microtiter plate. Two classes of mutants, named *sad* (surface attachment defective), were described, one class constitutes flagellar-motility mutants while the other class consists of mutants defective in the biogenesis of the adhesive type IV pili (123). While a pili mutant was able to form a wild type-like monolayer of cells on the surface, they were unable to develop into microcolonies. Type-IV pili are required for twitching motility, a mode of surface locomotion used by *P. aeruginosa* and other bacteria in which the polar pili are believed to extend and retract, and thereby propelling bacteria across a surface. Thus, the findings by O'Toole and Kolter (123) suggested that surface based motility is required for the second step of biofilm formation.

Vibrio cholerae- In a similar genetic analysis performed by Watnick and Kolter (190), three classes of *V. cholerae* El Tor *sad* mutants were described (190). The first class of genes is required for the biosynthesis of the mannose-sensitive haemagglutinin type-IV pilus (MSHA); the second group of mutants was defective in flagellar motility, including both mutants lacking flagella and mutants with paralyzed flagella. The third group of *sad* mutants had transposon insertions in *vps* genes. The phenotypes of these mutant classes suggested that pili and flagella accelerate attachment to and mediate the spread along the abiotic surface, while exopolysaccharide synthesis by the *vps* genes is required for the formation of the three-dimensional biofilm architecture. In contrast to mutants lacking pili and flagella, EPS mutants were unable to form a detectable biofilm even after extended incubation time (190).

E. coli- A study by Pratt and Kolter reveled three classes of attachment-deficient *E. coli Tn*10 mutants (135). The mutations isolated included flagellar biogenesis and motor function genes, and

genes which were involved in the biogenesis and regulation of type-I pili (135). Interestingly, in a strain overproducing curli background, flagella were dispensable for initial adhesion and biofilm development (137), arguing that at least part of the role of flagella in surface colonization might be of a regulatory nature.

Biofilm maturation

P. aeruginosa- Klausen *et al*. have shown that flagella and type-IV pili take part in shaping the architecture of *P. aeruginosa* biofilms, although they are not essential for biofilm formation (87). The model which they proposed suggests that the formation of mushroom-shaped structures in *P. aeruginosa* biofilms is caused by bacteria which climb on the top of the microcolony stalks using of type-IV pili mediated twitching motility (86); according to this model, type-IV pili driven bacterial migration plays a key role in structure formation in the late phase of biofilm development.

V. cholera- *V. cholerae* strain which is defective in EPS synthesis fails to form a mature biofilm architecture (189, 202). Moorthy and Watnick have recently shown that the cell monolayers formed on surfaces represent a distinct stage of this microorganism biofilm development (112). They have demonstrated that while MSHA pilus is only required for the monolayer formation, *vps* is required for formation and maintenance of the mature biofilm and that the maturation of these monolayers to three-dimensional biofilm structure, requires monosaccharides such as mannose, which induce the expression of *vps* genes (112).

E. coli- Molin and co-workers have shown recently that the maturation of *E. coli* K12 biofilms requires the presence of an *incF* plasmids (145). They have demonstrated that while surface attachment, clonal growth and microcolony formation were not affected in the plasmid plasmid-free strains, the efficient biofilm maturation could only occurred in strains carrying the conjugation pilus proficient plasmid (145) and that *E. coli* strains lacking these plasmids were not able to form the elaborated three-dimensional biofilm architecture that include pillars and channels (145). They have

shown that the final shape of the mature biofilm seemed to be determined by the pilus configuration, when various mutants affected in the processing or in the activity of these transfer pili, displayed differently structured biofilms. In addition to that, flagella, type 1 fimbriae, curli and cell-to-cell signalling did not seem to be required for biofilm maturation in *E. coli* K12 carrying the *incF* plasmids (145). This work was with a complete agreement with a previous work published by Ghigo (53), which has demonstrated the involvement of conjugative plasmids in the competence of the bacterial host to form a biofilm (53).

Regulation of biofilm formation

Complex regulatory pathways such as the global carbon metabolism regulator (CRC) (122) and stationary-phase sigma factors (σ^s) (60, 197) have been shown to play an important role in biofilm development despite the fact that these systems are not exclusively committed for biofilm development. High-cell density, high osmolarity, scarce nutrients as well as oxygen limitation are only some of the situation which a biofilm embedded cell and a stationary phase cell might encounter; this similarity could explain some of the convergent regulation circuits that control biofilm formation in addition to stationary phase and stress respond. Besides being subjected to global metabolic control, biofilm components underlie specific regulation at the transcriptional and post-transcriptional level. For example, the *Salmonella typhimurium* CsgD, a transcriptional regulator of the LuxR superfamily, has been shown to positively control the expression of cellulose and curli fimbriae (15). The expression of *csgD* itself is modulated by a variety of stimuli, including, osmolarity, oxygen, nutrient availability, pH, temperature, and the subject of control by many cellular factors, such as RpoS, RpoD, IHF and others (15, 136). In *P. aeruginosa* GacS/GacA proteins of the two-component signal transduction system which controls the production of many secondary metabolites and extracellular enzymes and involved in pathogenicity in plants and animals (58), were shown to also control biofilm formation when *gacA* mutant failed to aggregate and form microcolonies (128). Although the signals that activate the GacS/GacA circuit are not known, it was demonstrated that the *gac* genes are activated during the transition from exponential to stationary phase of the growth (58); and since the expression of *rpoS* is positively regulated by GacS/GacA, some of the GacS/GacA-dependent phenotypes may be related to RpoS activity (117, 195). In addition to the GacA/GacS, a three-component regulatory system specifically required for biofilm maturation was identified (90). This system is comprised of genes *sadARS* coding for a putative sensor histidine kinase and two response regulators; mutations in any of these genes, blocked biofilm maturation of *P. aeruginosa* without affecting growth, early biofilm formation, swimming, or twitching motility (90). The

expression of *sadR* and *sadS* is very similar in planktonic and biofilm cells, while *sadA* expression is slightly decreased (~2-fold) in biofilm cells. The authors have postulated that the SadARS system acts as a regulator of both biofilm formation and for genes involved in type III secretion (TTSS) and it may function to promote biofilm formation, possibly in part by repressing the expression of the TTSS (90).

In addition to the species-specific control mechanisms, biofilm formation is also regulated by two global signal transduction networks. The first, quorum sensing (QS) allows transmitting information between cells and has been shown to regulate cellular processes in response to cell density or crowdedness (129). Since biofilms comprise arrays of dense microbial populations, it was not surprising to find that QS influences biofilm related processes. Davies *et al.* (30) showed that *P. aeruginosa* PAO1 requires the *lasI* gene product $3OC_{12}$-HSL in order to develop a normal biofilm; *lasI* mutant formed flat, undifferentiated biofilms which remain sensitive to SDS (30); interestingly, mutant biofilms appeared normal when supplemented extracellularly with a synthetic $3OC_{12}$ signal molecule (30). Similarly, *Burkholderia cepacia* mutants defective in the *cep* quorum sensing system were able to form microcolonies on a glass surface, but were unable to develop into a mature biofilm (68). In *E. coli*, biofilm formation was shown to be stimulated by the auto-inducer 2 signal (AI-2) (201). It was suggested that AI-2 stimulates biofilm formation through a regulatory cascade including novel motility quorum sensing regulator, MqsR, the two component system QseBC which then promotes cell motility via the master regulon *flhDC*, stimulating MotA and FliA and leads to biofilm formation (201). QS-dependent biofilm formation regulation in *E. coli* was demonstrated also by the deletion of *ydgG* (a putative transport protein that either enhances AI-2 secretion or inhibits AI-2 uptake) which increased the intracellular concentration of AI-2 as turn resulted in a 7,000-fold increase in biofilm thickness and 574-fold increase in biomass in flow cells (59). In contrast, in *V. cholerae*, a reciprocal relationship between quorum sensing and biofilm formation was described (205). *V. cholerae* strains lacking HapR, a LuxR homolog, forms thicker biofilms; microarray

analyses of biofilm-associated bacteria showed that the expression of the *V. cholerae vps* genes is increased in *hapR* mutants when CqsA, one of two known autoinducer synthases in *V. cholerae*, acts through HapR to repress *vps* gene expression (205).

The second global regulator controlling cell adhesiveness and biofilm formation is cyclic di(3'→5')-guanylic acid (c-di-GMP). C-di-GMP is emerging as a global second messenger in bacteria controlling "social behavior." As described above, cell surface appendages mediate bacterial aggregation and facilitate biofilm formation; flagella and pili which are involved in biofilm formation were shown to be regulatory targets of c-di-GMP (reviewed in (25, 74, 148)). Genetic studies have implicated c-di-GMP in the regulation of motility, the production of extracellular polysaccharide, biofilm establishment and maintenance as well as host persistence in a wide range of bacteria (74, 148). Biochemical studies have reveled that cellular levels of cyclic-di-GMP are inversely controlled by the activity of diguanylatecyclases (GGDEF domain) and phosphodiesterases (EAL domain) (Figure 2) (19, 130, 153, 158, 183). GGDEF and EAL domain proteins are abundant and found in most bacteria, covering all branches of the phylogenetic tree (148). C-di-GMP was first described as an allosteric activator of the enzyme cellulose synthase of the bacterium *Gluconacetobacter xylinum* (151). In *Caulobacter crescentus*, c-di-GMP was shown to orchestrate the controlled transition of a flagellated into a "sticky" cell pole which secretes an unknown form of polysaccharide (4, 130). The production of cellulose or derivatives thereof, is activated by GGDEF domain proteins in several other bacteria including *E. coli*, *S. enterica*, *Rhizobium leguminosarum* and *P. fluorescence* (7, 170, 208), in addition to polysaccharides, the biosynthesis of adhesive fimbriae, another component of extracellular matrix also depends on the activity of GGDEF domain proteins (24, 161). In the current working model, high levels of c-di-GMP favor the production of adhesive organelles and blocks different forms of cell motility (162). Hickman *et al.* (62) have recently shown that an increase in cellular levels of c-di-GMP elicited by a specific diguanylatecyclase, WspR, results in higher expression of the *pel* and *psl* EPS gene clusters of *P. aeruginosa* and led to the formation of mature

biofilms (62). Similarly, *vps* expression in *V. cholerae* is controlled by c-di-GMP (183). When the enzymatic activity of the VieA phosphodiesterase is required to repress EPS production under non-biofilm conditions (183). The deletion of *vieA* results in increased cell attachment, probably as a consequence of up-regulation of VpsR, a positive regulator of *vps* gene expression (183). Signature-tagged transposon mutagenesis in *Salmonella* have led to the identification of CdgR, an EAL domain protein which its mutagenesis resulted in lower resistance to hydrogen peroxide and accelerated killing of macrophages in mice model (65). Hoffman *et al.* have shown that alterations in the intracellular levels of c-di-GMP caused by the addition of sub inhibitory concentrations of the antibiotic tobramycin, induced a specific, defensive reaction in both in *E. coli* and *P. aeruginosa* (66). Tobramycin induces the expression of *arr* phosphodiesterase which results in reduced levels of c-di-GMP, increased biofilm formation and increased resistance to tobramycin (66). These studies implicate a complex relationship between c-di-GMP intracellular levels and regulation of biofilm formation.

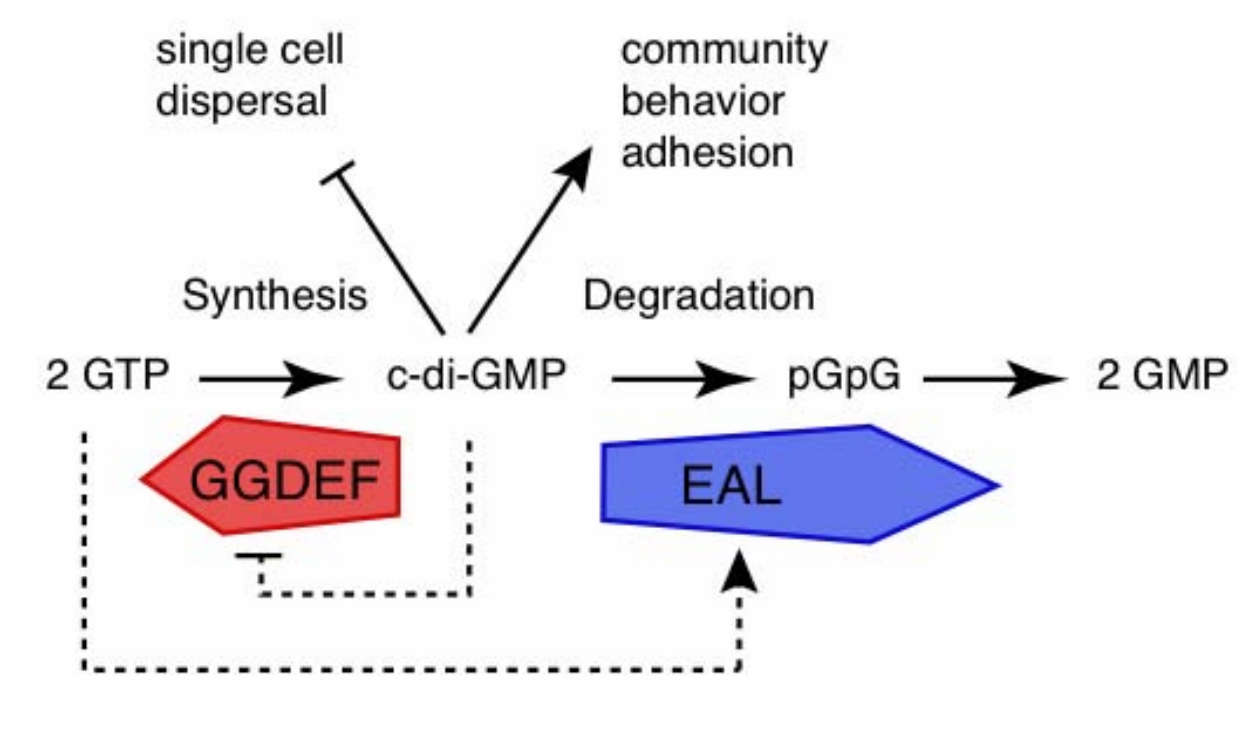

Figure 2) The conversion of GTP into c-di-GMP is catalyzed by the diguanylatecyclases, which reside in the GGDEF domain. Increased intracellular levels of c-di-GMP promote biofilm formation and the biosynthesis of adhesive organelles and inhibit different types of cell motility (reviewed in (162) . Degradation of c-di-GMP is catalyzed by the activity of EAL domain of phosphodiesterases. The illustration was taken from a poster ("Biochemical and genetic identification of a c-di-GMP binding motif") presented by Beat and Mathias Christen and Marc Folcher).

Caulobacter crescentus as a model organism for studying controlled surface attachment and biofilm formation

The genus *Caulobacter* consists of a collection of Gram-negative, hetero-oligotrophic aerobe, rod-like shaped cells that are equipped with a single polar flagellum and polar pili. *Caulobacter crescentus* possesses a stalk, a thin cylindrical extension of the cell containing cell wall and cytoplasm, with an adhesive material, the holdfast, located at its tip. The holdfast mediates strong irreversible attachment of *Caulobacter* cells to solid substrates (110). *Caulobacter* are generally found in aquatic environments, where they attach to biotic and abiotic surfaces (133, 204) and participate in biofouling processes (204). The unique life cycle of *C. crescentus* with its asymmetric cell division and obligatory cell differentiation has made it one of the preferred model organisms to study microbial development and the mechanisms underlying bacterial cell cycle control (152). The dimorphism is established by an asymmetric cell division that gives rise to two genetically identical, but morphologically and physiologically distinct daughter cells with different developmental programs: a sessile stalked cell equipped with an adhesive holdfast and a motile swarmer cell bearing a single flagellum and adhesive pili (16). The stalked cell is competent to start a new replicative cycle immediately after cell division, whilst the swarmer cell is engaged in chemotaxis while the replicative program is being blocked. Before the swarmer cell re-enters replication and cell division it differentiates into a stalked cell, a process during which it loses the flagellum, retracts its pili, and forms a holdfast and a stalk at the pole previously occupied by the flagellar motor. Dimorphism is believed to have evolved to allow *Caulobacter* to cope with life in dilute, nutrient-poor environments (70). The swarmer cell stage allows rapid dispersal and the scavenging of new nutrients resources, while the surface adherent form permits growth where nutrients are available.

The nature of *C. crescentus* cell poles is constantly changing during its development (Figure 3). Pole differentiation is regulated by a complex regulatory network which includes several members of two-

component signal transduction proteins (57, 72, 76, 125). Some of these regulators interlink cell-cycle progression and pole development. E.g., The response regulator CtrA directly controls the initiation of chromosome replication as well as several aspects of polar morphogenesis and cell division (42). The intrinsic asymmetry and microscopically visible appendages make it possible to monitor cell cycle progression and pole differentiation and allow the analysis of temporal and spatial control of polar organelles like flagellum, pili, holdfast, and stalk.

Developmental control of *C. crescentus* polar appendages

The synthesis of *C. crescentus* flagellum requires about 50 different genes. Flagellar gene expression underlies cell cycle control with the temporal activation of CtrA (36, 144). In addition flagellar gene transcription is controlled by hierarchical regulatory system in which the expression and productive assemblage of gene products are required for the expression of gene products which participate successively in the multistep flagellar assembly (118, 141). This regulatory cascade consists of four hierarchical classes. The cascade initiates with class I genes, namely CtrA, which promotes the transcription of the class II genes encoding the MS ring of the basal body, the flagellar switch, and the flagellum-specific type III secretion system (36, 139). The transcription of the flagellar class III and IV is dependent on the proper assembly of the class II components (116). In addition, the expression of class III and IV flagellar genes requires σ^{54} and the transcriptional activator FlbD, which in addition to being subjected to cell cycle-regulated phosphorylation (199), FlbD activity is also subjected to the hierarchical regulation system (115). The ejection of the flagellum during the swarmer-to-stalked cell transition coincides with the degradation of the FliF flagellar anchor. The activity of the diguanylatecyclase response regulator PleD was shown to be required for efficient removal of FliF, ejection of the flagellum, and stalk biogenesis (2). PleD activity is regulated through cell-cycle dependent phosphorylation by PleC and DivJ kinases (4, 130).

These elaborate regulatory mechanisms ensure the linking of flagella assembly and disassembly to the cell cycle and to the development of *C. crescentus.*

Pili are extracellular filaments, found in a wide variety of bacteria. Pili were shown to play a major role in adhesion of bacteria to surfaces, biofilm formation, conjugation, twitching motility, and host infection (165). *Caulobacter crescentus* pili are extracellular surface appendages, 1–4 μm in length and ~4 nm in diameter and are located exclusively at the flagellated pole (165). The pili composed of polymerized pilin subunit (PilA) which is assembled by proteins encoded by a cluster of pilus assembly genes (*cpaA-F*) that are closely related to the tight-adherence genes (TAD) from *Actinobacillus actinomycetemcomitans* (82, 131). The transcription of *cpaB–F* is induced in the late predivisional cell, followed by *cpaA* and, finally the CtrA-dependent transcription of *pilA* with peak of expression in the progeny swarmer cells (96). The timing of pilus assembly can be shifted from the swarmer cell to the predivisional cell stage by expressing *pilA* from a constitutive promoter, suggesting that the temporal transcription is the main type of regulation that prevents premature assembly of the pili (165). It was demonstrated that the PleC histidine kinase, which is localized to the piliated pole during the pilus assembly time window, controls the accumulation of PilA (186). PleC was shown to be responsible for the asymmetric distribution of CpaC (a putative outer membrane pilus secretion channel) and its assembly factor, CpaE (186).

The adhesive holdfast is located at the tip of the stalk at the pole previously occupied with the flagellum. The exact biochemical composition of the holdfast is unknown, however, lectin binding and glycolytic enzymes sensitivity experiments suggest that the holdfast is composed of polysaccharides containing *N*-acetylglucosamine (GlcNAc) oligomers (110). Janakiraman and Brun used an *hfaA-lacZ* fusion to show that the transcription of *hfaA* (part of the *hfaA-D* gene cluster which required for holdfast attachment to the cell envelope (21, 93)) is temporally regulated during the cell cycle. *hfaA* exhibit maximal transcription levels in predivisional cells (73). The authors however, have failed to observe the holdfast before differentiation of the swarmer cell had occurred. How the spatial and temporal regulation of holdfast expression is achieved is still unclear.

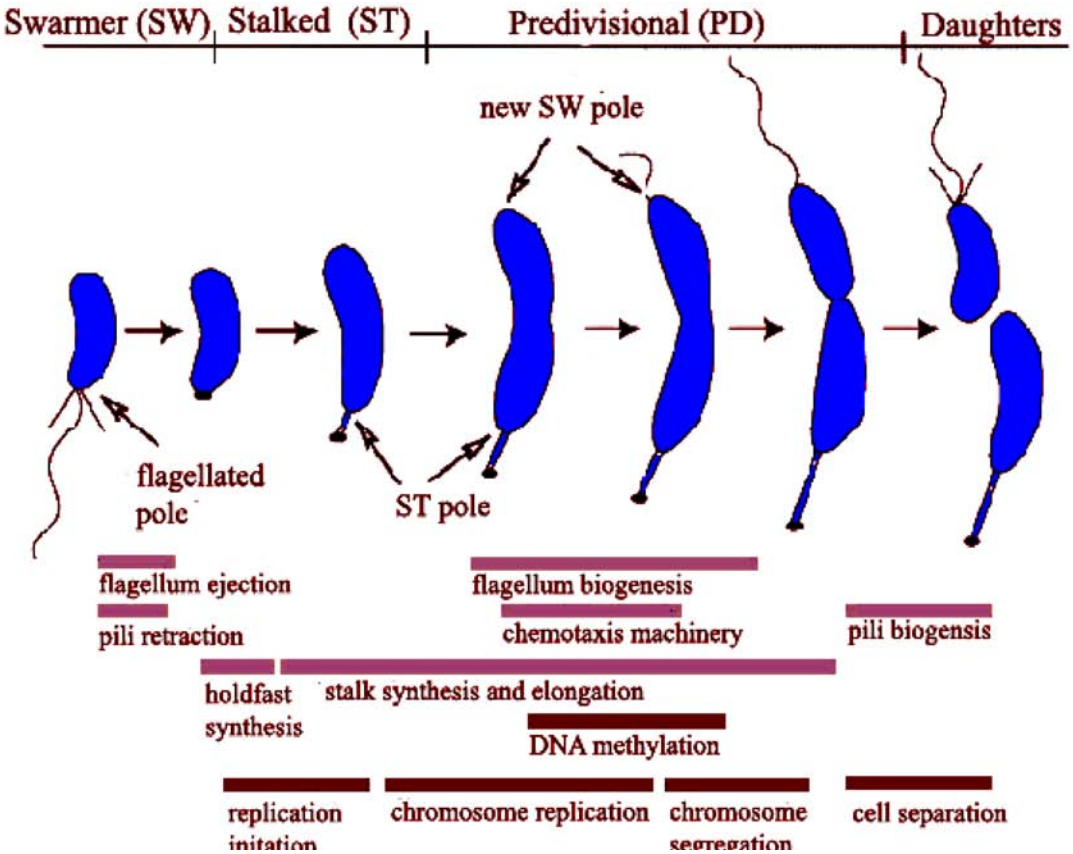

Figure 2) A schematic representation of the *Caulobacter crescentus* cell cycle. The replication-incompetent swarmer cell is equipped with a polar flagellum and flp-like pili. After a defined period, the swarmer cell differentiates into a stalked cell in successive of developmental steps, including the ejection of the flagellum, the retraction of the pili, the synthesis of the holdfast, and the elongation of the stalk. Chromosome replication initiation coincides with the formation of the stalked cell. The timing of several morphogenetic and cell cycle events is shown by the light and dark grey bars respectively. The flagellated, stalked (ST) and new swarmer (SW) poles are indicated. The relative duration of each phase is indicated on top as horizontal axis. This figure was adapted from (72)

AIM OF THESIS

The aim of this work was to genetically identify components involved in *C. crescentus* surface binding and colonization. New structural and regulatory components of *C. crescentus* pole development and surface adhesion should be analyzed with respect to their function, their temporal and spatial coordination, and the specific molecular mechanisms facilitating surface colonization.

CHAPTER 1

The coincident exposure of polar organelles optimizes surface attachment during *Caulobacter crescentus* development

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ABSTRACT

During its biphasic life cycle, *Caulobacter crescentus* oscillates between a planktonic and a surface attached life style. A hallmark of this transition is the temporally and spatially regulated assembly and disassembly of polar organelles like flagellum, pili, and an adhesive holdfast. A genetic screen for mutants affected in surface binding and colonization revealed a large number of known and novel components of flagellar motility, pili formation, and holdfast biogenesis, arguing that these organelles are required for optimal surface adhesion of *C. crescentus*. Several new holdfast genes were identified, which are potentially involved in the formation and polymerization of polysaccharide precursors. Together with experiments that implicate a cellulose-like polymer as a main constituent of holdfast structure and function, this provides the basis for future analyses on the formation and exact composition of this adhesive organelle. Several lines of evidence suggested that the coincident exposure of polar organelles optimizes surface attachment during *Caulobacter crescentus* development. i) The holdfast is synthesized and exposed on the cell surface very early during the swarmer-to-stalked cell transition and, during a defined time window, coincides with an active flagellum and adhesive pili at the same pole. ii) Cell cycle-dependent surface attachment showed a prominent peak coinciding with the surface exposure of all three polar organelles, and mutants lacking any one of these subcellular structures exhibited basal levels of attachment. iii) Active growth, as well as passage through development, greatly enhanced surface colonization. iv) A delay of holdfast biogenesis observed in a *pleD* mutant resulted in a strong reduction of surface binding during development. In cells lacking PleD, a developmentally controlled diguanylate cyclase, holdfast biogenesis was delayed by almost one third of a cell cycle equivalent, indicating that PleD and its readout signal, c-di-GMP, are used as timing device for holdfast formation. Based on these results we propose a model for *C. crescentus* surface colonization that involves the successive and concerted

activity of flagella, pili, and holdfast. The model provides a rational framework for the precise temporal and spatial control of these cellular appendices during development.

INTRODUCTION

In most natural environments, microbial cells are found attached to surfaces and associated in communal structures known as biofilms. The formation of biofilms from single planktonic cells, widely studied in a few model organism (*Vibrio cholerae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *E. coli*), involves several discrete stages, including reversible and irreversible attachment to surfaces, formation of cell monolayers, microcolony formation, and biofilm maturation (113, 120, 156, 190). This process is associated with a dramatic change of the cells' genetic program and physiology (155-157, 171, 194, 196). Initial stages of surface colonization are facilitated by cellular appendages like flagella and pili that can mediate initial attachment and accelerate biofilm development (8, 35, 82, 84, 135, 156). Later stages of biofilm formation are associated with the formation of an extracellular matrix, which mediates surface anchoring and provides structural support for the cell community (177). While all major classes of macromolecules can be present in biofilm matrices, increased synthesis of exopolysaccharide (EPS) is generally associated with biofilm formation (27, 47, 48).

The contribution of flagella and pili to various stages of biofilm formation have been demonstrated independently for several bacteria but it remains to be shown whether flagella, pili and EPS are part of a coordinated program for surface attachment and colonization rather than contributing to biofilm formation in a stochastic and independent manner. If these distinct organelles and mechanisms are indeed interlinked and are part of a program dedicated to surface colonization, how would these interactions be regulated in time and maybe space? How would cell motility and adhesive properties be coordinated to optimize surface attachment early during biofilm formation and to ensure the escape or detachment of cells from biofilms at a later stage? One possibility is that different components of this multicellular behavior are co-regulated (149). A number of environmental signals, including nutrients, temperature, osmolarity, pH, iron, and oxygen influence

biofilm formation (reviewed in: (120)), but little is known about mechanisms that integrate these inputs and transduce them into an altered bacterial behavior required for surfaces colonization.

During *Caulobacter crescentus* development surface adhesion is coupled to cell growth and division. Each cell division is intrinsically asymmetric and generates a sessile, replicative stalked cell and a motile, flagellated swarmer cell. A single flagellum is assembled in the predivisional cell at one pole and is activated prior to cell division (200). Upon separation of the two daughter cells, pili are formed at the flagellated pole of the swarmer cell (165, 168). The newborn swarmer cell performs chemotaxis for a defined period (5) before it sheds the flagellum, loses its pili and differentiates into a stalked cell. During this process, an adhesive holdfast structure and a stalk are assembled at the pole previously occupied by pili and flagellum. The exact role of the polar pili in *C. crescentus* and its temporal and spatial control are unknown but it has been proposed that they might facilitate surface interaction and cell attachment (13, 168). Irreversible anchoring of *C. crescentus* cells to surfaces requires an intact holdfast structure (126). Genetic screens have identified several genes required for holdfast secretion and anchoring (21, 167). While some of these genes encode homologs of polysaccharide export components in other gram-negative bacteria, the exact structure and composition of the holdfast remains unclear (167). Staining and lectin binding experiments had proposed that it is composed of an acidic polysaccharide, which contains *N*-acetylglucosamine (GlcNAc) residues (110, 167, 185).

The observation that in *C. crescentus* swarmer cells are able to attach to surfaces (13, 132, 133) suggested that the model for surface attachment as being mediated by stochastic and independent adhesion events might be too simplistic and has indicated that all polar organelles might contribute to this process in a concerted manner. Here we show that in a static system, flagella, pili, and holdfast substantially contribute to *C. crescentus* surface attachment. Using a new method to detect holdfast we could demonstrate for the first time that holdfast biogenesis occurs much earlier in development than reported previously. Consequently, all three polar organelles are concomitantly exposed at the

same cell pole during a defined time window of swarmer cell differentiation. This developmental stage coincides with a sharp peak of surface binding activity during the *C. crescentus* life cycle. This attachment peak was reduced or eliminated in mutants lacking pili, flagellum, or holdfast. Moreover, in a mutant that shows delayed holdfast synthesis during development, attachment is dramatically reduced. Together with the observation that optimal surface binding is coupled to growth and cell differentiation this lead us to propose a model for *C. crescentus* attachment in which rapid surface binding is optimized by the careful temporal and spatial coordination of all three organelles during development.

MATERIALS AND METHODS

Media and Strains

Strains and plasmids used in this study are listed in Table 1. *E. coli* DH10B and S17-1 were used as host strain for molecular cloning experiments and as donor strain for conjugational transfer of plasmids into *Caulobacter*. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth (154) supplemented with kanamycin (50 μg/ml) or tetracycline (12.5 μg/ml), when necessary. *C. crescentus* strains were grown at 30°C in either PYE complex medium (132) or in M2 minimal glucose medium (M2G) (79) supplemented with kanamycin (5 μg/ml), tetracycline (2.5 μg/ml), chloramphenicol (1 μg/ml) or nalidixic acid (20 μg/ml) when necessary. Semisolid agar plates for motility assays contained 0.3% agar (DIFCO®).

Synchronization of *C. crescentus* was done as described earlier (172). Isolated swarmer cells were released into fresh minimal medium at an OD_{660} of 0.3. Samples were removed for microscopic analysis, attachment assays, and holdfast staining at 15 minutes intervals. For surface binding assays, cells were allowed to attach to polystyrene in microtiter plates for 15 minutes. Cell cycle progression was monitored by light microscopy.

DNA manipulations

Plasmid and chromosomal DNA preparation, DNA ligation, electroporation, agarose gel electrophoresis, and PCR amplifications were carried out by using standard techniques (154). All PCR products used for cloning were amplified with “Expand high-fidelity PCR system®” form Roche. Restriction enzymes were from New England Biolabs, Inc.

Random Tn*5* mutation analysis

The mini-Tn*5* transposon delivery vector pUT_Km2 (32) was inserted into *C. crescentus* wild type by conjugation. Approximately 2,000 colonies were grown in 96-well plates in 200 µl of PYE medium supplemented with kanamycin. Cells were discarded and the microtiter plates were washed under a gentle stream of distilled water. The plates let to air-dry and surface attached cells were quantified as described below. Mutants with a reproducible and significant increase or decrease (>25%) of attachment were selected for further analysis.

Genomic DNA sequencing

Genomic DNA was extracted from *C. crescentus* wild type or mutant strains using ethanol precipitation following lysis of cells in 5M Guanidinium thiocyanate, 0.1M EDTA. Tn*5* insertion sites were mapped by direct sequencing of chromosomal DNA using oligonucleotide primers complementary to the ends of the kanamycin resistance cassette of Tn*903*: primer #698 (TCT AGA GTC GAC CTG CAG GC) and #699 (TAC CGA GCT CGA ATT CGG CC). Sequencing reactions were set up using ~500 ng genomic DNA as template and 10 pmol of sequencing primer in a total reaction volume of 20 µl following the BigDye Terminator protocol with few modifications: Annealing temperature was raised to 58^{o} C and the number of cycles was increased to 99 (Big Dye; Perkin-Elmer®). The sequencing reactions were run on an ABI Prism 310 DNA Sequencer or ABI Prism 3100 Avant Genetic Analyzer (Perkin-Elmer®).

Construction of deletion mutants

In frame deletions of the chromosomal copies of *flgH*, *fliFG*, *flgDE*, *flgFG*, *fliL*, CC2277 and CC0095 were constructed in *C. crescentus* wild type strain and in UJ590 (Δ*pilA*) using pNPTS138 based constructs carrying in frame deletions in the respective genes. Plasmids (see below) were introduced into the recipient strains by conjugation and recombinants were selected on PYE plates supplemented

with kanamycin and nalidixic acid. Resulting single colonies were then grown overnight in liquid PYE medium and plated on PYE containing 3% sucrose. Sucrose-resistant colonies were screened by PCR for recombinants that had lost the chromosomal copy of the respective gene.

Construction of plasmids for chromosomal deletions

Plasmid pAL8 was constructed for an in-frame deletion of the complete *flgFG* coding region. PCR amplification of a 1.0 kb region upstream of *flgFG* was done with primers #655 and #656 (5'-GGA TCC GGC GTT CGA GCT GCT GCT GA-3' and 5'-GAA TTC TCA CCT GGC GGG TGA GTG AG-3'). PCR amplification the 1.0 kb region downstream of *flgFG* was done with primers #657 and #658 (5'-GAA TTC CGC TCG CCT AAG CGA ACG TC-3' and 5'-ACT AGT GGC CGA GAT CTT GCC GTC GA-3'). Ligation of both fragments into pNPTS138 (*Sph*I/*Spe*I) resulted in plasmid pAL8.

Plasmid pAL2 was constructed for an in-frame deletion of the complete *flgH* coding region. PCR amplification of a 1.0 kb region upstream of *flgH* was done with primers #637 and #638 (5'-GGA CTA GTC CCG GCG ACA ACC TGT TCC TGG-3' and 5'-CGG GAT CCC GGA CGA CGC ATG ATC TGG TCC-3'). PCR amplification a 1.0 kb region downstream of *flgH* was done with primers #639 and #640 (5'- CGG GAT CCC GGG TCG AGA AGT TCT CGC CCT-3' and 5'-CGG AAT TCC GGG AGC GCA TTC GAC GTC TGG-3'). Ligation of both fragments into pNPTS138 (*Spe*I/*Eco*RI) resulted in plasmid pAL2.

Plasmid pAL6 was constructed in order to create an in-frame deletion of the complete *fliL* coding region. PCR amplification of a 1.0 kb region upstream the *fliL* was done with primers #710 and #711 (5'-ACT AGT CCA TCA TCT TGG CGA CGC-3' and 5'- GAA TTC CGT ACT CAT GCG CGA AGC -3'). PCR amplification a 1.0 kb region downstream of *fliL* was done with primers #712 and #713 (5'- GAA TTC GCG AAC GAT CAT GGC GGA-3' and 5'-GCA TGC ACC TGC ATG TTC AGC ACG-3'). Ligation of both fragments into pNPTS138 (*Spe*I/*Sph*I) resulted in plasmid pAL6.

Plasmid pAL7 was constructed in order to create an in-frame deletion of the complete *flgDE* coding region. PCR amplification of a 1.0 kb region upstream the *flgDE* coding sequences was done with primers #563 and #564 (5'- GCA TGC GAC CGC CGC GAC CGC CCC GC-3' and 5'- GAA TTC CCG GTC AAG GAC CGA GGC GG -3'). PCR amplification a 1.0 kb region downstream of *fliL* was done with primers #565 and #566 (5'- GAA TTC TCG AAG ATC ATC ACG ACC GC-3' and 5'- CTA GTT GGC GAC CTT GTC GCG CGG C-3'). Ligation of both fragments into pNPTS138 (*Spe*I/*Sph*I) resulted in plasmid pAL7.

An in-frame deletion of the chromosomal copies of *fliF* and *fliG* genes was generated in wild-type strain CB15 using plasmid pBG22 (56).

Plasmid pAL21 was constructed in order to create an in-frame deletion of the complete CC2277 coding region. PCR amplification of a 1.0 kb region upstream the CC2277 was done with primers #625 and #626 (5'-cgg aat tcC GGG CTT CCT GCC GTT CCA CCG-3' and 5'- gct cta gaG CGC GCC ATC AGG CCT CCG TGT-3'). PCR amplification a 1.0 kb region downstream of CC2277 was done with primers #627 and #628 (5'- gct cta gaG CCG CGT GAG CGT GAT CAC CGA-3' and 5'-ggg gta ccC CCC AGG CCA AAG GTG ACC GGC-3'). Ligation of both fragments into pNPTS138 (*Eco*RI/*Kpn*I) resulted in plasmid pAL21.

Plasmid pDM25 was constructed in order to create an in-frame deletion of the complete CC0095 coding region. PCR amplification of a 550 bp region upstream of CC0095 was done with primers #1380 and #1381 (5'- GAA TTC TTC GAC CGT TCC CAG CCC-3' and 5'- GGA TCC CGC TGT CCA GAC GCT CTA-3'). PCR amplification a 550 bp region downstream of CC0095 was done with primers #1382 and #1383 (5'- GGA TCC TGA GGA ACG AAC ATC TCC GCA G-3' and 5'- AAG CTT CGA CAA GGA CGG CCA GAA GGA-3'). Ligation of both fragments into pNPTS138 (*Eco*RI/*Hin*dIII) resulted in plasmid pDM25.

Microscopy techniques

Cell morphology, motility, and rosette formation were analyzed by light microscopy using a Nikon Eclipse 6000 with a planApo 100x phase contrast objective or an Olympus AX70 with an UplanApo 100x phase contrast objective. Pictures were taken with a charge-coupled device camera (Hamamatsu®) connected to the Olympus microscope and analyzed with Open-lab (Improvision®) software.

Microtiter plate attachment assay

For *C. crescentus* surface attachment assays the protocol described by O'Toole was slightly modified (124). Stationary-phase cultures were diluted with fresh PYE (plus supplements when mentioned) into 96, 24 or 12 well polystyrene microtiter plates (at final volumes of 0.2, 1.0, or 2.0 ml, respectively) to an OD_{660} of 0.05 and incubated at 30ºC on a shaker (200 rpm) until cultures reached an OD_{660} between 0.9-1.2. Cells were discarded and the wells were washed gently under a stream of distilled H_2O to remove unattached cells. Plates were air dried and a culture-volume of 0.1% crystal violet (CV) was added and incubated with shaking for 15 min. Wells were washed again several times with distilled H_2O and CV was dissolved in 20% acetic acid. The color intensity was measured with a microplate reader spectrophotometer at 600 nm. Rapid attachment assays were performed as described above with the following modifications: Aliquots of 150 µl of mid-logarithmic phase cultures (OD_{660} of 0.4-0.6) were transferred to microtiter plates and incubated for short period (15-120 minutes, as indicated) at room temperature. CV stain (0.1%, final concentration) was added to the cells in order to fix and stain the cells. The wells were washed and analyzed as described above.

Attachment assay with microscopy cover-slides

Sterile cover-slides (Ø 18 mm) were placed in 12 well polystyrene microtiter plates (Falcon®) and 1 ml of culture (OD_{600} ~ 0.05) was added. The plates were incubated at 30º C with shaking (100 RPM) for different periods. Calcofluor white stain (Sigma-Aldrich®) was added when needed to a final

concentration of 0.002%. Cover-slides were washed several times in distilled H_2O to remove unattached cells. For calcofluor staining experiments, cells were incubated for 15 minutes in the dark, washed again with distilled H_2O, and analyzed microscopically.

Holdfast staining and visualization

A mixture of Oregon green 488 conjugated wheat-germ agglutinin (Molecular Probes®) and calcofluor white (Sigma) at final concentrations of 0.2 mg/ml and 0.1 mg/ml, respectively, were added to a *C. crescentus* liquid cultures and incubated at room temperature for 15 minutes in the dark with occasional manual stirring. The cells were washed with distilled water and were resuspended with SlowFade® antifade (Molecular Probes). Stained holdfasts were visualized and recorded microscopically as indicated above, using DAPI filter setting.

Cellulase and protease assay

Cellulase (1,4-(1,3:1,4)-β-D-Glucan 4-glucano-hydrolase) from *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich) was added to cell cultures or to a glass cover-slide assay at dilutions of 1:100 (≥ 1.5U; 1U=1 µmol glucose from methylcellulose per minute at 40ºC). Removal of attached cells with cellulase was done in 50 mM sodium acetate buffer pH 5.5 @ 37^{o} C for 5 hrs. Proteinase K (Endopeptidase K, recombinant from *Pichia pastoris,* PCR grade; Roche Diagnostics, Mannheim, Germany) was added to cell cultures or to a glass cover-slide assay at a final concentration of 0.01 mg /ml. Removal of attached cells with Proteinase K was done in 50 mM sodium acetate buffer pH 5.5 @ 37^{o} C for 5h. All other hydrolytic enzyme were tested as described above for the cellulase, *i.e*., enzymes were added to the growth media or assayed with 50 mM sodium acetate buffer. All enzymes were used at concentrations that did not affect growth or viability of *C. crescentus*.

Table 1: Strains and plasmids used in this study

Strain/ plasmid	Description	Source/reference
Strains:		
E. coli		
S17-1	M294::RP4-2 (tet::Mu) (Kan::Tn7)	(163)
DH10B	F⁻*mcrA* Δ(*mrr*⁻ *hsd* RMS⁻*mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lac*X74 *endA*1 *recA*1 *deoR* Δ(*ara, leu*)7697 *araD*139 *galU galK nupG rpsL*	GIBCO BRL®
UJ1288	*E. coli* S17-1 with pUT_Km2	(32)
UJ606	MT607 containing pRK600	(160)
C. crescentus		
CB15	*Caulobacter* wild type	(132)
NA1000	Synchronizable mutant of CB15 lacking holdfast	(45)
YB2862	CB15 Δ*hfsB*	(167)
UJ590	CB15 *ΔpilA*	M. Ackermann
UJ730	CB15 *ΔpleD*	(4)
UJ1847	CB15 *ΔfliFG*	This work
UJ2430	CB15 *ΔflgH*	This work
UJ2438	CB15 *ΔflgDE*	This work
UJ2440	CB15 *ΔflgFG*	This work
UJ2441	CB15 *ΔpilA ΔflgFG*	This work
UJ2442	CB15 *ΔfliL*	This work
UJ2591	CB15 *motA*::Tn*5*	This work
UJ2982	CB15 ΔCC0095	This work
UJ2984	CB15 ΔCC2277	This work
Plasmids:		
pAL2	pNPTS138 with *Spe*I/*Eco*RI 2kb fragment designed for in-frame deletion of *flgH*	This work
pAL6	pNPTS138 with *Spe*I/*Sph*I 1.8kb fragment designed for in-frame deletion of *fliL*	This work
pAL7	pNPTS138 with *Sph*I/*Spe*I 2.1kb fragment designed for in-frame deletion of *flgDE*	This work
pAL8	pNPTS138 with *Sph*I/*Spe*I 2.2kb fragment designed for in-frame deletion of *flgFG*	This work
pAL21	pNPTS138 with *Eco*RI/*Kpn*I 1.9 kb fragment designed for in-frame deletion of CC2277	This work
pDM25	pNPTS138 with *Eco*RI/*Hin*dIII 1.05 kb fragment	This work

	designed for in-frame deletion of CC0095	
pNPTS138	Kan^R pLitmus38 derived vector with *oriT* and *sacB*	Dickon Alley
pUT_Km2	Mini-Tn*5* transposon delivery vector	(32)

RESULTS

Isolation and characterization of *C. crescentus* surface attachment mutants

In order to identify components involved in surface colonization, a Tn*5* insertion library of *C. crescentus* wild type strain was screened for mutants with an altered ability to attach to a plastic surface using the assay first described by O'Toole and Kolter (124). A total of 96 mutants were isolated that were not affected in growth but showed significantly altered surface binding efficiency. For 65 of these mutants the transposon insertion site was mapped to a total of 49 independent genes by direct sequencing of chromosomal DNA (see Materials and Methods). The identity of the mutated genes and the relative attachment of the corresponding mutant strains are shown in Figure 1A. In many cases, polar effects on clustered downstream genes cannot be excluded. Strikingly, a large fraction of the insertions affected polar organelle function or biogenesis with the largest subgroup mapping to flagellar genes. All motility mutants showed a similar surface attachment phenotype with a reduction of 50-70% compared to wild type (Figure 1A). Most of these mutants affected the assembly of the hook-basal-body structure or, in the case of *motA*, resulted in fully assembled but paralyzed flagellum.

Several novel genes required for motility were identified: CC0934, CC1064, CC2058, and CC2059 (Figure 1A). While CC1064 codes for a hypothetical protein with no known homologs, CC2058 and CC2059 have orthologs in *Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, and other α-purple bacteria (COG0457 and COG3334). In all three species these two orthologous genes are part of a chromosomal gene cluster that also contains the known flagellar genes *fliL* and *fliM* (203). This suggested that CC2058 and CC2059 represent novel flagellar genes specific for the α-purple group of gram-negative bacteria. The observation that insertions in CC2059 result in a *fla*$^-$ phenotype while CC2058 mutants are *mot*$^-$ indicated that both genes have distinct roles in flagellar assembly or function (data not shown). A transposon insertion in CC0934, which encodes one of the

61 *C. crescentus* histidine protein kinase paralogs (119), resulted in cells lacking a polar flagellum (data not shown). The observation that the downstream genes of both CC0934 and CC1064 have opposite orientations, excludes polar effects of the Tn insertions mapped in these two genes.

To confirm the effect of motility mutants on surface binding, defined in-frame deletions of selected flagellar genes were generated in wild type strain. Mutants lacking inner ring (*fliFG*), rod (*flgFG*), hook (*flgDE*), or outer ring (*flgH*) components of the flagellum were all non-motile (data not shown) and showed a 50-70% reduction in surface attachment (Figure 1B). A *fliL* deletion mutant, that assembles a complete but paralyzed flagellum (78), showed a similar reduction in surface binding. Together, this indicated that motility provides a strong benefit for surface colonization and confirms the earlier finding that it is not the flagellum per se that is necessary for optimal attachment but the motility imparted by the flagellum (13).

Skerker and Shapiro (165) had genetically identified a seven clustered genes (CC2948-CC2942) required for the biogenesis of swarmer pole-specific pili. Our screen for altered surface binding identified mutational insertions in CC2948, the gene coding for the major pilin subunit PilA (165), and in two flanking genes of this cluster, which are also potentially involved in pili biogenesis (Figure 1A). CC2941 and CC2950 code for homologs of the *tadB* and *tadG* genes, both of which are required for the synthesis of Flp pili (82). Mutations in all three genes reduced surface attachment by about 60% (Figure 1A) and resulted in resistance to bacteriophage CbK (data not shown), indicative of a failure to assemble functional polar pili (95). Similarly, an in-frame deletion of *pilA* (CC2948) encoding the major pilin subunit (165) reduced surface binding efficiency by about 90% (Figure 1B). These findings suggest that polar pili are required for optimal surface attachment and are in agreement with the observation that a *C. crescentus* mutant lacking the pre-pilin peptidase CpaA also showed significantly lower binding affinity to polystyrene (13).

A third class of polar organelle mutants mapped to genes involved in holdfast synthesis and showed the strongest change in surface binding (Figure 1A). While some of these insertions were

mapped to regulatory genes known to be required for polar development (*pleC* (187), *podJ* (41)), two insertions were found in genes identified recently as being involved in holdfast synthesis (*hfsD*, CC2432) (167) and anchoring (*hfaB*, CC2630) (21). Two additional Tn insertions mapped to novel genes required for holdfast synthesis. The first, CC2277, codes for an ortholog of ExoM, a glycosyl transferase 2 family protein involved in succinoglycan biosynthesis in *Sinorhizobium meliloti* (146). The second, CC0095, is part of a chromosomal region, which contains several candidate genes for oligosaccharide biosynthesis, including a putative UDP-*N*-acetylmannosaminuronic acid transferase (CC0095, COG1922), UDP-glucose epimerase (CC0092, COG1087), and a GGDEF/EAL family protein (CC0091, COG2200), orthologs of which have been implicated in the control of cellulose polymerization in *Acetobacter xylinum* (178). Both mutant strains failed to synthesize a visible holdfast structure (Figure 1C) and were unable to attach to a plastic (Figure 1A) and glass surface (Figure 2, data not shown).

We also identified mutants with an increased propensity to attach (Figure 1A) and most of these displayed a more intense holdfast staining. Two of the mutated genes, orthologs of *manB* (CC2264, COG1109) and *manC* (CC3618, COG836), are of particular interest, because their insertional inactivation had a dramatic effect on holdfast formation (Figure 1C). The *manB* gene codes for phosphomannomutase (converts mannose-6-P into mannose-1-P) and *manC* codes for mannose-1-P guanylyltransferase (converts mannose-1-P into GDP-mannose). Since we observed a comparable increase in attachment and holdfast formation when mannose was added to the growth medium (data not shown), we conclude that mannose or mannose-6-P is a precursor for holdfast biogenesis and that *manC* and *manB* mutants show an increased holdfast formation because they accumulate mannose or mannose-6-P.

The identification and analysis of a large number of surface colonization mutants confirm other findings on the importance of polar organelles for surface attachment of *C. crescentus* and emphasize that the holdfast structure is most critical but not sufficient for optimal surface binding

(13). The requirement for these polar organelles is not specific for plastic surface. As shown in Figure 2, cells that lacked a functional flagellum, polar pili, or holdfast showed decreased colonization of glass surface at the broth/air interphase in non-shaking cultures. This result is similar to the attachment phenotype observed for plastic surfaces and is consistent with a general role for the three polar appendices in surface colonization.

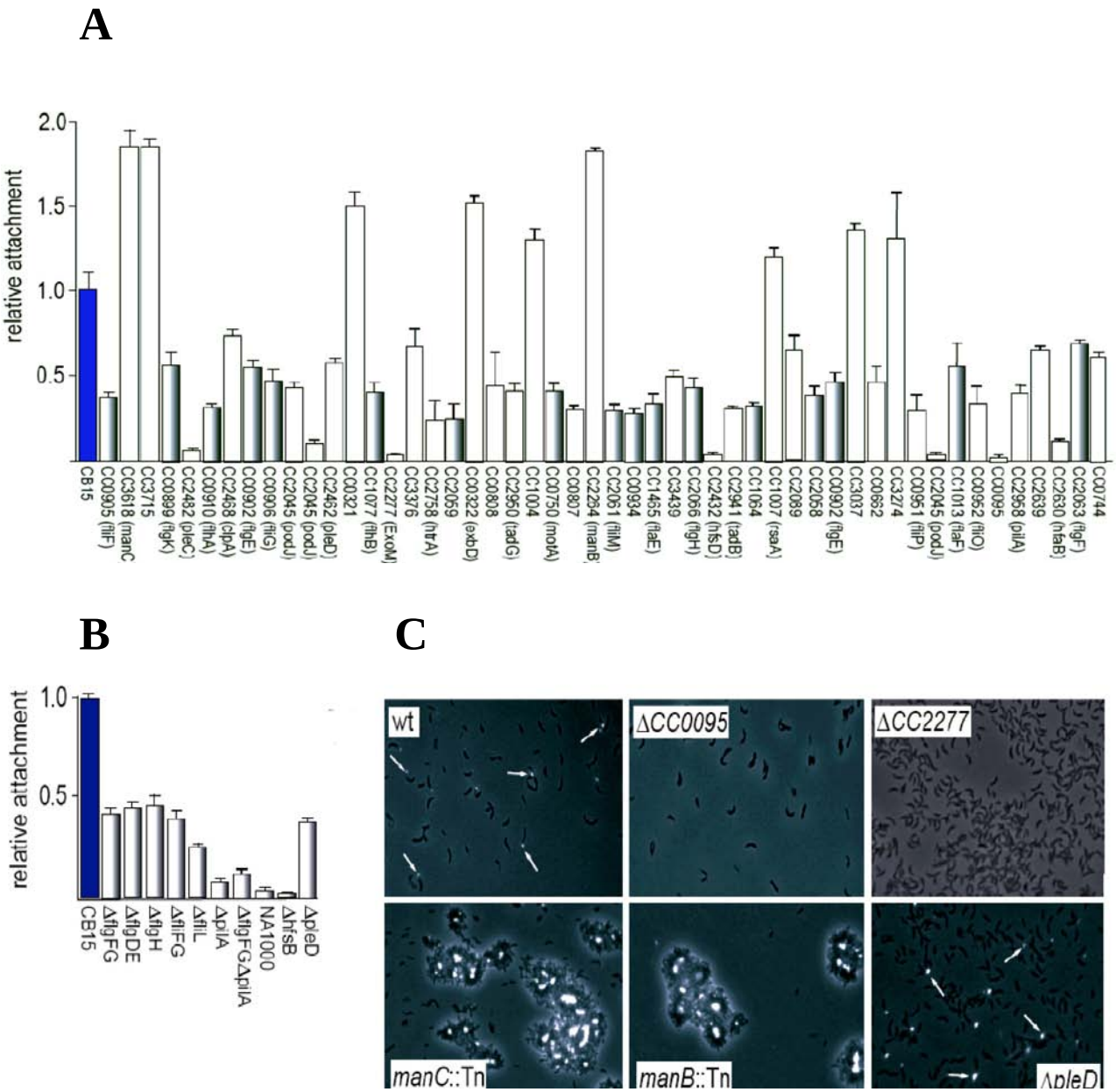

Figure 1) Surface attachment and holdfast formation of *C. crescentus* wild type and transposon-derived mutants. **A)** Relative attachment of *C. crescentus* wild type and selected transposon mutants. Cultures were grown overnight in polystyrene 96–wells microtiter plates and surface attachment was quantified as described in the Methods section. Attachment is shown as relative value of wild type. Mutated genes are indicated with the standard *C. crescentus* gene designation (CC numbers). For Tn*5* insertions in known genes or in paralogs with significant sequence conservation, gene names are indicated in brackets. The attachment of wild type (blue bar) was arbitrarily set to 1.0. Gradient bars indicate mutants with a motility defect. **B)** Relative attachment of *C. crescentus* wild type and selected in-frame deletion mutants. **C)** Holdfast staining of *C. crescentus* wild type and selected mutants. Holdfasts were stained and visualized as described in the Methods section. Arrows indicate

holdfast staining in wild type. Mutants with a deletion in CC0095 and CC2277 have no detectable holdfast, while *manB* and *manC* mutants show a dramatic overproduction of holdfast polysaccharide.

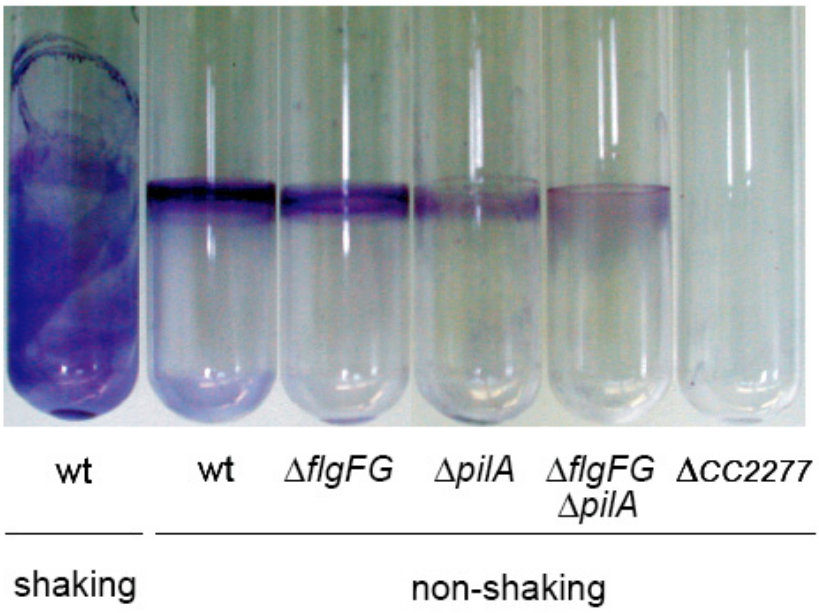

Figure 2) Polar organelles contribute to the attachment of *C. crescentus* to glass. Cultures of CB15 wild type and mutants lacking flagellum, pili or holdfast were grown for 72 hrs in glass tubes with shaking (left panel) or without shaking (right panels). Cells were discarded, tubes were washed with water, and surface attachment was visualized by crystal violet staining. Attachment is uniformly distributed over the entire glass surface in shaking cultures. In contrast, non-shaking conditions cause *C. crescentus* to attach at the air-broth interphase. A cellulose-like polymer contributes to irreversible surface anchoring of *C. crescentus* cells.

Holdfast tightly binds the lectin wheat germ agglutinin (WGA) (110) and specifically binds to calcofluor white (this work). To demonstrate the importance of holdfast for *C. crescentus* surface adhesion and to probe holdfast composition we measured attachment in the presence of compounds that might bind to or interfere with the integrity of the holdfast. When cells were allowed to adsorb to polystyrene for one hour in the presence of calcofluor (Figure 3A) or WGA (data not shown) surface attachment was significantly decreased. In contrast, Congo red (data not shown) and concanavalin A (Figure 3A), both that compounds bind different polysaccharides but do not bind holdfast (110), did not interfere with attachment. In addition, attachment of cells in the presence of chitinase or α-amylase did not reduce surface binding. In contrast, when cellulase or Proteinase K was present at concentrations that did not affect growth, attachment to plastic was strongly reduced (Figure 3A). Similarly, when polystyrene wells were first colonized with *C. crescentus* cells for 12 hours and then treated with cellulase, the total number of attached cells was also significantly reduced (Figure 3A), indicating that the hydrolytic enzyme is able to release already surface anchored cells. A similar reduction was observed when cells attached to glass surface were treated with cellulase (Figure 3B). Finally, treatment with either cellulase or proteinase almost completely abolished rosette formation (Figure 3C), arguing that the reduction of attachment observed in Figure 3A and 3B is indeed the result of a damaged component of the holdfast structure. While treatment with cellulase clearly reduced the intensity of holdfast staining with calcofluor and fluorescent WGA (Figure 3C), treatment with proteinase did not diminish fluorescent labeling of the holdfast, but holdfast structures were often found detached from cells (Figure 3C). This is consistent with the idea that holdfast is composed of at least two different components, a cellulase-sensitive polysaccharide and a proteinaceous component that might help to anchor the sugar moiety in the cell envelope.

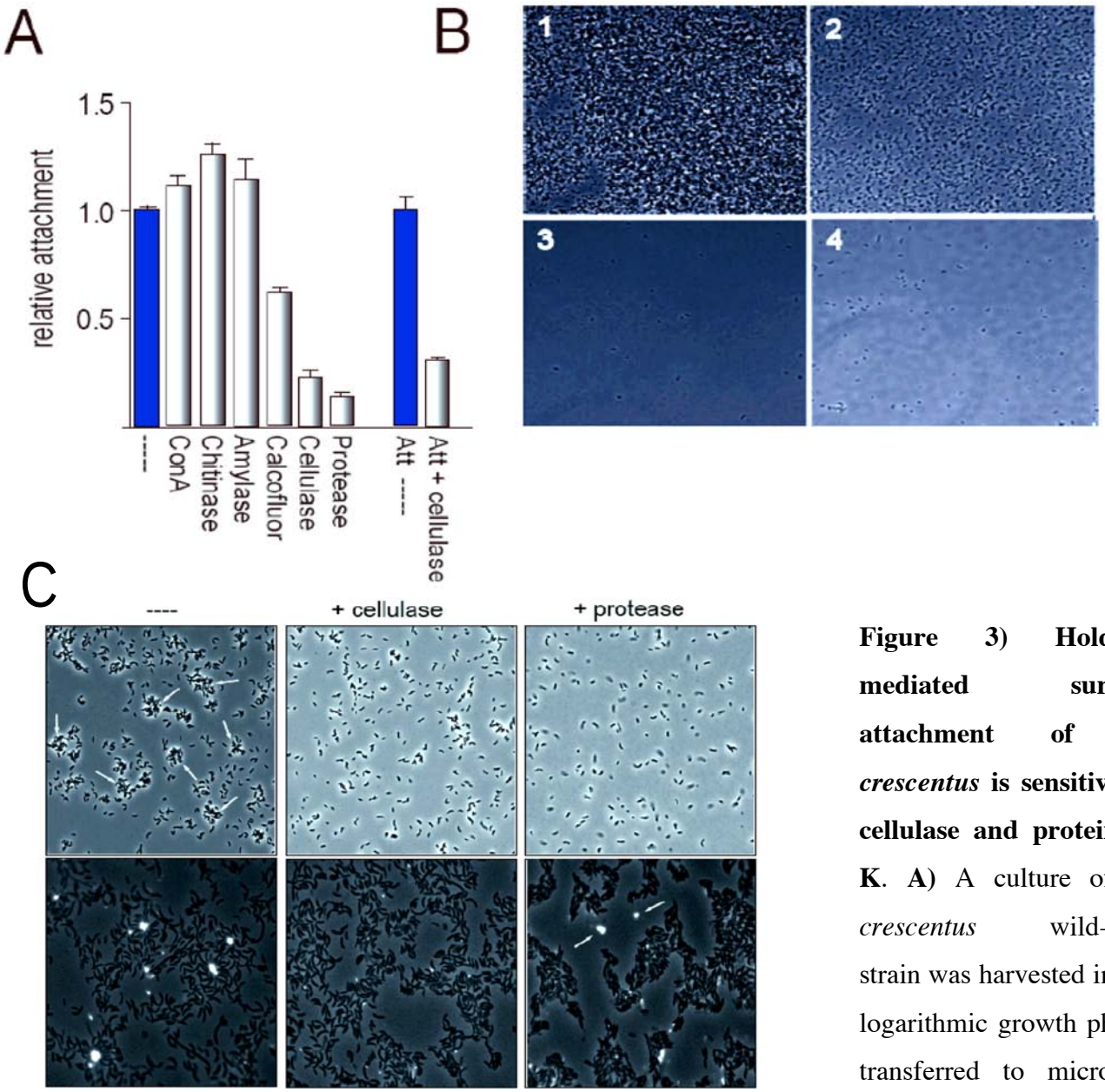

Figure 3) Holdfast mediated surface attachment of C. *crescentus* is sensitive to cellulase and proteinase K. A) A culture of C. *crescentus* wild-type strain was harvested in the logarithmic growth phase, transferred to microtiter plates and incubated for one hour in the presence of the following compounds: ConA: 0.02% concanavalin A; Calc: 0.1% calcofluor; Chit: 0.03% chitinase; Amyl: 2% amylase; Cell: 5% cellulase; Prot: 0.02% proteinase K. Cells were then washed and surface attachment was quantified by crystal violet staining. The attachment level of CB15 wild type (blue bar) was arbitrarily set to one. **B)** Phase-contrast images of *C. crescentus* wild type strain CB15 attached to a glass surface. Cells were allowed to attach to microscopy cover-slides in the presence (3) or absence of cellulase (1, 2, and 4) for 24 hours and analyzed microscopically. Cultures shown in panels 2 and 4 were washed with water and then incubated for five hours with buffer alone (2) or with buffer containing cellulase (4) (see Methods section). **C)** Phase contrast (upper row) and fluorescence images (lower row) of *C. crescentus* wild-type strain untreated (left panels) and treated with cellulase (middle panels) or

proteinase K (right panels). Cellulase and proteinase K treatment was as described in the Methods section and holdfast was stained with fluorescent WGA.

Optimal *Caulobacter* surface attachment correlates with active growth

The observation that formation and loss of flagellum, pili and holdfast occur in succession, together with the finding that the cellular appendices all play a critical role in surface adhesion, prompted us to test if optimal surface attachment might depend on consecutive steps in *C. crescentus* development. One assumption of such a model is that optimal surface attachment correlates with active growth and passage through the cell cycle. To test this, we quantified binding of *C. crescentus* wild-type cells to polystyrene during batch culture growth. Attachment increased in parallel with increasing optical density of the culture during logarithmic growth but rapidly declined under growth limiting conditions (Figure 4A, open bars). The correlation between optimal attachment and rapid growth is demonstrated most clearly when attachment is normalized to the optical density of the culture (Figure 4A, gradient bars). In agreement with this, blocking protein synthesis also noticeably reduced *C. crescentus* surface attachment (Figure 4B). When cells were allowed to attach to polystyrene for two hours in the presence of inhibitory concentrations of tetracycline, surface binding was reduced by about 70% (Figure 4B). In contrast, when cells were allowed to pre-adhere for two hours before tetracycline was added, attachment was not affected (Figure 4B)

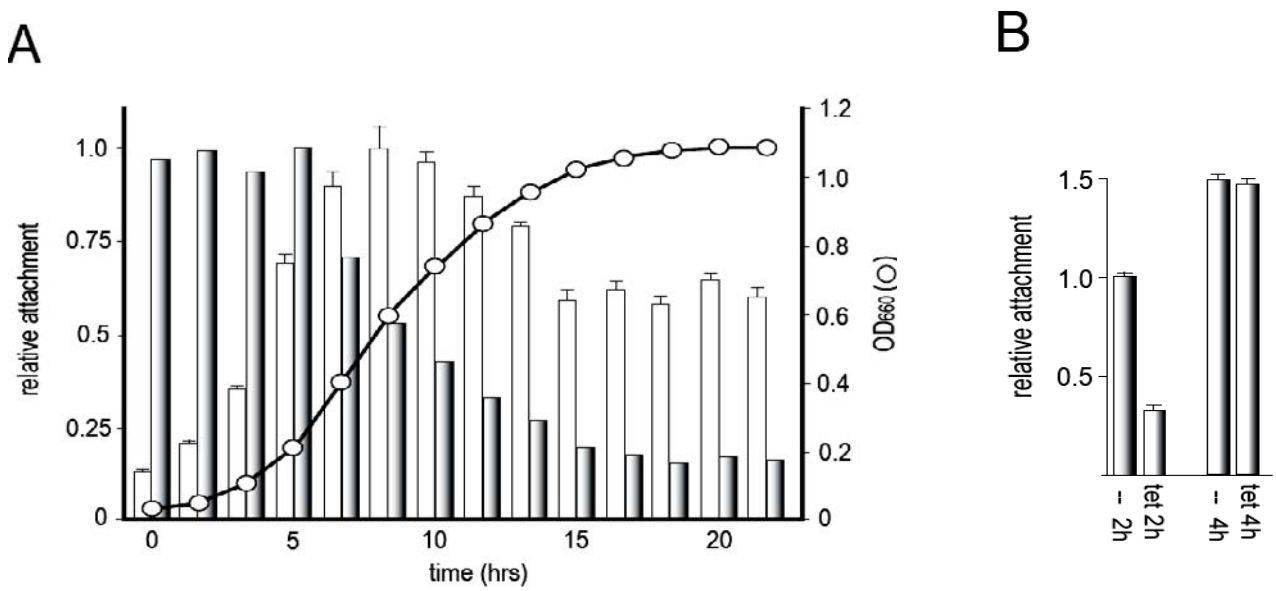

Figure 4) Optimal *C. crescentus* surface attachment correlates with rapid growth. **A)** Aliquots of cells from a growing batch culture (PYE) of *C. crescentus* wild-type strain were transferred at the indicated time points to a microtiter plate and allowed to adhere to the plastic surface for 45 minutes. The medium was discarded and attachment was quantified by crystal violet staining. Relative attachment (bars) is shown as a function of the optical density (open circles) of the culture. The white bars represent non-normalized levels of attachment while gradient bars show attachment normalized to the optical density of the culture. **B)** Aliquots of a logarithmically growing culture of *C. crescentus* CB15 were transferred to microtiter plates and allowed to attach to the plastic surface for two hours in the absence (2h) or presence of tetracycline (tet-2h). Attachment was then quantified by crystal violet staining. As a control, cells were first allowed to attach to the plastic surface for two hours and were then incubated for an additional two hours in the absence (4h) or presence of tetracycline (tet-4h).

Surface attachment peaks with the coincident exposure of polar organelles

While surface attachment drops considerably when cells enter stationary phase, holdfast formation is not affected under these conditions as the percentage of *C. crescentus* cells with a polar holdfast structure even slightly increases under non-growing conditions (data not shown). This confirms that the adhesive holdfast is not sufficient for optimal surface attachment and argues that for irreversible holdfast-mediated surface anchoring the successive appearance and disappearance of flagellum and pili during development might be necessary. To test this, we used synchronized *C. crescentus* cells to assay surface attachment throughout the cell cycle (Figure 5). At each time point indicated, aliquots of cells were transferred to microtiter plates and allowed to bind to the plastic surface for 15 minutes before attachment was measured by crystal violet staining. Surprisingly, motile swarmer cells rather than holdfast-bearing stalked cells showed the highest attachment activity. Attachment peaked 15-30 minutes after purified swarmer cells were released into fresh medium (Figure 5) (note that the temporal resolution of this experiment is limited by the 15 min time window used to quantify attachment). Since the holdfast structure is clearly critical for irreversible surface anchoring of cells (see Figure 1), this finding is at odds with the current believe that holdfast is not present in swarmer cells and is synthesized only during the swarmer-to-stalked cell transition after cells have ejected the flagellum (73). To assess the possibility that holdfast is synthesized already at an earlier, motile phase of the cell cycle, we developed an improved holdfast staining method based on a mixture of calcofluor and FITC-WGA or OG-WGA (see Materials and Methods). When synchronized cells were analyzed with this technique, the appearance of holdfast at the cell pole could be confined to the first 15 minutes after release of isolated swarmer cells into fresh medium (Figure 6A, B). While no holdfast structures were visible at time zero (data not shown), under these conditions, the majority of the swarmer cells had already developed a detectable holdfast after 15 minutes of development (Figure 6A, B). At this time point, cells are still fully motile and equipped with pili (Figure 5) indicating that during a short window of development flagellum, pili, and holdfast co-exist at the

same pole of the differentiating cell and that optimal attachment observed at this stage might be a consequence of these co-existing functionalities. After passing this stage of development, attachment levels quickly dropped and reached their lowest level at the stalked cell stage, only to increase again towards the end of the cell cycle (Figure 5). A pili mutant showed a similar but reduced cell cycle-dependent attachment behavior with a peak coinciding with the phase when holdfast and flagellum co-exist (Figure 5). A non-motile mutant showed basal, stalked cell-like attachment levels throughout the cell cycle and cells that lacked a holdfast structure were unable to bind to plastic surface (Figure 5). Thus, the surface binding kinetics during development seems to be determined by the presence and activities of the flagellar motor and holdfast, while polar pili seem to play a prominent role in making the original surface contact more efficient. The data in Figure 5 also suggest that if cells are non-motile, the beneficial role of pili for surface binding does not come into play. Obviously, cell motility is important to move cells efficiently closer to the surface where pili and holdfast catalyzed adhesion and anchoring can occur.

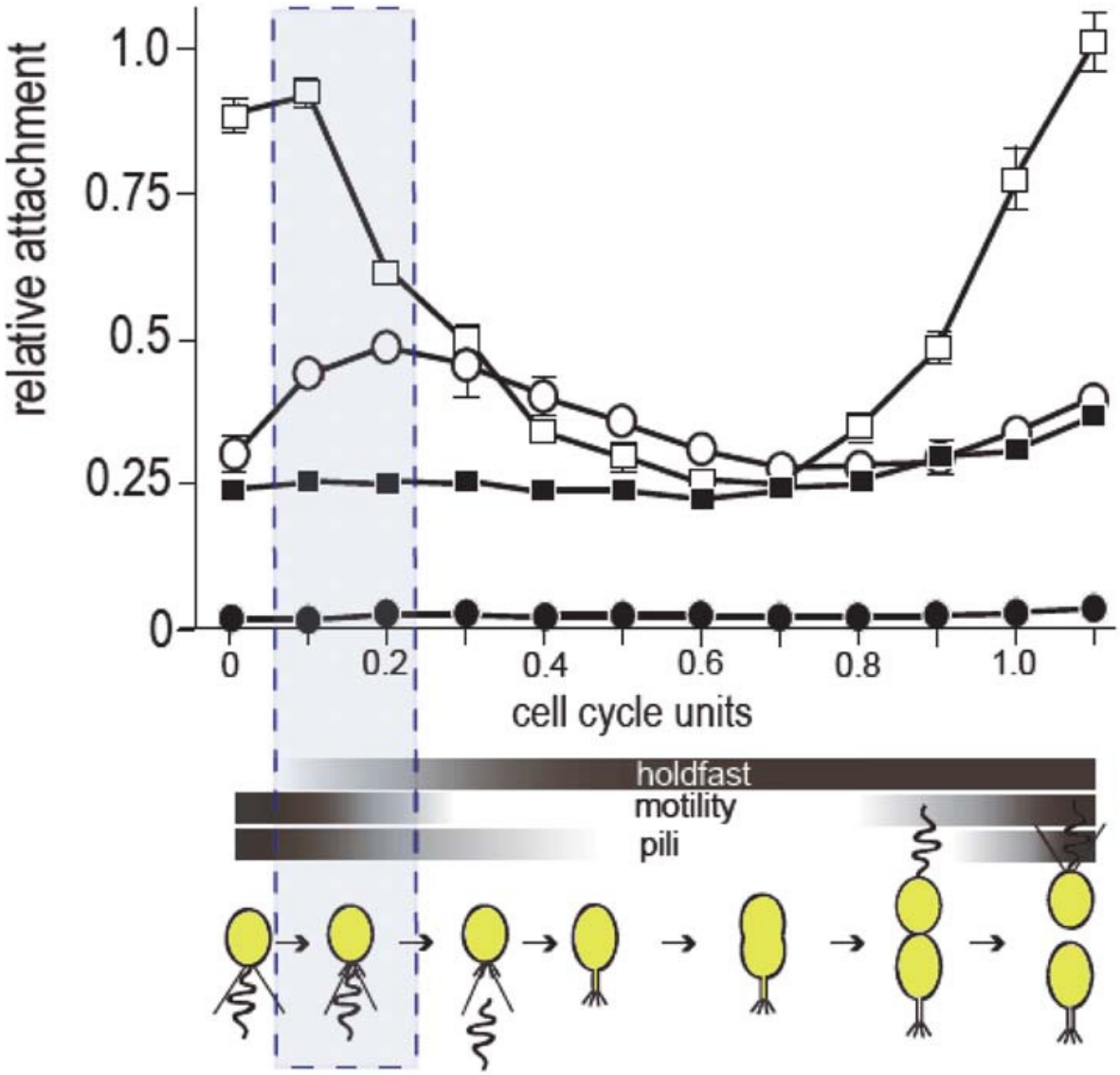

Figure 5) **Surface attachment during the *C. crescentus* cell cycle**. Swarmer cells of CB15 wild type (open squares) and isogenic Δ*pilA* (open circles), Δ*flgFG* (closed squares), and ΔCC2277 (closed circles) mutants were purified and suspended in fresh PYE medium. Aliquots were removed from the synchronized culture throughout the cell cycle in 15-minute intervals, transferred to microtiter plates, and allowed to attach to the plastic surface for 15 minutes. Attachment was then quantified by crystal violet staining. The presence and activity of polar organelles is indicated with horizontal bars below the time scale. Appearance and disappearance of pili was taken from Sommer and Newton (168), motility was monitored microscopically throughout the cell cycle, and the presence of a polar holdfast

was determined by fluorescence staining as described in the Methods section (see also Figure 6). Cell cycle progression is indicated as cell cycle units and schematically below the graph. The time window of development during which flagellum, pili and holdfast are exposed concomitantly at the same cell pole is boxed.

One of the transposons isolated in the screen for surface binding mutants (Figure 1) mapped to the *pleD* gene. PleD is a diguanylate cyclase that, upon phosphorylation by the polar kinases PleC and DivJ, sequesters to the developing pole (4, 130). Mutations in *pleD* show a pleiotropic phenotype in that they fail to efficiently eject the flagellum and to synthesize stalks during development (2, 57). Both a *pleD* Tn insertion and an in-frame deletion of the *pleD* gene reduced surface binding by 50-70% (Figure 1A, B). This was surprising since earlier results not only had indicated that this mutant shows increased motility compared to wild-type but also suggested that neither pili nor holdfast biogenesis was affected in strains lacking *pleD* (4, 57) (Figure 1C). We therefore analyzed a *pleD* null mutant more closely by comparing its surface binding capacity during the *C. crescentus* cell cycle with surface attachment of a wild-type strain. Surface binding of the *pleD* mutant strain was exclusively affected during the early stages of development, while at later stages of the cell cycle attachment was similar to wild type (Figure 6A). Reduced attachment of the mutant during the swarmer-to-staked cell transition correlated with a considerable delay in holdfast biogenesis. While wild-type swarmer cells acquire a holdfast more or less immediately after entry into development, the exposure of a visible holdfast was delayed for almost one third of the entire cell cycle in the *pleD* mutant (Figure 6A). Together with the observation that the cell cycle length of wild type and *pleD* mutant were similar (data not shown), this argued that pole development, rather than the replicative program is altered in the mutant and that PleD is a timing device for the formation of the adhesive organelle during *C. crescentus* cell differentiation. Two observations indicated that neither pili formation nor pili disappearance during development is altered in a *pleD* mutant. First, a *pleD* null mutant is sensitive towards bacteriophage øCbK and produced phage titers indistinguishable from wild type (data not shown). Second, the major pilin subunit PilA rapidly disappears during the swarmer-to-stalked cell transition coincident with the loss of pili from the cell surface (186). When analyzing PilA levels during the cell cycle, we found that the kinetics of PilA loss in wild type and the *pleD* mutant were identical (data not shown). This suggested that the surface adhesion defect of a

pleD mutant is primarily due to a timing defect of holdfast synthesis and, as a result, the temporal uncoupling of the two adhesive organelles, pili, and holdfast.

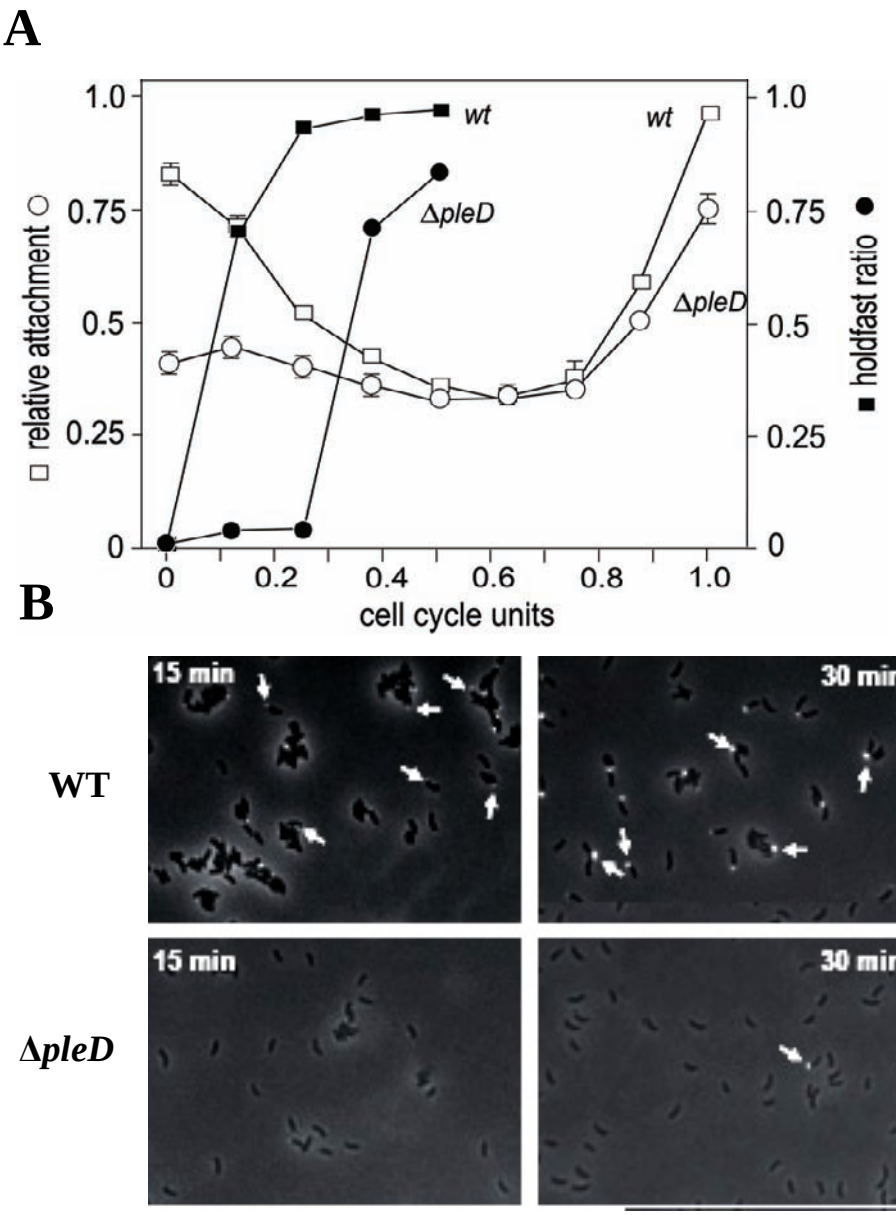

Figure 6) A pleD mutant shows reduced surface binding and delayed holdfast formation. A) Swarmer cells of CB15 wild type (open squares) and an isogenic pleD (open circles) mutant were purified and suspended in fresh PYE medium. Aliquots were removed from the synchronized culture throughout the cell cycle in 15-minute intervals, transferred to microtiter plates, and allowed to attach to the plastic surface for 15 minutes. Attachment was then quantified by crystal violet staining. The fraction of cells possessing a polar holdfast was determined for wild type (closed squares) and for the pleD mutant (closed circles) by fluorescence staining as described in the Methods section. Cell cycle progression is indicated as cell cycle units. B) Holdfast staining of wild type (top panels) and pleD mutant cells (bottom panels) recovered from a synchronous population. Cells were stained 15 and 30 minutes after swarmer cells were released into fresh medium.

Optimal surface attachment requires cell differentiation

To provide more evidence for the idea that in *C. crescentus* surface binding is developmentally controlled, we analyzed attachment of a homogenous population of swarmer cells under conditions that block the swarmer-to-stalked cell transition. It has been reported that the swarmer-to-stalked cell transition is blocked in the absence of a nitrogen or carbon source (18, 54). In agreement with this, we found that swarmer cells released into M2 minimal medium (79) without nitrogen or with a 100-fold reduced glucose concentration (0.002%) failed to differentiate, but fully retained their motility (data not shown). Under these nutrient-limiting conditions, the attachment of swarmer cells was reduced significantly (Figure 7A). In contrast, when swarmer cells were first allowed to differentiate into stalked cells for 90 minutes in M2G minimal medium before they were transferred to a medium lacking nitrogen, surface binding, even though reduced to the level typically observed for stalked cells (Figure 5), was no longer dependent on nitrogen (Figure 7A). This argues that the observed reduction in surface binding is not a direct consequence of limited nutrients but is caused by an indirect effect on swarmer cell development. The reduction in surface binding in the absence of nitrogen or at low glucose concentrations correlated with a significant drop in holdfast formation during the first 30 minutes of development (Figure 7B-C). The observation that low glucose concentrations had a more dramatic effect on holdfast formation and surface binding than the absence of nitrogen (Figure 7B-C) suggested that either low glucose causes a more stringent block in development, or, alternatively, might reflect the obvious limitation of sugar precursors required for the formation of the holdfast polysaccharide under these conditions. The addition of kanamycin at growth inhibitory concentrations also prevented swarmer-to-stalked cell differentiation (data not shown). In contrast to nitrogen and carbon limitations, kanamycin had no effect on attachment or on holdfast biogenesis (Figure 7A-C). This indicated that protein synthesis, while being required for a later stage of development is not critical for the differentiation into adhesion competent cells. Newborn swarmer cells, while lacking a visible holdfast, seem to be fully equipped with the components required for holdfast biosynthesis.

The activation of this machinery early during swarmer cell development must occur at the post-translational level. The observation that mutants lacking the PleD diguanylate cyclase show a dramatic delay in holdfast synthesis indicates that c-di-GMP signaling is likely to be involved in this developmental transition.

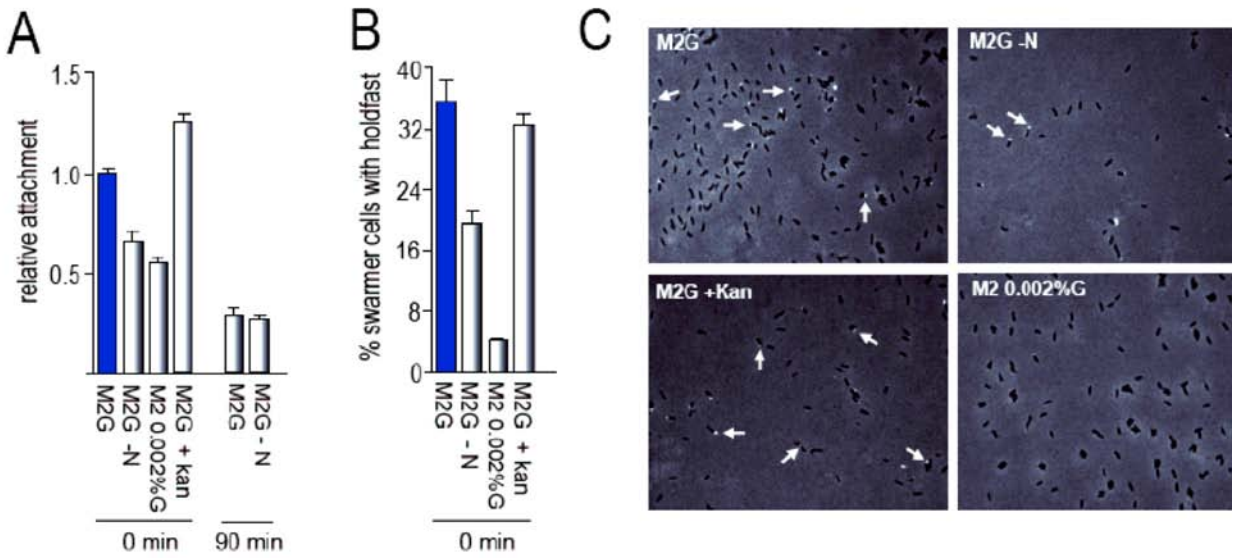

Figure 7) Optimal *C. crescentus* surface attachment correlates with cell cycle progression and development. **A)** Purified swarmer cells of C. *crescentus* CB15 were released into glucose minimal medium (M2G), M2G lacking nitrogen (M2G –N), M2 with a 100-fold lower than normal glucose concentration (M2 0.002%G), and M2G containing kanamycin (50 µg/ml). Culture aliquots were immediately (0 min.) transferred to microtiter plates and allowed to attach to the plastic surface for 30 minutes. Attachment was quantified as described in the Methods section. As a control, purified swarmer cells were released into M2G and allowed to go through the swarmer-to-stalked cell transition for 90 minutes (90 min.) before cells were harvested, washed and released into either M2G or M2G lacking nitrogen (M2G -N). Cell were transferred to microtiter plates and allowed to attach to the plastic surface for 30 minutes. **B)** Purified swarmer cells were released into M2G, M2G lacking nitrogen (M2G –N), M2 with a 100-fold reduced glucose concentration (M2 0.002%G), and M2G containing kanamycin (50 µg/ml), incubated for 30 minutes at 30ºC, before the fraction of cells with a visible holdfast was determined by fluorescent labeling. Examples of stained cultures are shown in **C)** with arrows indicating polar labeling of holdfast structures.

DISCUSSION

We have used a genetic screen to define the components required for *C. crescentus* surface attachment and colonization. More than two thirds of the mutants that showed significantly altered surface binding properties mapped to genes involved in the assembly or function of flagellum, pili, and holdfast. While this corroborates the importance of these surface components for *C. crescentus* cell adhesion (13), we also addressed the question if these structures contribute to surface colonization in an independent and stochastic manner or if optimal attachment requires their concerted activity. Based on our experiments we propose a model for the initial stages of *C. crescentus* surface colonization in which efficient and irreversible surface anchoring requires the active passage of cells through a defined window of development during which all three organelles coincide at the cell pole (Figure 8). This model suggests that the careful timing and maybe spatial arrangement of polar organelles during *C. crescentus* development is critical for optimal and rapid surface colonization of this aquatic organism.

The role of the flagellum for *C. crescentus* surface binding seems to be functional rather than structural and may be to help breaking the surface charge barrier or to increase effective collisions with the surface. This is inferred from the observation that *fla* and *mot* mutants result in a similar reduction of surface attachment. The surface-binding defect of non-motile mutants was most dramatic in very short attachment intervals (e.g. Figs. 5 & 6). When the incubation time during the attachment assays was increased, the surface binding phenotype of *fla* and *mot* mutants was alleviated, while attachment of pili mutants remained low compared to wild type (data not shown). This suggested that motility contributes to attachment merely by increasing the cells' chance to collide with the surface. Once the cell is close to the surface, pili mediate direct contact and facilitate surface anchoring of *C. crescentus* cells. However, pili-mediated adhesion is reversible and cannot withhold strong shearing forces in the absence of a holdfast structure. Pili could be playing a role in surface binding by

bringing the holdfast occupied pole into close proximity of the solid substratum or by positioning the cell so that it is in the correct orientation for binding. Consequently, pili could be mere cell adhesins or could play a more active role, as indicated by the observation that pili disappear during the swarmer-to-stalked cell transition by retraction or by some other unknown mechanism (168). However, there is no evidence that *C. crescentus* pili and their homologs of the Flp subfamily (83, 131) are able to retract like other type IV pili (101, 111, 164).

The most important component for irreversible surface anchoring of *C. crescentus* is the holdfast. Mutants that are unable to synthesize a visible holdfast show dramatically reduced binding to different surfaces both in over-night and rapid attachment assays (Figs. 1, 2, 5) (13, 21, 167). Staining properties and enzyme sensitivity studies had suggested that holdfast is a complex acidic polysaccharide, which contains *N*-acetylglucosamine (GlcNAc) residues (110, 167, 185). The most convincing evidence for an involvement of GlcNAc is the observation that holdfast efficiently binds wheat germ agglutinin, a lectin specific for GlcNAc polymers (110). We found that holdfast could also be very efficiently bind calcofluor, which is specific for β-linked polysaccharides such as chitin and cellulose. Cellulase treatment of *C. crescentus* cells not only prevented surface attachment very effectively, but also resulted in the release of already surface anchored cells. In addition, treatment of cells with cellulase severely reduced holdfast staining with calcofluor and fluorescently labeled lectin, providing a plausible explanation for its negative effect on cell surface binding. It is important to note that at the concentrations used in these experiments, cellulase had no adverse effect on cell growth or morphology. In contrast, chitinase or amylase showed no effect on attachment or holdfast integrity. From this, we concluded that a cellulose-like polymer contributes to structure and function of the holdfast. However, no homologs of cellulose biosynthesis genes (147) are found in *C. crescentus*. A cellulase sensitive, glucose-rich polymer has recently been identified as a component of the *P. aeruginosa* biofilm matrix (47). While *P. aeruginosa* also lacks cellulose biosynthesis genes, a gene cluster named *pel*, which contains functional homologs of carbohydrate processing genes, was shown

to be responsible for the production of this EPS matrix (47). While *pel* orthologs are also absent in *C. crescentus*, our experiments have defined novel genes, which seem to be required for holdfast polysaccharide biosynthesis. The gene CC2277 codes for an ExoM homolog, one of several glycosyltransferases involved in the polymerization of succinoglycan (EPS I) in *Sinorhizobium meliloti* (9, 98). A recombinant form of ExoM has been demonstrated to transfer glucose from UDP-glucose to the native polyprenyl-pyrophosphate trisaccharide substrate (Glc1-4Glc1-3GalP-P-lipid) *in vitro* (98). The gene CC0095 codes for a homolog of UDP-*N*-acetylmannosaminuronic acid transferase (COG1922), which could also be involved in polymerization reactions leading to a precursor oligosaccharide, which is then exported to the cell surface. Two other genes of the cluster containing CC0095 are potentially involved in polysaccharide production. CC0092 has homology to enzymes that convert UDP-glucose into UDP-galactose (COG1087), and CC0091 encodes a protein with a GGDEF and an EAL domain. Members of this family have recently been implicated in the control of EPS production and biofilm formation (162, 179, 182). It remains to be shown if multiple genes of this cluster play a role in holdfast synthesis or control.

Earlier studies had suggested that motile swarmer cells are able to attach to surfaces and are primarily responsible for rosette formation, a phenomenon of holdfast mediated cell-cell attachment (132). But while these studies had proposed that holdfast should appear already in swarmer cells at the base of the flagellum (133), staining with fluorescent WGA allowed detection of holdfast only in stalked and predivisional cells (13, 73). We obtained a similar result when using calcofluor or WGA alone. However, when applying a mixture of calcofluor and fluorescent WGA (see Materials and Methods) a large proportion of swarmer cells exhibited polar holdfast staining very early during development, at a time when cells were still fully motile. Interestingly, inhibition of protein synthesis in synchronized swarmer cells did not affect holdfast biogenesis and surface attachment, arguing that the components responsible for secretion and assembly of holdfast material are already present in newborn swarmer cells. While this is in agreement with the observation that known holdfast genes are

transcribed in the predivisional cell before the motile swarmer cell is released (73), it raised the question of the molecular mechanisms and signals involved in the initiation of holdfast synthesis during swarmer cell development. We have presented evidence that the response regulator PleD constitutes a timing device for holdfast biosynthesis. In agreement with such a role, PleD is activated by phosphorylation during the swarmer-to-stalked cell transition and as a consequence sequesters to the differentiating pole (130). Phosphorylation of PleD results in the activation of the C-terminal diguanylate cyclase domain, which catalyzes the conversion of two molecules GTP into c-di-GMP (130). The signaling molecule c-di-GMP plays a prominent role in the transition between the planktonic and surface attached mode of bacterial growth (reviewed in (74, 148)). While c-di-GMP effector proteins are still largely unknown, signaling by c-di-GMP seems to take place, at least in part, at the post-translational level (67, 192, 208). It is thus conceivable that a PleD-catalyzed burst of c-di-GMP is responsible for the correct temporal control of holdfast formation during *C. crescentus* development.

Work by Bodenmiller et al. (13) had also provided evidence that the initial attachment could be developmentally controlled in *C. crescentus*. However, the surface binding kinetics reported in this study was noticeably different from the results shown in Figure 5 with attachment being constant and at a relatively low level throughout the swarmer-to-stalked cell transition (13). In addition, while the number of swarmer cells at time zero should be equivalent to the number of swarmer cells after cell division, the authors reported that surface binding after cell division was three-fold higher than at the beginning of the cell cycle. This obvious discrepancy was explained by the possible damage or loss of flagellar motility during the synchronization process (13). This assumption seems reasonable in view of our findings that a non-motile mutant indeed shows a constant but low attachment level throughout the cell cycle (Figure 5). The observed timing of holdfast formation early in development offers a reasonable explanation for the pronounced peak of surface binding during the *C. crescentus* cell cycle. Considering that for technical reasons surface binding had to be measured during a 15-minute

time window (*e.g.* the value determined for the 15 minute time point actually corresponds to the window between 15 and 30 minutes), the actual peak of attachment in Figure 5 is shifted about 15 minutes to the right. For the same reason, the surface binding capacity of swarmer cells at time zero has most likely been overestimated in that experiment (Figure 5). Considering that at this stage of development holdfast has not been assembled yet, might be very low in reality. Thus, the model for surface attachment of *C. crescentus* schematically depicted in Figure 8, proposes that the developmental program defines a relatively short window during which efficient surface binding can take place. It is likely that environmental factors (*e.g.* nutritional status, see Figure 7) can in principle override this developmental control of surface adhesion. In particular, the model in Figure 8 does not exclude the possibility that upon initial contact, the surface itself could act as signal that would then trigger rapid formation of the holdfast and cell anchoring. While cell cycle-dependent pole development might be a unique feature of *C. crescentus*, it is possible that cell poles play a similar role in surface binding of other bacteria, *e.g.* in the attachment of *Bradyrhizobium* to plant root hairs or in the adherence of *Pseudomonas aeruginosa* to tracheal epithelial cells (103, 209).

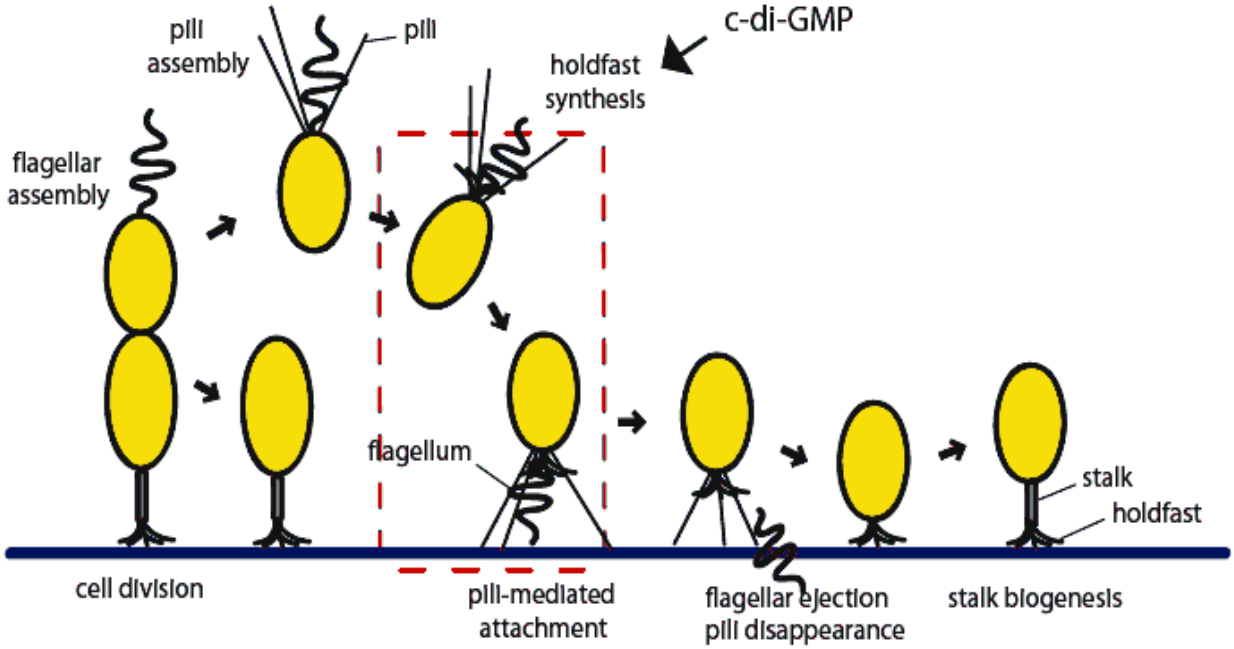

Figure 8) Model for surface attachment during *C. crescentus* development. The figure schematically depicts the transition of events of pole development during the *C. crescentus* cell cycle. The polar flagellum is assembled and activated in the predivisional cell at the pole opposite the stalk and is ejected during the swarmer-to-stalked cell transition (75). Flagellar motility facilitates surface adhesion merely by increasing the cells' chance to collide with the surface. Pili are formed upon completion of cell division and disappear during the swarmer-to-stalked cell transition by an unknown mechanism (168, 186). Pili-mediated surface binding requires both active motility and the exposure of the adhesive holdfast on the cell surface. While flagellar motility brings the adhesive pili in close contact with the solid substratum, holdfast is critical for the irreversible anchoring of cells. Holdfast is synthesized during the motile stage of the swarmer cell, and for a considerable amount of time, coincides with flagellum and pili at the same pole. PleD and possibly its signaling-readout, c-di-GMP, is required for the correct timing of holdfast formation. During this time window (boxed), rapid surface attachment of *C. crescentus* is optimized by the presence and activity of all three polar organelles.

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CHAPTER 2

PleD diguanylatecyclase and a novel phosphodiesterase control holdfast biosynthesis during *Caulobacter crescentus* development

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ABSTRACT

The correct timing of morphogenetic events is a hallmark of all forms of cell differentiation. During the *Caulobacter crescentus* swarmer-to-stalked cell differentiation the polar flagellum is ejected and replaced by an adhesive holdfast and a stalk. This study identifies two genes, CC0091 and CC0095, which are expressed almost exclusively during the swarmer stage of *C. crescentus* development and are required for holdfast synthesis and its temporal control. CC0091 codes for a GGDEF-EAL domain protein, which *in vitro* showed a strong c-di-GMP specific phosphodiesterase (PDE), but not diguanylatecyclase (DGC) activity. Analysis of pole development in strains that are lacking or overexpressing CC0091 indicated that the CC0091 PDE is an antagonist of the PleD DGC, which has been implicated in *C. crescentus* pole development control. While holdfast formation is delayed in a *pleD* mutant, it occurs prematurely in cells lacking CC0091. In contrast, overexpression of CC0091 not only showed a delay in holdfast formation, but also caused a hyper-motile phenotype and stabilization of the flagellar anchor protein FliF, indicative of a delay in flagellar ejection. CC0095 codes for a glycosyltransferase of the WecB family, which is required for holdfast biogenesis, and when overexpressed, leads to massive holdfast synthesis, increased surface attachment, and loss of motility. This indicated that CC0095 catalyzes a rate-liming step of holdfast polysaccharide synthesis. The observation that the CC0095 overexpression phenotype is modulated by the CC0091 PDE and by the PleD DGC argues that the activity of CC0095 is modulated, directly or indirectly by this second messenger and that this is the first example of a developmental process being regulated by the novel bacterial second messenger, c-di-GMP.

INTRODUCTION

The dimorphic bacterium *Caulobacter crescentus* divides asymmetrically and progresses through an obligate developmental transition that allows it to switch between a motile and an adhesive, sessile cell types. For that purpose, cell poles are adjusted during cell differentiation to facilitate gain and loss of motility and the acquisition of surface adherence organelles at the right time and in the correct order (159). A flagellum and pili are being assembled in the pre-divisional (PD) cell at pole opposite to the stalk pole. Cell division generates two cell types with distinct properties: a surface-attached stalked cell and a motile swarmer cell. The swarmer progeny differentiates into a stalked cell before it initiates DNA replication and cell division. During this transition, it loses its pili and sheds the flagellum, and replaces theses organelles with a polysaccharidic holdfast and a long extension of the cell body, the stalk. Flagellar motility, pili and holdfast contribute to *C. crescentus* surface adherence, which was shown by us and by others to be cell cycle regulated (13, 100). While motility and pili adhesion positively modulate surface colonization, the holdfast is critical for the irreversible attachment of the cells. The finding that optimal attachment occurs during a short window of development in which active flagellum, pili and holdfast coincide, suggested that the correct timing of polar organelles assembly is an important aspect of *C. crescentus* surface adhesion (100). Pole development in *C. crescentus* is a complex process that requires the coordinated activity of many signal transduction and proteins (72, 107, 130). One of the major readouts of this regulatory network is the response regulator PleD (2, 4, 57, 130). Cells that lack a functional PleD are hyper-motile, are unable to eject the flagellum, fail to synthesize a complete stalk structure, and show a delay in holdfast synthesis (3, 57, 100). In contrast, the presence of a constitutively active form of PleD, PleD*, results in elongated stalks and paralyzed motility (4). Recently, it was shown that PleD is a diguanylatecyclase (DGC), which is activated by phosphorylation during the swarmer cell differentiation, and as a result, dynamically localized to the differentiating cell pole (4, 74, 130). Cells

lacking PleD showed a dramatic delay in holdfast synthesis, and, as a consequence displayed serious impairment of cell adhesion (100). These observations have led us to the idea that c-di-GMP might be involved in temporal control of *Caulobacter* pole developmental process.

C-di-GMP is hypothesized to inversely regulate motility and cell adhesion in a wide range of bacteria and to modulate the bacterial cell surface, arguing that it could act as a master second messenger to facilitate the transition from motility to sessility in microorganisms (reviewed in (25, 74, 148)). C-di-GMP cellular levels are controlled by the opposing activities of diguanylatecyclases (DGCs) and phosphodiesterases (PDEs), which resume in GGDEF and EAL domains, respectively (19, 130, 148, 158, 180). In *Gluconacetobacter xylinum* c-di-GMP-dependent extracellular cellulose production results in cell aggregation and pellicle formation (150). In *Pseudomonas fluorescens* and *P. aeruginosa* the DGC protein, WspR mediates cell aggregation, pellicle formation and biofilm development (24, 62, 169). In *Salmonella enterica sv Typhimurium*, cellulose biosynthesis is controlled by the presumable DGC protein, AdrA (149); in *V. cholerae* and *V. parahaemolyticus*, the activity of RocS and ScrC, two GGDEF domain proteins was shown to be responsible for the rugose phenotype of these organisms (14, 142), moreover, recently it was shown that in response to an elevated level of c-di-GMP, there is an increase in *V. cholerae* transcription of vps, eps, and msh genes and decrease of flagellar genes (11, 102) . The GGDEF protein HmsT of *Yersinia pestis* is responsible for autoaggregation and polysaccharide biosynthesis (80, 85).

Here we show that the PleD guanylatecyclase, CC0091, a novel c-di-GMP specific PDE, and a holdfast-synthesis glycosyltransferase, CC0095 are all involved in timing of flagellar ejection and holdfast synthesis during *C. crescentus* swarmer cell differentiation. We show that PleD and CC0091 inversely control pole development during the swarmer-to-stalked transition, arguing that c-di-GMP is an important element of the signal transduction network of *C. crescentus* development.

MATERIALS AND METHODS

Media and Strains

Strains and plasmids used in this study are listed in Table 1. *E. coli* DH10B or S17-1 were used as host strains for molecular cloning experiments and as donor strains for conjugational transfer of plasmids into *Caulobacter*. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth (154) supplemented with kanamycin (50 μg/ml), chloramphenicol (12.5 μg/ml), tetracycline (12.5 μg/ml) when necessary. *C. crescentus* strains were grown at 30°C in either PYE complex medium (132) or in M2 minimal glucose medium (M2G) (79) supplemented with kanamycin (5 μg/ml), tetracycline (2.5 μg/ml), chloramphenicol (1 μg/ml) or nalidixic acid (20 μg/ml) when necessary. Semisolid agar plates (motility plates) for motility assays contained 0.3% agar (DIFCO®). Antibiotics concentrations, when added, were the same concentration as the corresponding solid agar plate contained.

Synchronization of Caulobacter crescentus

Synchronization of *C. crescentus* cultures was done as described earlier (172). Isolated swarmer cells were released into fresh minimal medium at an OD_{660} of 0.3. Samples were taken for microscopic analysis, attachment assays, or holdfast staining at 10 or 15 minutes time intervals. For surface binding assays, cells were allowed to attach to polystyrene microtiter plates for 15 minutes. Cell cycle progression was monitored by light microscopy.

DNA manipulations

Plasmid and chromosomal DNA preparation, DNA ligation, electroporation, agarose gel electrophoresis, and PCR amplifications were carried out by using standard techniques (154). All PCR products used for cloning were amplified with “Expand high-fidelity PCR system®” form

Roche®. Restriction enzymes were from New England Biolabs®, Inc. Mini and midi plasmid preparations were performed using Sigma GeneElute™ plasmid minipreps or midipreps kits, respectively.

Immunoblots

Immunoblots were performed as previously described (77). The secondary antibody (goat anti-rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase (GibcoBRL®) was used in a 1:10'000 dilution. The "western lightning™" chemiluminescence kit (Perkin Elmer®, Boston, USA) was used for detection.

Construction of chromosomal in-frame deletion mutants and plasmids delivery

In frame deletions of the chromosomal ORFs CC0091, CC0095, CC0091-96 and *ple*D were constructed in *C. crescentus* wild type strain (ATCC 19089) using pNPTS138 based constructs carrying in frame deletions fragments in the respective genes. Plasmids pDM1 (ΔCC0091), pDM25 (ΔCC0095), pAL13 (ΔCC0091-96), pPA24 (Δ*pleD*), pPA114-47 (*pleD** allele), pDM13 (CC0095 overexpression) and pAL17 (CC0091 overexpression) were introduced into the recipient strains by conjugation, and recombinants were selected on PYE plates, supplemented with kanamycin and nalidixic acid. Resulting single colonies were then grown overnight in liquid PYE medium and plated on PYE agar plates containing 3% sucrose. Sucrose-resistant colonies were then screened by PCR for recombinants that had lost the chromosomal copy of the respective gene.

Construction of plasmids for chromosomal deletions

Plasmid pDM1 was constructed by PCR amplification of a 1.0 kb region upstream of CC0091 with primers: 5'- GGA TCC ATG ATC TGT CGA ACG GCC ATC-3' and 5'- GAA TTC CGT TCC TTG AGG GTC ACT CGC-3', and a 1.0 kb region downstream of CC0091, using primers 5'-GAA TTC GAG CAA GAC CAG ACG TTC CGC-3' and 5'- GCT AGC CGG CTG ATC GTC TGA TCC

AGT-3'. Both fragments were cloned into pNPTS138 using *BamH*I, *Nhe*I, and *EcoR*I that had been introduced into the PCR primers.

Plasmid pDM25 was constructed by PCR amplification of a 0.55 kb region upstream of CC0095 with primers: 5'- GAA TTC TTC GAC CGT TCC CAG CCC-3' and 5'- GGA TCC CGC TGT CCA GAC GCT CTA-3' and a 0.55 kb region downstream of CC0095 using primers: 5'- GGA TCC TGA GGA ACG AAC ATC TCC GCA G -3' and 5'- AAG CTT CGA CAA GGA CGG CCA GAA GGA -3'). Both fragments were cloned into pNPTS138 using *EcoR*I, *Hind*III, and *BamH*I that had been introduced into the PCR primers.

Plasmid pAL13 was constructed by PCR amplification of a 1.0 kb region upstream of CC0091, with primers: 5'- CGG GAT CCC GCG GCA CGC AGT ATG GCA ATG TT -3' and 5'- CGG AAT TCC GCC GCC TAG GGT CGA TTG CGC CG -3', and PCR amplification a 1.0 kb region downstream of CC0096, using primers 5'- CGG AAT TCC GCC GCC TTC ATA TCG CCT CTC CC -3' and 5'- GAA GGC CTT CGG CCG GCT CAA CCG AAC TTC CT -3'). Both fragments were cloned into pNPTS138 using *EcoR*I, *Stu*I, and *BamH*I that had been introduced into the PCR primers.

Construction of plasmid for ectopic expression on a high copy number plasmid

CC0091 ORF was amplified with primers: 5'-AAG CTT CGA GTG ACC CTC AAG GAA CGT-3' and 5'- GAA TTC GCC TTG TTG ATC TTC GCC AAG-3', and subcloned into pGEM®-T Easy Vector (Promega®), restricted with *Hind*III/*Ecor*I sites which were incorporated into the primers and inserted into *Hind*III/*Ecor*I cut pBBRMCS2, resulting with plasmid pAL17.

CC0095 ORF was amplified with primers: 5'- GAA TTC AGT CGT CAT GTT AGC GCG -3' and 5'- AAG CTT CCG CAG AAT GTC TCC AAG-3', and subcloned into pGEM®-T Easy Vector (Promega®), restricted with *Hind*III/*Ecor*I sites which were incorporated into the primers and inserted into *Hind*III/*EcoR*I cut pBBRMCS2, resulting with plasmid pDM13.

Xylose inducible expression of CC0095

CC0095 coding sequence was amplified with primers: 5'-GAA TTC AGT CGT CAT GTT AGC GCG-3' and 5'-AAG CTT CCG CAG AAT GTC TCC AAG-3' and subcloned into pGEM®-T Easy Vector (Promega®), restricted with *EcoR*I and polished using T4 DNA polymerase. The polished fragment was subsequently cloned into pHRXLT (pPHU281 suicide plasmid which contains 2.3 kb DNA fragment of *C. crescentus* P*xylX* promoter locus) that was cut with *EcoR*I and polished with T4 DNA polymerase as well, resulting in plasmid pHRXLT95.

Quantitative reverse transcriptase PCR (QC RT-PCR)

Total RNA was isolated from cell pellets of synchronized or non-synchronized cultures of *C. crescentus* using the "High Pure RNA Isolation Kit" from Roche® according to the manufacturer's instructions. 2 µg of RNA and 1 µl of random nonamer primers were added to a 500 µl PCR reaction tube and diluted in sterile water to a volume of 15 µl. The sample was boiled at 70^{o} C for 5 minutes and centrifuged at 14'000 rpm at 4^{o} C for 2 minutes. The reaction mix was brought to a volume of 25 µl by adding dNTPs to a final concentration of 250 µM, 1 µl of Reverse Transcriptase, 1 µl or 40 units of RNase Inhibitor (Ambion®) and 5 µl of 5 x reaction buffers. The reaction was carried out at 37^{o} C for one hour. Finally the reaction mix containing the 40 µg/ml cDNA was boiled at 95^{o} C for 5 minutes and stored at -80^{o} C. Quantitative real time PCR was performed with 0.2 ng template cDNA, 2 gene specific primers, 300 nM each and SYBR® Green PCR Master Mix (Applied Biosystems®). Each reaction plate (96-well Optical Reaction Plate from Applied Biosystems®) included non-template control (NTC) to exclude primer-primer hybridization and DNA contaminations in the water, and an internal reference (house-keeping gene). The reaction was carried out using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). 60^{o} C "Dissociation Protocol" was used with the following settings: 50^{o} C for 2 minutes (step 1), 95^{o} C for 10 minutes (step 2), 95^{o} C for

15 seconds and 60^{o} C for 60 seconds (step 3; 50 cycles). Computer analysis was carried out using the ABI PRISM® 7000 SDS software.

Microtiter plate attachment assay

For *C. crescentus* surface attachment assays, we used the protocol described by O'Toole with some modifications (124). Over-night grown cultures were diluted with fresh PYE into 96, 24, or 12 well polystyrene microtiter plates (at final volumes of 0.2, 1.0, or 2.0 ml, respectively) to an OD_{660} of 0.05 and incubated at 30^{o} C on a shaker (200 rpm) until cultures reached an OD_{660} between 0.9-1.2. Cells were discarded and the wells were washed under a gentle stream of distilled water to remove unattached cells. Plates were let to air dry and a culture-equivalent volume of 0.1% crystal violet (CV) was added and incubated on a shaker for 15 min. Wells were washed again several times with distilled water and bound CV was dissolved with 20% acetic acid. The color intensity was measured with a microplate reader spectrophotometer at 600 nm. Rapid attachment assays were performed as described above with the following modifications: Aliquots of 150 µl of mid-logarithmic phase cultures (OD_{660}= 0.4-0.6) were transferred to microtiter plates and incubated for short time periods (15-120 mintes, as indicated) at room temperature. Cells were stained, washed, and analyzed as described above.

Microscopy techniques and image processing

Cell morphology, motility, and rosettes formation were analyzed by light microscopy using a Nikon Eclipse 6000 with a planApo 100x phase contrast objective or an Olympus AX70 with an UplanApo 100x phase contrast objective. Pictures were taken with a charge-coupled device camera (Hamamatsu®) connected to the Olympus microscope and analyzed with Open-lab (Improvision®) software. Processing, pixel surface and intensity quantification and other image manipulations were carried out, either by Adobe® Photoshop® CS2 or by ImageJ 1.34s (National Institute of Health, USA). Staining of the holdfast was done with a mixture of Oregon green 488® conjugated wheat-

germ agglutinin (OG-WGA) (Invitrogen™, Molecular Probes™) and Calcofluor white (Sigma) at final concentrations of 0.2 mg/ml and 0.1 mg/ml, respectively. This mixture was added to *C. crescentus* liquid cultures and incubated at room temperature for 15 minutes in the dark with occasional manual stirring. The cells were washed with distilled water and resuspended with SlowFade® antifade (Molecular Probes™). Stained holdfasts were visualized and recorded microscopically as indicated above, using a DAPI filter setting.

Overexpression and purification of CC0091

A DNA fragment coding for a truncated CC0091 (Δ1-338) was amplified using primers: 5'-GAC ATA TGG ACG ACG GGG CGC GCG TAG AAA CCT CGG-3' and 5'-GAA TTC GCC CTC GAG GGC GGA ACG TCT GGT CTT GCT C-3', cloned into pET42b(+) vector (Novagen®) using *Nde*I/*Xho*I sites which had been introduced into the PCR primers. The resulting plasmid, pDM18, allowed the induced expression of the truncated, 471AA long protein, which consists of the GGDEF and EAL domains of CC0091. *E. coli* BL21 cells carrying pDM18 were grown in LB medium with kanamycin (50 μg/ml), and expression was induced at OD_{600} 0.4 by adding IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation, resuspended in lysis buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM EDTA, 5 mM β-Mercaptoethanol, and an aliquot of *Complete™ Protease Inhibitor* from *Roche*), and lysed by passage through a French pressure cell. The lysed cell suspension was clarified by 30 minutes, 10,000 X g centrifugation step. Soluble and insoluble protein fractions were separated by a high-spin centrifugation step (100,000 X g, 1 h). The supernatant was loaded onto Ni-NTA affinity resin (Qiaexpressionist™ kit from Qiagen®), washed twice with washing buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM β-Mercaptoethanol, 20 mM imidazole), and eluted with elution buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM β-Mercaptoethanol, 250 mM imidazole). Protein preparations were examined for purity by SDS-PAGE and fractions containing pure protein were pooled and dialyzed for 12 h at 4 °C with storage buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM β-Mercaptoethanol, 10% glycerol).

Synthesis and Purification of [^{33}P]cyclic-di-GMP

[^{33}P] labelled c-di-GMP was produced enzymatically using α–labelled [^{33}P]GTP as described in (19).

DGC (Diguanylate Cyclase) and PDE (Phosphodiesterase) Assays

Diguanylatecyclase assays were conducted as described in (1, 19). Briefly, reaction mixtures with purified hexahistidine-tagged protein contained 25 mM Tris-HCl, pH 8.0 250 mM NaCl, 10 mM $MgCl_2$ and were started by the addition of 100µM [^{33}P]GTP (3000 Ci/mmol, Amersham Biosciences®). At regular time intervals the reaction was stopped with an equal volume of 0.5 M EDTA pH 8.0.

c-di-GMP specific phosphodiesterase activity was measured by monitoring the decrease of [^{33}P]c-di-GMP and the increase of [^{33}P]pGpG by thin-layer chromatography as described in (19). Briefly, purified preparation of hexahistidine tagged CC0091 was pre-incubated with or without GTP in a buffer contains 250 mM NaCl, 25 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 5 mM β-Mercaptoethanol for 2 min prior to the addition of radio-labelled c-di-GMP. The reactions were carried out at 30° C; aliquots were removed at different time points and the reaction was stopped by transferring to an equal volume of 0.5 M EDTA, pH 8.0.

PEI-Cellulose Chromatography

Samples were dissolved in 5 µl running buffer containing 1:1.5 (vol/vol) saturated NH_4SO_4 and 1.5 M KH_2PO_4, pH 3.60 and blotted on Polygram® CEL 300 PEI cellulose thin-layer Chromatography Plates (Macherey-Nagel®). Plates were developed in 1:1.5 (vol/vol) saturated NH_4SO_4 and 1.5 M KH_2PO_4, pH 3.60 (R_f(c-di-GMP) 0.2, R_f(pGpG) 0.4), dried and exposed on a Storage Phosphor Screen (Molecular Dynamics®). The intensity of the various radioactive species was calculated by quantifying the intensities of the relevant spots using ImageJ software version 1.33.

UV Cross-linking of proteins with [^{33}P]c-di-GMP

UV-crosslinking of proteins with radio labelled c-di-GMP was performed as described in (19). Protein samples were incubated for 10 minutes on ice in PDE reaction buffer containing either 10 µM c-di-GMP, 100 µM GTP and [^{33}P]c-di-GMP (0.75 µCi, 6000 Ci/mmol). Samples were irradiated at 254 nm for 20 min on an ice-cooled, parafilm-wrapped 96-well aluminium block in an RPR-100 photochemical reactor with a UV lamp RPR-3500 (The Southern New England Ultraviolet Company®). After irradiation, samples were mixed with 2x SDS PAGE sample buffer and heated for 5 min at 95 °C. Labelled Proteins were separated by SDS-PAGE and quantified by autoradiography.

Table 1. Strains and plasmid used in this study

Strains	Genotype	Reference
Caulobacter		
CB15	*C. crescentus* wild type	ATCC (#19089)
UJ2448	CB15 ΔCC0091-CC0096	This work
UJ3193	CB15 with *pleD** allele	This work
UJ3183	CB15 + pAL17	This work
UJ3194	CB15 + pAL17 with *pleD** allele	This work
UJ2982	CB15 ΔCC0095	(99)
UJ3195	CB15 ΔCC0091 with *pleD** allele	This work
UJ3055	CB15 ΔCC0091	This work
YB2833	CB15 *ΔhfsA*	(167)
YB2837	CB15 *ΔhfsB*	(167)
YB2841	CB15 *ΔhfsC*	(167)
YB2845	CB15 *ΔhfsD*	(167)
UJ730	CB15 *ΔpleD*	(2)
UJ3055	CB15 + pDM13	This work
UJ3104	UJ3055 + pHRXLT95	This work
E.coli		
DH10B	F$^-$*mcrA* Δ(*mrr*$^-$ *hsd* RMS$^-$*mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lac*X74 *endA*1 *recA*1 *deoR* Δ(*ara, leu*)7697 *araD*139 *galU galK nupG rpsL*	GIBCO BRL®
UJ606	MT607 containing pRK600	(160)

Plasmids	Description	Source
pNPTS138	pUC based plasmid with M13 ori and pLITMUS38 polylinker + *sacB* + *nptI* + RK2 oriT, suicide vector	Dickon Alley
pMR10	RK2-based broad host range and low copy number plasmid based on pGL10 (RK2 *oriV* + *trfA*, *oriT*, *nptI*) with modified polylinker of pUCBM20BSX	
pBBR1MCS-2	Medium copy number plasmid similar	M. Kovach
pHRXLT	pPHU281 (*Hind*III-*Xba*I) + PxylX (2.3 kb long genomic fragment) region (*Hind*III-*Spe*I)	This work
p*fljL-lacZ*	Plac290 derivative, contains *fljL* promoter fused to β-Galactozidase gene	(198)
pAL17	pBBR1MCS-2 + CC0091	This work
pAL53	pBBR1MCS-2 + CC3396 (pdeA gene)	This work
pAL71	pBBR1MCS-2 + CC0091 E609A mutated allele	This work
pPA114-28	pMR20 + *pleD** allele	(4)
pDM1	pNPTS138 + 2kb fragment (*Bam*HI-*Nhe*I) designed for clean deletion of CC0091	This work
pDM2	pNPTS138 + 1kb fragment (*Eco*RI-*Sph*I) designed for chromosomal integration upstream CC0096	This work
pDM3	pNPTS138 + CC0091-0096	This work
pDM5	pMR10 + CC0091-0096 from CB15	This work
pDM13	pBBR1MCS-2 + CC0095 (*Hind*III-*Eco*RI)	This work
pDM26	pMR10 + CC0091 (*Hind*III-*Eco*RI)	This work
pDM14	pMR10 + CC0092 (*Hind*III-*Eco*RI)	This work
pDM15	pMR10 + CC0093 (*Hind*III-*Eco*RI)	This work
pDM16	pMR10 + CC0094 (*Hind*III-*Eco*RI)	This work
pDM17	pMR10 + CC0095 (*Hind*III-*Eco*RI)	This work
pDM18	pET42b(+) + CC0091 Δaa1-338 (*Nde*I-*Xho*I)*	This work
pDM21	pMR10 + CC0096 (*Hind*III-*Eco*RI)	This work
pHRXLT95	pHRXLT + CC0095 (*Eco*RI)*	This work
pDM24	pET42b(+) + CC0095 (*Nde*I-*Xho*I)*	This work
pDM25	pNPTS138 + 1kb fragment (*Eco*RI-*Hind*III) designed for clean deletion of CC0095	This work

RESULTS

A WecG homolog is required for *C. crescentus* holdfast formation

A screen for surface attachment deficient transposon insertion mutants led to the isolation of a Tn*5* insertion in an open reading frame CC0095 (100). This mutant strain showed a specific defect in holdfast biogenesis (100). Gene CC0095 codes for a homolog of the WecG UDP-N-acetyl-D-mannosaminuronic acid transferase, which is involved in synthesis of the enterobacterial common antigen (ECA) (44). Some of the neighboring genes of CC0095 are coding for potential bacterial polysaccharide biosynthesis components as well (Figure 1A). CC0091 encodes a GGDEF/EAL family protein, members of which are involved in the turnover of the novel bacterial second messenger, c-di-GMP (reviewed in (74)); CC0092 encodes a UDP-glucose 4-epimerase (GalE homolog), enzymes that converts UDP-galactose into UDP-glucose. In order to analyze the role of each gene in this cluster and a possible contribution to holdfast synthesis, an in-frame deletion spanning all six genes was created in the wild type strain CB15 creating strain UJ2448. Like the CC0095::Tn*5* mutant, UJ2448 failed to synthesize a holdfast and completely lost its ability to adhere to surfaces (Figure 1B and 1C). Complementation with CC0095 alone was sufficient to restore holdfast formation and surface attachment in UJ2448 (Figure 1B ad 1C), arguing that CC0095 is the only gene of this gene cluster that is essential for holdfast biogenesis.

CC0091 is a c-di-GMP specific phosphodiesterase

Open reading frame CC0091 codes for a multi-domain protein with N-terminal MHYT and PAS sensory domains that are fused to C-terminal GGDEF and EAL domains (Figure 2A). This suggested that in response to some unknown signal(s), CC0091 might activate a C-terminal DGC and/or PDE domain. In addition, the observation that a strain lacking the DGC, PleD, exhibits a similar phenotype like a strain overexpressing CC0091 (Figure 4), led us to presume that this protein has a phosphodiesterase activity. To analyze this we have purified a truncated derivative of CC0091 protein, which lacks amino acids 1-338, thereby trimming the membrane spanning MHYT and the PAS domains. UV-crosslinking of purified CC0091Δaa1-338 with radiolabeled c-di-GMP indicated that CC0091 specifically binds this second messenger (Data not shown). Enzymatic assays performed with purified CC0091Δaa1-338 confirmed that this truncated protein indeed possesses a PDE activity, but not CDG activity (Figure 2B and C). Christen *et al*. (19) have shown that *C. crescentus* CC3396, a similar GGDEF-EAL composite protein has a PDE activity which is allosterically controlled by GTP through its GGDEF domain (19). However, when performing the PDE activity assays with CC0091 in the presence or absence of GTP, no effect of the nucleotide on enzymatic activity was observed (Figure 2C). The specific activity of the CC0091 PDE was calculated to be 11.66 ± 0.39 µmol c-di-GMP / (µmol*min). This rate is comparable with the cleavage rate reported for CC3396, without GTP (2.42 ± 0.28) and in the presence of GTP (106.8 ± 1.5 µmol c-di-GMP / (µmol*min)) (19).

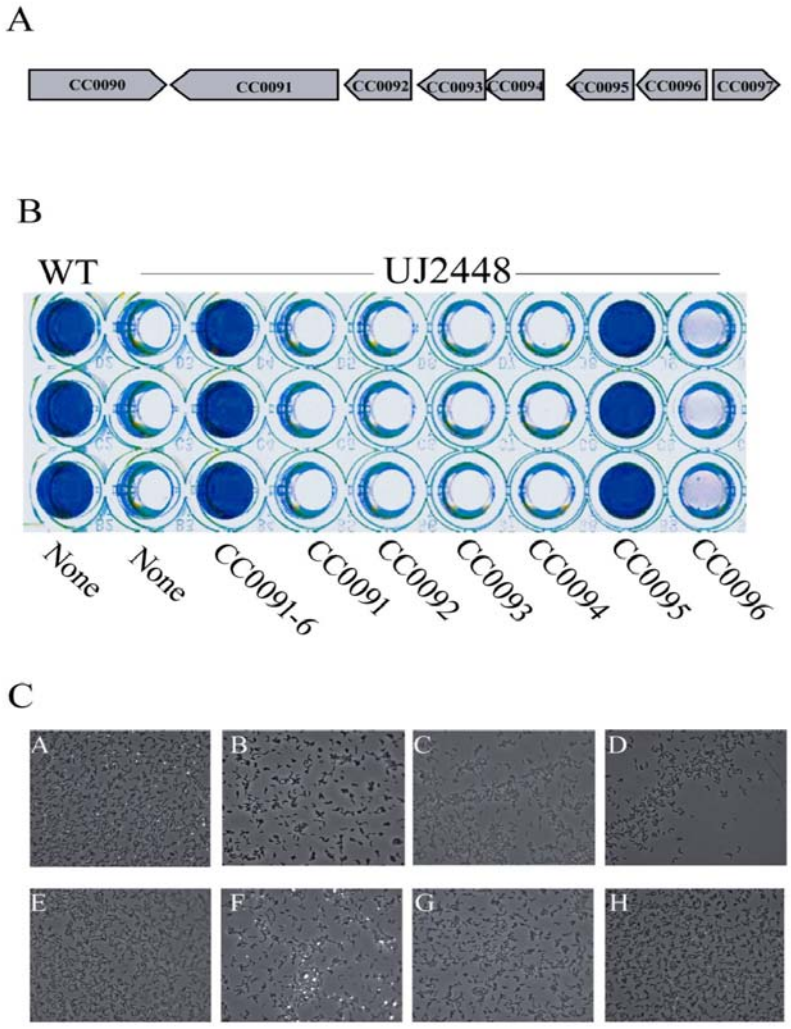

Figure 1) CC0095 but not its neighboring genes are required for holdfast synthesis A) Map of the genomic region containing CC0095 and its neighboring genes. **B)** Crystal violet attachment assay of triplicates CB15 wild type and mutant strain UJ2448 complemented by each of the ORFs in the gene cluster CC0091-6. Genes carried on complementing plasmids are indicated below the microtiter plate. This assay shows that CC0095 alone is sufficient to restore wild-type attachment levels in the mutant strain UJ2448. **C)** Holdfast staining with Oregon green-conjugated wheat germ agglutinin illustrates that complementation with CC0095 restores holdfast formation in strain UJ2448. (A-F CB15, UJ2448 with CC0091, CC0092, CC0093, CC0094, CC0095, CC0096, and UJ2448 complemented with the entire gene cluster, all on pMR10, respectively).

PleD and CC0091 are antagonistic regulators of holdfast biogenesis and surface attachment

We have shown recently that PleD Diguanylatecyclase is required for the correct timing of holdfast formation during *C. crescentus* cell differentiation (100). Here we show that CC0095, which codes for a glycosyltransferase homolog, is a critical component of the holdfast biogenesis (Figure 1), and that one of its neighboring genes, CC0091 codes for a c-di-GMP specific phosphodiesterase (Figure 2). We hypothesized that the opposing activities of CC0091 and PleD might cooperate in timing the formation of the adhesive holdfast at the developing pole. To test this model we assayed surface attachment and holdfast formation in the presence and absence of CC0091 and compared the contribution of this PDE with the role of PleD in surface binding. Consistent with the data in Figure 1, an in-frame deletion of CC0091 had a minor effect on *C. crescentus* surface attachment (Figure 3A). However, attachment was reproducibly increased by about 25% as compared to the wild type strain. In contrast, attachment is reduced more than 50% in the absence of PleD (Figure 3A; (100)). Consistent with its prominent role in holdfast biogenesis, we found that overexpression of CC0095 dramatically increased surface attachment (Figure 3A). Similar to the situation in the wild-type background, the increase of attachment was strongest in the ΔCC0091 background strain and was only modest in a strain lacking PleD (Figure 3A). In line with the observed increase in attachment, a ΔCC0091 mutant strain showed up-regulated holdfast production and cellular aggregation (Figure 3B, panel D). This was particularly evident in a strain overexpressing the presumable glycosyltransferase CC0095 (Figure 3B, panels E and F). In contrast, a strain lacking PleD showed a modest down-regulation of holdfast production even when overexpressing CC0095 (Figure 3B, panels B and C), probably due to a temporal delay of holdfast formation during swarmer cell differentiation (100). To test if the CC0091 PDE also contributes to the temporal control of holdfast formation, we analyzed the appearance of holdfast during the initial stages of swarmer cell differentiation. In wild-type cells, holdfasts can be detected as early as 10 minutes after release purified swarmer cells into fresh

medium (Figure 4). 30 minutes after release of fresh swarmers, 30% of the cells had acquired a visible holdfast. When PleD was absent, number of cells with a visible holdfast increased only marginally during this window of development (Figure 4). In contrast, a mutant lacking the CC0091 PDE shows premature holdfast formation, similar to a strain that over-expresses the CC0095 protein (80% and 95% already at time point 15 min., respectively) (Figure 4). In contrast, when the CC0091 PDE is over-expressed cells show a holdfast timing phenotype similar to Δ*pleD* mutant (Figure 4). Overexpression of another PDE protein from *C. crescentus*, PdeA (CC3396) did not have such a considerable affect on the timing of holdfast synthesis (Figure 4), suggesting that CC0091 is a holdfast-dedicated PDE. Similar analysis in cells containing the PleD* allele could not be done due to the inability to synchronize such strains (4). Together, these data indicate that the PleD DGC and CC0091 PDE activities inversely regulate holdfast biogenesis and that this antagonistic control fine-tunes holdfast appearance during the swarmer cell differentiation in *C. crescentus.*

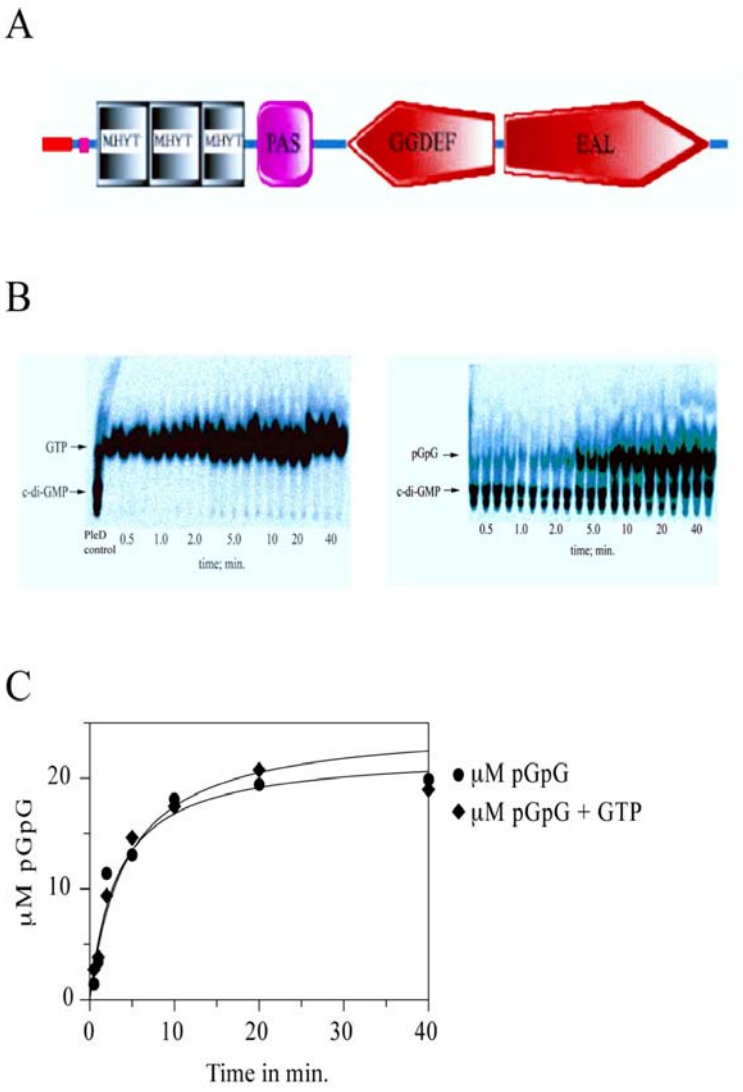

Figure 2) CC0091 is a c-di-GMP specific phosphodiesterase A) Schematic representation of the multidomain protein encoded by CC0091 ORF. The MHYT module represents a membrane integral domain, which was postulated to sense a diverse range of signals outside the cell (51). The PAS module is a cytoplasmic domain with a binding pocket for variety of small molecule ligands, such as, heme, flavin, or nucleotides. PAS domains have been implicated in sensing light, oxygen, or redox state. **B)** TLC radiograms of diguanylatecyclase (left panel) and phosphodiesterase (right panel) assays performed with purified CC0091Δ1-338 fragment. Reactions were spotted on TLC plates, separated by chromatography, and then stopped at regular time intervals (0-40 minutes) by adding 0.5M EDTA, and exposed to a phosphor-imaging screen (for assays details see Material and Methods section. The TLC plates were quantified by pattern processing with ImageJ software. Arrows indicate the position of the nucleotides. PleD served as a positive control for the diguanylatecyclase assay. **C)** Graphic representation of the kinetics of CC0091 PDE activity in the absence and presence of 100 µM GTP. The curve was fitted using Pro Fit software bundle based on radiogram intensity of the TLC as was quantified with ImageJ software.

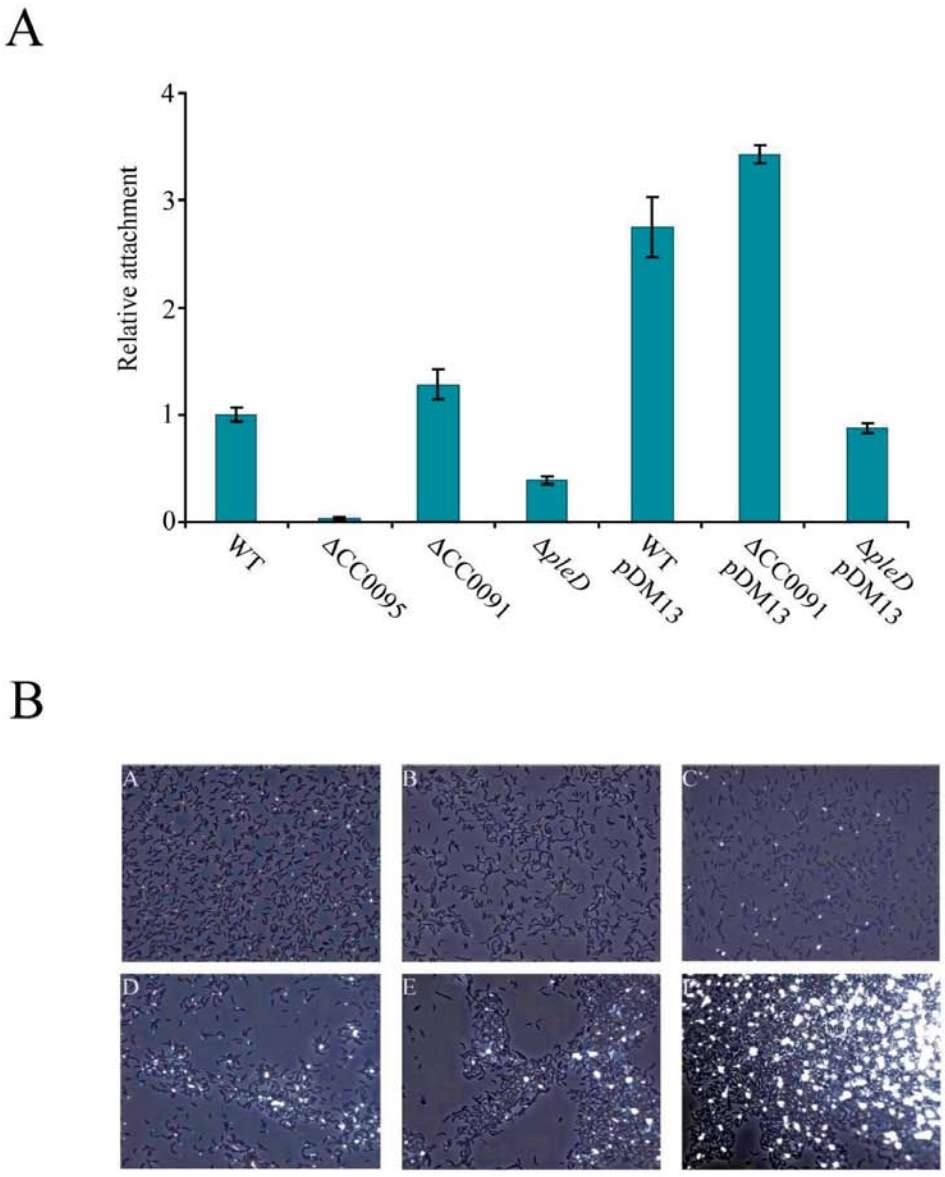

Figure 3) PleD and CC0091 inversely regulate holdfast biogenesis and attachment. **A)** Graphic representation of crystal violet attachment assay testing the following strains: CB15 wild type; ΔCC0095; ΔCC0091; Δ*pleD*, with or without pDM13 (medium copy plasmid carries CC0095 under the control of the *lac* promoter). Triplicates of the tested cells were grown for 24 hrs in 96-wells microtiter plate before the supernatant was discarded, plate was washed, and surface attachment was quantified by crystal violet staining. **B)** Different *C. crescentus* strains were stained with Oregon green-conjugated wheat-germ agglutinin and examined by fluorescence microscopy using FITC filter setting. A: WT; B: Δ*pleD*; C: Δ*pleD* with pDM13; D: ΔCC0091; E: CB15 pDM13; F: ΔCC0091 pDM13.

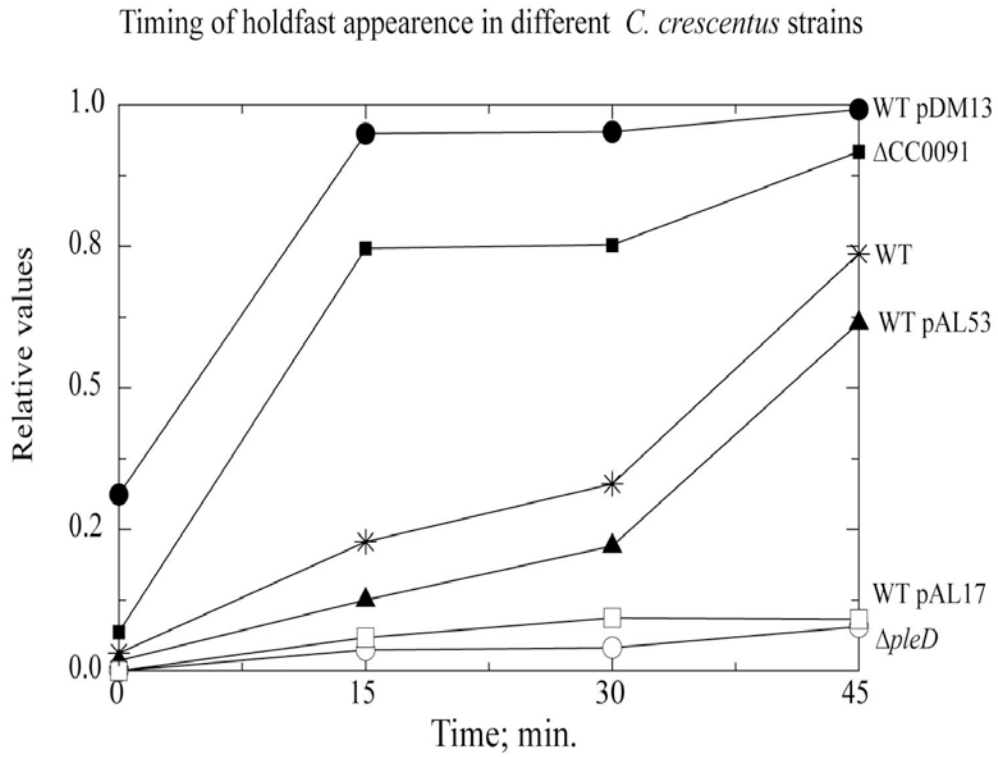

Figure 4) Appearance of a visible holdfast during the swarmer to stalked cell transition. Synchronized swarmer cells of the following strains were analyzed for the presence of a visible holdfast structure as described in Materials and methods section: WT (star); CB15 with pAL53 (*pdeA* on pBBR1MCS2) (filled triangles); CB15 with pAL17 (CC0091 on pBBR1MCS2) (empty squares); ΔCC0091 (filled squares); CB15 with pDM13 (filled circles), and Δ*pleD* (empty circles). Holdfast ratios were determined as the number of cells decorated with a visible holdfast divided by the total number of cells observed. Time point 0 is immediately after synchronized cells were released into fresh media.

PleD and CC0091 inversely regulate *C. crescentus* motility and stalk biogenesis

Mutants lacking PleD are hyper-motile, at least in part due to the delayed flagellar ejection and resultant loss of motility (2). The expression of constitutively active form of PleD, commonly known as PleD* has the opposite effect and blocks *C. crescentus* flagellar based motility (4, 130). To test the possibility that CC0091 is also involved in motility regulation, swarm colonies were analyzed on a semisolid agar plates. Although The swarm colony size of a ΔCC0091 mutant was only slightly tighter than the wild type, a strain overexpressing CC0091 showed a significantly larger swarm colony as compared to the wild type strain, and when analyzed microscopically, showed a similar hyper-motile phenotype behavior as had been reported for a Δ*pleD* mutant (data not shown). Here also overexpression of PdeA did not cause any affect (Figure 5A). Since the hyper motility phenotype of cell overexpressing CC0091 seemed similar to the phenotype of Δ*pleD* strain, we have postulated that cells overexpressing CC0091 might not eject the flagella correctly and the FliF, the flagella motor protein will be stabilized, like it was shown to be the case for Δ*pleD* (2). Synchronized culture of a strain overexpressing CC0091 were analyzed microscopically at different time points during the cell cycle and a large population of these cells seemed not to have lost its motility at any time point (data not shown); in addition a Western blot analysis using α-FliF antibodies was performed on corresponding aliquots and compared to the cell cycle degradation profile of FliF of the WT strain. The latter analysis has shown that while FliF was almost completely disappeared from the WT cells at time points 40-80 minutes (Figure 7A), the levels of this protein never really disappeared when CC0091 is overexpressed (Figure 7A), suggesting that the hyper motility phenotype of cells overexpressing CC0091 is at least in part due to a delayed loss of the flagellar motor protein FliF (Figure 7A); interestingly, a similar stabilization of FliF protein was observed in a Δ*pleD* mutant background (2), reinforcing the hypothesis that CC0091 and PleD have a counter effect on *C. crescentus* development. Overexpression of CC0091, but not of PdeA was also able to override the motility block of cells containing PleD*, while the negative effect of the PleD* allele on motility was

intensified in a ΔCC0091 mutant strain background (Figure 5B). These results support the idea that in the strains tested, fluctuating levels of c-di-GMP are responsible for the different degrees of motility observed. In addition to causing a motility block, un-controlled activity of PleD* resulting in significantly elongated stalks, while in the absence of PleD, stalk growth is severely affected (4). This argues that stalk growth is also controlled by c-di-GMP. To test whether CC0091 PDE has a role in controlling also this process we determined the relative average stalk length of different *C. crescentus* strains by measuring the stalks images of indicated strains photographed with Electron Microscope. While overexpression of CC0091 had no effect on the wild-type stalk length, it could restore a wild type-like stalk length of PleD* strain (Figure 5C), while overexpression of PdeA failed to do so (Figure 5C). Deletion of CC0091 was enough to cause about 70% increase of stalk length and when this strain was carrying *pleD** allele it showed also here in intensification of the PleD* related phenotype (Figure 5C), corroborating the idea that CC0091 is a specific PleD antagonist and that stalk elongation is controlled by cellular levels of c-di-GMP.

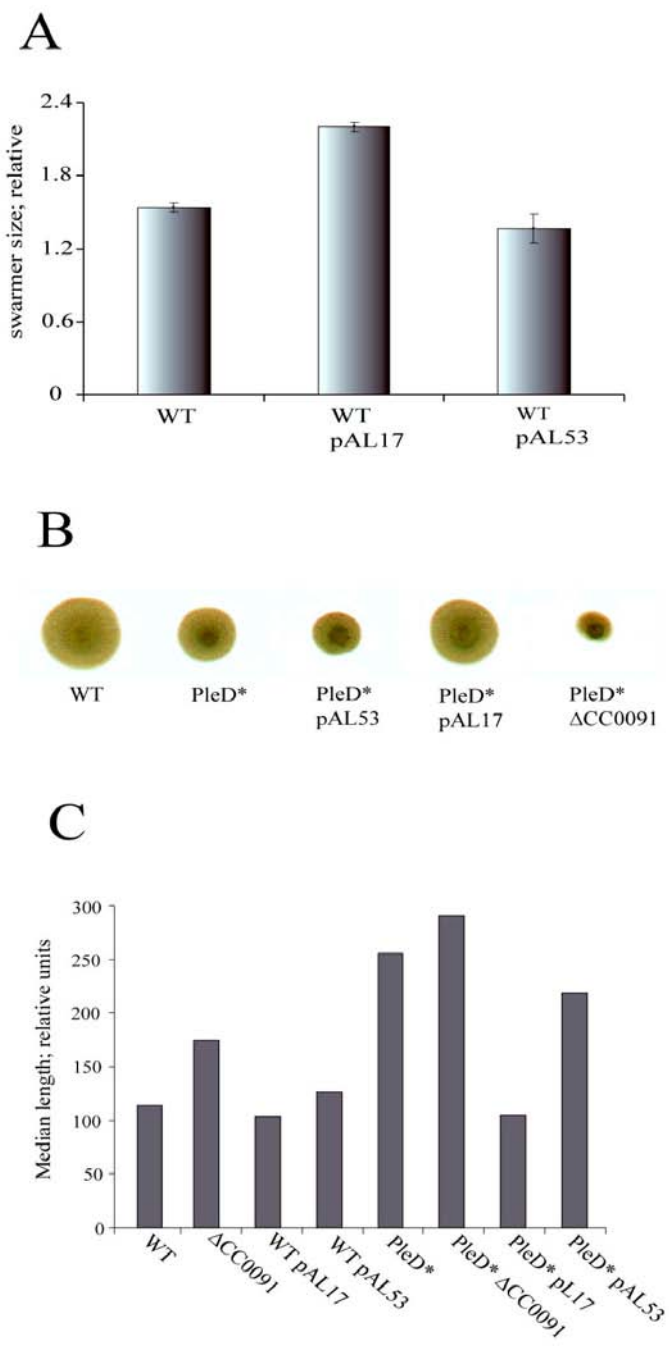

Figure 5) PleD and CC0091 inversely affect motility and stalk length A) Overexpression of CC0091, but not of PdeA, results in a hyper motile behavior, as indicated in this graph. Swarm size of triplicates colonies of WT, WT with pAL17 (overexpressing CC0091), and WT with pAL53 (overexpressing CC3396) was measured by using ImageJ software on a scanned image of the 5 days old swarmer plate and the arbitrary values were plotted in this graph. **B)** While a deletion of CC0091 PDE intensified the motility block caused by PleD*, overexpression of this PDE, but not of PdeA restores normal motility of PleD* strain as presented here in an image of swarmer plate. The strains indicated in the figure were stubbed on a semi solid (0.3%) agar plate and incubated at 30˚ C for 5 days. **C)** The stalks' length of the indicated strains was measured using Photoshop® software ruler tool applied on an Electron Microscopy images.

C. crescentus motility is regulated by c-di-GMP levels and by holdfast structure

Overexpression of the glycosyltransferase CC0095 is not only increased holdfast formation and surface attachment (Figure 3), but also had a strong negative effect on motility (Figure 6A). Interestingly, overexpression of the CC0091 has completely suppressed this phenotype and restored a hyper-motile phenotype, in a similar way it did for PleD* strain (Figure 6A). In contrast, as for the case for PleD* strain, the motility block caused by CC0095 overexpression was intensified in the ΔCC0091 mutant background (Figure 6A). Together this argued that, directly or indirectly, over-production of holdfast exopolysaccharides negatively affects motility and that this effect is dependent on c-di-GMP. The motility block caused by overexpression of the glycosyltransferase CC0095 was dramatically enhanced when the medium was supplemented with glucose (data not shown), arguing that holdfast production and negative effect on motility is not only controlled by c-di-GMP, but also dependent on the availability of sugar precursors. To further analyze the link between holdfast production and motility, CC0095 was inducibly expressed in *C. crescentus* holdfast mutant strains, including, NA1000, UJ2984 (ΔCC2277), YB2833 (CB15 Δ*hfsA*), YB2837 (CB15 Δ*hfsB*), YB2841 (CB15 Δ*hfsC*), and YB2845 (CB15 Δ*hfsD*). CC0095 expressed from a chromosomal copy was introduced to *C. crescentus* genome as transcriptional fusion with *Caulobacter*'s native xylose promoter, which is activated upon addition of xylose to the growth media. Colonies of the wild type and the different holdfast mutant strains carrying the P*xyl*::CC0095 were stubbed on semisolid agar plates supplemented with 0.2% glucose or with 0.2% glucose + 0.2% xylose. As demonstrated in Figure 6C, the swarm size of the different holdfast-lacking strains was similar to the WT strain in the absence of the xylose inducer. Induction of CC0095 expression by xylose addition resulted with partially or fully restored wild-type like motility in some, but not all of these mutants background (Figure 6C), arguing that increased synthesis of the holdfast (and not just overexpression of the CC0095 glycosyltransferase) is responsible for the motility block in these mutants. At this point

however, we were not able to determine the exact mechanism in which the holdfast interferes with *C. crescentus* motility.

To examine the basis for the motility block of strains overexpressing CC0095 and especially of those that in addition lacking CC0091 we have analyzed the promoter activity of different flagella structural genes. For that purpose we used plasmids which contain different flagella promoters that are transcriptionally fused to β-Galactosidase gene (*fliF*, *flgH*, and *fljL*) and measured their activity in strain lacking CC0091 (UJ3055); overexpressing CC0095 (CB15 + pAL13), or overexpressing CC0095 in a ΔCC0091 background (UJ3055 + pAL13) (Data not shown), showed that *fljL* has the only promoter that has exhibited a significant transcription reduction in CC0091 deletion mutant background. A second experiment set measuring β-galactosidase activity, this time only of the *fljL-lacZ* fusion construct in these genetic background strains, showed that this repression is enhanced by the addition of glucose to the growth media (Figure 7B) and overexpression of CC0095 glycosyltransferase further represses the *fljL* promoter activity (Figure 7B). Deletion of CC0091 reduced *fljL* promoter activity by 15% when cells were grown on PYE and by 20% when glucose was added (Figure 7B); stronger decrease was observed when CC0095 was overexpressed, 25% in PYE and 40% when glucose was added; the strongest promoter repression was obtained when CC0095 was overexpressed in the background of CC0091 deletion mutant, 40% in PYE and 90% when glucose was added (Figure 7B).

To prove that CC0091 PDE activity is important for its function, we created a single amino acid substitution (E609A) at the conserved glutamate of this domain and asked whether a medium copy-number plasmid carrying this mutant gene (pAL71) could complement the ΔCC0091 phenotype and cause a hyper motile phenotype as the WT copy does. An analogous mutation was shown to inactivate the phosphodiesterase activity in other phosphodiesterases (12, 69, 180). ΔCC0091 cells harboring a plasmid with the wild-type CC0091 gene exhibited a hypermotile phenotype similar to the WT strain harboring the same plasmid; on the contrary ΔCC0091 carrying pAL71 had an even

stronger motility block than the un-complemented ΔCC0091 (Figure 6B), suggesting that the mutant allele could interfere in an unknown motility regulation pathway. Taking together, these data suggest that CC0091 PDE activity is involved in regulation of motility through FliF stabilization and *fljL* transcription; and that holdfast synthesis might also interfere with that regulation.

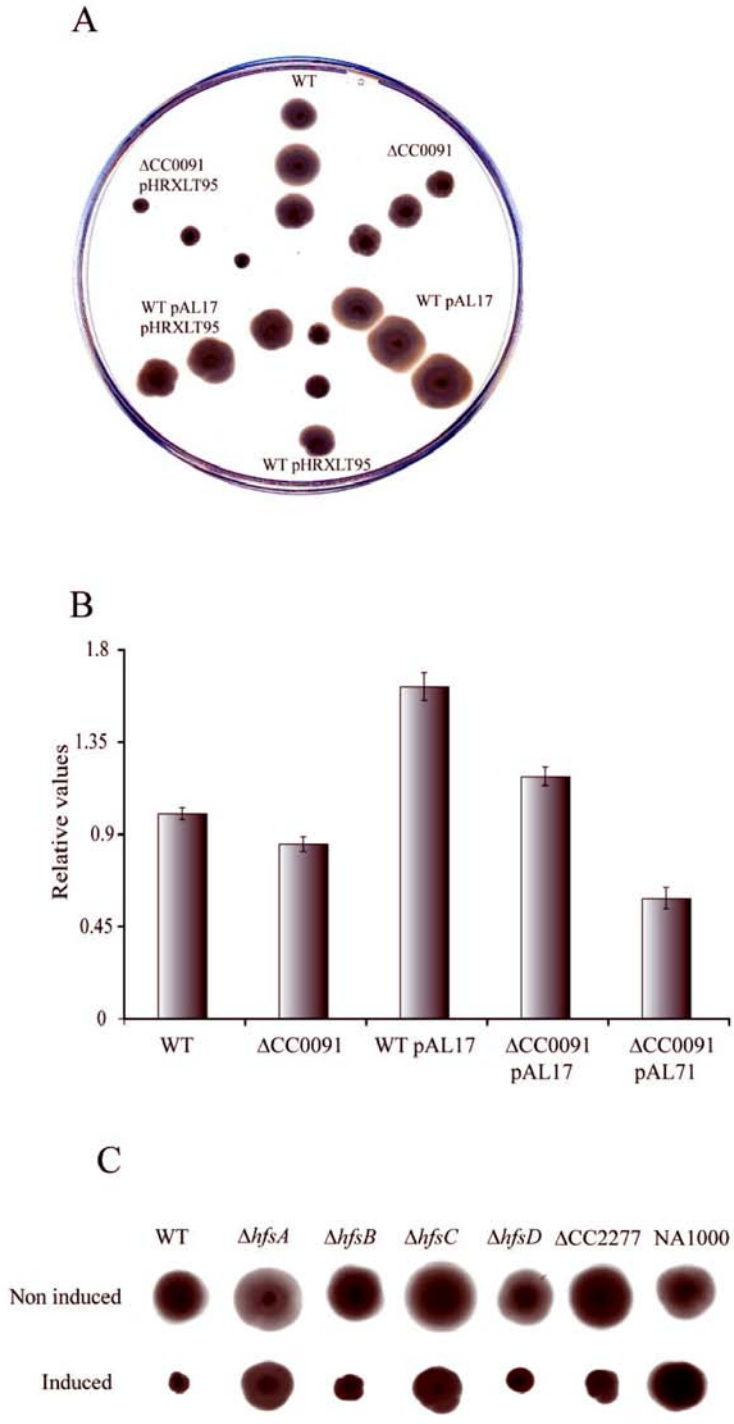

Figure 6. **CC0091 PDE and CC0095 glycosyltransferase inversely affect motility A)** The following strains: CB15 wild type; ΔCC0091; WT with pAL17 (CC0091 on pBBR1MCS2); CB15 pHRXLT95 (P*xyl*::CC0095); CB15 pHRXLT95 + pAL17; CB15 ΔCC0091 pHRXLT95 were stubbed (3 independent colonies) on a large (ø 150 mm) swarmer plate (0.3% agar PYE supplemented with 0.1% xylose) and incubated at 30˚ C for 5 days. A scanned image of this plate is presented here. **B)** CC0091 EAL domain is essential for its affect on motility. WT strain carrying pAL17 (CC0091 overexpression) exhibits a hypermotile phenotype. CC0091 deletion mutant strain complemented with pAL17 exhibited a hypermotile phenotype as well; though to a

lesser extend than the WT carrying the same construct. In contrast, CC0091 deletion mutant carrying CC0091 mutant variant (E609A) on pBBR1MCS2 (pAL71) displayed a severe block of motility. **C)** The motility block caused by overexpressing the presumable glycosyltransferase CC0095 is dependent on holdfast synthesis genes. Motility assay on semisolid PYE agar plates supplemented with 0.2% glucose with the following strains: CB15 P*xyl*::CC0095, NA1000 P*xyl*::CC0095, CB15 Δ*hfsA* P*xyl*::CC0095, CB15 Δ*hfsB* P*xyl*::CC0095, CB15 Δ*hfsC* P*xyl*::CC0095, CB15 Δ*hfsD* P*xyl*::CC0095, and CB15 ΔCC2277 P*xyl*::CC0095 was carried out in absence (upper strip, non induced) and in the absence (lower strip, induced) of 0.2% xylose.

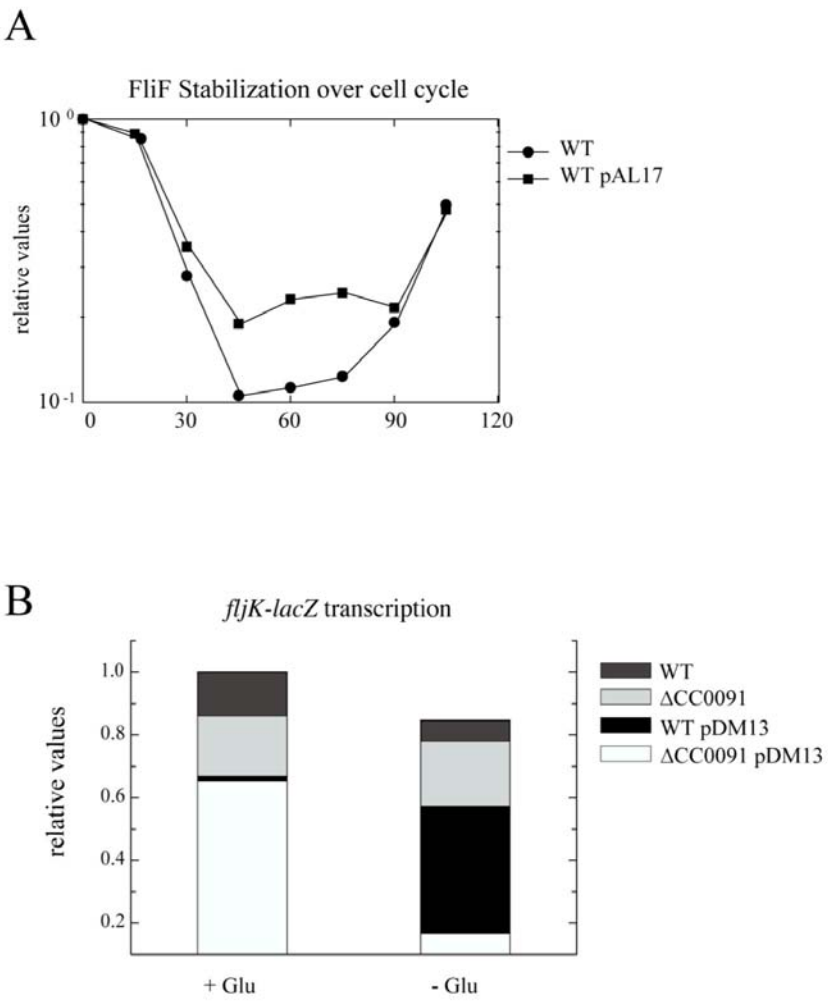

Figure 7. Both c di GMP levels and holdfast synthesis regulate flagella genes stability and transcription. A) Synchronized cultures of WT and WT with pAL17 were analyzed at different time points over the cell cycle for the presence of the flagellum motor protein, FliF. A Western blot analysis using α-FliF monoclonal antibody was used to detect the levels of FliF during the cell cycle. The intensity of the bands in the blot image was quantified with ImageJ® software package; levels were normalized to the levels of ClpP protein which is known to remain constant during *C. crescentus* cell cycle. **B)** *fljK* promoter activity of different strains: WT (dark grey); ΔCC0091 (light grey); WT with pDM13 (CC0095 overexpression) and ΔCC0091 pDM13, all contain P*fljK-lacZ* (plasmid harboring a transcriptional fusion of *fljK* to β –Galactosidase gene) was measured as described in

Material and Method section. Columns are aligned in front of each other so that the lowest value is in front and the highest value is at the back.

Swarmer cell specific expression of CC0091 and C0095

The finding that CC0091 and CC0095 are involved in controlled synthesis of holdfast during *C. crescentus* development raised the question if their own expression would be temporally controlled during *C. crescentus* cell cycle. Quantitative Real Time PCR (QRT-PCR) experiment was performed in order to quantify mRNA levels of both genes throughout the cell cycle in synchronized cell populations. Relative gene expression levels were determined using the *rpoD* housekeeping gene as the endogenous control (50) (for details refer to the Materials and Methods section). Both CC0091 and CC0095 exhibited a distinct cell cycle-dependent expression profile with a profound peak of transcriptional activity in the swarmer cell (Figure 8). CC2277, another glycosyltransferase encoding gene which was shown to be also essential for holdfast biosynthesis (100), exhibited a similar swarmer cell-specific expression profile (Figure 8). The holdfast and motility phenotype observed when CC0095 was expressed constitutively from the xylose promoter; indicate that this pronounced temporal expression control is an important part of pole development in *C. crescentus*.

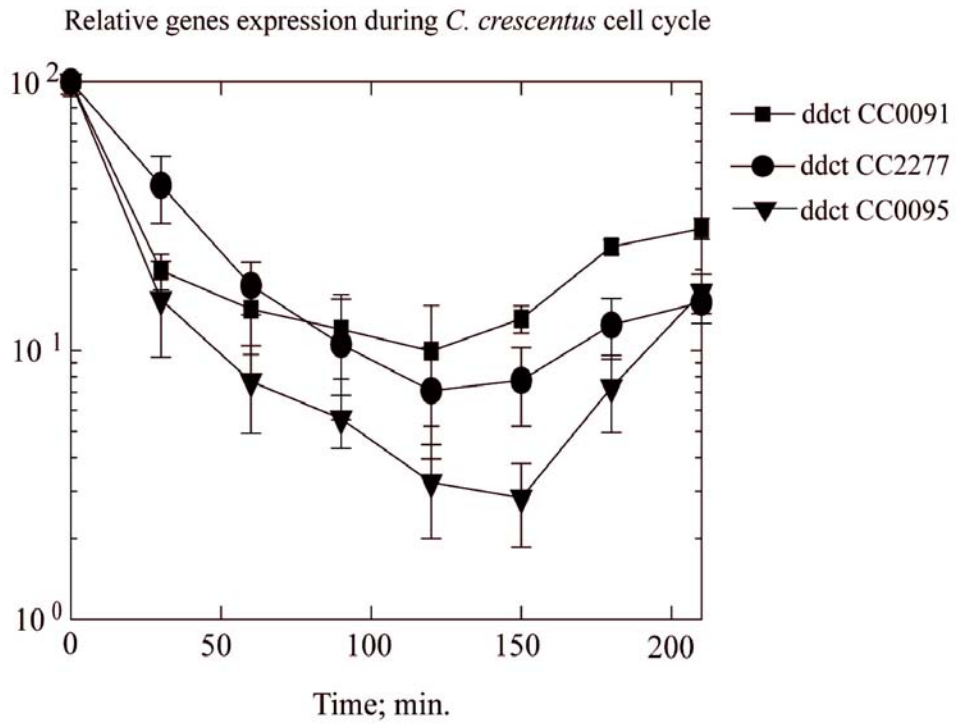

Figure 8. Cell cycle dependent expression of genes required for holdfast synthesis and control. Quantitative Real Time PCR was employed to quantify mRNA levels of CC0091 (squares), CC0095 (triangles), and CC2277 (circles) during *C. crescentus* cell cycle. The ct-values were normalized with respect to those of the housekeeping gene, *rpoD*, giving the ddct values indicated in the graph. Results of 3 independent mRNA samples are presented.

DISCUSSION

Here we have analyzed regulatory mechanisms, which act as a timing device for holdfast formation during *C. crescentus* cell differentiation. Our results suggest that one of the key components involved in holdfast control is the novel bacterial second messenger, c-di-GMP, making this the first example of a developmental process being controlled by this regulatory compound. The first evidence that c-di-GMP could be involved in *C. crescentus* cell differentiation came from the work by Paul *et al.* (130), which indicated that the response regulator PleD was a diguanylatecyclase. Mutations in *pleD* had been shown earlier to cause a pleiotropic pole development phenotype, including the failure to eject the flagellum and to produce normal-length stalks (2, 57). A molecular marker of delayed flagellar ejection in *pleD* mutants is the stabilization of the flagella anchor protein FliF, which is normally degraded during motor loss. In contrast, the presence of a constitutively active form of PleD, PleD*, blocked motility and caused a marked increase in stalk length (4). Recently, it was demonstrated that cells lacking PleD, also showed a dramatic delay in holdfast formation and, as a result, exhibit reduced surface binding (100). While these indicated clearly that the second messenger c-di-GMP is responsible for the temporal control of *C. crescentus* pole development, a number of important questions regarding the mechanism of c-di-GMP signaling remained unanswered. How would levels of c-di-GMP be tightly controlled during development in time and space? Is the observed coupling of activation of PleD by phosphorylation with the subcellular sequestration of this response regulator to the differentiating pole (130) important for c-di-GMP-dependent pole development? Moreover, which cellular structures would the signaling compound interfere with to direct pole development?

Here we have identified a novel c-di-GMP phosphodiesterase, CC0091, which is involved in the control of holdfast formation during the swarmer-to-stalked cell transition. Our analysis suggests that PleD and CC0091 have antagonistic roles during *Caulobacter* cell differentiation and that these opposing activities are responsible for the accurate temporal succession of pole development events. Most importantly, a CC0091 mutant showed increased and premature synthesis of holdfast. In contrast, overexpression of CC0091 resulted in pleiotropic defects similar to those observed in *pleD* mutants, including hypermotility, stabilization of FliF, and delayed holdfast synthesis. Also, while overexpression of CC0091 was able to suppress the pleiotropic phenotype of a *pleD** allele, the dominant effects caused by this gain-of-function allele were clearly more pronounced in a CC0091 mutant. These observations and the finding that *in vitro* CC0091 showed a strong PDE but not DGC activity, suggested that PleD and CC0091 are responsible for the correct timing of pole development through the antagonistic control of cellular c-di-GMP levels (Figure 9).

CC0091 is a multi-domain sensory protein with two distinct N-terminal input domains, MHYT and PAS, fused to the C-terminal GGDEF and EAL domains. The fact that a soluble fragment of CC0091 consisting of GGDEF and EAL domain showed PDE but not DGC activity *in vitro* provided a functional explanation for the EAL, but not for the GGDEF domain. We have shown recently that GGDEF domains, aside from being catalytically active, can also act as GTP sensor domains, and upon binding of GTP can activate the neighboring EAL PDE (19), but failed to show GTP induction for the CC0091 PDE *in vitro*. Thus, the biochemical activity and *in vivo* role of the GGDEF domain remains elusive. The MHYT domain is thought to function as transmembrane sensor domain in bacterial signaling proteins (51). It consists of six predicted membrane-spanning segments and three-conserved Met-His-Tyr motifs (after which the domain has been named) located in the periplasmic loops of the domain. Signals perceived or ligands bound by MHYT domain proteins have so far not been identified. PAS are abundant signaling domains that by virtue of a bound heme, flavin, or chromophore, can sense various signals including oxygen, redox status, or light (34, 134, 206).

While the exact role of these two sensory domains for *C. crescentus* pole development is unclear, it is possible that they respond to some key internal and/or external cues, thereby integrating critical information about the environment, or the metabolic and cell cycle status of the differentiating cell to reach an appropriate developmental decision. Similarly, it has been suggested that PleD, through activation by its cognate sensor kinase(s) also responds to internal and maybe external signals. Thus, the antagonistic activities of CC0091 and PleD might allow the cell to fine-tune the developmental transition of a motile swarmer to an adhesive and sessile stalked cell with critical internal and external parameters (Figure 9).

The observation that the glycosyltransferase gene CC0095 is strictly required for holdfast biogenesis and, when overexpressed, leads to massively increased holdfast synthesis, stronger surface attachment and colonization, and an almost complete loss of motility, suggested that its product catalyzes a rate-liming step of holdfast polysaccharide synthesis. CC0095 encodes a putative UDP-*N*-acetyl-D-mannosaminuronic acid transferase of the WecB/TagA/CpsF family (COG1922). WecG, an *E. coli* homolog of CC0095, was shown to catalyze an early step in enterobacterial common antigen (ECA) production (44). ECA is a linear heteropolysaccharide consisting of repeats of the trisaccharide 4-acetamido-4,6-dideoxy-D-galactose, *N*-acetyl-D-mannosaminuronic acid, and *N*-acetyl-D-glucosamine, (44). WecG catalyzes the transfer of *N*-acetyl-ß-D-mannosaminuronic acid to undecaprenylpyrophosphate-*N*-acetyl-α-D-glucosamine thereby converting lipid I into lipid II. After transfer of the sugar moieties through the cytoplasmic membrane, the ECA chains are anchored in the outer membrane via phosphoglyceride chains (44). It is interesting to note that the ECA and LPS share the same sugar precursors and the same carrier lipid (91). It is reasonable to assume that the so far uncharacterized holdfast polysaccharide, which has been proposed to be anchored in the outer membrane, shares some of the properties of ECA and LPS. Several genes critical for holdfast assembly were identified genetically and their products, HfaA, HfaB, and HfaD, were proposed to be involved in anchoring the holdfast structure in the outer membrane at the stalk tip (21, 92).

The finding that CC0095 could catalyze a rate-limiting step of holdfast biogenesis, together with the observation that CC0095 activity requires the DGC protein PleD and is strongly repressed by the PDE CC0091, not only confirmed the critical role of c-di-GMP in holdfast control, but also indicated that this glycosyltransferase might constitute a regulatory target of the second messenger. A direct allosteric control of key enzymes involved in exopolysaccharides biosynthesis by c-di-GMP has also been proposed for the regulation of cellulose synthesis in *Gluconacetobacter xylinus*, *Salmonella enterica,* and *E. coli* (151, 207). The binding the c-di-GMP second messenger could allosterically regulate CC0095 activity directly or indirectly (through a shuttle or a receptor protein); CC0095 might have specific binding site for c-di-GMP or to c-di-GMP receptor/shuttle protein and is subjected to allosteric regulation by the second messenger. If so, the specific activation of CC0095 and possibly other enzymes of holdfast biosynthesis upon increasing concentrations of c-di-GMP might lead to the initiation of holdfast formation during the swarmer cell differentiation (Figure 9). Such a post-translational control mechanism would be consistent with the observation that newborn swarmer cells are able to synthesize adhesive holdfast structures even if *de novo* protein biosynthesis is blocked (100). Future experiments should be geared at the identification of the c-di-GMP binding site of CC0095 or its interactions with another c-di-GMP binding protein and the isolation and characterization of c-di-GMP independent forms of the enzyme.

Genetic studies with *pleD*, CC0091, and CC0095 indicated that all three genes not only affect holdfast biosynthesis but, directly or indirectly, also influence cell motility. It is generally assumed that c-di-GMP itself, negatively influences flagellar motility (74). In a recent paper Beyhan *et al*. have shown that in response to an elevated level c-di-GMP *V. cholerae* increase the transcription of the vps, eps, and msh genes and suppress the expression of flagellar genes (11). In another work published by Huitema *et al.* a novel phosphodiesterase protein, TipF was shown to be involved in flagella biosynthesis and its correct positioning (69). In line with this view, mutations in *pleD* or overexpression of CC0091 results in *C. crescentus* hyper motility, while the constitutive form PleD*

blocks motility (4). Surprisingly, ectopic expression of the CC0095 glycosyltransferase gene from a xylose-inducible promoter also showed a strong negative effect on cell motility as assayed on semisolid agar plates (see Figure 6). One explanation for this unexpected result is that the presence of holdfast and flagellum at the same pole can functionally interfere with each other and that premature or increased production of holdfast polysaccharides could obstruct with flagellar rotation. Consistent with this view is the observation that the motility block caused by uncontrolled expression of CC0095 is dependent on functioning holdfast export and assembly machinery. Mutations in most, but not all of the known holdfast genes suppressed this motility block, arguing that it is indeed the adhesive holdfast or a substructure of it, which negatively influences flagellar performance. It is possible that the loss of flagellar motility during the *C. crescentus* swarmer-to-stalked cell transition, directly or indirectly, is coupled to the appearance of the holdfast at the same pole. The ability to differentially label flagellum and holdfast with fluorescent dyes in a differentiating population of swarmer cells would allow to directly analyzing the dynamics of organelle turnover during *C. crescentus* development and its control by c-di-GMP.

An interference of motility and adhesion organelles during *C. crescentus* development would be in line with the inverse regulation of EPS biosynthesis and flagellar motility reported in other microorganisms. In *Pseudomonas aeruginosa* alginate production and synthesis of flagellar proteins are inversely controlled by AlgT, the alternative sigma factor (σ^{22}). AlgT downregulates most of the flagellar genes of *P. aeruginosa* through the repression of FleQ, an essential flagellar regulator (181). Similarly, biofilm-associated cells of *E. coli* K-12 repress transcription of flagellar genes and simultaneously increase the transcription of colanic acid biosynthetic genes (138). In one reported case, the flagellum itself is hypothesized to be the sensor for the upregulation of EPS components. In *V. cholerae* mutations that blocked the assembly of a complete flagellar filament caused a rugose colony morphology and increased exopolysaccharide production (97, 191). It has been postulated that upon initial attachment of *Vibrio* cells to surfaces the increased flagellar drag would be used to sense

surface contact and rapidly initiate downstream events leading to irreversible adhesion and surface colonization. It is possible that similar mechanisms are required for successful surface attachment of differentiating *C. crescentus* swarmer cells.

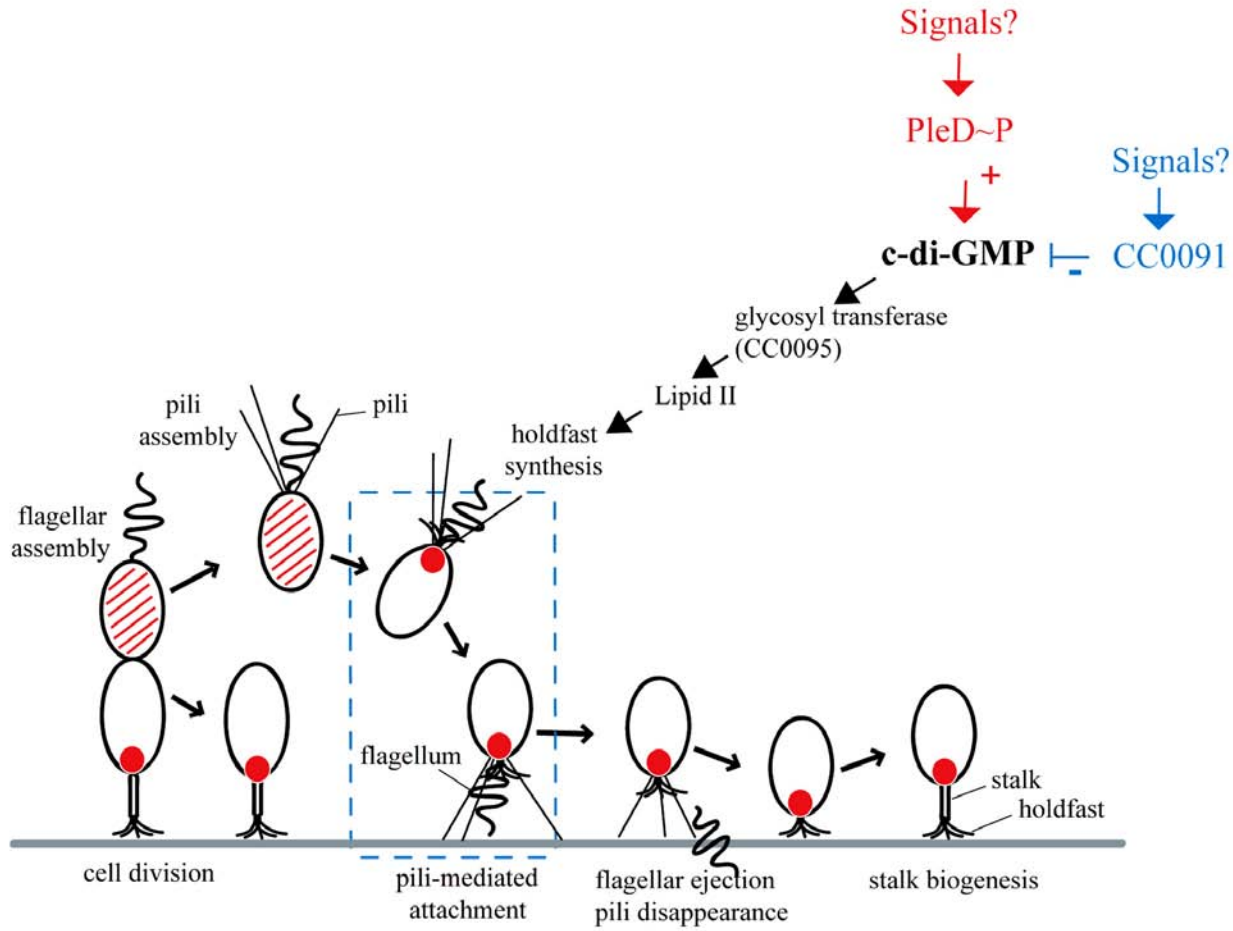

Figure 9) Schematic of the *C. crescentus* cell cycle and pole development. A polar flagellum is assembled and activated in the predivisional cell at the pole opposite the stalk and is ejected later during cell differentiation (75). Pili are assembled following cell separation and were proposed to retract in order to mediate cell-surface interaction (94, 100). Both flagellar motility and pili facilitate surface colonization (13, 100). Holdfast is synthesized during the motile stage of the swarmer cell and during a short time window coincides with the flagellum and pili at the same pole (100). A working model for the temporal control of holdfast biogenesis during the *C. crescentus* cell cycle is indicated. The correct timing of holdfast formation during cell differentiation is controlled by the global second messenger c-di-GMP, possibly through the activation of the holdfast-specific glycosyltransferase

CC0095. Levels of c-di-GMP are modulated by the opposing activities of PleD (DGC) and CC0091 (PDE). The polar localization pattern of PleD is indicated (red color). While CC0091 is expressed specifically in swarmer cells (Figure 8), no information is available with respect to temporal and spatial protein distribution of CC0091.

ACKNOWLEDGEMENTS

We thank Matthias Christen for his help with the biochemical characterization of CC0091 phosphodiesterase.

CHAPTER 3

Additional results

ADDITIONAL RESULTS

Biofilm associated *C. crescentus* cells exhibit increased antibiotic resistance

Biofilms resist antibiotic treatment and contribute to bacterial persistence in chronic infections(40, 49, 104, 173). The protective mechanisms at work in biofilms seem to be multitalented and consist of such general traits as poor antibiotic penetration due to cell encapsulation, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells. (reviewed in (173)). In addition to these more general resistance mechanisms, several examples are known that would suggest that biofilm embedded bacteria utilize distinct mechanisms to resist antimicrobial action. Hoffman *et al.* showed that tobramycin, an aminoglycoside antibiotic induces biofilm formation in *P. aeruginosa* and *E.coli* (66). An EAL domain protein with a presumable c-di-GMP specific phosphodiesterase activity was found to contribute to biofilm-specific tobramycin resistance (66), indicating that antibiotic induced biofilm formation and resistance development is controlled by c-di-GMP. Furthermore, NdvB catalyzed synthesis of periplasmic glucans was associated with resistance against tobramycin (104). These periplasmic glucans were shown to physically interact with tobramycin, sequestering the drug to the periplasm and thereby preventing it from reaching its target (104). In order to test if biofilm-embedded *Caulobacter crescentus* cells also show increased resistance to anti-microbial substances, cultures were grown over-night in 96 wells microplate, and the planktonic and the surface bound cell populations were challenged with different antimicrobial substances. As shown in Figure 1, biofilm embedded cells revealed an increased resistance to a variety of toxic materials, to some of them with more than a 100-fold factor. *Caulobacter* biofilm-embedded cells exhibited increased resistance to all antimicrobial tested, however the resistance factor was different from one substance to another. Further analyzes are required for better understanding the resistance mechanism of *C. crescentus* biofilms.

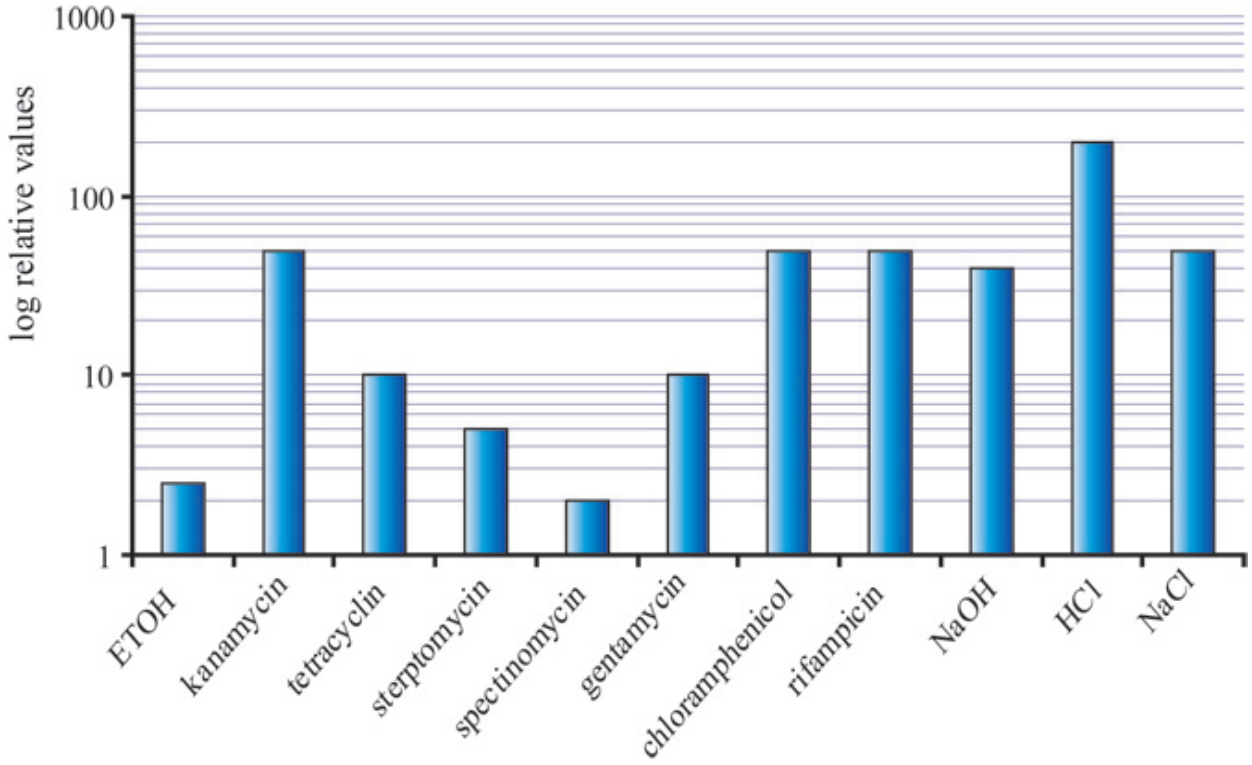

Figure 1) Increased resistance against antimicrobial substances by *Caulobacter crescentus* biofilms. *C. crescentus* wild type was grown in 96-wells polystyrene microplates for 48 hours before the supernatant was transferred into fresh wells containing increasing concentrations of the indicated anti-microbial substances. The remaining surface associated cell fraction was supplied with fresh media containing the same antimicrobials. Both cell fractions were incubated for additional 12 hours at 30° C with gentle shaking. Antibiotics were removed and cells were washed with fresh media and incubated for 12 hours at 30° C. Cultures from both 96-wells microplates (first contained the planktonic cells went through this treatment and the second contained the and biofilm cells) were stamped on PYE agar plate using a sterile comb, incubated for 72 hrs at 30° C, and wells which had had viable cells in them, appeared as large colony on the plate. The graphs represent the susceptibility difference between biofilm-associated and planktonic cells on a logarithmic scale.

Analysis of *C. crescentus* biofilm maturation in dynamic flow chambers

When grown in a dynamic flow chamber system, *C. crescentus* wild-type cells first form stable monolayers of cells on the surface within 24 hours. These monolayers consist mostly of predivisional cells, which are attached to the surface through their polar holdfasts (Figure 2). High cell density monolayers have been shown earlier to represent a well-defined stage of *C. crescentus* (43, 166) and *Vibrio cholera* biofilm maturation (112). At later stages of the biofilm development (72-96 hours after incubation), cells organized in monolayers tend to aggregate and assemble into 3-dimensional structures in which the holdfast polysaccharide material is no longer constrained to the zone of attachment of cells with the glass surface but rather is homogenously distributed forming the matrix of the cell aggregates (microcolonies). These structures are characterized by massive polysaccharide production as observed by light and fluorescent microscopy (Figure 4A), scanning light microscopy (SCLM) (Figure 4B), and intimate cell-to-cell contact and as seen by scanning electron microscopy (SEM) (Figure 3B). The regulatory mechanisms and signals involved in the transition from holdfast attached cell monolayers to mature biofilms with cells embedded in an extensive extracellular matrix have yet to be discovered.

Flagellar motility, adhesive pili, and the polar holdfast structure are important for the initial stages of surface adhesion and colonization (100). To determine a possible role of the three polar appendices in biofilm maturation, we have used the flow chamber system to compare biofilm formation of *C. crescentus* wild type with strains lacking one of the organelles. Figure 3 illustrates a time course of surface colonization under hydrodynamic conditions. 12 hours after inoculation wild-type cells had attached to the surface and by 36 hours had formed a dense monolayer with most cells standing upright in direct contact with each other. Between 36 and 72 hours after inoculation of the flow chamber, the attached cell layer had solidified 2-3 fold and characteristic cell mounds had formed indicative of a three-dimensional biofilm structure typically observed for other bacterial species (86, 87). Consistent with the results obtained in a static system (100), a mutant lacking

holdfast was unable to mediate surface attachment in the flow chamber and was washed away by the media flow (data not shown). Mutants lacking pili or flagellum showed a delayed attachment and failed to develop into thicker 3-dimensional structures (Figure 3A). To analyze this stage of biofilm in more detail, we used scanning electron microscopy analysis of cells attached to glass (Figure 3B). While characteristic microcolony formation was observed for wild type, flagellar and pili mutants failed to do so in the course of the experiment. While surface colonization of the *flgFG* mutant seemed to be delayed compared to wild type (possible due to the slower attachment kinetics), a mutant lacking pili formed unordered aggregates of filamentous cells on the surface with no signs of microcolonies formation (Figure 3B). This indicated that pili are required not only for the initial attachment to surfaces, but might also play a role in the later stages of *C. crescentus* biofilm formation and maturation. Pili play an important role in different steps of biofilm formation in several bacteria. Pili facilitate surface binding in *E. coli*, *P. aeruginosa*, and *V. cholerae* (31, 123, 135, 189), although this activity is dependent on the experimental system and nutritional conditions (31, 61). Later steps of biofilm formation, including surface migration and mushroom formation are also dependent on the presence of pili (86, 123). Klausen *et al.* (86) have proposed that pili based twitching motility helps *P. aeruginosa* to aggregate on top of a stalk of non-motile peers, leading to the formation of the typical caps of mushroom structures. While no evidence exists for twitching motility in *Caulobacter*, it is possible that pili-based cell migration contributes to microcolony formation. Alternatively, if the formation of microcolonies results from clonal growth (87) pili could have a more structural role in forming and/or stabilizing cell aggregates in microcolonies. Related type IV pili from *Actinobacillus actinomycetemcomitans* (131) are not only required for tight surface adherence but also seem to form an extracellular matrix of large fibril structures that interconnect cells in biofilms (83).

The holdfast is the critical organelle for *C. crescentus* surface anchoring. Mutants lacking holdfast completely fail to bind to surfaces and develop biofilms in a static or dynamic system.

Staining properties and enzyme sensitivity studies indicated that holdfast is a complex acidic polysaccharide, which contains *N*-acetylglucosamine (GlcNAc) residues and β-linked polysaccharides (100, 126, 167, 185). Comparison of the EPS staining properties using calcofluor and fluorescent lectins suggested that the main matrix polysaccharide of fully developed *C. crescentus* biofilms might be similar the polysaccharide component of the holdfast. As illustrated in Figure 4, holdfast material is not restricted only to the tip of the stalk, but eventually is distributed throughout the extracellular biofilm space. Lateral sectioning of the lectin-stained biofilm (Figure 4B) demonstrates that the holdfast material is present underneath, between, and above the biofilm-associated cells.

To assess if genes critical for holdfast formation are also required for the production of the biofilm matrix at a later stage of surface colonization, we used a strain, that allowed conditional expression of the *hgtA* gene (from a xylose-dependent promoter), which codes for a presumable glycosyltransferase involved in holdfast synthesis (100). The addition of xylose to the growth medium induced *hgtA* expression and restored holdfast production and surface attachment (Figure 5) in this test strain. When cells were allowed to pre-bind to the plastic surface in the presence of xylose for two hours, the accumulation of biomass on the surface during overnight incubation still required the presence of xylose in the medium (Figure 4). This indicated that although cells could initially adhere to the surface (in the presence of the inducer, xylose), they could not remain attached when xylose was removed from the media. This could suggest that the holdfast is not only required for initial attachment, but also for later stages of the biofilm development. An alternative explanation for this observation is that the new progeny of swarmer cells, which had been released from the attached predivisional cells upon cell division, could not re-initiate surface attachment in the absence of holdfast synthesis. In order to be able to distinguish between these two possibilities, a similar experiment should be conducted in a flow chamber in order to eliminate the “re-attaching” cells effect, simply by washing them away.

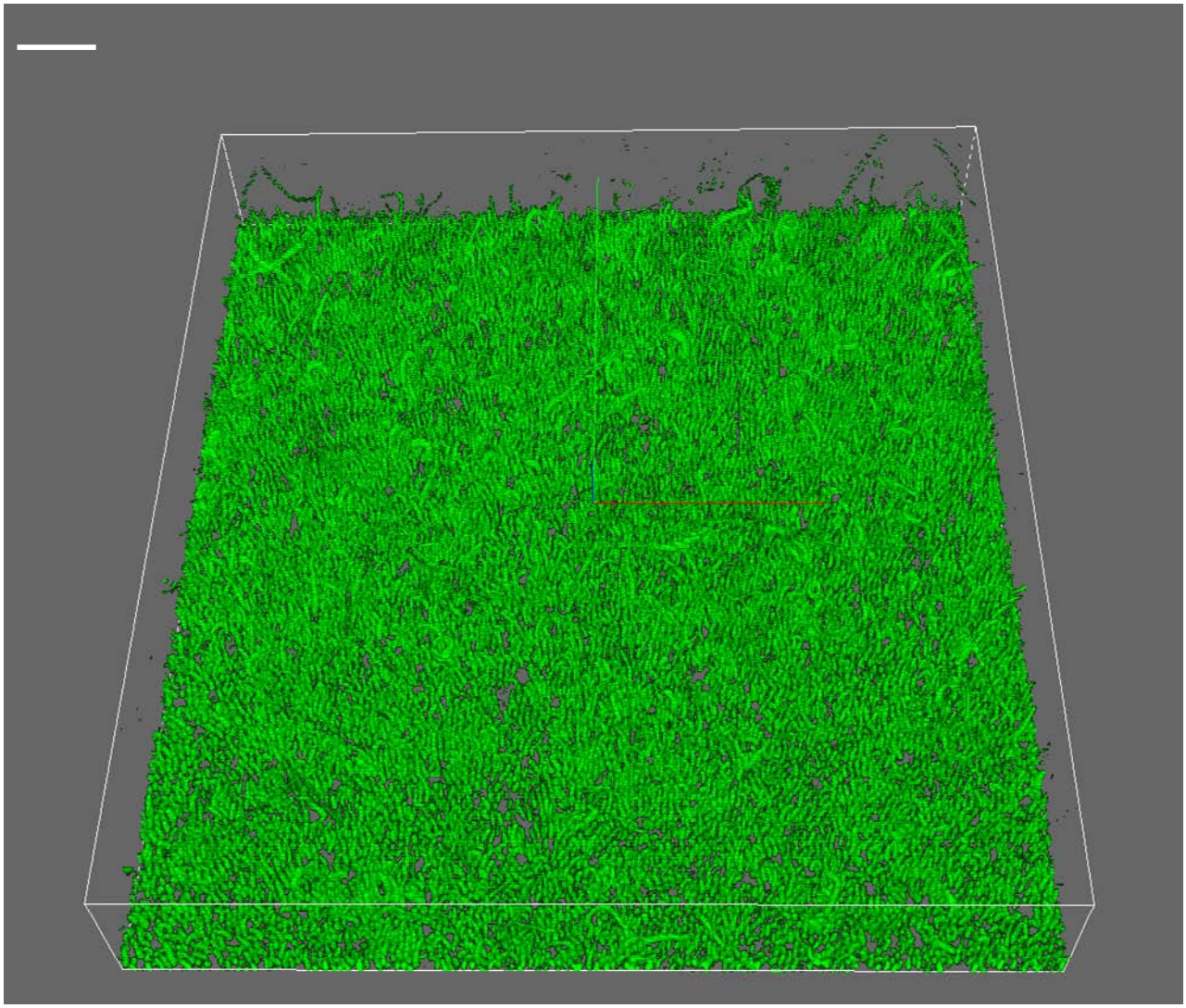

Figure 2) *C. crescentus* biofilm development analyzed by confocal laser scanning microscopy (CLSM). Biofilms of *C. crescentus* wild type were grown in a flow chamber for 24 hours before cells attached to the glass surface were analyzed by CLSM. Cells were attached in an up-right arrangement and reproducibly formed a typical high-density monolayer. At this stage of biofilm development there was little evidence of an extracellular matrix. White bar represent 20 μm.

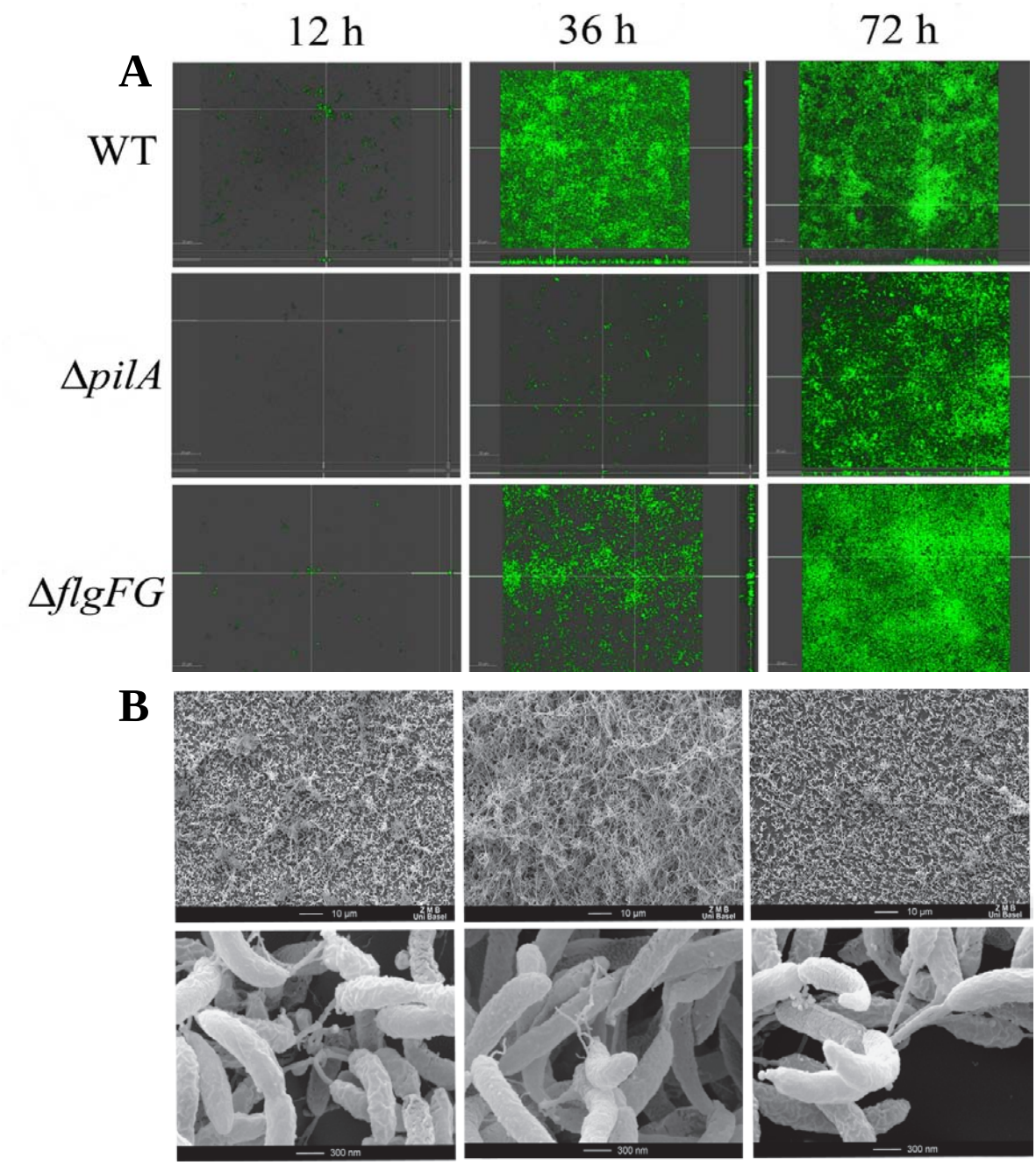

Figure 3) Biofilm formation of *C. crescentus* wild type and mutants lacking polar organelles. **A)** Biofilms were analyzed in a flow chamber using confocal laser scanning microscopy (CLSM). Images were taken 12, 36, and 72 hours after inoculation with GFP-tagged *C. crescentus* wild type strain CB15, a Δ*pilA* (pili mutant), and a Δ*flgFG* (flagellar) mutant, respectively (see experimental procedures). The bars represent 20 µm. Vertical sections through the biofilms collected at the positions indicated by the white cross are shown on the right and below the frames. **B)** Scanning electron microscopy (SEM) analysis of surface grown *C. crescentus* CB15 (left panels), Δ*pilA* (middle panels), and Δ*flgFG* (right panels) cultures 96 hours after inoculation. Biofilms were developed on a glass cover slide in

microtiter plates, fixed and analyzed microscopically. The bars represent 10 µm in the upper panels and 300 nm in the lower panels. The bottom panels represent a 50-fold magnification of the corresponding upper panels.

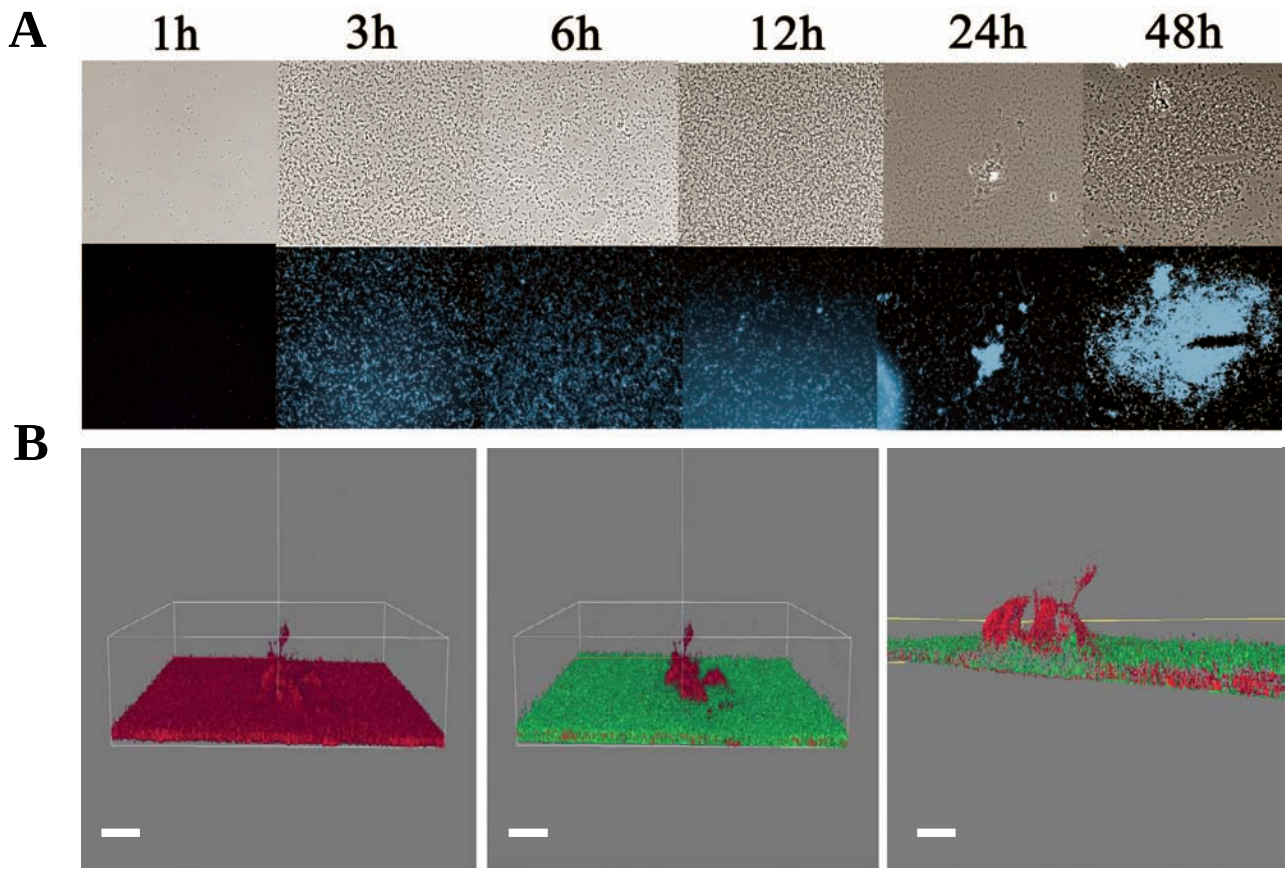

Figure 4) Polysaccharide staining during *C. crescentus* biofilm development. **A)** Polysaccharide build-up during biofilm maturation as visualized by calcofluor staining. Glass slides were incubated with *C. crescentus* wild-type cells in 12-well polystyrene microplates. At each indicated time point, a glass slide was removed from a well, rinsed in distilled water to remove unbound cells, and stained with calcofluor. Stained biofilms were analyzed by fluorescent microscopy. **B)** Polysaccharide staining of surface-associated *C. crescentus* wild-type cells. Biofilms were grown in flow chamber and stained with TRITC-WGA (see experimental procedures). Images were recorded with a two filter set system, which allows the simultaneous observation of the gfp-labeled cells (green channel) and the TRITC-WGA stained holdfast material (red channel). During the initial stages of biofilm formation, cell monolayers (green channel) are attached to the glass surface via adhesive holdfasts (red channel). Microcolonies formed at later stages of biofilm formation appear embedded in a red matrix, arguing that a holdfast-like exopolysaccharide is responsible for matrix formation. A lateral view of a cross-section through the biofilm (right panel) indicates that the holdfast material is present underneath and in between cells and seems to wrap around cell aggregates of the biofilm (left panel).

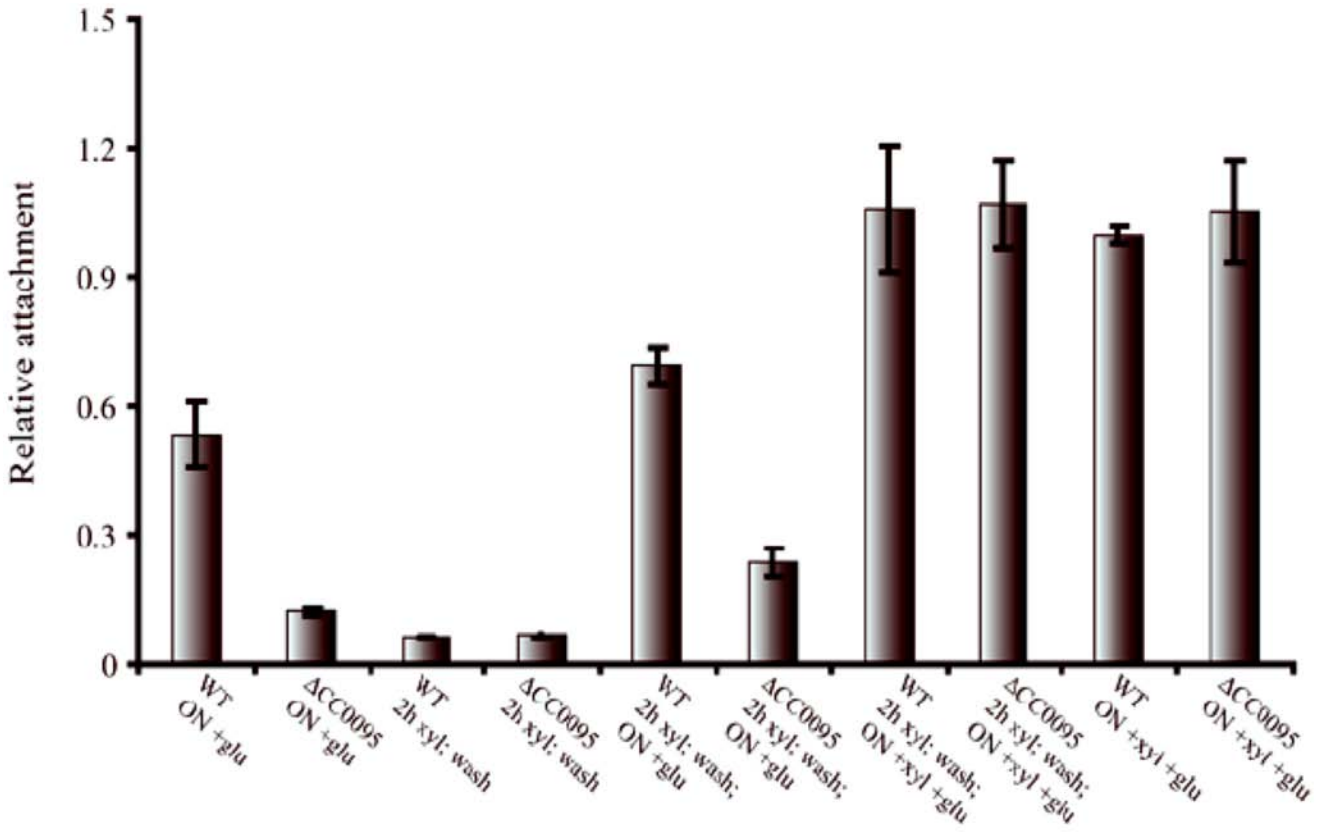

Figure 5) Evidence for a role of the holdfast EPS in biofilm maturation. A copy of the *hgtA* (holdfast glycosyltransferase) gene under the control of the P*xylX* promoter (108) was introduced into the chromosome of *C. crescentus* wild type and an *hgtA* deletion strain. Surface attachment of the resulting strains grown in the presence or absence of the inducer xylose, was assayed in microtiter plates as indicated in Experimental Procedures. Before quantification of surface binding, cells were grown overnight (ON) or for two hours (2h) in PYE complex medium supplemented with 0.1% glucose (+glu) and/or xylose (xyl). Pre-incubation of cells with xylose for 2 hours, followed by a washing step and a second overnight growth phase in the presence of fresh media supplemented with either glucose or with glucose and xylose, revealed a significant difference in surface colonization as a function of *hgtA* expression during the second growth/attachment phase.

Identification of *Caulobacter crescentus* genes specifically expressed during biofilm development using recombination-based *in vivo* expression technology (RIVET)

The RIVET system was designed for single-cell gene expression profiling experiments. It consists of two elements, a promoterless resolvase gene (*tnpR*), which can be fused transcriptionally or translationally to promoter regions of interest, and a *neo-sacB* cassette flanked by resolvase recognition sequences (RES) (Figure 6) (6, 127). The latter two genes confer resistance to kanamycin and sensitivity to high concentrations of sucrose and thus allow selection for and counter-selection against this genetic element. Thus, when the *sacB-neo* cassette is excised from the host DNA molecule by the action of TnpR, the resulting strain becomes sensitive to kanamycin and resistant to sucrose. Because the excision of the cassette requires the expression of *tnpR*, the system permits the isolation of promoters, which are in planktonic cells and are specifically induced during biofilm development. The use of three different *tnpR* reporter genes with different translational efficiencies and two different TnpR recognition sites (RES) with different resolution frequencies should in principle allow isolating promoters, which are active in planktonic cells, but up regulated in biofilms.

The following modifications were made to adapt the RIVET system for the use in *C. crescentus*: i) the ampicilin resistance marker was replaced by a chloramphenicol resistance gene (*cat*) due to *Caulobacter*'s natural β-lactamse activity. ii) The *lacZ* reporter upstream of *tnpR* was replaced by a copy of *gfp*, which was fused transcriptionally to *tnpR*. The *gfp* reporter should facilitate the analysis of temporal and topological aspects of gene expression in mature biofilm structures. iii) The *tnpR-gfp cat* cassette was inserted between the flanking inverted repeats of the *mariner* transposon delivery-vector (pLRS60) in order to improve the transposition frequency. We have found that the transposition frequency of the *mariner* element is considerably higher than Tn*5* (data not shown). v) The *neo-sacB* selection cassette with flanking resolvase recognition sequences (RES) was introduced

in the intragenic region downstream of two converging genes (CC0575 and CC0576) in order to be sure that the integration of the construct had no adverse effect on the host.

In order to test the resolution frequencies and efficiencies of the six different RIVET constructs (three different *tnpR* alleles combined with two RES sites), the three *tnpR-egfp-cat* cassettes were cloned behind the xylose-inducible promoter PxylX (108) and introduced into strains UJ3341 and UJ3342, which contain chromosomal copies the *neo-sacB* cassette with two different RES sites. Pre-cultures of the six resulting strains were diluted in PYE, containing increasing concentrations of xylose and were incubated for 3 hours at 30º C. Aliquots of each culture were harvested and scored on PYE agar plates containing sucrose and chloramphenicol. We had expected that the number of colonies obtained should correlate with the xylose concentration in the medium. However, such a correlation was only observed for the strains carrying the "wild-type" *tnpR* allele (Figure 7). Strains carrying the two *tnpR* mutant alleles mut168 and mut135, showed small number of colonies under all conditions (data not shown). Resolution of the *neo-sacB* cassette increased with increasing concentrations of xylose, although with a different dose response for strain UJ3341 and UJ3342 (Figure 7). The *neo-sacB* cassette resolved more readily in strain UJ3341, which harbors the more sensitive *res* cassette, than in the isogenic strain UJ3342, which contained the *res1* mutant cassette. The wild type RES sites were obviously used at a relative high frequency even in the absence of the inducer xylose (Figure 7), suggesting that the intact RES sites in strain UJ3341 are very sensitive for TnpR activity. The basal level activity of P*xylX* in the absence of xylose (108) could explain this result. Thus, the use of the wild type RES sites for RIVET analysis might be adequate only for the identification of extremely tight promoters, which are completely turned off under planktonic conditions. For this reason, we have chosen to work with strain UJ3342, which appeared to be less sensitive in our experiment.

In order to conduct a saturated genetic analysis with the RIVET system in *C. crescentus* there was a need for high numbers of biofilm cells, which allow a higher diversity of Tn insertions in the

initial biofilm inoculums. The *tnpR mariner* construct was introduced into strain UJ3342 by conjugation and the resulting chloramphenicol resistant mutants were pooled and used to inoculate silicon tubing, which were used to develop biofilms under dynamic flow conditions (see Experimental Procedures section for details). To avoid premature resolution, transformed cells were not plated on PYE nalidixic acid plates for counter selection, but were resuspended in liquid medium containing nalidixic acid, chloramphenicol, and kanamycin. Biofilms were allowed to develop on the surface of the silicon tubes for five days under constant flow of medium containing chloramphenicol, to avoid contamination of the biofilm during the prolonged incubation time. Cells were then scratched off the tube walls, resuspended in 10% glycerol, and frozen at -80º C. Due to the time constraints the rest of the analysis was not yet carried-out. This would include analyzing isolated clones for sucrose resistance and kanamycin sensitivity, and mapping of the transposon insertion site by direct genomic sequencing.

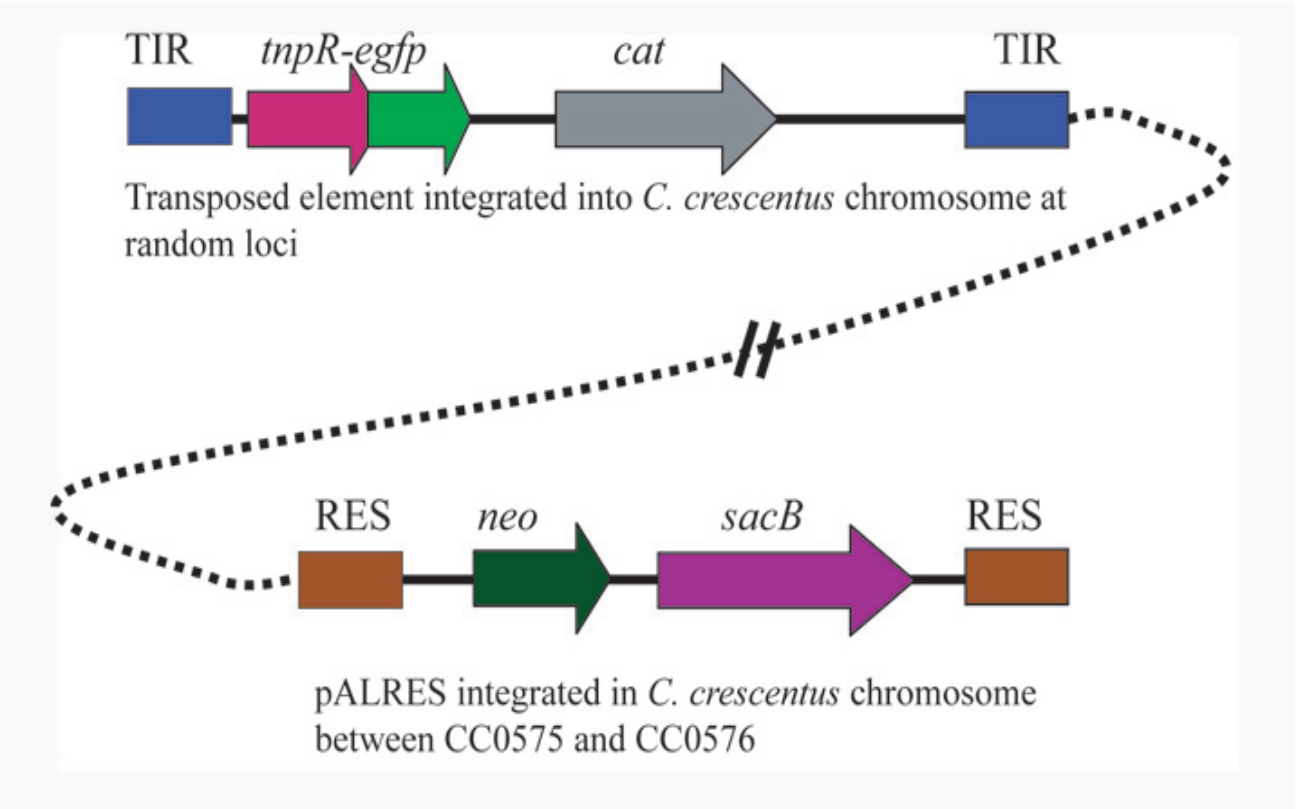

Figure 6) Schematic of the modified RIVET delivery system. Transposition of the *mariner* transposon carrying the *tnpR-gfp* cassette from the suicide plasmid pMRTNPR onto the *C. crescentus* chromosome randomly generates fusions of the promoterless resolvase gene (*tnpR*) with *C. crescentus* transcription units. Expression of *tnpR* allows splicing of the *neo-sacB* cassette by site-specific recombination at the resolvase recognition sites (RES). The two chromosomal regions are unlinked, as indicated by the dashed line. Genes are shown by filled arrows and are labeled (*tnpR*, resolvase; *egfp*, EGFP; *cat*, chloramphenicol acetyl transferase; *neo*, neomycin phosphotransferase; *sacB*, levansucrase). *Cis* acting elements are shown as filled rectangles (TIR, terminal inverted repeats; RES, resolvase recognition sequence).

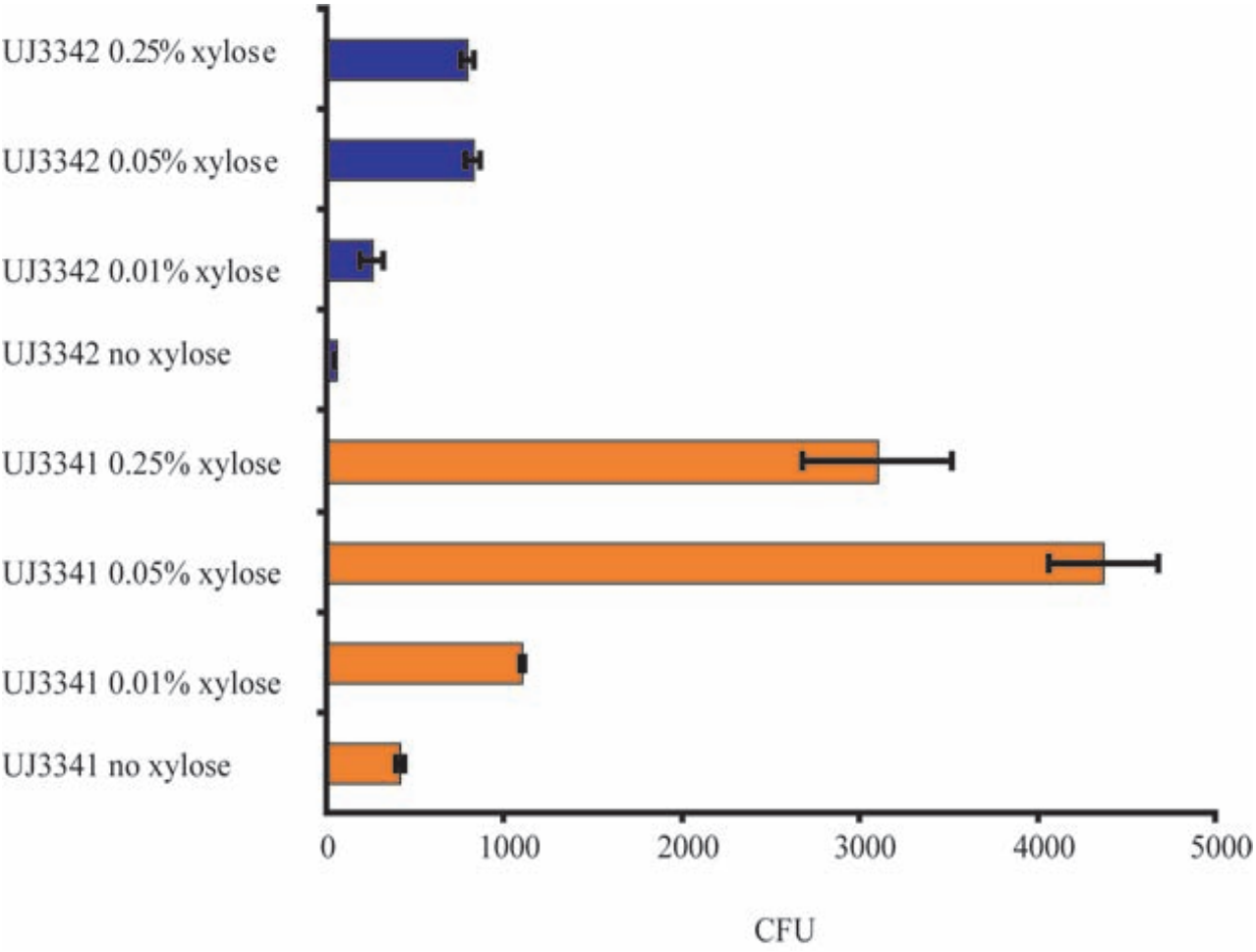

Figure 7) Controlled expression of *tnpR* leads to resolution of the reporter gene cassette. Colony forming units (CFUs) on PYE sucrose plates are indicated for each experiment. A P*xylX*-driven copy of *tnpR* was introduced into the reporter strains UJ3341 (RES) and UJ3342 (RES1). Resolution of the *neo-sacB* cassette was determined after growth of the tester strains in PYE complex medium containing increasing concentrations of xylose at 30º C for 3 hours. Cells were then scored by spotting dilutions of cells onto PYE sucrose plates. Xylose concentrations used are indicated on the left of each column. Orange column, strain UJ3341 (*res-neo-sacB-res* P*xylX*::*tnpR-egfp*); blue column, strain UJ3342 (*res*1-*neo-sacB-res*1 P*xylX*::*tnpR-egfp*).

EXPERIMENTAL PROCEDURES

Media and Strains

Strains and plasmids used in this study are listed in Table 1. *E. coli* DH10B and S17-1 were used as host strain for molecular cloning experiments and as donor strain for conjugational transfer of plasmids into *Caulobacter*. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth (154) supplemented with kanamycin (50 μg/ml) or tetracycline (12.5 μg/ml) when necessary. *C. crescentus* strains were grown at 30°C in either PYE complex medium (132) or in M2 minimal glucose medium (M2G) (79) supplemented with kanamycin (5 μg/ml), tetracycline (2.5 μg/ml), chloramphenicol (1 μg/ml) or nalidixic acid (20 μg/ml) when necessary. Swarmer plates for motility assays contained 0.3% agar (DIFCO®).

DNA manipulations

Plasmid and chromosomal DNA preparation, DNA ligation, electroporation, agarose gel electrophoresis, and PCR amplifications were carried out by using standard techniques (154). All PCR products used for cloning were amplified with"Expand high-fidelity PCR system" form Roche. Restriction enzymes were from New England Biolabs, Inc.

Construction of deletion mutants

The Δ*flgFG* chromosomal deletion mutation was constructed in *C. crescentus* wild type CB15 and UJ590 (Δ*pilA*) using pNPTS138 based constructs carrying in frame deletions in the respective genes. Plasmids (see below) were introduced into the recipient strains by conjugation and recombinants were selected on PYE plates supplemented with kanamycin and nalidixic acid. Resulting single colonies were then grown overnight in liquid PYE medium and plated on PYE containing 3% sucrose.

Sucrose-resistant colonies were screened by PCR for recombinants that had lost the respective genes on their chromosome.

Construction of plasmids for chromosomal deletions

Plasmid pAL8 was constructed for an in-frame deletion of the complete *flgFG* coding region. PCR amplification of a 1.0 kb region upstream of *flgFG* was performed with primers: 5'-GGA TCC GGC GTT CGA GCT GCT GCT GA-3' and 5'-GAA TTC TCA CCT GGC GGG TGA GTG AG-3'. PCR amplification the 1.0 kb region downstream of *flgFG* was done with primers: 5'-GAA TTC CGC TCG CCT AAG CGA ACG TC-3' and 5'-ACT AGT GGC CGA GAT CTT GCC GTC GA-3'. Ligation of both fragments into pNPTS138 (*Sph*I/*Spe*I) resulted in plasmid pAL8.

Construction of plasmids for RIVET analysis

pAL10 was created as follows: 680bp DNA fragment was amplified using primers: 5'-ACT AGT GGC AAA CGT CGC CTT TCG-3' and 5'-ACT AGT CTT CAT TCT CGG CGC GAC -3' , cut with *Spe*I restriction enzyme and cloned into the suicide plasmid pNPTS138 which was previously cleaved by *SpeI* as well.

pALRES *and* pALRES1 were created as follows: *res-neo-sacB-res* (*res1-neo-sacB-res1* in the case of pALRES1*)* cassette was cut from pRES or pRES1 (127) using *Nco*I and *Not*I restriction enzymes, polished with T4 polymerase and ligated into pAL10, which was previously cleaved by *Xho*I and *Hpa*I to remove its own *nptI-sacB* cassette (2.7 kb), and was polished with T4 DNA polymerase. The oriT, *lacZ*, colE1, and M13ori features are from pAL10 (pNPTS138 based plasmid) and the res-*neo-sacB-res* cassette is from pRES (or pRES1).

pMRTNPR was created as follows: pALMAR-1 was cleaved by *Xba*I restriction enzyme and ends were polished using T4 DNA polymerase and *tnpR-egfp-cat* cassette which was subcloned into pGEMT-easy was cut from pGEMT-easy using *Bst*BI and *Afl*III restriction enzymes, polished with T4 DNA polymerase and cloned between the two repeats sequences of the delivery vector. Egfp

source was pEGFP vector (Clontech®). Chloramphenicol resistance cassette was excised from pKPR10 (143) using *Xba*I restriction enzyme.

pBGSXYL was created as follows: 2.3kb long fragment containing the *xylX* promoter region was cleaved excised from pUJ83 using *Hind*III and *SpeI* restriction enzymes and cloned into pBGS18T in *Hind*III/*XbaI* sites.

Biofilm growth for RIVET analysis:

For increased yield of biofilm biomass, silicon tubes were used to cultivate biofilms. Each silicone tube was 100 cm in length and 0.3 cm in diameter. Cultures of UJ3342 transduced with *mariner* Tn (pMRTNPR) were pooled directly from the mating filters by vortexing and grown for 48 hours in glass tubes with PYE-kanamycin-nalidixic acid-chloramphenicol prior to the inoculation of silicone tubing by syringe injection. 5 milliliters of these cultures were injected into the tubing and allowed to attach for 3 hours before the flow of PYE-chloramphenicol (0.5 ml/min) was initiated. The residence time in the tubing was around 15 min, considerably shorter period than the doubling time of *C. crescentus*, allowing only attached cells to remain in the tubing. After 5 days, attached cells were squeezed out, collected in polystyrene tube, resuspended with 10% glycerol and deep-frozen (-80º C) until usage.

Microscopy techniques

Cell morphology, motility, and rosette formation were analyzed by light microscope using a Nikon Eclipse 6000 with a planApo 100x phase contrast objective or an Olympus AX70 with an UplanApo 100x phase contrast objective. Pictures were taken with a charge-coupled device camera (Hamamatsu®) connected to the Olympus microscope and analyzed with Open-lab (Improvision®) software.

Attachment assay with microscopy cover-slides

Sterile cover-slides (Ø 18 mm) were placed in 12 well polystyrene microtiter plates (Falcon®) and 1 ml of culture (OD_{600} ~ 0.05) was added. The plates were incubated at 30º C with shaking (100 RPM) for different periods. Cover-slides were dipped in sterile deionized H_2O to remove unattached cells and when needed, a drop of DAPI (10 µg/ml) or Calcofluor (0.002%) was added to the upper side of the cover-slide. Cells were incubated for 15 minutes in the dark, washed again with deionized H_2O and analyzed microscopically.

Microtiter plate attachment assay

Attached cells were quantified as described (124) with a few modifications. Stationary phase cultures were diluted with fresh PYE (plus supplements where mentioned) into 96, 24 or 12 well polystyrene microtiter plates (at final volumes of 0.2, 1.0, and 1.0 ml, respectively) to an OD_{600} of 0.05 and incubated at 30ºC on a shaker (200 rpm) until cultures reached an OD_{600} between 0.9-1.2. Cells were discarded and the wells were washed twice under a gentle stream of deionized H_2O to remove unattached cells. Plates were air dried, a culture volume of 0.1% crystal violet (CV) was added and incubated with shaking for 15 min. Wells were washed again several times with deionized H_2O and CV was dissolved in an equal volume of an ethanol:acetone solution (80:20). The color intensity was measured with a spectrophotometer at 600 nm.

Flow chamber experiments

Biofilms were grown at 30º C in three-channel flow chambers, with channel dimensions of 1 by 4 by 40 mm. The flow system was assembled and prepared as described (20). A flow velocity of 5 ml/hour was applied. Each channel was supplied with PYE medium containing 5 µg/ml kanamycin**.** Each channel was inoculated with an overnight culture of *C. crescentus* wild type or mutant strains containing plasmid pAL9 diluted 100-fold in PYE. Inoculation of the channels was done by injection

of the diluted culture with a small syringe. Cells were allowed to settle for 60 minutes before the flow was initiated using a 205S peristaltic pump (Watson Marlow).

Staining of biofilms in flow chamber

The biofilms inside the flow chamber were stained with wheat germ agglutinin-conjugated tetramethylrhodamine isothiocyanate (TRITC-WGA, Sigma). TRITC-WGA binds specifically the holdfast; stained holdfast were visualized by CLSM. 50 µl of TRITC-WGA (0.5 mg/ml) was injected into the flow chamber cell after the flow was stopped. After 15 minutes incubation at 30° C in the dark, the flow was reinstated and the unbound stain was washed out.

Image acquisition and processing

All 3-dimensional image acquisitions were done with a Zeiss LSM 510 CLSM (Carl Zeiss, Jena, Germany) equipped with detectors and filter sets for GFP fluorescence. Images were obtained using a 63x/1.4 objective or a 40x/1.3 objective. Processing of 3-D images and sections was done with the IMARIS® software package (Bitplane AG, Zürich, Switzerland).

Scanning electron microscopy

C. crescentus cultures (PYE) were incubated overnight on borosilicate slides at 30^{o} C with shaking (100 rpm). The resulting biofilms were fixed with 2% glutaraldehyde for 30 minutes, washed twice with PBS and twice with water for 20 minutes. Cells were then dehydrated with 30%, 50%, 70% and 90% acetone, each for 20 minutes, then twice with 100% acetone for 20 minutes and critical point dried and coated with gold palladium. Samples were examined with a XL30 FEG ESEM scanning electron microscope, Philips Electron Optics (The Netherlands).

Table 1) Strains and plasmids used in this work.

Strain or plasmid	Description	Source/reference
***E. coli* strains**		
DH10B	F⁻*mcrA* Δ(*mrr*⁻ *hsd* RMS⁻*mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX*74 *endA1 recA1 deoR* Δ(*ara, leu*)7697 *araD139 galU galK nupG rpsL*	GIBCO BRL
UJ606	MT607 containing pRK600	(160)
***C. crescentus* strains**		
CB15	*Caulobacter* wild type	(132)
NA1000	synchronizable mutant of CB15 lacking holdfast	(45)
UJ590	CB15 *ΔpilA*	M. Ackermann
UJ2430	CB15 *ΔflgH*	This work
UJ2440	CB15 *ΔflgFG*	This work
UJ2441	CB15 *ΔpilA ΔflgFG*	This work
UJ2982	CB15 ΔCC0095	(100)
UJ3341	CB15 with *res-neo-sacB-res* genomic insert between CC0575 and CC0576	This work
UJ3342	CB15 with *res*1-*neo-sacB-res*1 genomic insert between CC0575 and CC0576	This work
Plasmids		
pAL2	pNPTS138 with *Spe*I/*Eco*RI 2kb fragment designed to in-frame deletion of *flgH* coding region	This work
pAL8	pNPTS138 with *Sph*I/*Spe*I 2.2kb fragment designed to in-frame deletion of *flgFG* coding region	This work
pAL9	pBBRMCS2 with EGFP	This work
pAL10	pNPTS138 with *C.crescentus* 0.68 kb long intragenic region (between CC0575 and CC0576)	This work
pHRXLT95	pHRXLT + CC0095 (*Eco*RI)	Chapter 2
pBBRMCS-2	Medium copy number vector in *Caulobacter crescentus.*	(88)
pNPTS138	Kan^R pLitmus38 derived vector with *oriT* and *sacB*	Dickon Alley
pBGSXYL	pBGS18T with PxylX region	This work
pRES	RES resolution sites bordering Kan-SacB cassette. Source elements for pRES. *oriR6K mobRP4* Ap^r	(127)
pRES1	RES1 resolution sites bordering Kan-SacB cassette. Source elements for pRES1. *oriR6K mobRP4* Ap^r	(127)
pGOA1193	*oriR6K mobRP4 lacZ* Ap_r *tnpR*	(127)
pGOA1194	*oriR6K mobRP4 lacZ* $tnpR^{mut168}$ Ap_r	(127)
pGOA1195	*oriR6K mobRP4 lacZ* $tnpR^{mut135}$*Ap*.	(127)
pALRES	pRES and pNPTS138 derived plasmid which contains *re1-neo-sacB-res* cassette from pRES and 500 bp of	This work

pALRES	pRES and pNPTS138 derived plasmid which contains *re1-neo-sacB-res* cassette from pRES and 500 bp of homologous sequence to *C.crescentus* chromosomal region between CC0575 and CC0576	This work
pALRES1	pRES1 and pNPTS138 derived plasmid which contains *res1-neo-sacB-res1* cassette from pRES1 and 500 bp of homologous sequence to *C.crescentus* chromosomal region between CC0575 and CC0576	This work
pAL29	*tnpR*-GFP translational fusion with chloramphenicol resistance cassette inserted in pALMAR-1. Facilitate the delivering of TnpRI-GFP-CM fusion randomly into *Caulobacter* genome. The *tnpR* originated from pGOA1193	This work
pAL30	$tnpR^{mut168}$-GFP translational fusion with chloramphenicol resistance cassette inserted in pALMAR-1. Facilitate the delivering of TnpRImut1-GFP-CM fusion randomly into *Caulobacter* genome. The $tnpR^{mut168}$ originated from pGOA1194	This work
pAL31	$tnpR^{mut135}$ translational fusion with chloramphenicol resistance cassette inserted in pALMAR-1. Facilitate the delivering of TnpRImut2-GFP-CM fusion randomly into *Caulobacter* genome. The $tnpR^{mut135}$ originated from pGOA1195.	This work
pALMAR-1	pLRS60 based Tn *mariner* delivery vector	Modified from Henri Saenz and Ralf Schülein

*See plasmid map in Addendum.

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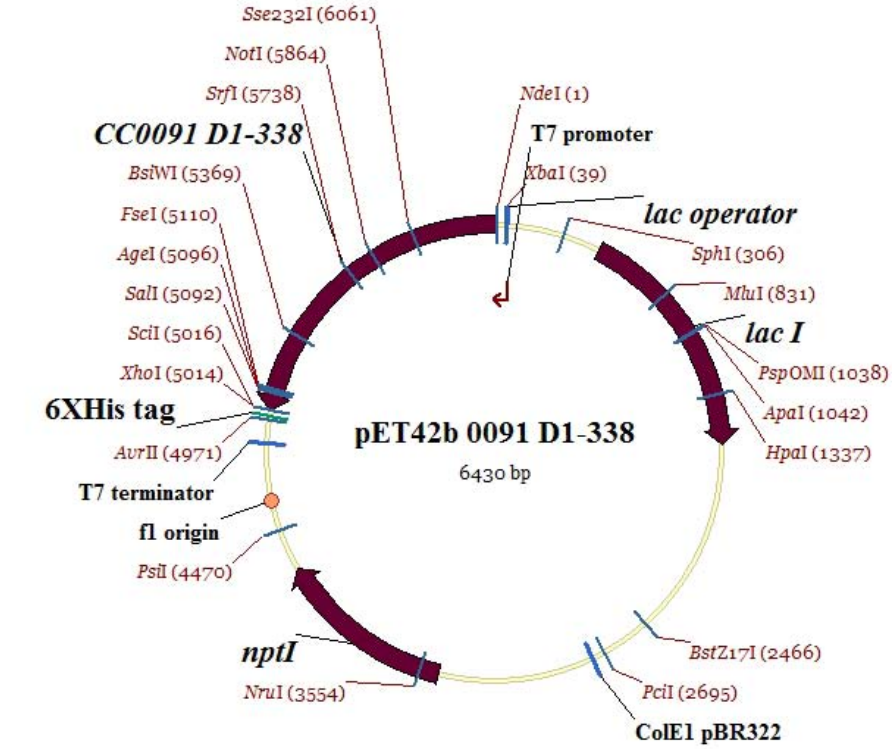
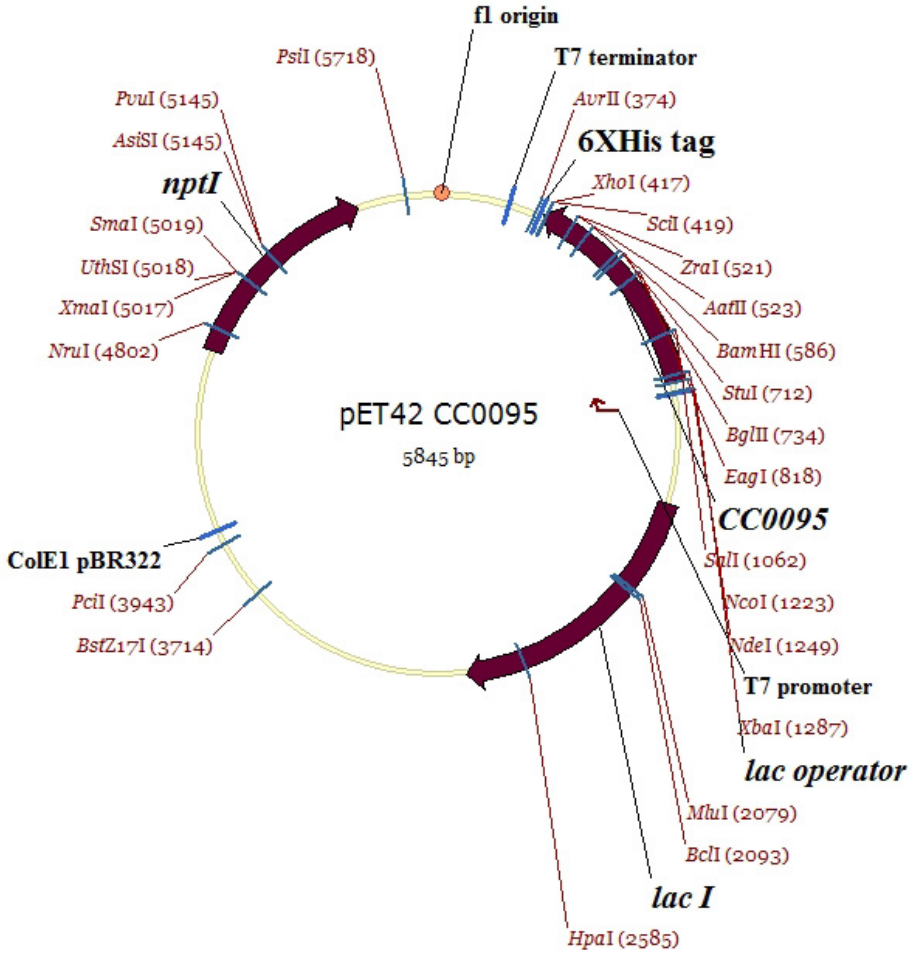
ADDENDUM

Addendum

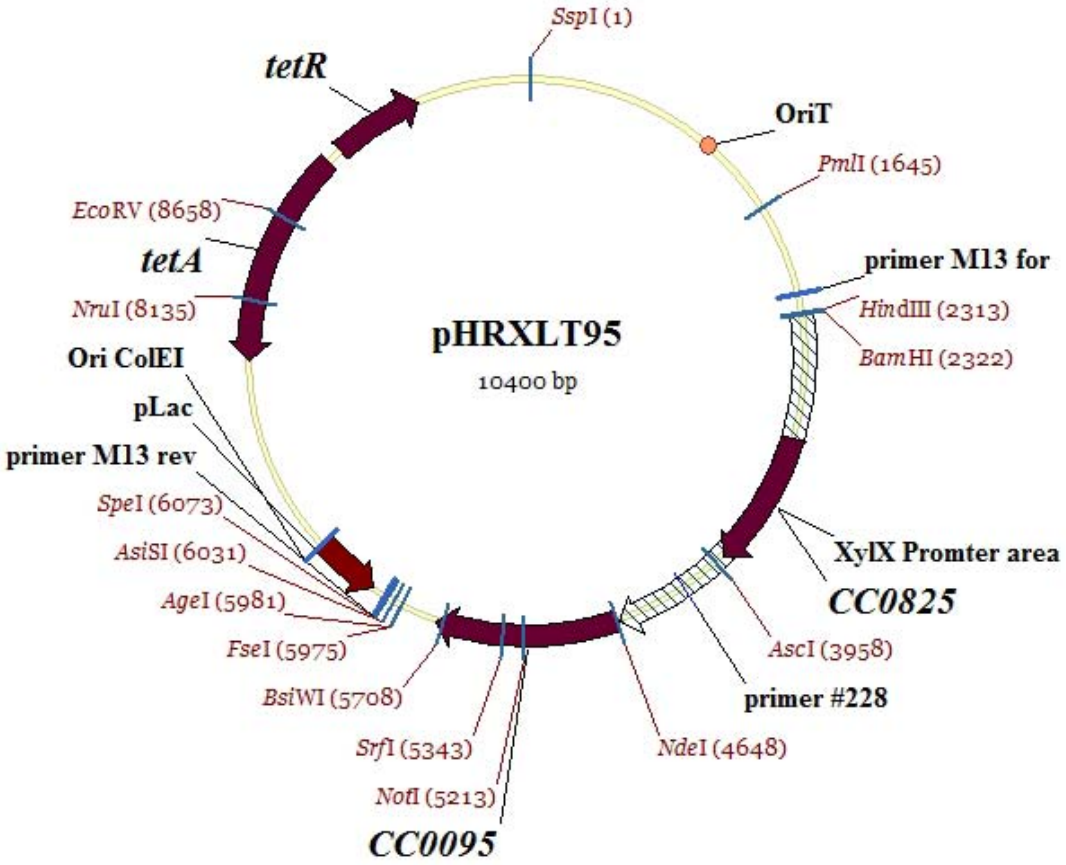
Plasmid maps of selected constructs



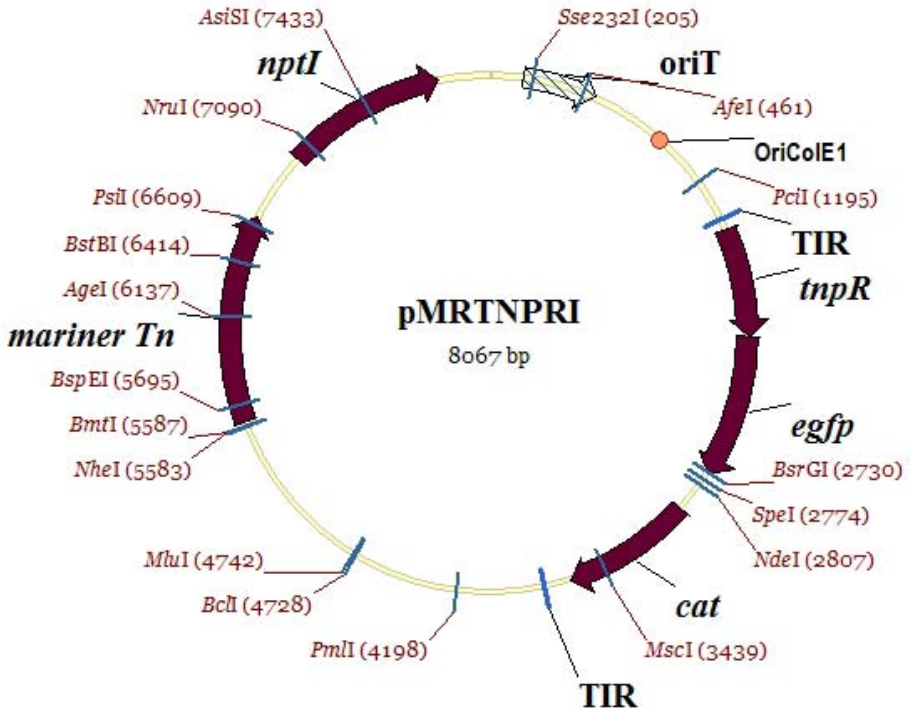
pET42b 0091 D1-338 (pDM18) was used to purify the truncated HfrA (Δaa1-338) protein utilizing the c-terminal 6XHis tag fusion.



pET42 CC0095 (pDM24) was used to purify CC0095 based on the C-terminal 6XHis tag fusion.



pHRXLT95 was used to induce the expression of CC0095 by xylose. pHRXLT95 is a suicide plasmid which introduced to *C. crescentus* chromosome based on 2.3kb homology region up stream of *PxylX*



pMRTNPRI was used as a delivery vector for the resolvase-Gfp-chloramphenicol resistance cassette. Insertion of this cassette to C. *crescentus* genome occurred by the activity of the *mariner* Tn.

A complete list of strains used in the PhD work

Srain		Genotype		Source	Plasmid
UJ	730	*C. crescentus*	CB15 (LS1250) ΔpleD	Assaf Levi	none
UJ	1807	*C. crescentus*	Δ(cheYVII-cheRIII)che103	Dickon Alley	none
UJ	1891	*E. coli*	S17-1	Steve Atkinson	mini Tn5 LuxCDABE
UJ	1892	*E. coli*	S17-1	Steve Atkinson	mini Tn5 LuxCDABE
UJ	2265	*E. coli*	DH5a	Andrew Camilli	pIVET5n (pAC1193)
UJ	2266	*E. coli*	DH5a	Andrew Camilli	pIVET5nMut135 (pAC1194)
UJ	2267	*E. coli*	DH5a	Andrew Camilli	pIVET5nMut168 (pAC1193)
UJ	2622	*E. coli*	DH10B	Henri Saenz	pALMAR1
UJ	2623	*E. coli*	DH10B	Assaf Levi	pALMAR2
UJ	2624	*E. coli*	DH10B	Assaf Levi	pALMAR3
UJ	2625	*E. coli*	DH10B	Assaf Levi	pALMAR4
UJ	2845	*E. coli*	BTH101: F- cya-99, araD139, galE15, galK16, rpsL1 (Strr), hsdR2, mcrA1, mcrB1.	Daniel Ladant	none
UJ	2846	*E. coli*	DHM1: F-, cya-854, recA1, endA1, gyrA96 (Nalr), thi1, hsdR17, spoT1, rfbD1, glnV44(AS).	Daniel Ladant	none
UJ	2858	*E. coli*	DH10B	Daniel Ladant	pUT18
UJ	2858	*E. coli*	DH10B	Daniel Ladant	pUT18
UJ	2859	*E. coli*	DH10B	Daniel Ladant	pUT18C
UJ	2860	*E. coli*	DH10B	Daniel Ladant	pUT18C-zip
UJ	2861	*E. coli*	DH10B	Daniel Ladant	pKT25
UJ	2862	*E. coli*	DH10B	Daniel Ladant	pKT25-zip
UJ	2867	*E. coli*	BTH101: F- cya-99, araD139, galE15, galK16, rpsL1 (Strr), hsdR2, mcrA1, mcrB1.	Daniel Ladant	pUT18C-zip +pKT25-zip
UJ	2868	*E. coli*	DHM1: F-, cya-854, recA1, endA1, gyrA96 (Nalr), thi1, hsdR17, spoT1, rfbD1, glnV44(AS).	Daniel Ladant	pUT18C-zip +pKT25-zip
UJ	3163	*C. crescentus*	Caulobacter crescentus ATCC 19089. Caulobacter Vibrioides. Genome sequencing strain	American Type Culture collection (ATCC)	none
UJ	3182	*E. coli*	DH10B	Assaf Levi	pAL17
UJ	3183	*C. crescentus*	LS1250 CC0091 OE	Assaf Levi	pAL17
UJ	3184	*C. crescentus*	LS1250 ΔpleD CC0095 Overexpression	Assaf Levi	pDM13
UJ	3185	*C. crescentus*	LS1250 ΔCC0091 CC0095 Overexpression	Assaf Levi	pDM13
UJ	3193	*C. crescentus*	CB15 WT (LS1250) pleD*	Assaf Levi	pPA114-47
UJ	3194	*C. crescentus*	CB15 WT (LS1250) pleD* CC0091 Overexpression	Assaf Levi	pPA114-47 + pAL17
UJ	3195	*C. crescentus*	CB15 WT (LS1250) pleD* ΔCC0091	Assaf Levi	pPA114-47
UJ	3241	*C. crescentus*	WT	Assaf Levi	none

UJ	3242	*C. crescentus*	ΔpilA	M. Ackermann	none
UJ	3243	*C. crescentus*	NA1000	Assaf Levi	none
UJ	3244	*E. Coli*			pRK600
UJ	3245	*E. Coli*	S-17	Steve Atkinson	miniTn5 LuxCDABE
UJ	3246	*E. Coli*	S-17	Steve Atkinson	miniTn5 LuxCDABE
UJ	3247	*E. Coli*	DH10B	Dickon Alley	miniTn5
UJ	3248	*E. Coli*	DH10B	Dickon Alley	miniTn5
UJ	3249	*C. crescentus*	CB15 ΔfliFG	Assaf Levi	none
UJ	3250	*C. crescentus*	CB15 ΔpilA; ΔfliFG	Assaf Levi	none
UJ	3251	*C. crescentus*	NA1000 ΔrsaA	Assaf Levi	none
UJ	3252	*C. crescentus*	NA1000 ΔpilA	Assaf Levi	none
UJ	3253	*C. crescentus*	NA1000 ΔfliFG	Assaf Levi	none
UJ	3254	*E. Coli*	DH10B	Assaf Levi	pAL1
UJ	3255	*C. crescentus*	CB15 ΔrsaA	Assaf Levi	none
UJ	3256	*E. Coli*	DH10B	Assaf Levi	pAL2
UJ	3257	*C. crescentus*	CB15 ΔflgH	Assaf Levi	none
UJ	3258	*E. Coli*	DH10B	Assaf Levi	pAL3
UJ	3259	*C. crescentus*	CB15 ΔfljK	Assaf Levi	none
UJ	3260	*E. Coli*	DH10B	Michael Kovach	pBBR1MCS-2
UJ	3261	*E. Coli*	DH10B	B. Grünenfelder	pBG22
UJ	3262	*C. crescentus*	NA1000 ΔflgH	Assaf Levi	none
UJ	3263	*C. crescentus*	NA1000 ΔpilA; ΔflgH	Assaf Levi	none
UJ	3264	*C. crescentus*	NA1000 ΔrsaA; ΔflgH	Assaf Levi	none
UJ	3265	*C. crescentus*	CB15 ΔpilA; ΔflgH	Assaf Levi	none
UJ	3266	*C. crescentus*	CB15 ΔpilA; ΔrsaA	Assaf Levi	none
UJ	3267	*C. crescentus*	NA1000 ΔpilA; ΔrsaA	Assaf Levi	none
UJ	3268	*C. crescentus*	CB15 ΔfliFG; ΔrsaA	Assaf Levi	none
UJ	3269	*C. crescentus*	CB15 ΔpilA; ΔfliFG; ΔrsaA	Assaf Levi	none
UJ	3270	*E. Coli*	DH10B	M.Ackermann	pNPTSRXH2
UJ	3271	*E. Coli*	DH10B	B. Grünenfelder	pBG60
UJ	3272	*C. crescentus*	CB15	Assaf Levi	pAL4
UJ	3273	*C. crescentus*	CB15	Assaf Levi	pAL5
UJ	3274	*E. Coli*	DH10B	Assaf Levi	pAL6
UJ	3275	*E. Coli*	DH10B	Assaf Levi	pAL4
UJ	3276	*E. Coli*	DH10B	Assaf Levi	pAL5
UJ	3277	*C. crescentus*	CB15 ΔrsaA; ΔflgH	Assaf Levi	none
UJ	3278	*C. crescentus*	CB15 ΔpilA; ΔfljK	Assaf Levi	none
UJ	3279	*C. crescentus*	CB15 ΔrsaA; ΔfljK	Assaf Levi	none
UJ	3280	*C. crescentus*	CB15 ΔpilA; ΔrsaA; ΔfljK	Assaf Levi	none
UJ	3281	*E. Coli*	DH10B	Assaf Levi	pAL7
UJ	3282	*E. Coli*	DH10B	Assaf Levi	pAL8
UJ	3283	*C. crescentus*	CB15 ΔflgDE	Assaf Levi	none
UJ	3284	*C. crescentus*	CB15 ΔpilA; ΔflgDE	Assaf Levi	none
UJ	3285	*C. crescentus*	CB15 ΔrsaA; ΔflgDE	Assaf Levi	none
UJ	3286	*C. crescentus*	CB15 ΔflgH; ΔflgDE	Assaf Levi	none
UJ	3287	*C. crescentus*	CB15 ΔflgFG	Assaf Levi	none
UJ	3288	*C. crescentus*	CB15 ΔpilA; ΔfliFG	Assaf Levi	none
UJ	3289	*C. crescentus*	CB15 ΔrsaA; ΔflgFG	Assaf Levi	none
UJ	3290	*C. crescentus*	CB15 ΔflgH; ΔflgFG	Assaf Levi	none
UJ	3291	*C. crescentus*	CB15 ΔfliL	Assaf Levi	none
UJ	3292	*C. crescentus*	CB15 ΔpilA; ΔfliL	Assaf Levi	none
UJ	3293	*C. crescentus*	CB15 ΔrsaA; ΔfliL	Assaf Levi	none

UJ	3294	*C. crescentus*	CB15 ΔpilA; ΔrsaA; ΔfliL	Assaf Levi	none
UJ	3295	*C. crescentus*	CB15 PleD*	Assaf Levi	pPA114-32
UJ	3296	*C. crescentus*	NA1000 ΔpilA; ΔrsaA; ΔflgH	Assaf Levi	none
UJ	3297	*C. crescentus*	CB15 Ca5 -Kac	John Smit	
UJ	3298	*C. crescentus*	EGfp	Assaf Levi	pAL9
UJ	3299	*C. crescentus*	NA1000 ΔflgDE	Assaf Levi	none
UJ	3300	*C. crescentus*	NA1000 ΔflgFG	Assaf Levi	none
UJ	3301	*C. crescentus*	CB15 ΔfliL; ΔfljK	Assaf Levi	none
UJ	3302	*C. crescentus*	CB15 ΔhfsB	Assaf Levi	none
UJ	3303	*C. crescentus*	NA1000 CC2077::Tn5	Assaf Levi	none
UJ	3304	*C. crescentus*	NA1000 CC3618:: Tn5 (manC)	Assaf Levi	none
UJ	3305	*C. crescentus*	NA1000 CC3146:: Tn5	Assaf Levi	none
UJ	3306	*C. crescentus*	NA1000 CC2718:: Tn5	Assaf Levi	none
UJ	3307	*E. Coli*	DH10B	Assaf Levi	pAL9
UJ	3308	*E. Coli*	DH10B	Assaf Levi	pAL11
UJ	3309	*C. crescentus*	CB15 ΔflgDE; ΔflgH	Assaf Levi	none
UJ	3310	*C. crescentus*	CC0744:: Tn5 (single domain response regulator)	Assaf Levi	none
UJ	3311	*C. crescentus*	CC2277:: Tn5 (glycosyltransferase, homolog to ExoM from Sinorhizobium).	Assaf Levi	none
UJ	3312	*C. crescentus*	CC2264:: Tn5 (phosphoglucomutase/phosphomann omutase family protein).	Assaf Levi	none
UJ	3313	*C. crescentus*	CC3618:: Tn5 (manC)	Assaf Levi	none
UJ	3314	*C. crescentus*	NA1000 EGFP	Assaf Levi	pAL9
UJ	3315	*C. crescentus*	CB15 ΔpilA EGFP	Assaf Levi	pAL9
UJ	3316	*C. crescentus*	CB15 ΔflgFG EGFP	Assaf Levi	pAL9
UJ	3317	*C. crescentus*	CB15 ΔrsaA EGFP	Assaf Levi	pAL9
UJ	3318	*E. Coli*	DH10B	Andrew Camilli	pIVET5n
UJ	3319	*E. Coli*	DH10B	Andrew Camilli	pIVET5n mut1
UJ	3320	*E. Coli*	DH10B	Andrew Camilli	pIVET5n mut2
UJ	3321	*E. Coli*	DH10B	Andrew Camilli	pRES
UJ	3322	*E. Coli*	DH10B	Andrew Camilli	pRES1
UJ	3323	*C. crescentus*	CB15 ΔpilA ΔflgFG GFP	Assaf Levi	pAL9
UJ	3324	*E. Coli*	DH10B	Assaf Levi	pAL10
UJ	3325	*C. crescentus*	CB15 ΔpilA ΔrsaA ΔflgFG	Assaf Levi	none
UJ	3326	*C. crescentus*	CB15 LS1250 ΔflgFG	Assaf Levi	none
UJ	3327	*E. Coli*	DH10B	Assaf Levi	pAL11
UJ	3328	*E. Coli*	DH10B	Assaf Levi	pAL12
UJ	3329	*C. crescentus*	CB15 CC2277 OE	Assaf Levi	pAL11
UJ	3330	*C. crescentus*	CB15 with hfsA driven lacZ	Assaf Levi	pAL12
UJ	3331	*C. crescentus*	CB15 ΔflgH with hfsA driven lacZ	Assaf Levi	pAL12
UJ	3332	*C. crescentus*	CB15 ΔflgH OE with hfsA driven lacZ	Assaf Levi	pAL12
UJ	3333	*C. crescentus*	CB15 CC2277 OE with hfsA driven lacZ	Assaf Levi	pAL12
UJ	3334	*C. crescentus*	CB15 CC3037::Tn5 with hfsA driven lacZ	Assaf Levi	pAL12
UJ	3335	*C. crescentus*	CB15 ΔCC0091-0096 first recombinant	Assaf Levi	pAL13
UJ	3336	*C. crescentus*	CB15 ΔCC0091-0096	Assaf Levi	none
UJ	3337		DH10B	Assaf Levi	pAL13
UJ	3338	*C. crescentus*	GFP-CC2277 C-terminal fusion	Assaf Levi	pAL14
UJ	3339	*E. Coli*	DH10B	Assaf Levi	pALRES
UJ	3340	*E. Coli*	DH10B	Assaf Levi	pALRES1

UJ	3341	*C. crescentus*	CB15 CC0575_pALRES_CC0576	Assaf Levi	none
UJ	3342	*C. crescentus*	CB15 CC0575_pALRES1_CC0576	Assaf Levi	none
UJ	3343	*E. coli*	DH10B	Assaf Levi	pAL15
UJ	3344	*E. coli*	DH10B	Assaf Levi	pAL16
UJ	3345	*C. crescentus*	motA:: Tn5 overexpression	Assaf Levi	none
UJ	3346	*E. coli DH5a*	pUC based plasmid with Chloramphenicol resistance	Gregory Philips	pKRP10
UJ	3347	*E. coli DH5a*	pUC based plasmid with Kanamycin resistance	Gregory Philips	pKRP11
UJ	3348	*E. coli DH5a*	pUC based plasmid with Tetracycline resistance	Gregory Philips	pKRP12
UJ	3349	*E. coli DH5a*	pUC based plasmid with Spec/Strep resistance	Gregory Philips	pKRP13
UJ	3350	*E. coli*	DH10B	Assaf Levi	pAL17
UJ	3351	*E. coli*	DH10B	Assaf Levi	pAL18
UJ	3352	*CB15*	CC0091 overexpression	Assaf Levi	pAL17
UJ	3353	*CB15*	CC0857 overexpression	Assaf Levi	pAL18
UJ	3354	*CB15*	fljK-gfp promoter fusion	Assaf Levi	pAL16
UJ	3355	*E. coli*	DH10B	Assaf Levi	pAL19
UJ	3356	*C. crescentus*	CB15 ΔCC2378-CC2385	Assaf Levi	none
UJ	3357	*E. coli*	DH10B	Assaf Levi	pAL20
UJ	3358	*C. crescentus*	CB15 WT CC0744 overexpression	Assaf Levi	pAL20
UJ	3359	*C. crescentus*	CB15 WT with pBBRMCS2	Assaf Levi	pBBRMCS2
UJ	3360	*E. coli*	DH10B	Assaf Levi	pAL21
UJ	3361	*E. coli*	DH10B	Assaf Levi	pAL22
UJ	3362	*E. coli*	DH10B	Assaf Levi	pAL23
UJ	3363	*E. coli*	DH10B	Assaf Levi	pAL24
UJ	3364	*E. coli*	DH10B	Assaf Levi	pALMAR_1
UJ	3365	*E. coli*	DH10B	Assaf Levi	pALMAR_2
UJ	3366	*E. coli*	DH10B	Assaf Levi	pALMAR_3
UJ	3367	*E. coli*	DH10B	Assaf Levi	pALMAR_4
UJ	3368	*E. coli*	DH10B	Assaf Levi	pAL25
UJ	3369	*C. crescentus*	CB15 WT	Assaf Levi	pAL25
UJ	3370	*E. coli*	DH10B	Assaf Levi	pAL26
UJ	3371	*E. coli*	DH10B	Assaf Levi	pAL27
UJ	3372	*E. coli*	DH10B	Assaf Levi	pAL28
UJ	3373	*C. crescentus*	CB15 CC0091-GFP overexpression	Assaf Levi	pAL26
UJ	3374	*C. crescentus*	CB15 CC0744-GFP overexpression	Assaf Levi	pAL27
UJ	3375	*C. crescentus*	CB15 CC0857-GFP overexpression	Assaf Levi	pAL28
UJ	3376	*C. crescentus*	CB15 CC2277-GFP overexpression	Assaf Levi	pAL29
UJ	3377	*C. crescentus*	CB15 ΔCC2277	Assaf Levi	none
UJ	3378	*E. coli*	DH10B	Assaf Levi	pAL29
UJ	3379	*E. coli*	DH10B	Assaf Levi	pAL30
UJ	3380	*E. coli*	DH10B	Assaf Levi	pAL31
UJ	3381	*E. coli*	DH10B	Assaf Levi	pAL32
UJ	3382	*E. coli*	DH10B	Assaf Levi	pAL33
UJ	3383	*E. coli*	DH10B	Assaf Levi	pAL34
UJ	3384	*C. crescentus*	CB15 CC0744 D51E overexpression	Assaf Levi	pAL33
UJ	3385	*C. crescentus*	CC1064::Tn5	Assaf Levi	none
UJ	3386	*C. crescentus*	CC1064::Tn5 non-motility suppressor	Assaf Levi	none
UJ	3387	*E. coli*	DH10B	Assaf Levi	pAL35
UJ	3388	*C. crescentus*	CB15 WT Pxyl::CC0744 transcriptional fusion	Assaf Levi	pAL35
UJ	3389	*E. coli*	DH10B	Assaf Levi	pAL36

UJ	3390	*C. crescentus*	CB15 WT Pxyl::CC2277 transcriptional fusion	Assaf Levi	pAL36
UJ	3391	*C. crescentus*	CB15 WT pleD*	Assaf Levi	pPA114-47
UJ	3392	*C. crescentus*	CB15 ΔCC0091-0096	Assaf Levi	pBBR2
UJ	3393	*C. crescentus*	CB15 ΔCC0091-0096 CC0091 overexpression	Assaf Levi	pAL17
UJ	3394	*C. crescentus*	CB15 ΔCC0091-0096 CC2277 overexpression	Assaf Levi	pAL11
UJ	3395	*C. crescentus*	CB15 ΔCC0091-0096 pleD*	Assaf Levi	pPA114-32
UJ	3396	*E. coli*	DH10B	Assaf Levi	pAL37
UJ	3397	*C. crescentus*	CC1064:: Tn5 non-motility suppressor pleD*	Assaf Levi	pPA114-32
UJ	3398	*C. crescentus*	CC1064::Tn5 pleD-GFP C-terminal	Assaf Levi	pPA53-4
UJ	3399	*C. crescentus*	CB15 ΔCC2277 with CC2277-YFP C-terminal fusion	Assaf Levi	pAL38
UJ	3400	*C. crescentus*	CB15 with CC0744-YFP C-terminal fusion	Assaf Levi	pAL39
UJ	3401	*C. crescentus*	CB15 ΔCC0091-0096 Pxyl::CC2277	Assaf Levi	pAL36
UJ	3402	*C. crescentus*	CB15 ΔCC2277 Pxyl::CC2277	Assaf Levi	pAL36
UJ	3403	*C. crescentus*	CB15 ΔhfsB	Assaf Levi	pAL11
UJ	3404	*C. crescentus*	CB15 CC0744 deletion 1st recombinant	Assaf Levi	pAL32 (genomic)
UJ	3405	*E. coli*	DH10B	Assaf Levi	pAL40
UJ	3406	*E. coli*	DH10B	Assaf Levi	pAL41
UJ	3407	*C. crescentus*	CB15	Assaf Levi	pAL40
UJ	3408	*C. crescentus*	CB15 WT CC0744D51N 1st recombinant	Assaf Levi	pAL41 (genomic)
UJ	3409	*C. crescentus*	Ccr CB15 CC0744 D51N	Assaf Levi	none
UJ	3410	*C. crescentus*	CB15 Pxyl::CC0744D51N	Assaf Levi	none
UJ	3411	*C. crescentus*	Ccr CB15 CC0744 D51E	Assaf Levi	none
UJ	3412	*C. crescentus*	CB15 Pxyl::CC0744D51E	Assaf Levi	none
UJ	3413	*E. coli*	DH10B	Assaf Levi	pHRXLT-0744D51E
UJ	3414	*E. coli*	DH10B	Assaf Levi	pHRXLT-0744D51N
UJ	3415	*E. coli*	DH10B	Assaf Levi	pHRXLT-0744
UJ	3416	*E. coli*	DHMI (Bacterial Two Hybrid System strain, recA-)	Assaf Levi	pKT25CC0744+ pUT18
UJ	3417	*C. crescentus*	CB15 CC0744	Assaf Levi	none
UJ	3418	*C. crescentus*	CB15 CC3037AAA/DDD	Assaf Levi	pAL42
UJ	3419	*E. coli*	DH10B	Assaf Levi	pAL42
UJ	3420	*E. coli*	DH10B	Assaf Levi	pAL43
UJ	3421	*E. coli*	DH10B	Assaf Levi	pAL44
UJ	3422	*C. crescentus*	CB15 ΔpleC	Assaf Levi	
UJ	3423	*E. coli*	DH10B	Assaf Levi	pAL45
UJ	3424	*E. coli*	DH10B	Assaf Levi	pAL46
UJ	3425	*E. coli*	DH10B	Assaf Levi	pAL47
UJ	3426	*E. coli*	DH10B	Assaf Levi	pHRXLT2277
UJ	3427	*C. crescentus*	CB15 WT CC0095 overexpression	Assaf Levi	pDM13
UJ	3428	*C. crescentus*	CB15 ΔpilA CC0095 overexpression	Assaf Levi	pDM13
UJ	3429	*C. crescentus*	CB15 ΔflgF GCC0095 overexpression	Assaf Levi	pDM13
UJ	3430	*C. crescentus*	CB15 ΔflgH CC0095 overexpression	Assaf Levi	pDM13
UJ	3431	*C. crescentus*	CB15 ΔfliL CC0095 overexpression	Assaf Levi	pDM13
UJ	3432	*C. crescentus*	CB15 ΔpilA ΔflgFG CC0095	Assaf Levi	pDM13

			overexpression		
UJ	3433	*C. crescentus*	CB15 ΔCC2277 CC0095 overexpression	Assaf Levi	pDM13
UJ	3434	*C. crescentus*	NA1000 CC0095 overexpression	Assaf Levi	pDM13
UJ	3435	*C. crescentus*	CB15 ΔhfsB CC0095 overexpression	Assaf Levi	pDM13
UJ	3436	*E. coli*	DH10B	Assaf Levi	pDM13
UJ	3437	*C. crescentus*	CB15 WT (LS1250, synchronizable) CC0095 overexpression	Assaf Levi	pDM13
UJ	3438	*C. crescentus*	CB15 WT (LS1250, synchronizable) ΔCC0091	Assaf Levi	pBBR2
UJ	3439	*C. crescentus*	CB15 WT (LS1250, synchronizable) CC0091 overexpression	Assaf Levi	pAL17
UJ	3440	*C. crescentus*	CB15 WT (LS1250) synchronizable; holdfast bearing; non-introducible strain.	Assaf Levi	none
UJ	3441	*C. crescentus*	CB15 WT (LS1250) synchronizable; holdfast bearing; non-introducible strain.	Assaf Levi	pBBR2
UJ	3442	*C. crescentus*	CB15 ΔCC0744 CC0744-no stop-codon YFP	Assaf Levi	pAL45
UJ	3443	*C. crescentus*	CB15 DCC0744 CC0744D51E- no stop-codon YFP	Assaf Levi	pAL46
UJ	3444	*C. crescentus*	CB15 DCC0744 CC0744D51N- no stop codon-YFP	Assaf Levi	pAL47
UJ	3445	*C. crescentus*	CB15 WT	Assaf Levi	pHRXLT2277 (genomic)
UJ	3446	*C. crescentus*	CB15 ΔCC2277	Assaf Levi	pHRXLT2277 (genomic)
UJ	3447	*C. crescentus*	CB15 CC0091 overexpression	Assaf Levi	pHRXLT2277 (genomic)
UJ	3448	*C. crescentus*	CB15 ΔCC0091-6	Assaf Levi	pHRXLT2277 (genomic)
UJ	3449	*C. crescentus*	CB15 CC0091 overexpression pleD*	Assaf Levi	pPA114-32 +pAL17
UJ	3450	*C. crescentus*	CB15 CC0095 overexpression pleD*	Assaf Levi	pPA114-32 +pDM13
UJ	3451	*C. crescentus*	CB15 (LS1250) ΔpleD	Assaf Levi	none
UJ	3452	*C. crescentus*	CB15 CC3037AAA/DDD	Assaf Levi	none
UJ	3453	*E. coli*	DH10B	Assaf Levi	pAL48
UJ	3454	*C. crescentus*	CB15 WT	Assaf Levi	pAL48 (in the genome)
UJ	3455	*C. crescentus*	CB15 DCC0744	Assaf Levi	pAL48 (in the genome)
UJ	3456	*E. coli*	DH10B	Assaf Levi	pDM25
UJ	3457	*C. crescentus*	CB15 DCC0095	Assaf Levi	none
UJ	3458	*C. crescentus*	CB15 pleD* ΔCC0095	Assaf Levi	pPA114-47
UJ	3459	*C. crescentus*	CB15 ΔpleC DCC0095	Assaf Levi	none
UJ	3460	*C. crescentus*	CB15 ΔpleC	Assaf Levi	pBBR2
UJ	3461	*C. crescentus*	CB15 ΔpleC CC0091 overexpression	Assaf Levi	pAL17
UJ	3462	*C. crescentus*	CB15 ΔpleC CC0095 overexpression	Assaf Levi	pDM13
UJ	3463	*C. crescentus*	CB15 ΔpleD (LS1250) (UJ730)	Assaf Levi	pBBR2
UJ	3464	*C. crescentus*	CB15 ΔpleD CC0091 overexpression	Assaf Levi	pAL17
UJ	3465	*C. crescentus*	CB15 ΔpleD CC0095 overexpression	Assaf Levi	pDM13
UJ	3466	*C. crescentus*	CB15 pleD*	Assaf Levi	pBBR2+ pPA114-47
UJ	3467	*C. crescentus*	CB15 ΔCC0091 pleD* pBBR2	Assaf Levi	pBBR2+ pPA114-47
UJ	3468	*C. crescentus*	CB15 ΔCC0091 pleD* CC0095	Assaf Levi	pDM13+ pPA114-

			overexpression		47
UJ	3469	*C. crescentus*	CB15 ΔCC0091pBBR2	Assaf Levi	pBBR2
UJ	3470	*C. crescentus*	CB15 ΔCC0091 CC0095 overexpression	Assaf Levi	pDM13
UJ	3471	*C. crescentus*	CB15 WT with Pxyl::CC0095	Assaf Levi	pHRXLT95 and pBBR2
UJ	3472	*C. crescentus*	CB15 ΔCC0091 with Pxyl::CC0095	Assaf Levi	pHRXLT95 and pBBR2
UJ	3473	*C. crescentus*	CB15 ΔCC0091-0096 with Pxyl::CC0095	Assaf Levi	pHRXLT95 and pBBR2
UJ	3474	*C. crescentus*	CB15 CC0091 overexpression with Pxyl::CC0095	Assaf Levi	pHRXLT95 and pAL17
UJ	3475	*C. crescentus*	NA1000 with Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3476	*C. crescentus*	CB15 hfaB::pNPTS138 Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3477	*C. crescentus*	CB15 hfaC::pNPTS138 Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3478	*C. crescentus*	CB15 hfaD::pNPTS138 Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3479	*C. crescentus*	CB15 ΔhfsA with Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3480	*C. crescentus*	CB15 ΔhfsB with Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3481	*C. crescentus*	CB15 ΔhfsC with Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3482	*C. crescentus*	CB15 ΔhfsC with Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3483	*C. crescentus*	CB15 ΔhfsA with CC0095 overexpression	Assaf Levi	pDM13
UJ	3484	*C. crescentus*	CB15 ΔhfsC with CC0095 overexpression	Assaf Levi	pDM13
UJ	3485	*C. crescentus*	CB15 ΔhfsD with CC0095 overexpression	Assaf Levi	pDM13
UJ	3486	*C. crescentus*	CB15 CC3036 overexpression	Assaf Levi	pAL49
UJ	3487	*C. crescentus*	CB15 CC3037 overexpression	Assaf Levi	pAL50
UJ	3488	*C. crescentus*	CB15 CC3037-8 overexpression	Assaf Levi	pAL51
UJ	3489	*E. coli*	DH10B	Assaf Levi	pAL49
UJ	3490	*E. coli*	DH10B	Assaf Levi	pAL50
UJ	3491	*E. coli*	DH10B	Assaf Levi	pAL51
UJ	3492	*E. coli*	DH10B	Assaf Levi	pAL52
UJ	3493	*E. coli*	DH10B	Assaf Levi	pAL53
UJ	3494	*C. crescentus*	CB15 hfaA::pNPTS138 Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3495	*C. crescentus*	CB15 ΔCC0091-0096 PleD-Gfp	Assaf Levi	pPA53-4
UJ	3496	*E. coli*	DH10B	Assaf Levi	pDM1
UJ	3497	*C. crescentus*	CB15 ΔCC0091-0096 pleD*	Assaf Levi	pSW7
UJ	3498	*E. coli*	DH10B	Assaf Levi	pHRXLT95
UJ	3499	*C. crescentus*	CB15 WT with CC3036::pNPTS138 insertion	Assaf Levi	pAL52
UJ	3500	*C. crescentus*	CB15 ΔCC0095 CC0091 overexpression	Assaf Levi	pAL17
UJ	3501	*C. crescentus*	CB15 ΔCC0095	Assaf Levi	pBBR2
UJ	3502	*C. crescentus*	CB15 CC3396	Assaf Levi	pAL53
UJ	3503	*C. crescentus*	LS1250 ΔCC0091 ΔCC0095	Assaf Levi	none
UJ	3504	*C. crescentus*	CB15 ΔCC0095 with pBBR2 harboring CC0095(aaΔ1-41)	Assaf Levi	pAL54

UJ	3505	*E. coli*	DH10B	Assaf Levi	pAL55
UJ	3506	*E. coli*	DH10B	Assaf Levi	pAL54
UJ	3507	*E. coli*	BL21 Rosetta™	Novagen	pAL55
UJ	3508	*E. coli*	DH10B	Assaf Levi	pAL56
UJ	3509	*E. coli*	DH10B	Assaf Levi	pAL57
UJ	3510	*E. coli*	DH10B	Assaf Levi	pAL58
UJ	3511	*E. coli*	DH10B	Assaf Levi	pAL59
UJ	3512	*E. coli*	DH10B	Assaf Levi	pAL60
UJ	3513	*C. crescentus*	CB15 ΔCC0095 Pxyl::CC0095	Assaf Levi	pHRXLT95
UJ	3514	*C. crescentus*	LS1250 WT pleD*	Assaf Levi	pPA114-47
UJ	3515	*C. crescentus*	LS1250 WT pleD* CC0091 overexpression	Assaf Levi	pPA114-47 + pAL17

The complete Tn*5* insertion library (surface adherent deficient strains)

Tn*5* insertion site*	Cell morphology**	Motility†	Holdfast‡
CC0095	normal	yes	no
CC0321-CC0321	normal	yes	yes++
CC0322, *exbD*	normal	low motility	yes+
CC0662, *iscc2*	elongated	yes	yes
CC0744	bit elongated	low motility	yes
CC0750, *motA*	normal	no	yes
CC0750, *motA*	normal	no	yes
CC0807	low conc.	no	nd
CC0808	normal	yes	yes
CC0899, *flaN*	bit elongated	no	yes
CC0902, *flgE*	normal	no	yes
CC0902, *flgE*	bit elongated	no	yes
CC0905, *fliF*	spiral elongated	no	yes
CC0906, *fliG*	spiral elongated	no	nd
CC0906, *fliG*	elongated	no	yes
CC0910, *flhA*	spiral elongated	no	yes
CC0934	spiral-elongated	no	nd
CC0951, *fliP*	elongated	no	yes
CC0952, *fliO*	spiral-elongated	no	no
CC1004	bit elongated	yes	yes
CC1007, *rsaA*	normal	yes	yes++
CC1064	normal	no	yes
CC1077, *flhB*	spiral elongated	no	yes
CC1077, *flhB*	elongated	no	yes
CC1459, *flaF*	bit elongated	no	yes
CC1465, *flaEY*	spiral-elongated	no	yes
CC1465, *flaEY*	normal	no	yes
CC2045, *podJ*	normal	low motility	no
CC2045, *podJ*	normal	no	no
CC2045, *podJ*	normal	low motility	no
CC2045, *podJ*	normal	low motility	no
CC2045, *podJ*	normal	low motility	no
CC2058	bit elongated	no	yes+
CC2059	elongated-spiral	no	no
CC2059	normal	yes	yes
CC2059	elongated	yes	yes
CC2061, *fliM*	bit elongated	No motility	yes
CC2063, *flgF*	normal	No motility	yes+++
CC2066, *flgH*	normal	no	yes
CC2089,	normal	yes	yes
CC2264	normal	low motility	yes+++
CC2277	normal	yes	no
CC2432	bit elongated	yes	no
CC2462, *pleD*	normal	yes	yes
CC2468, *clpA*	spiral-elongated	yes	no
CC2482, *pleC*	elongated	no	no
CC2482, *pleC*	bit elongated	no	no
CC2482, *pleC*	normal	no	no

CC2482, *pleC*	bit elongated	no	no
CC2630, *hfaD*	normal	yes	few
CC2630, *hfaD*	normal	yes	few
CC2639,	normal	yes	yes
CC2758, *htrA*	normal	yes	yes
CC2759-CC2758	bit elongated	yes	yes
CC2941	normal	low motility	yes
CC2950	normal	yes	yes
CC2958, *pilA*	normal	yes	yes
CC2958, *pilA*	bit elongated	yes	yes
CC2958, *pilA*	normal	yes	yes
CC3037	bit elongated	yes	yes
CC3274	normal	yes	yes+++
CC3376	normal	yes	yes
CC3439	normal	yes	yes
CC3618, *manC*	sick (low conc.)	low motility	yes
CC3618, *manC*	sick (low conc.)	low motility	yes
CC3715	normal	yes	yes
NS^{+}	normal	yes	yes
NS	bit elongated	low motility	nd
NS	normal	yes	yes
NS	normal	no	yes
NS	?	yes	?
NS	elongated	yes	yes
NS	normal	yes	yes
NS	normal	low motility	yes+++
NS	normal	yes	yes
NS	normal	no	yes
NS	normal	yes	yes+++
NS	spiral elongated	yes	no
NS	normal	yes	yes
NS	normal	low motility	yes+++
NS	elongated	low motility	no
NS	normal	yes	yes
NS	normal	yes	yes
NS	normal	yes	no
NS	bit elongated	yes	yes
NS	normal	yes	yes
NS	normal	yes	yes
NS	elongated	yes	nd
NS	elongated	low motility	yes
NS	normal	yes	no
NS	bit elongated	low motility	yes
NS	elongated	yes	yes
NS	normal	yes	yes
NS	elongated	yes	yes+++
NS	normal	yes	yes
NS	elongated	yes	yes
NS	normal	yes	yes

*Insertion site was determined by genomic sequencing.

** Cell morphology was determined visually by light microscopy of overnight, stationary phase cultures that were grown in PYE medium supplemented with kanamycin.

† Cell motility was determined by swarm circumference on semi sold PYE agar plates, which were incubated at 30º C for 5 days.

‡ Holdfast was determined by rosettes formation or by lectin binding assay with FITC-conjugated WGA.

[+]NS Not yet sequenced

Complete list of plasmids used in the PhD work

Plasmid	Created for
pAL1	Knockout of *rsaA*. pNPTS138 with *BamHI/SpeI* 2kb fragment designed to in-frame deletion of rsaA coding region
pAL10	pNPTS138 with CB15 genomic fragment from neutral location (between CC0575 and 0576). This fragment allows site directed homologous recombination.
pAL11	CC2277 on pBBRMCS2
pAL12	*hfsA* promoter fused to lacZ in order to quantify and compare *hfsA-D* operon activity in different strains.
pAL13	Knocking-out CC0091-0096
pAL14	EGFP C-terminally fused to CC2277
pAL15	Multi-copy plasmid without lac promoter
pAL16	Multi-copy plasmid without lac promoter with *fljK* promoter area C-terminally fused to EGFP.
pAL17	CC0091 overexpression
pAL18	CC0857 overexpression
pAL19	Knockout CC2378-CC2385, loci suspected to be involved in polysaccharide synthesis
pAL2	Knockout *flgH*. pNPTS138 with *SpeI/EcoRI* 2kb fragment designed to in-frame deletion of *flgH*
pAL20	CC0744 overexpression, a single domain response regulator
pAL21	Knockout of CC2277, a glycosyltransferase that is required for holdfast synthesis
pAL22	TnpRI_RsaA-RBS_EGFP_CM
pAL23	TnpRIMut1_RsaA-RBS_EGFP_CM
pAL24	TnpRIMut2_RsaA-RBS_EGFP_CM
pAL25	CC1162 overexpression, a LasI homolog (aminotransferase)
pAL26	CC0091-EGFP N-terminal fusion
pAL27	CC0744-EGFP N-terminal fusion
pAL28	CC0857-EGFP N-terminal fusion
pAL29	TnpRI_RBS_EGFP_CM construct in pALMAR1. Tool for delivering the TnpRI-GFP-CM in high efficiency into *Caulobacter* or another organism.
pAL3	Knockout of *fljK*. pNPTS138 with BamHI/SpeI 2kb fragment designed to in-frame deletion of fljK
pAL30	TnpRI1_RBS_EGFP_CM construct in pALMAR1. Tool for delivering the TnpRI-GFP-CM in high efficiency into *Caulobacter* or another organism.
pAL31	TnpRI2_RBS_EGFP_CM construct in pALMAR1. Tool for delivering the TnpRI-GFP-CM in high efficiency into *Caulobacter* or another organism.
pAL32	Knockout CC0744, a single domain response regulator
pAL33	pAL33 overexpressing CC0744 D51E allele
pAL34	pNPTS138 suicide plasmid with CC0744 D51E
pAL35	CC0744 overexpression
pAL36	CC2277 (a glycosyltransferase essential for holdfast synthesis) under xylose promoter
pAL37	Knockout CC0756, a glycosyltransferase 1 family protein. The location of this ORF close to *motA* (CC0750) and CC0744, CC0740 and *fixL* (CC0758), makes it interesting gene for the connection between motility, chemotaxis and attachment.
pAL38	CC2277-YFP C-terminal fusion on a low copy number plasmid. Created to study the localization of CC2277 (a glycosyltransferase which is essential for holdfast synthesis).
pAL39	CC0744-YFP C-terminal fusion on a low copy number plasmid. Created to study the localization of CC0744 (a single domain response regulator)
pAL4	*flgH* overexpression. 0.85 kb BamHI/SpeI PCR product containing the entire flgH coding region in pBBR1MCS2
pAL40	Overexpression of CC0744 D51N allele
pAL41	pNPTS138 suicide plasmid carrying the CC0744 D51N allele

pAL42	CC3037 on pBBRMCS2. CC3037 is a Cro/CI family transcriptional regulator which is probably involved in EPS regulation in *Caulobacter* (Tn*5* insertion in this gene, caused an aggregative phenotype)
pAL43	pNPTS138 suicide plasmid which carries CC3037AAA/DDD CC3037 is a Cro/CI family transcriptional regulator which is probably involved in EPS regulation in *Caulobacter* (Tn*5* insertion in this gene, caused an aggregative phenotype). CC3037 protein ends with 3 alanin residues (AAA), modifying these residues to DDD will presumably will stabilize the protein and produce a phenotype.
pAL44	Overexpression of CC3037AAA/DDD allele
pAL45	CC0744-YFP C-terminal fusion
pAL46	CC0744D51E-YFP C-terminal fusion
pAL47	CC0744D51N-YFP C-terminal fusion
pAL48	CC0744 is a single response regulator (CheY-like). CC0744 RR domain exhibit high homology to the RR domain of CckA. This construct switch the RR domain of CC0744 with the CckA one. According to the literature, CckA is essential in *caulobacter*, probably due to its requirement in phosphorylating CtrA. The question that this hybrid allele can answer is whether CckA can phosphrylate CC0744? Moreover, could CC0744 phosphrylate CtrA?
pAL49	CC3036 on pBBRMCS-5. CC3036 codes for LytR like DNA binding Response regulator, which might be involved in biofilm formation. Tn*5* insertion in CC3037 caused an aggregative phenotype, high attachment and increased staining with calcofluor.
pAL5	*fljK* overexpression. 0.92 kb BamHI/SpeI PCR product containing the entire *fljK* coding region in pBBR1MCS2
pAL50	Over expression of CC3037, a lambda like transcriptional regulator. A Tn*5* insertion in that ORF caused a severe aggregation phenotype and high surface attachment in *Caulobacter* CB15.
pAL51	CC3037-8 overexpression. CC3038 is fused to CC3037 (frame shift). Tn insertion in CC3037 caused an aggregative phenotype, high attachment and increased staining with calcofluor.
pAL52	CC3036 overexpression
pAL53	Overexpression of CC3396, a PDE, which is responsible for most of the phosphodiesterase activity of *Caulobacter* cell extract.
pAL54	CC0095Δaa1-41. Cell attachment of ΔCC0095 strain carrying this plasmid was slightly restored (~30% of WT).
pAL55	CC0095Δaa1-41 in *E. Coli* BL21 cells, created for 6XHis tag purification of this protein
pAL56	CC0468 overexpression. CC0468 is 4-amino-4-deoxy-L-arabinose transferase and related glycosyltransferases of PMT family [Cell envelope biogenesis, outer membrane].
pAL57	CC0469 overexpression. CC0469 is glycosyl transferase family protein involved in cell wall biogenesis [Cell envelope biogenesis, outer membrane].
pAL58	CC2889 overexpression. CC2889 is glycosyl transferase family protein involved in cell wall biogenesis.
pAL59	CC2425 overexpression. CC2425, HfsE, encodes a glycosyl transferase (undecaprenyl-phosphate galactosephosphotransferase) which probably catalyzes the first reaction of holdfast biosynthesis.
pAL6	Knockout of *fliL*. pNPTS138 with SpeI/SphI 1.8kb fragment designed to in-frame deletion of *fliL* coding region
pAL60	CC0095-GFP C-terminal fusion overexpression
pAL7	Knockout of *flgDE*. pNPTS138 with SphI/SpeI 2.1kb fragment designed to in-frame deletion of *flgDE* coding region
pAL8	Knockout of *flgFG*. pNPTS138 with SphI/SpeI 2.2kb fragment designed to in-frame deletion of *flgFG* coding region
pAL9	pBBRMCS2 contains HindIII/EcoRI EGFP coding region from pEGFP vector (BD Biosciences Clontech) Catalog #6077-1.
pALMAR1	Tn mariner delivery vector with kanamycin resistance cassette. This transposon delivery plasmid is non replicative in *Caulobacter*. The transposition frequency is around 25000 per mating or more. Can be used for random mutagenesis.
pALMAR2	Tn mariner delivery vector with chloramphenicol resistance cassette . This transposon delivery plasmid is non replicative in *Caulobacter*. The transposition frequency is around 25000 per mating or more. Can be used for random mutagenesis.

pALMAR3	Tn mariner delivery vector with tetracycline resistance cassette. This transposon delivery plasmid is non replicative in *Caulobacter*. The transposition frequency is around 25000 per mating or more. Can be used for random mutagenesis.
pALMAR4	Tn mariner delivery vector with streptomycin/spectinomycin resistance cassette . This transposon delivery plasmid is non replicative in *Caulobacter*. The transposition frequency is around 2500000 per mating or more. Can be used for random mutagenesis.
pALRES	This plasmid is a modification of pRES in order to make it suitable for RIVET analysis in *Caulobacter*.
pBGSXYL	pBGS18T with P*xylX* region. 2.3Kb *HindIII/SpeI* from pUJ83 ligated in pBGS18T suicide vector.
pBBR1MCS-2	replicative medium copy number plasmid for *Caulobacter*
pALRES1	This plasmid is a modification of pRES1 (AL82) in order to make it suitable for RIVET analysis in *Caulobacter*. (resolution frequency is only 10% of pRES).
pDM1	Knockout the CC0091 PDE gene.
pDM13	Overexpression of CC0095, a WecB/TagA type glycosyltransferase which is essential for holdfast synthesis.
pDM18	pDM24 is a pET42b+ based plasmid, used to purify CC0091Δaa1-338, based on the C-terminal 6XHis tag fusion. CC0091Δaa1-338 was inserted in *NdeI/XhoI* sites of this vector.
pDM24	pDM24 is a pET42b+ based plasmid, used to purify CC0095 based on the C-terminal 6XHis tag fusion. CC0095 was inserted in *NdeI/XhoI* sites of this vector.
pDM25	Knockout CC0095 coding region
pHRXLT95	pHRXLT95 was created in order to introduce CC0095 into *Caulobacter* genome based on homologous recombination of the 2.3Kb long fragment derived from the xylose loci in *Caulobacter crescentus*
pHRXLT0744	CC0744 on pHRXLT vector (pPHU281 based suicide vector)
pHRXLT0744D51E	CC0744 D51E allele on pHRXLT. This construct allows the genomic expression of this allele from the xylose promoter
pHRXLT0744D51N	CC0744 D51N allele on pHRXLT. This construct allows the genomic expression of this allele from the xylose promoter
pHRXLT2277	CC2277 on pHRXLT. This construct allows the genomic expression of CC2277 from the xylose promoter
pIVET5n	pIVET vector contain oriR6K, mobRP4 bla and *tnpR-lacZ E. coli* promoterless operon
pIVET5n mut1	TnpRI; lacZ Source elements for RIVET constructs. Base change in TnpRI RBS.
pIVET5n mut2	Amp r; TnpRI; lacZ Source elements for RIVET constructs. Base change in TnpRI RBS
pIVET5nMut135	pIVET vector contain *oriR6K, mobRP4 bla* and *tnpRmut135-lacZ E. coli* promoterless operon.
pIVET5nMut168	pIVET vector contain *oriR6K, mobRP4 bla and tnpRmut168-lacZ E. coli* promoterless operon.
pKRP10	Chloramphenicol resistance cassette between two polylinkers
pKRP11	Kanamycin resistance cassette between two polylinkers
pKRP12	Tetracycline resistance cassette between two polylinkers
pKRP13	Streptomycin/Spectinomycin resistance cassette between two polylinkers
pKT25	pKT25 encodes the T25 fragment of *B. pertussis* adenylate cyclase, corresponding to the first 224 amino acids of CyaA. This vector is a derivative of the low copy-number plasmid pSU40 (expressing a kanamycin resistance selectable marker). A multicloning sequence was inserted at the 3' end of T25 to allow construction of fusions in frame at the C-terminal end of the T25 polypeptide.
pKT25CC0744	This strain used in BTHS as a negative control for false positive results. CC0744 in

	inserted in pKT25 in Hind/EcoRI sites and transformed into DHMI.
pMRTNPR	pMRTNPRI was used as a delivery vector for the resolvase-Gfp-chloramphenicol resistance cassette construct exploiting the *mariner* Tn for random insertion throughout *C. crescentus* genome.
pKT25-zip	pKT25-zip is a derivative of pKT25 in which the Leucine zipper of GCN4 (1) is genetically fused in frame to the T25 fragment
pNPTSRXH2	knocking out *pilA*
pPA114-32	Carries the *pleD* allele that was isolated as a compact colony on a SW plate. Showed a non-motile, stalked, no SW band phenotype.
pPA53-4	*pleD*-GFP fusion
pRES	RES resolution sites bordering Kan-SacB cassette. Source elements for pRES.
pRES1	RES resolution sites bordering Kan-SacB cassette. Source elements for pRES1. Point mutation in RES sequence, resulted in 1:10 resolution frequency.
pRK600	match maker strain. For mobilization of *IncP* oriT plasmids (triparental conjugation) (pRK2013 nptI::Tn9)
pSW7	*pleD*-egfp*
pUT18	pUT18 is a derivative of the high copy number vector pUC19 (expressing an ampicilin resistance selectable marker) that encodes the T18 fragment (amino acids 225 to 399 of CyaA). The T18 open reading frame lies downstream of the polylinker with 9 unique restriction sites. This plasmid is designed to create chimeric proteins in which a heterologous polypeptide is fused to the N-terminal end of T18
pUT18C	pUT18C is a derivative of the high copy number vector pUC19 (expressing an ampicilin resistance selectable marker) that encodes the T18 fragment (amino acids 225 to 399 of CyaA). The T18 open reading frame lies upstream of the polylinker with 9 unique restriction sites. This plasmid is designed to create chimeric proteins in which a heterologous polypeptide is fused to the C-terminal end of T18
pUT18C-zip	pUT18C-zip is a derivative of pUT18C in which the leucine zipper of GCN4 is genetically fused in frame to the T18 fragment. The plasmids pKT25-zip and pUT18C-zip serve as positive controls for complementation.
pUT-Km_1	Mini Transposon mini-Tn*5* Km2: kka1-nptI; length -B381.84 kb - used for random transposon mutagenesis in *Caulobacter*
pUT-Km_1-rev	Mini Transposon mini-Tn*5* Km2: kka1-nptI (reverse orientation!), length -B381.84 kb - used for random transposon mutagenesis in *Caulobacter*

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