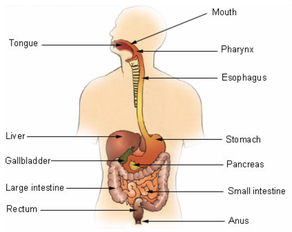
Engineering Multi-Functional Probiotic Bacteria

Caltech 2008 iGEM Team

Abstract:

The human gut houses a diverse collection of microorganisms, with important implications for the health and welfare of the host. We aim to engineer a member of this microbial community to provide innovative medical treatments. Our work focuses on four main areas: (1) pathogen defense either by expression of pathogen-specific bacteriophage or by targeted bursts of reactive oxygen species; (2) prevention of birth defects by folate over-expression and delivery; (3) treatment of lactose intolerance by cleaving lactose to allow absorption in the large intestine; and (4) regulation of these three treatment functions to produce renewable subpopulations specialized for each function. Our research demonstrates that synthetic biology techniques can be used to modify naturally occurring microbial communities for applications in biomedicine and biotechnology.

Microbial Flora:



The human intestinal track is a perfect environment for bacteria. It is a 37°C mobile incubator with a constant stream of food. While bacteria are present in all parts of the intestinal track downstream of the stomach, the majority of those bacteria reside in the large intestine. There are approximately 10^{12} bacterial/mL in the large intestinal lumen, composed of between 500-1000 different species of bacteria.

Most of the bacteria in our gut have yet to be characterized because they are difficult to culture, owing to their sensitivity to oxygen. However, several important species are known. One such bacterium, *Bacteroides fragilis*, plays an important role in proper development of the immune system and in controlling intestinal inflammation. Several species are known to produce vitamin K, an important cofactor in blood clotting which humans are unable to synthesize. Other species play an important role in pathogen defense by out-competing pathogenic bacteria.

For these and many other reasons, maintenance of the gut flora has become an important medical frontier, as evidenced by the recent interest in prebiotics (supplements to encourage the growth of 'good' microbes) and probiotics (direct supplementation with beneficial microbes). Nissle 1917 is a commercially available non-pathogenic, probiotic strain of *E. coli*. It has been successfully used to treat gastrointestinal disorders including colitis and intestinal bowel disease and shows little immunostimulatory activity. Engineered versions of the Nissle 1917 strain have been developed as anti-HIV[1] and anti-cholera agents. The ability of Nissle 1917 to efficiently colonize the gut without provoking an inflammatory response[2] makes it an ideal chassis for *in situ* applications in biomedicine and biotechnology.

Engineering Opportunities:

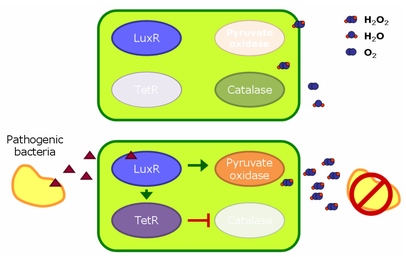
Natural gut flora do many important things for their human hosts. However, there are many potential applications that are not being served by existing microbes. Pathogen prevention, for instance, is generally only limited to passive competitive inhibition, rather than active pathogen detection and destruction. Similarly, while microbes provide sufficient vitamin K for human needs, there are many other vitamins they could be providing but are not. Finally, these microbes offer the unrealized potential for *in situ* delivery of therapeutic proteins in the intestine.

Biosafety:

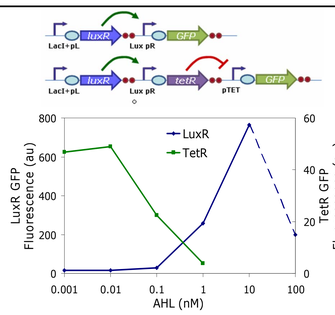
The safety of the unmodified chassis, Nissle 1917, has been consistently demonstrated across a range of conditions[2]. However, our engineered modifications might introduce new risks. Specific issues for each subproject are described below.

- Oxidative Burst: The concentrations of H_2O_2 produced by our engineered system are comparable to the concentrations used to preserve milk (~1 mM). However, the effects of persistent *in situ* H_2O_2 production in the human gut will need to be investigated.
- Phage Pathogen Defense: Phage which target pathogenic species may also lyse closely related, non-pathogenic species, disrupting the gut composition. Lysogeny will also need to be tightly controlled, since lysogeny can often induce pathogenesis.
- Lactose Intolerance: Lactase is 'Generally Recognized as Safe' by the FDA, so we expect few safety concerns.
- Folate Production: Neither folate nor its major intermediate, PABA, are known toxicity risks. However, little research has been done on the effects of persistent PABA exposure.
- Population Variation: This subproject regulates the other projects, and therefore introduces few new risks into the system. Loss of function mutations are the most likely failure mode and pose little risk to the host.

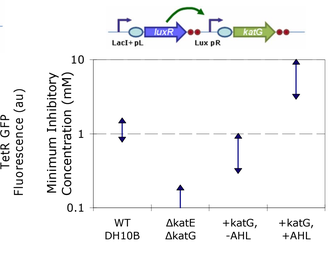
Oxidative Burst:



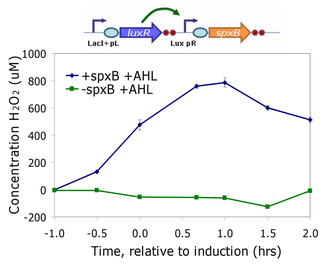
Specialized white blood cells called neutrophils defend us from illness by killing bacteria using hydrogen peroxide. However, pathogens of the human large intestine are able to cause serious illness while being sheltered from neutrophils. We engineered a strain of *E. coli* that is able to mimic a neutrophil by producing cytotoxic amounts of hydrogen peroxide in a controlled, inducible manner. Our engineered *E. coli* use the transcriptional activator LuxR to detect the presence of AHL molecules secreted by invading pathogens. LuxR activates production of the pyruvate oxidase of *S. pneumoniae*, which produces large amounts of hydrogen peroxide by oxidizing pyruvate. The engineered *E. coli* is capable of killing certain strains of antibiotic resistant E. coli within six hours. AHL-repressed production of a LVA-tagged catalase, KatG, protects the cell from peroxide until a pathogen is detected and peroxide production is induced.



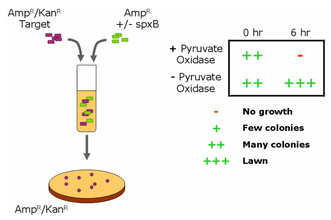
Characterization of LuxR inducer and TetR inverter by flow cytometry. The LuxR inducer, shown in blue, corresponds to SpxB expression; the TetR inverter, in green, will be used for KatG. At 100 nM AHL, cells grew very poorly.



Minimum inhibitory concentration (MICs) ranges of native and engineered strains. Upper arrow shows lowest concentration which inhibited growth; lower arrow shows highest concentration which did not inhibit growth. Expression of KatG-LVA increases the MIC of a catalase knockout above wildtype levels.

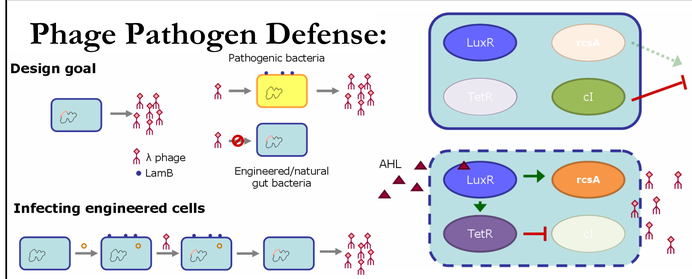


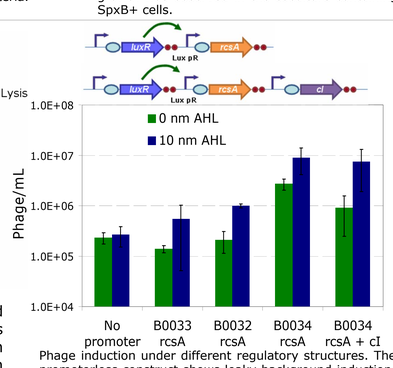
H_2O_2 production by SpxB in a catalase/peroxidase mutant grown in SOC. 800uM H_2O_2 is bacteriostatic to many pathogenic bacteria.



Coculture inhibition assay. Cells expressing SpxB were mixed with target cells. After 6 hours, no growth was observed in the coculture containing SpxB+ cells.

Phage Pathogen Defense:



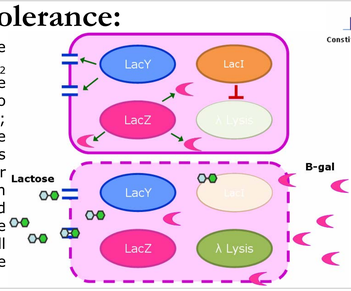


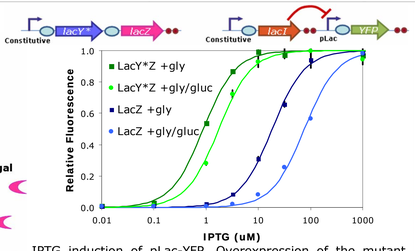
Bacteriophage are able to specifically lyse target strains of bacteria. We have introduced a bacteriophage (λ) into a bacterium which is not normally susceptible (lamB- *E. coli*) as a model system for expression of a non-native bacteriophage (P22 from *Salmonella*) in our engineered *E. coli*. Lysis will be triggered by the population maintenance system described later, here replaced by an AHL receiver. Lysis is then controlled by expression of a specific inducer, RscA, and decreased expression of the cI repressor.

Phage induction under different regulatory structures. The promoterless construct shows leaky background induction. Varying the RBS in front of *rcsA* changed the basal induction level but maintained the same dynamic range. Constitutive cI expression increased the dynamic range.

Treatment of Lactose Intolerance:

Lactose intolerance is caused by excess lactose in the large intestine, leading to CH_4 and H_2 production and an osmotic imbalance. We propose to produce β-galactosidase *in situ* to cleave the lactose into glucose and galactose; these monosaccharides can be absorbed by the host. β-gal will be constitutively expressed, as will a mutant form of the lactose transporter which is no longer inhibited by glucose. When lactose is present in the gut, it will be imported by the mutant LacY and de-repress the bacteriophage λ lysis cassette. Lysis will release β-gal into the gut, where it can cleave the available lactose.

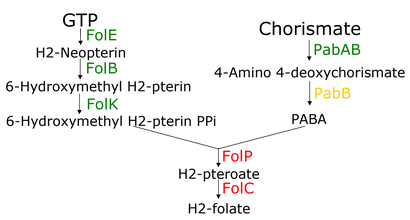




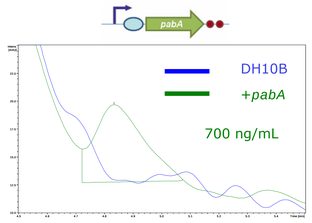
IPTG induction of pLac-YFP. Overexpression of the mutant LacY raises the IPTG sensitivity and reduces the catabolite repression from glucose.

Folate Production:

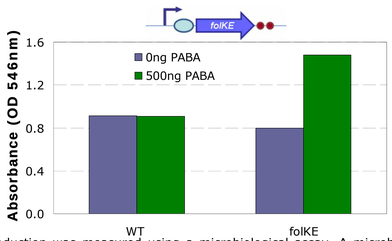
Folate is an essential vitamin, with particular importance during pregnancy. We have engineered *E. coli* to increase folate synthesis by inserting the *folKE* and *folB* genes from *Lactobacillus lactis*. Next we increased the biosynthesis of a key synthetic intermediate, PABA, by overexpressing the *pabA* gene from *L. lactis*. This system can be used to provide bioavailable folate *in situ* in the human gut.

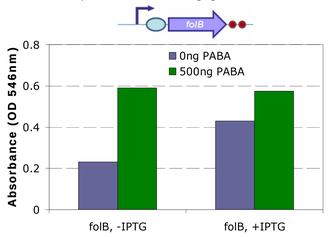


Folate biosynthetic pathway. FolKE, FolB, and PabA have been tested. PabB was cloned, but not characterized. Efforts to clone FolP and FolC were unsuccessful.



PABA was detected using high performance liquid chromatography (HPLC). PABA elutes at 4.8 minutes. PabA expression increased PABA production from negligible levels to 700 ng/mL.

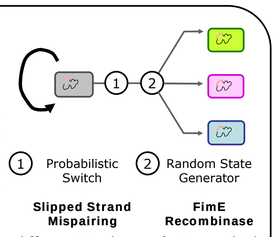




Folate production was measured using a microbiological assay. A microbe was used which displayed folate-limited growth; increased absorbance indicates increased folate in the initial sample. FolKE produced more folate than a control, but only when external PABA was added.

As with FolKE, folate production was measured using a microbiological assay. Increasing the FolB copy number (with IPTG) increased folate production, as did addition of PABA. However, addition of both IPTG and PABA was no better than PABA alone, suggesting the presence of a bottleneck at FolP.

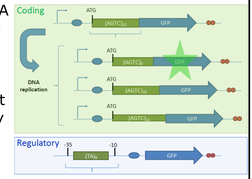
Population Maintenance:



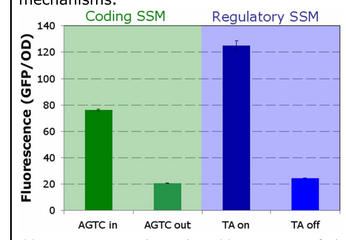
Differentiation can be used to combine multiple non-compatible functions into a single cell line. In this case, only one function will be turned on in each cell at any time, but the cell population will express the entire set of functions. In such a system, cells start off in an undifferentiated state. As undifferentiated cells replicate, their offspring can stay in the current state or switch into one of the mutually-exclusive states. As long as cells divide faster than they differentiate, the reproduction of undifferentiated cells maintains the undifferentiated population, which ensures that the entire cell line is kept alive. Thus, fatal and mutually-exclusive functions can be incorporated into cell lines by utilizing cellular differentiation.

It is also important to have cells differentiate at random times. Having asynchrony ensures that a large portion of the population will not differentiate at once. Doing so might leave too few undifferentiated cells to maintain the population. In addition, if the environment the cells are in continually changes, cells may differentiate into a particular state that is incompatible with the current environment. For example, that particular function may be useless in the current environment, or performing that function in such an environment may lead to the cell's death. As such, spreading out the time during which cells differentiate increases the chances that an individual cell committed to a state finds itself in a hospitable environment. Thus, both random state and random time are needed for differentiation.

Slipped-Strand Mispairing:

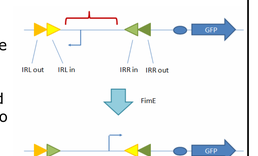


When a DNA polymerase copies a long repetitive sequence, it occasionally slips and inserts or deletes one repeat, a phenomenon known as slipped-strand mispairing (SSM). If this repetitive region is placed in the protein coding sequence or between the -10 and -35 regions of a promoter, SSM can lead to changes in gene expression. We have implemented an example of each of these mechanisms.

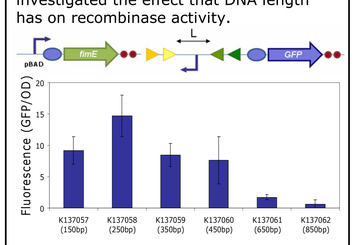


SSM activity. For the coding SSM, repeats of the tetranucleotide AGTC were placed immediately after the start codon. 9 repeats (AGTC in), yielding an in-frame sequence, showed significantly higher fluorescence than an out of frame sequence of 10 repeats (AGTC out). Similarly, the dinucleotide TA was inserted between the -10 and -35 regions of a constitutive promoter. 10 repeats (TA on) gave ideal spacing and higher expression than 9 repeats (TA off)

FimE Recombinase:



The FimE recombinase inverts a region of DNA located between two inverted repeats (IRs). This protein will be used to choose a random state for the differentiated cell. We first investigated the effect that DNA length has on recombinase activity.

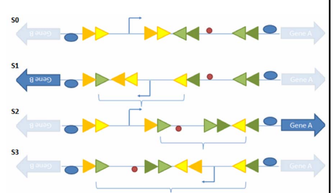


Effect of IR spacing on FimE activity. FimE was used to invert a region of DNA containing a strong constitutive promoter (R0040). When inverted, the promoter activated GFP expression. Maximum fluorescence was observed at 250bp, indicating a balance between enthalpic and entropic effects.

Having shown that we can tune the FimE recombination frequency, our next step will be to test whether the protein can randomly recombine between one right repeat (IRR) and two possible left repeats (IRLs). Doing so will allow the cell to randomly select one of several differentiated states.

System Integration:

First, FimE will be placed under the control of an SSM mechanism described above. Polymerase slipping will lead to FimE expression and therefore differentiation. FimE production will then be used to drive differentiation using the construct shown to the left. Folate production is the default state. In state S1 the promoter is inverted, Gene B (LuxR) is expressed and the cell is in the oxidative burst phenotype. In state S2 the transcriptional terminator is moved to the opposite strand, Gene A (LacYZ) is expressed, and the cell is committed to the treatment of lactose intolerance. In the final state the entire region inverts and the cells differentiate but remain in the folate-producing state. A separate SSM mechanism will be used to induce bacteriophage production.



FimE differentiation construct. The undifferentiated state is shown at the top. The red circle is a unidirectional transcriptional terminator. The IRL and IRR at the center of the construct are too close to allow recombination. In each differentiated state, S1-S3, the bracket indicates the region inverted by FimE.

Acknowledgements:

Funding:
Caltech SURF, Amgen Scholars, Howard Hughes Medical Institute
Materials:
Professor Jim Imlay (JI377), Dr. Sankar Adhya (λcm), Dr. Sidney Cox (pLac)

References:

1) Rao S, *et al*. **Toward a live microbial microbicide for HIV: commensal bacteria secreting an HIV fusion inhibitor peptide**. *PNAS* 2005 Aug 23; 102(34) 11993-8.
2) Westendorf AM, *et al*. **Escherichia coli NISSLE 1917: a safe carrier for therapeutic molecules**. *FEMS Imm Med Microbiol* 2005 Mar 1; 43(3) 373-84.