Cling-*E. coli*: Bacteria on target

A system for targeting bacteria to a specific substrate and effecting a cellular response

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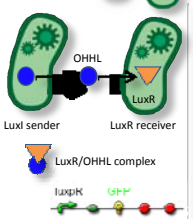
Ellenor Brown, Stephanie Lo, Alexander Pickett, Sammy Sambu, Kevin Shee, Perry Tsai, Shaunak Vankudre, George Xu

Quorum Sensing

The motivation for this part of the project was to effect downstream activity after *E. coli* bind to a particular substrate, using the luxI/luxR quorum-sensing system from *Vibrio fischeri*, which would turn on after the bacteria localize to the target.

Lux quorum-sensing works like a system of senders and receivers. In the sender, LuxI codes for a protein that catalyzes the synthesis of 3-oxo-hexanyl homoserine lactone (OHHL) which can diffuse freely out of the sender cell and into other cells. In the receiver, LuxR encodes for a non-permeating protein which, when bound to OHHL, upregulates the lux pR promoter. This only occurs at a high enough OHHL concentrations, so a certain concentration of cells (quorum) is required.

Initial characterizations of the luxI/luxR system and quorum activity were made using GFP and RFP reporters. Two approaches were taken to quorum sensing. (1) A luxI/luxR production system in one cell acting as both sender and receiver would be simpler, but it's possible that the cells might self-induce. (2) luxI and luxR production in separate cells would ensure no self-induction, but it requires monitoring two populations of cells.



Methods

We used a sender construct with LuxI and RFP under one constitutive P_{tet} promoter, and a receiver construct with LuxR under constitutive P_{tet} promoter and GFP under lux pR promoters. We transformed these constructs into *E. coli* to create constitutive-RFP constitutive-sender cells and inducible-GFP constitutive-receiver cells.

To characterize one-cell quorum activity, a non-RFP constitutive-sender construct and an inducible-GFP constitutive-receiver construct were assembled onto a single plasmid and transformed into *E. coli*. An overnight culture was diluted and grown to OD 0.3, rediluted and grown to OD 0.3, etc. and fluorescence was detected after each dilution.

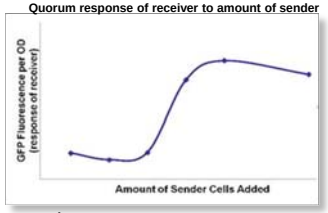
To characterize two-cell quorum activity, constitutive-RFP senders were mixed with inducible-GFP receivers. Fluorescence and OD600 readings were taken every 15 minutes during incubation at 37 degrees Celsius.

Results

In the one-cell system, we found that the overnight culture exhibited high GFP fluorescence, but with each successive dilution, the fluorescence decreased to a level comparable with no-GFP cells. When the culture was allowed to grow past OD 0.3, the fluorescence increased again at around OD 0.6.

In the two-cell system, we found that at a specific concentration of sender cells added to a mixed culture, the GFP fluorescence per OD (per cell) in receiver cells increased greatly.

Quorum response of receiver to amount of sender

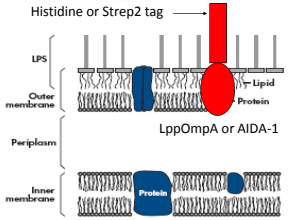


Conclusion and Future Plans

We have constructed both one-cell and two-cell models of quorum-sensing activity. We determined that the one-cell model was not self-inducing and does exhibit a quorum response, making it a better candidate for future quorum-sensing applications. We determined that quorum-sensing activity can also be divided between two cells, one sender and one receiver. We will continue to characterize the one-cell system.

Bacterial Targeting

The motivation for this part of the project was to **engineer bacteria to adhere to targets with a high degree of specificity**. Initial targeting was done by displaying **histidine** and **strep2 tags** on the E. coli surface via fusion with the proteins **LppOmpA** and **AIDA-1**, and screens were performed with binding to their known nickel and streptavidin targets, respectively. After characterization and high enrichment with these known substrates, random libraries were inserted into LppOmpA and AIDA-1 constructs for screening peptides with affinity for novel targets. As we proceed with this experiment, we hope to characterize sequences that have specificity for calmodulin and EGF.



Methods

Bacteria were engineered with histidine and strep2 tags displayed on the *E. coli* surface via fusion with LppOmpA and AIDA-1 vehicles: LppOmpA with C-terminus insertion, LppOmpA with a loop 1 insertion, and AIDA-1 with a N-terminus insertion.

In order to test the tags and their ability to bind to specific antibodies and beads (nickel/streptavidin), two cell sorting assays were performed to ascertain the binding strength of the tagged cells against a background of untagged cells.

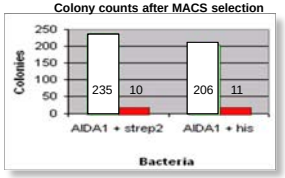
In Magnetic Activated Cell Sorting (MACS), cultures of white cells expressing histidine (nickel-targeting) or strep2 (streptavidin-targeting) tags on the surface were mixed with cultures of RFP-expressing non-tagged cells. The mixture was incubated with nickel- or streptavidin-coated magnetic beads, then run through a magnetic column, so that non-tagged cells would flow through, and bead-bound tagged cells would stick to the column. After removal of the magnet, the bound fraction of cells was eluted and spread on agar plates. After overnight incubation, the numbers of white and red colonies were counted.

In Fluorescence Activated Cell Sorting (FACS), we added anti-his and anti-strep2 fluorescent antibodies to the mixed cultures. The fluorescent fraction of cells was separated from the mixture with a flow cytometer and spread on agar plates. After overnight incubation, the numbers of white and red colonies were counted.

Results

We were able to construct LppOmpA and AIDA-1 constructs with histidine or strep2 tags. We significantly enriched histidine and strep2 tagged cells through MACS, as there were many more white colonies (from tagged cells) than red (from non-tagged) on the plates spread with bound fractions. Similar results were found with FACS as well (data not shown).

Colony counts after MACS selection



Conclusions and Future Plans

LppOmpA and AIDA-1 have proven to be effective vehicles for expressing tags on the surface of *E. coli* to bind specific targets, as shown by enrichment of tagged cells through MACS and FACS. We plan to try out new peptides specific for other targets, such as calmodulin (CaM, a calcium binding protein). We will also explore using a random library to select for novel targeting peptides, by which you introduce fixed-length random nucleotide sequences into the construct, express the random tag, and select for peptides with affinity to your target. This has important medical implications since we may be able to target "microbial factories" to harmful toxins or microbes, or to various areas of the body.

Bringing Things Together

Methods

Cells were cotransformed with the constitutive-RFP constitutive-sender plasmid and the AIDA-strep2 construct plasmid. Then they were enriched by MACS with magnetic streptavidin beads against a background of non-tagged constitutive-GFP cells. The bound fraction of cells was eluted and spread on agar plates. After overnight incubation, the numbers of green and red colonies were counted.

The same strep2-tagged RFP-sender cells and non-tagged GFP cells were mixed with streptavidin beads, and then observed under a microscope for RFP and GFP fluorescence.

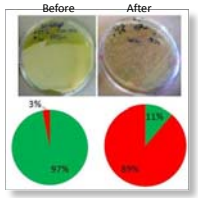
A lawn of inducible-GFP constitutive-receiver cells was spread on an agar plates. An aliquot from the RFP-sender MACS elution was dropped in the center of the plate. The plate was incubated overnight.

Results

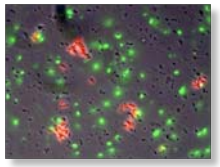
The same enrichment was observed with tagged RFP-sender cells as with the tagged white cells in "Bacterial Targeting." There was a increased percentage of red colonies (from RFP-sender cells) after MACS selection. Targeted localization can also be observed under the microscope as RFP-sender cells clump around streptavidin beads, while non-tagged GFP cells do not. Finally, the enriched tagged RFP-sender cells do produce OHHL, as there is a green circle (arrow 2) of GFP-induced receiver cells around the drop where enriched RFP-sender cells grew (1).

Conclusions and Future Plans

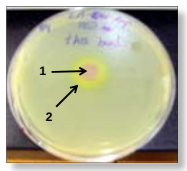
We have demonstrated that we can create bacteria that can both target and produce a quorum signal. We will need to characterize cell and bead concentrations required to produce a quorum response in tagged one-cell or two-cell systems.



Selection of AIDA-strep2/RFP-sender cells with streptavidin beads



Clumping of AIDA-strep2/RFP-sender cells around streptavidin beads



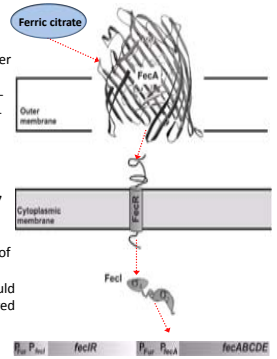
Evidence of OHHL quorum signal from AIDA-strep2/RFP-sender cells after selection with streptavidin beads

Fec Signal Transduction

The motivation for this project was to create a system of targeting and direct signal transduction/gene expression. The Fec system was chosen because it is the only known well-characterized signaling system with an outer membrane receptor, able to bind to extracellular targets. The Fec system receptor is the outer membrane protein FecA, whose wild-type ligand is dinuclear ferric citrate. When binding occurs in iron-limiting conditions, FecA activates the inner membrane protein FecR, which activates cytoplasmic sigma factor FecI; and FecI induces gene expression under the P_{fecA} promoter.

Structural papers detail the conformational changes that FecA undergoes when binding to ferric citrate. The alpha helix in loop 7 unravels, and the loop moves by up to 11 angstroms, and loop 8 moves up to 15 angstroms. The motion of these two loops closes over the ferric citrate. These large changes imply the importance of loops 7 and 8 for binding.

We propose inserting a tag into loop 7 of FecA such that it would bind to a target and potentially transduce a signal. We also explored a computational approach with the Maranas lab (Penn State) to produce sequences for binding a target, and a random-library approach to select for binding sequences for a target.



Methods

The constructs we used came from Volkmar Braun at the University of Tuebingen, Germany. He provided AA93 cells, strain of *E. coli* with the Fec system knocked out to isolate a re-engineered Fec system; a plasmid expressing GFP under P_{fecA} promoter; and the pLCIRA plasmid containing all the Fec system genes.

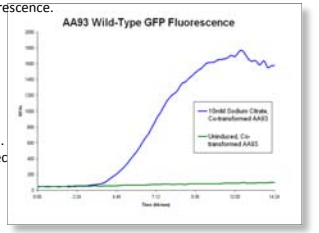
To test P_{fecA} induction, we transformed AA93 cells with the P_{fecA}-GFP and pLCIRA plasmids, and induced with sodium citrate. The Fec system is repressed via the P_{fur} repressor by free iron in LB. Sodium citrate was used instead of ferric citrate, so that the citrate could chelate free iron from the media without adding new iron. GFP fluorescence was detected over time with a plate reader.

Because pLCIRA is not well-characterized and the expression of the Fec system is controlled by its own P_{fecA} promoter, we thought it valuable to be able to control levels of FecA expression. We attempted to use a T7-regulated system by cloning the FecI, R, and A genes into a pColA duet vector, lysogenizing the AA93 cells into AA93(DE3) cells, and transforming with the P_{fecA}-GFP plasmid, so that Fec expression could be induced by IPTG/T7, and P_{fecA} induction could be assayed by GFP fluorescence.

Results

We found significant increases in GFP fluorescence with P_{fecA}-GFP / pLCIRA-transformed AA93 cells, after sodium citrate induction. Having tried different concentrations, we found that 10mM sodium citrate worked best.

The FecIRA/pColA system proved difficult to work with. Our cells had trouble surviving both leaky and induced Fec system expression. We believe this toxicity might be due to membrane disruption. So far, our assays have not yielded significant results.



Conclusions and Future Plans

We have confirmed that the P_{fecA}-GFP / pLCIRA system works in AA93 cells. Sodium citrate can effectively transduce a signal into the cell and upregulate P_{fecA}-GFP expression. We are still working on inserting tags (histidine and strep2) into loop 7 of FecA in an attempt to re-engineer FecA for targeting nickel and streptavidin, and potentially transducing a signal. If this is successful, we will explore the use of computers and/or random libraries to select for novel targeting/signaling sequences.

References

Quorum-sensing

Anderson JC, et al. Environmentally controlled invasion of cancer cells by engineered bacteria. J Mol Biol 2006 Jan 27; 355(4) 619-27.

Basu S, et al. Spatiotemporal control of gene expression with pulse-generating networks. Proc Natl Acad Sci U S A. 2004 Apr 27;101(17):6355-60.

Sayut DJ, et al. Construction and engineering of positive feedback loops. ACS Chem Biol. 2006 Dec 20;1(11):681-2.

Voigt CA. Genetic parts to program bacteria. Curr Opin Biotechnol 2006 Oct; 17(5) 548-57.

Bacterial targeting

Cheong I, et al. A bacterial protein enhances the release and efficacy of liposomal cancer drugs. Science. 2006 Nov 24;314(5803):1308-11.

Georgiou G, et al. Display of heterologous proteins on the surface of microorganisms: from the screening of combinatorial libraries to live recombinant vaccines. Nat Biotechnol. 1997 Jan;15(1):29-34. Review.

Jose J.Autodisplay: efficient bacterial surface display of recombinant proteins. Jose J. Appl Microbiol Biotechnol. 2006 Feb;69(6):607-14. Review.

Pasqualini R, et al. Organ targeting in vivo using phage display peptide libraries. Nature. 1996 Mar 28;380(6572):364-6.

Fec signal transduction

Braun V, et al. Gene regulation by transmembrane signaling. Biometals 2006 Apr; 19(2) 103-13.

Ferguson AD, et al. Signal transduction pathway of TonB-dependent transporters. Proc Natl Acad Sci U S A 2007 Jan 9; 104(2) 513-8

Ferguson AD, et al. Structural basis of gating by the outer membrane transporter FecA. Science 2002 Mar 1; 295(5560) 1715-9.

Sauter A and Braun V. Defined inactive FecA derivatives mutated in functional domains of the outer membrane transport and signaling protein of Escherichia coli K-12. J Bacteriol 2004 Aug; 186(16) 5303-10.

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