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Engineered *E. coli* That Detect and Respond to Gut Inflammation through Nitric Oxide Sensing

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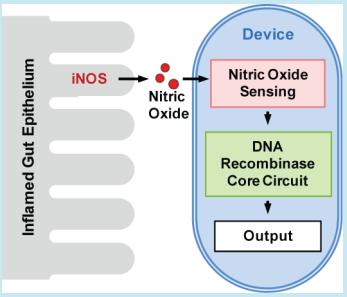
Supporting Information

ABSTRACT: Advances in synthetic biology now allow for the reprogramming of microorganisms to execute specific tasks. Here, we describe the development of an engineered strain of *E. coli* capable of sensing and responding to the presence of a mammalian inflammatory signal. The synthetic gene regulatory circuit is designed to permanently alter gene expression in response to the well characterized inflammatory signal nitric oxide. The detection of nitric oxide initiates the expression of a DNA recombinase, causing the permanent activation of a DNA switch. We demonstrate that *E. coli* containing this synthetic circuit respond to nitric oxide from both chemical and biological sources, with permanent DNA recombination occurring in the presence of nitric oxide donor compounds or inflamed mouse ileum explants. In the future, this synthetic genetic circuit will be optimized to allow *E. coli* to reliably detect and respond to inflammation *in vivo*.



KEYWORDS: *inflammatory bowel disease, medical microbes, nitric oxide sensing, inflammation sensing, DNA recombinase, DNA switch, engineered bacteria, synthetic genetic circuit*

Engineered biological systems are currently being developed that use synthetic genetic circuits to generate a wide variety of biological behaviors. Recent advances show that microorganisms containing synthetic genetic circuits can perform simple and useful functions.[1,2] Several engineered microbes are designed for medically relevant tasks, including strains of *E. coli* that detect the pathogen *P. aeruginosa* and respond by secreting antimicrobial peptides[3] and *E. coli* designed to localize to tumors and attack cancerous cells.[4] In the present study, we describe the construction and testing of a synthetic genetic circuit capable of detecting and responding to nitric oxide, an important marker of inflammation in inflammatory bowel disease (IBD).

IBD is a group of incurable disorders of unknown etiology that affect the gastrointestinal tract.[5] The current standard of care focuses on preventing inflammatory flare-ups and on symptom management.[6] We suggest that engineered microorganisms may be ideal for the treatment of inflammation in IBD management. The intestinal lumen is readily accessible to microorganisms, including thousands of species of commensal bacteria.[7] Additionally, because IBD patients suffer from intermittent disease flare-ups, the immediate detection and treatment of inflammation could arrest disease progression. Moreover, localizing the production and delivery of anti-inflammatory drugs could decrease the risks and side-effects associated with high dose systemic drug treatment.[8,9]

The treatment of IBD may be an ideal application for engineered microbes, but predicting the exact properties of a synthetic genetic circuit can be difficult. Modeling methods are currently being explored for predicting the behavior of synthetic genetic circuits.[10] In other engineering disciplines, the concept of modularity simplifies the design of complex systems. Theoretically, modularity allows the components of synthetic genetic circuits to be interchangeable.[11] Here, we present an engineered *E. coli* strain that uses a modular synthetic genetic circuit to control gene expression with a FimE recombinase-based DNA switch in response to the inflammatory signal nitric oxide.

Construction and Characterization of a FimE Recombinase-Based DNA Switch. We constructed a bidirectional fluorescent reporter switch (Figure 1A) using elements of the *E. coli* fimbriae (Fim) phase variation system. In *E. coli*, the expression of the fimbriae component, FimA, is controlled in a binary fashion through the inversion of a 314 bp segment of DNA (*fimS*) that contains the *fimA* promoter.[12] The inversion of *fimS* is performed by the DNA recombinase FimE, which binds to two inverted repeat sequences (Inverted Repeat Left and Right, IRL and IRR, respectively) that flank the *fimS* element. FimE has different binding affinities for IRL and IRR depending on the orientation of *fimS*, and as a result FimE is able to efficiently cause recombination only when the promoter faces IRR.[13,14] Therefore, switch inversion is permanent and heritable.

The bidirectional fluorescent reporter switch constitutively expresses yellow fluorescent protein (YFP) when *fimS* is oriented toward IRR, corresponding to the OFF state. Following unidirectional inversion (activation) of the switch by FimE, *fimS* is reoriented toward IRL and causes the constitutive expression of cyan fluorescent protein (CFP), which corresponds to the ON state. Activation of the switch can be detected in single cells (Figure 1B) or at a population level (Figure 1C) when cells change from YFP production to expression of CFP. Additionally, inversion of the DNA switch

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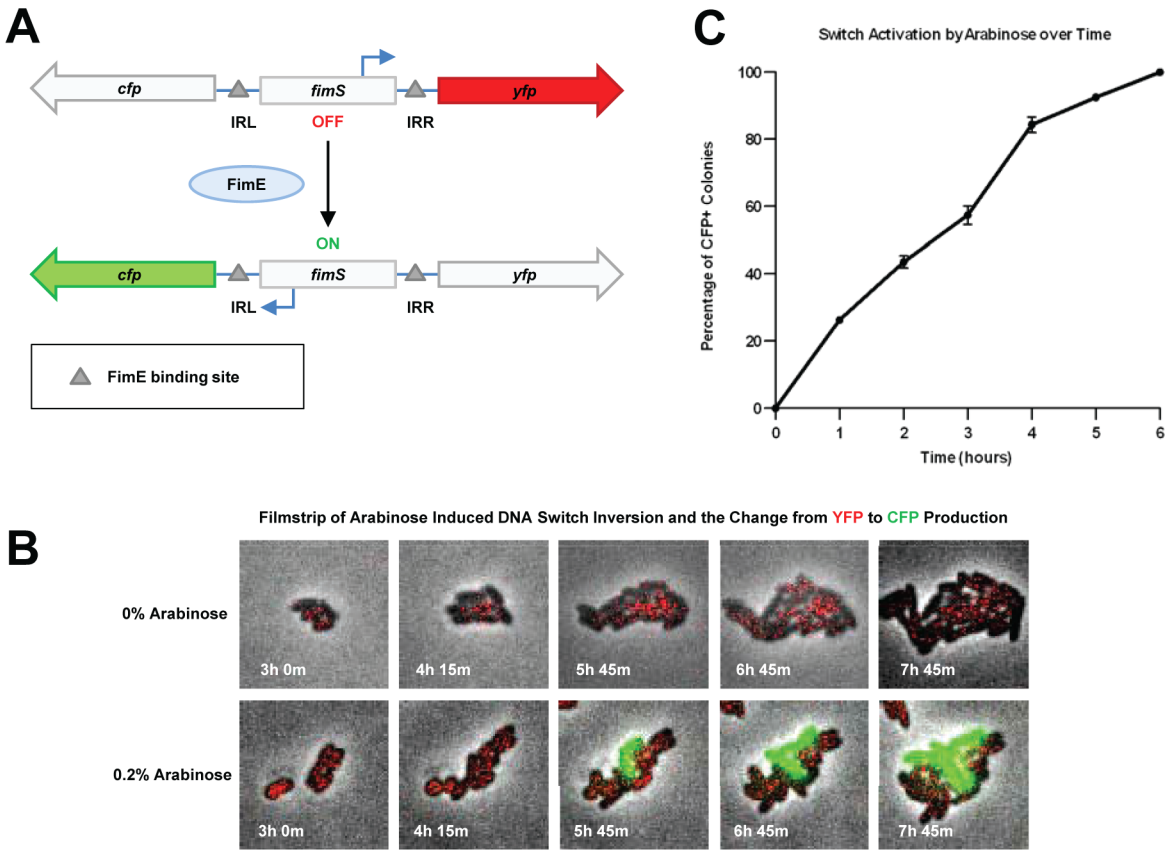

Figure 1. Construction and characterization of the Fim invertible bidirectional switch. (A) Cartoon representing the response of the FimE invertible bidirectional switch. Initially, *fimS* constitutively drives transcription of *yfp* mRNA. Activation of the switch is achieved by site-specific inversion of *fimS* by FimE, resulting in loss of *yfp* transcription and constitutive *cfp* expression. (B) Single cell time-lapse fluorescence microscopy of strain EA2029, containing both the bidirectional switch and arabinose inducible *fimE*. Cells grown on agarose pads containing 0.2% arabinose lose YFP fluorescence (pseudo colored red) while increasing CFP fluorescence (pseudo colored green) as switch activation occurs. (C) The response of strain EA2029 to arabinose over time was determined by calculating the percentage of CFP+ colonies after serial dilution and plating of the batch culture on selective antibiotic media.

can be detected through the use of orientation specific primers for PCR analysis (Supplemental Figure 1).

To characterize the bidirectional fluorescent switch, we controlled FimE expression using the arabinose inducible *Pbad* promoter[15,16] in *E. coli* strain EA2029. This strain should switch to the ON state when grown in the presence of arabinose. As expected, when grown on agarose pads containing 0.2% arabinose, YFP signal decreases while CFP increases after ~5 h, as observed in single cells by fluorescence microscopy (Figure 1B). In liquid culture, permanent inversion occurs in over 95% of EA2029 cells (Figure 1C) after 6 h exposure to 0.4% arabinose.

As expected, switch activation is heritable and can be detected even after overnight culture in the absence of arabinose. After overnight growth the number of colonies with CFP fluorescence above background was counted and divided by the total number of colonies formed to determine the percentage of CFP+ colonies. Bacterial colonies formed from a single cell are typically considered to be clonal, but colony level heterogeneity could arise if the founding cell contains both activated and inactivated DNA switches on multiple plasmids. Because heterogeneous colonies still indicate that switch activation occurred within the founding cell, these colonies are counted as CFP+ colonies. After induction, EA2029 colonies that are homogenously CFP positive after streaking for isolation produce clonal populations of CFP positive cells through further serial passaging (data not shown), consistent with previous studies of FimE function.[13,16]

The use of this recombinase-based core circuit provides multiple benefits in designing this engineered microorganism. Even transient input signals can induce permanent DNA recombination, causing heritable changes in gene expression. Previous studies have suggested that the use of DNA recombinases as an intermediate between sensing and circuit output prevents background promoter activity (leaky expression) from causing noisy activation of the final system output[16,17] and is supported by the absence of CFP+ cells detectable without arabinose induction.

Additionally, other inducible or regulated promoters can be used to control FimE production and trigger recombination.[14,16] This suggests that many cellular sensors and signaling pathways could be used as system inputs to induce switch activation. Further, both the circuit output gene (in this case, CFP) and the promoter driving its expression (*fimS*) are interchangeable with other sequences. For instance, the invertible *fimS* region can be replaced with another promoter, provided it is flanked by recombinase binding sites.[14,16] The modular design of the DNA switch allows replacement or tuning of the circuit components.

Nitric Oxide Sensing As the Input for the Recombinase-Based Genetic Switch. Nitric oxide is used for cell−cell communication in eukaryotes and is a natural marker of

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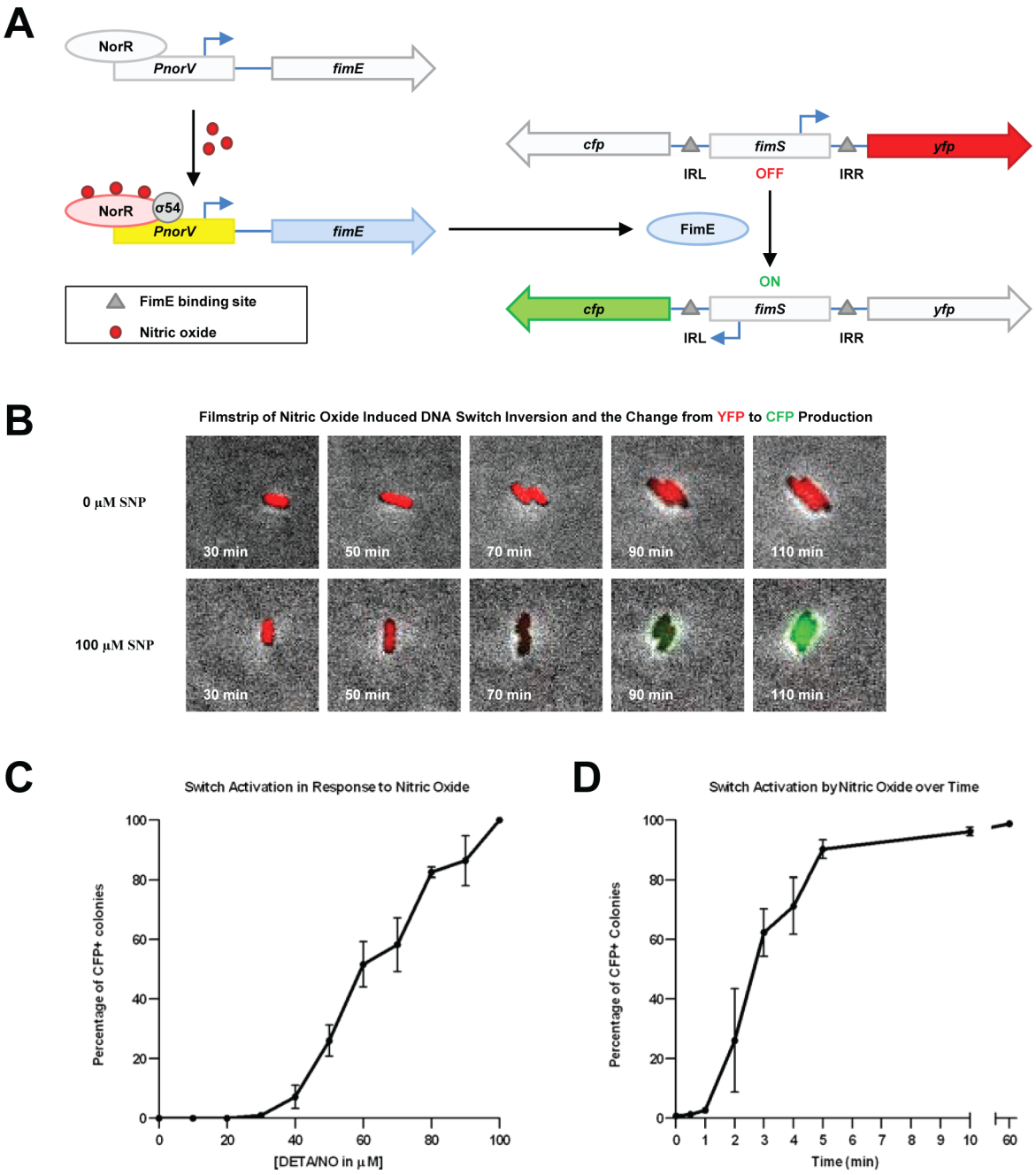

Figure 2. Nitric oxide sensing as the input for the synthetic microbial device. (A) The core DNA recombinase circuit is coupled to nitric oxide sensing by driving FimE production from *PnorV*. Binding of nitric oxide to NorR results in σ^{54} binding and subsequent expression of FimE, driving switch activation. (B) Single cell time-lapse fluorescence microscopy of strain EA3020 containing both the bidirectional switch and *PnorV-fimE*. Cells treated with 100 μM of the nitric oxide donor SNP lose YFP signal (pseudo colored red) while increasing CFP fluorescence (pseudo colored green) as FimE activates the DNA switch. (C) The dose response of strain EA3020 to nitric oxide from the chemical donor DETA/NO over 1 h was determined by calculating the percentage of CFP+ colonies after serial dilution and plating of the batch culture on selective antibiotic media. (D) The response of strain EA3020 to nitric oxide (100 μM DETA/NO) over time was determined by calculating the percentage of CFP+ colonies after serial dilution and plating of the batch culture on selective antibiotic media.

inflammation,[18] making it an ideal input signal for this engineered microorganism. Inflamed epithelial cells produce nitric oxide by up-regulating inducible nitric oxide synthase (iNOS), an enzyme that produces nitric oxide from L-arginine.[18]

Though nitric oxide is used as an inflammatory signal in mammals, many bacteria possess nitric oxide sensors, often to control the expression of cognate nitric oxide reductases that metabolize and detoxify nitric oxide. Several *E. coli* nitric oxide sensors exist,[19] but the bacterial enhancer binding protein NorR was selected because it reacts solely with nitric oxide and not other reactive nitrogen species.[20,21] NorR binds to 3 conserved sites in the promoter for *norV* (*PnorV*) in the *norR-norV* intergenic region. In the absence of nitric oxide, the NorR N-terminal GAF domain blocks the NorR AAA+ domain from binding to the bacterial transcription factor σ^{54} and prevents transcription of the nitric oxide reductase NorV. When nitric oxide binds to NorR, the GAF domain relaxes repression of the

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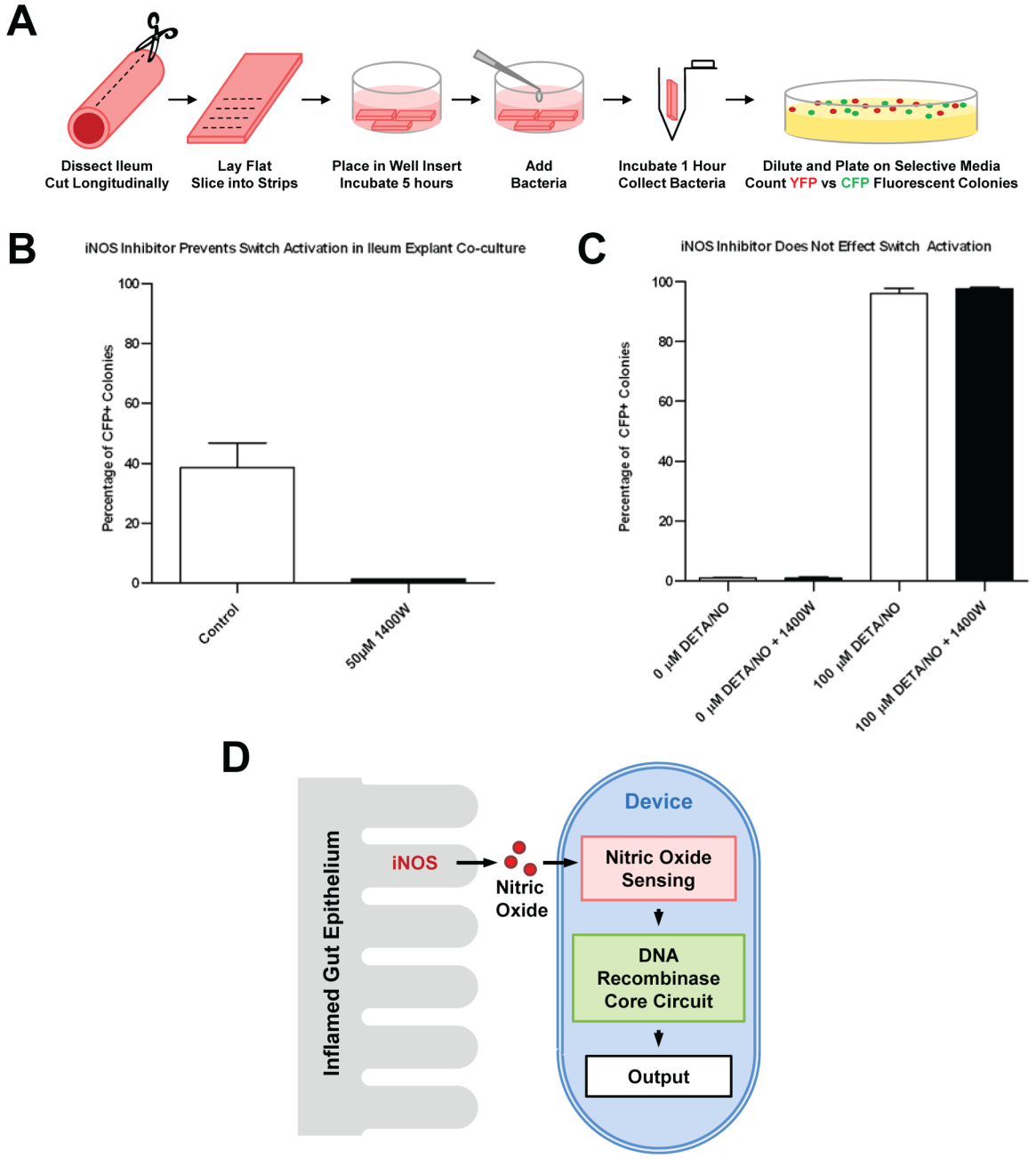

Figure 3. The coupled circuit is capable of responding to nitric oxide produced by iNOS. (A) Co-culture of strain EA3020 with mouse intestinal explants. (B) Percentage of CFP+ colonies recovered after co-culturing EA 3020 with mouse intestinal explants. Switch activation in EA 3020 was decreased ~30-fold by treatment with iNOS inhibitor (50 μM 1400W dihydrochloride). (C) Percentage of CFP+ colonies recovered in the presence or absence of iNOS inhibitor (100 μM) and/or DETA/NO (100 μM). (D) Strain EA3020 responds to mammalian gut inflammation using the sensor NorR to detect nitric oxide produced by iNOS. The production of FimE activates switching of the core circuit resulting in a permanent change in gene expression output.

AAA+ domain, allowing binding to σ^{54} and transcription of NorV to occur.[22]

We combined nitric oxide sensing through NorR with the DNA recombinase core circuit by placing *fimE* transcription under the control of *PnorV* (Figure 2A). The following design strategies were necessary to successfully couple nitric oxide sensing to switch activation. The sequence used for *PnorV* extended into the coding sequence of *norR*, as reported previously.[23] Additionally, rather than using the sequence for the native ribosomal binding site (RBS) for *norV*, we used a stronger synthetic RBS and spacer (AAGGAGGAAAGTCACATT). We also inserted an ATG start codon before the native *fimE* GTG start codon. Finally, we constitutively expressed the NorR sensor (part K256007 kindly provided by the BioBrick Parts Registry) in *E. coli* strain EA3020 to eliminate the possibility of stoichiometric imbalance between genomic NorR expression levels and additional copies of *PnorV* on exogenous plasmids.

To characterize the switching properties of the nitric oxide responsive engineered *E. coli* (strain EA3020), we used the nitric oxide donors DETA/NO (diethylenetriamine/nitric oxide adduct) and SNP (sodium nitroprusside) as sources of

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nitric oxide. After exposure to 100 μM SNP, cells decreased YFP signal and increased CFP fluorescence within 1 cell division, ~70 min (Figure 2B). We determined that strain EA3020 responds to concentrations between 40 and 100 μM DETA/NO within 1 h (Figure 2C). To characterize the speed of switch activation, we determined that EA3020 undergoes permanent inversion in more than 95% of cells within 5 min of exposure to 100 μM DETA/NO (Figure 2C). PCR amplification using ON state specific primers demonstrates that switch inversion can be detected in a population of cells after only 3 min of exposure to nitric oxide (Supplemental Figure 3), confirming that switch activation occurs very rapidly. This demonstrates the successful construction of a nitric oxide induced switch capable of responding to chemically produced nitric oxide.

Strain EA3020 Responds to Biologically Produced Nitric Oxide. In the gut, nitric oxide freely diffuses in the lumen and across cell membranes, and has a short half-life due to its high reactivity.[24] In healthy controls measurements of nitric oxide levels in the colon (~60 ppb nitric oxide) are up to 90 times lower than from patients with active inflammation caused by ulcerative colitis (~5675 ppb), a form of IBD.[25] The bacterial nitric oxide sensor NorR has been reported to induce a 30-fold increase in gene expression from *PnorV* in the presence of 120 ppm nitric oxide.[26] However, because of differences in experimental setup, this concentration of nitric oxide is not directly comparable to measurements obtained from the entire lumen compartment as described above. Additionally, iNOS localization studies on biopsies from IBD patients indicate that nitric oxide production is non-uniform within the colon and is highest in inflamed tissues.[18] Furthermore, nitric oxide is rapidly degraded, thus it is expected that the highest levels of nitric oxide are present in the microenvironment immediately adjacent to inflamed tissue.

To determine if NorR is capable of sensing and responding to nitric oxide in this microenvironment, strain EA 3020 was co-cultured with mouse ileum explants (Figure 3A). After co-culture with intestinal explants, we found that a fraction (~38%) of EA 3020 colonies recovered had induced recombination and were CFP positive (Figure 3B). To confirm that iNOS is the source of nitric oxide that induces switch activation, mouse ileum explants were treated during culture with the selective iNOS inhibitor (1400W dihydrochloride, 50 μM)[27] and co-cultured with strain EA 3020. Explants treated with iNOS inhibitor exhibited a 30-fold decrease in the percentage (from ~30% to ~1%) of cells that responded to nitric oxide during co-culture (Figure 3B). This supported the hypothesis that strain EA3020 can sense and respond to nitric oxide produced by iNOS in mammalian tissues.

To verify that 1400W dihydrochloride does not affect nitric oxide sensing or FimE recombination, strain EA3020 was grown in the presence or absence of 100 uM 1400W dihydrochloride and assayed for responsiveness to DETA/NO in 1 h (Figure 3C). The resulting data showed that iNOS inhibitor treatment has no significant effect (Student's t test p = 0.79 and 0.57 for 0 μM DETA/NO and100 μM DETA/NO, respectively) on the ability of EA3020 to respond to nitric oxide produced by the chemical donor DETA/NO.

We expected that the explants culture conditions cause an increase in iNOS expression due to mechanical disruption during surgical dissection. We confirmed that iNOS is upregulated during explant culture using qRT-PCR (Supplemental Figure 4A). Corresponding with iNOS upregulation during culture, nitric oxide concentrations directly adjacent to ileum explants are higher in explants measured after 6 h in culture (13.0 ± 4.8 nM) when compared to measurements taken immediately after dissection (54.3 ± 15.3 nM) (Supplemental Figure 4B). The nitric oxide concentration measured in explants cultured for 6 h is comparable to the previously published response range of NorR.[26]

In summary, we designed and characterized a synthetic genetic circuit that detects and responds to an inflammatory signal produced by the mammalian gut. In the future, this synthetic genetic circuit can be optimized to ensure the reliable detection and treatment of IBD associated inflammation in patients. Because the recombinase-based core circuit can be used to control the expression of any output gene, in the future this synthetic genetic circuit can be redesigned to produce and secrete anti-inflammatory protein drugs.[8,9] The eventual goal is to localize the production and delivery of medications near target tissues, avoiding side effects by decreasing systemic exposure to anti-inflammatory drugs.

METHODS

Bacterial Strains. Bacteria were maintained at 37 °C in LB Miller broth (EMD Chemicals Inc.) and plates containing 1.2% agar with appropriate antibiotics (ampicillin 100 μg/mL, kanamycin 25 μg/mL, chloramphenicol 30 μg/mL). All bacterial strains (Supplemental Table 1) were constructed using standard molecular cloning procedures.

Fluorescent Time Lapse Microscopy. For imaging, bacteria were cultured to mid-logarithmic phase in M9Media (6.8 g/L sodium phosphate, 3 g/L potassium phosphate, 0.5 g/L sodium chloride, 1 g/L ammonium chloride, 2 mM magnesium sulfate, 0.1 mM calcium chloride) supplemented with 0.4% casamino acids, 0.4% glutamate, and appropriate antibiotics and then placed on a 1.5% low melting point agarose pad containing 100 μM SNP (sodium nitroprusside, Sigma). Pads were then transferred to a coverslip-bottom dish. Cells were observed using fluorescence time-lapse microscopy at 37 °C with an Olympus IX-71 inverted microscope with a motorized stage (Applied Precision) and an incubation chamber. Image sets were acquired every 20 min with an Evolve 512 EMCCD camera. DeltaVision softWoRx software was used to automate image acquisition and microscope control.

Dose Response and Time Course Experiments. Saturated bacterial cultures were diluted 1:50 in M9Media supplemented with 0.4% casamino acids, 0.4% glutamate, and appropriate antibiotics and allowed to grow to mid-log phase. DETA/NO (diethylenetriamine/nitric oxide, Sigma), 0.4% arabinose (ACROS Organics), and 100 μM iNOS inhibitor (1400W dihydrochloride, Sigma) were added to appropriate cultures at time zero. At the indicated time points, cells were harvested, serially diluted, and plated on antibiotic selective media. The following day, colonies were assessed for CFP expression and counted.

Mouse Ileum Explant Culture. Protocol adapted from Schmidt et al.[28] Six-week-old C57B/6 mice were purchased from the UT Southwestern mouse breeding core facility. All animals were housed in the same specific pathogen-free facility. Animals were maintained with *ad libitum* access to water and autoclaved rodent chow (5K67, LabDiet). Mice were killed by isoflurane inhalation and cervical dislocation before dissection; ~5 cm of the terminal ileum was collected and flushed with phosphate-buffered saline to remove the contents. Segments (1

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mm × 3mm) of the ileum were removed and cultured at 37 °C and 95% oxygen for 5 h in Dulbecco's modified Eagle's medium (containing 4 g/L glucose and L-glutamine; Invitrogen) supplemented with 10% charcoal-stripped heat-inactivated fetal bovine serum, 25 mM HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin, and in the presence or absence of 50 μM iNOS inhibitor. Each condition contained three 1 mm × 3 mm segments of ileum. Following 5 h of incubation, ~ 3×10^7 cells bacteria were pipetted onto the ileum segments. The co-culture was then centrifuged at 3000*g* for 1 min and incubated for an additional 1 h at 37 °C and 95% oxygen. Ileum segments were transferred to a microcentrifuge tube with 1 mL of sterile phosphate buffered saline and vortexed to dissociate bacteria. The supernatant was then serially diluted and plated on antibiotic selective media. The following day, colonies were assessed for CFP expression and counted.

PCR Detection of Switch Activation. Activation of the switch state can be detected by PCR amplification using the *fimS* primer (CTATATGTAAAGCTAACGTTTCTGTGG-CTCGAC) and *yfp* reverse primer (GCAGTTTGCCGGTGG-TACAG). Amplification can occur only if *fimS* has been inverted and the *fimS* primer binding site is on the cDNA strand from the *yfp* reverse primer.

To detect switch activation in response to nitric oxide in Supplemental Figure 3, EA 3020 cells were grown in liquid culture at 37 °C in LB Miller broth. At the indicated times after the addition of 100 μM DETA/NO, 50 μL aliquots of cells were heat killed at 100 °C for 5 min. DNA recombination was detected using PCR.

qRT-PCR. Ileum segments were collected (immediately after dissection and after 6 h of explant culture) and flash frozen in liquid nitrogen before proceeding to RNA extraction using standard protocols. qRT-PCR and analysis (ΔCt method) were performed using standard protocols as in ref 29. U36B4 (primers CGTCCTCGTTGGAGTGACA and CGGTGCGT-CAGGGATTG) was used as a reference gene to determine iNOS expression (primers CAGGAGGAGAGAGATCC-GATTTA and GCATTAGCATGGAAGCAAAGA).

Nitric Oxide Measurement from Ileum Explants. Measurements of nitric oxide produced by explant cultures of mouse ileum were performed using the nitric oxide specific ISO-NOPF-L10 sensor, TBR1025 analyzer, and LabScribe2 software (World Precision Instruments Inc.). Sensor readings were calibrated using Method 2 from the manufacturer instructions, using copper(II) chloride to catalyze the decomposition of *S*-nitroso-*N*-acetyl-DL-penicillamine (Sigma-Aldrich) into known concentrations of nitric oxide. Readings from tissue were performed in phosphate buffered saline (PBS, pH 7.4, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4) at room temperature.

■ ASSOCIATED CONTENT

Supporting Information

Table of strains and plasmids (Supplemental Table 1) and verification of switch inversion by PCR amplification (Supplemental Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

E.J.A. and G.M.S. devised the study. E.J.A. and A.B.R. designed and performed experiments. E.J.A. analyzed data and wrote the manuscript, with editing from A.B.R. G.M.S. supervised and directed the study and edited the manuscript.

Notes

The authors declare no competing financial interest.

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