Guiding Bacteria with Small Molecules and RNA

Shana Topp and Justin P. Gallivan*

Contribution from the Department of Chemistry and Center for Fundamental and Applied Molecular Evolution, Emory University, 1515 Dickey Drive, Atlanta, Georgia 30322

Received December 22, 2006; E-mail: justin.gallivan@emory.edu

Abstract: Chemotactic bacteria navigate their chemical environment by coupling sophisticated information processing capabilities to molecular motors that propel the cells forward. The ability to reprogram bacteria to follow entirely new chemical signals would create powerful new opportunities in bioremediation, biomanufacturing, and synthetic biology. However, the complexities of bacterial signaling and limitations of current protein engineering methods combine to make reprogramming bacteria to follow novel molecules a difficult task. Here we show that by using a synthetic riboswitch rather than an engineered protein to recognize a ligand, E. coli can be guided toward and precisely localized to a completely new chemical signal.

Introduction

Chemotactic bacteria navigate complex chemical environments by coupling sophisticated information processing capabilities to powerful molecular motors that propel the cells forward.\(^1\)\(^-\)\(^3\) A general method to reprogram the ligand sensitivity of the bacterial chemo-navigation system would enable the production of cells that autonomously follow arbitrary chemical signals, such as pollutants or disease markers. Equipping bacteria that can degrade pollutants,\(^4\) synthesize and release therapeutics,\(^5\) or transport loads\(^6\) with the additional ability to localize to a specific chemical signal would open new frontiers in bioremediation, drug delivery, and synthetic biology. For example, such cells could be engineered to follow specific pollutants in soil and to degrade them. Alternatively, such cells could be engineered to selectively target small-molecule signals of disease, thus providing a powerful drug-delivery system.\(^7\) However, the complexity of the bacterial chemosensory system makes reprogramming a cell to follow a completely new chemical signal a formidable challenge.

\textit{Escherichia coli} recognize chemoattractants using five transmembrane receptor proteins, which cluster with one another\(^8\)\(^-\)\(^11\) and interact with a set of well-characterized cytosolic proteins to effect changes in the directional rotation of the flagellar motor.\(^3\)\(^,\)\(^12\) In the absence of a chemical gradient, individual \textit{E. coli} execute a random walk characterized by smooth runs punctuated by tumbles that often result in a change of direction.\(^1\)\(^-\)\(^3\) When a cell moves up a chemoattractant gradient, the flagellar motor preferentially rotates counterclockwise, resulting in less frequent tumbling and longer runs toward the attractant; when the chemoattractant concentration becomes constant, the cell resumes a random walk.\(^2\) Similarly, when a cell moves down a chemoattractant gradient, the tumbling frequency increases. A defining feature of \textit{E. coli} chemotaxis is that individual cells do not simply respond to the absolute concentration of a stimulus at a given time. Rather, individual \textit{E. coli} cells integrate the concentration of a chemical stimulus over a 1–3 s period\(^2\) and migrate up attractant gradients by adjusting the frequency of tumbling based on the concentration differences between these time points.

In principle, bacteria can be programmed to respond to a new chemical signal by engineering an existing chemoreceptor protein to recognize a new ligand. Although \textit{E. coli} have only 5 chemoreceptor proteins, they perform chemotaxis toward greater than 30 compounds,\(^1\) indicating that some chemoreceptors recognize multiple compounds. A recent effort to engineer the Tar (aspartate) receptor\(^13\) produced relatively modest changes in ligand specificity, consistent with the tendency of engineered proteins to display broadened rather than shifted ligand specificities.\(^14\)\(^,\)\(^15\) Although it may be possible to produce more dramatic changes in receptor specificity using rational design, directed evolution,\(^13\) or computational methods,\(^16\) such efforts are ultimately limited by structural constraints enforced by the receptor scaffolds and the need to interface with the existing...
signaling network. Thus, engineering cells to follow a new stimulus presents a difficult molecular recognition problem. Faced with these challenges, we sought to bypass the chemoreceptors entirely by developing an effective, generic stimulus that presents a difficult molecular recognition problem.

Figure 1. (Top) Proteins involved in wild-type E. coli chemotaxis. The direction of rotation of the flagellar motor is controlled by the protein CheY. When CheY is not phosphorylated, the flagellar motor rotates counterclockwise (CCW). When CheY is phosphorylated (CheY-P), it binds to the flagellar motor protein FlIM, causing the cell to tumble. Wild-type E. coli can migrate on semisolid agar (top right; cells grown for 10 h at 37 °C). (Bottom) Cells lacking the protein CheZ (strain RP1616) cannot dephosphorylate CheY-P and these cells tumble incessantly (bottom right; cells grown for 10 h at 37 °C). Cartoon adapted from ref 34.

Figure 2. Model for how the theophylline-sensitive synthetic riboswitch controls the translation of the CheZ protein. In the absence of theophylline (left), the mRNA adopts a conformation in which the ribosome binding site is exposed and CheZ is expressed, thus allowing the cells to run and tumble. Model adapted from ref 28.

We anticipated that using a ligand-inducible expression system to control the production of CheZ would enable us to guide cells toward higher concentrations of a new ligand in a...
process known as pseudotaxis. This system would differ from classical *E. coli* chemotaxis in that cell motility toward the new ligand would be dictated by the absolute ligand concentration at a specific time, rather than the concentration differences between two points in time.

Key to these efforts is the development of a tunable ligand-inducible expression system that can precisely control the production of the CheZ protein. Although a variety of bacterial expression systems are ligand-inducible, many of these, such as the lac repressor and the araC transcriptional regulator, use proteins to recognize ligands. Consequently, engineering these protein-based expression systems to respond to new ligands presents many of the same challenges that make engineering the chemoreceptor proteins difficult. In contrast to inducible expression systems that use proteins to recognize ligands, riboswitches control gene expression in a ligand-dependent fashion by using RNA aptamers to recognize ligands. Using powerful in vitro selection techniques, it is possible to generate aptamers that tightly and specifically recognize new ligands without the need for a pre-existing RNA scaffold. We and others have shown that a variety of aptamers generated by in vitro selection can be engineered into synthetic riboswitches that regulate gene expression in a ligand-dependent fashion. Here we show that a synthetic riboswitch can guide *E. coli* toward a new, nonmetabolized ligand without protein engineering. These reprogrammed cells migrate preferentially up a ligand gradient and have the unique ability to localize to a specific chemical signal, which enables precise spatial patterning. We anticipate that the ability to engineer cells to follow new chemical signals will provide new opportunities in bioremediation and synthetic biology.

**Results and Discussion**

Because *E. coli* lacking a single gene in the signaling pathway (strain RP1616, ΔcheZ) tumble incessantly and are essentially nonmotile (Figure 1, bottom), we first asked whether a synthetic riboswitch could restore ligand-dependent motility to *E. coli* RP1616 by activating the translation of CheZ in response to the alkaloid theophylline (Figure 2). Reprogramming a cell to follow theophylline is a challenging test: Theophylline is neither chemoattractive, nor extensively metabolized, and because it is structurally dissimilar to natural chemoreceptor ligands, which are predominantly amino acids, dipeptides, and sugars, it would be difficult to reengineer a chemoreceptor to selectively bind it.

To investigate ligand-inducible motility, we introduced the *cheZ* gene under the control of a theophylline-sensitive synthetic riboswitch into *E. coli* RP1616 cells (hereafter referred to as “reprogrammed cells”), plated the reprogrammed cells onto semisolid media containing various ligand concentrations, and measured their migration radius after 10 h (Figure 3a). The distance that the cells migrated increased as a function of ligand concentration until reaching a maximum at 2 mM theophylline, after which further concentration increases led to cell death (Figure 3b). When theophylline was replaced with caffeine, which is structurally similar but does not bind to the riboswitch, the reprogrammed cells were nonmotile (Figure 3b), demonstrating that motility changes are riboswitch-dependent.
E. coli grammed cells recapitulate many features of chemotactic changes in motility are observed in this concentration range. This behavior is consistent with the data in Figure 3b, in which significant changes in motility are observed in this concentration range.

Taken together, the data show that populations of reprogrammed cells recapitulate many features of chemotactic E. coli, including the ability to migrate up a ligand gradient. However, unlike wild-type E. coli, which rarely stop moving, populations of these engineered cells do not migrate in the absence of theophylline. To investigate the mechanisms that influence cell migration and to explain the phenotypic similarities and differences between the reprogrammed cells and wild-type E. coli, we assessed the ability of these cells to perform chemotaxis toward a natural chemoattractant in the presence of theophylline, as well as their ability to respond to various static concentrations of theophylline in the absence of chemoattractant gradients.

To determine if theophylline restored chemotaxis toward natural chemoattractants, we plated reprogrammed and wild-type cells onto semisolid minimal-media plates that presented a gradient of the chemoattractant L-aspartate and a static theophylline concentration (0 or 2 mM). As expected, reprogrammed cells do not perform chemotaxis toward aspartate in the absence of theophylline (Figure 5). When theophylline is present, however, the reprogrammed cells perform chemotaxis toward aspartate in a manner similar to wild-type cells. Taken with the data in Figure 3b, these results indicate that the reprogrammed cells exhibit stronger chemotaxis toward natural chemoattractants at higher static theophylline concentrations, suggesting that the theophylline concentration may serve to establish the steady-state tumbling frequency of these cells. Thus, chemo-navigation is achieved, in part, because the riboswitch acts as a molecular brake. In the absence of theophylline, the brake is engaged and the reprogrammed cells tumble in place. Addition of theophylline releases the brake in a dose-dependent fashion by inducing the expression of CheZ, which allows the cells to navigate gradients of natural chemoattractants.

To investigate additional mechanisms influencing taxis, we used video microscopy to observe and track the motility of individual reprogrammed cells in rich liquid media containing various static theophylline concentrations. Because rich liquid media largely eliminates chemoattractant gradients, the effects of different theophylline concentrations can be studied independently of the gradient-induced chemotaxis that occurs on semisolid media. The tracking data reveal that a prime determinant of the population behavior is a sharp concentration-dependent rise in the fraction of motile cells (Figure 6a). At low theophylline concentrations, most cells tumbled in place, consistent with the behavior of cells lacking CheZ. At higher concentrations, a greater fraction of the population was motile, consistent with theophylline-induced expression of CheZ and a transition from a tumbling to a running phenotype. These observations help explain both the theophylline-dependent increases in motility on semisolid media (Figure 3b) and the gradient-sensing behavior seen in Figure 4.

To test whether motile cells exhibit pseudotaxis toward theophylline, we determined the average maximum run speeds (Figure 6b) and tumbling frequencies (Figure 6c) for the motile populations of the wild-type and the reprogrammed cells as a function of theophylline concentration. Although motile cells from both strains showed modest theophylline-dependent changes in maximum run speed (Figure 6b), only the reprogrammed cells showed dramatic changes in tumbling frequency (Figure 6c). Whereas wild-type E. coli maintain a steady-state tumbling
frequency of approximately 0.5 s\(^{-1}\) regardless of the absolute concentration of a chemoattractant,\(^{18}\) reprogrammed cells adopt lower steady-state tumbling frequencies at higher theophylline concentrations, which allows cells to migrate up theophylline gradients via pseudotaxis.

Because reprogrammed cells migrate further at higher ligand concentrations and essentially stop migrating at low concentrations, we anticipated that these cells might also navigate paths containing the ligand. To test this idea, we plated cells onto semisolid media patterned with the various compounds shown in Figure 7a. Wild-type cells migrate radially without regard for the patterned compounds (Figure 7b), whereas RP1616 cells lacking the riboswitch are essentially nonmotile (Figure 7c). In contrast, the reprogrammed cells exclusively follow the theophylline-containing path and eschew the other paths (Figure 7d). This behavior is possible because, unlike wild-type E. coli, reprogrammed cells become less motile as they move down a concentration gradient and, in the limit, are nonmotile. Thus, these cells have the unique ability to localize to a specific target, which may be particularly useful for targeting cells to a specific disease site for applications in biomedicine.

The reprogrammed cells described here capture many of the features displayed by naturally chemotactic bacteria such as gradient sensing, but do so through different mechanisms. Unlike natural chemotaxis in which cells detect gradients by integrating the concentration differences between chemoattractants at two points in time, reprogrammed cells respond to theophylline gradients via pseudotaxis, in which the motility of a cell changes as a function of the local theophylline concentration. Because motility changes are mediated by protein synthesis induced by the synthetic riboswitch, these cells respond to changes in the concentration of new ligands more slowly than wild-type cells, which respond to chemoattractants over a period of seconds by adjusting the methylation state and the degree of clustering of the chemoreceptor proteins. Although the response rate of our reprogrammed cells to new ligands is slower than the natural chemotaxis response of E. coli, these cells show unique behaviors that are not displayed by wild-type bacteria. As an example, reprogrammed cells become less motile as the ligand concentration decreases, which allows these cells to not only detect gradients of a new ligand (Figure 4), but also to precisely target a patterned ligand (Figure 7). Finally, because the displacement of a reprogrammed cell is proportional to the ligand concentration and cells that migrate the farthest are easily identified in large populations, we anticipate that motility-based screens using synthetic riboswitches may provide a powerful and inexpensive technique to detect the production of small molecules through biocatalysis.

**Conclusion**

In summary, we demonstrated that E. coli can be reprogrammed to detect, follow, and precisely localize to a completely new chemical signal by using a synthetic riboswitch, rather than a protein, to recognize a ligand. These reprogrammed cells not only retain the gradient sensing behavior of chemotactic E. coli, they also have the unique ability to localize to a specific chemical signal. Because ligand recognition in the reprogrammed cells is performed by RNA aptamers, which can be selected to recognize new compounds\(^{24–26}\) and incorporated into synthetic riboswitches using established methods,\(^{27–31}\) we anticipate that it will be straightforward to reprogram bacteria to follow a variety of new ligands for applications in bioremediation and medicine. This new ability to equip motile bacteria with a precise and tunable chemo-navigation system greatly enhances the impressive arsenal of natural and engineered cell behaviors.

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**Supporting Information Available:** Full experimental procedures and sample Quicktime movies of tracked bacteria. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Material

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Shana Topp and Justin P. Gallivan*

Department of Chemistry and Center for Fundamental and Applied Molecular Evolution, Emory University, 1515 Dickey Drive, Atlanta, GA 30322

*To whom correspondence should be addressed.
email: justin.gallivan@emory.edu, phone: 404-712-2171, fax: 404-727-6586

Experimental

General Considerations. Synthetic oligonucleotides were purchased from Integrated DNA Technologies. Culture media was obtained from EMD Bioscience. Amino acids were purchased from Sigma. Ampicillin was purchased from Fisher. DNA polymerase, restriction enzymes, and the pUC18 cloning vector were purchased from New England Biolabs. The pGFPuv and pDsRed-Express vectors were purchased from Clontech. Purifications of plasmid DNA, PCR products, and enzymatic digestions were performed using kits from Qiagen. Plasmid manipulations were performed using E. coli TOP10F’ cells (Invitrogen) that were transformed by electroporation. All new constructs were verified by DNA sequencing at the NSF-supported Center for Fundamental and Applied Molecular Evolution at Emory University. Experiments were performed with E. coli strains RP437 and RP1616, both of which were kindly provided by J. S. Parkinson.

Plasmid Construction. The cheZ gene was cloned from E. coli TOP10F’ cells using PCR. A cassette containing the tac promoter, a theophylline-sensitive riboswitchS1, and the cheZ gene was assembled using PCR and was subcloned into the BamHI and SacI sites of SKD1248, a derivative of pUC18 (ApR), to create the riboswitch-containing plasmid. A fluorescent reporter gene, GFPuv, flanked by a constitutively active promoter and a transcriptional terminator was

amplified from the plasmid pGFPuv and subcloned into the SapI site of the riboswitch-containing plasmid.

**Dose-Dependent Migration on Semi-Solid Media.** To perform the migration experiments shown in Figure 3, selective media (tryptone broth with 0.25% agar, 50 µg/mL ampicillin, and various concentrations of theophylline) was prepared in Petri dishes (85 mm dia.). Diluted cell suspensions from mid-log-phase cultures (1.5 µL, ~2×10^5 cells/µL) were applied to the center of the plates, which were dried in air for 15 min, and incubated at 37 °C for 10 h. The migration radii were determined by measuring the diameter of the outermost ring of growth, dividing by two, and subtracting the radius of migration of non-motile RP1616 cells grown under the same conditions. The motility of the parent strain (RP437) was unaffected by concentrations of caffeine or theophylline below 2 mM; above this concentration, motility was decreased as a result of cell death.

**Gradient Response to Theophylline on Semi-Solid Media.** Macroscopic motility experiments using a gradient of theophylline were performed as above, with the following modifications: Media was prepared in 100 mm square Petri dishes. Layers (15 mL) of selective 0.25% agar containing theophylline (1 mM, 0.25 mM, 0 mM) were poured in the pattern shown in Figure 4. Each layer was allowed to solidify for 50 min before applying the following layer. After all layers were applied, the media was allowed to equilibrate at room temperature for 3.5 h, after which diluted cell suspensions from mid-log-phase cultures (3 µL, ~2×10^5 cells/µL) were applied to the locations shown in Figure 4. The plates were dried in air for 15 min, and the cells were grown at 37 °C for 13.5 h.

**Gradient Response to Aspartate on Semi-Solid Minimal Media.** Square Petri dishes (100 mm) were filled with 50 mL of M9 minimal media containing 0.25% agar, 2% (v/v) glycerol, 50 µg/mL ampicillin, and 1 mM each of methionine, leucine, histidine, and threonine. Plates also contained a static concentration of theophylline (0 mM or 2 mM). After two hours, 40 µL of a 10 mM solution of L-aspartate dissolved in M9 salts was evenly distributed along a thin line in the center of each plate, as indicated in Figure 5. The plates were left at 21 °C for 3.5 h to permit diffusion of aspartate. Cells were grown to mid-log-phase in selective tryptone
broth (50 µg/mL ampicillin). Cells were pelleted at 4,000 rcf and resuspended with M9 salts. After two washes with M9 salts, diluted suspensions of cells (2.0 µL, \( \sim 2 \times 10^5 \) cells/µL) were applied approximately 13 mm from the center of the plates. For each plate, the reprogrammed cells were spotted to the left of center, while wild-type cells were spotted to the right of center. Plates were dried in air for 10 min and incubated at 30 °C for 16 h.

**Microscopic Behavior on a Glass Surface.** To track the behavior of individual cells, cells were grown to mid-log-phase (\( \text{OD}_{600} = 0.55 \)) in selective tryptone broth containing various concentrations of theophylline. A square (~1 cm/side) was drawn on a glass microscope slide using a wax pencil, the cell suspension (3 µL) was placed at the center of the square, and a glass coverslip was placed on top of the wax to seal the chamber. Samples were imaged in two dimensions using differential interference contrast microscopy (DIC) with an inverted microscope (Leica) equipped with a C.C.D. camera and a 40× objective (N.A. = 1.4). A red filter was used to improve DIC images and to protect cells from high-intensity blue light. Digital images at the surfaces of the coverslip and the slide were captured at 30 Hz and the positions of individual cells were identified using macros within the Interactive Data Language (IDL)\(^{30}\). Cells that were visibly stuck to the glass surface or were tracked for less than 1 s were discarded from further data analysis. To differentiate motile cells from non-motile cells within the complex populations, the mean-square displacement (MSD) of each cell was determined at lag times of 0.2 s and 0.6 s. A cell was considered motile if the following three conditions were met: MSD \( \geq 4.7 \) µm at 0.2 s; MSD \( \geq 9.4 \) µm at 0.6 s; MSD at 0.6 s \( \geq 2.5 \times \) MSD at 0.2 s. These values were determined empirically by examining the behavior of wild-type (RP437, motile) and \( \Delta \text{cheZ} \) (RP1616, non-motile) cells.

Determining the fraction of cells that contribute to the overall motility of the population is complicated by the fact that motile cells appear on both the top and bottom of the chamber, while dead and non-motile cells tend to collect on the bottom of the chamber. At low theophylline concentrations, most cells on the bottom of the chamber are non-motile, while at high concentrations, a large fraction of these cells are dead. While these cells do not move in either case, their existence impacts the motility of the population as a whole. To account for this contribution, data were collected from locations on both the top and bottom of the chamber for
equal lengths of time, and the fraction of motile cells in the combined population was determined as a function of theophylline concentration for wild-type and reprogrammed cells (Figure 6a).

To determine the behavior of motile cells, data were collected on the top of the chamber, where cell motion was generally not obstructed by dead or non-motile cells. To minimize tracking errors, the x and y coordinates of each track were independently filtered by calculating the mean of the 3 median points of a 5 point moving window as previously described \(^28\). Using this filtered data, the maximum run speed of each track was calculated by averaging the top 10% instantaneous velocities in the track. A cell was considered to be tumbling when its instantaneous velocity was less than 30% of its maximum run speed, and its rate of change of direction (RCD) exceeded 5.9 rad/s for at least 2 consecutive frames. The tumbling frequency of the motile population was determined by dividing the total number of tumbling events by the total tracking duration summed over all tracks. The run speeds and tumbling frequencies are shown as a function of theophylline concentration for wild-type and reprogrammed cells (Figure 4).

**Supporting Video.** Supporting videos of reprogrammed cells in the presence of theophylline are available: 0.05 mM theophylline: SW_0_05mM.mov; 0.25 mM theophylline: SW_0_25mM.mov; 2.0 mM theophylline: SW_2_00mM.mov.

**Spatial Localization on Semi-Solid Media.** To perform the spatial localization experiments shown in Figure 7, selective media (tryptone broth with 0.25% agar, 50 µg/mL ampicillin) was prepared in Petri dishes (85 mm dia.). After solidification of the media, solutions of caffeine or theophylline (10 µM in tryptone broth), or tryptone broth alone, were applied in the pattern shown in Figure 7a by spotting with a micropipet (1 µL/mm), and the plates were air-dried for 90 min. Diluted cell suspensions from mid-log-phase cultures (2 µL, \(~2\times10^5\) cells/µL) were applied at the location shown in Figure 7a, the plates were dried in air for 15 min, and incubated at 37 °C for 10 h. Plates were imaged on a Kodak transilluminator with NEN filters #25 (red) or #61 (green). False color (red or green) was applied using ImageJ; adjustments to brightness and contrast were performed to the whole image using Adobe Photoshop 8.0.