

Algorithmic Programming of Sequential Logic and Genetic Circuits for Recording Biochemical Concentration in a Probiotic Bacterium

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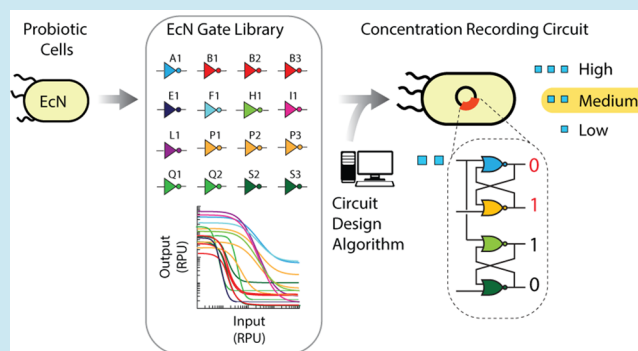
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ABSTRACT: Through the implementation of designable genetic circuits, engineered probiotic microorganisms could be used as noninvasive diagnostic tools for the gastrointestinal tract. For these living cells to report detected biomarkers or signals after exiting the gut, the genetic circuits must be able to record these signals by using genetically encoded memory. Complex memory register circuits could enable multiplex interrogation of biomarkers and signals. A theory-based approach to create genetic circuits containing memory, known as sequential logic circuits, was previously established for a model laboratory strain of *Escherichia coli*, yet how circuit component performance varies for nonmodel and clinically relevant bacterial strains is poorly understood. Here, we develop a scalable computational approach to design robust sequential logic circuits in probiotic strain *Escherichia coli* Nissle 1917 (EcN). In this work, we used TetR-family transcriptional repressors to build genetic logic gates that can be composed into sequential logic circuits, along with a set of engineered sensors relevant for use in the gut environment. Using standard methods, 16 genetic NOT gates and nine sensors were experimentally characterized in EcN. These data were used to design and predict the performance of circuit designs. We present a set of genetic circuits encoding both combinational logic and sequential logic and show that the circuit outputs are in close agreement with our quantitative predictions from the design algorithm. Furthermore, we demonstrate an analog-like concentration recording circuit that detects and reports three input concentration ranges of a biochemical signal using sequential logic.

KEYWORDS: genetic circuit design, living diagnostics, engineered probiotic, genetically encoded biosensor, whole cell biosensor, nonmodel bacteria, synthetic gene regulation



INTRODUCTION

A multitude of biochemicals are found within the human gastrointestinal tract whose presence, overabundance, or deficiency can indicate or impact disease and human physiology.^{1–5} Detecting these transient signals in the intestines often proves difficult or currently requires invasive procedures by conventional approaches. Engineered living microorganisms, also referred to as “smart probiotics”, have emerged as a promising alternative approach to repurpose the dynamic biological processes of cells for living diagnostic and therapeutic applications.^{6–8} The probiotic bacterial strain *Escherichia coli* Nissle 1917 (EcN) has been considered a well-suited chassis for therapeutic applications^{9–16} and in vivo detection of biomarkers and metabolites,^{17–24} such as those used in clinical trials for conditions affecting the human gastrointestinal tract.^{25–29} In order to effectively perform as a living diagnostic or therapeutic device, it would be advantageous for cells to be able to detect multiple biochemical signals found in the gastrointestinal tract,³⁰ as well as record transient signals and dynamically respond to changing signals. This requires more complex decision making that can be imparted

through the implementation of synthetic regulatory networks and genetically encoded logic circuits.^{31–34}

Model-guided approaches have been developed to create complex genetically encoded circuits, allowing for the design and prediction of dynamic signal processing in engineered cell populations.^{35–42} Numerous types of protein-based and RNA-based circuitry have been employed to construct these synthetic regulatory networks and compute Boolean logic in cells, such as transcription factors,^{43–46} protein–protein interactions,^{47–49} sequestration approaches,^{50,51} RNA-based regulation (e.g., trans-acting RNA, toehold switches, STARS),^{52,53} CRISPR interference systems,^{54–57} and riboswitches.^{58–60} This has generated a large set of genetic parts from which to choose when designing a circuit. However,

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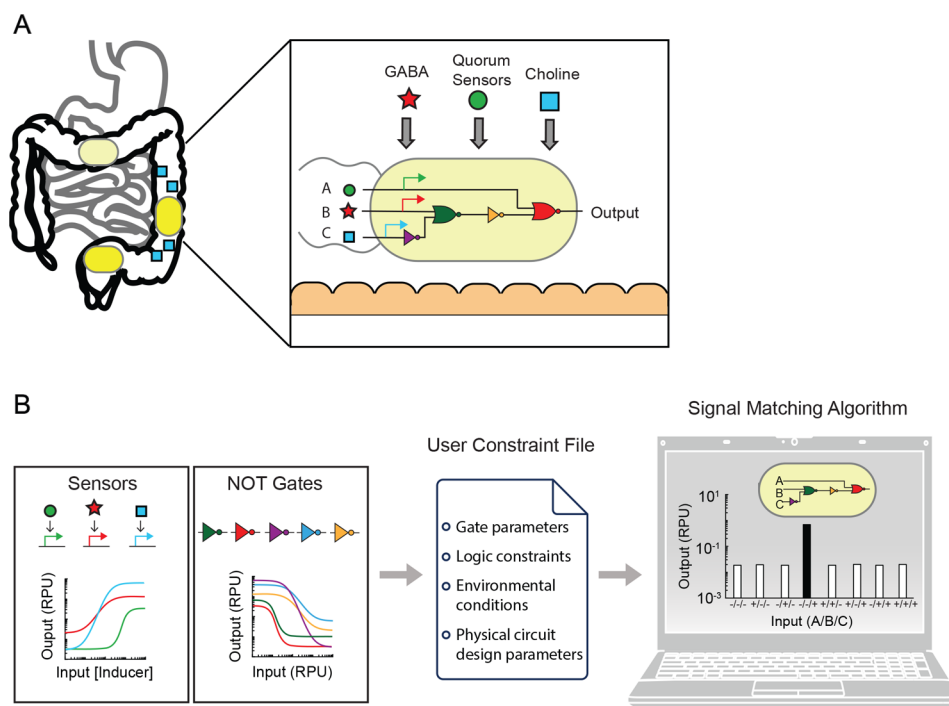


Figure 1. Genetic circuit design in EcN using characterized components. (A) An engineered probiotic microorganism can be used to dynamically sense and respond to multiple signals found in the human gut. A genetically encoded circuit can program the microbial cells to have a specified transcriptional output to a combination of sensed signals. (B) A library of genetic NOT gates and sensors is characterized to determine their standard response functions and is used as the input to the circuit design algorithm, which predicts the circuit signal processing when gates are combined. The gate characterization data and sensors can be incorporated into a customized user constraint file for automated circuit design.

applying the genetic circuit components used in previous works in a different microorganism, such as a probiotic bacterium, and integrating them with other genetic parts and sensors for the new host is often not straightforward and can require significant engineering to reacquire functionality.

To create a genetic circuit, it is critical that the specified set of genetically encoded sensors (i.e., for sensing the biochemicals and biomarkers) can be integrated with the necessary circuitry for the required response without disrupting the circuit's signal processing. A library of highly insulated genetic gates having standardized DNA architecture and a common signal carrier between them allows for more easily mixing and matching genetic sensors and logic gates together into the required logic circuits, while applying a uniform set of design rules and predictive modeling parameters or algorithms to guide the circuit design.^{61–64} In this work, we focus on insulated repressor-based logic gates built from a set of orthogonal transcriptional repressors⁶⁵ and in which the input and output of a gate are promoters regulated by transcription factors. A framework for algorithmically designing complex logic circuits, achieved by signal matching of the characterized response functions for the repressor-based NOT gates and characterized sensors, has been implemented in *E. coli* NEB 10-beta, *Bacteroides thetaiotamicron*, and *Saccharomyces cerevisiae*.^{61,62,66–68} The output promoter activity of one gate is used as the input to the next when gates are interconnected in layers, and the RNA polymerase flux along DNA, quantified in standard relative promoter units, is the common signal between gates.^{69,70} Using this approach, layered gates have been used to design genetic circuits in multiple microbes,^{61,66,67} including sequential logic circuits containing memory, in *E. coli* NEB 10-beta.⁶² Whether the performance of

these genetic circuit components is changed when transferred to another bacterial strain and what effect these changes might have on the designed circuits have not been examined in EcN.

Here, we establish a framework for predictive genetic circuit design in EcN using a signal matching algorithm and a data set of standard characterized components (Figure 1). We characterize repressor-based genetic NOT gates in EcN, which can be used to generate strain-specific user constraint file. We further show that the observed differences in NOT gate response functions between *E. coli* strains can prevent genetic circuit designs from being functionally transferable between different strains of *E. coli*. Using small molecule sensors characterized in EcN and the library of repressor-based genetic NOT and NOR gates, we design and test multi-input combinational logic circuits, including the use of a genetic 3-input NOR gate, to validate a signal matching design algorithm. We also design and test a library of 28 set-reset latches (SR latches) to validate a circuit design algorithm in the probiotic strain EcN, as well as propose new design rules for this strain. Furthermore, we demonstrate that predicting the switching threshold of SR latch circuits and combining them into a memory register can achieve an analog-like concentration recorder circuit in EcN. We create engineered EcN cells that contain a concentration-recording circuit, which enables the cells to sense, record, and report three distinct concentration ranges of a small molecule. With this, we provide an approach for programming EcN by algorithmic genetic circuit design that may one day be used to potentially design living therapeutic and diagnostic devices with multi-input sensing to probe the gut environment.

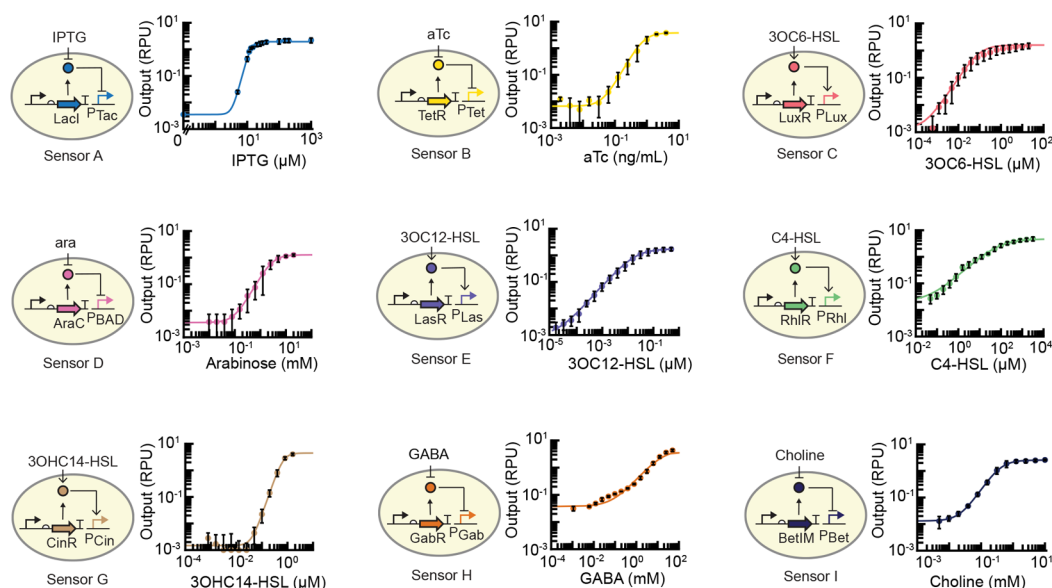


Figure 2. Sensor characterization of genetically encoded small molecule sensors in EcN. The measured sensor response for nine small molecule sensors. The concentrations of IPTG used were 1000, 200, 150, 100, 40, 30, 25, 20, 15, 13, 11.5, 10, 5, and 0 mM. The maximum inducer concentrations for other sensors were: 4 ng/mL aTc, 20 μ M 3OC6-HSL, 20 mM ara, 3.2 mM C4-HSL, 0.5 μ M 3OC12-HSL, 1.6 μ M 3OHC14-HSL, 50 mM GABA, and 10 mM choline. Serial dilutions of 2-fold were performed for each subsequent inducer concentration. Cell fluorescence was measured via flow cytometry, and arbitrary fluorescence units were converted to RPU (Materials and Methods). The experimental measurements were fit to the Hill equation. The markers represent the average of the measured median fluorescence of a population of at least 5,000 cells assayed in three identical experiments performed on three different days. All error bars represent the standard deviation.

RESULTS

Characterizing a Set of Small Molecule Sensors for EcN. To facilitate multiplex sensing and to validate the interchangeability of sensors in genetic circuits in EcN, nine different genetically encoded small molecule sensors, including two metabolite sensors and 4 quorum sensors, were assembled into backbone plasmids on which circuits would later be built. The sensors were constructed for use in EcN using an identical plasmid architecture and by replacing the appropriate sensor block on the low-copy (p15A origin) circuit backbone plasmid. Each sensor consisted of a single allosteric transcription factor that regulated its cognate sensor output promoter in response to a small molecule. The sensor output promoters $P_{T_{ac}}$, $P_{T_{et}}$, $P_{B_{ad}}$, and $P_{L_{ux}}$ have been used in previous work as circuit inputs in *E. coli* NEB 10-beta, and here, were tested on the circuit backbone plasmid pLW555,⁶² which constitutively expresses the transcription factors LacI, TetR, AraC, and LuxR to regulate each promoter (Figure S1) and are induced by isopropyl β -D-1-thiogalactopyranoside (IPTG), anhydrotetracycline (aTc), L-arabinose (ara) and 3-oxohexanoyl-homoserine lactone (3OC6-HSL), respectively. Additionally, sensors were constructed for gamma-aminobutyric acid (GABA) using GabR,²¹ choline (cho) using BetI, N-butyl-homoserine lactone (C4-HSL) using RhlR, N-3-oxododecanoyl-homoserine lactone (3OC12-HSL) using LasR, and N-(3-hydroxytetradecanoyl)-homoserine lactone (3OHC14-HSL) using CinR.

To quantify each sensor's response function, the sensor output promoter was placed upstream of an insulated yellow fluorescent protein (eYFP) transcriptional unit fragment from the relative promoter unit (RPU) standard plasmid (pAN1717)⁶¹ on a plasmid also containing the corresponding sensor block (Figure S1). Each constructed sensor characterization plasmid was transformed into EcN to assay the

response function for each sensor. The single-cell fluorescence output was measured via flow cytometry, converted to RPU, and fit to the Hill equation to determine the response function (Materials and Methods). The RPU standard plasmid contains the BBA_J23101 reference promoter, which is set to 1 RPU in the conversion from arbitrary fluorescence units to RPU (Materials and Methods). The purpose of converting the output to RPU is to allow for quantitative comparison of response functions between flow cytometer instruments, laboratories, and measurement parameters (e.g., cytometer laser voltage).^{70,71} The response functions for each of the nine sensors in EcN were determined (Figure 2).

When comparing the sensor responses in EcN and *E. coli* NEB 10-beta, differences in the response functions were observed (Figure S2 and Table S1). The $P_{T_{ac}}$ sensor in particular had a steeper transition from the uninduced to the induced state, possibly due to EcN expressing the *lac* operon from its genome.⁷² Though not surprising, these changes in sensor response functions demonstrate that it can be inaccurate to directly use the response function parameters from a different strain of a microorganism. Notably, an inaccurate sensor response is problematic not only because it affects the genetic circuit predictions but also because it can affect the determination of response functions for the genetic logic gates if that sensor is used. Despite differing average cell fluorescence (in arbitrary units) for the RPU standard plasmid between strains (EcN = 1143 ± 41 au; 10-beta = 1427 ± 130 au), the single-cell output in a population showed less relative cell-to-cell variation in EcN than in *E. coli* NEB 10-beta. The standard deviation for a cell population of EcN was $50 \pm 2\%$ of the average fluorescence as opposed to $64 \pm 4\%$ for *E. coli* NEB 10-beta ($p < 0.01$), supporting that the plasmid system is stable and provides a consistent output in EcN (Figure S3).

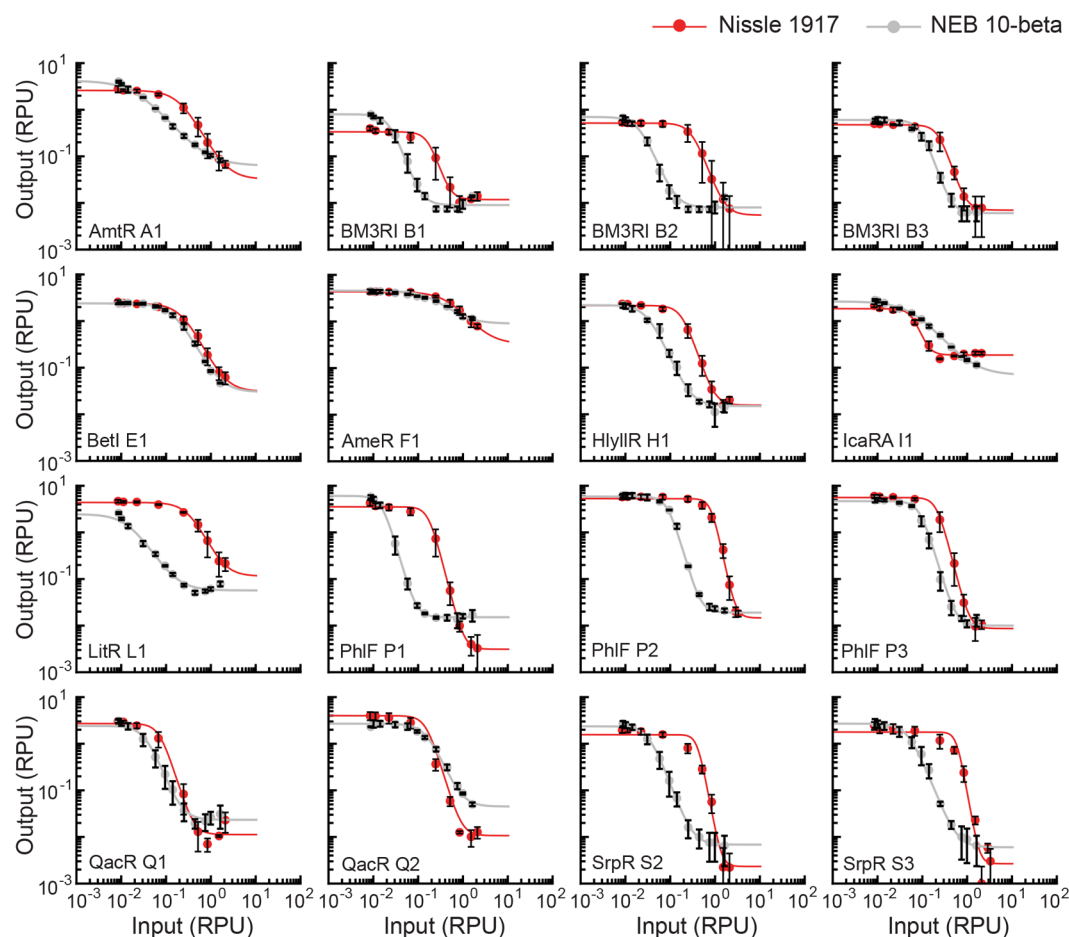


Figure 3. Comparison of NOT gate response functions in EcN and laboratory *E. coli* strains. NOT gate characterization in *E. coli* NEB 10-beta (gray) used the P_{Tac} input sensor and in *E. coli* Nissle 1917 (red) used the P_{Tet} input sensor. The aTc concentrations used were 1.00, 0.75, 0.50, 0.375, 0.25, 0.125, 0.0625, 0.0313, and 0.0156 ng/mL. Additional concentrations of 0.185 ng/mL for IcaRA I1 and 2.0 and 1.5 ng/mL for SrpR S3 and PhlF P2 were used. The concentrations of IPTG used were 1000, 200, 150, 100, 70, 50, 40, 30, 20, 10, 5, and 0 μ M. Cell fluorescence was measured via flow cytometry, and arbitrary fluorescence units were converted to standard RPU (Materials and Methods). The experimental measurements were fit to the Hill equation. The markers represent the average of the measured median cell fluorescence for a population of at least 5,000 cells assayed in three identical experiments performed on three different days. All error bars represent one standard deviation from the mean.

In addition, four different homoserine lactone (HSL) sensors were tested in EcN. HSL quorum sensing systems are commonly found in Gram-negative bacteria, which utilize an allosteric transcriptional activator (e.g., LuxR) to detect HSL biochemical signals and regulate the cognate promoter, often facilitating population-level responses using bacterial intercellular communication.^{73,74} Previously, HSL quorum sensors have been widely used to engineer cell–cell communication⁷⁵ and program multicellular functions in microbial consortia.^{76,77} In EcN, both LuxR and LasR have been used previously.^{78,79} To construct HSL sensors in EcN, *luxR* in the sensor block was replaced with either *rhlR*, *lasR*, or *cinR*, which sense C4-HSL, 3OC12-HSL, or 3OHC14-HSL and activate the promoter P_{Rhl} , P_{Las} , or P_{Cin} ^{73,80} respectively (Figure S1).

The last two sensors characterized respond to two metabolites present in the gastrointestinal tract, gamma-aminobutyric acid (GABA) and choline. GABA produced by bacteria found in the human intestines⁸¹ is a neurotransmitter that has been shown to affect neurological conditions including depression and epilepsy^{82–84} as well as affect mood and sleep disorders.^{82,85} The GABA sensor used the GabR protein from

B. subtilis to regulate an engineered promoter P_{Gab105} .²¹ In *B. subtilis*, GabR has been shown to be a pyridoxal 5'-phosphate-dependent transcriptional activator of its native promoter,⁸⁶ and in EcN, GabR has been reported to act as a repressor for the synthetic P_{Gab105} .²¹ Choline is a precursor to a variety of chemicals in the body such as neurotransmitters and membrane phospholipids.^{87,88} It is an important nutrient for pregnant and breast feeding women as it is vital for brain development.^{3,87} Additionally, choline deficiency has been associated with liver and muscle damage.⁸⁸ While the essential nutrient choline can be synthesized in the body,⁸⁷ it still must be supplemented by dietary intake from sources such as eggs, meat, or milk.³ Choline is detected by the allosteric transcriptional repressor BetI, which de-represses its cognate promoter P_{BetI} when sufficient choline is present.⁸⁰ Here, we used BetIM, an engineered variant of BetI incorporating a point mutation that has been shown to improve the dynamic range of the sensor.⁸⁰ Three designs of the choline sensor were constructed by varying the strength of the promoter expressing BetIM (Figure S4). The variant with the highest dynamic range (200-fold induction) was chosen as the choline sensor for subsequent genetic circuit design.

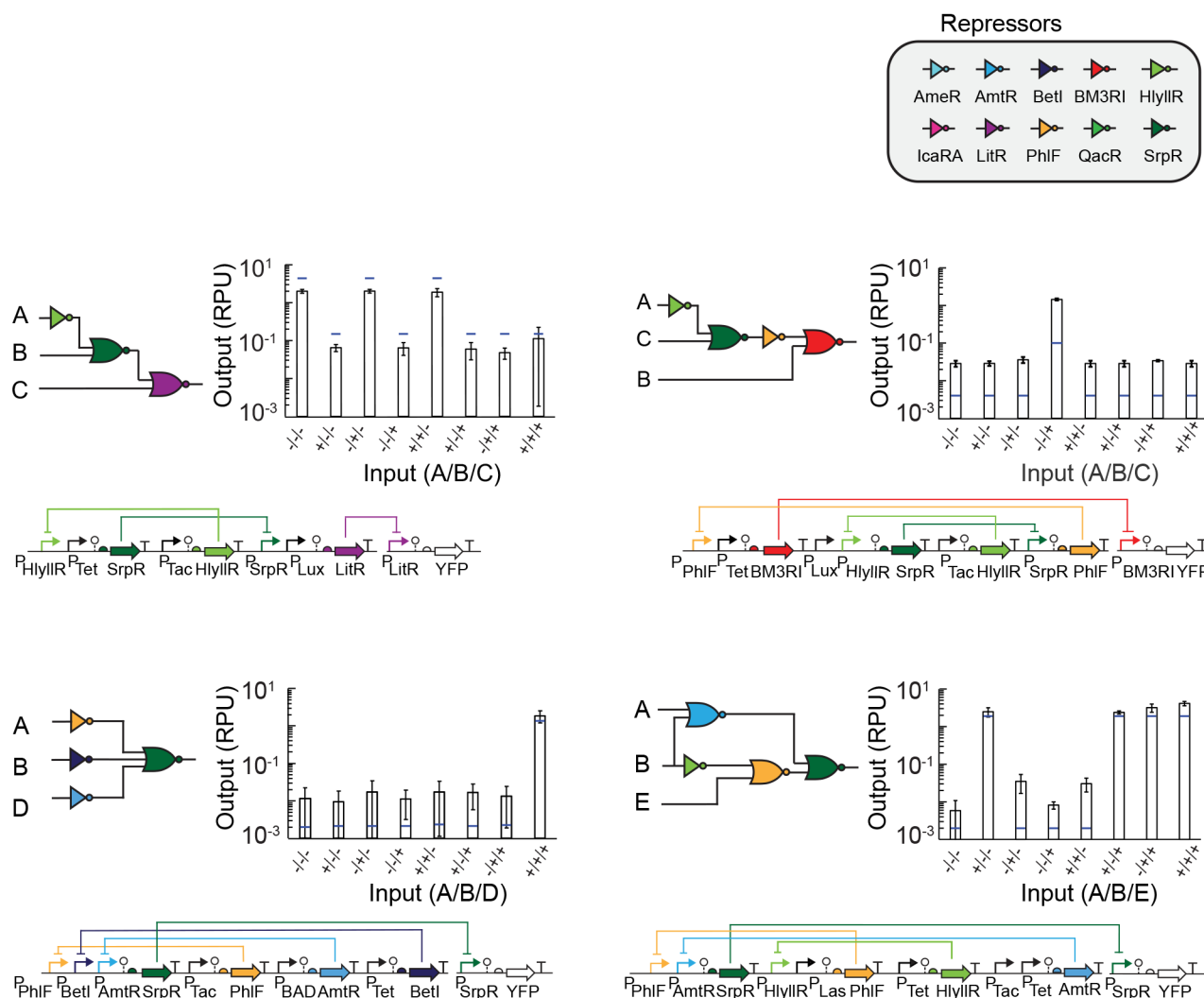


Figure 4. Combinational logic circuits in *E. coli* Nissle 1917. EcN cells containing genetic circuits were grown in the presence (+) or absence (–) of the small-molecule inducers. The inducer concentrations used for each sensor are 1 mM IPTG (Input A), 2 ng/mL aTc (Input B) 10 μ M 3OC6-HSL (Input C), 10 mM arabinose (Input D), and 0.008 μ M 3OC12-HSL (Input E). Colored symbols represent genetic gates with each color representing a different repressor (Figure S5). Cell fluorescence was measured via flow cytometry, and arbitrary fluorescence units were converted to standard RPU (Materials and Methods). The bars represent the average of the measured median fluorescence of a population of at least 5,000 cells assayed in three identical experiments performed on three different days. All error bars represent one standard deviation from the mean. The predicted output from signal matching is overlaid on the corresponding bar (— blue marker). For each circuit, there is a significant difference between each ON state and each OFF state ($p < 0.01$ for paired two-tailed Student's *t*-tests).

Each sensor was characterized to determine the response function relating the sensor's output (i.e., sensor promoter activity) to the concentration of the small molecule input (Table S1). For the set of nine sensors, the basal promoter activity was 0.010 ± 0.009 RPU with an induced promoter activity of at least 1.2 RPU. Each sensor had ON (induced) and OFF (uninduced) states compatible with switching the states of our genetic NOT gates. All characterized sensors were able to achieve a dynamic range of at least 115-fold induction (645 ± 520 -fold induction for the 9 sensors).

A Library of Repressor-Based NOT Gates for EcN.

Using only layered NOT and NOR gates, all logic functions are theoretically accessible from the perspective of Boolean logic. Here, we used a set of 10 orthogonal TetR-family repressors⁶⁵ for our genetic NOT and NOR gates and circuit designs (Figure 3). The genetic NOT gate has one transcriptional input (i.e., input promoter) controlling the

expression of the insulated gene for the repressor, which subsequently regulates its cognate promoter (i.e., gate output promoter) (Figure S5). With the output of one gate becoming the input to another gate as they are layered in a circuit design, it is imperative to accurately know the signal processing performed by each to utilize circuit design algorithms. While large libraries of characterized repressor NOT gates have been reported for other bacteria, including *E. coli* NEB 10-beta, they have not been examined for EcN. However, a library of 7 repressor-based NOT gates on the chromosome was recently reported for EcN.²⁴ For four repressors in our study (BM3R1, PhlF, QacR, SrpR), multiple gates were tested with a different ribosome binding site (RBS) for each.^{61,62} To characterize the set of 16 NOT gates, we integrated a characterized sensor as the input to the insulated gate and used the gate output promoter to express the standard eYFP transcriptional fragment,⁶¹ which notably contains a ribozyme insulator

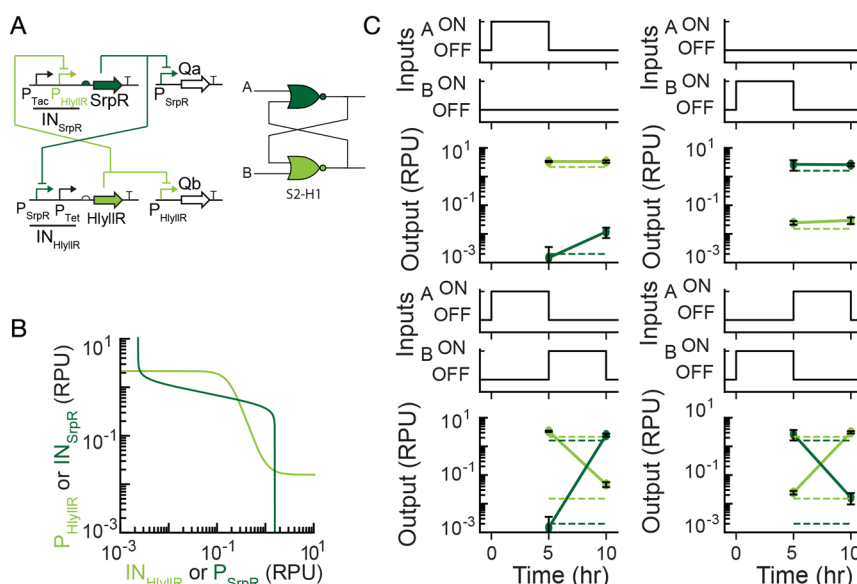


Figure 5. A functional genetic SR latch in *E. coli* Nissle 1917. (A) Genetic schematic of the SR latch using the repressors SrpR and PhlF with input promoters P_{Tac} and P_{Tet} which sense IPTG and aTc, respectively. The output of P_{SrpR} (Qa) and P_{HlylIR} (Qb) were measured. (B) The nullclines of the two gates showing predicted bistability. (C) One inducer was added to the cells and grown for 5 h (either 1 mM IPTG for Sensor A or 2 ng/mL aTc for Sensor B). Cells were then diluted into fresh media without inducer (memory recording assay) or with the other inducer (state switching assay) and grown for 5 h longer. Cell fluorescence was measured via flow cytometry at 5 and 10 h (Figure S9), and circuit output is reported in standard RPU (Materials and Methods). Dashed and solid lines represent predicted and measured outputs, respectively. Markers represent the average of the measured median fluorescence of a population of at least 5,000 cells assayed in three identical experiments performed on three different days. All error bars represent the standard deviation.

(RiboJ). We applied previously reported gate characterization protocols^{61,62} to measure each gate's response function, which describes the input–output relationship for the transcriptional NOT gate in standard units for promoter activity. The promoters P_{Tac} and P_{Tet} were used as input promoters for characterization of the gates in *E. coli* NEB 10-beta and EcN, respectively. While P_{Tac} was previously used to assay gates in *E. coli* NEB 10-beta,^{61,62} P_{Tet} was chosen for EcN due to its more gradual transition in the response function as compared to P_{Tac} , which facilitated sampling the range of RPU input values at more even intervals.

Each of the 16 NOT gate constructs was characterized in both the laboratory strain (*E. coli* NEB 10-beta) and the nonmodel EcN strain on a circuit backbone plasmid containing the *lacI* and *tetR* genes. The cell fluorescence was measured by flow cytometry and converted to standard relative promoter units (RPU). The data for each gate were fit to the Hill equation to determine the gate response function (Materials and Methods), creating a library of characterized NOT gates for constructing genetic circuits in EcN (Table S2). When comparing the gate response functions between EcN and *E. coli* NEB 10-beta, we observed shifts for most but not all NOT gates (Figure 3). These changes included the input thresholds for state switching, the ON state promoter output, and the OFF state promoter output for a gate. While not surprising, this further reinforces the need for characterized circuit components for nonlaboratory strains (e.g., strain-specific user constraint files⁶¹) and demonstrates that genetic gates may not function identically when transferred to another strain, which would affect circuit performance and signaling.⁸⁹ The library of gates for EcN contains multiple gates for some repressors that transition between ON and OFF promoter states at different input values, which is useful to provide a

greater number of gates that can be functionally interconnected in a circuit.

Combinational Logic in EcN. Once the response functions of all sensors and NOT gates were determined, we applied a genetic circuit design algorithm to select the genetic circuit components for a set of combinational logic circuits for EcN that were then built and assayed. A combinational logic circuit is composed of logic gates but does not contain memory, and therefore, the signal processing and output of the circuit are dependent on the combination of input signals present. Here, we applied the signal matching algorithm⁶¹ and NOT/NOR gates for genetic circuit design. The signal matching algorithm predicted the circuit signaling in the cell by computing the output of each gate wired to the specific input promoter(s) strictly from the corresponding gate response function and using the input promoter activity in RPU. Then, the predicted output of the gate (i.e., gate output promoter activity in RPU) was used as the input to the next gate in series for a given circuit design. This was repeated for each subsequent layer of gates in the circuit until the final layer was reached and the circuit output was predicted. For a genetic NOR gate, the identical response function as the repressor NOT gate was used since their DNA sequences are the same aside from a second input promoter placed in tandem, the effects of which we assumed to be additive.⁶¹ The algorithm also applied design rules to determine if an input promoter is functionally compatible with a downstream NOT or NOR gate, meaning that the high and low promoter strengths are sufficient to switch the downstream gate between the OFF and ON states (i.e., 2-fold input threshold as previously applied),^{61,66} and to exclude two roadblocking promoters in a NOR gate.⁶¹ Signal matching has been previously used to predict the functionality of multi-input combinational circuits

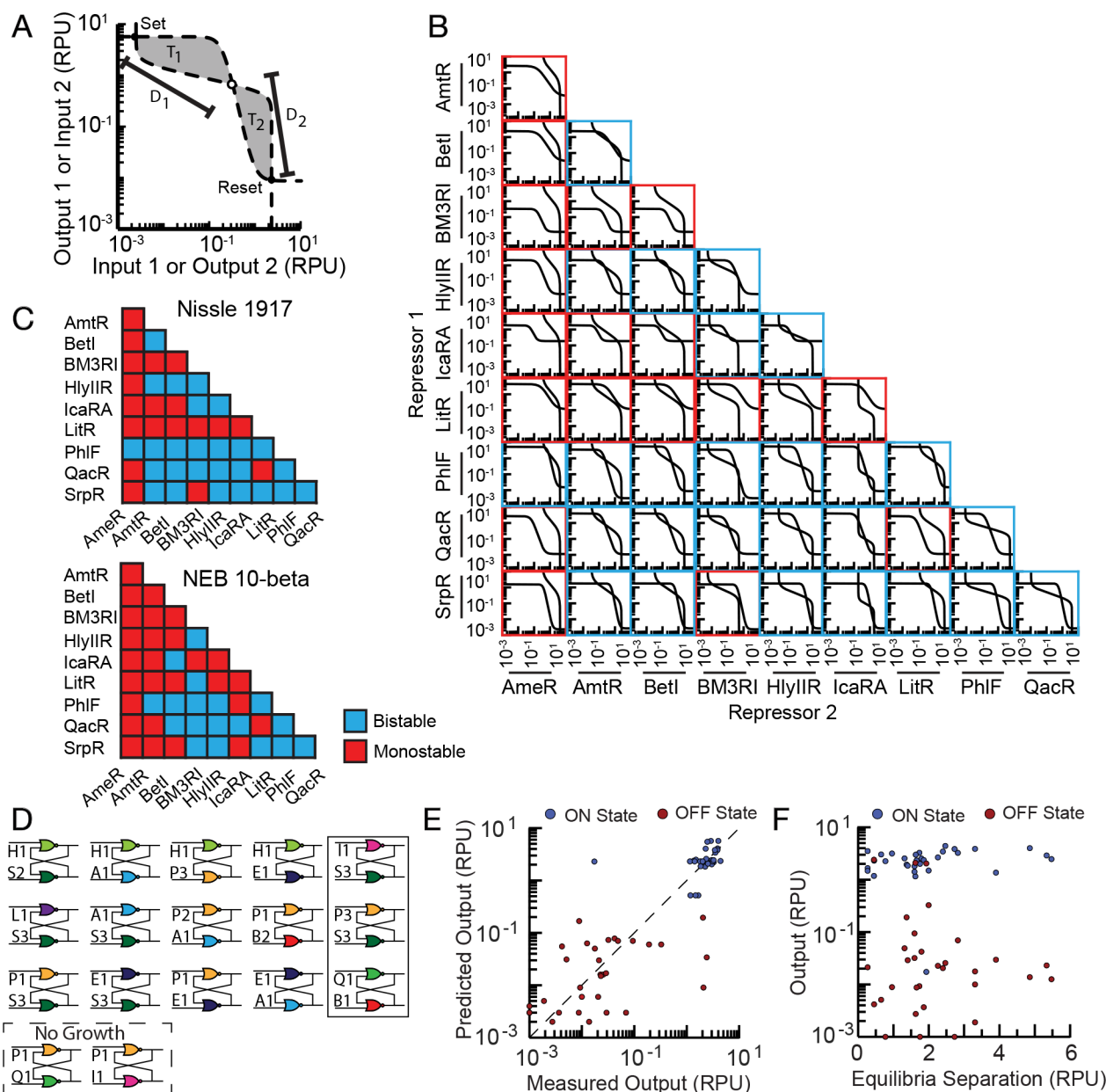


Figure 6. Library of SR latches in EcN to refine design criteria. (A) An example of the phase plane analysis for the design of an SR latch is shown. Equilibria separations (D_1 , D_2) are the distance in phase space between either stable equilibrium (SET or RESET state) and the unstable saddle point (open circle). The transversality is the area shaded gray (T_1 and T_2). (B) Phase plane analysis of each combination of repressors in EcN showing both bistable (blue) and monostable (red) latches. The pairs of gates shown had the largest transversality among different RBS variants for a repressor. (C) Combinations of gates that are predicted to be theoretically bistable (blue) and monostable (red) SR latches in both *E. coli* Nissle 1917 and NEB 10-beta. (D) The set of SR latches used in this study with the ribosome binding site variant of each gate shown. Boxed latches were empirically determined to be nonfunctional. (E) A comparison between the observed ON (blue) and OFF (red) states of the output promoters from each latch (Pearson coefficient = 0.85, $R^2 = 0.69$, $n = 15$ SR latches with cell growth). The dashed line is $y = x$. (F) The equilibria separations for each latch (D_1 and D_2) plotted against its corresponding measured output for each state.

comprised of insulated genetic sensors and gates in *E. coli* NEB 10-beta and other microbes.^{61,66–68} Key to this relatively simple design approach is the use of insulated sensors and genetic gates. For example by applying self-cleaving ribozymes and strong terminators, the effects of genetic context on expression can be reduced.^{61,62} For genetic circuit design, we used the response functions from our characterized library and applied signal matching to compute the signal processing in EcN, which allowed us to assign a set of suitable gates for genetic circuits in EcN.

To test the signal matching design algorithm in EcN, four combinational logic circuits each having three inputs were constructed by using a subset of the sensors developed above (Figure 4). Each circuit was assembled onto a plasmid backbone that contained sensor blocks for IPTG, aTc, ara, and either 3OC6-HSL or 3OC12-HSL, as appropriate for the circuit inputs. The plasmid containing the genetic circuit was transformed into EcN, along with an output plasmid containing eYFP under the control of the output promoter of the final gate in the circuit (Figure S1). The EcN cells were

grown in media containing a three-input combination of the appropriate small molecule inducers for all eight combinations of the 3 inputs, and the cell fluorescence of each sample was analyzed via flow cytometry and output was converted to RPU (Materials and Methods).

We observed a strong correlation and fairly close agreement between the predictions from the design algorithm and the measured circuit output for the 4 circuit designs and 32 conditions (Pearson coefficient = 0.94, $R^2 = 0.72$) (Figure S6). However, cells containing the three-input AND circuit had a small population (5–10% of cells) of highly fluorescent cells for each of the seven OFF states (Figure S7). We designed an additional 3-input AND circuit using a different set of sensors (substituting P_{Lux} for P_{BAD}) and reassigned the gates. Testing of this circuit showed that the subpopulation of spuriously activated cells was eliminated (Figure S8). Each circuit was able to achieve each predicted state and an observed dynamic range of at least 16-fold between the lowest ON state and highest OFF state for each circuit (16-fold to 482-fold range).

Genetic SR Latches in EcN. A genetic circuit requires memory to store an output signal after cells leave the environment in which their sensed target or small molecule was present. Logic circuits that have memory are known as sequential logic circuits. They can record the history of input signals and are the basis of modern computing. Among the simplest sequential logic circuits is the bistable set-reset latch (SR latch), which utilizes the feedback from two cross-coupled NOR gates to record one bit of information, such as the presence of a small molecule after it is removed or no longer present⁶² (Figure 5A). In order for a genetic switch or SR latch to store memory, it must exhibit bistability.^{43,62} Therefore, SR latches require nonlinear response functions, achieved in our work by using a set of repressors (TetR homologues) that have cooperative ligand binding and empirical Hill coefficients greater than one ($n > 1$) in their gate response functions. As expected, all 16 genetic NOT gates in EcN meet this criterion (Table S2). Theoretical bistability for an SR latch comprised of cross-coupled NOR gates can be evaluated by plotting the response function of one NOT gate against another reflected over the diagonal to generate the phase plane diagram and nullclines⁶² (Figure 5B). Then by phase plane analysis, the intersections of the response functions (nullclines) represent equilibria. If there are three intersection points, then there are two stable equilibria separated by an unstable saddle point between them. If the curves intersect only once, then the combination of NOR gates is monostable and not a functional genetic SR latch. We first tested the application of this theory to EcN by building a genetic SR latch. As predicted, the circuit switched states when grown sequentially with either inducer. The cells also maintained and stored the output after the triggering input signal was no longer present, demonstrating functional memory (Figure 5C). The combination of repressors able to form bistable latches can be expanded by using a different RBS to shift the response function of a gate in a manner dependent on the RBS strength.³¹ We next sought to use the library of characterized gates to refine the design criteria for biological SR latches in EcN.

We performed a phase plane analysis to assess the bistability criterion for the design of SR latches in EcN (Figure 6A). For each pair of repressors, all combinations of gates were analyzed by plotting the steady state response functions as nullclines to determine whether they were predicted to be monostable or bistable (Figure 6B). From the 16 NOT gate response

functions for each *E. coli* strain, we identified 26 repressor pairs (54 gate combinations) that met the theoretical bistability criterion in EcN, and for comparison, only 18 repressor combinations (44 gate combinations) were identified to be bistable in *E. coli* NEB 10-beta (Figure 6C). Additionally, 16 pairs of gates predicted to be bistable in *E. coli* NEB 10-beta do not meet the bistability criterion in EcN.

We constructed a set of 17 SR latches each containing a different pair of gates on our 4-input (Sensors A–D) backbone pLW555 to test the design predictions for EcN using sensors from our characterized set (Figure 6D). For an SR latch to be functional, it must have the ability to switch between states and hold either output state with the appropriate input signals. Here, we assayed the SR latches using previously established protocols.⁶² To test its functionality, the circuit was first induced to one state by growing the cells in the presence of one input signal (small molecule sensed by the sensor) for 5 h, and this was performed in parallel for each of the two input signals separately. To determine whether the latches were able to store memory, the cells from each sample were then washed or diluted and grown for another 5 h without inducer. To determine whether the latches were able to switch states, the cells were grown for a further 5 h with the small molecule for the previously uninduced sensor promoter. Separate samples were used to analyze each output of the circuit, both transformed with a plasmid containing the circuit and a plasmid expressing eYFP from one of the two output promoters. Samples were analyzed via flow cytometry to measure single cell fluorescence after each 5 h interval (Figure S10). For two SR latch designs, there was no observed cell growth for one cell state, and analysis could not be performed (Figure 6D). Of the remaining 15 SR latches, 12 designs were functional latches, meaning that after induction to an initial state, they were able to hold their current state for 5 h and switch states when provided with other inducers. To be considered a functional latch, a cutoff of a 7-fold dynamic range between the ON and OFF states was applied. For these 12 SR latches, the average dynamic range of each promoter output was 423-fold (7–3000-fold range), and the measured outputs had strong agreement with their predicted values (Pearson coefficient = 0.92, $R^2 = 0.85$) (Figure 6E).

To test whether the SR latches were composable with different sensor combinations, a set of 11 additional SR latches were constructed with different sensors using circuit backbones that incorporated the choline, GABA, or other HSL sensors (Figure S10). The interchangeability of the sensors for SR latch designs built from insulated genetic gates has been previously demonstrated in *E. coli* NEB 10-beta.⁶² The phase plane analysis assumes that the NOT gate response functions would remain unchanged. Ten of the SR latches were able to hold their states within 7-fold for 5 h and switch states after a 5 h induction (Figure S10). For all ten of these SR latch designs, at least a 7-fold difference between the average ON and OFF state for each output was observed. For three pairs of gates and using three different pairs of sensors for each (9 SR latches), we analyzed the effect of changing the sensors and found strong agreement between the measured and observed output of the SR latches (Pearson coefficient = 0.92–0.98, $R^2 = 0.86$ –0.96 for 3 gate pairs) (Figure S11). This demonstrates that generally, sensors can be interchanged in the SR latch circuit design and remain functional in EcN.

We next evaluated whether we could determine design rules to improve the accuracy of SR latch design in EcN from our set

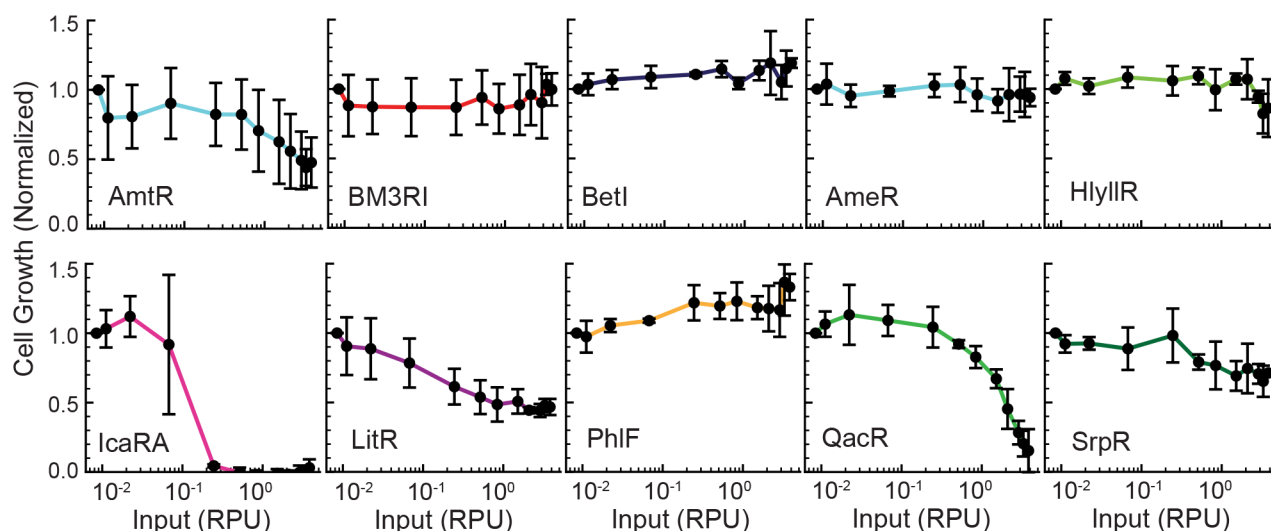


Figure 7. Repressor toxicity measured in *E. coli* Nissle 1917. NOT gates containing each repressor were induced with increasing concentrations of aTc as in the initial NOT gate characterization assay. The concentrations of aTc used were 4, 2, 1.5, 1, 0.75, 0.5, 0.375, 0.25, 0.1875, 0.125, 0.0625, and 0.03125 aTc. 100 μ L of liquid was taken from the samples after 5 h of induction, and the OD₆₀₀ was measured using a plate reader. The absorbance was normalized to the uninduced sample for each gate. Markers represent the average of the measured OD₆₀₀ in three identical experiments performed on three different days. All error bars represent the standard deviation.

of assayed SR latches. It has been previously shown that adding design criteria for the separation of the equilibria or transversality (Figure S12) can prevent designing nonfunctional SR latches when theoretical bistability is determined from the gate response functions, which describe the average cell output and do not represent the inherent cell-to-cell variation. Here, an equilibria separation (i.e., distance between the stable and unstable steady states in the phase plane) cutoff of 2.0 RPU appeared sufficient to remove nonfunctional states (Figure 6F). However, each of the designed latches had one state with an equilibria separation of less than 2 RPU. A less stringent equilibria separation cutoff of 0.8 RPU, approximately what was used for *E. coli* NEB 10-beta (0.75),⁶² removed all but one nonfunctional design. After applying the design requirement for an equilibria separation ≥ 0.8 RPU, the agreement with the predicted circuit performance improved (without cutoff: Pearson coefficient = 0.85, R^2 = 0.69; with cutoff: Pearson coefficient = 0.91 and R^2 = 0.82) (Figure S13). There was no obvious transversality cutoff to eliminate nonfunctional latches. For the subset of SR latches (5 out of 17) that were nonfunctional or had negligible cell growth in at least one circuit state, all but one latch contained the repressor QacR, IcaRA, or LitR. Notably, these repressors displayed high growth inhibition in EcN when induced at an input level comparable to that in the latch design (49–99% reduced cell growth) (Figure 7). Particularly the combination of P_{PhIF} , the strongest of our set of repressible promoters, or P_{Tet} with one of these repressors, often inhibited cell growth. The toxicity of these repressors has been previously reported in *E. coli* NEB 10-beta,⁶¹ and suggests that a toxicity score criterion would also improve the accuracy of genetic circuit design in EcN.

Memory Registers and Concentration Recording Circuits in EcN. We next sought to test whether we could construct more complex sequential logic circuits that could be used for recording concentration in EcN. Utilizing our library of functional SR latches, a pair of SR latches was chosen to be combined to create a memory register circuit that could record 2 bits of memory or two separate chemical signals. Within a

memory register, the SR latches must be composed of different pairs of repressors. The memory register circuit was grown overnight and assayed in the same way as the SR latches, with all four outputs being recorded after 5 h of induction as well as after 5 h of switching or removing inducers. The 2-bit memory register was able to both switch states and hold its states as predicted (within 2-fold for ON states, 12-fold for OFF states) (Figure 8).

To achieve concentration recording, we hypothesized that by choosing SR latches with different input thresholds for state switching (i.e., the unstable saddle point), we could apply the memory register circuit as an analog-like concentration recording device for sensed small molecules. Whereas an SR latch typically has a binary output to sense whether the input signal (small molecule) is present or absent, here we could leverage the distinct response functions of the gates (nullclines) to design latches with specified and different state switching input thresholds. In this way, the biological concentration recording circuit in EcN was designed to have a distinct multibit output that could indicate the range of the concentration of input used. As the number of SR latches in the register increases, smaller concentration intervals can theoretically be distinguished. In this initial demonstration, we designed a two-bit memory register circuit that could store three concentration ranges for a sensed small molecule, which we refer to as low, medium, or high concentration (Figure 9A). For diagnostic applications, this concentration recording circuit is initially induced so that both SR latches are in the RESET state, which indicates a low concentration range. Once the cells are deployed, the circuit will maintain the RESET state for both latches if the input signal (small molecule) is below the state switching threshold for both SR latches. As the concentration increases, the input signal will reach the lower state switching threshold and be sufficient to switch the state of one SR latch but not yet sufficient to switch the other. Once the concentration exceeds the input thresholds for state switching for both SR latches, both latches will be in the SET state and indicate a high concentration. By choosing which

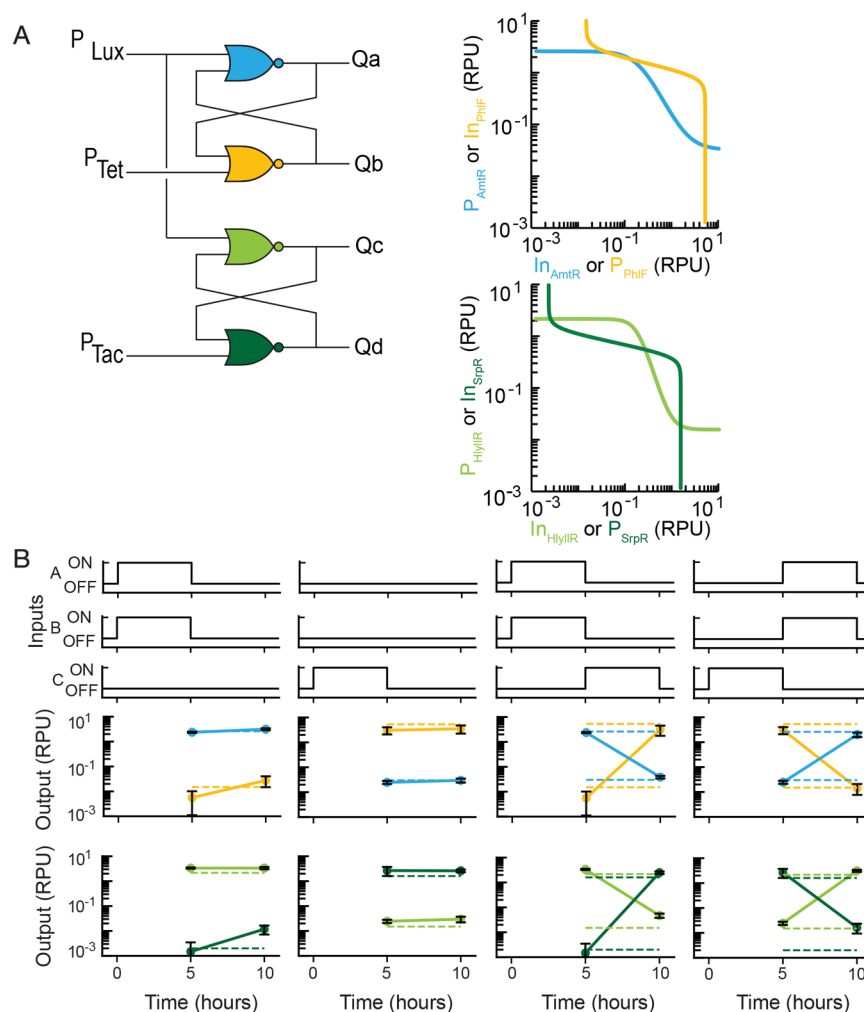


Figure 8. Two-bit memory register circuit. (A) Circuit wiring diagram for the two-bit memory register circuit. (B) Samples were induced with either 1 mM IPTG (Input A) and 2 ng/mL (Input B) or only 10 μ M 3OC6-HSL (Input C). After 5 h of growth the inducers were either switched or removed completely. Cell fluorescence was measured via flow cytometry at 5 and 10 h, and arbitrary fluorescence units were converted to standard RPU (Materials and Methods). Dashed and solid lines represent predicted and measured outputs, respectively. The markers represent the average of the measured median fluorescence of a population of at least 5,000 cells assayed in three identical experiments performed on three separate days. Error bars represent the standard deviation.

latches are used, one can tune the specific ranges of input concentrations detected by the circuit.

We constructed a two-bit memory register for concentration recording with predicted input thresholds for state switching at 0.13 RPU and 0.29 RPU, (0.030 and 0.078 μ M 3OC6-HSL, respectively) and integrated the sensor for 3OC6-HSL (Figure 9B). The 3OC6-HSL has a relatively gradual response and is not a roadblocking promoter. To test whether the switching thresholds were accurate, the circuit was first grown with the reset signals (1 mM IPTG and 2 ng/mL aTc) to initialize both SR latches in the RESET state. Cells were then washed and transferred into aliquots of the media containing an amount of 3OC6-HSL (with 10 samples spanning a concentration range) and grown for 5 h before being washed and transferred again to fresh media with no inducers present and grown for another 5 h. Flow cytometry was used to measure the fluorescence of each sample (Figure S14). Both SR latches had a state switching threshold at approximately the predicted state switching input threshold for each (0.21 and 0.42 RPU) input for half-maximal average output (Figure 9C). After the 3OC6-HSL was removed, we observed that the circuit stored

the state for the different input levels, respectively (Figure 9D). This demonstrates the functionality of the theoretical circuit design for concentration recording. However, we observed that both SR latches had a state switching threshold slightly higher input values than predicted yet within 2-fold. One factor for this may be cell-to-cell variation (i.e., noise), exhibiting a response that is distributed around the mean value, and not a single value for all cells that we assume when applying the response functions as nullclines for determining the state switching input threshold. We expect this contributed to the bimodal distribution observed at certain input values close to the predicted switching threshold since output values greatly differ when the input is close to this threshold (Figure 9D). We expect this circuit design approach could be refined using stochastic models.⁹⁰ Our threshold predictions did not account for the fact that there are now two P_{Lux} input promoters in the system, each of which shares the pool of the activator LuxR with the other. Nevertheless, the circuit was able to record three concentration ranges (less than 0.28 RPU, 0.28–0.57 RPU, and greater than 0.57 RPU).

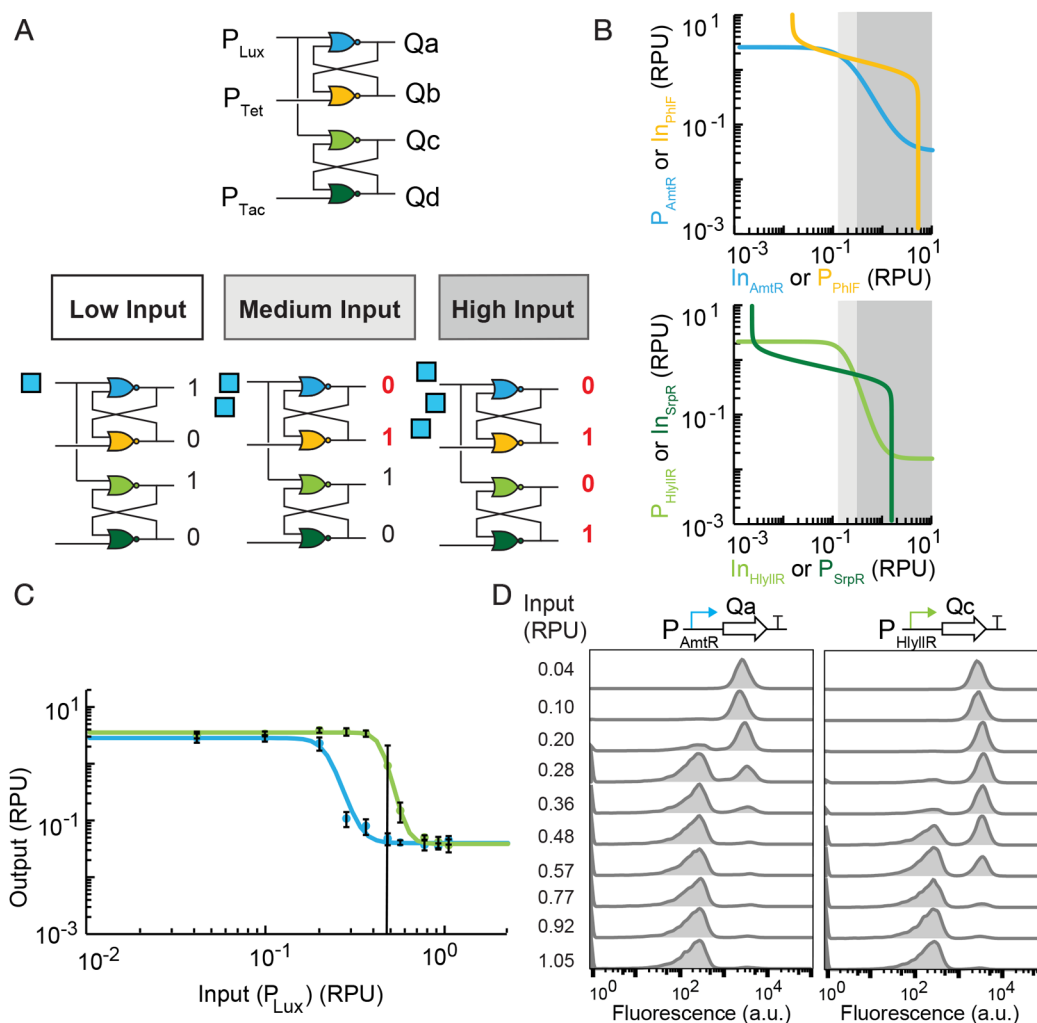


Figure 9. Analog-like concentration recording circuit. (A) Schematic of the concentration recording circuit designed to sense and record three concentration ranges of 3OC6-HSL. Increasing the input will first switch the state of one SR latch, and increasing further will switch the state of both. (B) Utilizing the phase planes for each SR latch, we can predict the input value for each to switch states. (C) The hold states of the circuit outputs P_{AmtR} (blue) and P_{HlyIR} (green) under the control of P_{Lux} induced by 3OC6-HSL. Samples were first grown in 1 mM IPTG and 2 ng/mL aTc to set the initial state of the circuit. At time = 0 h, samples were diluted into fresh media with 3OC6-HSL. Samples were induced for 5 h before being diluted into media with no inducer present. After 5 h of growth cell fluorescence was measured via flow cytometry. Markers represent the average of the measured median fluorescence of a population of at least 5,000 cells assayed in three identical experiments on three separate days. Error bars represent the standard deviation. (D) Representative histograms for the P_{AmtR} and P_{HlyIR} circuit outputs in the hold state after they were previously induced with different concentrations of 3OC6-HSL. Histograms were normalized to 1,000 events.

DISCUSSION

In this study, we present a set of standardized and characterized circuit components and an approach to predictive design of genetic circuits in the EcN bacterium. We demonstrate that these circuits can impart the ability to sense and respond to multiple inputs for a variety of logic functions, record memory via sequential logic, and record the concentration range of an input biochemical signal by using a novel circuit design. The library of components characterized in EcN includes 16 transcriptional NOT gates and 9 biochemical sensors. The transcriptional signal processing of genetic sensors and NOT gates was shown to differ between EcN and *E. coli* NEB 10-beta, highlighting the importance of establishing characterized circuit components in nonlaboratory strains. Characterizing genetic NOT gates and sensors in EcN was critical for using predictive circuit design, as signal processing is highly dependent on the response functions.

Using the strain-specific response functions, we validated that circuit design algorithms previously demonstrated in the laboratory *E. coli* NEB 10-beta strain could be extended to EcN. Necessary design constraints became apparent from the assayed sequential logic circuit library. Requiring a minimum equilibria separation cutoff for SR latch design improved the accuracy of the predictions, similar to a constraint previously implemented for laboratory *E. coli*.^{61,62} SR latches with an equilibrium distance of less than 0.8 RPU had a greater likelihood of having a low dynamic range. Genetic circuits that contained highly toxic repressors (i.e., QacR, IcaRA) proved to be both difficult to build and fully inhibited cell growth when expressed at high levels, which is in agreement with toxicity reported in laboratory *E. coli*.^{61,62} By implementing these design constraints, we can more accurately predict how memory latches function in EcN bacterium.

Here we also present a framework for the design of analog-like concentration recording devices using predictive sequential

logic circuit design from a library of characterized NOT gates. This theoretical approach could be applied to other biochemicals such as gut-microbiota-derived metabolites or neurotransmitters, which have proven difficult to study in their native environment, provided a biosensor for the analyte is developed or available. While other analog memory recording devices have used DNA recording devices (e.g., recombinases, CRISPR) to record signal strength in bacterial cells,^{91–95} our transcriptionally encoded memory can be directly integrated with established genetic circuit design algorithms and does not require DNA sequencing to read the output. Further supporting the potential use of transcriptionally encoded memory and sequential logic circuits in vivo, a genetic toggle switch was previously shown to be functional in vivo in the gut of a mouse model for over 7 days and record memory in *E. coli* MG1655 and an endogenous murine *E. coli* bacterium.⁹⁶

While we present a general framework to achieve combinational and sequential logic circuit design in EcN here, challenges remain and warrant further investigation in future studies. The signal processing and response functions of the circuit components are dependent on growth conditions. Such dependency may necessitate the characterization of circuit components under other relevant conditions when considering the use of a probiotic microorganism in the intestines, such as varying oxygen level, pH, and nutrient availability as well as variable growth rate and interactions with other cells.^{97–100} Future work could aim to establish robustness criteria to assign parts to retain signal processing in conditions more closely resembling the gut environment than those of this study and account for cell-to-cell variability using stochastic models.^{101,102}

Implementing signal recording and multiplex sensing in EcN provides a tool that can potentially be used to probe the gut environment for biomarkers and metabolites indicative of health conditions.^{2,17,19,22,103,104} While the sensors characterized in this work respond to a limited set of small molecules and metabolites, other sensors that have been developed to respond to a wide range of additional stimuli¹⁰⁵ such as inflammation markers,^{17,19} short chain fatty acids found in the intestines,^{21,106} cancer markers,¹⁸ and various other metabolites^{80,107,108} that may be able to be incorporated into the library of circuit components presented. Importantly, due to the modularity of this system, circuit designs can draw from the large array of sensors and outputs developed. This work provides preliminary groundwork for designing sensing and recording circuits in the chassis EcN to probe the gut environment.

MATERIALS AND METHODS

Strains, Media, and Inducers. *E. coli* Nissle 1917 was used for experimentally assaying sensors, genetic gates, and circuits. *E. coli* NEB 5-alpha (New England Biolabs) was used for cloning. *E. coli* NEB 10-beta (New England Biolabs) was used to compare sensor and NOT gate response functions to EcN. Genetic circuits and sensors were assayed in M9 minimal media (Sigma-Aldrich; 6.78 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 1 g/L NH₄Cl, 0.5 g/L NaCl final concentration) with 0.34 g/L thiamine hydrochloride (Sigma-Aldrich), 0.2% Casamino acids (Acros), 2 mM MgSO₄ (Sigma-Aldrich), 0.1 mM CaCl₂ (Sigma-Aldrich), and 0.4% D-glucose (Sigma-Aldrich). Antibiotics used to select for circuit plasmids were 50 µg/mL kanamycin (GoldBio), and 100 µg/mL spectinomycin (GoldBio). The inputs used for the sensor promoters were isopropyl

β-D-1-thiogalactopyranoside (GoldBio), anhydrotetracycline hydrochloride (Sigma-Aldrich), L-arabinose (Sigma-Aldrich), choline chloride (Sigma-Aldrich), gamma-aminobutyric acid (Sigma-Aldrich), N-butyryl-DL-homoserine lactone (Sigma-Aldrich), 3-oxohexanoyl-L-homoserine lactone (Sigma-Aldrich), N-(3-oxododecanoyl)-L-homoserine lactone (Sigma-Aldrich), and N-(3-hydroxytetradecanoyl)-DL-homoserine lactone (Sigma-Aldrich). The stock solutions were aqueous solutions, except those used for HSLs and aTc. The stock solutions for HSLs were dissolved in 100% dimethyl sulfoxide, and aTc was dissolved in 100% ethanol.

Construction of Genetic Circuits and Sensors. Genetic circuits were constructed by hierarchical Type IIS DNA assembly in two sequential DNA assembly reactions.⁶² In the first assembly reaction, transcriptional unit constructs were assembled by joining one or more input promoter parts and an insulated repressor part (ribozyme, RBS, repressor gene, terminator) using BsaI-HFv2 (New England Biolabs) for the DNA assembly reaction. The destination vector was supplied as a purified PCR product, and the genetic parts used were purified part plasmids. In the second Type IIS DNA assembly reaction, these transcriptional unit constructs were assembled into the circuit backbone containing appropriate sensors to generate the final circuit plasmid constructs using BbsI (New England Biolabs). Type IIS DNA assembly reactions were performed in 5 µL total volume containing 20 fmol of each purified part or transcription unit plasmid, 10 fmol of the purified destination vector PCR product, 5 U of the appropriate Type IIS restriction enzyme, and 250 U T4 DNA ligase (2000 U/µL; New England Biolabs) in 1× T4 DNA Ligase Buffer (New England Biolabs). The reaction mixture was incubated in a thermal cycler (Bio-Rad C1000 thermal cycler, 105 °C lid) with the following protocol: 37 °C for 6 h, followed by 50 °C for 30 min, and inactivated at 80 °C for 15 min. Then, 2 µL of the assembly reaction was transformed into 5 µL chemically competent cells (*E. coli* NEB 5-alpha, New England Biolabs). Circuit constructs were analyzed by PCR. All transcriptional unit plasmids were sequenced by Sanger sequencing (Azenta, formerly Genewiz).

To construct circuit backbones containing quorum sensors, the *luxR* gene and its RBS on the pLW555 backbone were replaced with another quorum sensing regulator (*lasR*, *cinR*, or *rhlR* each with a unique RBS) using SapI (New England Biolabs) in a Type IIS DNA assembly reaction. A purified PCR fragment containing the regulator was assembled with an inverse PCR product of pLW555, excluding *luxR*. The gene *lasR* was amplified from Bsr078-LasR, which was a gift from Jeff Hasty (Addgene plasmid #85147). The gene *cinR* was amplified from pAJM.1642, which was a gift from Christopher Voigt (Addgene plasmid #108535). The gene *rhlR* was ordered as a synthetic DNA fragment (Twist Bioscience). The circuit backbones containing the choline sensor were constructed in two steps. First, a Type IIS DNA assembly reaction using BbsI was performed to self-ligate a purified PCR product of the BetI E1 repressor part that contained the mutation to create *betIM*. Next, a Type IIS DNA assembly reaction using SapI (New England Biolabs) was performed to assemble three purified PCR fragments together: a constitutive promoter found in Table S2, the *betIM* fragment (*betIM*, ribozyme, RBS, repressor, terminator) and a PCR product of pAN1201⁶¹ designed to insert the transcriptional unit containing *betIM* downstream of *tetR* (Figure S1). All primers were commer-

cially ordered (IDT). Genetic parts and plasmids used in this work are listed in Table S3 and Table S4, respectively.

Genetic Sensor and NOT Gate Characterization Assays.

Plasmids containing a sensor characterization or gate characterization construct (Figure S1) were transformed into the appropriate strain of *E. coli* (EcN or NEB 10-beta). To assay genetically encoded sensors and NOT gates, one colony was inoculated into 200 μ L of M9 media with the appropriate antibiotics in a sterile U-bottom 96-well microtiter plate sealed with a sterile breathable seal and incubated at 37 °C for 16 h in an ELMI DTS-4 digital thermostatic microplate shaker at 1,000 rpm. Two serial dilutions were performed each with 15 μ L of cell suspension added to 185 μ L of M9 media with the appropriate antibiotics. The cells were then incubated for 3 h in a plate shaking incubator using identical growth conditions. Samples were then further diluted with two serial dilutions, the first one being 15 μ L of cells into 185 μ L of M9 media with the appropriate antibiotics and the second being 3 μ L of the diluted samples into 145 μ L of M9 media with the appropriate antibiotics and inducers. To assay different input values, different inducer concentrations were tested in separate wells in parallel. The cells were incubated for 5 h in a plate shaking incubator. Next, an aliquot of cell suspension was diluted into sterile phosphate buffered saline (pH 7.4) (Fisher Scientific) with 2 mg/mL kanamycin to arrest cell growth and incubated at room temperature for 30 min before measuring cell fluorescence by flow cytometry analysis.

Genetic Circuit Assays. Each plasmid containing a genetic circuit was cotransformed with a corresponding output plasmid containing eYFP expressed by the output promoter of the circuit. For circuits containing multiple outputs, each output was measured independently in separate strains. To assay combinational logic circuits, one colony was inoculated into 200 μ L of M9 media with the appropriate antibiotics in a U-bottom 96-well microtiter plate sealed with a breathable seal and incubated for 16 h at 37 °C and 1,000 rpm in a plate shaking incubator. Two serial dilutions were performed each with 15 μ L of cell suspension added to 185 μ L of M9 media with the appropriate antibiotics, and the cells were incubated for 3 h in a plate shaking incubator using identical growth conditions. Samples were then diluted by performing two serial dilutions. In the first dilution, 15 μ L of cell suspension was added to 185 μ L of M9 media with the appropriate antibiotics. In the second dilution, 3 μ L of the diluted sample was added to 145 μ L of M9 media with the appropriate antibiotics and the appropriate combination of inducer inputs for the circuit. All combinations of inputs for a circuit were assayed in parallel. The inducer concentrations used were as follows; 1 mM IPTG (Sensor A), 2 ng/mL aTc (Sensor B), 10 mM 3OC6-HSL (Sensor C), 10 mM L-arabinose (Input D), 0.008 μ M 3OC12-HSL (Input E), 400 μ M C4-HSL (Sensor F), 2 μ M 3OHC14-HSL (Sensor G), 50 mM GABA (Sensor H), and 10 mM choline (Sensor I). After adding the inducers, cells were incubated for 5 h in a plate shaking incubator. For each sample, an aliquot of cells was diluted into sterile phosphate buffered saline (pH 7.4) with 2 mg/mL kanamycin and incubated at room temperature for 30 min before flow cytometry analysis.

Sequential logic circuits were assayed using the same procedure described above for the combinational logic circuits. To test state switching and memory recording, the assay was continued after the cells were incubated with the first set of inducers. After the 5 h incubation with the inducers, the cells were pelleted and washed for samples containing GABA,

3OC6-HSL, 3OC12-HSL, or 3OC14-HSL and then resuspended in M9 media. The cells were diluted in two serial dilutions in which first 15 μ L of cell suspension was added to 185 μ L of M9 media with the appropriate antibiotics, and then 3 μ L of the diluted culture was added to 145 μ L of M9 media with the appropriate antibiotics and the next set of inducers (for testing state switching) or without inducers (for testing memory recording). Cells were then incubated for another 5 h in a plate shaking incubator. An aliquot of cells was diluted into sterile phosphate buffered saline (pH 7.4) with 2 mg/mL kanamycin and incubated at room temperature for 30 min before flow cytometry analysis. To assay the concentration recording circuit (3OC6-HSL recording), the cells were grown overnight and in the initial 3 h incubation with 1 mM IPTG and 2 ng/mL aTc to initialize the state of all SR latches to the RESET state. The assay then proceeded as described for sequential logic circuit analysis.

Flow Cytometry Analysis. Cell fluorescence was measured by using a BD DUAL LSRFortessa flow cytometer with a 488 nm blue laser and the FITC detection channel or a BD Accuri C6 flow cytometer using a 480 nm blue laser and the FL1-A detection channel. The data for each sample was collected with a cutoff of 10,000 gated cell events at a flow rate of less than 1,000 events/s and at least 5,000 gated events collected per sample. For data analysis, the events were gated with a gate for cell-sized particles by using the FlowJo software. The median cell fluorescence for each sample was calculated by using FlowJo. Histograms of cell fluorescence were generated by using FlowJo. In all flow cytometry assays, the autofluorescence of the *E. coli* cells (without plasmid) and the fluorescence of cells containing the RPU standard plasmid were measured for samples from three separate colonies using identical dilutions and growth conditions as the assayed constructs.

Fitting of the Sensor and NOT Gate Response Functions. The measured cell fluorescence in arbitrary units was converted to relative promoter units (RPU) as previously described⁶¹ and using eq 1 with the arbitrary unit values for the sample cell fluorescence (YFP), autofluorescence of cells containing no plasmid (YFP₀), and fluorescence of cells containing the RPU standard plasmid pAN1717⁶¹ (YFP_{RPU}), which constitutively expresses eYFP. The sample fluorescence on each day's experiment was converted to RPU. For each experiment, the average of the cell fluorescence from three separate colonies was used for the autofluorescence and RPU plasmid standard. The limit of detection was set to 0.001 RPU, and an output below this cutoff was set to this minimum value.

$$\text{RPU} = (\text{YFP} - \text{YFP}_0) / (\text{YFP}_{\text{RPU}} - \text{YFP}_0) \quad (1)$$

For a sensor response function, the data set in RPU for the sensor characterization construct assayed with different small molecule concentrations was fit to Hill equation¹⁰⁹ (eq 2). For a NOT gate response function, the input in RPU was determined using the corresponding sensor response function and inducer concentration. The data set for the measured output of the gate characterization construct with a range of input promoter activities was then fit to the Hill equation for repression (eq 3) to determine the gate response function. The response function relates an input signal, either small molecule concentrations or another input promoter activity, to an output promoter signal. In these equations, the output promoter activity (y) is a function of the total input activity (x) and the parameters for the minimum observed output promoter

activity ($y_{\min}$), maximum observed output promoter activity ($y_{\max}$), Hill coefficient (n), and activation or repression coefficient (K), which is the input value for half-maximal output. Curve fitting was performed using the least-squares method by minimizing the sum of the $\log_{10}$ of the error between the predicted curve fit and the measured data points using the Solver add-in in Microsoft Excel with the GRG nonlinear solving method. Fitted parameters for the sensors and library of NOT gates in EcN are listed in Table S1 and Table S2 respectively.

$$y = y_{\min} + (y_{\max} - y_{\min}) \frac{x^n}{(x^n + K^n)} \quad (2)$$

$$y = y_{\min} + (y_{\max} - y_{\min}) \frac{K^n}{(x^n + K^n)} \quad (3)$$

Genetic Circuit Predictions. Combinational logic circuits were simulated using a signal matching algorithm.⁶¹ The input promoter activity to the gate was substituted into the corresponding gate response function to calculate the output of the gate at steady state. For gates having tandem input promoters (i.e., NOR and OR gates), the sum of both the input promoter activities was used for the input. For the first layer in the circuit, the input promoter activity is calculated from the sensor response function for each corresponding sensor. For subsequent layers and gates in the circuit, the gate output is the input value for the next gate in the circuit when two gates are wired together. This simulation of the signal processing was done for each gate in the circuit until the last was reached, and the circuit output was calculated.

The steady-state predictions for the SR latch circuits with two cross-coupled NOR gates, X and Y , were modeled by a pair of coupled differential equations, which were derived and validated in a previous work for this genetic circuit topology.⁶²

$$\frac{dm_{Qa}}{dt} = \xi \left[y_{\min X} + (y_{\max X} - y_{\min X}) \frac{K_X^{n_x}}{K_X^{n_x} (uA + Qb)^{n_x}} \right] - \gamma_m m_{Qa} \quad (4)$$

$$\frac{dm_{Qb}}{dt} = \xi \left[y_{\min Y} + (y_{\max Y} - y_{\min Y}) \frac{Y^{n_y}}{K_Y^{n_y} (uB + Qa)^{n_y}} \right] - \gamma_m m_{Qb} \quad (5)$$

Here, m_{Qa} and m_{Qb} represent the concentration of mRNA for the outputs Qa and Qb , produced from the output promoters of gates X and Y , respectively (each of which serves as one of the two input promoters for the opposite gate). The degradation rate of mRNA, γ_m has been set to 0.025 min^{-1} as in previous work.⁶² A conversion factor ξ has been set to 0.025 min^{-1} with $Qa = m_{Qa} \xi^{-1} \gamma_m$.⁶² The inputs uA and uB are the sensor promoters used with the latch for gates X and Y , respectively. The parameter values of $y_{\min}$, $y_{\max}$, K , and n are the values for the corresponding gate's response function. For predicting the hold state of the circuits, uA and uB were set to the basal expression level of each input promoter, as there would be no inducer present. The circuits were analyzed at steady state. The resulting nullclines have the form of the gate response functions. Equations were solved using the ode45 function in MATLAB.

Toxicity Analysis. Growth inhibition was analyzed using cells containing a NOT gate characterization plasmid and the

identical assay described previously for the NOT gate characterization. After the 5 h induction, 100 μL of sample was taken from each well and put into a clean well of a new U-bottom 96-well plate. The optical density at 600 nm (OD_{600}) was measured using an Epoch microplate spectrophotometer (BioTek). The OD_{600} of the blank media without cells was subtracted from the measured OD_{600} for each sample. To normalize the cell growth, the sample OD_{600} was divided by the OD_{600} for the corresponding gate without an inducer added. For repressors with multiple gates, the following gates were assayed: AmtR A1, BM3RI B1, BetI E1, AmeR F1, HlylIR H1, IcaRA I1, LitR L1, PhlF P1, QacR Q1, and SrpR S2.

■ ASSOCIATED CONTENT

Data Availability Statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.3c00232>.

Supporting Figure S1 (Plasmid backbones used in this study), Supporting Figure S2 (Response functions for P_{Tac} , P_{Tet} and P_{BAD}), Supporting Figure S3 (Fluorescence distribution for the RPU plasmid in *E. coli* NEB 10-beta and Nissle 1917), Supporting Figure S4 (Choline sensor tuning), Supporting Figure S5 (Genetic schematics of a NOT and NOR gate), Supporting Figure S6 (Comparing measured and predicted outputs for combinational logic circuits), Supporting Figure S7 (Representative populations of cells for three input circuit designs), Supporting Figure S8 (Improved design for the 3-input AND circuit after debugging), Supporting Figure S9 (Representative populations of cells for an SR latch), Supporting Figure S10 (The full set of SR latches tested for this work), Supporting Figure S11 (Measured vs predicted outputs for select SR latches with different input sensor combinations), Supporting Figure S12 (Transversality of the SR latches), Supporting Figure S13 (Measured vs predicted outputs for latches with equilibria separation ≥ 0.8), Supporting Figure S14 (Concentration recorder outputs), Supporting Table S1 (Hill function parameters for sensors in EcN), Supporting Table S2 (Hill function parameters for NOT gates in EcN), Supporting Table S3 (Genetic part sequences used in this work), and Supporting Table S4 (Plasmid constructs used in this paper) (PDF)

E. coli Nissle 1917 SBOL file (ZIP)

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Author Contributions

LBA and ML conceived of the study, designed experiments, and analyzed data. ML and MZ performed the experiments. LBA, ML, and MZ wrote the manuscript.

Notes

The authors declare no competing financial interest.

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