Tunable Low-Rate Genomic Recombination with Cre-*lox* in *Escherichia coli*: A Versatile Tool for Environmental Biosensing and Synthetic Biology

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ABSTRACT

The ability to induce heritable genomic changes in response to environmental cues is valuable for environmental biosensing, for experimentally probing microbial ecology and evolution, and for synthetic biology applications. Site-specific recombinases provide a route to genetic memory via targeted DNA modifications, but their high specificity and efficiency are offset by leaky expression and limited tunability in prokaryotes. We developed a tightly regulated, titratable Cre recombinase system for *Escherichia coli* that achieves low recombination rates and minimal basal activity. Implemented on both plasmids and the chromosome, the latter showed superior retention of genetic memory across generations. These features make the system broadly useful for environmental biosensing and other applications. To demonstrate applicability to environmental biosensing, we developed a whole-cell recombination-based biosensor for arsenite, a toxic and ubiquitous pollutant that is primarily mobilized in anoxic environments such as flooded soils, sediments, and aquifers. However, existing arsenite whole-cell biosensors face limitations in sensitivity and workflow in anaerobic settings. Our biosensor reliably recorded anoxic arsenite exposure as a stable genetic memory for delayed fluorescence readout in aerobic conditions, with detection sensitivity comparable to conventional wet chemical methods. By decoupling exposure from measurement, this approach offers a foundation for arsenite biosensing under field-relevant conditions, including redox variability and other physicochemical gradients, without the constraints of anoxic measurement. More broadly, the ability to induce low-rate, heritable genetic changes expands the genetic toolkit for environmentally responsive systems, with applications in environmental monitoring, bioproduction, bioengineering, as well as experimental studies of microbial ecology, evolution, and host-microbe interactions.

IMPORTANCE

Arsenic is a toxic and globally prevalent pollutant, mobilized primarily under anoxic conditions where detection is challenging. Whole-cell biosensors offer a promising route for monitoring bioavailable arsenic *in situ*, but their development has largely focused on aerobic conditions, with anoxic assays limited by sensitivity and workflow constraints. Genetic tools that enable heritable, low-frequency genomic changes in bacteria can expand biosensor capabilities by recording transient exposures and supporting applications in environmental monitoring, synthetic biology, and quantitative microbial population dynamics research. Here, we developed a tightly regulated, chemically inducible Cre-*lox* system in *Escherichia coli* that enables recombination at low, tunable rates. We demonstrate its utility by constructing an arsenite biosensor that reliably detects low concentrations and records exposures under both aerobic and anoxic conditions. This approach is broadly applicable for biosensors designed for field deployment and for experiments investigating microbial ecology and evolution, where controllable genetic diversification may be desirable.

KEYWORDS

Recombinase, Biosensor, Genetic Memory, Genetic Switch, Arsenic, Bioavailability

INTRODUCTION

Controlling microbial responses to environmental signals is central to developing biosensing strategies suited to complex environmental settings (1) and to developing and validating quantitative models of microbial ecology and evolution (2–4). Environmental biosensors designed to detect chemical contaminants would benefit from systems capable of stably recording transient exposures, particularly in field conditions where continuous monitoring is impractical. Similarly, tools that enable phenotypic or genotypic diversification at controlled rates can be used to simulate mutational processes (3), track lineages, engineer controlled diversification (2), or probe community responses to controlled perturbations. All of these can be used to test ecological and evolutionary theory. Both use cases require genetic systems that can induce heritable changes at low, tunable rates in response to specific environmental inputs.

Recombinase-based systems offer a powerful approach for designing environmental biosensors (5,6) because site-specific recombination produces permanent, heritable DNA changes that can serve as a genetic record of transient environmental exposure without the need for continuous signal input. This capability is particularly valuable for field deployment, where exposures may occur under conditions that are challenging for real-time measurement (e.g., anoxic environments or fluctuating physicochemical gradients) and where sample analysis often takes place well after collection. For example, a recombinase-based biosensor for arsenite could detect exposure events in anoxic settings relevant to arsenic mobility (7) and allow the record to be read out later under aerobic conditions, avoiding the need for specialized anaerobic measurement setups (8). These practical advantages, combined with the ability to tune recombination rates, make recombinases well-suited for environmental monitoring and other applications. To realize this potential, it is important to understand and overcome their current limitations in bacterial systems.

Site-specific recombinases and nucleases enable heritable genetic changes by allowing targeted genome modifications at specific loci *in vivo*. These enzymes can be used to induce reversible or irreversible phenotypic changes, often coupled to reporter systems such as fluorescent proteins. Among them, Cre is a tyrosine recombinase that recognizes two short DNA sequences, called *lox* sites, and catalyzes recombination between them with high specificity and efficiency. When two *lox* sites are oriented in the same direction in the same DNA molecule, Cre excises the DNA between them (9,10). Depending on the configuration of *lox* sites, Cre can also mediate DNA inversion, integration, or translocation, expanding its utility in genetic circuit design. The resulting genotype is heritable and maintained even after Cre expression is interrupted. This feature, shared by other recombinases such as Flp and Bxb1, has been exploited in synthetic genetic logic gates and circuits with a memory component (6,11). Unlike nucleases such as CRISPR-Cas, recombinases do not rely on host double-strand break repair machinery, allowing them to carry out precise recombination events autonomously. This characteristic is particularly advantageous in prokaryotes like *E. coli*, where DNA repair options, though varied, predominantly depend on homology-directed mechanisms that may be less efficient or more error-prone. Because recombinase-mediated DNA changes are permanent and do not require ongoing expression, Cre-*lox* systems offer a robust means of encoding long-term memory of environmental inputs.

Despite these advantages, applying recombinase systems like Cre in *E. coli* remains challenging, particularly in scenarios that require precise control over recombination rates. In bacteria, recombinases have direct access to chromosomal and plasmid DNA, and even minimal basal expression can lead to rapid and irreversible switching in a large fraction of the population. This makes it difficult to implement systems that require slow, partial, or tunable transitions, which are valuable for experiments involving controlled genotypic diversification (2), lineage tracing (3), or temporally integrated biosensing (6,12,13). In eukaryotic hosts, recombinase activity can often be tightly regulated through compartmentalization or ligand-dependent nuclear import. For instance, the yeast Mother Enrichment Program uses β-estradiol to control Cre nuclear import via a fusion with a hormone-binding domain, enabling tight regulation of recombinase activity (14). In contrast, *E. coli* lacks these regulatory layers, making transcriptional leakiness a critical issue. Recent work by Williams *et al.* (15) demonstrated that the effective rate of Bxb1-mediated differentiation can be tuned by controlling integrase expression levels. Most existing implementations of Cre have been optimized for rapid and complete switching, leaving a need for systems that support persistent, low-frequency recombination under precise regulatory control. To control Cre recombinase activity in bacteria, Sheets *et al.* (16,17) constructed and characterized different variants of an optogenetic system that regulates it at the post-translational level. Although using a light signal to regulate Cre activity brings benefits such as temporal control over recombination, it also limits the experimental setup that can be employed, due to the necessity of controlling sample illumination at all times. These illumination requirements restrict deployment outside the lab and make the approach ill-suited to applications such as environmental biosensing.

Here, we developed and characterized titratable Cre-*lox* reporters with low background expression in *E. coli*. By regulating Cre at both the transcriptional and post-translational levels and implementing these systems either on plasmids or on the chromosome, we enable phenotypic switching at low, chemically inducible rates. This makes them suitable for a range of uses beyond traditional synthetic biology applications, such as metabolic engineering and biosensing, and extends to experiments in cell physiology, ecology, and evolutionary biology.

We demonstrate the broad applicability and advantages of these constructs by developing a Cre-recombinase-based biosensor for quantification of bioavailable arsenite, and compare its performance to more conventional transcription-based fluorescent reporters. Arsenic (As) is a widespread environmental contaminant that poses major risks to both human health and ecological systems. In anaerobic environments such as waterlogged rice paddies, sediments, and groundwater aquifers, microbial processes mobilize arsenic primarily in its trivalent form, arsenite (As(III)), which is highly toxic and mobile. Detecting arsenite under these conditions is critical for understanding its biogeochemical cycling (7) and assessing exposure risks. However, relatively few whole-cell arsenite biosensors have been developed for anaerobic conditions assays (18), and existing ones show lower sensitivity than aerobic systems employing oxygen-dependent fluorescent reporters (19,20), largely due to the reduced brightness of flavin-based fluorescent proteins. Moreover, they require fluorescence measurements to be performed within anaerobic chambers, or potentially *in situ* under anoxic conditions. These constraints make their deployment more challenging than measurements conducted in aerobic conditions after exposure. To address this gap, we engineered a Cre-recombinase-based biosensor that can record transient arsenite

exposure under anoxic conditions, preserve this information in the genome, and allow delayed readout under aerobic conditions.

RESULTS

This section is organized as follows: First, we describe the optimization of Cre-*lox* constructs for titratable, low-rate recombination by expressing *cre* from a promoter inducible by 4-isopropylbenzoic acid (cuminic acid), supplied as its cumate salt. Second, we characterize the inducer response of these constructs at both the population and single-cell levels. Third, we present the development of a Cre-recombinase-based arsenite biosensor and assay its behavior in aerobic and anoxic growth conditions. Finally, we examine the broader applicability of our constructs and test the reporters for potential loss of genetic memory or fluorescence readout over multiple generations.

Aiming to reduce and control Cre activity, our first construct mounted on a low copy number plasmid (pEG36, with origin of replication *pA15* as all other plasmid unless otherwise noted) expressed *cre* from the cumate-sensitive promoter *cymRCp* obtained from the "Marionette" sensors collection (21,22). This promoter is repressed by the regulator $CymR^{AM}$ (constitutively expressed from the chromosome of the host strain Marionette-Clo, sAJM.1504 (21)) in the absence of cumate. Both $cymR^{AM}$ and *cymRCp* are mutated with respect to the wild-type to provide lower leakage and increased dynamic range. In addition, this construct includes $cymR^{AM}$, which maintains the ratio between the copy numbers of the regulator $cymR^{AM}$ and the promoter *cymRCp* close to one. Finally, we added the C-terminal *ssrA-LVA* degradation tag (23) to *cre* to reduce the Cre half-life in the cell and thus its activity (Fig. 1).

To test the inducer-response curve of this circuit and other variants, we assembled a construct composed of a constitutive promoter, two transcriptional terminators flanked by two *loxP sites* (part of phage P1's wild-type Cre-*loxP* system) with the same orientation, followed by the enhanced yellow fluorescent protein gene (*eyfp*). In the presence of both *loxP* sites, the two transcriptional terminators interrupt transcription, leaving *eyfp* unexpressed (Fig. 1A). Upon Cre-induced recombination of the two *loxP* sites, the transcriptional terminators are excised (Fig. 1B), enabling expression of the fluorescent protein that we detected using a fluorescence plate reader or a flow cytometer, depending on the assay. Simultaneous measurements of optical density at 600 nm (OD600) and fluorescence at the plate reader allowed us to identify cumate concentrations that did not significantly affect growth rates (Table S1), while promoting strong Cre activity.

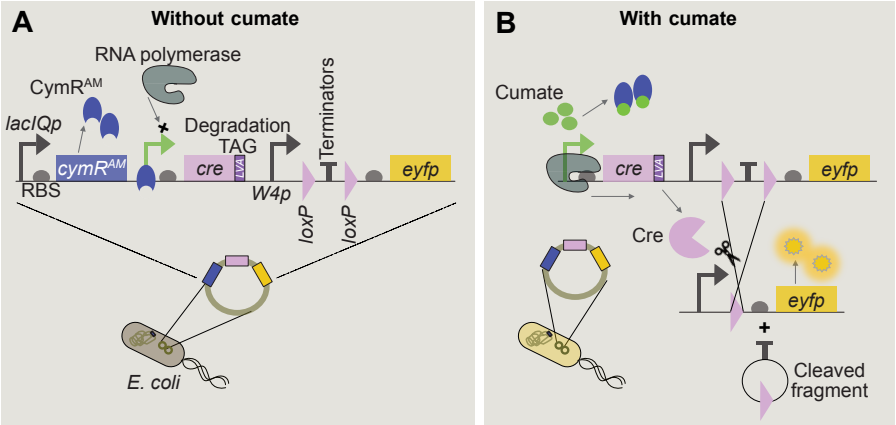

Fig. 1 Schematic of the Cre-*loxP* reporter system in *E. coli* on a plasmid. (A) In the absence of cumate, the constitutively expressed repressor CymR^{AM} occupies the *cymRCp* operator site, inhibiting *cre* transcription. (B) When cumate is present, it binds to the repressor, which detaches from the operator site allowing *cre* transcription. If the *loxP* sites are positioned in the same orientation, Cre excises the terminators located between the *loxP* sites and transcription of *eyfp* is initiated.

Fig. 2A shows that this system (pEG36) has low leakage and titratable Cre activity across a reproducible range. To further vary the titrability range of the construct, we tested different regulatory strategies at both the transcriptional and the post-translational level (Fig. 2). Specifically, we explored if we could alter the activity of our low-rate Cre-*loxP* system by adopting heterologous *lox* sites (pAG56), by removing the Cre degradation tag (see additional notes in Supplemental Materials), or by reducing the gene copy number of *cymR^{AM}* by removing it from the plasmid (pEG39). Recombination rates increased, as expected, when the regulator gene *cymR^{AM}* was removed from the plasmid (pEG39, Fig. 2C), leaving only the chromosomal copy. This may be of interest for applications that require a reporter with high sensitivity and quick response times. Conversely, we observed a dramatic drop in the fraction of fluorescent cells in the population, and thus in Cre activity, when the first wild-type *loxP* was replaced with *lox511* (pAG56, Fig. 2B), which differs from *loxP* by a single nucleotide in the spacer, the central region of *lox* sites that determines recombination specificity and directionality (24,25). Although we were able to obtain higher fractions of fluorescent cells by inducing this construct with concentrations of cumate higher than the upper limit we had set, this had a sizable fitness cost (Table S1). Since this construct had a reduced titrability range compared to other variants, we did not characterize it further. Still, it may be of interest for longer assays, for example for growth in chemostats in which recombination of homologous *lox* sites constructs such as pEG36 would likely reach saturation (100% fluorescent cells) in a short time. Removal of the Cre degradation tag resulted in frequent recombination even in the absence of the inducer, making it impossible to obtain clonal populations with two intact *loxP* sites, unless heterologous *lox* sites were used. The various plasmid variants are listed in Table 1 (Materials and Methods).

After constructing the plasmids described above and characterizing their inducer-response curves, we integrated the Cre-*loxP* reporter sequence with homologous *loxP* sites from pEG55 into the chromosome at *attTn7* (strain bEG57). pEG55 differs from pEG39 (construct with two *loxP*, no *cymR^{AM}*) by substituting *eyfp* with the yellow super fluorescent protein gene *syfp2* and using a stronger promoter and ribosome binding site to increase the fluorescence signal.

Chromosomal integration guarantees a more homogeneous gene copy number in the population and longer stability of the Cre-*loxP* reporter construct across generations (Fig. 6), even in the absence of selective agents.

We decided to further characterize reporters of interest (pEG36 and bEG57) at the single-cell level using flow-cytometry, as we reasoned that it would allow us to detect recombination events earlier than with the fluorescence plate reader. To capture the kinetics of these reporters, we collected samples of the cell population during the growth phase (Fig. 3A). Cytometry measurements allowed us to carry out a high-throughput analysis of the fraction of cells expressing the fluorescence reporter and to observe fluorescence heterogeneity at the single cell level (Fig. 3D), even when the population fluorescence was well below the detection limit of the plate reader. In addition, we performed colony PCR on samples treated with different inducer concentrations and collected at various time-points to confirm the presence of both the original (two *loxP*) and recombined (one *loxP)* sequences in populations that appeared mixed by flow-cytometry (Fig. S2).

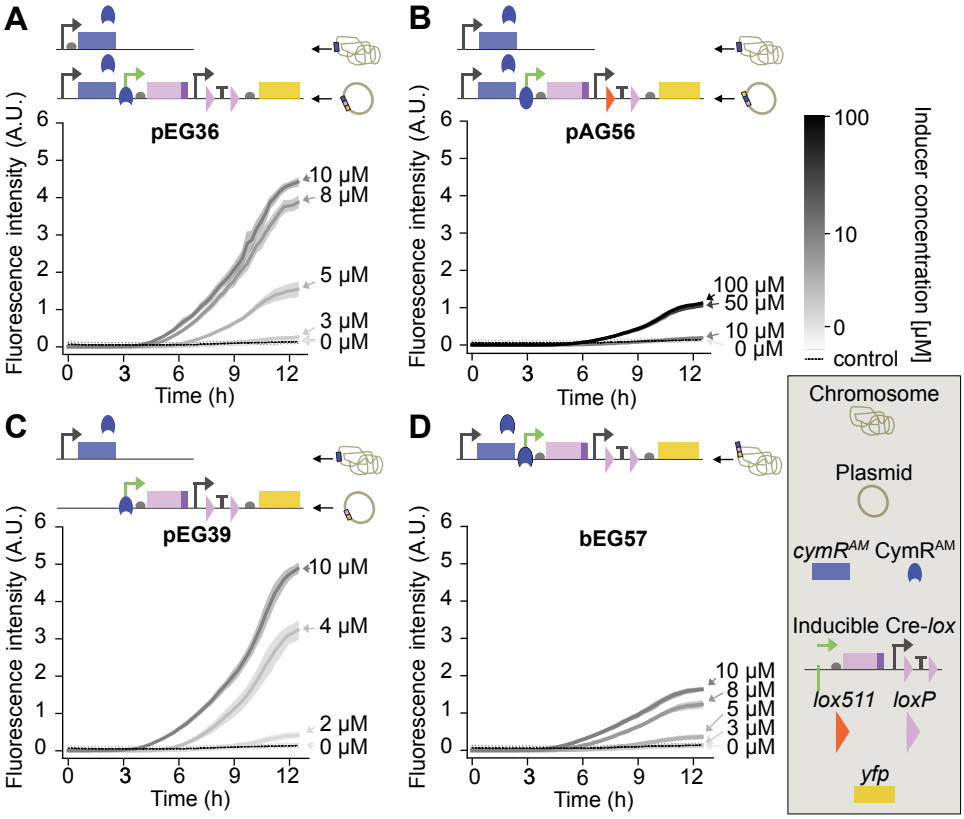

Fig. 2 Inducer-response curves of four Cre-*lox* reporter variants measured at the plate reader. The fluorescence intensity and range of titrability of the reporters varied depending on the copy number of the repressor gene *cymR*^{AM} (A vs C), on the identity of *lox* sites (homologous in A vs heterologous in B), and on the genomic location of the circuit (plasmid in A-C vs chromosome in D). The negative control is the fluorescence intensity recorded for the parent strain Marionette-Clo which contains a copy of *cymR*^{AM} on the chromosome and neither a Cre-*lox* circuit, nor *eyfp*. Each curve shows the average of three biological replicates, with error bars indicating 95% confidence intervals computed by bootstrapping. For pEG39, only two biological replicates were included due to a setup error in the third replicate, which used incorrect inducer concentrations.

We identified plasmid pEG36 (with *cymR*^{AM} and two *loxP*) as a good candidate to quantitatively characterize the response of the reporter, due to the range of titrability we could explore by employing reasonably low concentrations of inducer. Both pEG36 and pEG39 (two *loxP*, no *cymR*^{AM}) reached saturation of cells that cut the *loxP* site in a single growth curve by adding 10 μM of cumate. However, pEG36 showed slower recombination rates than pEG39, allowing us to follow the reporter kinetics by taking measurements at more widely spaced time points. Together with pEG36, we characterized the chromosomally integrated reporter bEG57. We considered pEG36 to be the ideal plasmid reporter to compare with the chromosomal Cre-*loxP* reporter, due to the approximately equal ratio between gene copy numbers of the repressor and of Cre expressed by the cumate-sensitive promoter. From this point onwards, we will only focus on these two constructs and refer to pEG36 as the *plasmid reporter* and to bEG57 as the *chromosomal reporter*. To estimate recombination rates (Fig. 3C), we fit a logistic or exponential curve (Materials and Methods) to the fraction of cells with one *loxP* measured with flow-cytometry and report the corresponding rate parameters in Table S2 (see also Fig. S1).

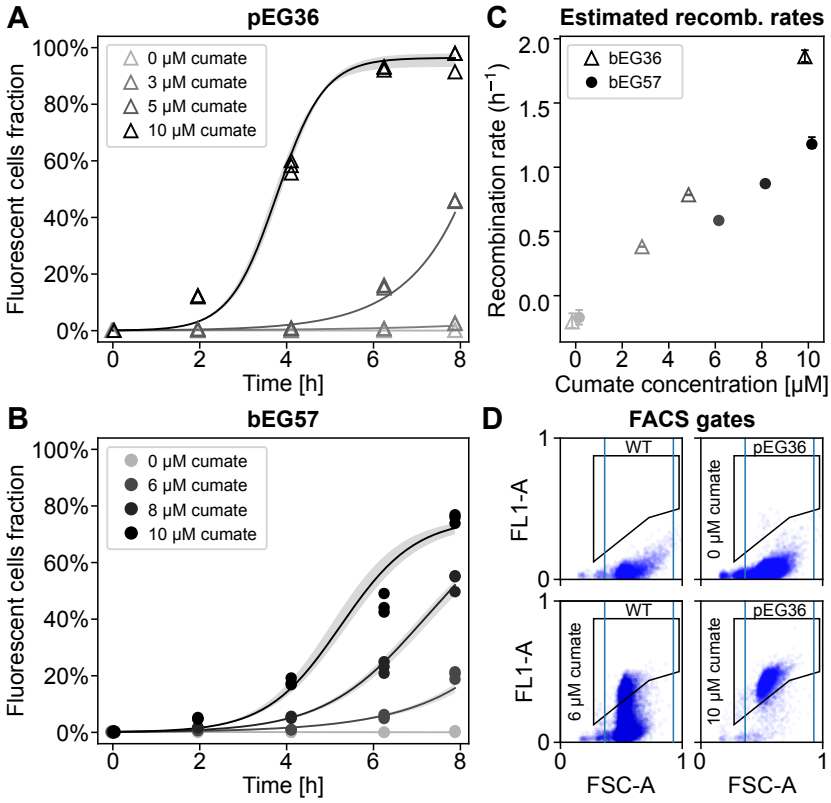

Fig. 3. Inducer-response curves measured at the flow-cytometer for the low-rate Cre-*loxP* reporter systems on plasmid pEG36 (with *cymR*^{AM} and two *loxP*) and on the chromosome, bEG57 (with a single chromosomal copy of *cymR*^{AM} and two *loxP*). (A-B) Fraction of fluorescent cells in the population with the plasmid (A) or chromosomal (B) reporter. Each point represents a technical replicate. Time point 0 h is the same for all treatments and is the fraction of fluorescent cells in the starting culture after exiting the lag phase and before adding the inducer. Solid curves show the mean of the best-fit model predictions (Materials and Methods) across technical replicates; shaded bands show the min-max across technical replicates. Corresponding plots for two additional biological replicates are reported in Fig. S1. (C) Best-fit estimates of recombination rates as a function of the inducer concentration. Error bars show the minimum and maximum estimated rates across technical replicates. (D) Example flow-cytometry data for plasmid reporter samples, showing

single-cell fluorescence signal area (FL1-A) versus forward-scattered area (FSC-A), in arbitrary units. Threshold gates are indicated by blue vertical lines (for the FSC-A channel) and black polygons (for FL1-A).

To demonstrate the applicability of these designs for environmental biosensing, we built a proof-of-concept Cre-*loxP* reporter in which *cre* transcription is induced by arsenite. To this end, we expressed both *cre* and *E. coli*'s transcriptional repressor *arsR* (26) from the promoter *arsRp* in a bicistronic design (pEG69). We refer to this reporter as ArsRBS2-Cre-*loxP*-SYFP2*,* where ArsRBS stands for ArsR Binding Site, and the number 2 highlights that, following (27,28), an auxiliary copy of ArsRBS is present before the *cre* gene to reduce leakage, in addition to the ArsRBS before *arsR* (Fig. 4A). We grew this recombinase-based arsenite-sensitive reporter in MOPS for growth medium (MPG) with concentrations of As(III) as low as 15 nM, comparable to the sensitivity of existing whole-cell arsenic biosensors and standard wet-chemical methods, including inductively coupled plasma mass spectrometry (ICP-MS) (29). To compare the sensitivity of our recombinase-based reporter pEG69 to that of a transcription-based reporter, we used a modified version of the plasmid reporter pMP01 from Pothier *et al.* (28), ArsRBS2-SYFP2, carrying *syfp2* instead of *mCherry*, with the same backbone as pEG69 (Fig. 4B). Unlike ArsRBS2-SYFP2 where *syfp2* transcription is regulated via the promoter *arsRp*, expression of *syfp2* in ArsRBS2-Cre-*loxP*-SYFP2 is regulated by a strong, constitutive promoter. To compare the two biosensors, we took endpoint, single-cell fluorescence measurements at the flow-cytometer for both reporters, grown with arsenite concentrations from 0 to 60 nM in. Since the transcription-based reporter returns a continuous response, we took the median single-cell fluorescence intensity as the representative measure of the overall expression level. We observed limited leakage in ArsRBS2-Cre-*loxP*-SYFP2, with an increase in the fraction of fluorescent cells in the population of 0.091%±0.006% (mean±SEM) in the assay without arsenite. We expected some basal expression due to ArsR-mediated negative feedback on *arsRp*, which drives the bicistronic *arsR*-*cre* transcriptional unit. For comparison, in the treatments with added arsenite, the fraction of fluorescent cells ranged from 0.157±0.007% to 0.66±0.01%, corresponding to the minimum and maximum inducer concentrations, respectively. We found that both the ArsRBS2-Cre-*loxP*-SYFP2 and ArsRBS2-SYFP2 reporters could detect 15 nM arsenite (p-value < 0.01), which is lower than the EPA threshold values (29) for arsenic contamination in drinking water (10 ppb As, equivalent to ~134 nM As).

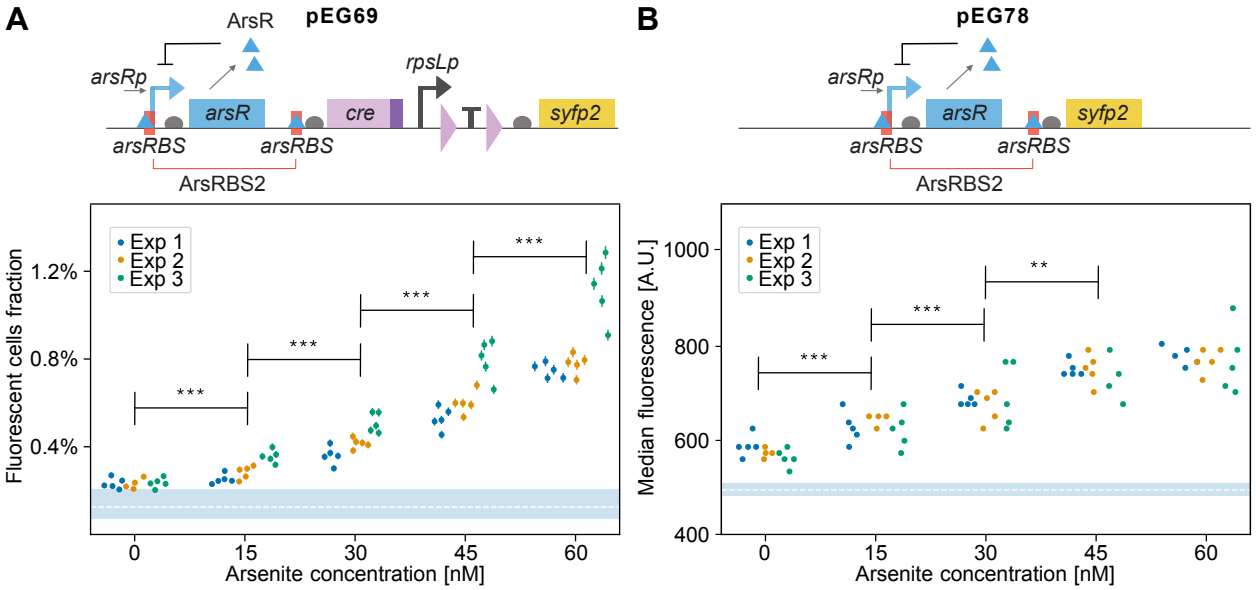

Fig. 4 Schematics of arsenite whole-cell biosensors and endpoint fluorescence measurements collected at the flow-cytometer. (A) Recombinase-based biosensor schematics (top) and fraction of fluorescent cells at the end of the assay (bottom; error bars report the Poisson counting error). (B) Transcription-based biosensor schematics (top) and median cell fluorescence in each sample (bottom); error bars computed via bootstrapping are smaller than symbols. In both panels, each point is a technical replicate, with three biological replicates shown in different colors. The light blue regions mark baseline values at the start of the experiment, before induction with arsenite. The region limits are respectively the minimum and maximum values of the fraction of fluorescent cells (A) or median cell fluorescence (B) while the white dashed line represents the intermediate measured value. The third biological replicate (green symbols) was incubated an hour longer (18 vs 17 hours) with respect to the two other replicates, which might explain the larger fraction of fluorescent cells compared to the other two experiments. Note that due to different designs, the measured response is different depending on the bioreporter: for the recombinase-based biosensor, we report on the y-axis the fraction of fluorescent cells, counted by cytometry gating; for the transcription-based reporter, we report the median cell fluorescence intensity.

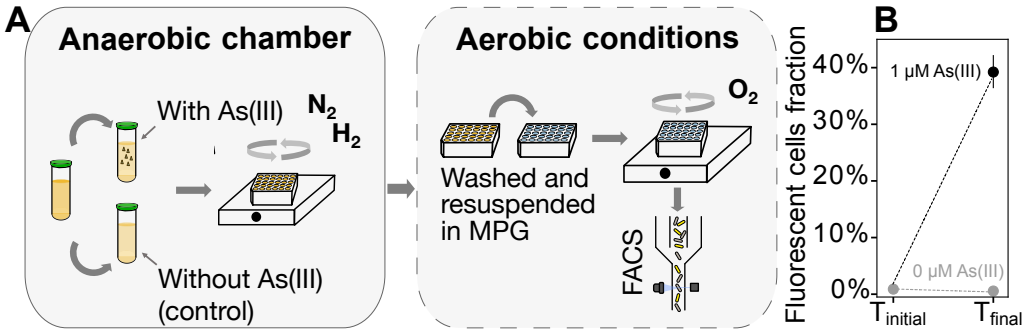

Fig. 5 Schematics of the anoxic assay and mean endpoint fluorescence measurements collected at the flow-cytometer. (A) Schematics of the experimental setup: in the anaerobic chamber (left), cells were grown in well-mixed liquid LB cultures with fumarate and glycerol, re-diluted in the same medium with and without arsenite, and grown in a 96-well plate placed on a microplate shaker for ~60 h. Samples were then removed from the anaerobic chamber (right), washed to remove arsenite, and resuspended in fresh MPG medium supplemented with dextrose, while lacking arsenite and fumarate. (B) Fraction of fluorescent cells sampled from cultures before transfer into the exposure medium ($T_{initial}$) and at the end of the exposure phase, with or without arsenite (T_{final}). Flow-cytometry measurements were performed after cells were resuspended in

arsenite- and fumarate-free medium and incubated in aerobic conditions for 24 h, allowing reporter expression to develop. Data points are averages across 3 biological replicates; error bars represent 95% confidence intervals computed via bootstrapping.

Unlike ArsRBS2-SYFP2, the Cre-recombinase based biosensor maintains a memory of arsenite exposure, allowing the user to decouple exposure from measurement. This is an advantage over existing biosensors that use anaerobically compatible fluorescent proteins, which require all aspects of the experiment, including plate reader fluorescence measurements, to be performed inside an anaerobic chamber, and typically suffer from low signal-to-noise ratios due to the reduced brightness of these fluorophores (8,20). We demonstrate this advantage by successfully detecting arsenite exposure in anoxic conditions using the ArsRBS2-Cre-*loxP*-SYFP2 biosensor. In a Coy anaerobic chamber, we grew three biological replicates at room temperature in liquid LB cultures that had been degassed overnight, using fumarate as an electron acceptor (Fig. 5A). We used LB as a rich nutrient medium to favor *E. coli*'s switch to anaerobic respiration (30) and to increase its growth rate relative to MPG. Cells were exposed for 61 hours to 1 μM arsenite in LB with fumarate and glycerol (to enhance GlpF activity (31)). Cells grown in the same conditions, minus the arsenite, were used as controls. Following exposure, cells were washed in phosphate-buffered saline (PBS) and incubated in MPG in aerobic conditions, at 37 °C, for 24 hours, allowing full maturation of the fluorescent protein and enabling expression in cells that had just recombined the plasmid. Finally, samples were analyzed at the flow-cytometer (Fig. 5B). The fraction of fluorescent cells in samples exposed to arsenite (39%) was significantly higher (p-value ~ 10^{-16}) than in the control (0.6%), indicating that the recombinase-based biosensor can be used in experimental settings without specialized equipment for anoxic fluorescence measurements, as such high fractions produce fluorescent signals easily detectable even without a flow cytometer. We further observed that the subpopulation with only one *loxP* site remained far from saturation after being exposed to a high concentration of arsenite for two days in anoxic conditions. This may indicate that, under anoxic conditions, the biosensor can resolve a wide range of contaminant concentrations or be deployed for long periods in the exposure phase without reaching full recombination of the population. As the resuspended population contains a mixture of fluorescent and non-fluorescent cells, differences in growth rate between the two may bias the end-point flow cytometry measurement. To account for this possibility, we collected flow cytometry data at three time points during growth in aerobic conditions after arsenite removal: 1) immediately after sampling from the anaerobic chamber, 2) at 6 hours, and 3) 24 hours post-incubation in arsenite-free medium (Fig. S4). Fluorescent cell fractions were similar across all time points, indicating that the observed signal is stable and not strongly affected by potential growth rate differences following exposure. Longer term stability of the reporter after several generations of growth in aerobic conditions is discussed below.

Long-term stability of sensing-reporter systems is essential for deploying engineered microbes as reliable environmental biosensors. However, these systems remain vulnerable to failure over successive generations due to mutations or fitness costs associated with the expression of exogenous genetic constructs. To assess the robustness of our designs, we examined the plasmid (pEG36) and chromosomal (bEG57) cumate-sensitive reporters, and the arsenite reporter ArsRBS2-Cre-*loxP*-SYFP2 (pEG69) for two potential failure modes. First, we wanted to monitor a possible decline in the fraction of fluorescent cells after exposure, which may occur due to fitness costs associated with expressing the fluorescent protein and potentially allow cells that

did not perform Cre recombination (with two *lox* sites) to outcompete those that did (with one *lox* site). Alternatively, mutations arising within the latter could silence fluorescent protein expression and sweep through the population. For practical purposes, spontaneous recombination and fluorescent protein expression should be sufficiently rare to avoid false detection of the inducer, although proper controls can help mitigate these issues. Such events compromise the fidelity of the biosensor's memory and reduce its reliability for long-term quantification of contaminants.

To probe these effects, we measured the fraction of fluorescent cells in the population immediately after transient inducer exposure and tracked this fraction over 50 generations in inducer-free media. We induced the reporters using 2 μM cumate or 500 nM arsenite to generate a subpopulation of fluorescent cells. For the cumate-sensitive reporters, we prepared an additional treatment (5 μM cumate) in which most cells in the population were recombined, allowing us to examine dynamics from both low and high initial recombined fractions. Parallel uninduced cultures were used to monitor background recombination over time. Cells were grown in LB (pEG36 and bEG57) or MPG (pEG69), exposed for 12 h, and then diluted every 12 h, with each growth phase corresponding to 13.5 and 12.5 generations, respectively (Materials and Methods). We observed a 60% and 50% decline in the fluorescent cell fraction after around 50 generations for pEG36 (Fig. 6A) and pEG69 (Fig. 6C), respectively. Conversely, the chromosomal reporter showed no reduction in the fluorescent cells fraction, likely reflecting the reduced fitness cost of expressing a single-copy fluorescent protein gene and avoiding plasmid replication costs. After removing the inducer and growing cells in fresh media, we observed an initial increase of the fraction of fluorescent cells in both the chromosomal cumate-sensitive reporter and the recombinase-based arsenite-sensitive reporter. This increase likely reflects recombination that occurred toward the end of the exposure phase, when cells in stationary phase may not yet have had time to express and mature the fluorescent protein.

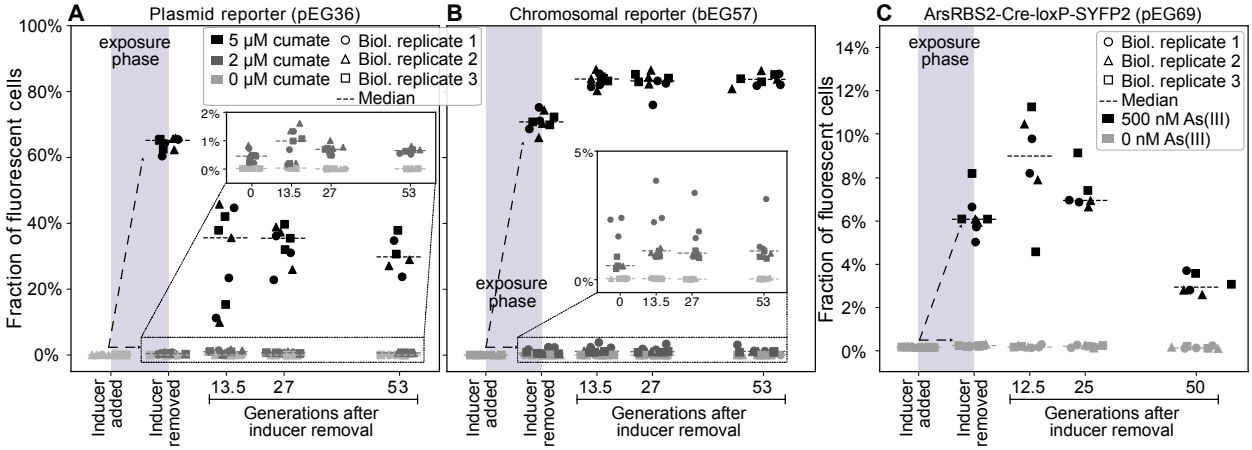

Fig. 6 Fraction of fluorescent cells in cultures passaged in inducer-free media after exposure; different panels show data for different Cre reporters: (A) pEG36 (cumate repressor and homologous *loxP* sites on plasmid), (B) bEG57 (cumate-sensitive reporter on the chromosome), and (C) ArsRBS2-Cre-loxPSYFP2, the recombinase-based arsenite-sensitive reporter. Cells were measured at the flow-cytometer before the addition of the inducer, after the exposure phase, before washing the cells and removing the inducer, and at several passaging points. Each point is a technical replicate of one of three biological replicates, and it is scattered around the x-axis tick to aid visualization. Error bars are smaller than data points.

DISCUSSION

To summarize, in this work we explored how different regulatory strategies of a Cre-*lox* system affect the response of cumate-inducible reporters in *E. coli*, on both plasmids and the chromosome. To assess the performance of the engineered bioreporters, we examined their basal expression, sensitivity, titrability, and stability over multiple generations. We observed that the use of an optimized inducible promoter and protein degradation tag effectively limited leakage. Furthermore, we showed that recombination rates could be manipulated by altering the ratio of gene copy numbers of the regulator and transcriptional promoter, and that extremely low recombination rates could be achieved by hindering Cre activity by substituting homologous *loxP* sites with the heterologous *lox511*-*loxP* pair. Mutated Cre recombinases (32) with reduced efficiency could also be explored to expand the tunability range. Our constructs provide an alternative to optogenetic Cre-*loxP* (16) systems and require only low concentrations of cumate, an inexpensive, non-toxic inducer. Moreover, the dynamic range of induction could potentially be expanded through the addition of salicylic acid, which antagonizes the cumate sensor (21).

To better understand the persistence of genetic memory, we examined reporter stability over multiple generations. As expected, fluorescent protein expression imposes a fitness burden, even when on a low-copy-number plasmid. These fitness costs became detectable after approximately 13 generations following inducer removal. Chromosomal integration improved stability, likely due to reduced gene copy number, lower expression burden, and avoidance of plasmid replication costs. Because our primary goal was to characterize Cre titrability rather than long-term memory stability, we used a constitutively expressed fluorescent protein to facilitate real-time detection of rare recombination events. Future designs could mitigate fitness costs by placing the reporter under inducible control, activated only immediately before measurement.

Recombinases are widely employed in synthetic biology applications for their efficiency, specificity, and ability to encode genetic memory. However, tunable recombinase systems present challenges in prokaryotes, particularly with efficient enzymes like Cre, where tight regulation is needed to prevent unintended genomic changes. Being able to experimentally tune recombinase-mediated recombination rates *in vivo* across a titratable range opens up new possibilities for applications in genetic circuit design (15), cell physiology (14), microbial ecology, and experimental evolution (2) research. For example, low-rate recombination could be used to simulate mutational drift, create stochastic genotypic diversification in structured populations, or trace spatially segregated lineages in microbial colonies and biofilms.

To demonstrate the broad applicability of our circuits, we applied the same design principles to construct a recombinase-based arsenite biosensor. This reporter reliably detected low nanomolar concentrations of arsenite and retained its functionality under anoxic growth conditions. The majority of existing whole-cell arsenite biosensors (27,28) adopt traditional designs comprising fluorescence-based reporter circuits in which transcription of a fluorescent protein is regulated by *arsRp*. In contrast, our system uses recombinase-mediated excision to store the exposure signal in DNA, enabling decoupled measurement.

Our biosensor's capability to detect recombination events at small rates and in anoxic conditions could be particularly relevant for monitoring arsenic pollution in real-world environments, such as rice paddy waters, where microbial uptake of dissolved arsenic can be inhibited due to factors

including complexation with dissolved organic matter, association with colloidal phases, or repression of arsenic uptake by organic carbon (8,31). Arsenic uptake into microbial cells is a prerequisite for enzymatic arsenic methylation, which produces methylated arsenic species that can represent a significant fraction of the arsenic that accumulates in rice grains (7). Biogeochemical interactions between arsenic, iron, and sulfur that govern arsenic chemical speciation and fate depend strongly on redox conditions, highlighting the need for experimental methods to characterize microbial availability of arsenite under anaerobic conditions where it is most mobile, as found for example in waterlogged paddy soils, wetlands, marine sediments, and deep subsurface environments (7). Although the use of flavin-based fluorescent proteins allows traditional biosensor designs to function in anoxic or anaerobic assays (8,18), the low signal-to-noise ratio of these measurements can significantly reduce the sensitivity and accuracy of detecting low concentrations of analytes. In contrast, recombinase-based biosensors, such as the one developed here, are particularly suitable for these assays thanks to the decoupling of environmental sensing from measurements. This genetic memory brings interesting new functionalities compared to traditional biosensors and can be advantageous for obtaining temporally-integrated measurements in the field or laboratory (13), with cells transitioning to the recombined state at low rates over extended periods of time. In the future, the reporter construct could be further optimized to reduce leakage, for example by disrupting the *arsR* negative feedback loop (33,34), using an engineered version of *arsRp*, or integrating the synthetic construct in the chromosome. This would further benefit the inheritance of the inserted sequence across generations without providing a selective agent.

While recombinase-based biosensors are well-established in synthetic biology, their application to environmental sensing remains limited (5). Our findings show that Cre-*lox* systems can be tuned to operate at low recombination rates, maintain stable genetic memory, and function reliably under both aerobic and anoxic conditions. These properties make them well-suited for applications that require temporally integrated sensing, post-exposure readouts, or compatibility with environmentally relevant settings. Further optimization, such as chromosomal integration in environmentally adapted strains, reducing expression burden, or alternative signal outputs, could enhance their suitability for field applications.

As synthetic biology continues to expand into environmental and ecological domains, titratable recombinase systems offer a robust and adaptable platform for recording microbial exposure to environmental stimuli and contaminants in dynamic settings.

MATERIALS AND METHODS

Strain editing

We assembled and characterized the cumate reporter in the Marionette-Clo *E. coli* strain (21), which has DH10B background. On plasmids, *eyfp* transcription is controlled by the constitutive, medium-strength promoter *W4p* (16). In the chromosomal reporter, *syfp2* (10, 27) transcription is regulated by the high-strength constitutive promoter *rpsLp*. *rpsLp-syfp2* was amplified from strain eDE31, which was a gift from Johan Paulsson. Cre was a gift from Niels Geijsen (Addgene plasmid #62730) (36). The terminators *ECK120033736 and L3S2P11* and *lox511* site were synthesized by Twist Bioscience (Table S5). *loxP* and *W4p* were a gift from Mary Dunlop (Addgene plasmid #134405) (16). Plasmids were constructed using FastCloning (37) or Gibson

assembly (38). Oligos used are reported in Table S4. Chromosomal insertions were achieved via the mTn7 (MCS MS26) mobile element, inserting at *attTn7*, downstream of *glmS*, following the protocol in Sibley & Raleigh (39). Plasmids were isolated with the Omega Bio-Tek E.Z.N.A. Plasmid DNA Mini Kit I (Q - spin) and sequenced via Oxford Nanopore long-read sequencing. Chromosomal insertions were amplified with primers oAG70/oEG103 (Table S4) surrounding *attTn7* and sequenced via Oxford Nanopore long-read sequencing. After substituting *mCherry* in pMP01 with the Cre-*loxP*-SYFP2 construct, we transferred the reporter sequence on a vector with low copy number (pAJM.717, with *pA15* origin of replication). This decreased the leakage of the reporter. The ArsRBS2-Cre-*loxP* and ArsRBS2-SYFP2 constructs were assembled via Fastcloning, except for the last step, consisting in swapping the backbone with the low copy number vector, which was performed via enzymatic restriction cloning.

Table 1 Summary of relevant plasmids and strains used in this study

Strain	**Source**	**Genotype**
sAJM.1504	Meyer *et al.* (21)	*E. coli* Marionette-Clo (DH10B background), *cymR*^{AM} on chromosome
bEG57 (*chromosomal reporter*)	This work	sAJM.1504; *attTn7*::*cymRCp – cre*-LVA, *TT – rpsLp – loxP – TT – loxP – syfp2*
Plasmid	**Source**	**Key Inserts**
pAJM.657	Meyer *et al.* (21)	*cymR*^{AM}, *cymRCp – eyfp*
pAJM.717	Meyer *et al.* (21)	*kanR, tetp* – yfp* (p15A backbone)
pAG56	This work	*cymR*^{AM}*, cymRCp – cre-LVA, TT – W4p – lox511 – TT – loxP – eyfp*
pEG36 (*plasmid reporter*)	This work	*cymR*^{AM}*, cymRCp – cre-LVA, TT – W4p – loxP – TT – loxP – eyfp*
pEG39	This work	*cymRCp – cre-LVA, TT – W4p – loxP – TT – loxP – eyfp*
pEG55	This work	*cymRCp – cre-LVA, TT – rpsLp – loxP – TT – loxP – syfp2*
pMP01	Pothier *et al.* (28)	ArsRBS2 – *mCherry* (pUCP19 backbone)
pEG69	This work	ArsRBS2 – *cre-LVA, TT – rpsLp – loxP – TT – loxP – syfp2*
pEG78	This work	ArsRBS2 – *syfp2*
Units	**Source**	**Genetic Elements**
TT	Synthesized by Twist Bioscience, sequence from Chen *et al.* (40)	Transcriptional terminators *ECK120033736 – L3S2P11*
ArsRBS2	Pothier *et al.* (28)	*arsRBS – arsRp – arsR – arsRBS*

Media and chemical inducers

Characterization of the cumate-inducible constructs was performed in Lysogeny Broth (LB) medium with the appropriate selective antibiotic, whereas the arsenite-inducible constructs characterization in aerobic conditions was done in MOPS for growth medium (MPG), following the recipe used in Pothier *et al.* (28), supplemented with 50 µg/µL ampicillin sodium salt. Super Optimal broth with Catabolite repression (SOC) medium and plates made with Lysogeny Broth (LB) medium with 1.5% agar and 50 µg/µL kanamycin sulfate were used for cloning and strain maintenance. Unless specified otherwise, cells were grown in LB with the appropriate selective antibiotic. To induce *cre* transcription, we added appropriate amounts of a stock solution of 100 mM cuminic acid (4-isopropylbenzoic acid) ≥ 96% purity (Sigma-Aldrich 268402) in 100% ethanol to the medium.

Cumate-sensitive reporter response characterization

Plate reader assays: Overnights of strains of interest were grown from fresh single colonies on agar at 37 °C in 1 mL of LB in a roller drum. The following day, cultures were diluted 1:200 in glass culturing tubes and incubated at 37 °C on the roller drum for 2 hours. Afterwards, cells were further diluted 1:100 in pre-warmed LB with antibiotic and inducer and, for each sample and treatment, a total of 1.5 mL was distributed in equal volumes of 125 µL into 12 wells of a sterile, flat-bottom, non-treated, 96-well tissue culture plate with lid (VWR 10861-562). Three technical replicates were used for each treatment condition and time-step recovery. The samples were distributed on the plate in a random configuration. Cells were then grown in the plate reader at 37 °C with continuous double orbital shaking at 807 cpm. Strains were induced with a range of cumate concentrations ranging from 0 µM to the maximum concentration of cumate as recommended in Meyer *et al.* (21), i.e. 100 µM. Some biological replicates showed a fitness cost when grown at this maximum concentration (Table S1). OD600 and fluorescence (excitation at 501 nm and emission at 533 nm) were measured every 15 minutes over 21 hours of incubation. OD600 and fluorescence data of 3 wells inoculated with only medium and no cells were averaged at each timestep and used as a measure of background expression. At four time points, after 1 h, 2 h 30 min, 5 h and 21 h from the beginning of the assay, the plate was shortly removed from the plate reader, between 2 and 5 µL were transferred from each well into a volume ranging from 125 to 198 µL of Tris-HCl pH 7.5 in a 96-well U bottom plate to perform flow-cytometry measurements (10,000 single cells recorded per sample).

Flow-cytometry and colony PCR: Flow-cytometry was performed by collecting forward and side scattering (FSC/SSC), as well as fluorescence intensity with excitation at 488 nm and emission at 533/30nm (FL1). We performed data analysis using the Python package FlowCytometryTools (41) to carry out the data analysis. In the biological replicates experiment shown in Fig. S1(D-F), three technical replicates for each treatment at different times were pooled and used as template for colony PCRs to amplify the DNA region enclosing the two *loxP* sites. After qualitatively comparing the bands length difference, the DNA was purified from the PCR product with the Omega Bio-Tek E.Z.N.A. Cycle Pure Kit (V-Spin) and Sanger sequenced. The generated chromatograms were inspected to confirm the presence of the recombined and non-recombined variants. In all experiments, the parent strain Marionette-Clo was used as a negative control and

Sanger sequenced. In all experiments, the parent strain Marionette-Clo was used as a negative control.

Recombination rates estimation: To estimate recombination rates for pEG36 and bEG57 as a function of cumate concentration, we fitted a logistic curve to flow cytometry data (Fig. 3):

$$f_{1loxP}(t, k, f_{max}) = \frac{f_{max}}{1+\left(\frac{f_{max}}{f_0} - 1\right)e^{-kt}} \qquad \text{(Eq. 1)}$$

where:

- f_0 is the experimentally measured fraction of fluorescent cells at time 0,
- f_{max} is a fitting parameter, bounded between the maximum measured fraction and 1,
- k is a fitting parameter representing the Cre recombination rate (h^{-1}),
- t is time (h).

At low cumate concentrations, the parameters of the logistic growth model are not identifiable because a time-invariant fraction of fluorescent cells can arise either by setting $f_{max} = 0$ (for any k), or setting $k = 0$ (for any f_{max}) in Eq. 1. Furthermore, if $f \ll f_{max}$ throughout the time course, f_{max} cannot be determined. Thus, for each technical replicate, we fit both the logistic Eq. 1 and its asymptotic expansion in the small parameter f/f_{max}, whose leading term is the exponential growth $f(t, k) = f_0 e^{kt}$, which has one fewer fitting parameter compared to Eq. 1. Accordingly, for each technical replicate we fit both the full logistic model (maximum likelihood fit of Eq. 1) and its early-growth exponential approximation, and we select the estimate of k from the model with the lowest corrected Akaike Information Criterion (AICc) value. A non-linear fit was performed for the exponential model, while Markov chain Monte Carlo was used for the logistic model fit. Only the exponential fit was performed for replicates with no supplemented cumate. Figs. 3 and S1 and Table S2 report the average across technical replicates of such best fits.

Arsenite-sensitive reporter response characterization and analysis

Aerobic conditions

A stock solution of 7 mM of arsenite was prepared by dissolving sodium arsenite (LabChem, Zelienople, PA) in Milli-Q water, followed by 0.22 µm syringe-filtration. The preparation was conducted in a Coy anaerobic chamber (98% N_2 and 2% H_2), and the stock solution was stored anaerobically at 4 °C after its concentration was verified using ICP-MS (Agilent 7800). Negligible oxidation of arsenite to arsenate was confirmed before use by high-performance liquid chromatography (HPLC; Agilent 1260 Infinity) hyphenated to ICP-MS. From this stock, we collected 15 µL diluted in a total volume of 15 mL sterile water in a 15 mL falcon tube. Experiments were carried out in the same week the 70 µM arsenite stock was prepared, to minimize the solution exposure to air and the risk of oxidation into As(V). Yoon *et al.* (42) verified the absence of arsenite speciation in their biosensor experiments via HPLC-ICP-MS analysis, with sampling conducted during the first 10 hours of their experiment, in conditions similar to our experiments.

The host strain used for the arsenite biosensors is *E. coli* NEB 10-beta, which has DH10B background. Strains used for the arsenite-sensitive reporter assay were streaked from the cryostock on an LB agar plate with 2% dextrose, 50 µg/mL kanamycin sulfate, and 50 µg/mL ampicillin sodium salt and incubated at 37 °C overnight. At least three colonies per strain were picked and inoculated in 2 mL-deep 96 well plates (VWR 76397-576) with 1 mL of MPG

supplemented with 50 µg/mL ampicillin sodium salt and 100 µL of 20% dextrose. The plate was covered with a rayon film and lid and placed on an orbital shaker rotating at 400 rpm in a 37 °C incubator. The following day, samples were measured at the flow cytometer. Dextrose helped reduce Cre expression leakage, probably due its inhibitory action on the glycerol facilitator transporter protein (GlpF), which mediates the uptake of arsenite by the cell (31,43). The biological replicates with the least leakage were selected for the assay. Cells were washed in MPG to remove dextrose and diluted 10,000 fold in MPG with ampicillin and different concentrations of arsenite. Technical replicates were inoculated in 2 mL-deep 96-well plates and kept in the incubator with 400 rpm orbital shaking until they reached stationary phase. Samples were measured at the flow cytometer after 17 hours of incubation (18 hours for the third biological replicate) by sampling between 10 and 12 µL of liquid, which were then diluted in 50 mM Tris-HCl pH 7.5. For each well, we collected 150,000 single-cell measurements. The binary response of the recombinase-based biosensor was measured at the single-cell level by measuring the fraction of cells contained within a gate in the FSC-A vs FL1-A that was drawn by comparing cells grown in the absence of As(III) to fully recombined cells.

To assess statistical significance (p-value < 0.05) of the sensors' sensitivity, within the same biological replicate, we performed two-sided Welch's t-tests between treatments at successive inducer concentrations (i.e., 0 nM with 15 nM, 15 nM with 30 nM, etc). The p-values obtained from the three different experiments (biological replicates) for the same arsenite concentration were then combined following Fisher's method. The corresponding p-values are reported in Table S3.

Anoxic conditions

Experiments were conducted at room temperature in a Coy chamber with 97.7% N_2 and H_2 2.3% and O_2 < 20 ppm. ArsRBS2-Cre-*loxP*-SYFP2 was streaked on plates prepared by mixing in aerobic conditions one part of 4% agar with one part of MPG medium with 10mM sodium fumarate and grown in low oxygen conditions (O_2 around 1000 ppm). Plates were supplemented with 2% dextrose (to repress GlpF expression), 50 µg/mL ampicillin sodium salt, and 50 µg/mL kanamycin sulfate. Growth and exposure were performed in liquid LB medium supplemented with 80 mM fumarate, 0.001% resazurin and 50 µg/mL ampicillin sodium salt. Additionally, the exposure medium was supplemented with 0.4% v/v glycerol. Media were sterilized by autoclaving, allowed to cool below 60 °C before adding ampicillin, and then transferred into the anaerobic chamber. Bottle lids were left loosely closed overnight to promote degassing, and a rayon film was placed over the opening to prevent contamination. Degassing was confirmed using a resazurin dye test, which also served as a visual indicator of anoxic conditions during cell growth (Fig. S3). Three isolated colonies were picked and inoculated into 2 mL of medium in 10 mL Falcon tubes and placed on a shaker rotating at 160 rpm. After 6 days of growth in liquid LB, 10 µL/mL of the cultures were transferred to a 2 mL-deep 96-well plate, containing 1 mL per well of exposure medium, with or without 1 µM arsenite. We prepared five technical replicates for each of the three biological replicates. The plate was covered with rayon film and a lid and incubated on a shaker set at 150 rpm. A 50 µM arsenite stock solution was prepared under an air atmosphere and sterile-filtered into an autoclaved serum bottle. The bottle was then capped, crimped, and its headspace was purged with argon for 15 minutes. Cells were exposed to the treatment conditions for 61 hours. At the end of the exposure period, the cultures were removed from the anaerobic chamber, immediately washed in PBS, diluted 1:100, and transferred to MPG supplemented with 2%

dextrose for aerobic culturing. The 2 mL-deep 96-well plate was incubated at 37 °C on a shaker rotating at 400 rpm for 24 hours to allow full maturation of the fluorescent protein. For the initial time point t_0, 100 µL of the LB culture (prior to inoculation) was taken out of the chamber, resuspended in MPG without fumarate (10 uL in 1 mL total volume), and grown under the same conditions for 24 hours. After incubation, cells were diluted 10-fold in PBS supplemented with 15 µg/mL propidium iodide and analyzed by flow cytometry to assess viability. No differences in viability were observed in the presence and absence of arsenite (*p*-value 0.41, *Fig*. S5).

Reporters' stability assay

The plasmid and chromosomal cumate reporters were streaked on LB plates supplemented with 50 µg/mL kanamycin sulfate. ArsRBS2-Cre-*loxP*-SYFP2 was streaked on an LB plate with 50 µg/mL of kanamycin sulfate, 50 µg/mL of ampicillin sodium salt, and 2% dextrose. The day after streaking, three colonies from each plate were inoculated into 1 mL of medium (for ArsRBS2-Cre-*loxP*-SYFP2 MPG with kanamycin and 2% dextrose). Overnights were diluted 10,000-fold into 1 mL of medium without inducers and distributed in a 2 mL-deep 96-well plate, covered with rayon film and placed on a rotating roller drum. After 12 hours of incubation, cells were diluted in fresh media with inducers: 0, 2 or 5 µM of cumate, and 0 or 500 nM of arsenite. From here onwards, dextrose was absent from MPG. After 12 hours, cells were diluted 100-fold in PBS, followed by another 100-fold dilution in medium without inducers. Dilution and propagation into fresh media were carried out every 12 hours for five cycles. Every 24 hours, cells from the intermediate 100-fold dilution in PBS were measured at the flow cytometer. We measured 200,000 cells per sample, or up to 140 uL, whichever occurred first. Following this protocol, bEG36 and bEG57 went through ~53 generations after exposure. In MPG, ArsRBS2-Cre-*loxP*-SYFP2 grows slower than the other bioreporters in LB and does not reach carrying capacity after 12 hours. Therefore, the number of cell generations per cycle was computed as $\log_2(\rho_i - \frac{\rho_{i-1}}{\text{dilution factor}})$, where ρ_i is the total number of cells counted at the flow-cytometer, divided by the volume at the i-th cycle.

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Author Contributions

E.G. and A.G. conceived the study. E.G. performed the experiments and data analysis. A.G. supervised the experiments and data analysis. H.Y. and M.C.R. provided the arsenite stock solution, gave technical and conceptual advice for the arsenite biosensor assays, and provided support for anoxic experiments. E.G. and A.G. wrote the manuscript. H.Y. and M.C.R. edited the manuscript.

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SUPPLEMENTAL MATERIALS

Supplemental materials include supplementary tables reporting strains' growth rates measured in the experiments carried out to characterize the cumate-sensitive reporter population, *p*-values of all statistical tests performed, the main oligonucleotides used in this study, and additional notes on data analyses.

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