A bacterial extracellular DNA inhibits settling of motile progeny cells within a biofilm

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Summary
In natural systems, bacteria form complex, surface-attached communities known as biofilms. This lifestyle presents numerous advantages compared with unattached or planktonic life, such as exchange of nutrients, protection from environmental stresses and increased tolerance to biocides. Despite such benefits, dispersal also plays an important role in escaping deteriorating environments and in successfully colonizing favourable, unoccupied habitat patches. The α-proteobacterium Caulobacter crescentus produces a motile swarmer cell and a sessile stalked cell at each cell division. We show here that C. crescentus extracellular DNA (eDNA) inhibits the ability of its motile cell type to settle in a biofilm. eDNA binds to the polar holdfast, an adhesive structure required for permanent surface attachment and biofilm formation, thereby inhibiting cell attachment. Because stalked cells associate tightly with the biofilm through their holdfast, we hypothesize that this novel mechanism acts on swarmer cells born in a biofilm, where eDNA can accumulate to a sufficient concentration to inhibit their ability to settle. By targeting a specific cell type in a biofilm, this mechanism modulates biofilm development and promotes dispersal without causing a potentially undesirable dissolution of the existing biofilm.

Introduction
Biofilms are multicellular, surface-associated complexes formed by different microorganisms. Bacteria are predominantly organized in such structured communities in natural environments (Hall-Stoodley et al., 2004). The chemical composition within biofilms is highly dynamic, promoting solute gradient formation and nutrient exchange (Spormann, 2008; Stewart and Franklin, 2008; Moons et al., 2009). Furthermore, biofilms represent a protected mode of growth, as bacteria within a biofilm typically exhibit greater resistance to deleterious agents, such as antibiotics, biocides or predators, than their planktonic counterparts (Davey and O’Toole G, 2000; Harrison et al., 2007).

An extracellular matrix provides the structural basis and many of the protective properties of mature biofilms (Tart and Wozniak, 2008). The distribution of matrix compounds varies from one microorganism to another (Kolter and Greenberg, 2006), but it typically comprises primarily exopolysaccharides, though proteins, lipids and nucleic acids also serve important roles (Goller and Romeo, 2008; Tart and Wozniak, 2008; Karatan and Watnick, 2009). Work on Pseudomonas aeruginosa first identified an essential role of extracellular DNA (eDNA) in stabilizing the biofilm matrix; addition of DNase to P. aeruginosa biofilms inhibits biofilm formation and dissolves existing biofilms (Whitchurch et al., 2002). This stabilizing eDNA derives from cell lysis within the biofilm (Allesen-Holm et al., 2006). Numerous other studies have since reported similar properties of eDNA in biofilms formed by a wide array of Gram positive and Gram negative bacteria (Spoering and Gilmore, 2006; Karatan and Watnick, 2009; Lappann et al., 2010), suggesting that bacterial biofilm stabilization by eDNA is widespread.

Although biofilm-associated bacteria may enjoy certain benefits not available to planktonic counterparts, cells must nonetheless disperse at least occasionally in order to colonize new habitat patches. Dispersal becomes particularly important upon deterioration of local habitat quality within the biofilm, e.g. due to overcrowding, phage infection or a change in the physical environment (Spormann, 2008). Ideally, bacteria should adjust their behaviour to suit the current environmental context, contingent on the ability to detect and respond to environmental cues in a timely manner (Meyers and Bull, 2002). Such behaviours are indeed observed, whereby accumulation of nitric acid or depletion of oxygen or nutrients triggers bacterial dispersal from a biofilm (Karatan and Watnick, 2009). Specific biofilm inhibitory molecules often mediate the dispersal response, including quorum-sensing signalling analogues, excreted proteins and polysaccharides (Karatan and Watnick, 2009). Although details of the dispersal process remain unresolved for many bacterial...
systems, described mechanisms typically involve regulatory changes in response to an environmental cue, whereby signal transduction pathways effect inhibition of exopolysaccharide synthesis and/or induction of motility genes (Goller and Romeo, 2008; Spormann, 2008). Thus, biofilm-associated cells achieve a balance between attachment and planktonic dispersal (Moons et al., 2009), and this balance may be specifically tuned in response to the current conditions encountered in the biofilm.

_Caulobacter crescentus_ is an oligotrophic α-proteobacterium commonly found in aquatic environments. This stalked bacterium has a dimorphic life cycle where each cell division produces a motile swarmer cell and a sessile stalked cell (Fig. 1). A flagellum and several pili occupy a single pole of the nascent swarmer cell, which cannot initiate DNA replication. Irrespective of whether cells reside in suspension or attached to a surface, differentiation into a division-competent stalked cell begins after a developmentally programmed delay. The swarmer cell sheds its flagellum, retracts its pili and elaborates a membranous stalk at the former flagellar pole. Like many α-proteobacteria, _C. crescentus_ adheres to surfaces by means of a polar polysaccharide called the holdfast (Brown et al., 2009). The holdfast is synthesized in the late stage of the swarmer phase and resides at the tip of the stalk once the stalk is synthesized at the same pole during cell differentiation.

Fig. 1. _Caulobacter crescentus_ life cycle. Each cell division produces a motile swarmer cell and a sessile stalked cell. After an obligatory delay during which it is unable to start the next cell cycle, swarmer cells differentiate into a division-competent stalked cell. The stalked cell has a polar adhesin called the holdfast that is required for permanent adhesion to surfaces and for biofilm formation. The holdfast is synthesized at the flagellated pole in the late stage of the swarmer phase and is found at the tip of the stalk once the stalk is synthesized at the same pole during cell differentiation.

does not occur with non- _Caulobacter_ DNA. By acting via direct interaction between eDNA and holdfast, this dispersal mechanism differs substantially from those described previously in that it requires no intermediate intracellular response, and our findings identify a novel role of eDNA as a specific inhibitor of bacterial biofilm formation. Finally, the specific targeting of the swarmer cell type allows the modulation of biofilm development and the promotion of dispersal without causing a potentially undesirable deleterious dissolution of the biofilm.

**Results**

eDNA inhibits biofilm formation in _C. crescentus_

To determine whether _C. crescentus_ produces factors that enhance dispersal from a biofilm, we tested the effect of filtered spent medium from saturated planktonic cultures and found that it inhibited biofilm formation. In an attempt to identify and characterize the molecules involved in biofilm inhibition, we tested different conditions that could remove or destroy extracellular compounds. The inhibitory activity was not dialysable using two different molecular weight cut-offs (3500 and 10 000 Da) (Fig. S1A). The pH of the spent medium was 6.4 as compared with a pH of 7.0 for fresh medium. Adjusting the pH of the spent medium to pH 7.0 or that of the fresh medium to pH 6.4 did not affect the biofilm inhibition activity (Fig. S1B). The inhibitory effect persisted after both heat treatment of the spent medium (100°C for 15 min) and both proteinase K and pronase treatment (Fig. 2A). However, treatment with DNase I completely abolished the inhibition, whereas treatment of the spent medium with RNase I had no impact on its inhibitory activity, suggesting that the inhibitory molecule was DNA (Fig. S1B). The inhibitory effect persisted after both heat treatment of the spent medium (100°C for 15 min) and both proteinase K and pronase treatment (Fig. 2A). However, treatment with DNase I completely abolished the inhibition, whereas treatment of the spent medium with RNase I had no impact on its inhibitory activity, suggesting that the inhibitory molecule was DNA (Fig. 2A).
Purified eDNA from the spent medium had a relatively low molecular weight, mostly below 500 bp (Fig. 2B). Addition of intact purified genomic DNA (gDNA) from *C. crescentus* cells did not inhibit biofilm formation (Fig. 2C). Progressive digestion of gDNA increased its inhibitory activity in a manner inversely proportional to its average molecular weight (Fig. 2C and D). We conclude that low molecular weight DNA from *C. crescentus* is an inhibitor of biofilm formation in *C. crescentus*.

We examined the growth rate of *C. crescentus* in the presence of DNA in order to determine whether reduced biofilm formation resulted from a decline in reproductive rate. The growth rate of *C. crescentus* was not affected by the presence of 66% or less spent medium (containing ~20 μg ml⁻¹ eDNA) or 20 μg ml⁻¹ or less purified, sheared gDNA (Fig. S2), despite strong biofilm inhibition at similar concentrations of DNA or spent medium (see below, Fig. 3B). This result indicates that the biofilm inhibition did not result from bacteriostatic or bacteriocidal activity. eDNA inhibited biofilm formation in a variety of media with different carbon sources at different concentrations, demonstrating that inhibition of biofilm formation by eDNA operates both under-good and poor growth conditions of this facultative oligotroph (Fig. S3).

*eDNA is released during cell death*

As shown in Fig. 3A, measurable cell death occurs during growth of *C. crescentus* and is correlated with the accumulation of eDNA and biofilm inhibitory activity. Filter-sterilized spent medium, eDNA purified from the spent medium or sheared gDNA purified from *C. crescentus* cells gave indistinguishable dose–response curves (Fig. 3B). This result indicates that components other than eDNA are not required for biofilm inhibition, and supports the conclusion that eDNA is a product of cell death rather than secretion of specific DNA fragments.

Small amounts of free DNA are detectable in the environment, up to 20 ng ml⁻¹ for aquatic environments (Lorenz and Wackernagel, 1994), and *C. crescentus* eDNA concentrations likely remain low outside of biofilms. In biofilms, however, high cell density and local diffusion may result in sufficient *C. crescentus* eDNA accumulation to inhibit new attachment. Live/dead staining of a static biofilm grown on a plastic coverslip shows that substantial cell death occurs during biofilm formation even under favourable growth conditions (Fig. 4A). Quantification of eDNA in the biofilm reveals that ~6 μg ml⁻¹ is present within the biofilm matrix after 24 h (Fig. 4B); this concen-
tration is sufficient to inhibit biofilm formation (Fig. 3B). The amount of eDNA measured under these conditions is an underestimate of the total eDNA present in the biofilm because the quantification method only measures double-stranded DNA using PicoGreen, whereas we have shown above using nuclease S1 treatment that single-stranded DNA also inhibits biofilm development (Fig. 2A). Furthermore, this method requires the complete removal of the supernatant, thereby removing some of the diffusible eDNA. Indeed, extensive pre-washing of the biofilms prior to quantification of eDNA removes most of the eDNA from the biofilm matrix (Fig. 4B), showing that eDNA is not tightly bound to the biofilm extracellular matrix. These results also demonstrate that, unlike species where eDNA plays an important role in biofilm structural integrity, firmly bound eDNA is not an abundant component of the *C. crescentus* biofilm matrix, as discussed below.

**eDNA is not required for the stability of *C. crescentus* biofilm**

Numerous studies indicate that eDNA is a major component of the biofilm extracellular matrix in many Gram positive and Gram negative bacteria and is required for biofilm stability (Karatan and Watnick, 2009). In all the reported cases, DNase I treatment causes inhibition and/or dispersion of the biofilm. However, *C. crescentus* biofilm formation is not inhibited by the presence of 20 µg ml⁻¹ DNase I in a static biofilm assay (Fig. 4C). In fact, the biofilm matures faster in the presence of DNase I than in its absence (Fig. 4C). We suggest that in the presence of DNase, eDNA is degraded as soon as it is released by cell lysis and cannot prevent biofilm formation, whereas without DNase, endogenously produced eDNA inhibits biofilm formation. Following maturation at ~30 h, biofilm dispersion appears more effective in the sample without DNase I treatment (Fig. 4C). Furthermore, DNase I treatment is not able to disperse established biofilms, as shown when biofilms at different stages of maturation were treated with DNase I (Fig. 4D). Finally, quantification of the eDNA firmly bound to the extracellular matrix reveals only traces of DNA (Fig. 4B). We therefore conclude that diffusible eDNA has a net inhibitory effect on biofilm formation and persistence rather than serving as a stabilizing component of the biofilm matrix.

**eDNA inhibits swarmer cell attachment by inhibiting holdfast adhesiveness**

The initial and reversible stage of *C. crescentus* attachment to surfaces is mediated by flagellar motility and pili, whereas permanent attachment requires the holdfast (Bodenmiller *et al.*, 2004). Previous research has identified a role for pilin–eDNA interactions in biofilm integrity (Allesen-Holm *et al.*, 2006; Jurcisek and Bakalatz, 2007), albeit a net stabilizing effect of eDNA rather than the inhibitory effect reported here. eDNA still inhibited biofilm formation of a pilus- and flagellum-deficient mutants at a magnitude comparable with wild type, suggesting that eDNA does not inhibit the reversible attachment stage mediated by these structures (Fig. 5).
In order to determine how the initial attachment of cells to surfaces was affected by eDNA, we monitored single cell attachment to glass by microscopy. eDNA significantly decreased the binding efficiency of synchronized single swarmer cells (Fig. 6A), even in the presence of kanamycin, indicating that new protein synthesis is not required for the inhibition (Fig. S4). In contrast, eDNA had no effect on the reversible attachment of a holdfast synthesis mutant (Fig. 6B), indicating that attachment inhibition by eDNA requires the holdfast.

In order to determine whether eDNA inhibits *C. crescentus* adhesion by interacting with the holdfast, we labelled sonicated *C. crescentus* gDNA or purified eDNA using AlexaFluor (AF) 488 and determined their localization by fluorescence microscopy. Similar results were obtained with both types of DNA (not shown). When added to a preformed biofilm, the AF 488-labelled DNA was associated with cells in the biofilm and exhibited a punctate staining pattern (Fig. 6C). Co-staining of the holdfast using AF 594-labelled wheatgerm agglutinin (WGA) indicated that DNA co-localized with the holdfast in the biofilm (Fig. 6C). Observation of planktonic cells confirmed that eDNA bound at the location of the holdfast in stalked cells and in swarmer cells harbouring a
The biofilm inhibition is not due to interaction between eDNA and pili or eDNA and flagellum. Static biofilm assays were performed using 10 μg ml⁻¹ sheared gDNA extracted from CB15 (black solid bars), CB15 ΔpilA (striped bars) or CB15 ΔflgE (dotted bars) in 3 ml M2G (12-well plates). Control biofilm grown in the absence of eDNA are represented with white bars. Tested bacteria were incubated for 16 h at 30°C and the biofilm attached to the coverslips was quantified with crystal violet. Results are given as the biofilm score (amount of biofilm formed on the coverslip corrected by the culture cell density). Despite the fact that the ΔpilA and ΔflgE mutants are already deficient in biofilm formation, the addition of eDNA reduces their biofilm formation by ~70%, as is the case for wild type. The error bars represent the SEM of 2 independent experiments run in duplicate.

We conclude that the holdfast is necessary and sufficient for the binding of the inhibitory eDNA to cells and that eDNA limits swarmer cell attachment by inhibiting holdfast adhesiveness. In addition, these results and the results of flow cell experiments presented later indicate that an adhesive contact already made with a surface cannot be readily disrupted by eDNA or WGA-lectin when they bind to exposed areas of the holdfast.

**Biofilm inhibition by eDNA is specific to Caulobacter species eDNA**

To determine whether the observed biofilm inhibition is specific to *C. crescentus* DNA, we tested four additional *Caulobacter* strains and 14 other bacterial species (Fig. 7B). Only spent media from *Caulobacter* strains, eDNA purified from these spent media or sonicated gDNA from these strains inhibited *C. crescentus* CB15 biofilm formation (Table 1). Thus, *C. crescentus* biofilm inhibition is a specific property of *Caulobacter* DNA and not a general property of DNA or negatively charged molecules.

*Caulobacter crescentus* CB15 DNA has a high GC content (67%); however, the inhibitory activity is not specific to high-GC DNA as DNA from *Rhodobacter capsulatus* or *Brevundimonas diminuta*, whose genomes also have a GC content of 67%, do not inhibit *C. crescentus* biofilm formation (Table 1). Biofilm assays using hemi-methylated DNA extracted from a *C. crescentus* strain depleted of the DNA methylase CcrM (Stephens et al., 1996) indicated that the inhibition specificity did not result from DNA modification (Fig. S5).

Although DNA from non- *Caulobacter* species failed to inhibit biofilm formation, we sought to determine whether any inhibition might be detected by the more sensitive assay using isolated holdfasts in suspension. eDNA from the closely related species *B. diminuta* inhibits holdfast binding to some extent in the purified holdfast adhesion assay, with around 60% inhibition at 25 μg ml⁻¹ (Fig. 7A). A weaker attachment inhibition can also be detected with the other tested eDNA, with a positive correlation between phylogenetic distance and the degree of inhibition (Fig. 7B). The results indicate that the magnitude of *Caulobacter* holdfast binding inhibition correlates directly with the relatedness of the gDNA donor bacterium, resulting in strong binding inhibition only in response to DNA from *Caulobacter* and close relatives.

We examined bacterial genomic sequence data for enrichment of particular motifs that may impart the observed specificity of biofilm inhibition by *Caulobacter* DNA. We sought to identify any correlations between the genomic abundance of particular sequences and the magnitude of holdfast binding inhibition (Fig. 7A) across four bacterial species: *C. crescentus* CB15, *Rhodopseudomonas palustris* CGA009, *B. diminuta* and *Asticacaulis biprosthecum*. In order to identify candidate inhibitory sequences, we used MEME 4.3.0 (Bailey and Elkan, 1994) to search for repeated sequence elements in the *C. crescentus* genome. We then compared the frequency of each motif in the various query genomes as determined by MAST. None of the motif abundance patterns reflects the large differences in holdfast-binding inhibition observed among the corre-
sponding gDNA treatments (Table S1). Even in cases where the relative ordering of genomes based on motif abundance agrees with their ordering based on holdfast inhibition (e.g. Motif Max16-7), the differences in motif frequencies appear inconsistent with the corresponding magnitude of inhibition (Fig. 7A). Quite possibly, an enriched inhibitory sequence exists in Caulobacter, but the search space rapidly becomes intractable with increasing sequence length and complexity (e.g. mixtures of specific and non-specific sites in a signature sequence and/or variable spacing between specific residues).

Fig. 6. eDNA prevents the reversible attachment of cells and binds specifically to the C. crescentus holdfast.
A. Attachment of wild-type C. crescentus CB15 synchronized swarmer cells to a glass surface in the presence (open circles) or absence (solid diamonds) of 10 μg ml⁻¹ eDNA. The swimming cells are given as a percentage of the overall cell population at various times. Results are averages from duplicate samples in 3 independent experiments.
B. Swimming cell quantification of the holdfast-deficient C. crescentus CB15 ΔhfsDAB mutant in the presence (open circles) or in the absence (solid diamonds) of 10 μg ml⁻¹ eDNA. Results are given as a percentage of swimming cells at t = 0 for 2 independent replicates.
C. Biofilm and planktonic cells of C. crescentus wild-type strain CB15 and a holdfast-minus ΔhfsDAB mutant in the presence of AF 488-labelled eDNA purified from C. crescentus CB15 (green), and AF 594-labelled WGA (red), to visualize holdfast.
D. Coverslip binding of cells of C. crescentus CB15 and holdfasts left on the surface following the removal of the holdfast-shedding mutant CB15 ΔhfaB and fluorescence localization of eDNA (green) and holdfast (red).
eDNA prevents the attachment of newborn cells but does not disperse existing biofilm

We have shown that eDNA acts as a competitive inhibitor of holdfast adhesion by coating its adhesive surface, thereby reducing its ability to bind to new surfaces, but that eDNA does not dislodge previously bound holdfast from a surface. This suggests that eDNA might specifically prevent adhesion of newborn cells in a biofilm without disrupting stalked cells already bound by their holdfast. Indeed, static biofilm experiments showed that eDNA does not cause dispersal of pre-established biofilms (Fig. 8A), but instead blocks the increase of biofilm biomass over time (Fig. 8B). Furthermore, as described earlier, biofilm dispersion is enhanced in the presence of eDNA compared with a DNase I-treated biofilm (Fig. 4C), showing that newborn cells are prevented from settling within the biofilm when the eDNA concentration is sufficient.

Under dynamic flow conditions, no biofilm development could be detected in the presence of eDNA; only a few cells were found on the surface of flow cells irrigated with a spent:fresh medium mixture even after 4 days (Fig. 9A, compare flow cells a and e), indicating that eDNA inhibits new adhesion to the surface as shown by surface coverage quantification (Fig. 9B). eDNA stopped the increase of biomass regardless of the maturation stage of the biofilm, but did not disrupt established biofilms (Fig. 9C). Therefore, eDNA acts in a cell-specific manner to prevent growth of a biofilm without causing its dissolution.

Discussion

Association with a sessile biofilm and dispersal into new environments provide different benefits to bacteria that depend on the current ecological context. Mechanisms that maintain a balance between these two strategies should enhance bacterial fitness, particularly when such mechanisms tune bacterial behaviour to suit the current environmental state. Specifically, favourable local conditions should tend to favour biofilm maturation and persistence, whereas deterioration of the local environment due to crowding, predation or other deleterious factors should promote dispersal into alternate habitat patches. Here we report that eDNA can modulate this balance by specifically stimulating the dispersal of newborn swarmer cells in a *C. crescentus* biofilm. We show that eDNA...
Species specificity of the biofilm inhibition.

Table 1. Species specificity of the biofilm inhibition.

<table>
<thead>
<tr>
<th>Tested bacterium</th>
<th>CB15 + tested bacterium spent medium</th>
<th>CB15 + tested bacterium eDNA</th>
<th>CB15 + tested bacterium gDNA</th>
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<tr>
<td>C. crescentus CB15</td>
<td>44.7 (±1.3)</td>
<td>44.8 (±1.3)</td>
<td>40.1 (±1.3)</td>
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<td>C. crescentus NA1000</td>
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<td>43.9 (±6.1)</td>
<td>41.6 (±3.9)</td>
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<td>C. crescentus ATCC 19089</td>
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<td>39.8 (±3.5)</td>
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<td>Caulobacter sp. K31</td>
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<td>C. crescentus CB13</td>
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<td>S. meliloti</td>
<td>n.t</td>
<td>n.t</td>
<td>94.5 (±5.1)</td>
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a. Biofilm formation was calculated as a percentage of biofilm formation in the absence of spent medium or sheared gDNA. Percentages were calculated from at least three independent experiments done in duplicate. SEM are indicated between parentheses.

b. The concentration of eDNA was normalized to provide 15 μg ml⁻¹ of eDNA for all the biofilm assays.
c. eDNA was extracted from spent media and added to a final concentration of 15 μg ml⁻¹ for all biofilm assays.
d. The final concentration of purified and sonicated gDNA was 15 μg ml⁻¹ for all the biofilm assays.
e. Only purified sheared gDNA addition was tested for these strains.

Inhibits single cell attachment and biofilm growth irrespective of the stage of biofilm maturation but that it does not dissolve existing biofilm. eDNA binds specifically to the adhesive holdfast and the holdfast is both necessary and sufficient for eDNA binding. Interestingly, our observation that eDNA can inhibit biofilm formation stands in stark contrast with previous studies showing that eDNA often plays an important role in biofilm formation and stabilization as a major compound of the extracellular biofilm matrix in many bacterial species (Allesen-Holm et al., 2006; Spoering and Gilmore, 2006; Karatan and Watnick, 2009).

As cell death occurs at a sufficient frequency in a C. crescentus biofilm even under favourable growth condi-

Fig. 8. Effect of spent medium on different stages of biofilm formation.
A. Addition of spent medium to mature biofilms. Biofilms of C. crescentus were grown on coverslips for 24 h in fresh M2G medium (before addition) and then placed in the presence of spent medium or fresh M2G medium for an additional 24 h.
B. Time course study of the effect of C. crescentus CB15 eDNA on C. crescentus CB15 biofilm formation under static conditions. 10 μg ml⁻¹ of eDNA was added (open circles) or not (solid diamonds) to M2G for static biofilm assays. The bacteria were incubated for various times at 30°C and the biofilm attached to the coverslips was quantified with crystal violet. The error bars represent the SEM of at least 3 independent experiments run in duplicate.
tions, we hypothesize that the inhibitory effect of eDNA increases dispersal of swarmer cells born in a biofilm. The specific targeting of the swarmer cell type in the developing biofilm allows the stimulation of dispersal without the simultaneous destruction of the biofilm. Holdfast-bearing stalked cells are non-motile and associate tightly with the biofilm through their holdfast, and we have shown that pre-bound holdfasts cannot be dislodged from a surface by eDNA. Therefore, stalked cells are not able to disperse, even when the eDNA concentration increases within the biofilm, explaining why mature biofilms are not disrupted. Our results indicate that eDNA functions by coating the adhesive surface of the holdfast of swarmer cells, thereby reducing their ability to bind to surfaces. Even if a dispersing swarmer cell with bound eDNA will not be able to attach efficiently to a surface, it will eventually differentiate into a stalked cell and produce a progeny swarmer cell free of eDNA and capable of settling in a new environment.

This method of biofilm inhibition permits rapid tuning of the degree of dispersal in response to environmental cues. Previous studies have identified various dispersion mechanisms, including active degradation of the extracellular matrix, induction of motility, production of surfactants or cell lysis, in each case mediated by signal transduction and/or regulatory changes within the cell (Goller and Romeo, 2008; Karatan and Watnick, 2009). In contrast, our study shows that eDNA acts via direct interference with cell attachment and requires no intermediate intracellular response. Because the magnitude of inhibition is roughly proportional to the eDNA concentration, this mechanism may provide a rheostat control of biofilm inhibition: cells should be less affected by small quantities of DNA released through sporadic cell death, but they should be strongly inhibited under conditions when cell death is more frequent. This mechanism can also promote swarmer cell dispersal without causing a potentially undesirable dissolution of the biofilm. Particularly in the case of overcrowding, cells currently residing in the biofilm may continue to occupy a productively colonized habitat patch, albeit at a saturating density; whereas inhibition of new attachment by eDNA promotes dispersal of newborn swarmer cells that would otherwise stretch the biofilm beyond its carrying capacity.

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While our data support the role of eDNA as a cue that prevents settling of swarmer cells, it is unclear if this mechanism constitutes cooperation (Nadell et al., 2009), or is simply a consequence of random cell death occurring within the biofilm. Cooperative behaviours involve a fitness cost to the actor while benefiting other individuals (Nadell et al., 2009). In biofilm inhibition by eDNA, such behaviours could include active lysis via programmed cell death (Bayles, 2007; Claverys and Havarstein, 2007; Rice and Bayles, 2008; Lopez et al., 2009; Thomas et al., 2009) or production of endonucleases that specifically digest the released DNA. On the other hand, if eDNA only arises from non-regulated cell death and is not cleaved by an endonuclease produced specifically for that process, there is no direct cost to the dying cells and therefore no cooperation.

The species-specific nature of biofilm inhibition by eDNA provides the ability to respond to close relatives, an advantage as DNA is an abundant molecule in all environments that support life (Lorenz and Wackernagel, 1994). Therefore, the species specificity of the inhibition should allow C. crescentus to colonize permissive environments that contain eDNA from other organisms. Furthermore, this specificity may increase the likelihood that Caulobacter cells avoid settling in environments already densely inhabited by closely related bacteria, thereby reducing intraspecific resource competition.

The mechanism underlying species specificity of the eDNA–holdfast interaction remains undetermined. The holdfast is composed in part of β-1,4-N-acetylglucosamine polysaccharides (Merker and Smit, 1988), but its detailed chemical composition is not known. Interestingly, β-1,3-glucan polysaccharides can specifically interact with certain polynucleotides by forming triple-stranded and helical macromolecular complexes (Sakurai et al., 2005); similar DNA–polysaccharide interactions may mediate the sequence specificity of the eDNA–holdfast interaction. The next challenge is to determine how species-specific inhibition is achieved.

### Experimental procedures

#### Bacterial strains, plasmids and growth conditions

Bacterial strains used in this study are listed in Table 2. C. crescentus was grown at 30°C in complex peptone-yeast extract (PYE) medium (Poindexter, 1964) or in minimal M2 medium supplemented with 0.2% glucose (M2G) (Johnson and Ely, 1977). For some experiments, different concentrations of glucose (0.2%, 0.1%, 0.05%, 0.02% and 0.01% w/v) were used in M2G as well as xylose and alanine as carbon sources.

#### Table 2. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>C. crescentus</td>
<td>Wild type</td>
<td>Poindexter (1964)</td>
</tr>
<tr>
<td>CB15 (YB135)</td>
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<td>Hardy et al. (2010)</td>
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<tr>
<td>CB15 ΔhfsB (YB4251)</td>
<td>Clean deletion of hfsB</td>
<td>C. S. Smith, unpublished</td>
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<tr>
<td>CB15 ΔhfsDAB (YB2857)</td>
<td>Clean deletion of hfsDAB</td>
<td>Entcheva-Dimitrov and Spormann (2004)</td>
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<tr>
<td>AS110 (YB4784)</td>
<td>CB15::miniTn7gfp</td>
<td>YB127</td>
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<tr>
<td>YB4789</td>
<td>CB15::miniTn7gfp</td>
<td>YB1309</td>
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<tr>
<td>ATCC 19089 (YB1360)</td>
<td>NA1000, holdfast deficient</td>
<td>Evinger and Agabian (1977)</td>
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<td>Caulobacter sp. K31 (YB4571)</td>
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<td>Asticcacaulis excentricus C48 (YB258)</td>
<td>Wild type</td>
<td>Pate and Ordal (1965)</td>
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<td>S. Kaplan</td>
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sources in M2 medium. PYE diluted 50% v/v (PYE 1/2) was used in some experiments. When using GFP-expressing C. crescentus CB15 (YB4789), kanamycin was added to the culture medium at a final concentration of 5 μg ml⁻¹. YB4789 was constructed by ϕCr30-mediated transduction (West et al., 2002) of the miniTn7-gfp from AS110 (Entcheva-Dimitrov and Spormann, 2004) into C. crescentus CB15.

Preparation of spent medium

Bacteria were grown for 24 h with shaking at 30°C in M2G or in PYE medium. At that point, cultures were in stationary phase (OD₆₀₀ of 1.2–1.8). Bacteria were removed by centrifugation and the spent medium was filter-sterilized using a 0.2 μm filter and kept at –20°C until needed.

DNA extraction and sonication

Genomic DNA was extracted from 1 ml overnight culture in PYE using the Bactozol kit for Bacterial DNA Isolation (Molecular Research Center) and was resuspended in 500 μl dH₂O. DNA was sonicated using a Misonix 3000 sonicator apparatus and a cup horn attachment filled with iced water for 2 cycles (10 s on, 2 s off for 1 min) at power 2.

Fluorescent labelling

Extracellular DNA and gDNA were labelled with AF 488 using the ULYSIS Nucleic Acid labelling system (Molecular Probes) according to the manufacturer’s instructions. C. crescentus holdfasts were labelled using AF 594 conjugated WGA (Molecular Probes). WGA binds specifically to the N-acetyl glucosamine residues of the holdfast (Merker and Smit, 1988). Fluorescent and phase contrast microscopy were performed using a Nikon Eclipse E800 microscope.

Static biofilm experiments

Bacteria were grown to mid-log phase in PYE or M2G medium and diluted to A₆₀₀ = 0.05 in the same medium. Biofilm assays were conducted in two different experimental set-ups: (i) in 12-well polystyrene plates containing polyvinyl chloride coverslips placed vertically in the wells where the biofilm formed on the coverslip is measured; or (ii) in 24-well polystyrene plates without coverslips where the biofilm formed inside the well is measured. For biofilms grown in 12-well plates with coverslips, the final volume of diluted cultures was 3 ml. The final volume in the 24-well plates without coverslips was 500 μl. Both methods gave comparable results. When specified, 33% of filtered spent medium, purified eDNA or sheared DNA resuspended in dH₂O was added directly to the medium in the wells. Plates were incubated at 30°C overnight. After incubation, coverslips or wells were rinsed with dH₂O to remove planktonic cells, stained with a 0.1% crystal violet solution for 5 min, and rinsed again with dH₂O to remove excess crystal violet. The crystal violet was dissolved using 10% acetic acid and quantified by measuring the absorbance at 600 nm (A₆₀₀).

Hydrodynamic biofilm experiments

Five sterile flow cells (three 40 mm × 5 mm × 1 mm channels) were equilibrated for 24 h at 30°C prior to inoculation (4 flow cells were equilibrated with M2G and 1 with a mixture 33% spent medium : M2G). Mid-log phase cultures of C. crescentus CB15::miniTn7gfp (YB4089) were adjusted to A₆₀₀ = 0.05 and 200 μl was inoculated per chamber. Initial attachment was performed in the absence of flow for 1 h, followed by a constant flow of 3 ml h⁻¹. Surface colonization of the glass surface covering the flow cells was monitored for 5 days at 30°C. The medium was switched from M2G to the spent medium mixture at different times after inoculation (0, 24, 48 and 72 h). Images of the biofilms grown in the flow cells were recorded by confocal scanning laser microscopy and quantitative analyses were performed using autoCOMSTAT, a modified version of the COMSTAT software (Merritt et al., 2007). Each experimental condition was performed twice in parallel triplicate chambers.

Coverslip binding assay

Coverslip binding assays were performed as described (Cole et al., 2003) with some modifications. Sterilized coverslips (22 × 22 mm) were placed at the bottom of a 6-well polystyrene plate containing 1.5 ml M2G-grown bacteria diluted to A₆₀₀ = 0.2. Plates were then incubated at 30°C for 4 h under constant agitation (50 r.p.m.). After incubation, coverslips were rinsed with dH₂O to remove all unattached cells. AF 488-labelled sheared C. crescentus CB15 gDNA (10 μg ml⁻¹) and/or AF 594-labelled WGA (5 μg ml⁻¹) were added to the rinsed coverslips and incubated in the dark for 30 min at room temperature. Coverslips were then rinsed with dH₂O, mounted on a microscopy glass slide with a drop of M2G under a large coverslip (24 × 50 mm) and sealed with nail polish. To determine if the DNA binds directly to the holdfast, we used a C. crescentus ΔhfaB holdfast-shedding mutant, YB4251, that can synthesize and export holdfast polysaccharide but cannot keep it attached to the tip of the stalk (Kurtz and Smith, 1992; Cole et al., 2003; Hardy et al., 2010), to coat microscope coverslips with holdfasts without cells. We then incubated the coverslips with labelled C. crescentus DNA and stained the holdfast with WGA as above.

Cell synchronization and initial attachment of C. crescentus single cells to surfaces

Small-scale synchronization of C. crescentus CB15 was performed as described previously (Degnen and Newton, 1972) with some modifications. An overnight culture of C. crescentus CB15 in M2G was diluted 10 times in fresh M2G and incubated shaking at 50 r.p.m. at room temperature in a 150 mm diameter Petri dish. After overnight incubation, the medium was removed and the monolayer biofilm formed in the dish was thoroughly washed. 60 ml of fresh M2G medium was added to the dish and the incubation was carried out for 4 h under the same conditions. The dish was then rinsed 10 times using 10 ml of fresh M2G medium to remove unattached cells from the biofilm. One millilitre of M2G medium was added to the dish and the incubation was carried out...
5 min under the same conditions to release the newly born swarmer cells. The homogeneity of the synchronized swarmer population was checked by microscopy.

Initial attachment of *C. crescentus* cells to surfaces was recorded by dark-field microscopy. A 1 µl aliquot of synchronized swarmer cells was observed by dark-field microscopy (10X objective, stage warmed at 30°C). A stack of 100 frames (100 ms exposure time) was recorded every minute during 60 min. To be able to track attached cells over time, images were processed as follows: the average of the 100-frame stack at t = 0 was calculated. This average file was subtracted from the entire stack to eliminate background noise. The maximum intensity of the 10 first frames of each stack was calculated in order to visualize motile cells with a swimming trajectory. All the calculations were performed using the built-in functions in the Metamorph version 7 software package (Molecular Devices).

The attachment of *C. crescentus* CB15 ΔhfsDAB cells (YB2857) was studied with non-synchronized cells, as this holdfast-minus mutant cannot be synchronized under the conditions mentioned above. An aliquot of cells grown to exponential phase was used instead of the synchronized swarmer cells.

**Purified holdfast attachment assay**

This assay was performed using 12-well glass multitest slides (MP Biomedicals). To maximize holdfast binding, the multitest slides were soaked overnight in 100% Micro-90 detergent, carefully rinsed with dH2O and cleaned with ethanol prior to use.

The holdfast-shedding mutant *C. crescentus* CB15 ΔhfaB strain (YB4251) was grown to late exponential phase in M2G and cells were pelleted by centrifugation (30 min at 4000 g). The supernatant contains free holdfasts shed by the ΔhfaB mutant. Different concentrations of purified sonicated gDNA were added to 100 µl of holdfast-containing supernatant. 50 µl samples was spotted on a precleaned 12-well glass multitest slide and incubated for 2–5 h at room temperature in a humid chamber. After incubation, the slides were rinsed with dH2O to remove unbound material. AF 488-labelled WGA (50 µl at 5 µg ml\(^{-1}\)) was added to the rinsed wells and incubated in dark for 20 min at room temperature. Slides were then rinsed with dH2O, topped with a large glass coverslip (24 × 50 mm) and sealed with nail polish. Holdfast attachment was visualized by epifluorescence microscopy and quantified using Image J analysis software.

**Biofilm extracellular matrix and DNA extraction**

*Caulobacter crescentus* CB15 wt cells were grown in 2 ml microtubes (1 ml final culture in M2G, starting OD\(_{600}\) = 0.05) and sealed with a piece of sterile breathable sealing film (AeraSeal, Excel Scientific). After incubation at 30°C for different amounts of time, the planktonic phase was carefully removed and biofilm extracellular matrix was extracted as described previously (Schooling and Beveridge, 2006) with some modifications. One millilitre M2 was added and the treated tube was incubated with DNase I for 1 h at 37°C to degrade eDNA. The other tube received the same treatment but without DNase I. Both tubes were heated at 70°C for 30 min to inactivate DNase I in the treated tube. Controls indicated that heating did not change the eDNA quantification results. The biofilms formed inside the microtubes were resuspended in sterile 0.9% NaCl by vortexing 3 min. Crystal violet staining was used to confirm that the biofilms were completely resuspended. The tubes were then centrifuged at 12 000 g at 4°C for 20 min. The supernatants were filtered through 0.2 µm filter (Millipore) to remove any remaining cells. eDNA was quantitated using a PicoGreen assay (Molecular Probes) following the manufacturer’s instructions. The total eDNA concentration was determined as the difference between the non-treated and the DNase I-treated samples at each time point and includes both diffusible eDNA and eDNA attached to the matrix. The amount of eDNA attached to matrix was determined using the same procedure but after the biofilms were washed with sterile water three times prior to the procedure.

The effect of DNase I treatment on biofilm formation and stability was determined in similar experiments in which the biofilm was quantified after DNase I treatment using crystal violet as described above.

**Live/dead staining**

Biofilms were grown on plastic coverslips as described above and incubated for different times. After incubation, the coverslips were carefully rinsed with dH2O, 0.5 µl of Live/Dead stain mixture (Live/Dead BacLight Bacterial Viability kit L7007, Molecular Probes) was added to 500 µl dH2O placed on top of the coverslip and incubated for 15 min at room temperature in the dark. The stained coverslips were then rinsed with dH2O and observed under epifluorescent microscopy.

**Bioinformatic analysis**

Bioinformatic searches utilized genome sequences of *C. crescentus* CB15 (NC_002696), *R. palustris* CGA009 (NC_005296), *B. diminuta* (GDSub11081) and *A. biprosthecum* (GDSub11080). To select candidate biofilm inhibitory sequences from *Caulobacter*, we sampled a 24 kb region of the *C. crescentus* CB15 genome and searched for repeated sequence elements using MEME 4.3.0 (Bailey and Elkan, 1994) to identify the 20 best-scoring elements with 10 to 500 occurrences in the sampled region. As the MEME scoring strategy favours longer matches, we iterated the analysis with maximum motif lengths of 6, 9, 12, 16, 24 and 50 bases in order to consider a range of target sequence lengths, also constraining minimum match lengths to avoid overlap with searches that used shorter maxima. MAST (Bailey and Gribskov, 1998) then determined the frequency of each motif in the query genomes.

**Acknowledgements**

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Ghigo and Clay Fuqua for their critical reading of this manuscript and helpful discussions, and to Sarah Schooling and Sima Setayeshgar for helpful discussions. This work was supported by the National Institutes of Health grant GM077648 and by the Indiana METACyt Initiative of Indiana University, funded in part through a major grant from the Lilly Endowment, Inc.

References


**Supporting information**

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