Programmable bacteria detect and record an environmental signal in the mammalian gut

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The mammalian gut is a dynamic community of symbiotic microbes that interact with the host to impact health, disease, and metabolism. We constructed engineered bacteria that survive in the mammalian gut and sense, remember, and report on their experiences. Based on previous genetic memory systems, we constructed a two-part system with a “trigger element” in which the lambda Cro gene is transcribed from a tetracycline-inducible promoter, and a “memory element” derived from the cl/Cro region of phage lambda. The memory element has an extremely stable cl state and a Cro state that is stable for many cell divisions. When Escherichia coli bearing the memory system are administered to mice treated with anhydrotetracycline, the recovered bacteria all have switched to the Cro state, whereas those administered to untreated mice remain in the cl state. The trigger and memory elements were transferred from E. coli K12 to a newly isolated murine E. coli strain; the stability and switching properties of the memory element were essentially identical in vitro and during passage through mice, but the engineered murine E. coli was more stably established in the mouse gut. This work lays a foundation for the use of synthetic genetic circuits as monitoring systems in complex, ill-defined environments, and may lead to the development of living diagnostics and therapeutics.

Significance

The human microbiota represents the trillions of bacteria that live on the skin, in the oral, nasal, and aural cavities, and throughout the gastrointestinal tract. The species that live in the gastrointestinal tract, the gut microbiota, closely interact with host cells and have a profound impact on health. To develop tools to effectively monitor the gut microbiota and ultimately help in disease diagnosis, we have engineered Escherichia coli to sense and record environmental stimuli, and demonstrated that E. coli with such memory systems can survive and function in the mammalian gut. This work demonstrates that E. coli can be engineered into living diagnostics capable of nondestructively probing the mammalian gut.


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Here, we show that *Escherichia coli* engineered with a synthetic memory system based on the phage lambda Cl/Cro genetic switch can sense and record antibiotic exposure during passage through the mouse gut. This work lays the foundation for the use of synthetic genetic circuits in living diagnostics.

**Results**

To engineer a bacterium to record an environmental signal in the mammalian gut, we set the following design specifications: (i) the initial "nonmemory" state should be highly stable, only failing as a result of mutation of the system; (ii) the "memory" state should also be highly stable; (iii) the engineered elements should be integrated into the chromosome rather than on plasmids to minimize the chance of loss, and (iv) the engineered elements should not impose a large fitness burden on the host (Fig. 1A).

We used the well-characterized cI/cro genetic switch from bacteriophage lambda (22–24) to construct a memory element for the circuit (Fig. 1B). Natural selection has already tuned the repressed cI state to be so stable that in an induction-deficient cI<sup>mid</sup>-lysogen; the repressor state only fails because of spontaneous mutation of cI and not to fluctuations in cI protein levels (25). The presence of a lambda prophage causes little burden on the bacterial host as only 100–200 Cl monomers per cell are present in a lysogen (26, 27). The well-characterized wild-type tetA promoter (tetP) was placed upstream of cro to generate a trigger element (Fig. 1C). The Tn10 tetracycline repressor is particularly sensitive to anhydrotetracycline (ATC), such that a low dose of 100 ng/mL ATC will cause full derepression of the promoter without inhibiting growth of tetracycline-sensitive *E. coli* (28).

To identify a well-behaved memory element, we constructed more than 10 candidate memory elements with the general structure as shown in Fig. 1B, based on the elements of cI/cro regulation (22, 29). We inserted a DNA fragment from phage lambda from the left operator (O<sub>L</sub>), including the *rexAB* genes, cI, and cro, upstream of *lacZ*, these replaced lacI. The constructs should thus reproduce exactly the elements of cI expression, including the interaction between the O<sub>L</sub>-O<sub>R</sub> operator sites (29) and the natural downstream genes and terminators of the cI transcript, which may influence mRNA stability (Table 1 and Figs. S1–S4).

*E. coli* MG1655 was also engineered to contain a trigger element driving Cro expression. tetP was placed upstream of cro, and a trigger element consisting of CAM<sup>+</sup>–<sup>-</sup>tert-R-tetP-cro was integrated between the divergent *araC* and *araB* promoters (Fig. S5) to minimize transcriptional effects from outside the element. When the concentration of cI falls below about 10% of its steady-state value in a lysogen (30), lambda switches from the lysogenic to lytic state, which leads to derepression of the *P<sub>R</sub>* promoter and expression of Cro. When Cro levels reach about 100 molecules per cell, the activity of the *P<sub>R</sub>M* promoter decreases (30, 31). In the presence of Cro-mediated *P<sub>R</sub>M* repression, about four cell divisions are required for cI to be sufficiently diluted to allow switching from the cI state to the cro state (12, 23, 29). Thus, we expected that if tetP is induced via ATC for four consecutive cell divisions the memory element will switch from the cI state to the cro state.

The candidate memory elements were chromosomally inserted by "recombinering" into strain TB10, which automatically sets the element into the cro state (27). Unexpectedly, the Cro state of lambda prophages was unstable and not to fluctuations in cI protein levels (25). The trigger and memory elements were not significantly deleterious to growth of *E. coli* in diverse conditions, as inferred from competitive growth experiments in mixed cultures with the parental strain of *E. coli* (Fig. 2E) and growth curves (Fig. S7). Multiple independent mixed cultures with an initial ratio of about 1:1 *E. coli* MG1655<sup>strR</sup> and PAS132 were subcultured with and without ATC for about 50 cell divisions, and titered on indicator plates to distinguish the two strains. The change in ratios of parent cells to engineered cells varied from culture to culture, but did not show a consistent overgrowth of parental cells (Fig. 2E). This observation suggests that in some cultures a spontaneous mutation enhancing growth under the conditions tested was arising in one strain or the other, and outgrowing the culture (33), rather than a fitness effect because of our engineered
behaved similarly to the engineered K12 strain PAS132 in vitro, their environment. Mouse weight was not affected by antibiotic treatment or administration of PAS132 (34); some mice also received ATC (0.1 mg/mL) in the water. About 10^7 bacteria were administered by oral gavage. Fecal samples were collected and titered on MacConkey lactose plates with streptomycin to select for PAS132, and on brain-heart infusion (BHI) plates (anaerobic) to titer culturable microbes. All of PAS132 isolated from mice that were given ATC stably switched from the cI state to the Cro state within 1 d of exposure (<1/10^6 Lac^-) (Fig. 3B). The culturable endogenous gut flora began recolonizing the gut as soon as the streptomycin treatment ended, and the titer of the engineered bacteria decreased slowly thereafter (Fig. 3 C and D).

PAS132 in ATC-treated mice remained in the Cro state for more than a week after termination of ATC treatment. After 24 h of ATC exposure, at which point 100% of the PAS132 in the mouse gut had switched to the Cro state (Fig. 3B), ATC was removed from the drinking water. More than 50% of the surviving PAS132 maintained a stable Cro memory state after more than a week in the mouse gut without further exposure to ATC (Fig. 3B).

In separate in vivo experiments, mice given ATC after PAS132 had colonized the mouse gut and streptomycin was removed (Fig. 3E). Again, PAS132 switched from the cI state to the Cro state within 24 h and remained in the Cro state for several days (Fig. 3E). This result indicates that PAS132 cells that have already colonized the gut are able to record subsequent changes to their environment. Mouse weight was not affected by antibiotic treatment or administration of PAS132 (Fig. S9), indicating that these bacteria are not grossly deleterious to their host. After ATC removal, there was not sufficient ATC in the filtered fecal samples to activate the memory circuit in cultured PAS132. Tetracycline (Tc) is undetectable in the serum, kidneys, and liver of female mice after less than 8 h of administration (35). Thus, the ATC was likely cleared from the mouse when we evaluated our engineered bacteria for memory at later times.

The genetic memory circuit functioned essentially identically in an uncharacterized coliform bacterium from the mouse gut. From an untreated mouse we isolated a bacterium that fermented lactose on MacConkey Lactose plates, and confirmed that its 16S ribosomal RNA matched that of E. coli (Fig. 4A and Fig. S10). PlvIR transduction was used to insert the memory circuit, trigger, and streptomycin resistance mutation into this isolate from natural gut flora, termed NGF-1. The engineered NGF-1 strain (PAS133) behaved similarly to the engineered K12 strain PAS132 in vitro, registering ATC exposure within 4 h (Fig. 4B).

PAS133 sensed and remembered exposure to ATC in the mouse gut. Female Balb/C mice were given PAS133 and treated as described above. Cells were collected and analyzed as above for lacZ expression on lactose indicator streptomycin plates. PAS133 detected ATC exposure within 1 d, and remembered exposure of mice to ATC for more than 7 d after ATC withdrawal (Fig. 4C). Moreover, PAS133 stably colonized the mouse gut longer than PAS132, the K12-derived strain. Although we administered roughly equal amounts of PAS132 and PAS133, after 1 d in the mouse we
recovered 10-fold more PAS133 per milligram of fecal sample (Fig. 4D). Between 5 and 8 d postinoculation, the PAS133 population stabilized to around 1,000 colony-forming units per milligram, which was comparable to the coliform titers in most of the pretreated mice obtained from Charles River Laboratories over the course of these experiments. In contrast, PAS132, the K12 based-strain, was almost completely outcompeted by the natural gut flora after 8 d.

Discussion

Microbe-based recording systems have the potential to be used as diagnostics in health care, environmental monitoring, and other applications. In this work, we sought to construct a bacterium that could sense and record exposure to a chemical cue, ATC, in the mammalian gut. Based on the strengths and limitations of previously constructed genetic memory systems, we built a system in E. coli strains based on the bistable lambda cl/Cro switch (4–10, 12–14, 17–23, 31). This circuit was designed to begin in the cl state and switch to theCro state upon induction of a “trigger” element in which a tetracycline-responsive promoter directs transcription of a cI gene. We demonstrated that the cl andCro states were stable both in bacterial cultures and when the bacteria were passaged through the mouse gut. Switching from the cl to the Cro state occurred efficiently upon exposure to ATC in E. coli in laboratory culture or in the mouse gut. In addition, the entire circuit was transferred from E. coli K12 to an uncharacterized murine E. coli strain; in this context the system behavior was virtually identical, but the engineered murine strain was more stably established in the mouse gut compared with the engineered K12 strain.

DNA rearrangement, such as deletion, is an alternative method for genetic information storage, with the obvious advantage that the rearranged state is stable. Developmental biologists previously used trigger elements based on expression of a FLP recombinase from a promoter of interest, followed by permanent excision of a DNA to mark the event in daughter cells for fate-mapping (18, 19). These studies were carried out in the mouse, in which the expression of developmental regulatory proteins is tightly controlled and thus false-positive deletions because of low-level expression of FLP may have been avoided. Mammalian genes are generally highly cooperative, in part a result of self-reinforcing states of chromatin that involve large numbers of adjacent nucleosomes. Eu-bacteria lack these mechanisms and it remains to be seen if the “transfer function” relating levels of FLP protein expression to DNA excision is well-behaved.

In the context of a microbial gene-based sensor, stability of the starting state is particularly important so that false-positives can be avoided, and low-level stochastic expression from a trigger element must not be enough to flip the switch. The natural lambda-epigenetic switch is extremely stable. Gimble and Sauer (25) noted that lambda-lysogens with a DNA damage-insensitive lambda-epigenetic switch is extremely stable. Gimble and Sauer (25) noted that lambda-lysogens with a DNA damage-insensitive cI* mutation only spontaneously induce via inactivating a mutation in cI. Thus, “epigenetic failure,” which might be the result of a rare stochastic failure to express enough cI protein or segregate it evenly to both daughter cells, was not observed. Natural selection may have acted on this system to be particularly tightly regulated, with a highly cooperative structure defining the repressed state, involving eight cI proteins bound to operators left (O-L) and right (O-R) with DNA looping (29).

The use of the lambda cl/Cro system as a memory element also had the advantages that it imposed a low burden on the host and was largely predesigned by nature. In the lysogenic state, the 256- AA lambda cI protein is only present in 100–200 copies per cell (23), whereas the 66-A A Cro protein is likely present in <1,000 copies per cell (31); thus, the burden of expressing these proteins was expected to be minimal. We found no detectable selective burden on hosts carrying the trigger-memory system in either epigenetic state (Fig. 2F).
environmental detection and recording, this behavior will often

be adequate because the presence of state-switched bacteria will
defINITIvEly indicate that a detection event has occurred.

When orally administered to mice and then recovered, E. coli
cells carrying the memory circuit responded to ATC administrated
to the mice by switching the memory element into the Cro state.
This result occurred efficiently in several experimental permuta-
tions: for example, ATC was administered for varying periods
during or after establishment of the bacteria in the gut. The com-
plete circuit worked in both E. coli K12 and in an uncharacterized
E. coli strain isolated from a mouse during the course of the ex-
periments. The cI state was completely stable, and Cro state was
stable for many days in the context of the mouse gut. It is par-
cularly noteworthy that the memory system behaved as desired in an
uncharacterized murine bacterium infected with this bacterium, which colonized the gut more stably than E. coli K12. These results indicate
that artificial genetic circuits can be designed and characterized in
well-understood but attenuated laboratory strains, and then
transferred to a related isolate from the environment of interest.

The ability to engineer bacteria to report on environmental cues
in variable, incompletely understood environments has implica-
tions for the development of living diagnostics and therapeutics.
For example, bacteria native to the human gut or skin might be
used to monitor for exposure to chemicals characteristic of specific
disease states. The system described herein is sufficiently modular
that the trigger and memory circuits could be reengineered to
respond to chemical signatures of inflammation, cancer, par-
asites, or environmental toxins in the gut. In combination with
additional genetic circuits, such as the recent search-and-destroy
circuits (36–38), cells could be designed with the memory ele-
ment described here to diagnose a specific pathogen, and emit
a therapeutic. Together, these approaches may allow construction
of a new class of engineered probiotic bacteria that serve as benign
and transient diagnostics and therapeutics.

Methods

Strain Construction. The chromosomally integrated memory and trigger ele-
ments were constructed by a combination of commercial synthesis (Genscript)
and PCR amplification of component elements from source DNAs, assembly in
vitro through overlap extension PCR (39), and introduction directly into E. coli
TB10 (40) by recombination without plasmid intermediates (32). A sponta-
neous high-level streptomycin-resistance mutation was isolated in MG1655
and confirmed to be rpsL (lys24arg) (41, 42). Memory and trigger elements and
the rpsL mutation were moved between strains by P1vir transduction (43).

Memory element 14 consists of a kanamycin-resistance cassette trans-
scribed away from cI and cro, and phage lambda sequences from 35561 to
38241, including the cDTR mutation (44). This DNA was inserted between
bases 366802 and 365529 in the E. coli K12 MG1655 genome (45). The
resultant construct contains a constitutive promoter downstream of the mhp
gene upstream of lacI, but lacks sequences from the lacI promoter up to the
start codon for lacZ. These genes are replaced by phage sequences, including
Pm, Om, rexA, rexB, rexC, cI, Om, and cro through the cro stop codon, such that lacZ
is now transcribed from Pm after cro. Memory elements 11–13 were con-
structed similarly (Figs. S1–S4).

The trigger element consists of a chloramphenicol-resistance cassette, a
tet-tetP segment from Tn10 that includes the divergent tetracycline pro-
moters, and the cro gene transcribed from the teta promoter. This segment
was inserted into the MG1655 genome at base 70165, in a CAP binding site
between araB and araC promoters to minimize aberrant read-through from
external promoters (Fig. 5S).

Induction of Cro Expression with ATC (Triggering). Overnight M9 glucose
cultures of engineered bacteria in were diluted 100-fold into the same
medium with or without ATC (100 ng/mL) and grown aerobically with shaking
for up to 6 h. For each condition, at least three cultures were tested. At the
indicated times, aliquots were diluted and plated on M9 glucose X-gal plates
to evaluate the fraction of cells that switched from the cI state to the cro state
in response to ATC.

Maintenance of the Cro State in the Absence of Inducer (Memory). PAS132 that
had been induced into the cro state by prolonged exposure to ATC were
pelleted, washed, resuspended in M9 glucose media without ATC, and grown
aerobically with shaking for up to 5 d, with 1,000-fold dilutions performed
every 8 h. At the indicated times, aliquots were plated on M9 glucose X-gal
plates to evaluate the fraction of cells that remained in the cro state. PAS132 continuously grown in the presence of ATC or never exposed to ATC were used as controls.

Competition Growth Assays. To compare the relative growth rates of engineered and nonengineered bacteria, mixed cultures were grown as follows. Six overnight cultures of MG1655RpsL and six of PAS132RpsL were grown in M9 glucose or BHI media, from which six mixed cultures were created in the same media with and without 100 ng/mL ATC. Cultures were transferred on indicator plates immediately after mixing, and after 5 d of daily growth to saturation followed by 10-fold dilution.

Analysis of Engineered Bacteria in Mouse Fecal Samples. Because E. coli administered orally to mice will generally not colonize the gut unless the engineered bacteria is present, fecal samples were collected from acclimated female BALB/c mice (Charles River Laboratories) on days 1, 4, and 8, weighed, solubilized in 0.85% NaCl, and titrated on anaerobic BHI plates and on aerobic MacConkey Lactose Streptomyacin plates at 37 °C. On day 8 the mice were fasted overnight and given water with 5% sucrose, 0.5 mg/mL streptomycin (34), with or without 0.1 mg/mL ATC. On day 9, 10e11 engineered bacteria were administered by oral gavage, and food was returned. On day 10 the streptomycin was removed from the water. On day 11 the ATC and sucrose were removed from the water. Throughout all experiments, mice were fed a grain-based chow without lactose. Each mouse experiment represents the cumulative data from eight mice; four without and four with ATC. For some experiments, fecal samples were tested for residual activity by spotting filter-sterilized solubilized fecal samples onto indicator plates spread with PAS132; no ATC activity was observed. The animal protocol was approved by the Harvard Medical Area Standing Committee on Animals, protocol 04966.

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SI Methods

To determine the ratio of *Escherichia coli* in the cI and Cro states in a population, we titered the *E. coli* to yield at least 100 single colonies on lactose indicator plates, and also plated at a 100-fold lower dilution, yielding at least 10,000 colonies. On the latter plates colonies could not be counted but it was possible to recognize rare colonies of a distinct phenotype. Thus, in Figs. 2–4 and Fig. S6, where it is stated that 100% or 0% of cells have a certain phenotype, the limit of detection is about 1/10^4.
Fig. S1. Memory element in PAS129.
Fig. S3. Memory element in PAS131.
Fig. S5. The tetP-cro trigger element used in this study.
Fig. S6. Identification of a memory element with optimal switching properties. (A) Memory elements 11–14, which were integrated into strains PAS129-PAS132, were evaluated for switching in response to anhydrotetracycline (ATC). (B) PAS129-PAS132 were evaluated for switching in response to an incubation temperature of 42 °C, without ATC. For both panels, points represent the means ± SD of three or more independent samples.
Fig. S7. Growth curves of engineered strains in brain-heart infusion (BHI) media. (A) Growth of MG1655 and PAS132 in BHI media without ATC. (B) Growth of MG1655 and PAS132 in BHI media with ATC. (C) Growth of NGF1 and PAS133 in BHI media without ATC. (D) Growth of NGF1 and PAS133 in BHI media with ATC. (E) Growth of PAS132 and PAS133 in BHI media without ATC. (F) Growth of PAS132 and PAS133 in BHI media with ATC. (G) Growth of MG1655 and PAS132 in M9 glucose + casamino acids media without ATC. (H) Growth of MG1655 and PAS132 in M9 glucose + casamino acids media with ATC. (I) Growth of PAS132 and PAS133 in M9 glucose + casamino acids media without ATC. (J) Growth of PAS132 and PAS133 in M9 glucose + casamino acids media with ATC.
Fig. S8. Sequence of the rpsL mutation. The rpsL gene of PAS132 and MG1655 was amplified using 5′-CCA GCC AGA TGG CCT GG-3′ and 5′-GAC GCG ACG ACG TGG C-3′ primers, then sequenced. The sequences were compared using Lasergene software to identify the A430G mutation that resulted in a Lys42Arg mutation.
Fig. S9. Monitoring mouse weights. Mice were weighed on the indicated days to monitor their health. A drop in total body mass >20% would indicate that there was a potential health concern. From day 1 to day 18 of all in vivo experiments, all of the mice showed a net gain in total body mass. This gain indicated that administering two drugs, streptomycin and ATC, as well as our engineered bacteria, did not adversely affect mouse health. (A) In vivo experiment #1 corresponds to data presented in Fig. 3B. (B) In vivo experiment #2 corresponds to data presented in Fig. 3D. (C) In vivo experiment #3 corresponds to data presented in Fig. 4. Points represent the mass of an individual mouse on the specified day.
Fig. S10. Alignment of the 16S Sequence of PAS132 and PAS133 with MG1655. The gene encoding the 16S ribosomal subunits of PAS132 and PAS133 were amplified by PCR, then sequenced (1). The sequences were aligned against the reference sequence of MG1655 using Lasergene software. A phylogenetic tree was constructed comparing the reference sequences of the indicated bacteria using Lasergene software.