Signaling events that occur when cells of Escherichia coli encounter a glass surface

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Methods

Results

Microbial cells organized on solid surfaces are the most ancient form of biological communities. Yet how single cells interact with surfaces and integrate a variety of signals to establish a sessile lifestyle is poorly understood. We developed and used sensitive biosensors to determine the kinetics of second messengers’ responses to surface attachment. This allowed us to examine cell-by-cell variability of the initial signaling events and establish that some of these events depend on flagellar motor function while others do not. Environmentally determined factors, like the energetic status of the cell, can modulate all signaling events. The complex interplay between the surface interaction inputs and external conditions can now be studied using our system.

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Bacterial cells interact with solid surfaces and change their lifestyle from single free-swimming cells to sessile communal structures (biofilms). Cyclic di-guanosine monophosphate (c-di-GMP) is central to this process, yet we lack tools for direct dynamic visualization of c-di-GMP in single cells. Here, we developed a fluorescent protein–based c-di-GMP-sensing system for Escherichia coli that allowed us to visualize initial signaling events and assess the role played by the flagellar motor. The sensor was pH sensitive, and the events that appeared on a seconds’ timescale were alkaline spikes in the intracellular pH. These spikes were not apparent when signals from different cells were averaged. Instead, a signal appeared on a minutes’ timescale that proved to be due to an increase in intracellular c-di-GMP. This increase, but not the alkaline spikes, depended upon a functional flagellar motor. The kinetics and the amplitude of both the pH and c-di-GMP responses displayed cell-to-cell variability indicative of the distinct ways the cells approached and interacted with the surface. The energetic status of a cell can modulate these events. In particular, the alkaline spikes displayed an oscillatory behavior and the c-di-GMP increase was modest in the presence of glucose.

Free-swimming bacterial cells attach to solid supports and initiate a transcriptional program essential for the transition to a sessile lifestyle (1). The trigger event, surface sensing, is not well understood. Many cell surface proteins and cellular appendages have been shown to mediate a response to surface attachment (2). Ideally, one would want to link each such “surface detector” to an initial intracellular signal and then further link that signal to an established long-term response. It is challenging to study how single cells respond dynamically to the surface encounter, mainly because not all initial signals can be identified by genetics and suspected secondary messengers’ dynamics are difficult to detect with high temporal resolution (2).

Single-cell imaging experiments indicate that cyclic di-guanosine monophosphate (c-di-GMP), an established mediator of the planktonic-to-sessile lifestyle transition (3, 4), is also an initial signal to surface attachment (5, 6). The signaling networks that respond to surface attachment are species specific, yet the connection between surface attachment, c-di-GMP increase, and biofilm formation has been demonstrated in several bacteria (3, 4). For Pseudomonas aeruginosa, one of the most studied models of biofilm formation, the signaling networks appear to generate a hierarchical cascade of secondary messengers (7) and result in distinct developmental states within a bacterial population (5, 6, 8, 9). The ability to visually track c-di-GMP concentration changes in individual cells is essential to understanding how various inputs increase the c-di-GMP concentration in different bacteria.

Here, we developed a fluorescent protein–based c-di-GMP-sensing system that allowed us to visualize initial signals to surface attachment in single cells of Escherichia coli and assess the role of the flagellar motor input. E. coli possesses specific challenges to dynamic c-di-GMP detection. In particular, the rapid cell doubling requires fast folding proteins as part of a sensing system, and the low c-di-GMP concentration requires sensing in the 100 to 700 nM range (10, 11). Key to achieving the required sensitivity is a biosensor bright enough to be detected at low intracellular concentrations.

Significance

Microbial cells organized on solid surfaces are the most ancient form of biological communities. Yet how single cells interact with surfaces and integrate a variety of signals to establish a sessile lifestyle is poorly understood. We developed and used sensitive biosensors to determine the kinetics of second messengers’ responses to surface attachment. This allowed us to examine cell-by-cell variability of the initial signaling events and establish that some of these events depend on flagellar motor function while others do not. Environmentally determined factors, like the energetic status of the cell, can modulate all signaling events. The complex interplay between the surface interaction inputs and external conditions can now be studied using our system.

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Balleza et al. (14). For a ratiometric measurement, we coexpressed mScarletI, a bright, rapidly folding red fluorescent protein (19) (Fig. 1B). As a control, we introduced the R113A mutation in MrkH (numbering as in ref. 15) previously shown to eliminate its c-di-GMP–binding ability (15, 16). We thus produced two systems, a biosensor (Sensor) and a mutant biosensor (Sensor*) (Fig. 1B), with the expectation that the former is sensitive to c-di-GMP while the latter is not and thus serves as a control for fluorescence changes unrelated to c-di-GMP concentration changes.

The Biosensor Responds to c-di-GMP Concentration Changes in Motile E. coli. We created cell populations with low c-di-GMP concentrations (expressing the phosphodiesterase PdeH) and high c-di-GMP concentrations [expressing a constitutively active form of the diguanylate cyclase WspR (20)] and measured the ratio (R) of mVenusNB to mScarletI fluorescence emission for individual cells (Fig. 1C). We found that the average R value for cells that express the Sensor is ∼2-fold higher in the low c-di-GMP than in the high c-di-GMP regimen. This is consistent with our expectation that the c-di-GMP–bound biosensor has reduced mVenusNB fluorescence. In contrast, cells that express the mutant biosensor (Sensor*) have similar average R values between the two c-di-GMP regimes (low and high c-di-GMP concentrations, Fig. 1C and SI Appendix, Fig. S1A). This indicates that Sensor, but not Sensor*, responds to changes in c-di-GMP concentration and that the dynamic range of our sensing system is at least twofold.

Note that E. coli with unperturbed levels of c-di-GMP registers between the low and high c-di-GMP regimes (Fig. 1D). In addition, cells that lack stators (ΔmotAmotB) and are therefore not motile have lower than wild-type intracellular c-di-GMP (higher average R ratio, SI Appendix, Fig. S1B). This suggests that both increases and decreases in the c-di-GMP concentration could be detected with our system.

The Biosensor and the Mutant Biosensor Are pH Sensitive. YFPs with insertions suffer from pH sensitivity in the physiological pH range (17). In contrast, mScarletI fluorescence, as well as MrkH c-di-GMP–binding ability, are not sensitive to pH in that range (13, 19, 21) (SI Appendix, Fig. S3). We collapsed E. coli internal pH with the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and found that both biosensors (Sensor and Sensor*) are pH sensitive in the 7 to 8 pH range (SI Appendix, Fig. S2A, green and gray symbols, respectively). Therefore, fluorescence changes that occur for both biosensors likely reflect changes in internal pH, while changes that occur only in the Sensor’s fluorescence correspond to changes in c-di-GMP concentration. The pH sensitivity is in line with previous reports, such as refs. 17 and 22. However, the pKₐ of our sensors (∼7.7) is close to E. coli’s internal pH (∼7.6) (23–26), making them sensitive to physiological pH changes. Note that the Sensor platform exhibits similar pH sensitivity for both the low c-di-GMP and the high c-di-GMP regimens (SI Appendix, Fig. S2A, red and black symbols, respectively), possibly indicating that the two states (c-di-GMP bound and c-di-GMP unbound) have similar pH sensitivities.

Using Sensor* as a pH indicator, we found that internal pH decreased when the cells were transferred from tryptone broth (TB) to motility buffer (Methods and SI Appendix, Fig. S2B).
The Sensor’s R values varied accordingly between TB and motility buffer for both the low c-di-GMP and the high c-di-GMP regimens (Fig. 1D).

The c-di-GMP Concentration Increases after E. coli Surface Attachment.

To detect changes in the intracellular c-di-GMP concentration following surface attachment, we imaged cells attached to the coverslips of premade channel slides (from Ibidi, see Methods). We acquired mVenus^NB and mScarletI fluorescence using our total internal reflection fluorescence (TIRF) microscope setup for cells resuspended in motility buffer with 10 mM lactate as an external energy source (Fig. 2A). Following rapid attachment (less than 1 min), images were acquired every 15 s. Fig. 2B shows the average R ratios for cells expressing either Sensor (black symbols) or Sensor* (red symbols) as a function of time. The decrease observed for Sensor-expressing cells, but not for Sensor*-expressing cells, is consistent with an increase in c-di-GMP within the first 5 min of surface attachment. Interestingly, the c-di-GMP increase did not occur in the presence of 40 mM potassium benzoate and 40 mM methylamine (SI Appendix, Fig. S4), which dissipate the transmembrane pH gradient. Thus, the c-di-GMP increase depends on the presence of this proton concentration gradient. Note that the modest R decrease for Sensor*-expressing cells (Fig. 2B, red symbols) and for both Sensor- and Sensor*-expressing cells in SI Appendix, Fig. S4 is consistent with mVenus^NB bleaching at a faster rate than mScarletI during the experiment (SI Appendix, Fig. S5A).

Among individual cells, the signal descent (c-di-GMP increase) is heterogeneous, with some cells descending rapidly (within 1 min), some gradually, and some not at all. To display this heterogeneity and to directly compare individual traces of Sensor- and Sensor*-expressing cells, we fitted each trace to an exponential function \( R_0e^{-\alpha t} \) and plotted the descent constant \( \alpha \) (min^−1) versus the initial \( R_0 \) value. Notice that decent constants for most Sensor*-expressing cells (Fig. 2C, red circles) cluster around zero, while decent constants for Sensor-expressing cells (Fig. 2C, black circles) are heterogeneous. The Sensor-expressing population with low \( R_0 \) values (high c-di-GMP) and small decent constants (Fig. 2C, green dashed circle) suggested the possibility that some cells increase c-di-GMP within the first minute of surface attachment. We imaged Sensor-expressing cells every 10 s as they attached to the coverslip (Fig. 2D) and found, indeed, that some cells decreased their R value (increased their c-di-GMP concentration) within the first minute of surface attachment (Fig. 2D), in contrast to Sensor*-expressing cells (SI Appendix, Fig. S6). Interestingly, some of the Sensor-expressing cells that decreased their R value abruptly also “spiked” just before the decrease (Fig. 2D). Cells expressing Sensor* also spiked during the experiment (SI Appendix, Fig. S5B).
The Intracellular pH Spikes after *E. coli* Surface Attachment. To further evaluate these spikes, we acquired images with high temporal resolution (every 0.5 and/or 1 s) for both Sensor- and Sensor*-expressing cells. During the first 5 min of surface attachment, we detected spikes for both cell types. This suggests that the spikes represent pH changes, specifically, pH increases (alkaline spikes). The spikes’ duration, amplitude, and position vary widely from cell to cell. Some cells display well-defined narrow spikes, while others exhibit broad and/or low-amplitude events. Fig. 3 is an example of “spectacular” spiking, with data acquired every half second following cell attachment. Spikes with full width at half maximum (FWHM) under 2 s (blue trace), along with a spike with FWHM of ~15 s (red trace), can be seen. Fine structure can also be observed in many of the larger spikes (such as the red trace), suggesting that the larger spikes might result from temporal superposition of several more basic events. Note that at this acquisition rate (every 0.5 s), the main reason for the global decrease in R values is mVenusNB bleaching faster than mScarletI (SI Appendix, Fig. S5B).

Approximately 50% of the cells spike in the first 3 min of attachment, and only around 10% of cells spike after they have been attached for 5 min (SI Appendix, Table). This suggests that alkaline spiking is a transient event that occurs in the initial stages of surface attachment.

When 40 mM potassium benzoate and 40 mM methylamine were added to the motility buffer, no alkaline spikes were observed—as expected, since potassium benzoate and methylamine clamp the cytoplasmic pH to the external pH (SI Appendix, Fig. S7).

The Alkaline Spikes Display Periodicity and the c-di-GMP Increase Is Modest in Motility Buffer with Glucose. When glucose, rather than lactate, was used as the external energy source, elaborate, persistent spiking events were observed (Fig. 3B). Many spiking events were periodic with a ~20-s period (Fig. 3B), resembling the glycolytic pH oscillations observed in *Saccharomyces cerevisiae* (27). “Glucose spiking” is more persistent than “lactate spiking,” since many cells still display periodic spiking events 5 min after surface encounter (SI Appendix, Table). Like the lactate spikes, the glucose spikes were not observed when benzoate and methylamine were added to motility buffer (SI Appendix, Fig. S8). Thus, both spiking events are blocked by a pH clamp.

Cells attaching in lactate motility buffer increased the intracellular c-di-GMP concentration within 5 min of finding the surface (Fig. 2B). In contrast, we detected only a modest increase in intracellular c-di-GMP for cells attaching in glucose motility buffer. Specifically, the average R ratio decreased for both Sensor- and Sensor*-expressing cells when attaching in glucose buffer (SI Appendix, Fig. S9A), consistent with a decrease in intracellular pH. However, the R ratio decrease was more pronounced for Sensor- than for Sensor*-expressing cells, suggesting a modest increase in intracellular c-di-GMP in addition to the decrease in internal pH. To assay this differently and eliminate the impact of mVenusNB bleaching during the time course of the experiment, we measured R values for cell populations that have just found the surface and compared them to R values of cell populations that have been attached for 5 to 6 min at opposite ends of the channel slide. We found that Sensor* cells have lower R values after 5 to 6 min attachment but similarly shaped distributions (SI Appendix, Fig. S9B and C), consistent with a decrease in internal pH. The average R value for Sensor-expressing cells also decreased (SI Appendix, Fig. S9B). However, the distribution of R values tightened, suggesting that the initial population (broad distribution and diverse c-di-GMP concentrations) became more homogenous as it modestly increased the c-di-GMP concentration and decreased the cytosolic pH (SI Appendix, Fig. S9C, black and gray circles).

Note that individual traces show a wide range of behavior, with R values abruptly decreasing, R values spiking and gradually decreasing, and R values spiking with little downward trend (SI Appendix, Fig. S9D).

Flagellar Motor Function Is Required for c-di-GMP Increase in Lactate Buffer. Genetic analysis in *E. coli* revealed that motility is critical to normal biofilm formation (28). In addition, flagellar motor activity was shown to be essential for the c-di-GMP response in *Caulobacter crescentus* and *P. aeruginosa* (6, 29).

To address the role of the flagellar motor function in the initial surface-sensing events, we imaged cells that lacked stator unit function (cells with genomic deletions of either *motB* or both *motA* and *motB*). Flagella assemble in these cells, but they do not rotate, so the cells are not motile. We found that the
average R value decreased for both the Sensor- and the Sensor*-expressing cells, consistent with a decrease in pH following attachment (Fig. 4). There was no significant difference between the two average traces (Fig. 4), consistent with a lack of c-di-GMP increase within the attached cell population. In addition, as shown in Fig. 4, Inset, individual cell behavior is similar between the Sensor- and the Sensor*-expressing cells. Motor function is thus required for both maintaining intracellular pH and increasing the c-di-GMP concentration following attachment.

Images acquired every 1 s show that ΔmotAmotB cells display alkaline spikes when attached to glass coverslips (SI Appendix, Fig. S10). Therefore, E. coli does not require functional flagellar motors for the generation of alkaline spikes. However, ΔmotAmotB cells did not display clear, periodic events in glucose motility buffer (SI Appendix, Fig. S11), suggesting that the motor’s function is important for the complex spiking behavior of wild-type cells in glucose buffer.

Discussion

When bacterial cells attach to surfaces, a variety of “surface detectors” generate intracellular signals that inform the altered motility state (2). c-di-GMP stands out as an initial signal to surface encounter as well as a longer-term surface-sensing signal integrator. Indeed, its concentration increases within minutes of P. aeruginosa and C. crescentus surface attachment (6, 29, 30). In addition, c-di-GMP is essential to both forming and maintaining a biofilm structure in various bacteria (4).

Here, we developed a bright fluorescent protein–based biosensor to determine the kinetics of c-di-GMP concentration changes that follow surface attachment in single E. coli. Our sensing system, which consists of c-di-GMP–responsive and c-di-GMP–unresponsive platforms (Fig. 1B), informs us of changes in intracellular c-di-GMP as well as intracellular pH. We detected an increase in the c-di-GMP concentration within minutes of E. coli attachment to coverslips (Fig. 2D), consistent with reports from other species (6). In addition, transient pH increases (alkaline spikes) were observed within seconds of the surface encounter. These spikes can be immediately followed by an increase in c-di-GMP (Fig. 2D), which suggests that the alkaline spikes may trigger this increase. Indeed, DgcB, the C. crescentus enzyme responsible for c-di-GMP increase following attachment (29), has a pH-dependent activity that increases at alkaline pH (31). However, in our experimental setup, alkaline spikes per se are not sufficient to trigger the c-di-GMP rise, since cells lacking stator units do spike (SI Appendix, Fig. S10), yet they do not increase intracellular c-di-GMP (Fig. 4). As previously suggested (29), functional flagellar motors may be distinctively required to generate a higher-amplitude, local increase in pH (see next paragraph, SI Appendix, Supplementary Note) necessary to activate a motor-associated diguanylate cyclase.

How are the pH spikes generated? Similar pH transients have been observed in S. cerevisiae, in which they appear to be linked to glycolytic oscillations (27), in the mitochondrion as “mitoflash” (32, 33), and in plants (34, 35). While the mechanism is not well understood, the spikes are thought to be generated by imbalances in the energy metabolism that trigger a pH increase followed by a compensatory mechanism that returns intracellular pH to its homeostatic value (36). How could surface attachment trigger an increase in intracellular pH? One possibility is that an extracellular pH increase at the attachment interface (37) triggers an increase in intracellular pH (26), which is then brought back to the set pH (generating a spike) by the metabolic enzymes involved in pH homeostasis. Such spikes could be generated in both motile and nonmotile cells and would depend on the nature of the attachment surface (37). An intracellular pH spike could also be generated by stalling the flagellar motor through flagellar interactions with the surface, followed by a similar compensatory mechanism. Indeed, at 100 Hz rotation, a flagellar motor translocates around 50,000 protons per second (38, 39). Even though this is a small contributor to the total proton influx (38), the stall can produce measurable pH spikes, particularly if there is a delay in the proton homeostasis feedback mechanism (SI Appendix, Supplementary Note). A spike generated by the second mechanism would require functional flagellar motors and could display a higher amplitude in the vicinity of the motor.

We detected only a modest increase in intracellular c-di-GMP concentration for cells attaching in glucose motility buffer (SI Appendix, Fig. S9). Glucose inhibits E. coli biofilm formation (40, 41), and this effect is mediated in part by the decreased intracellular cyclic AMP (cAMP) that results from glucose utilization as a carbon source (40, 41). Interestingly, for P. aeruginosa, a CAMP increase is thought to precede and trigger the c-di-GMP increase (7, 42). P. aeruginosa initial attachment events do occur on a longer timescale than our attachment experiment (hours versus minutes) (7). However, the same hierarchical second messenger cascade (cAMP, then c-di-GMP) may occur for E. coli surface attachment, too. Alternatively, cAMP may play a permissive, carbon source–informative role in E. coli biofilm formation (43).

The periodic alkaline spikes observed in the glucose motility buffer (Fig. 3B) are striking and reminiscent of the pH oscillations observed in S. cerevisiae during glycolysis (27). Distinctly, though, E. coli need not be starved to trigger such oscillations, since we immediately transferred the cells from growth media to the buffer containing glucose. Why, then, would the internal pH oscillate upon attachment? Glycolytic enzymes do oscillate together with the internal pH (27, 44), and the key enzyme phosphofructokinase is pH sensitive (27). An initial transient increase in pH following surface attachment could trigger an oscillatory behavior of the glycolytic enzymes and of the internal pH.

Both the pH spiking behavior as well as the c-di-GMP increase display a lot of cell-to-cell variability (Figs. 2C and 2D).
and 3). The variability might be due, in part, to the heterogeneity of the E. coli–surface interactions, since some cells approach and interact with the surface over their entire length, while other cells interact closely only at the poles (as seen in our TIRF images, Fig. 24). It would be interesting to establish if this heterogeneity persists to create a low c-di-GMP population and a high c-di-GMP population, as for P. aeruginosa (5).

Methods

Strains and Plasmids. The motile MG1655 strain was used as a parent strain for all the experiments. motB, motA and motB deletions were made using the Datsenko and Wanner method and the pKD3 plasmid (45). Following site-directed recombination to eliminate the chloramphenicol resistance gene, an 85-bp scar was left in place of the disrupted gene. Deletions were verified using PCR and Sanger sequencing of the PCR products.

deh and wspRD70E were amplified using PCR either from MG1655 cells or, respectively, from the pCLOA-T7-wspD70E plasmid (Addgene No. 79164) (20). The resulting fragments were ligated into a pTrc99A backbone (46) for expression from the inducible hybrid trplac promoter.

mrkH (codon optimized, as in ref. 15) was amplified from a plasmid provided by Maria Schumacher (Duke University School of Medicine, Durham, NC); mVenusNSHNVY (green letters represent mVenus amino acids, blue letters represent MrkH amino acids, and GSG is the linker sequence). The ribosome-binding sites were the T7 phase ribosome binding site (for mScarletI expression) and the GGAGACAGAC nucleotide sequence between the stop codon (TAA) of mScarletI and the start codon (ATG) of mVenusNSHNVY for biosensor expression. The R113A mutation was introduced by PCR followed by the NEBuilder HiFi assembly method.

Cell Preparation and Experimental Setup. Cells for microscopy were grown in tryptone broth (TB, 10 g/L Bacto-Tryptone, 5 g/L sodium chloride) at 30°C from single colonies and inoculated into fresh TB media with necessary antibiotics at 1:1,000 dilution. The cultures were grown to 28 to 29°C for 35 to 45 s, and then 200 μl buffer was added to the reservoir to dilute the unattached population. Image acquisition was started ~1 min after cell addition to the channel slide. The second approach was preferred because signal to noise was better under that scheme (cells were already attached when image acquisition was started and the background was spatially uniform).

CCCP was used at 100 μM in 20 mM potassium phosphate and 100 μM EDTA buffers of various pH. Potassium benzoate and m ethylamine were used together, each at a concentration of 40 mM.

Microscope Setup and Imaging Assay. We used a Nikon Eclipse Ti-U microscope in TIRF mode with a 514-nm laser and an Apo 60x oil (numerical aperture 1.49). The emission was split and projected onto two halves of an Andor iXon camera. Exposure settings were the same for all the acquired channels of the camera. The camera exposure was at 200 ms, and the camera gain at 200. Images were acquired every 0.5 s or every 1 s for better resolution of the alkaline spikes. Note that in this regimen, bleaching is the dominant reason for the R ratio decrease, since mVenusNSHNVY bleaches faster than mScarletI. To minimize bleaching and assess longer-term changes in the R ratio, we acquired images every 10 or 15 s.

Image Analysis. The acquired images were processed in ImageJ (47). The background was subtracted from each stack; alignment was performed using linear stack alignment with Scale Invariant Feature Transform (SIFT) (48). Corresponding mScarletI and mVenusNSHNVY signals were measured and the measurements were processed and analyzed in Excel and MATLAB.

Data Availability. All study data are included in the article and/or SI Appendix.

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Channel slides from Ibidi (μ-Slide VI 0.5 Glass Bottom) were used for microscopy. The channels (40 μL volume) were coated with 0.1% poly-L-lysine solution (Sigma) and washed with at least 2 mL water. Cells were added either directly in growth media or after being washed two times and resuspended in motility buffer at OD600 0.5. Motility buffer is 10 mM potassium phosphate, 100 μM ethylenediaminetetraacetic acid (EDTA), and 10 mM lactate (or 0.2% glucose) pH 6.9.

A total of 200 μL cells were added to the channel reservoir, and image acquisition was started immediately (for imaging cells as they attached). Alternatively, 35 μL cells were added to the channel slide, cells were attached for ~45 s, and then 200 μL buffer was added to the reservoir to dilute the unattached population. Image acquisition was started ~1 min after cell addition to the channel slide. The second approach was preferred because signal to noise was better under that scheme (cells were already attached when image acquisition was started and the background was spatially uniform).

Pseudomonas aeruginosa: 2011