ENGINEERED E. COLI THAT DETECT AND RESPOND TO GUT INFLAMMATION THROUGH NITRIC OXIDE SENSING

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DEDICATION

I dedicate this dissertation to my parents Rodney and Kuni Archer, for their never-ending encouragement. I would also like to thank my collaborators Jamaal Benjamin and Dr. Katie Colbert-Coate for their help in completing this work. Lastly, I would like to especially acknowledge my former labmate Andra Robinson, without her critical thinking and help this project would not have been possible.

ENGINEERED E. COLI THAT DETECT AND RESPOND TO GUT INFLAMMATION THROUGH NITRIC OXIDE SENSING

by

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Within the last several years, advances in synthetic biology have allowed for the development of re-programmed microorganisms that perform useful tasks in areas like fuel production, bioremediation, and medicine. Several engineered microorganisms are in pre-clinical development for the treatment of human diseases, but may face critical limitations that decrease their utility in medicine due to adverse events like sepsis, caused by the introduction of bacteria within patients. Here I describe the design, construction, and characterization of a

synthetic genetic network that is intended for use by *E. coli* within lumen of the intestine, which is presumed to be a safer location than other tissues, such as blood, for the introduction of engineered microbes. The synthetic gene regulatory circuit described here regulates gene expression through the activation of a permanent DNA switch in response to nitric oxide produced by inducible nitric oxide synthase. The detection of nitric oxide initiates the expression of a DNA recombinase, causing the permanent genetic rearrangement of a short DNA segment containing a gene promoter, allowing for the regulation of output gene expression upon nitric oxide sensing. Here I demonstrate that *E. coli* containing this synthetic genetic circuit respond to nitric oxide as designed from both chemical nitric oxide donors and from injured mouse intestinal explants. This synthetic genetic circuit could be optimized for clinical use by allowing *E. coli* to reliably detect and treat inflammation in patients with inflammatory bowel disease, but the circuit described herein now serves as the proof-of-concept for both bacterial sensing of mammalian inflammation and for the use of DNA recombinases to translate transient environmental signals into permanent responses in engineered bacteria.

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PRIOR PUBLICATIONS

Archer, E., and Süel, G. (2013) Synthetic Genetic Networks, *Reports on Progress in Physics*. 76, 1-7

Archer, E., Robinson, A. B., and Süel, G. (2011) Engineered E. coli that detect and respond to gut inflammation through nitric oxide sensing, *ACS Synthetic Biology. 1*, 451–457.

Kim, HJ., **Archer, E.**, Escobedo, N., Tapscott, SJ., Unguez, GA. (2008) Inhibition of mammalian muscle differentiation by regeneration blastema extract of Sternopygus macrurus, *Developmental Dynamics. 237(10), 2830-42*.

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LIST OF DEFINITIONS

Synthetic biology – a multi-disciplinary field that combines traditional biology with engineering principles to design and construct of artificial biological systems for useful purposes

Synthetic genetic circuit – an artificially designed genetic circuit that controls gene expression through interactions of genes, proteins, and DNA regulatory elements

Inflammatory bowel disease (IBD) – a group of disorders characterized by periodic inflammatory episodes that primarily affect the gastrointestinal tract,

Flare-up – a periodic episode of acute inflammation in the gastrointestinal tract

Dextran sodium sulfate – a colitis inducing chemical used in animal models of inflammatory bowel disease

FimE – a well characterized *E. coli* DNA recombinase capable of causing the unidirectional inversion of a segment of DNA between two of its binding sites

NorR – a well characterized *E. coli* nitric oxide sensor

iNOS – inducible nitric oxide synthase, an inducible gene that releases nitric oxide through the breakdown of L-arginine during inflammatory states

TNF-α – tumor necrosis factor alpha

CHAPTER ONE
Project Rationale

Introduction

Synthetic biology is a developing multidisciplinary field that focuses on the design, construction, and characterization of synthetic biological networks[1]. Biological networks are the patterns of interactions that organize the behavior of living systems from the smallest scale to the very largest. Examples of biological networks include the interactions between individual amino acids that result in complex protein folding and structure, as well as the genetic regulatory networks made up of interacting DNA segments and corresponding protein and RNA gene products. Even the complex relationships between individuals or species within ecological networks or biomes can be viewed as biological networks.

In some ways, synthetic biology can be viewed as a method of learning by building, unraveling new principles that govern biological networks or testing understanding of natural biological networks in an echo of Dr. Richard Feynman's famous quote: "What I cannot create, I do not understand". While much of synthetic biology is dedicated to this academic pursuit of testing and understanding natural living systems, another common goal is the development of biological technologies that replace or improve upon current technologies[2-5]. This dissertation describes the design, construction, and characterization of a synthetic genetic network in *E. coli* that functions as an irreversible genetic switch that detects and responds to inflammation-induced

nitric oxide produced by injured mammalian gut tissue. The ultimate goal of this project is to develop a synthetic genetic circuit within *E. coli* that is clinically useful for the treatment of inflammatory bowel disease.

Inflammatory Bowel Disease: Epidemiology and Etiology

Within the United States alone, the prevalence of inflammatory bowel disease (IBD) is approximately 1.5 million patients, and rising incidence rates within the developed world have led to IBD becoming a growing public health concern[6-8]. IBD encompasses a diverse group of disorders with Crohn's disease and ulcerative colitis being the most commonly diagnosed. Patients diagnosed with an IBD disorder experience severely diminished quality of life[9-12]. While the etiology of inflammatory bowel disease has been intensely studied, there is currently no consensus regarding a single causal agent in IBD[6, 8, 13].

Several hypotheses regarding the pathogenesis of IBD have been investigated, but no single genetic or environmental factor has been identified that can account for a significant fraction of IBD cases. Numerous gene mutations have been correlatively linked to the pathogenesis of IBD, but few mutations alone have been found to cause IBD symptoms in animal models, and the total number of mutation-linked cases of IBD account for a very small (<5%) amount of overall diagnoses[14, 15]. Additionally, many environmental factors have been investigated as contributing or causative factors in IBD, and diverse mechanisms including the composition of patient's gut microbiota and patient diet may play some role[8, 16-19].

Due to evidence that nutritional changes are clinically beneficial in the treatment of IBD, and higher IBD prevalence within developed Western countries (the U.S. and Western Europe) supports the importance of diet in the development or maintenance of IBD. Multiple observations support the hypothesis that gut microbes and their interactions with the human gut are important in the pathogenesis of IBD. Analysis of the relative composition of gut microbe species demonstrates significantly different profiles of intestinal microbiota in IBD patients. Furthermore, antibiotic treatment can temporarily decrease disease symptoms, implicating intestinal bacteria and their interactions with the host in the pathogenesis of IBD[19-21]. Finally, the role of viral infection in IBD has been investigated, with several studies suggesting that viral infection acts as a trigger in the development of IBD in genetically or otherwise predisposed patients[6, 13].

As genetic, environmental, and infectious origins have been explored as possible causes for IBD without the identification of a single causal agent, the current consensus, known as the "multi-hit" hypothesis, is that the pathogenesis of IBD is multi-factorial, arising from a complex combination of contributing genetic and environmental elements[13]. One key finding in support of the multi-hit hypothesis was that in transgenic mice carrying an IBD linked mutation, mice only developed IBD symptoms after exposure to multiple disease triggers[13].

Mice harboring hypomorphic mutations in the yeast autophagy gene homolog Atg16L1 were found to demonstrate IBD symptoms and pathologies in response to dextran sodium sulfate (a colitis inducing chemical) only when infected with a murine norovirus. An additional finding of the study was that mice treated with broad spectrum antibiotics failed to develop an IBD-like

pathology, mirroring the finding that antibiotics in human IBD patients decrease symptoms[13]. This supports a complex origin for the pathogenesis of IBD, requiring a combination of genetic and environmental factors to induce disease development.

Inflammatory Bowel Disease: Clinical Features and Treatment

IBD disorders are chronic and characterized by recurring and periodic episodes of acute inflammation known as flare-ups, within gastrointestinal tract tissues[11, 22, 23]. The longevity and severity of these flare-ups can vary greatly, but flare-ups are uniformly associated with diminished quality of life for patients[6, 9, 11, 12, 22, 23]. Over the course of the disease, IBD patients will experience a range of symptoms, with initial flare-ups typically presenting with relatively mild events such as fatigue, abdominal pain, and bloody diarrhea at disease onset. However, as IBD progresses due to the cumulative effect of multiple flare-ups, patients can experience more severe symptoms including the formation of intestinal fistulas or impacted colons, and rarely death due to disease complications. As many IBD patients experience disease onset in early adolescence, pediatric patients may also suffer from serious malnutrition with associated growth deficits, including delays in pubertal onset. Lastly, IBD is a serious risk factor for colorectal cancer later in life[9, 10, 12].

Perhaps because the causal factors for IBD have not been identified, IBD is incurable and the current standards of care for IBD focus on the prevention of flare-ups, often through nutritional approaches and the prophylactic use of aminosalicylates, corticosteroids, or tumor necrosis factor alpha (TNFα) targeting immunosuppressants. When prophylactic measures fail,

symptom management to decrease the severity and longevity of flare-ups becomes the top priority[22-24].

In an effort to manage quality of life and side effects in IBD patients, the predominant therapeutic treatment strategy for IBD is a "bottom-up" approach, meaning that the course of treatment will escalate from relatively tolerable treatments with mild side effects to more effective anti-inflammatory medications that have higher risks of serious side effects. For example, as mentioned above, nutritional changes and treatment with anti-inflammatory medications such as aminosalicylates are commonly used for patients with mild symptoms[24]. In patients with more severe flare-ups or complications, treatment strategies are typically escalated to corticosteroids or cytokine targeting drugs such as the anti-TNF monoclonal antibody infliximab[24, 25]. In advanced IBD cases, as a last treatment option many IBD patients will eventually require colectomy (the surgical removal of affected portions of the large intestine) to control IBD symptoms. While individual flare-up prevention and management is often highly effective, over a lifetime approximately 75% of patients with Crohn's disease and 25% with ulcerative colitis will require colectomy[6].

In addition to disease symptoms, the long-term use of corticosteroids or anti-inflammatory drugs can lead to numerous side effects that severely decrease quality of life for IBD patients. These can include the development of Cushing's disease phenotype, osteoporosis and corresponding increased risk of bone fractures, type II diabetes, cataracts, and heightened risk of serious infection and cancer development[23, 26, 27]. Unfortunately for many patients, constant corticosteroid usage becomes a necessity to prevent immediate recurrence of IBD

symptoms. The decreased quality of life associated with IBD flare-ups, side effects with current therapies, and the high lifetime occurrence of surgical intervention in IBD patients suggests significant unmet need, with current treatment strategies being inadequate for the long term management of IBD.

Project Rationale: Unmet Need in Inflammatory Bowel Disease

IBD represents a significant disease burden and high unmet need, with no currently available long-term treatment strategy that is both highly effective in managing flare-ups and tolerable over long term use without significant side effects. In diseases such as IBD that are localized to specific tissues, organs, or anatomical locations, a highly effective strategy for decreasing side effects while increasing treatment efficacy is localizing treatment to the site of disease. This can be accomplished through either by direct drug delivery to affected tissues or organs, or through specific molecular targeting of disease drivers.

IBD shares many common features with another inflammatory disease, asthma. Like IBD, asthma is characterized by periodic flare-ups. Prior to the invention of the metered dose inhaler for the delivery of asthma medications directly to patient's lungs, the management of asthma required daily injections with corticosteroids, which are still part of the standard of care for the treatment of IBD[28]. Because of their prolonged use of corticosteroids, patients with asthma experienced many of the same debilitating side effects and decreased quality of life that are seen in corticosteroid dependent IBD patients today. Following the development of the metered dose inhaler, asthma could be treated reactively rather than proactively, decreasing

reliance on daily usage of corticosteroids and associated side effects. The direct delivery of medication directly to constricting bronchioles allowed for a relatively high and effective dose within the lungs, while the overall systemic dose of drug was greatly decreased. This localized approach increased treatment efficacy while minimizing side effects, and is an excellent example of the benefits of a targeted rather than systemic approach to disease treatment[28].

While the treatment of asthma was revolutionized by a simple medical device, several factors would prevent a similar device from being effectively used in IBD. However, the concept of localization and improved treatment targeting may still prove to be a useful approach in the treatment of IBD. By utilizing synthetic biology, it may be possible to develop an engineered microorganism that is capable of locally detecting and directly treating inflammation within the gastrointestinal tract.

Project Rationale: Medical Uses for Engineered Microorganisms

Engineered bacteria and other microorganisms are under constant development and are currently in use for both biofuel and pharmaceutical production[29-37]. Microbes that detect, neutralize, or sequester environmental pollutants are also being used for bioremediation[38, 39]. In medicine, several engineered microbes are being designed to perform medically relevant tasks. One recent example of this is a strain of *E. coli* that detects the pathogen *P. aeruginosa* and responds by secreting anti-microbial peptides, which may be of medical interest for the treatment of opportunistic *P. aeruginosa* infection in cystic fibrosis patients[40]. Likewise, attempts have been made to design *E. coli* that would travel within the bloodstream, localize to tumors using a

variety of natural and engineered systems, and attack or invade cancerous cells[41]. Growing lists of medical conditions are being targeted for treatments using synthetic biology approaches[42-49]. One promising synthetic biology approach for the treatment of various hematological malignancies is the development and use of chimeric antigen receptors in T-cells (CART). CARTs form an artificial link that causes the activation of T-cells in response to a cell surface marker, such as CART19 which activates T-cells in response to CD-19[50]. Synthetic biology approaches to disease treatment are currently in multiple stages of clinical development, leading to the possibility that in the near future several diseases may be treated with engineered microorganisms or mammalian cells carrying synthetic genetic circuits.

Synthetic genetic circuits within engineered bacteria are being investigated for the treatment of cancer and *P. aeruginosa* infection, but the use of engineered microorganisms for the treatment of these diseases faces several hurdles that are not present in IBD, which may be an ideal disease for demonstrating proof-of-principle for engineered bacteria in treating human diseases. To effectively treat cancer or bacterial infection of the lungs using bacteria would require the introduction of engineered bacteria within the lungs or blood, possibly leading to side effects such as sepsis or immunological reactions. In contrast, the localized treatment of IBD would take place within the intestinal lumen, a space that is readily accessible to microorganisms, and is home to thousands of species of commensal bacteria[51]. In fact, many patients currently use probiotic strains of bacteria as part of their therapeutic regimen, deliberately introducing "helpful" commensal bacteria[20, 21].

At least one randomized clinical trial has demonstrated that in patients with ulcerative colitis the ingestion of *E. coli* Nissle strain 1917 is as safe and effective a therapy in maintaining remission as mesalazine, the current standard of care[52]. Furthermore, clinical trials are currently underway to test the safety and efficacy of the use of genetically modified *Lactococcus lactis* that express and secrete a TNF-α neutralizing camelid antibody or recombinant interleukin 10 as an anti-inflammatory for the treatment of IBD[46, 47, 53]. The clinical benefits associated with probiotic use and currently available pre-clinical data from the use of genetically modified microbes strongly suggest that the use of engineered microorganisms for the treatment of IBD is a reasonable strategy.

In comparison to probiotic therapy or treatment with aminosalicylates like mesalazine, an ideal therapy would increase the length of remission in patients by preventing flare-ups, but also be capable of treating the symptoms of a flare-up in progress. Currently, escalation to more potent treatment only occurs after a flare-up is symptomatic, but an engineered microbe capable of sensing inflammation and responding through the production of an anti-inflammatory molecule would offer the advantage of beginning treatment immediately, possibly before a patient notices symptoms. This strategy may offer increased safety over both the use of genetically modified microbes that constitutively express anti-inflammatory molecules and conventional systemic treatments, as localization of the production and delivery of anti-inflammatory medications may decrease the risks and side-effects associated with high dose systemic drug treatment[46, 47].

CHAPTER TWO
Literature Review

Introduction

Since the discovery of the *lac* operon by Jaqcues Monod and Francois Jacob in 1961[54], an incredible variety of genetic regulatory circuits have been discovered that are responsible for a wide variety of cellular processes and behaviors. Genetic regulatory circuits are the network of interactions between segments of DNA and their corresponding RNA and protein gene products. Typically, genetic regulatory circuits are represented as a diagram of positive or negative interactions (edges) between circuit elements (nodes) like gene promoters and transcription factors[55].

Artificial genetic circuits, by contrast, are created either by re-wiring existing circuits or by creating completely new networks using elements from multiple unrelated genetic circuits. By creating artificial genetic circuits, synthetic biologists can attempt to alter or modulate existing cellular behaviors, change the inputs or outputs from a genetic circuit, or generate entirely novel cellular behaviors that are not seen in nature. While the ability to predictably control every aspect of cellular behavior using synthetic genetic circuits is an as yet unattained goal, synthetic biology may eventually allow for the programming of engineered cells to function as biological computers, allowing microbes to be designed for industrial, medical, or academic applications[2, 4, 56, 57].

Integration or Isolation of Synthetic Genetic Circuits from the Chassis' Native Circuitry

Many factors play a role in the success or failure of a synthetic genetic circuit's design. One of the most important initial factors to consider is the genetic background of the host organism (the chassis) in which synthetic genetic circuit will be characterized and optimized. While many bacterial, fungal, and even mammalian cells have been used as hosts for synthetic genetic circuits, the most commonly used chassis for the development of synthetic genetic circuits is *E. coli* because of its genetic tractability and extensive characterization[42, 58, 59].

An important consideration is that synthetic genetic circuits developed in one organism are typically not readily portable between different chassis due to a wide variety of species or strain specific factors. Variation in the rates of cell division and growth, basal protein degradation and production rates, the speed of protein folding and maturation, and direct interactions between synthetic and native network components can all prevent a synthetic genetic circuit developed in one chassis maintaining the same behavior in another chassis[59, 60]. Even relatively simple differences, such as species or strain differences in codon usage and plasmid copy number can greatly influence the behavior of synthetic genetic circuits. Lastly, unforeseen interactions between synthetic and natural genetic circuits (such as homologous promoter sequences or transcription factors) can cause unexpected behavior in synthetic circuits.

The result of these species specific differences is that even well characterized and robust circuits that are developed and optimized within one chassis often function completely differently when introduced into another host species or strain, making the initial choice of chassis an important consideration.

Initially, many researchers attempted to address this problem by isolating synthetic genetic circuits from the native chassis genetic circuitry[60]. This approach is commonly used in synthetic genetic circuits, and is accomplished by using exogenous circuit elements that are not present in the genome of the chassis species. The construction of synthetic genetic circuits using elements from species that are evolutionarily distant to the chassis helps to insulate effects caused by chassis' genetic background[60]. This works because the co-evolution of interacting partners increases binding specificity, decreasing the likelihood of non-specific protein-protein or protein-DNA interactions in a new chassis. When taken to the extreme, the approach of isolating genetic circuits from the chassis background has led to the systematic deletion of non-essential genes in pursuit of "barebones" microbes with simplified genomes that contain the minimal number of genes necessary to sustain life[61]. These minimalist bacteria may eventually be commonly used as a cellular chassis for biological devices, to minimize potential interactions between the host cell genome and synthetic genetic circuits.

As an alternative approach, many synthetic circuits are designed to seamlessly integrate and interact with the endogenous cellular circuitry of the host cell[60]. Synthetic genetic circuits can be designed to use the conditions in the chassis cell to determine the circuit's output. An example of this type of circuit is the cell state classifier, in which the cell's state can be determined based on the expression of a single output gene that is only expressed when specific miRNAs levels are high or low. This circuit was designed to match the miRNA expression profile of HeLa cells, and used Boolean logic to integrate 6 independent miRNA signals[49]. When a plasmid containing the synthetic genetic circuit was transfected into HeLa cells, the final

output gene was expressed. However, transfection of the same plasmid into other cell lines resulted in no expression of the final circuit output gene because the synthetic genetic circuit could not detect the triggering miRNA signals.

Another strategy for creating synthetic genetic circuits that interact with the endogenous chassis' components has been to alter the behavior of natural genetic circuits by rewiring the circuit. Typically this has been achieved through changing the sign of interactions (positive or negative) between circuit elements. This has been used to test the functional differences in behavior between specific network architectures, providing insight into evolutionary limits and tradeoffs inherent in specific network architectures[62]. Insights from re-wiring native genetic circuits may allow for the tuning or modulation of naturally useful behaviors in engineered microorganisms.

When synthetic genetic circuits are designed with a particular chassis in mind, naturally-evolved behaviors that would be difficult to engineer can be harnessed and integrated into the synthetic genetic circuit architecture to generate novel behaviors. With examples of bacteria that naturally localize to solid tumors when injected into blood[63], and probiotic organisms that demonstrate equivalent efficacy in prolonging the maintenance of remission in IBD patients[52], and extremophiles capable of withstanding high temperatures, radiation, osmotic pressures, and other extreme environments[64], the integration of synthetic genetic circuits into less common chassis may be a useful strategy in creating engineered microbes for specific industrial or medical applications.

Component Parts of Synthetic Genetic Circuits

While the choice of chassis is an important consideration in designing and constructing a synthetic genetic circuit, the component parts of synthetic genetic circuits are another key element for creating an engineered organism. Synthetic biologists adopt new components for genetic networks by either utilizing existing naturally evolved elements or by the mutation of existing ones[65-67]. Protein coding sequences are the most commonly considered circuit elements, but other less noticeable genetic circuit elements are also important, such as transcriptional terminators, ribosomal binding sites, promoters, and consensus sequences for DNA-protein interactions. These components allow for the tuning of a synthetic genetic circuit to achieve the desired output.

In addition to re-purposed naturally evolved elements, many circuit elements can be rationally designed with sufficient knowledge. Protein engineering approaches enable the creation of novel coding sequences for chimeric proteins with hybridized functions. Additionally, the direct synthesis of artificial RNA and DNA components allow for the generation of artificial 5' and 3' untranslated regions that can contain novel ribosomal binding sites, transcriptional terminators, RNA loops and other secondary RNA structures, or miRNA binding sites[68-71].

Using these methods, new "parts" are made available for use in synthetic biological networks. For example, naturally occurring sensors and receptors have been repurposed to create engineered bacteria that sense natural compounds and signals[40, 72]. Sensors and receptors have been artificially mutated or evolved to detect various novel compounds[66, 73, 74]. In some cases,

entirely novel chimeric receptors have been constructed that combine the sensing of cellular or environmental signals from one receptor with the signal transduction or downstream effect of another receptor or enzyme. One interesting application of the latter is the use of chimeric receptors to translate the exposure to different wavelengths of light into changes in gene expression in bacteria[69]. These examples show how synthetic biologists are increasing the possible applications for engineered organisms by widening the range of physical, chemical, and biological signals that can be used as inputs in synthetic genetic networks.

In engineering disciplines, the concept of modularity is commonly applied to simplify the design of complex systems. Modularity describes the property by which individual modules within a larger complex system can be interchangeable with other modules. In synthetic biology, modularity would describe the interchangeability of either synthetic genetic circuit components or of sub-circuits with well characterized behaviors[5, 67, 75].

To facilitate modularity in synthetic biology, a Massachusetts Institute of Technology initiative has created a widely used and invaluable tool for synthetic biologists: the Registry of Standard Biological Parts[76]. The Registry is an important repository of genetic components for use in synthetic circuits and is an open resource for designers of genetic circuits. The Registry can be viewed as somewhat analogous to reference books of standardized parts in electrical engineering that detail the properties of common electrical components and electrical circuits. The Registry contains thousands of documented and characterized DNA sequences that have been used by synthetic biologists as biological network elements, with components including coding sequences for proteins and RNA, gene promoters, transcriptional terminators, ribosome

binding sites, and combinations of the above. Using the Registry, users easily share and exchange components and network modules, as well as documentation for each part and their uses in different synthetic genetic circuits.

An important feature of the Registry is the standardization of restriction sites that allow all Registry components to be easily cloned into place with other components, greatly increasing their modularity while simplifying and accelerating the construction of synthetic genetic circuits.

In addition to the Registry, breakthroughs in *de novo* DNA synthesis have accelerated the development of new engineered networks, and have even led to the chemical synthesis of an entire bacterial genome[77]. These developments are important in synthetic biology and may eventually lead to the ability to design of new artificial bacterial species within computer models, followed by their actual construction with entirely synthesized genomes. With parts libraries like the Registry of Standard Biological Parts, these advancements in DNA synthesis, and software for genetic circuit design and modeling[78], the construction of genetic circuits has become much faster while being greatly simplified[71]. Once a circuit has been designed, researchers now have the option to quickly test multiple versions of synthetic genetic circuits by directly synthesizing the necessary DNA sequences, rather than individually cloning multiple constructs.

Synthetic Biology and Biological Design Principles

Despite vast amounts of data collection, we often lack a clear conceptual understanding of how interactions among components can give rise to complex behavior at the network level. While the construction of synthetic genetic circuits has become exponentially faster and cheaper,

synthetic biologists are unable to reliably create designs that function as predicted in living cells[4, 70, 75, 79]. Synthetic genetic circuits typically require multiple cycles of detailed characterization and changes to the circuit before functioning as originally designed. To increase the relevance and accuracy of genetic circuit modeling, synthetic biologists are attempting to uncover specific rules or principles of genetic circuit design. If such principles can be identified, they may be able to accurately guide the construction of engineered microbes in the same way that principles such as Ohm's law are used by designers of electric circuits[1, 3, 5, 60, 75, 80]. Unfortunately, while electrical engineers can reliably predict exactly how elements like resistors and capacitors will interact with each other in electronic circuits, biological networks are typically understood through the reverse engineering of complex networks and are only comprehensible within the limited context in which they are encountered. Because of this, principles or "laws" in biological networks are difficult to extract, making the role of individual biological interactions within a complex system difficult to accurately predict.

The fields of systems biology and network biology make use of vast amounts of experimental data and bioinformatics information in an attempt to uncover the design principles that apply to evolved biological networks. Because biological networks can contain hundreds of discrete elements, and must also describe the interactions between these elements, it is difficult or impossible to understand the function of an entire network at a glance. The evolution over millions of years in response to often unknown selection pressures has made many biological networks intractably complex. Many evolved biological networks contain elements that are functionally redundant, or interactions that are vestigial and no longer functionally useful. In

other cases, single genes, such as P53, have been shown to intersect with multiple genetic networks[81]. Because of these features, understanding these networks can be very difficult using traditional reverse engineering techniques. Gene knockout or overexpression experiments can fail to yield important information regarding the importance of individual circuit elements, because interactions of minor importance that modulate or tune the function of a genetic circuit may not yield a readily observable behavioral phenotype. Entire network modules may perform redundant functions, while core network properties can be hidden in a web of trivial or physiologically irrelevant interactions. In these cases, synthetic biology can be a tool for probing the design principles of natural systems, furthering our understanding of the basic rules that guide biological networks[1, 3, 5, 60, 75, 79].

Synthetic and systems biology share significant overlap in some areas of interest, but use different techniques to discover biological principles. A systems biologist might analyze libraries of data to identify and analyze biological networks, each composed of recurring network topologies or motifs, and make hypotheses about how they combine to carry out diverse cellular function. Hypothesis testing often includes using mathematical modeling in an attempt to recapitulate network properties and behavior in an *in silico* system[82, 83].

In contrast, a synthetic biologist can directly test and characterize the dynamic properties of different motifs or circuit architectures by building and characterizing simplified artificial genetic circuits[62, 82]. By creating, perturbing, and quantifying the dynamic behavior of engineered gene circuits, synthetic biology allows for the detailed study of simplified biological networks or modules[1, 3]. Synthetic biology researchers can increase the accuracy of computer modeling of

genetic circuits by identifying critical interactions found through fully characterizing these simplified networks and dissecting the effects caused by changes in the network components[84]. The use of simplified artificial genetic networks, rather than naturally evolved complex networks, allows the design principles of living systems to be probed in the absence of often poorly understood interactions within natural networks that have not decoupled co-evolved network elements. In turn, the biological design principles revealed in simplified genetic networks allow for both the construction and testing of more complex circuits and an increased ability to predictably and reliably design synthetic genetic circuits for engineering applications. In this way, the field of synthetic biology mirrors the history of chemistry, in which insights gained through artificial chemical reactions revealed principles that govern chemical reactions in nature[1, 3, 84].

The contribution that the patterns of interaction (topology or architecture) has on the behavior of genetic circuits is one question that is currently being approached with synthetic biology approaches[62, 80, 82, 83, 85, 86]. One common and widespread architectural motif that has been identified within genetic circuits is the feed forward loop, in which a single circuit element both directly and indirectly (through an intermediate node) interacts with another circuit element[82, 86]. Using a synthetic biology approach, a library of synthetic feed forward loop motifs was designed, constructed, and expressed in *E. coli*, which allowed different and comparable feed forward loop architectures to be extensively tested and characterized. These experiments identified the feed-forward loop architecture as a molecular noise filter, allowing gene expression to be tightly controlled in response to activation of the genetic circuit[82]. This

conclusion would have been difficult or impossible to arrive at using traditional reverse engineering biological techniques such as the individual knockdown or overexpression of different circuit components. The ability to directly compare the behaviors of multiple different artificial feed forward loop architectures simultaneously using a synthetic biology approach allowed this important circuit design principle to be uncovered. Synthetic biology approaches have also been used to reveal the importance of biochemical noise (stochasticity) and circuit feedback in genetic circuits[86-90].

The ability to accurately engineer behaviors in living systems has been tested in several synthetic genetic circuits that were designed as biological analogs of electrical circuits. For example, synthetic genetic circuits have been created that act as limit-cycle oscillators[91, 92], DNA toggle switches[93], genetic logic circuits that perform Boolean logic operations to act as genetic on-off switches[49, 94-97], and bacterial communities with programmed or reactive pattern formation[98, 99]. Mathematical models are often utilized to design and predict the function of these engineered circuits, but even with modeling each circuit requires several iterations of testing and redesign in order to produce a functioning circuit.

Strategies are currently being explored to increase the efficiency of mathematical modeling and decrease the number of testing cycles that are necessary before creating a functional synthetic genetic circuit that behaves as predicted. One of these strategies is to employ semi-random mutagenesis to create a library of circuit elements that are each extensively characterized[70]. The increased number of well characterized available parts allows mathematical

models a greater degree of flexibility in key parameters, allowing for more precise modeling of predicted circuit behavior that more readily translates into functional synthetic genetic circuits.

The construction of synthetic genetic circuits remains a process of trial and error, requiring multiple cycles of testing, characterization, and re-design. Advances have been made in understanding the importance of interactions between the chassis and synthetic genetic circuits, and biological parts are becoming increasingly available for use as circuit components. Growing understanding of the basic principles of genetic circuits design, more powerful and accurate mathematical modeling, and the rising availability of cheap *de novo* DNA synthesis may soon lead to the automation of synthetic genetic circuit design. Together with the invention of simpler and modular genetic circuit construction methods, and an increasing body of knowledge and experience, these advances give synthetic biologists today more powerful tools for the design and implementation of synthetic genetic circuits.

This project represents an attempt to leverage these tools to address a significant unmet need in medicine, namely, the safe and effective treatment of inflammatory bowel disease.

CHAPTER THREE
Methods

Strains and Plasmids

For maintenance, *E. coli* strains were grown in liquid suspension at 37^{o}C in LB Miller broth (EMD Chemicals Inc.) or on solid LB Miller media plates containing 1.2% agar containing appropriate concentrations of antibiotics to ensure plasmid maintenance and prevent contamination (ampicillin 10 μg/ml, kanamycin 25 μg/ml, chloramphenicol 30 μg/ml).

Glycerol stocks were prepared from saturated overnight cultures in LB Miller broth mixed 1:1 with 50% glycerol (final glycerol concentration 25%), and were kept at -80^{o}C.

Plasmids used in this study are represented in Table 1. All bacterial strains and plasmids were constructed using standard molecular cloning procedures. pEA2065 and pEA3017 were constructed by modifying the pUA66-IHFβ-GFP plasmid (a kind gift from Dr. Uri Alon). The backbone pUA66 plasmid was selectively amplified by PCR (removing the IHFβ-GFP construct) for use in cloning. pBAD33-FimE was constructed by cloning FimE into pBAD33 (a kind gift from Dr. Vanessa Sperandio). pSB1A2-K256007 was unmodified and procured from the BioBricks Parts Registry.

All *E. coli* strains are listed in Table 2, all strains are in the Top10 background, which lacks the *fim* operon (including *fimA, fimB, and fimE)*, precluding unintended fim switch activation through endogenous expression of FimB or FimE recombinase.

Table 1: Plasmids

Plasmid	Constructs	Antibiotic resistance
pEA2065	**YFP/FimS/CFP switch**	**Kanamycin**
pEA3017	**YFP/FimS/CFP switch + pNorV-(ATG) FimE**	**Kanamycin**
pBAD33-FimE	**Arabinose inducible FimE production**	**Chloramphenicol**
pSB1A2-k256007	**Constitutive NorR production**	**Ampicillin**

Table 2: Strains

Strain	Genotype	Purpose
EA2029	***fimA*$^{(-)}$ Top10 + pEA2065 + pBAD33-FimE**	**Arabinose inducible fim switch**
EA3020	***fimA*$^{(-)}$ Top10 + pEA3017 + pSB1A2-k256007**	**Nitric oxide inducible fim switch**

Fluorescence Time Lapse Microscopy

Prior to fluorescence time lapse microscopy, bacterial strains were cultured to mid-logarithmic growth phase in M9 media (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. Appropriate antibiotic concentrations for maintenance of strain plasmids were included. Following entry into log phase growth, cells were diluted 1:100 in fresh M9 media before being pipetted onto 1.5% low melting

point agarose M9 media pad. M9 media was used preferentially over LB Miller broth due to its lower optical density.

For relevant experiments, arabinose (ACROS Organics), or SNP (sodium nitroprusside, Sigma) were added to agarose pads prior to solidification.

After the pads were inoculated with bacteria they were transferred upside down onto a coverslip-bottom dish, with the bacteria placed between the agarose pad and the glass bottom. Cells were imaged using fluorescence time-lapse microscopy at 37^0C using an Olympus IX-71 inverted microscope with a motorized stage (Applied Precision) and a temperature controlled incubation chamber. Image sets were acquired every 10 or 20 minutes with an Evolve 512 EMCCD camera. DeltaVision softWoRx software controlled automatic image acquisition and stage motorization.

Dose Response and Time Course Experiments

Saturated bacterial cultures were diluted 1:100 in M9 Media 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 and serially diluted before being plated on antibiotic selective media and incubated at 37°C for 12 to 18 hours. Colonies were assessed for CFP expression under a dissecting microscope with a fluorescent light source. Both

CFP positive and negative colonies were counted. Percentage CFP expression was calculated using plates containing between 100 and 1000 colonies.

Mouse Ileum Explant Culture

Protocol was adapted from Schmidt et al.[100]. Six week old C57B/6 mice were purchased from the UT Southwestern mouse breeding core facility. All animals were housed in the same pathogen-free facility. Animals were maintained with *ad libitum* access to water and autoclaved rodent chow (5K67, LabDiet). Mice were sacrificed using isoflurane inhalation and cervical dislocation prior to dissection. Approximately 5 cm of the terminal ileum was collected and flushed with a syringe using phosphate-buffered saline to remove the feces within the collected intestinal segments. The ileum segments were cut longitudinally using a pair of surgical scissors, and then further cut into rectangles approximately 1 x 3 mm which were then removed and cultured in 6 well plates using well inserts at 37°C and 95% oxygen in Dulbecco's modified Eagle's medium (containing 4 g/liter 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 to four 1 x 3-mm ileum segments depending on the amount of tissue collected. Following 5 hours incubation, ~ 3 x 10^7 cells bacteria were pipetted onto the ileum segments. The co-culture was then centrifuged at 3000g for 1 min, and then incubated for an additional 60 minutes at 37°C and 95% oxygen. Ileum segments were then 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. Plates were incubated at 37°C for 12 to 18 hours. Colonies were assessed for CFP expression and counted as above.

PCR Detection of Switch Activation

Activation of the switch state can be detected by the PCR amplification of a 377 bp fragment using the internal *fimS* primer TATATGTAAAGCTAACGTTTCTGTGGCTCGAC) and the *yfp* reverse primer (GCAGTTTGCCGGTGGTACAG). Amplification can only occur if *fimS* has been inverted and the *fimS* primer binding site is on the complementary DNA strand from the *yfp* reverse primer. To detect switch activation in response to nitric oxide EA3020 or EA2029 cells were grown in liquid culture at 37^{o}C in LB Miller broth. At the indicated time points after the addition of 100 μM DETA/NO, 50 μL aliquots of cells were heat killed at 100^{o}C for 5 minutes. DNA recombination was then assayed using PCR.

qRT-PCR

Ileum segments were collected immediately after dissection and after 6 hours 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 described by Bookout et al[101]. U36B4 (primers CGTCCTCGTTGGAGTGACA and CGGTGCGTCAGGGATTG) was used as a reference gene to determine iNOS expression (primers CAGGAGGAGAGAGATCCGATTTA 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, 8g/L NaCl, 0.2g/L KCl, 1.44g/L Na_2HPO_4, 0.24g/L KH_2PO_4) at room temperature.

CHAPTER FOUR
Construction and Characterization of a FimE Recombinase-Based DNA Switch

Introduction

Recent studies demonstrate the potential usefulness of engineered microorganisms that contain synthetic genetic circuits for a wide variety of simple tasks[30, 33, 36-38, 43-45, 53, 56, 69]. Several of these studies have focused on the design of engineered microbes for medically relevant uses, including a strain of *E. coli* that is intended to detect and treat *P. aeruginosa* infection through the expression of *P. aeruginosa* quorum sensing receptors, and subsequent up-regulation and secretion of anti-microbial peptides[40]. Another study explores the use of engineered *E. coli* that are designed to localize to cancerous solid tumors following injection into the bloodstream, and directly infect tumor cells[41]. In this chapter I describe the construction and characterization of a synthetic genetic circuit capable of detecting and responding to nitric oxide, an important marker of inflammation in inflammatory bowel disease (IBD).

IBD disorders are incurable and of unknown etiology, primarily affecting the gastrointestinal tract[8, 9, 16, 18, 22, 24]. Current standards of care focus on prevention of flare-ups and symptom management[22-25]. Several lines of evidence suggest that engineered microorganisms may be an ideal treatment for IBD management. The intestinal lumen is occupied by thousands of species of commensal bacteria[51], and engineered organisms can be easily delivered to the gut through ingestion. In comparison to current treatments, engineered microbes offer the possibility of rapid detection of inflammation and immediate localized treatment. This site and temporal specificity may arrest the progression of intermittent disease flare-ups. The localization of anti-

inflammatory drug production to the intended site of action in the gastrointestinal tract may decrease the risks and side-effects associated with systemic drug treatment[46, 47].

In other engineering fields, the design of complex systems is simplified by applying the concept of modularity. In synthetic biology, the concept of modularity is applied to increase the interchangeability of components in synthetic genetic circuits[5, 67, 75]. I designed an *E. coli* strain that contains a synthetic genetic circuit utilizing the FimE DNA recombinase and a novel genetic switch to control gene expression. Using the FimE DNA recombinase and switch, the inputs and outputs are modular, with inducible control of FimE expression serving as the circuit input. Circuit output is determined by the coding sequences flanking the invertible fim switch. In the current study, I have used the inflammatory signal nitric oxide as the circuit input and fluorescent proteins as the circuit output, and have demonstrated that this circuit is capable of detecting and responding to inflammation.

Results

I constructed a bi-directional fluorescent reporter switch (Figure 1) using elements of the *E. coli* fimbriae (Fim) phase variation system in plasmid pEA2065. In *E. coli*, the expression of the fimbriae component FimA is entirely controlled through the inversion of a 314bp segment of DNA (*fimS*) that contains the *fimA* promoter[102-104]. The inversion of *fimS* is performed by the DNA recombinases FimE and FimB, which bind 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*. As a result

FimE is only able to efficiently cause recombination when the promoter faces IRR[103, 104]. Therefore, switch inversion by FimE is considered to be both permanent and heritable.

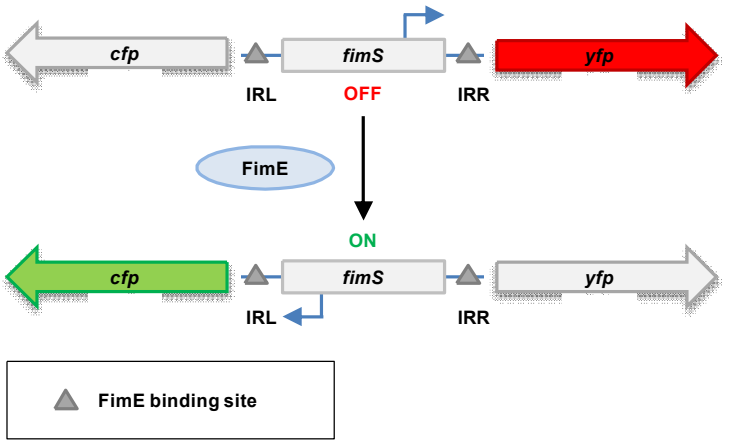

Figure 1: <u>Construction and characterization of the Fim invertible bi-directional switch</u>
Cartoon representing the response of the FimE invertible bi-directional switch. Initially, *fimS* constitutively drives transcription of *yfp* mRNA. Activation of the switch is achieved by site-specific inversion of *fimS* by FimE, resulting in loss of *yfp* transcription and constitutive *cfp* expression. YFP and CFP expression are represented as red and green, respectively

The bi-directional fluorescent reporter switch constitutively expresses yellow fluorescent protein (YFP) when fimS is oriented toward IRR, corresponding to the OFF state. The expression of FimE leads to unidirectional inversion (activation) of the switch, causing fimS to be re-oriented toward IRL and resulting in the constitutive expression of cyan fluorescent protein (CFP), which corresponds to the ON state. Activation of the switch can be detected in single cells using fluorescence microscopy or at a population level as cells change from constitutive YFP production to expression of CFP in response to switch activation.

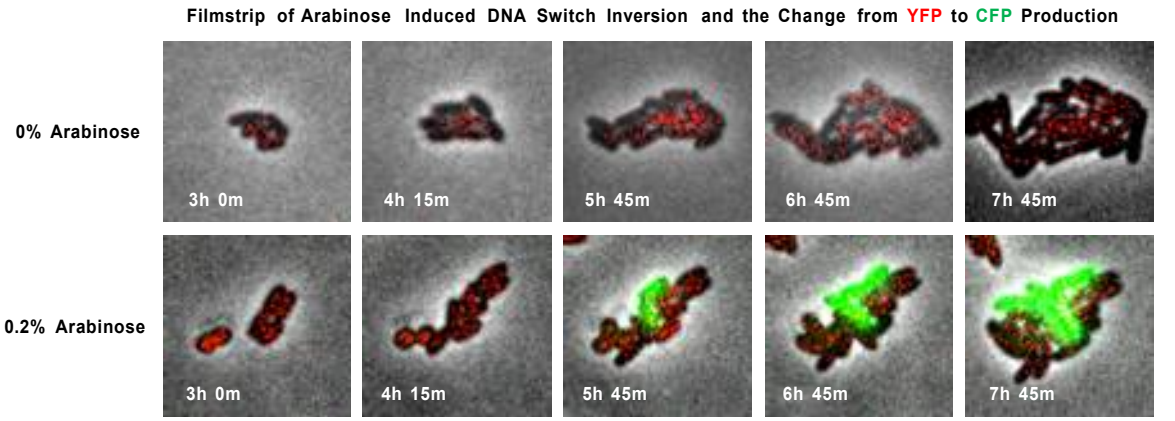

Figure 2: <u>Observation of fim switch activation in single cells in strain EA2029</u>
Single cell time-lapse fluorescence microscopy of strain EA2029, containing both the bi-directional switch and arabinose-inducible fimE. Cells grown on agarose pads containing 0.2% arabinose lose YFP fluorescence (pseudo colored red) while increasing CFP fluorescence (pseudo colored green) as switch activation occurs.

In order to characterize the bi-directional fluorescent switch, I controlled FimE expression using the arabinose inducible *Pbad* promoter[97, 105] from plasmid pBAD33-FimE in cells also harboring pEA2065 (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 hours, as observed in single cells by fluorescence microscopy (Figure 2).

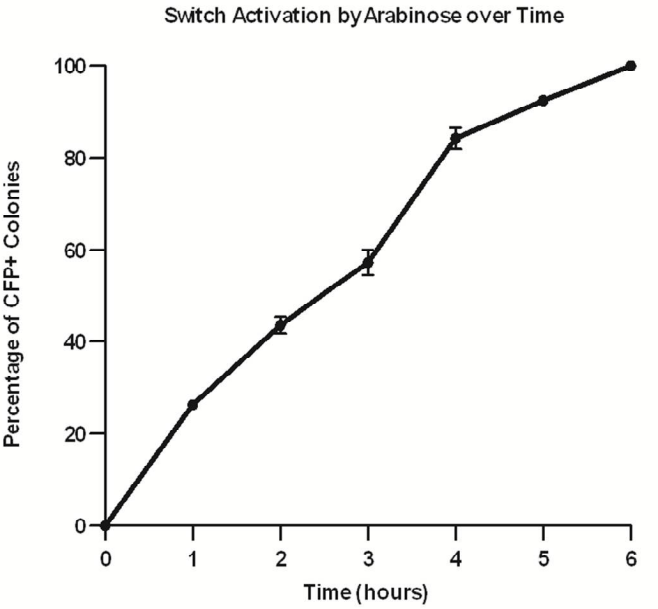

Figure 3: <u>Switch activation over time in strain EA2029</u>
The response of strain EA2029 to arabinose over time was determined by calculating the percentage of CFP+ colonies after serial dilution and plating of the batch culture on selective antibiotic media. Shown is the mean percentage of CFP colonies and standard error

In liquid culture, permanent inversion occurs in over 95% of EA2029 cells (Figure 3) after 6 hours 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. Inversion of the DNA switch was also detectable through the use of orientation specific primers for PCR analysis (Figures 4 and 5).

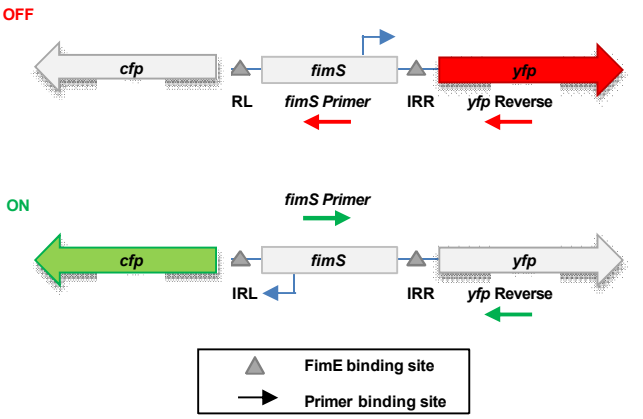

Figure 4: <u>Primer design for detection of switch activation</u>
Schematic cartoon of ON state specific primers for the detection of switch activation using PCR.

Bacterial colonies formed from a single cell are typically considered to be clonal populations, but colony level heterogeneity could arise if the founding cell contained a mixture of both activated and inactivated DNA switches on a multiple plasmid copies. Because heterogenous 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[96, 97, 103].

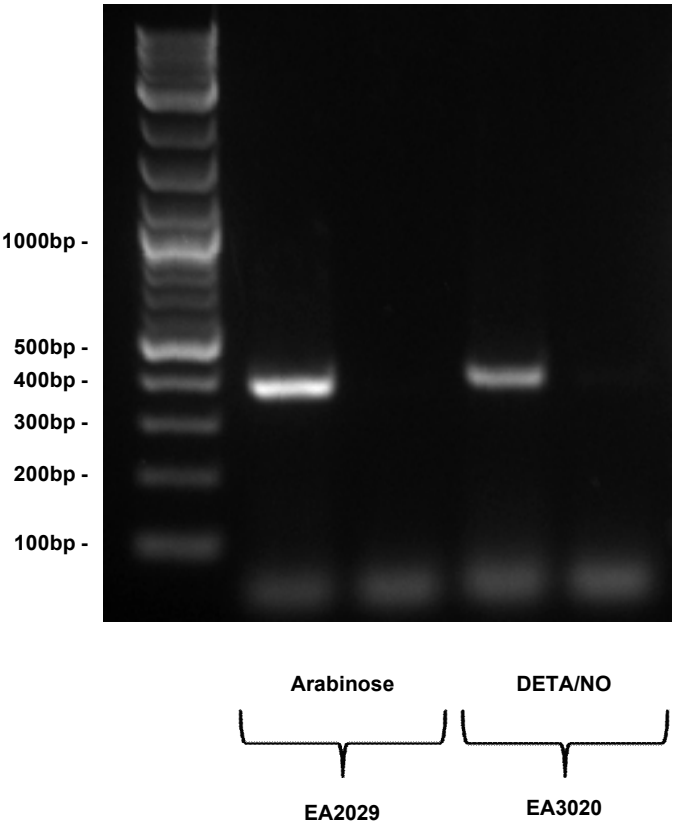

Figure 5: <u>PCR detection of switch activation</u>
PCR amplification of activated and inactivated colonies from strains EA2029 and EA3020 using *fimS* and *yfp* reverse primers. The 377 bp fragment present in EA2029 colonies exposed to arabinose or EA3020 colonies exposed to nitric oxide is not detected in unexposed colonies.

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[97, 106], and is supported by the absence of CFP+ cells detectable without arabinose induction.

CHAPTER FIVE
Nitric Oxide Sensing as the Input for the Recombinase-Based Genetic Switch

Introduction

Nitric oxide is used for cell-cell communication in eukaryotes and is a natural marker of inflammation[107], making it an ideal input signal for the engineered microorganism detailed here. Inflamed epithelial cells produce nitric oxide by up-regulating inducible nitric oxide synthase (iNOS), an enzyme that produces nitric oxide from L-arginine[107-109].

Though nitric oxide is used as an inflammatory signal in mammals, many bacteria possess nitric oxide sensors that often control the expression of cognate nitric oxide reductases that metabolize and detoxify nitric oxide. Several *E. coli* sensor proteins are responsive to nitric oxide[110], but the bacterial enhancer binding protein NorR was selected because it is believed to react solely with nitric oxide and no other reactive nitrogen species or small molecules[111-117].

NorR binds to 3 conserved sites in the promoter for the *E. coli* flavorubredoxin gene *norV* (*PnorV)* in the *norR-norV* intergenic region[117]. 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 AAA+ domain, allowing NorR binding to σ^{54} and transcription of n*orV* to occur[111, 115].

Results

In order to functionally link nitric oxide sensing with the response of activating the bi-directional fim switch, I placed control of *fimE* transcription under the control of the *E. coli* flavorubredoxin gene promoter *PnorV* (Figure 6). As is common in the construction of novel synthetic genetic circuits, my initial attempt at linking these two circuit elements did not cause switch activation in response to nitric oxide exposure. Through further research, I identified several elements within my first construct that could be changed with the goal of increasing the expression levels of FimE[118]. My first *PnorV-fimE* construct was constructed using the *norR-norV* intergenic region. Due to the possibility that additional regulatory sequences may have existed within the complement of the *norR* gene, I used a previously published sequence for the *PnorV* circuit element that included part of the coding sequence for *norR*[116].

This change did not lead to CFP expression or detectable inversion of the DNA switch, leading me to attempt other methods for increasing FimE levels within my bacterial strain in response to nitric oxide. I hypothesized that the use of an optimized RBS and spacer (AAGGAGGAAAGTCACATT) available through the Biobricks Registry would increase the translation rate of FimE from mRNA, and additionally that the addition of an ATG start codon before the native *fimE* GTG start codon would have a similar effect on protein expression. However, none of these changes resulted in effective inversion of the bidirectional fim switch.

The final necessary change needed to cause efficient recombination in response to nitric oxide was made after developing the hypothesis that stoichiometric imbalance might exist due to

the presence of an estimated additional 15 NorR binding sites on the low copy number plasmid pUA66, while the wildtype system contains only 3 copies per chromosome. To rectify this imbalance, I introduced the BioBrick part K2560007, a promoter that constitutively expresses *E. coli* NorR on the plasmid pSB1A2, resulting in the construction of strain EA3020 that robustly responds to nitric oxide exposure through recombination of the bidirectional fim switch. Notably, when K256007 was transformed into other strains with either GTG start codons or the native *norV* ribosomal binding site and spacer, no recombination was detected in response to nitric oxide exposure.

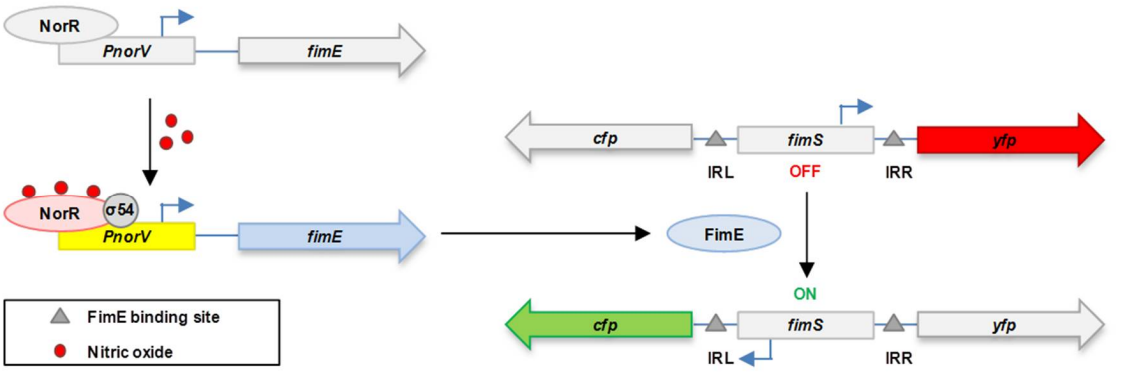

Figure 6: <u>Schematic of strain EA3020</u>
The core DNA recombinase circuit is coupled to nitric oxide sensing by driving FimE production from *PnorV*. Binding of nitric oxide to NorR results in σ^{54} binding and subsequent expression of FimE, driving switch activation

To characterize the switching properties of the nitric oxide responsive engineered *E. coli* (strain EA3020), I used the nitric oxide donors DETA/NO (diethylenetriamine/nitric oxide adduct) and SNP (sodium nitroprusside) as sources of nitric oxide. After exposure to 100 μM

SNP, cells decreased YFP signal and increased CFP fluorescence within 1 cell division, ~70 min (Figure 7).

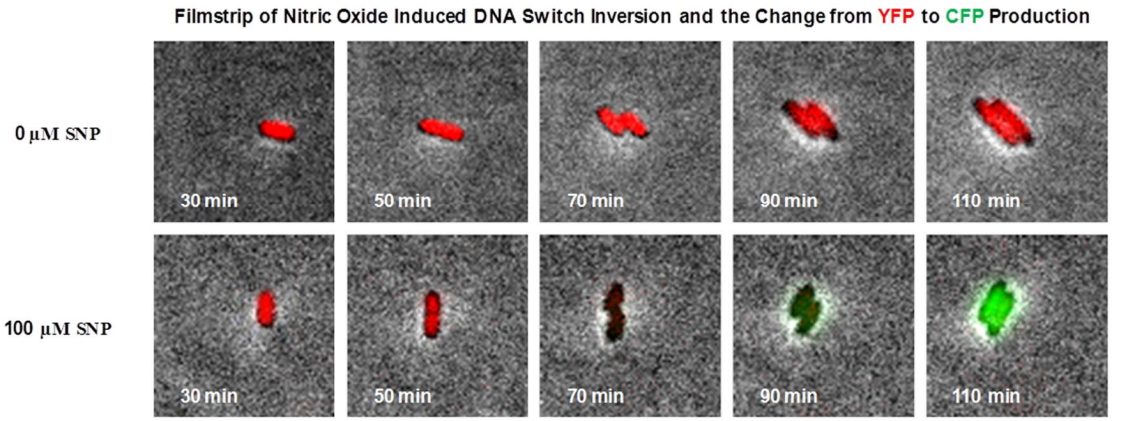

Figure 7: <u>Observation of fim switch activation in single cells in strain EA 3020</u>
Single cell time-lapse fluorescence microscopy of strain EA3020 containing both the bi-directional switch and *PnorV-fimE*. Cells treated with 100 µM of the nitric oxide donor SNP lose YFP signal (pseudo colored red) while increasing CFP fluorescence (pseudo colored green) as FimE activates the DNA switch.

To further characterize the response of strain EA3020 to nitric oxide, I exposed the strain to a range of concentrations of DETA/NO for a 1 hour period, before serial dilution and plating on antibiotic selective media. 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. Strain EA3020 was responsive to DETA/NO in a range from 40 to 100µM (Figure 8).

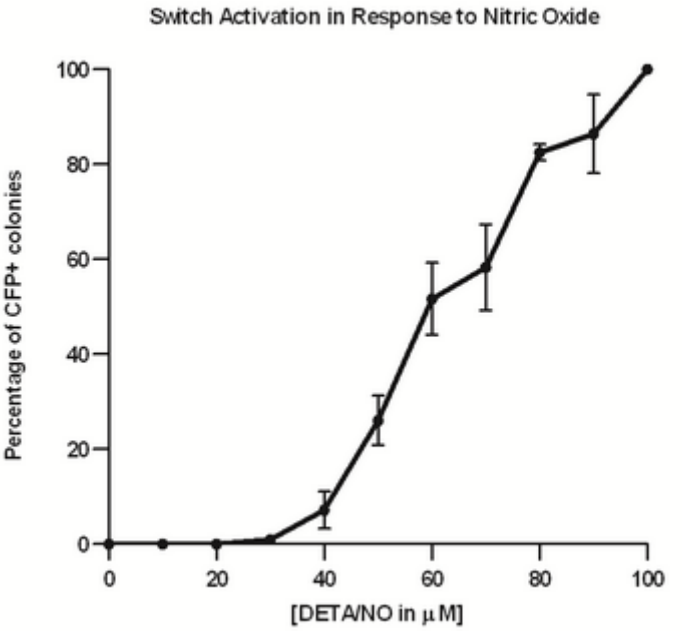

Figure 8: <u>Dose response of strain EA3020 to nitric oxide produced by DETA/NO</u>
The dose response of strain EA3020 to nitric oxide from the chemical donor DETA/NO over 1 hour was determined by calculating the percentage of CFP+ colonies after serial dilution and plating of the batch culture on selective antibiotic media. Shown is the mean percentage of CFP+ colonies and standard error.

To determine how quickly switch activation follows exposure to nitric oxide, I exposed a liquid culture of strain EA3020 to 100μM DETA/NO and took samples at times ranging from 30 seconds after the addition of DETA/NO to 1 hour. After each predetermined exposure period, cells were serially diluted before being plated on antibiotic selective media. 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.

Surprisingly, I found that strain EA3020 undergoes permanent inversion in more than 95% of cells within 5 minutes of exposure to DETA/NO (Figure 9A).

To determine the actual speed by which switch activation can occur, and to rule out the possible explanation that brief exposure to nitric oxide could lead to delayed switch activation, I designed an experiment that used PCR amplification using ON state specific primers as a sensitive test for switch activation. Cells were exposed to 100 μM DETA/NO for a predetermined time between 1 and 60 minutes. At each time point, aliquots of the liquid culture were removed and immediately placed in a 100^{o}C water bath to heat kill the cells. PCR amplification using ON state specific primers demonstrated that switch inversion is detectable in a population of cells after only 3 minutes of exposure to nitric oxide (Figure 9B), confirming that switch activation occurs very rapidly.

The construction and characterization of strain EA3020 links the sensing of an extracellular signal with the activation of an irreversible DNA-recombinase based switch, and demonstrates the modularity of inputs for the fim switch. These experiments also demonstrated that fim recombination can occur at a relatively fast rate, within less than three minutes of exposure to nitric oxide.

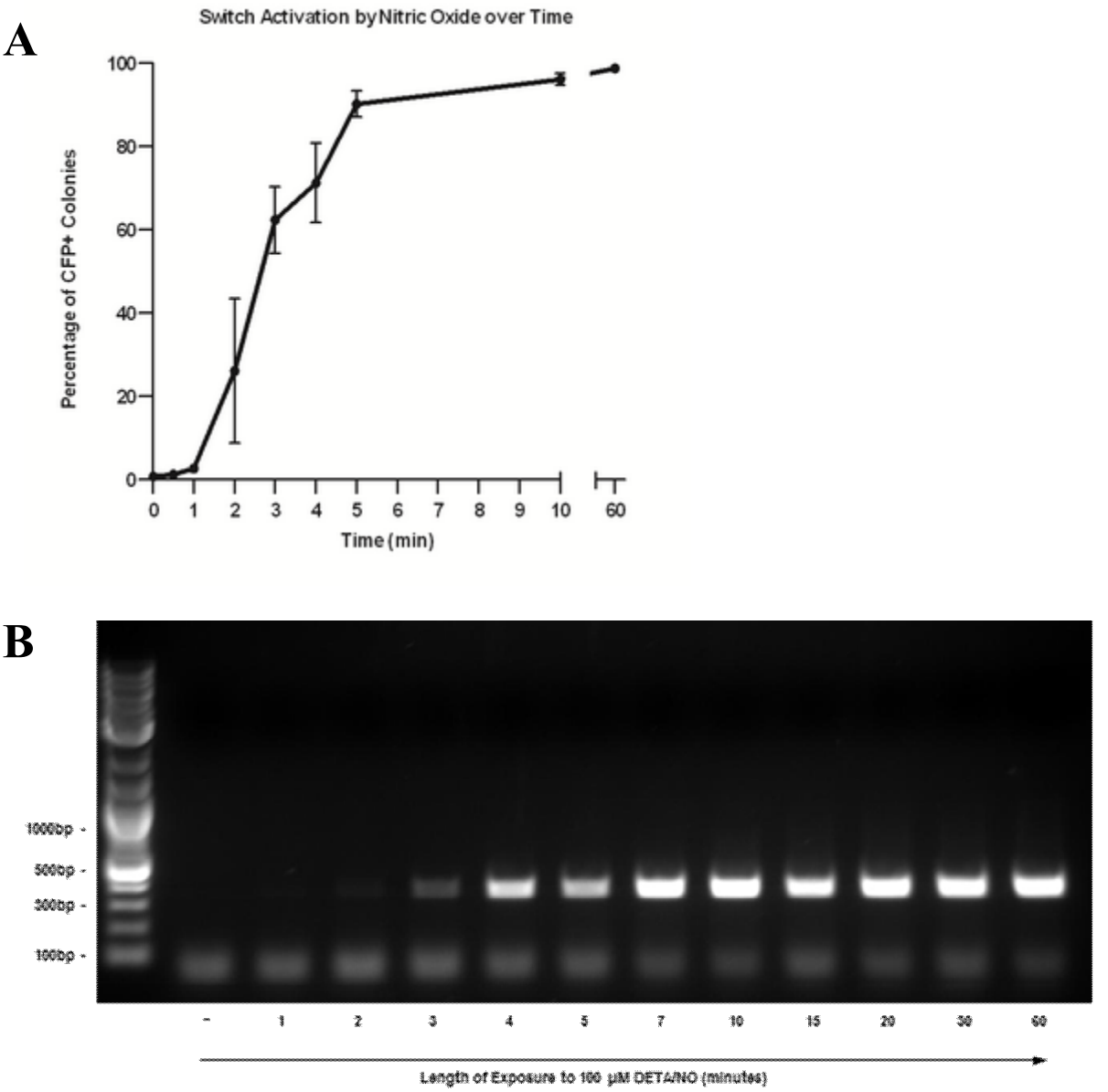

Figure 9: <u>Speed of switch activation in response to nitric oxide exposure in strain EA3020</u>

A) 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. Shown is the mean percentage of CFP+ colonies and the standard error

B) Using ON state specific primers (377bp fragment), DNA recombination is detected using PCR within 3 minutes of exposure to nitric oxide (100 µM DETA/NO).

CHAPTER SIX
Strain EA3020 Senses iNOS Derived Nitric Oxide from Inflamed Mammalian Gut Tissue

Introduction

Within the mammalian gut, nitric oxide molecules freely diffuse throughout the lumen and are capable of easily crossing cell membranes. Nitric oxide has a short half-life in these conditions due to its high reactivity, as well as the presence of host and bacterial enzymes that break down nitric oxide[103]. Gross measurements of nitric oxide levels in the colon of healthy patients (mean 60 ppb nitric oxide) are up to 90 times lower than levels in patients with active inflammation caused by ulcerative colitis (mean 5500 ppb), colorectally localized Crohn's disease (mean 5675 ppb), or ileocaecal Crohn's disease (mean 2625 ppb)[109].

The bacterial nitric oxide sensor NorR is reported to induce a 30 fold increase in gene expression from *PnorV* in response to nitric oxide concentrations of 120 ppm[109]. Several differences in experimental design prevented direct comparison of nitric oxide concentrations between these two studies, rendering it difficult to predict whether NorR would be capable of sensing nitric oxide produced by inflamed tissue. In human patients, nitric oxide concentrations were measured from the amount of nitric oxide that diffused into the balloon of a Foley catheter, before being measured by a chemiluminescent assay. To determine the response range of NorR to nitric oxide, bacteria carrying a PnorV promoter fusion to beta-galactosidase were grown in

liquid suspension, with nitric oxide supplied as a gas mix in atmosphere. After dissolving in liquid, nitric oxide concentrations in liquid were approximately 250 nM.

An additional factor in determining the responsiveness of NorR to nitric oxide concentrations produced by inflammatory tissue is the micro-environment within the gut lumen. Immunohistochemical labeling of inducible nitric oxide synthase in biopsies from IBD patients indicate that inducible nitric oxide synthase expression is localized to inflamed tissue, and it can be inferred that nitric oxide production is non-uniform within the gastrointestinal tract[102]. Further, nitric oxide rapidly diffuses and is degraded within the gut lumen, suggesting that the combination of non-uniform production and rapid degradation would lead to micro-environments of higher nitric oxide concentration immediately adjacent to inflamed tissue when compared to the lumen as a whole.

Results

To determine whether strain EA3020 is sensitive to iNOS derived nitric oxide in animals with inflamed gastrointestinal tissue, I utilized a chemically induced mouse model of colitis to generate an inflammatory response in the mouse gut. Dextran sodium sulfate (DSS) is well characterized as an intestinal irritant, and is commonly used to generate colitis in mouse models of IBD. Mice supplied with 2% DSS in drinking water have been shown to develop intestinal inflammation, with concomitant up-regulation of iNOS expression in previous studies using histology, immunohistochemistry, RT-PCR, and western blotting[119]. After seven days of treatment, mice exhibit externally visible symptoms of colitis, including bloody and loose stools,

poor vigor, anal prolapse, as well as shortening of the colon upon dissection and gross morphological examination.

Mice were separated into control and treated groups, with the treated group receiving 2% dextran sodium sulfate in drinking water. After one week of DSS treatment, both groups were orally gavaged with 0.3 mL of strain EA3020 grown to an optical density of 0.4. Fecal samples were later collected from both groups of mice and weighed before being serially diluted in water and plated on antibiotic selective plates. Several differences were noted between the fecal samples of DSS and control treated animals. Samples from control animals were generally dry and hard, and the total amount of feces easily collectable was higher in control animals. Feces from DSS treated animals was softer and more liquid, and dissolved easily prior to serial dilution.

Because of the differences in collected stools, it seemed prudent to determine if there were any differences in the total number of colonies recovered between the two treatment groups. The following day the number of colonies recovered was counted, and the number of recovered colonies per gram of feces were compared between DSS treated mice and controls. Samples were initially taken 4, 6, 8, 12, and 24 hours after oral gavage. The highest number of recovered colonies was from samples taken 6 hours after treatment, with no colonies detectable at 24 hours post-gavage, indicating that strain EA3020 does not colonize the mouse intestine. All data shown are from the 6 hour time point.

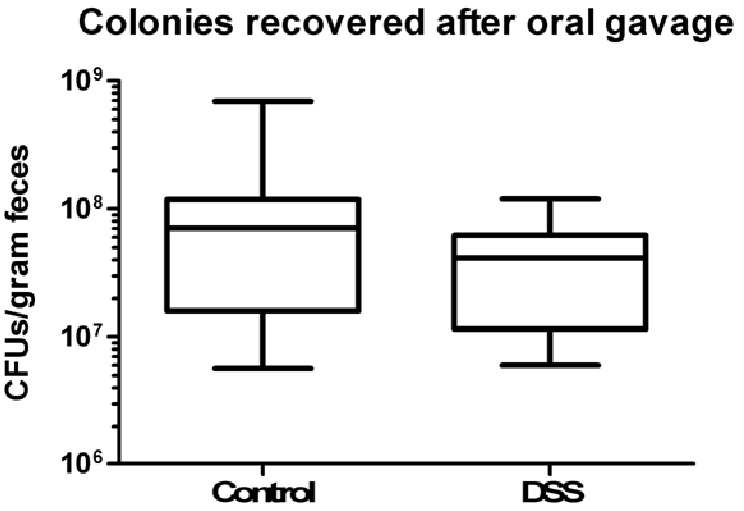

Figure 10: **Comparison of CFUs collected between DSS treated and control animals**
Following stool sample collection, stool samples were dissolved in sterile PBS and serially diluted before being plated on antibiotic selective media. No significant differences were noted in the number of CFUs per gram of feces collected. N=24

No significant differences were found between the DSS treated and control animals in the amount of recoverable EA3020 after oral gavage after adjusting for collected stool weights (Figure 10). To determine whether EA3020 strain is exposed to nitric oxide sufficient to drive recombination within the mammalian gut, 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. The percentage of CFP+ colonies in DSS treated animals were then compared to untreated controls.

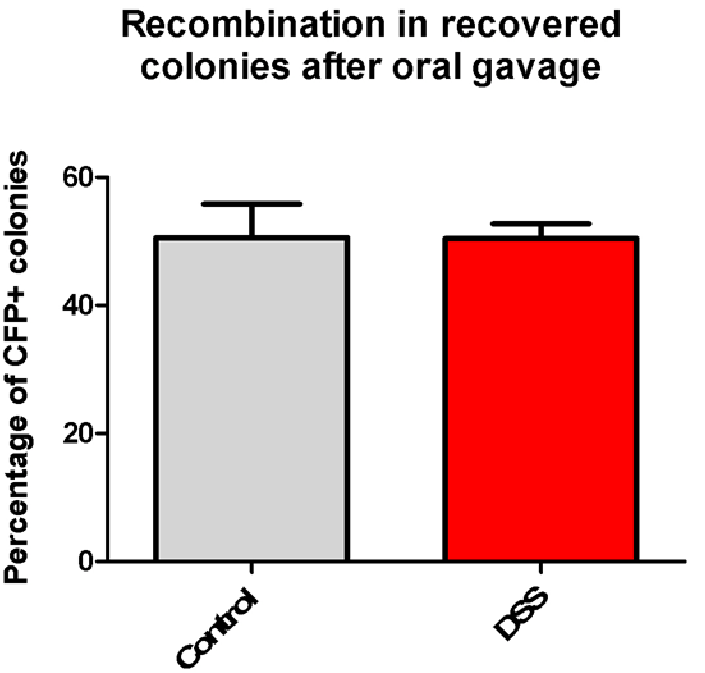

Figure 11: <u>Switch activation in strain EA3020 following oral gavage in mice</u>
Control and DSS treated (2% DSS in water, 7 days) mice were orally gavaged and stool samples were collected 6 hours later, serially diluted, and plated. After overnight culture, the number of CFP+ and total colonies were counted using a fluorescence dissecting scope, and the ratio of CFP+ to YFP+ colonies was counted.

No significant difference was found in the percentage of CFP+ colonies between DSS treated and control animals. This result was unexpected, as original expectations predicted that either no nitric oxide sensing would take place within either group, or that the EA3020 strain would be capable of differentiating between healthy control animals and those with experimentally induced colitis. Several possible causes could explain the response in control

animals, especially the possibility of high nitric oxide levels in the stomach due its chemical synthesis from dietary nitrogen sources.

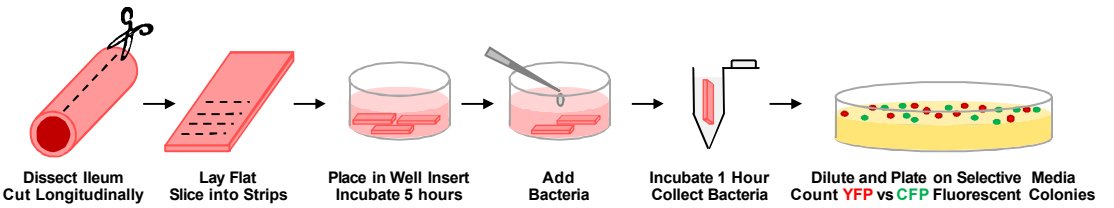

Figure 12: <u>Co-culture of strain EA3020 with intestinal explants</u>
Following sacrifice, approximately 5 cm of ileum was dissected, flushed with saline to remove residual feces, and cut longitudinally. Small strips were sliced off and placed in well inserts, and the tissue was incubated for 5 hours before strain EA3020 was added to the well inserts. Strain EA3020 was co-cultured with the intestinal explants for one hour before the explants were removed and vortexed in sterile PBS to resuspend strain EA3020. Finally, bacterial suspension was serially diluted and plated on selective media.

I determined that a simpler model might be appropriate for the characterization of strain EA3020's ability to sense nitric oxide. I designed an experiment in which strain EA3020 could be co-cultured with mouse ileum explants (Figure 12), to determine if strain EA3020 is sensitive enough to respond to nitric oxide produced by intestinal tissue.

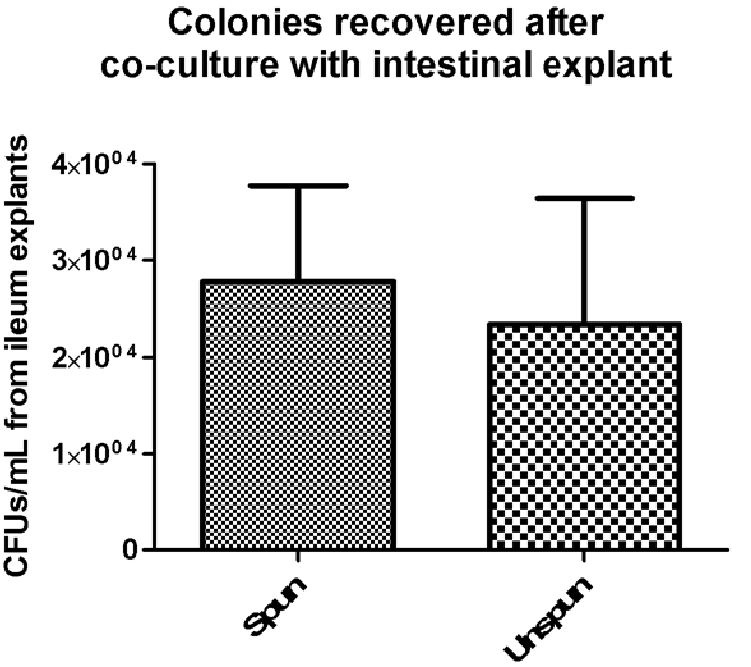

Figure 13: <u>Centrifugation doesn't affect the amount of recovered CFUs after co-culture</u> Plates were centrifuged at 2000 RPM immediately after the addition of bacterial suspension on the recovery of EA3020 following co-culture. I found no significant difference in the recovered CFU's between the spun and control culture plates. Shown is the mean CFUs/mL and standard error.

While refining my protocol for the co-culture of intestinal explants with bacteria, I centrifuged plates immediately after the addition of bacteria to the culture wells under the assumption that it would lead to a closer physical association between introduced bacterial cells and intestinal explants. After quantifying the number of recovered colonies following a one hour co-culture (Figure 13), centrifugation was found to have little or no effect on efficiency of recovering colonies post co-culture.

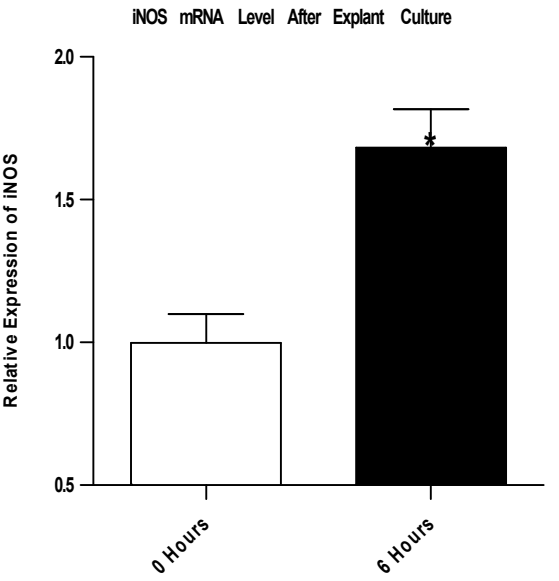

Figure 14: <u>Explant culture leads to up-regulation of iNOS mRNA</u>
A comparison of iNOS mRNA levels as measured by qRT-PCR immediately after dissection, and after 6 hours of explant culture demonstrates that iNOS mRNA expression levels increase by a modest but significant (p=0.01) factor of 1.7.

Following the co-culture of strain EA3020 with intestinal explants, I found that a fraction (~38%) of EA 3020 colonies recovered had successfully activated the fim recombination switch and were CFP positive, indicating that nitric oxide sensing had occurred.

I expected that the explant culture conditions would cause an increase in inducible nitric oxide synthase expression due to the mechanical injury to the tissue and resultant inflammation caused by surgical dissection and tissue processing for explant culture. To test the hypothesis that explant culture caused an increase in iNOS levels due to inflammation, I compared the level of inducible nitric oxide synthase expression in tissues immediately following dissection and

after 6 hours in explant culture using qRT-PCR to measure iNOS mRNA expression levels (Figure 14). As expected, iNOS expression increased during the 6 hours of explant culture, with a modest but significant increase in iNOS mRNA levels.

While iNOS mRNA levels indicated that iNOS was being up-regulated in explant cultures, whether this had a functional effect on the levels of nitric oxide being produced was unclear. While the Griess reaction is a common chemical assay used to determine the production of nitric oxide by iNOS, by determining the ratio of nitrite to nitrate in a liquid sample, it was unclear whether the reaction would be of any use for measuring nitric oxide production by these explant cultures. Problematically, the reaction would be measuring the entire volume of growth media, rather than the levels of nitric oxide that a bacterial cell might be exposed to in the microenvironment immediately near the explanted intestine.

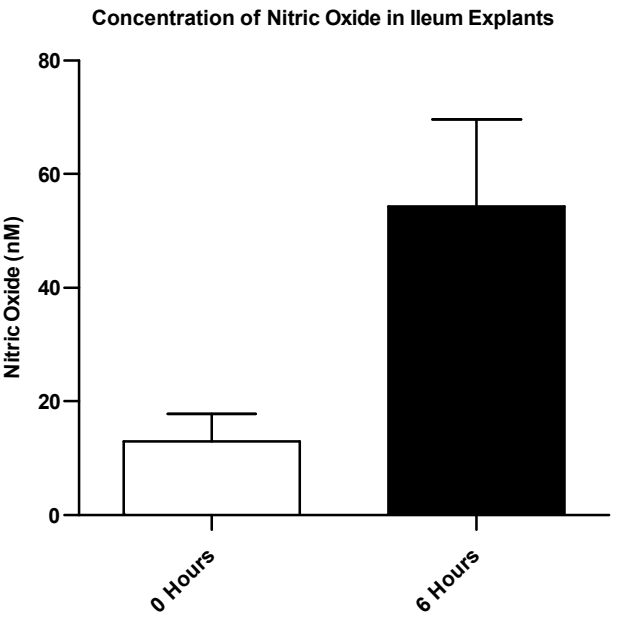

Figure 15: <u>Increased nitric oxide levels in cultured ileum explants</u>
Direct measurement of nitric oxide concentration from ileum explants using a nitric oxide microsensor. Mean nitric oxide concentration (immediately after dissection = 13.0 ± 4.8 nM, after 6 hours in culture = 54.3 ± 15.3 nM) and standard error.

To test whether nitric oxide concentrations correspond with inducible nitric oxide synthase up-regulation during explant culture, nitric oxide concentrations were measured directly adjacent to ileum explants using an L shaped nitric oxide specific sensor probe. I found that nitric oxide production is higher in explants after 6 hours in culture conditions (54.3 ± 15.3 nM) when compared to measurements taken immediately after dissection (13.0 ± 4.8 nM) (Figure 15). Interestingly, the nitric oxide concentration immediately adjacent to intestinal explants cultured for 6 hours is approximately one-fifth the concentration (~250 μM) of nitric oxide previously reported to elicit measurable response from the sensor NorR[109].

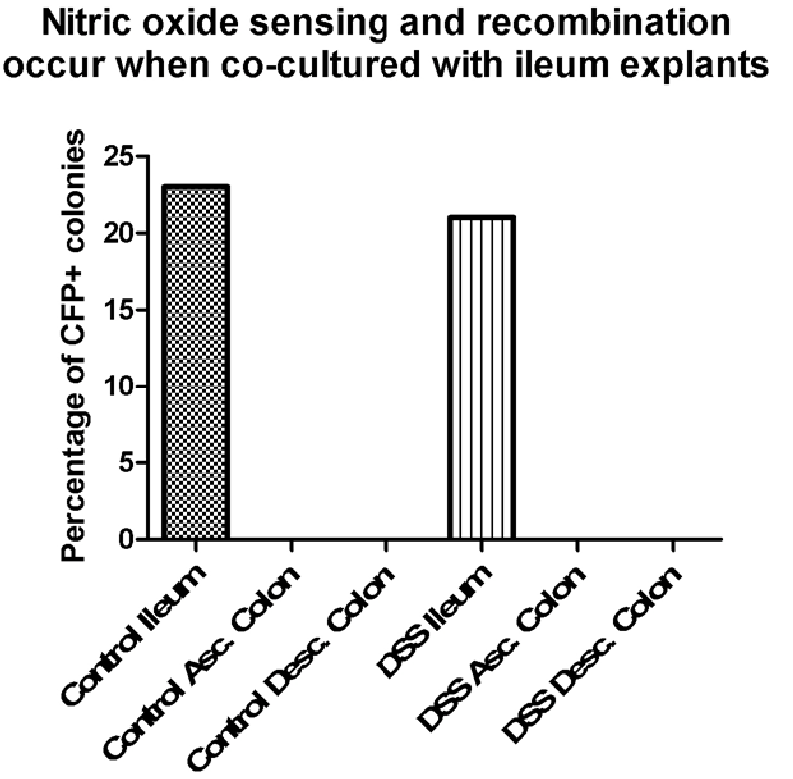

Figure 16: <u>Differences in nitric oxide sensing between intestinal tissues</u>
Following seven days of treatment with DSS, mice were sacrificed along with control animals to determine whether DSS pre-treatment would have any effect on nitric oxide production in explant culture. Additionally, intestinal explants were taken from the ascending and descending colon to determine whether nitric oxide production differs between different intestinal tissues.

Both the direct measurement of iNOS mRNA expression levels and a functional measurement of nitric oxide levels immediately adjacent to ileum explants support the hypothesis that the process of explant culture leads to inflammation. However, it is interesting to note that different intestinal tissues seem to have no ability to induce nitric oxide induced switching of the fim recombinase switch (Figure 16). Additionally, the pre-treatment of mice

with DSS for seven days prior to sacrificing the animals for explant culture seemed to have no effect on the level of nitric oxide being produced by intestinal tissue as measured by fim recombination after co-culture.

To confirm that inducible nitric oxide synthase is the source of nitric oxide leading to fim switch activation during co-culture, mouse ileum explants were treated during culture with the selective inducible nitric oxide synthase inhibitor (1400W dihydrochloride, 50 μM) before and during co-culture with strain EA 3020. Explants treated with inducible nitric oxide synthase inhibitor exhibited a 30 fold decrease in the percentage (from ~30% to ~1%) of cells that responded to nitric oxide during co-culture (Figure 17). This finding lends significant support to the hypothesis that strain EA3020 is capable of sensing and responding to nitric oxide produced by inducible nitric oxide synthase in mammalian tissues.

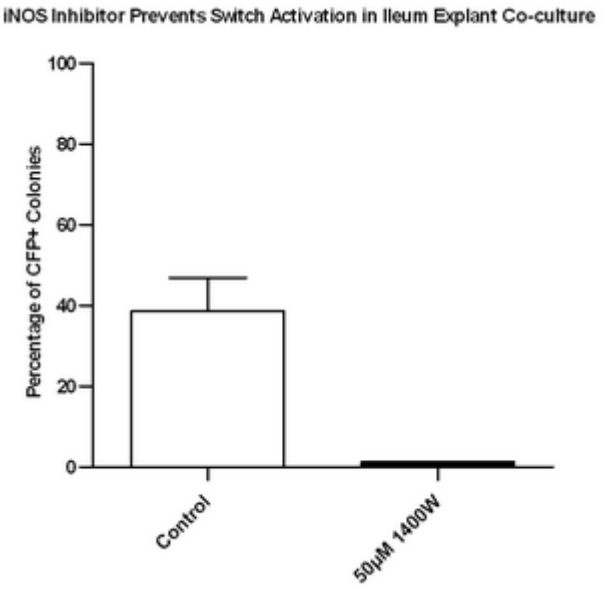

Figure 17: <u>iNOS inhibition prevents switch activation in strain EA3020 co-cultured with intestinal explants</u>

Intestinal explants were treated with a selective small molecule inhibitor of iNOS, 1400W dihydrochloride both before and during co-culture with strain EA3020. Treatment with an iNOS inhibitor led to an approximate 30 fold decrease in fim switch recombination when compared to untreated controls.

To ensure that 1400W dihydrochloride was not having off target effects on strain EA3020, possibly interfering with 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 nitric oxide produced by the chemical donor DETA/NO for 1 hour (Figure 18). The resulting data showed that inducible nitric oxide synthase inhibitor treatment has no significant effect (Student's t-test $p=0.79$ for 0 μM DETA/NO and $p=0.57$ for 100 μM DETA/NO) on the ability of EA3020 to respond to nitric oxide produced by the chemical donor DETA/NO.

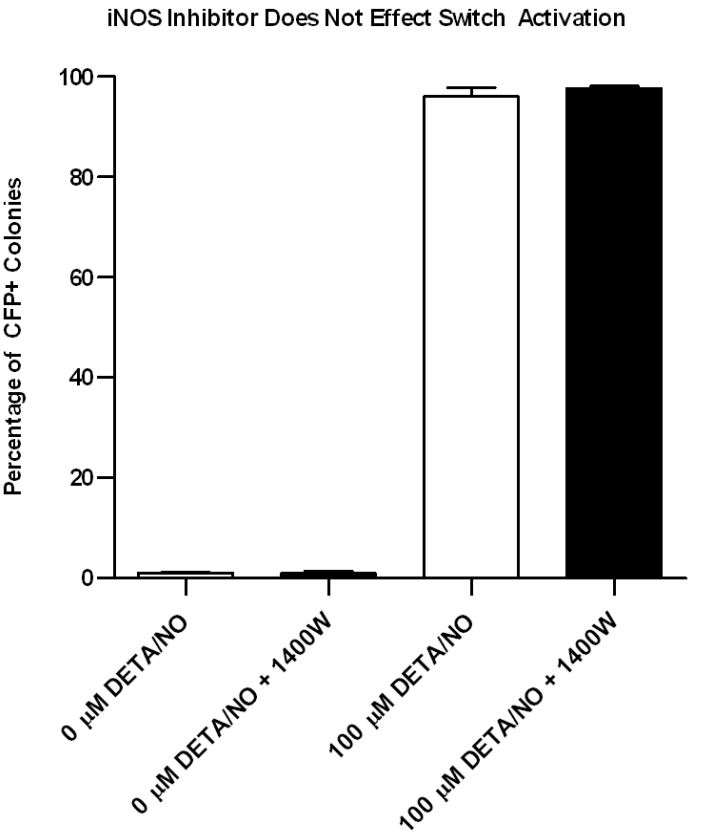

Figure 18: <u>1400W dihydrochloride does not prevent recombination in strain EA3020</u>
The ability of strain EA3020 to effectively activate the fim switch in the presence of 1400W dihydrochloride was tested using the nitric oxide donor DETA/NO.

CHAPTER SEVEN
Discussion and Recommendations for Future Study

The Benefits of a Recombinase-Based Synthetic Genetic Circuit

To be useful as a treatment for inflammatory bowel disease, this synthetic genetic circuit would require multiple additional iterations of design, construction, and testing. The primary reason for the use of an engineered microorganism in this setting is the promise of a highly effective but very well tolerated treatment that increases patient quality of life by decreasing disease burden and side effects associated with treatment. To meet this goal, the use of a DNA recombinase switch is especially beneficial because the core circuit limits the expression of its modular output gene.

In most genetic circuits, noisy or "leaky" gene expression can lead to stochastic bursts of unwanted gene expression, even from uninduced promoters. The additional step requiring a threshold concentration of DNA recombinase to cause DNA inversion acts as a filter for biological noise. Because the use of potent anti-inflammatory drugs can also lead to serious side effects like serious infections or the development of lymphomas and other cancers, the tight control of output gene expression may help prevent side effects in patients. This strategy may be much safer than other engineered microbes.

Because this recombinase-based core circuit is modular in design, in the future, this inflammatory sensor could be used to control the downstream expression of a potent anti-inflammatory molecule, such as recombinant anti-inflammatory cytokines like IL-10 or a TNF-α

neutralizing camelid antibody[45, 46], forming the basis of an effective therapy for inflammatory bowel diseases.

An additional beneficial feature of the recombinase circuit is that it can act as a permanent record of transient events. Even without the ability to produce and locally deliver anti-inflammatory medications directly at the site of inflammation, the ability to permanently alter gene expression in the engineered bacterium could be used as an early detection system for patients. If, for instance, the modular output of the recombinase switch were changed from a fluorescent protein into a chromophore or dye, patients could be alerted to an oncoming flare-up, allowing them to begin prophylactic treatment before the onset of symptoms.

While the goal of this project has been the development of a treatment for inflammatory bowel disease, the core features of this recombination based genetic circuit may eventually be useful in the treatment of other diseases. The same inherent properties that are being exploited here such as biological noise filtering, the permanent genetic recording of transient environmental events, and the tight control of output gene expression could someday be applied in bacteria designed to treat other diseases.

Biological Containment and Safety Issues

Because the recombinase switch causes permanent genetic changes, it would be necessary to ensure that an engineered microorganism carrying this circuitry would not be capable of continuous colonization of the intestines. Similarly to the use of steroids or other immunosuppressants, the ability to cease treatment in case of serious infection is an important

consideration. To this end, a prudent design feature would be the inclusion of biocontainment measures. This might include choosing a chassis organism that is unlikely to colonize the mammalian intestine. Many strains of probiotic microorganisms cannot colonize the human gut due to competition from co-evolved commensal organisms, and the choice of this type of chassis might also increase the clinical benefit of an engineered microorganism.

Luckily, with the use of engineered microorganisms in industrial and bioremediation settings, many strategies for biocontainment have already been well developed and characterized. This could include the knockout of essential metabolic genes through homologous recombination. It is even possible to increase the containment of the engineered genetic material, by surrounding the artificial genetic circuit with homologous sequences for critical metabolic genes. By doing so, organisms that escape from metabolic containment would simultaneously lose the artificial genetic circuit. Likewise, in the case of horizontal gene transfer of the artificial genetic circuit, recombination would cause the loss of a critical metabolic gene. This strategy has also been tested in engineered microorganisms designed for the treatment of colitis[47].

Other clever strategies for biocontainment include the use of bacterial toxins or viral lysins as suicide genes, with expression of an anti-toxin or control of a repressor gene being tied to a small molecule that would be unavailable within the gut. Additionally, genetic timer circuits have been developed, with the predictable expression of an output gene sometime after the addition or removal of an environmental signal or small molecule[70, 120]. This type of circuit

could be used to cause cell death using a bacterial toxin after a certain period of time within the patient.

Refining the Ability to Differentiate Healthy Patients from Sick Ones

A major obstacle for the implementation of this engineered microbe will be the tuning of nitric oxide sensing to allow reliable distinction between healthy patients in remission, and patients entering or experiencing inflammatory flare-ups. While much of the tuning of this synthetic genetic circuit will require modeling, as well as multiple iterations of design, construction, and testing, intriguing possibilities exist for the use of combinatorial logic to prevent unwanted activation of the recombinase switch. One strategy might be the use of another DNA recombinase, or the construction of a hybrid promoter, that would prevent FimE or another recombinase from being expressed in the absence of a quorum sensing signal like AI-2. This would result in the output of the entire synthetic genetic circuit requiring the presence of other gut bacteria, which might prevent premature activation in an environment such as the stomach, which can contain high levels of nitric oxide as dietary nitrogen sources are enzymatically broken down in the low pH environment.

While the use of quorum sensing to prevent premature activation of the recombinase switch may be an intriguing possibility, many other environmental signals might be used singly or in an integrated fashion to control activation of the recombinase circuit. In fact, while nitric oxide sensing is easily co-opted for use in this synthetic genetic circuit as a proxy for sensing

inflammation, other inflammatory signals may be recognizable by bacteria, which could allow for more stringent criteria to be met before circuit activation could take place.

Novel Finding: *E. coli* Can Sense Nitric Oxide Produced by Inflamed Tissues

The discovery that bacteria are capable of sensing nitric oxide may have multiple implications. Bacteria have already been shown to respond to a variety of mammalian signaling molecules, and the role of this communication between host organisms and their commensal or pathogenic microbiota is of great interest. For instance, enterohemorrhagic E. coli have been shown to respond to the mammalian signaling molecules epinephrine and norepinephrine, initiating changes in virulence in response to host stress[121]. Evidence exists for bacterial sensing of opioid and opiate molecules, also with changes in virulence[122]. Mounting evidence suggests that bacteria are capable of sensing complex signals from mammalian hosts, but most research focuses on the role that this communication plays in pathogenesis.

An interesting result of this work is the demonstration that *E. coli* are capable of sensing nitric oxide at concentrations being produced by gut epithelial tissues. While the concentrations of iNOS derived nitric oxide produced by macrophages during bacterial killing after phagocytosis have been known to be high enough to cause changes in bacterial physiology, the possibility that bacteria within the gut could detect inflammation is intriguing and was previously unknown to occur through this nitric oxide sensor. Additionally, while the nitric oxide sensing capability of NorR is dedicated to regulating the production of flavorubredoxin and flavorubredoxin reductase in *E. coli*, other nitric oxide sensors in a variety of other strains and

species, both pathogens and commensal organisms may also be capable of responding to mammalian inflammation in unknown ways. This may be another example of cross-kingdom signaling from mammalian hosts with their gut microbes.

Novel Finding: Rapid DNA Recombination Upon FimE Expression

While the goal of this work was not to investigate the biology of either FimE or the NorR-*PnorV* components, the process of characterizing a new genetic circuit component has high potential for uncovering novel information about biological systems.

The ability to rapidly detoxify nitric oxide has obvious benefits for bacteria that may be exposed to high levels of nitric oxide in unpredictable environments that can include phagocytosis by macrophages. The speed of this response is unsurprising, considering that nitric oxide can be toxic to cells at high concentrations due to its high reactivity. Because flavorubredoxin and flavorubredoxin reductase that enzymatically break down nitric oxide are the downstream targets of nitric oxide sensing, it makes sense that their production be rapidly initiated in conditions of nitric oxide stress.

In contrast, the speed with which FimE performs DNA recombination is somewhat surprising. Because FimE expression is being directly controlled using a tightly controlled but relatively fast-responding promoter, the speed at which FimE can fold and initiate DNA recombination can be directly measured from a fixed starting point. In my experiments, the speed at which FimE can be expressed, properly fold, and accomplish DNA recombination has now been clocked at 2-3 minutes after induction.

Conclusion

In addition to the construction of and characterization of a synthetic genetic circuit that enables engineered *E. coli* to detect and respond to inflammation produced by mammalian tissues, this project has yielded unexpected returns. This project has resulted in the characterization of two unique genetic circuit elements, the NorR-*PnorV* nitric oxide sensing system and the bidirectional fim switch. The characterization and testing of these elements has independently revealed new information about the biology of each, demonstrating that NorR is capable of sensing nitric oxide produced by inflamed tissue, and that FimE can cause DNA recombination much faster than was previously assumed. Together, the entire synthetic genetic circuit is a proof of principle for the conversion of a transient environmental signal into a permanent genetic recording.

REFERENCES

1. Elowitz, M., and Lim, W. A. (2010) Build life to understand it, *Nature 468*, 889-890.
2. Ferber, D. (2004) Synthetic biology. Microbes made to order, *Science 303*, 158-161.
3. Mukherji, S., and van Oudenaarden, A. (2009) Synthetic biology: understanding biological design from synthetic circuits, *Nat Rev Genet 10*, 859-871.
4. Tucker, J. B., and Zilinskas, R. A. (2006) The promise and perils of synthetic biology, *New Atlantis 12*, 25-45.
5. Schwille, P., and Diez, S. (2009) Synthetic biology of minimal systems, *Crit Rev Biochem Mol Biol 44*, 223-242.
6. Hanauer, S. B. (2006) Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities, *Inflamm Bowel Dis 12 Suppl 1*, S3-9.
7. Kappelman, M., Rifas-Shiman, S., Bousvaros, A., Grand, R. J., Ollendorf, D., Kleinman, K., and Finkelstein, J. (2007) Prevalence and geographical distribution of Crohn's disease and ulcerative colitis in the United States, *Gastroenterology 132*, A37-A37.
8. Loftus, E. V., Jr. (2004) Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences, *Gastroenterology 126*, 1504-1517.
9. Gupta, N., Bostrom, A. G., Kirschner, B. S., Ferry, G. D., Gold, B. D., Cohen, S. A., Winter, H. S., Baldassano, R. N., Abramson, O., Smith, T., and Heyman, M. B. (2010) Incidence of stricturing and penetrating complications of Crohn's disease diagnosed in pediatric patients, *Inflamm Bowel Dis 16*, 638-644.
10. Jevon, G. P., and Madhur, R. (2010) Endoscopic and histologic findings in pediatric inflammatory bowel disease, *Gastroenterol Hepatol (N Y) 6*, 174-180.
11. Karwowski, C. A., Keljo, D., and Szigethy, E. (2009) Strategies to Improve Quality of Life in Adolescents with Inflammatory Bowel Disease, *Inflammatory Bowel Diseases 15*, 1755-1764.
12. Lutgens, M. W., Vleggaar, F. P., Schipper, M. E., Stokkers, P. C., van der Woude, C. J., Hommes, D. W., de Jong, D. J., Dijkstra, G., van Bodegraven, A. A., Oldenburg, B., and

Samsom, M. (2008) High frequency of early colorectal cancer in inflammatory bowel disease, *Gut 57*, 1246-1251.

13. Cadwell, K., Patel, K. K., Maloney, N. S., Liu, T. C., Ng, A. C., Storer, C. E., Head, R. D., Xavier, R., Stappenbeck, T. S., and Virgin, H. W. (2010) Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine, *Cell 141*, 1135-1145.
14. Barrett, J. C., Hansoul, S., Nicolae, D. L., Cho, J. H., Duerr, R. H., Rioux, J. D., Brant, S. R., Silverberg, M. S., Taylor, K. D., Barmada, M. M., Bitton, A., Dassopoulos, T., Datta, L. W., Green, T., Griffiths, A. M., Kistner, E. O., Murtha, M. T., Regueiro, M. D., Rotter, J. I., Schumm, L. P., Steinhart, A. H., Targan, S. R., Xavier, R. J., Libioulle, C., Sandor, C., Lathrop, M., Belaiche, J., Dewit, O., Gut, I., Heath, S., Laukens, D., Mni, M., Rutgeerts, P., Van Gossum, A., Zelenika, D., Franchimont, D., Hugot, J. P., de Vos, M., Vermeire, S., Louis, E., Cardon, L. R., Anderson, C. A., Drummond, H., Nimmo, E., Ahmad, T., Prescott, N. J., Onnie, C. M., Fisher, S. A., Marchini, J., Ghori, J., Bumpstead, S., Gwilliam, R., Tremelling, M., Deloukas, P., Mansfield, J., Jewell, D., Satsangi, J., Mathew, C. G., Parkes, M., Georges, M., and Daly, M. J. (2008) Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease, *Nat Genet 40*, 955-962.
15. Weersma, R. K., Zhernakova, A., Nolte, I. M., Lefebvre, C., Rioux, J. D., Mulder, F., van Dullemen, H. M., Kleibeuker, J. H., Wijmenga, C., and Dijkstra, G. (2008) ATG16L1 and IL23R are associated with inflammatory bowel diseases but not with celiac disease in the Netherlands, *Am J Gastroenterol 103*, 621-627.
16. Andus, T., and Gross, V. (2000) Etiology and pathophysiology of inflammatory bowel disease--environmental factors, *Hepatogastroenterology 47*, 29-43.
17. Danese, S., Sans, M., and Fiocchi, C. (2004) Inflammatory bowel disease: the role of environmental factors, *Autoimmun Rev 3*, 394-400.
18. Xavier, R. J., and Podolsky, D. K. (2007) Unravelling the pathogenesis of inflammatory bowel disease, *Nature 448*, 427-434.

19. Sartor, R. B. (2008) Microbial influences in inflammatory bowel diseases, *Gastroenterology 134*, 577-594.
20. Sartor, R. B. (2004) Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics, *Gastroenterology 126*, 1620-1633.
21. Linskens, R. K., Huijsdens, X. W., Savelkoul, P. H., Vandenbroucke-Grauls, C. M., and Meuwissen, S. G. (2001) The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics, *Scand J Gastroenterol Suppl*, 29-40.
22. Caprilli, R., Angelucci, E., and Clemente, V. (2008) Recent advances in the management of Crohn's disease, *Dig Liver Dis 40*, 709-716.
23. Irving, P. M., Gearry, R. B., Sparrow, M. P., and Gibson, P. R. (2007) Review article: appropriate use of corticosteroids in Crohn's disease, *Aliment Pharmacol Ther 26*, 313-329.
24. Carter, M. J., Lobo, A. J., and Travis, S. P. (2004) Guidelines for the management of inflammatory bowel disease in adults, *Gut 53 Suppl 5*, V1-16.
25. Lichtenstein, G. R., Abreu, M. T., Cohen, R., and Tremaine, W. (2006) American Gastroenterological Association Institute medical position statement on corticosteroids, immunomodulators, and infliximab in inflammatory bowel disease, *Gastroenterology 130*, 935-939.
26. Geborek, P., Bladstrom, A., Turesson, C., Gulfe, A., Petersson, I. F., Saxne, T., Olsson, H., and Jacobsson, L. T. (2005) Tumour necrosis factor blockers do not increase overall tumour risk in patients with rheumatoid arthritis, but may be associated with an increased risk of lymphomas, *Ann Rheum Dis 64*, 699-703.
27. Piccin, A., Cortelazzo, S., Rovigatti, U., Bourke, B., and Smith, O. P. (2010) Immunosuppressive treatments in Crohn's disease induce myelodysplasia and leukaemia, *Am J Hematol 85*, 634.
28. Brown, H. M., Storey, G., and George, W. H. (1972) Beclomethasone dipropionate: a new steroid aerosol for the treatment of allergic asthma, *Br Med J 1*, 585-590.

29. Bokinsky, G., Peralta-Yahya, P. P., George, A., Holmes, B. M., Steen, E. J., Dietrich, J., Soon Lee, T., Tullman-Ercek, D., Voigt, C. A., Simmons, B. A., and Keasling, J. D. (2011) Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered Escherichia coli, *Proc Natl Acad Sci U S A 108*, 19949-19954.
30. Carothers, J. M., Goler, J. A., and Keasling, J. D. (2009) Chemical synthesis using synthetic biology, *Curr Opin Biotechnol 20*, 498-503.
31. Lee, S. K., Chou, H., Ham, T. S., Lee, T. S., and Keasling, J. D. (2008) Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels, *Curr Opin Biotechnol 19*, 556-563.
32. Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S. B., and Keasling, J. D. (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass, *Nature 463*, 559-562.
33. Widmaier, D. M., Tullman-Ercek, D., Mirsky, E. A., Hill, R., Govindarajan, S., Minshull, J., and Voigt, C. A. (2009) Engineering the Salmonella type III secretion system to export spider silk monomers, *Mol Syst Biol 5*, 309.
34. Zhang, F., Rodriguez, S., and Keasling, J. D. (2011) Metabolic engineering of microbial pathways for advanced biofuels production, *Curr Opin Biotechnol 22*, 775-783.
35. Prather, K. L., and Martin, C. H. (2008) De novo biosynthetic pathways: rational design of microbial chemical factories, *Curr Opin Biotechnol 19*, 468-474.
36. Neumann, H., and Neumann-Staubitz, P. (2010) Synthetic biology approaches in drug discovery and pharmaceutical biotechnology, *Appl Microbiol Biotechnol 87*, 75-86.
37. Ball, P. (2005) Synthetic biology for nanotechnology, *Nanotechnology 16*, R1-R8.
38. Brune, K. D., and Bayer, T. S. (2012) Engineering microbial consortia to enhance biomining and bioremediation, *Front Microbiol 3*, 203.
39. de Lorenzo, V. (2008) Systems biology approaches to bioremediation, *Curr Opin Biotechnol 19*, 579-589.

40. Saeidi, N., Wong, C. K., Lo, T. M., Nguyen, H. X., Ling, H., Leong, S. S., Poh, C. L., and Chang, M. W. (2011) Engineering microbes to sense and eradicate Pseudomonas aeruginosa, a human pathogen, *Mol Syst Biol 7*, 521.
41. Anderson, J. C., Clarke, E. J., Arkin, A. P., and Voigt, C. A. (2006) Environmentally controlled invasion of cancer cells by engineered bacteria, *J Mol Biol 355*, 619-627.
42. Aubel, D., and Fussenegger, M. (2010) Mammalian synthetic biology--from tools to therapies, *Bioessays 32*, 332-345.
43. Duan, F., Curtis, K. L., and March, J. C. (2008) Secretion of insulinotropic proteins by commensal bacteria: rewiring the gut to treat diabetes, *Appl Environ Microbiol 74*, 7437-7438.
44. Duan, F., and March, J. C. (2010) Engineered bacterial communication prevents Vibrio cholerae virulence in an infant mouse model, *Proc Natl Acad Sci U S A 107*, 11260-11264.
45. Rao, S., Hu, S., McHugh, L., Lueders, K., Henry, K., Zhao, Q., Fekete, R. A., Kar, S., Adhya, S., and Hamer, D. H. (2005) Toward a live microbial microbicide for HIV: commensal bacteria secreting an HIV fusion inhibitor peptide, *Proc Natl Acad Sci U S A 102*, 11993-11998.
46. Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W., and Remaut, E. (2000) Treatment of murine colitis by Lactococcus lactis secreting interleukin-10, *Science 289*, 1352-1355.
47. Vandenbroucke, K., de Haard, H., Beirnaert, E., Dreier, T., Lauwereys, M., Huyck, L., Van Huysse, J., Demetter, P., Steidler, L., Remaut, E., Cuvelier, C., and Rottiers, P. (2010) Orally administered L. lactis secreting an anti-TNF Nanobody demonstrate efficacy in chronic colitis, *Mucosal Immunol 3*, 49-56.
48. Westwater, C., Kasman, L. M., Schofield, D. A., Werner, P. A., Dolan, J. W., Schmidt, M. G., and Norris, J. S. (2003) Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for treatment of bacterial infections, *Antimicrob Agents Chemother 47*, 1301-1307.

49. Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R., and Benenson, Y. (2011) Multi-input RNAi-based logic circuit for identification of specific cancer cells, *Science 333*, 1307-1311.

50. Brentjens, R. J., Davila, M. L., Riviere, I., Park, J., Wang, X., Cowell, L. G., Bartido, S., Stefanski, J., Taylor, C., Olszewska, M., Borquez-Ojeda, O., Qu, J., Wasielewska, T., He, Q., Bernal, Y., Rijo, I. V., Hedvat, C., Kobos, R., Curran, K., Steinherz, P., Jurcic, J., Rosenblat, T., Maslak, P., Frattini, M., and Sadelain, M. (2013) CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia, *Science translational medicine 5*, 177ra138.

51. Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J. M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H. B., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E. G., Wang, J., Guarner, F., Pedersen, O., de Vos, W. M., Brunak, S., Dore, J., Antolin, M., Artiguenave, F., Blottiere, H. M., Almeida, M., Brechot, C., Cara, C., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Foerstner, K. U., Friss, C., van de Guchte, M., Guedon, E., Haimet, F., Huber, W., van Hylckama-Vlieg, J., Jamet, A., Juste, C., Kaci, G., Knol, J., Lakhdari, O., Layec, S., Le Roux, K., Maguin, E., Merieux, A., Melo Minardi, R., M'Rini, C., Muller, J., Oozeer, R., Parkhill, J., Renault, P., Rescigno, M., Sanchez, N., Sunagawa, S., Torrejon, A., Turner, K., Vandemeulebrouck, G., Varela, E., Winogradsky, Y., Zeller, G., Weissenbach, J., Ehrlich, S. D., and Bork, P. (2011) Enterotypes of the human gut microbiome, *Nature 473*, 174-180.

52. Kruis, W., Fric, P., Pokrotnieks, J., Lukas, M., Fixa, B., Kascak, M., Kamm, M. A., Weismueller, J., Beglinger, C., Stolte, M., Wolff, C., and Schulze, J. (2004) Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine, *Gut 53*, 1617-1623.

53. Steidler, L., Rottiers, P., and Coulie, B. (2009) Actobiotics as a novel method for cytokine delivery, *Ann N Y Acad Sci 1182*, 135-145.
54. Jacob, F., and Monod, J. (1961) Genetic regulatory mechanisms in the synthesis of proteins, *J Mol Biol 3*, 318-356.
55. Barabasi, A. L., and Oltvai, Z. N. (2004) Network biology: understanding the cell's functional organization, *Nat Rev Genet 5*, 101-113.
56. Benner, S. A., and Sismour, A. M. (2005) Synthetic biology, *Nat Rev Genet 6*, 533-543.
57. Clancy, K., and Voigt, C. A. (2010) Programming cells: towards an automated 'Genetic Compiler', *Curr Opin Biotechnol 21*, 572-581.
58. Wieland, M., and Fussenegger, M. (2012) Engineering molecular circuits using synthetic biology in mammalian cells, *Annu Rev Chem Biomol Eng 3*, 209-234.
59. Prindle, A., Selimkhanov, J., Danino, T., Samayoa, P., Goldberg, A., Bhatia, S. N., and Hasty, J. (2012) Genetic Circuits in Salmonella typhimurium, *ACS Synth Biol 1*, 458-464.
60. Nandagopal, N., and Elowitz, M. B. (2011) Synthetic biology: integrated gene circuits, *Science 333*, 1244-1248.
61. Glass, J. I., Assad-Garcia, N., Alperovich, N., Yooseph, S., Lewis, M. R., Maruf, M., Hutchison, C. A., 3rd, Smith, H. O., and Venter, J. C. (2006) Essential genes of a minimal bacterium, *Proc Natl Acad Sci U S A 103*, 425-430.
62. Cagatay, T., Turcotte, M., Elowitz, M. B., Garcia-Ojalvo, J., and Suel, G. M. (2009) Architecture-dependent noise discriminates functionally analogous differentiation circuits, *Cell 139*, 512-522.
63. Nuyts, S., Van Mellaert, L., Theys, J., Landuyt, W., Lambin, P., and Anne, J. (2002) Clostridium spores for tumor-specific drug delivery, *Anti-cancer drugs 13*, 115-125.
64. Horikoshi, K. (2008) Past, present and future of extremophiles, *Extremophiles : life under extreme conditions 12*, 1-2.
65. Benson, D. E., Haddy, A. E., and Hellinga, H. W. (2002) Converting a maltose receptor into a nascent binuclear copper oxygenase by computational design, *Biochemistry 41*, 3262-3269.

66. Looger, L. L., Dwyer, M. A., Smith, J. J., and Hellinga, H. W. (2003) Computational design of receptor and sensor proteins with novel functions, *Nature 423*, 185-190.
67. Boyle, P. M., and Silver, P. A. (2009) Harnessing nature's toolbox: regulatory elements for synthetic biology, *J R Soc Interface 6 Suppl 4*, S535-546.
68. Salis, H. M., Mirsky, E. A., and Voigt, C. A. (2009) Automated design of synthetic ribosome binding sites to control protein expression, *Nat Biotechnol 27*, 946-950.
69. Levskaya, A., Chevalier, A. A., Tabor, J. J., Simpson, Z. B., Lavery, L. A., Levy, M., Davidson, E. A., Scouras, A., Ellington, A. D., Marcotte, E. M., and Voigt, C. A. (2005) Synthetic biology: engineering Escherichia coli to see light, *Nature 438*, 441-442.
70. Ellis, T., Wang, X., and Collins, J. J. (2009) Diversity-based, model-guided construction of synthetic gene networks with predicted functions, *Nat Biotechnol 27*, 465-471.
71. Villalobos, A., Ness, J. E., Gustafsson, C., Minshull, J., and Govindarajan, S. (2006) Gene Designer: a synthetic biology tool for constructing artificial DNA segments, *BMC Bioinformatics 7*, 285.
72. Archer, E., Robinson, A., and Süel, G. (2012) Engineered E. coli That Detect and Respond to Gut Inflammation through Nitric Oxide Sensing, *ACS Synth. Biol. 1*, 451–457.
73. Allert, M., Rizk, S. S., Looger, L. L., and Hellinga, H. W. (2004) Computational design of receptors for an organophosphate surrogate of the nerve agent soman, *Proc Natl Acad Sci U S A 101*, 7907-7912.
74. Marvin, J. S., and Hellinga, H. W. (2001) Conversion of a maltose receptor into a zinc biosensor by computational design, *Proc Natl Acad Sci U S A 98*, 4955-4960.
75. Andrianantoandro, E., Basu, S., Karig, D. K., and Weiss, R. (2006) Synthetic biology: new engineering rules for an emerging discipline, *Mol Syst Biol 2*, 2006 0028.
76. Voigt, C. A. (2006) Genetic parts to program bacteria, *Curr Opin Biotechnol 17*, 548-557.
77. Gibson, D. G., Benders, G. A., Andrews-Pfannkoch, C., Denisova, E. A., Baden-Tillson, H., Zaveri, J., Stockwell, T. B., Brownley, A., Thomas, D. W., Algire, M. A., Merryman,

C., Young, L., Noskov, V. N., Glass, J. I., Venter, J. C., Hutchison, C. A., 3rd, and Smith, H. O. (2008) Complete chemical synthesis, assembly, and cloning of a Mycoplasma genitalium genome, *Science 319*, 1215-1220.

78. Hillson, N. J., Rosengarten, R. D., and Keasling, J. D. (2012) j5 DNA assembly design automation software, *ACS Synth Biol 1*, 14-21.
79. Hasty, J., McMillen, D., and Collins, J. J. (2002) Engineered gene circuits, *Nature 420*, 224-230.
80. Randall, A., Guye, P., Gupta, S., Duportet, X., and Weiss, R. (2011) Design and connection of robust genetic circuits, *Methods Enzymol 497*, 159-186.
81. Gu, B., and Zhu, W. G. (2012) Surf the post-translational modification network of p53 regulation, *Int J Biol Sci 8*, 672-684.
82. Mangan, S., and Alon, U. (2003) Structure and function of the feed-forward loop network motif, *Proc Natl Acad Sci U S A 100*, 11980-11985.
83. Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., and Alon, U. (2002) Network motifs: simple building blocks of complex networks, *Science 298*, 824-827.
84. Yeh, B. J., and Lim, W. A. (2007) Synthetic biology: lessons from the history of synthetic organic chemistry, *Nature chemical biology 3*, 521-525.
85. Sprinzak, D., and Elowitz, M. B. (2005) Reconstruction of genetic circuits, *Nature 438*, 443-448.
86. Kittisopikul, M., and Suel, G. M. (2010) Biological role of noise encoded in a genetic network motif, *Proc Natl Acad Sci U S A 107*, 13300-13305.
87. Locke, J. C., Young, J. W., Fontes, M., Hernandez Jimenez, M. J., and Elowitz, M. B. (2011) Stochastic pulse regulation in bacterial stress response, *Science 334*, 366-369.
88. Elowitz, M. B., Levine, A. J., Siggia, E. D., and Swain, P. S. (2002) Stochastic gene expression in a single cell, *Science 297*, 1183-1186.
89. Hanggi, P. (2002) Stochastic resonance in biology. How noise can enhance detection of weak signals and help improve biological information processing, *Chemphyschem 3*, 285-290.

90. Suel, G. M., Kulkarni, R. P., Dworkin, J., Garcia-Ojalvo, J., and Elowitz, M. B. (2007) Tunability and noise dependence in differentiation dynamics, *Science 315*, 1716-1719.
91. Elowitz, M. B., and Leibler, S. (2000) A synthetic oscillatory network of transcriptional regulators, *Nature 403*, 335-338.
92. Danino, T., Mondragon-Palomino, O., Tsimring, L., and Hasty, J. (2010) A synchronized quorum of genetic clocks, *Nature 463*, 326-330.
93. Gardner, T. S., Cantor, C. R., and Collins, J. J. (2000) Construction of a genetic toggle switch in Escherichia coli, *Nature 403*, 339-342.
94. Tamsir, A., Tabor, J. J., and Voigt, C. A. (2011) Robust multicellular computing using genetically encoded NOR gates and chemical 'wires', *Nature 469*, 212-215.
95. Anderson, J. C., Voigt, C. A., and Arkin, A. P. (2007) Environmental signal integration by a modular AND gate, *Mol Syst Biol 3*, 133.
96. Ham, T. S., Lee, S. K., Keasling, J. D., and Arkin, A. P. (2008) Design and construction of a double inversion recombination switch for heritable sequential genetic memory, *PLoS One 3*, e2815.
97. Ham, T. S., Lee, S. K., Keasling, J. D., and Arkin, A. P. (2006) A tightly regulated inducible expression system utilizing the fim inversion recombination switch, *Biotechnol Bioeng 94*, 1-4.
98. Basu, S., Gerchman, Y., Collins, C. H., Arnold, F. H., and Weiss, R. (2005) A synthetic multicellular system for programmed pattern formation, *Nature 434*, 1130-1134.
99. Tabor, J. J., Salis, H. M., Simpson, Z. B., Chevalier, A. A., Levskaya, A., Marcotte, E. M., Voigt, C. A., and Ellington, A. D. (2009) A synthetic genetic edge detection program, *Cell 137*, 1272-1281.
100. Schmidt, D. R., Holmstrom, S. R., Fon Tacer, K., Bookout, A. L., Kliewer, S. A., and Mangelsdorf, D. J. (2010) Regulation of bile acid synthesis by fat-soluble vitamins A and D, *J Biol Chem 285*, 14486-14494.
101. Bookout, A. L., and Mangelsdorf, D. J. (2003) Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways, *Nucl Recept Signal 1*, e012.

102. Klemm, P. (1986) 2 Regulatory Fim Genes, Fimb and Fime, Control the Phase Variation of Type-1 Fimbriae in Escherichia-Coli, *Embo J 5*, 1389-1393.
103. McClain, M. S., Blomfield, I. C., and Eisenstein, B. I. (1991) Roles of fimB and fimE in site-specific DNA inversion associated with phase variation of type 1 fimbriae in Escherichia coli, *Journal of Bacteriology 173*, 5308-5314.
104. Holden, N., Blomfield, I. C., Uhlin, B. E., Totsika, M., Kulasekara, D. H., and Gally, D. L. (2007) Comparative analysis of FimB and FimE recombinase activity, *Microbiology 153*, 4138-4149.
105. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter, *Journal of Bacteriology 177*, 4121-4130.
106. Sektas, M., Hasan, N., and Szybalski, W. (2001) Expression plasmid with a very tight two-step control: Int/att-mediated gene inversion with respect to the stationary promoter, *Gene 267*, 213-220.
107. Kimura, H., Miura, S., Shigematsu, T., Ohkubo, N., Tsuzuki, Y., Kurose, I., Higuchi, H., Akiba, Y., Hokari, R., Hirokawa, M., Serizawa, H., and Ishii, H. (1997) Increased nitric oxide production and inducible nitric oxide synthase activity in colonic mucosa of patients with active ulcerative colitis and Crohn's disease, *Dig Dis Sci 42*, 1047-1054.
108. Leonard, N., Bishop, A. E., Polak, J. M., and Talbot, I. C. (1998) Expression of nitric oxide synthase in inflammatory bowel disease is not affected by corticosteroid treatment, *J Clin Pathol 51*, 750-753.
109. Ljung, T., Lundberg, S., Varsanyi, M., Johansson, C., Schmidt, P. T., Herulf, M., Lundberg, J. O., and Hellstrom, P. M. (2006) Rectal nitric oxide as biomarker in the treatment of inflammatory bowel disease: responders versus nonresponders, *World J Gastroenterol 12*, 3386-3392.
110. Rodionov, D. A., Dubchak, I. L., Arkin, A. P., Alm, E. J., and Gelfand, M. S. (2005) Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks, *PLoS Comput Biol 1*, e55.

111. Tucker, N. P., D'Autreaux, B., Yousafzai, F. K., Fairhurst, S. A., Spiro, S., and Dixon, R. (2008) Analysis of the nitric oxide-sensing non-heme iron center in the NorR regulatory protein, *J Biol Chem 283*, 908-918.

112. Bush, M., Ghosh, T., Tucker, N., Zhang, X., and Dixon, R. (2011) Transcriptional regulation by the dedicated nitric oxide sensor, NorR: a route towards NO detoxification, *Biochem Soc Trans 39*, 289-293.

113. Bush, M., Ghosh, T., Tucker, N., Zhang, X., and Dixon, R. (2010) Nitric oxide-responsive interdomain regulation targets the sigma54-interaction surface in the enhancer binding protein NorR, *Mol Microbiol 77*, 1278-1288.

114. Gardner, A. M., Gessner, C. R., and Gardner, P. R. (2003) Regulation of the nitric oxide reduction operon (norRVW) in Escherichia coli. Role of NorR and sigma54 in the nitric oxide stress response, *J Biol Chem 278*, 10081-10086.

115. Tucker, N. P., D'Autreaux, B., Spiro, S., and Dixon, R. (2006) Mechanism of transcriptional regulation by the Escherichia coli nitric oxide sensor NorR, *Biochem Soc Trans 34*, 191-194.

116. Tucker, N. P., D'Autreaux, B., Studholme, D. J., Spiro, S., and Dixon, R. (2004) DNA binding activity of the Escherichia coli nitric oxide sensor NorR suggests a conserved target sequence in diverse proteobacteria, *J Bacteriol 186*, 6656-6660.

117. Tucker, N. P., Ghosh, T., Bush, M., Zhang, X., and Dixon, R. (2010) Essential roles of three enhancer sites in sigma54-dependent transcription by the nitric oxide sensing regulatory protein NorR, *Nucleic Acids Res 38*, 1182-1194.

118. Suel, G. (2011) Use of fluorescence microscopy to analyze genetic circuit dynamics, *Methods Enzymol 497*, 275-293.

119. Beck, P. L., Xavier, R., Wong, J., Ezedi, I., Mashimo, H., Mizoguchi, A., Mizoguchi, E., Bhan, A. K., and Podolsky, D. K. (2004) Paradoxical roles of different nitric oxide synthase isoforms in colonic injury, *American journal of physiology. Gastrointestinal and liver physiology 286*, G137-147.

120. Rosenfeld, N., Young, J. W., Alon, U., Swain, P. S., and Elowitz, M. B. (2007) Accurate prediction of gene feedback circuit behavior from component properties, *Mol Syst Biol 3*, 143.

121. Walters, M., and Sperandio, V. (2006) Quorum sensing in Escherichia coli and Salmonella, *International journal of medical microbiology : IJMM 296*, 125-131.

122. Lesouhaitier, O., Veron, W., Chapalain, A., Madi, A., Blier, A. S., Dagorn, A., Connil, N., Chevalier, S., Orange, N., and Feuilloley, M. (2009) Gram-negative bacterial sensors for eukaryotic signal molecules, *Sensors 9*, 6967-6990.