

# Flow Cytometry Reveals that Multivalent Chemoattractants Effect Swarmer Cell Dedifferentiation

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**ABSTRACT** Bacterial cells can differentiate into states that allow them to respond efficiently to their environment. An example of such a transformation is the differentiation of planktonic bacteria into highly motile swarmer cells. The hyperflagellated, filamentous swarmer cells can use coordinated movement to seek out and colonize new sites for pathogenic infection. Because the chemotaxis proteins are essential for swarmer differentiation, we sought to probe the relationship between differentiation and chemoattractants. To this end, we developed a method to screen large populations of swarmer cells using flow cytometry. Using this approach, we found that highly potent multivalent chemoattractants can induce the dedifferentiation of swarmer cells. Our results indicate that chemotactic signaling functions as a target for agents that interfere with bacterial swarming. In addition, the identification of ligands that promote the dedifferentiation of swarmer cells offers new strategies for modulating this multicellular behavior.

Bacteria have the ability to differentiate into a variety of forms that allow them to interact both with their environment and with other bacteria (1). Depending on the conditions, this ability allows them to survive or thrive. It also can have deleterious consequences for the host. For example, one well-studied differentiation process is that leading to bacterial biofilms, in which bacteria form sessile communities that resist treatment with common antibiotics (2). Similarly, some forms of bacteria differentiate during nutrient deprivation to protect their ability to survive and replicate (3). Additionally, bacteria can differentiate into highly motile swarmer cells that allow them to rapidly colonize a new environment. Differentiation of bacteria into swarmer cells correlates with elevated resistance to antibiotics (4) as well as the upregulation of virulence genes (5). In all cases, the response to extracellular signals promotes bacterial differentiation, and cells in the differentiated state exhibit multicellular behavior and interact more effectively with their environment.

Cells in the swarmer differentiation state are well-suited to scouting out new sites for colonization: they are elongated, multinucleoid, and hyperflagellated; they also run continuously. Although the swarmer state was first recognized in *Proteus mirabilis* (6), it is now known that a number of bacterial species can swarm, including *Escherichia*

*coli*, *Salmonella enterica* serovar Typhimurium, *Bacillus subtilis*, *Vibrio parahaemolyticus*, and *Serratia liquefaciens* (7). These species can exist as filamentous cells that align along their long axis as rafts and migrate as a population over a semisolid surface. Swarmer cells are usually found at the colony edge, which is consistent with their purported role in rapid colonization. Efforts to determine the triggers of swarmer differentiation (1) have led to the identification of the following factors: the wetness of the surface and the cells' ability to produce surfactants (8), environmental signals relating to cell density or quorum-sensing (9, 10), the condition and number of flagella (11), and proteins in the chemotaxis system (7, 12, 13).

Bacterial flagella are a crucial signaling and regulatory point in swarming. Deficiencies in flagellar production that decrease bacterial motility disrupt swarming (14). Another role for the flagella is as a mechanosensor, and impairment of flagellar rotation can lead to swarmer differentiation (1). Alternatively, the flagella can serve as a sensor of surface wetness to determine if conditions are favorable for swarming (1). The flagella also are the end point of the chemotaxis sensory system, which alters the switching between clockwise and counterclockwise flagellar rotation (15). Indeed, the proteins of the chemotaxis pathway are required for swarmer cell differentiation

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(12), and mutations in the corresponding genes result in swarming defects.

The relationship of chemotaxis and swarming is intriguing. We hypothesized that this connection could be exploited to modulate swarming. Bacteria sense chemoattractants using transmembrane receptors termed methyl-accepting chemotaxis proteins (MCPs), which are coupled to a two-component histidine kinase CheA. CheA catalyzes the phosphorylation of the response regulator CheY, which is responsible for interacting with the flagellar motor proteins and regulating flagellar switching (15). Attractants influence CheY phosphorylation to induce an “active” form, which elicits decreased flagellar switching and a smooth swimming response. In *E. coli* and *S. typhimurium*, active CheY is dephosphorylated, but it is phosphorylated in *B. subtilis* (16). In all species examined, CheY is required for swarming (7, 12). The production of a constitutively “inactive” phospho-CheY-mimic in *S. typhimurium* can completely rescue swarming in cells lacking the other chemotaxis components (13). The ability of CheY to induce switching of flagellar rotation appears to be critical for swarming behavior. These observations suggest that the chemotaxis pathway could serve as a target for agents that block swarming.

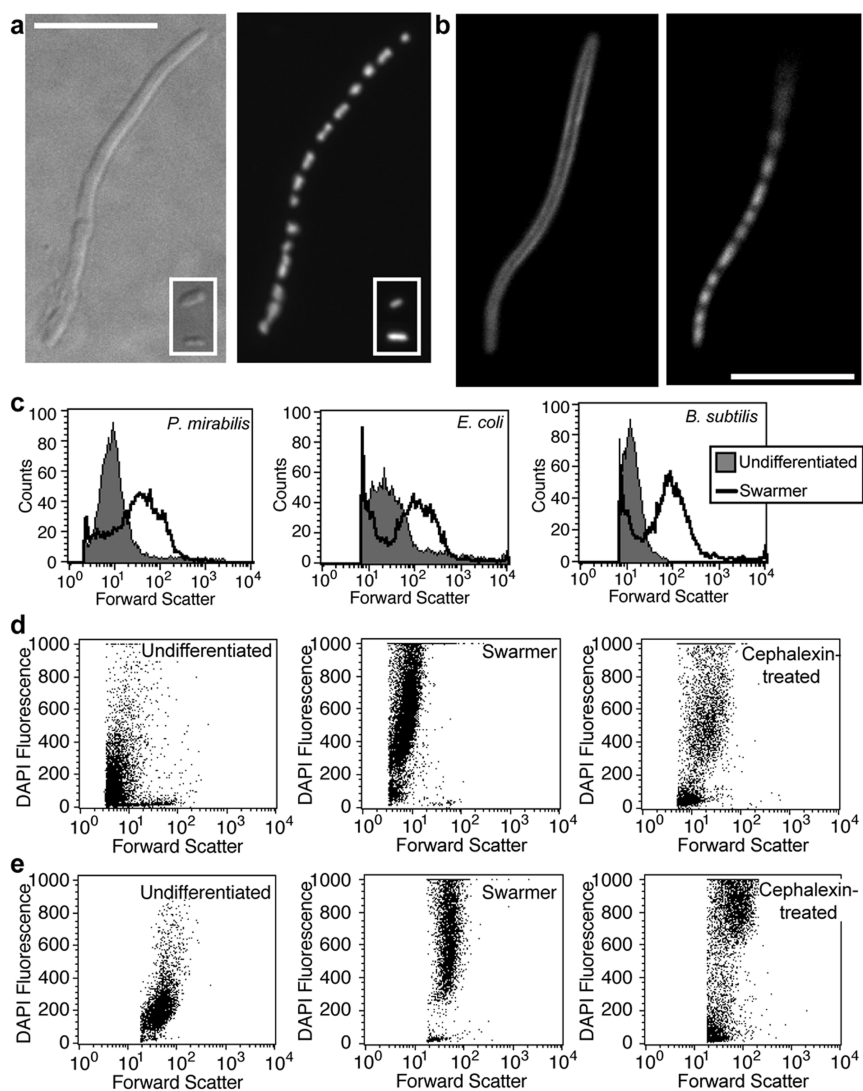
We found previously that multivalent chemoeffectors give rise to potent chemotactic responses (17–20). Polymer-based, multivalent saccharide displays can elicit chemotaxis at concentrations much lower than monovalent saccharide (17–19). The potency of this ligand class as attractants is underscored by our finding that multivalent repellents induce attractant-like behavior (20). In this study, we employed highly active glucose-bearing multivalent chemoattractants, which have been shown to be potent inducers of chemotaxis in several species of bacteria (17–19). In *E. coli*, these polymers can interact with the glucose-sensing MCP Trg through its adaptor protein glucose-galactose binding protein and

enhance chemotaxis by modulating chemoreceptor assembly (17–19). Because it has been shown that the kinase CheA and the response regulator CheY are important for swarmer cell differentiation (12), we hypothesized that agents that promote chemotactic signaling would also influence swarming. Specifically, we postulated that powerful attractants would increase the concentration of “active” CheY, decrease flagellar switching, and thereby promote swarmer dedifferentiation.

To test this hypothesis, we needed to develop an assay that can be used to monitor dedifferentiation of a population of swarmer cells in response to a compound of interest. Published techniques presented multiple drawbacks. First, most prior work with swarmer cells has focused on inhibiting differentiation rather than effecting dedifferentiation (21). Second, because bacteria are induced to differentiate into swarmer cells when they interact with a surface (*e.g.*, an agar plate), previous assays required bacteria be harvested and examined individually under a microscope. This approach has been used to evaluate the number of swarmer cells or the swarming ability in a population (22). In addition, mutants deficient in the ability to swarm (23) or their suppressors (13) could be identified. Despite its utility for these purposes, however, such an approach is not useful for evaluating the activity of different compounds, because of the large quantities that are needed for this type of plate assay. Thus, we sought an alternative approach. To target dedifferentiation, we wanted an assay that could minimize compound quantities employed but also rapidly characterize a whole population and quantify the relative number of swarmer and undifferentiated bacteria. Flow cytometry is a technique that, in principle, could address all of these criteria. Thus, we set out to determine whether this method could be applied to readily distinguish swarmer cells from planktonic bacteria.

We reasoned that swarmer cells possess two unique attributes that could be evaluated with flow cytometric analysis: increased DNA content and increased length. Swarmer cells contain multiple nucleoids in a non-septated cytoplasm and can be 20 times longer than undifferentiated bacteria (Figure 1, panels a and b). Previously, flow cytometry has been used to evaluate different bacterial populations harvested from lakes or seawater (24, 25) to determine which species of bacteria were present. DNA content was assessed in these studies using various nucleic acid stains, including specific fluorescently labeled rRNA probes to isolate different species (26). The cell-cycle progression of *Caulobacter crescentus* populations has also been assessed by measuring DNA content (3). It has been suggested that the forward angle light scatter signal may correlate with cell size or length (27); however, these results were difficult to interpret with bacteria of relatively similar size. Flow cytometric analysis of mean forward scatter of *E. coli* cells has been used to confirm an elongated growth state of the population at low temperatures in liquid culture (28). Together, the results provided impetus for us to examine swarmer cell differentiation using flow cytometry.

To determine if flow cytometry could be used to distinguish swarmer cells and their planktonic counterparts in mixed samples, we evaluated cell samples for DNA staining and forward scatter. The increases seen in the forward scatter histograms (Figure 1, panel c) demonstrate that the differences in length between swarmer cells and undifferentiated (*i.e.*, planktonic) *P. mirabilis*, *E. coli*, and *B. subtilis* are apparent. When DNA fluorescence is assessed by DAPI (4',6-diamidino-2-phenylindole) staining, swarmer cells from *P. mirabilis* (Figure 1, panel d) and *E. coli* (Figure 1, panel e) exhibit an increase in both forward scatter (length) and DAPI fluorescence (DNA content). The values obtained are comparable to those from planktonic cells treated with



**Figure 1. Characterization of swarmer cells by microscopy and flow cytometry.** a) Brightfield and fluorescence images of a DAPI-stained *E. coli* swarmer cell. Inset: Planktonic *E. coli* cells. b) FM 4-64-stained (left) and DAPI-stained (right) *P. mirabilis* swarmer cell. Bars = 0.010 mm. c) Forward scatter histograms of swarmer and undifferentiated bacteria from *P. mirabilis*, *E. coli*, and *B. subtilis* collected on a FACSCalibur flow cytometer. d,e) Planktonic, swarmer, or cephalixin-treated cells were stained with DAPI to highlight the DNA in *P. mirabilis* (panel d) or *E. coli* (panel e) cells and analyzed using a FACSvantage flow cytometer.

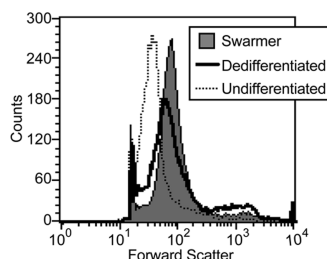
cephalexin, an inhibitor of cell septation, which induces a more homogeneous population of long, multinucleoid cells (29). Next, we tested whether flow cytometry could be used to monitor dedifferentiation of swarmer cells. Swarmer cells can be in-

duced to dedifferentiate when they are removed from the solid surface (plate) and shaken in liquid media. When we compared untreated swarmer cells to those that had been incubated in liquid media for five minutes, partial dedifferentiation was observed

(Figure 2). Dedifferentiation is not an instantaneous process, as the swarmer must form septa and divide to dedifferentiate. Complete dedifferentiation required incubation times of >2 h (data not shown). These results indicate that flow cytometry can be used to evaluate a population of bacteria based on their physical characteristics and that swarmer and planktonic cells can easily be distinguished.

With an assay suitable for identifying compounds with dedifferentiation activity, we investigated how swarmer cells respond to multivalent chemoattractants. The multivalent, glucose-bearing chemoattractants synthesized using ring-opening metathesis polymerization (ROMP) and used in this study (Figure 3, panel a) have been shown to bind chemoreceptors (MCPs) in undifferentiated bacteria (19). To assess their interactions with swarmer cells, we employed fluorophore-conjugated ligands in fluorescence microscopy. *P. mirabilis* swarmer cells were stained with fluorescein-labeled glucose 25mer **3**, and the MCPs were visualized with an anti-MCP antibody specific for the cytoplasmic portion of the MCPs and a Cy3-labeled secondary antibody (Figure 3, panel b). The images indicate that the multivalent glucose compounds do indeed colocalize with the MCPs. The staining pattern is consistent with previous studies indicating that swarmer cells have MCP patches along the length of the cell, not just at the poles as seen in planktonic bacteria (30). We confirmed the specificity of the MCP-polymer interaction by adding either glucose (Figure 3, panel c) or unlabeled glucose 25mer **2** (Figure 3, panel d) to the staining conditions. In the presence of competitor (glucose or compound **2**), the staining due to multivalent **3** was diminished substantially. These results highlight the specificity of the multivalent chemoattractants for the MCPs in swarmer cells.

To test our hypothesis that multivalent chemoattractants could disrupt swarming, we examined their influence on swarmer cell



**Figure 2.** Dedifferentiation of swarmer cells in media as monitored by flow cytometry. Forward scatter histogram of partially dedifferentiated *B. subtilis* swarmer cells compared to untreated swarmer cells and undifferentiated cells. Partially dedifferentiated cells were incubated at 37 °C with shaking in liquid media for 5 min.

differentiation state. We reasoned that if chemoeffectors could influence swarming, the most dramatic effects might be observed with multivalent chemoattractants, because they are not metabolized or taken up by the bacteria and are highly potent

(17). We treated swarmer cells from *B. subtilis*, *E. coli*, and *P. mirabilis* with a concentration of glucose-substituted polymer **2** (calculated as per-saccharide residue concentration) that would stimulate chemotaxis. For comparison, we also employed the monovalent attractant glucose. Samples were exposed to ligand with no shaking for 15–40 min and then subjected to analysis by flow cytometry (Figure 4). The results reveal that the exposure of swarmer cells to a glucose concentration that would induce chemotaxis in planktonic cells has no effect on swarmer cell differentiation. In contrast, treatment with a highly potent chemoattractant such as **2** results in a cell population with a size distribution that is significantly smaller. This change is indicative of dedifferentiation.

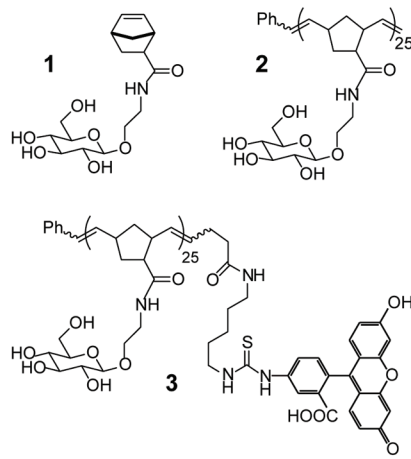
The ability of the multivalent chemoattractants to induce dedifferentiation adds to the evidence linking swarming and che-

motactic signaling. Interestingly, only highly potent multivalent attractants—not their monovalent counterparts—possess the ability to promote swarmer cell dedifferentiation. The unique activity of the multivalent ligands could arise from several factors. For example, the ability of CheY to trigger flagellar motor switching is critical. Based on their potency as attractants, compounds like **2** substantially decrease the concentration of active CheY (e.g., phospho-CheY in *E. coli*) available in the cell. Given the increased levels of flagella in swarmer cells, their requirement for active CheY should be higher. A related issue is the slowness of cells treated with multivalent ligands to undergo adaptation (17). Thus, the influence of these potent attractants on active CheY concentrations and therefore flagellar motor switching persists longer. Finally, although it is not known whether the chemoreceptor lattice plays a role in swarming, high concentrations of chemoattractants can alter the organization of the chemoreceptor lattice (31). Multivalent ligands appear to be especially effective at perturbing this intrinsic protein assembly (20).

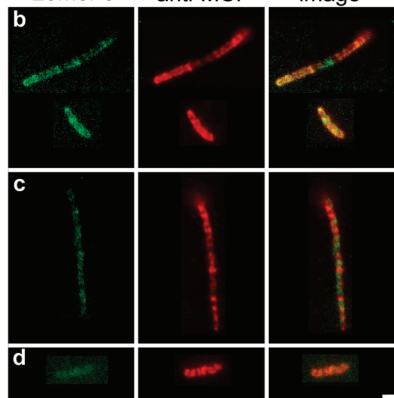
The ability of highly potent glucose-based attractants to elicit dedifferentiation provides impetus to explore further the relationship between chemotaxis and swarming. It also may have physiological implications. Specifically, swarmer colonization is a means to survey rapidly a new environment for nutrients that bypasses traditional chemotaxis. Still, when these bacteria encounter a nutrient-rich surface, it should be beneficial to take advantage of the environmental change by dedifferentiation.

Our data from the flow cytometry assay demonstrate that the chemotaxis pathway can serve as a target for dedifferentiation agents. We anticipate that agents that interfere with this pathway through other means may also disrupt swarming. Because swarming behavior is coupled to the expression of virulence factors, antibiotic resistance, and the production of quorum-

#### a Glucose-bearing chemoattractants

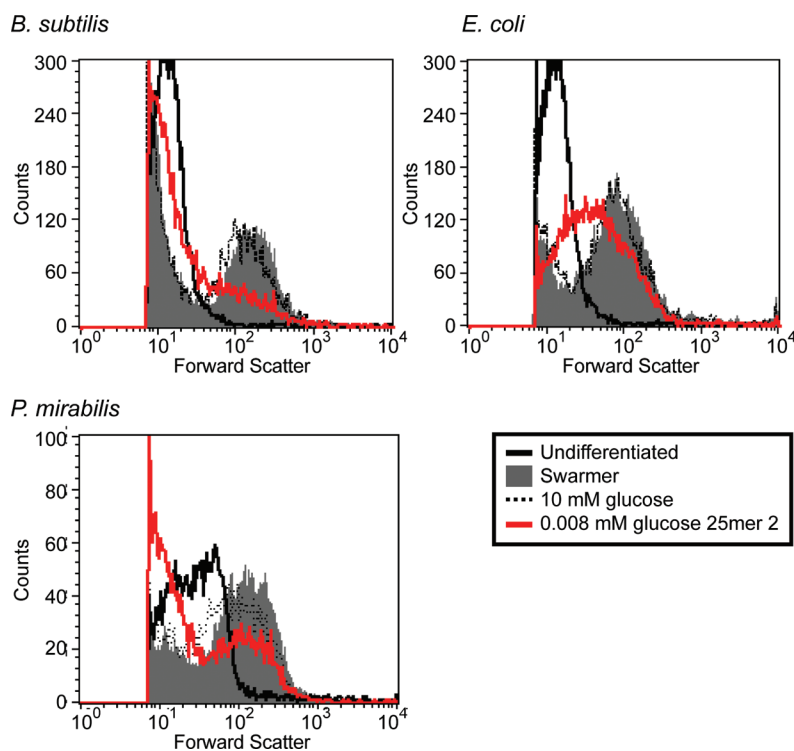


#### fluorescein-labeled glucose 25mer **3**



**Figure 3.** Co-localization of MCPs and fluorescent multivalent chemoattractants. a) Glucose-bearing chemoattractants (**2**, **3**) synthesized by ROMP from monomer **1**. Compound **3** was synthesized from a derivative of **2** using a bifunctional capping agent and further functionalized with fluorescein cadaverine. b) *P. mirabilis* swarmer cells stained with 0.5 mM fluorescein-labeled glucose-substituted 25mer **3** and an anti-MCP antibody. c) *P. mirabilis* swarmer cells stained with 0.5 mM fluorescein-labeled 25mer **3** and an anti-MCP antibody in the presence of 10 mM glucose. d) *P. mirabilis* swarmer cells stained with 0.5 mM fluoresceinated glucose 25mer **3** and an anti-MCP antibody in the presence of 0.5 mM unlabeled glucose 25mer **2**. In the merged images (far right), yellow represents co-localization. Bar = 0.002 mm.





**Figure 4. Dedifferentiation of swarmer cells by multivalent chemoattractants.** *B. subtilis*, *E. coli*, or *P. mirabilis* swarmer cells were treated with either 10 mM glucose or 0.008 mM glucose 25mer 2 for approximately 30 min to induce dedifferentiation. Forward scatter of undifferentiated bacteria are shown for comparison.

sensing signals, the ligands we have described provide a means to investigate multicellular behaviors, such as swarming and biofilm formation (32, 33). Our results provide new avenues to modulate and interfere with these behaviors in bacteria.

## METHODS

**Bacterial Strains and Growth Conditions.** Bacterial strains used include *P. mirabilis* BB2000 (R. Belas), *B. subtilis* O11085 (G. Ordal), and *E. coli* ATCC 25922 (R. Harshey). Undifferentiated cells were grown in Luria–Bertani (LB) liquid medium at 37 °C. Bacteria were induced to differentiate into swarmer cells on plates of LB supplemented with either 1.5% agar at 37 °C (*B. subtilis* and *P. mirabilis*) or with 0.55% agar and 0.5% glucose at 30 °C (*E. coli*). Swarmer cells were harvested by scraping the cells off the agar at the edge of the bacterial colony using a closed glass pipet (23). Cephalixin (0.01 mg mL<sup>-1</sup>) was added to liquid cultures 1 h before harvesting, as previously described (29). For dedifferentiation, cells harvested from swarmer plates were placed in LB

liquid culture with shaking at 37 °C. Chemicals were from Sigma unless otherwise noted.

**Multivalent Ligand Synthesis.** Compounds **1** and **2** were synthesized as described previously (17, 34, 35). Monomer **1** was utilized in ring-opening metathesis polymerization (ROMP) reactions to synthesize polymers **2** and **3** (35). Termination of the polymerization reaction with a bifunctional capping agent (36) provided the means to attach fluorophores, as in compound **3**. Briefly, fluorescein cadaverine was conjugated to the terminal amine revealed by hydrolysis of the ester-capped polymer (36). The valency ( $n = 25$ ) of **2** and **3** is reported here as the ratio between monomer and initiator used in the polymerization. Concentrations are reported as the molar concentration of saccharide.

**Fluorescence Microscopy.** Bacteria were prepared as described previously (19). Briefly, bacteria were harvested and washed in phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde, and allowed to adhere to polyllysine-treated coverslips. They were stained with an antibody (1:400) raised to the cytoplasmic portion of Trg (a generous gift of G. Hazelbauer (37)). MCPs were visualized with a goat anti-rabbit secondary antibody labeled with Cy3 (Molecular Probes). Cells stained with 0.5 mM fluorescein-

ated glucose 25mer **3** were allowed to incubate with the compound for 15 min on ice, in the presence of 10 mM glucose or 0.5 mM unlabeled glucose 25mer **2** where indicated. Live cells were stained with 0.02 mg mL<sup>-1</sup> DAPI (Molecular Probes) in addition to 0.002 mg mL<sup>-1</sup> FM4-64 (Molecular Probes) for 30 min at RT before being placed on slide-mounted pads composed of 0.5% agarose in LB as described previously (38). Bacteria were visualized using a Zeiss AxioScope microscope with the 60X oil-immersion objective and the MetaMorph Imaging System software package (Universal Imaging Corporation).

**Flow Cytometry and DNA Staining.** Bacteria were harvested and washed in PBS, pH 7.1. Each sample contained 0.25 mL ( $A_{600} = 0.083$ ). Cells then were fixed in a HEPES-buffered 1% paraformaldehyde solution for 30 min on ice. DAPI (0.002 mg mL<sup>-1</sup>) was added to cells as noted. DAPI-stained cells were analyzed on a FACSVantage cytometer (Becton Dickinson), and unstained samples were analyzed on a FACSCalibur cytometer (Becton Dickinson). Where noted, 0.008 mM glucose 25mer **2** or 10 mM glucose was added to bacteria and allowed to incubate on ice or at RT for 15–40 min before fixation.

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