21st Century Antibiotics

- Gram Negative Antibiotic
- Gram Positive Antibiotic
- Plasmid Library
- Software
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We can engineer better Gram-negative antibiotics using synthetic biology

Problems with traditional antibiotics:
- wipe out normal gut flora
- taken after symptoms are obvious
- widespread resistance

Engineered probiotic
- activated when pathogen is detected
- specific for Gram-negative organisms
- works before symptoms
- novel therapeutic
Engineered probiotic detects and destroys Gram-negative pathogens

Essential Probiotic Components
- Cell chassis
- Protein secretion system
- Inducible toxin/antitoxin system

- Probiotic Detects Pathogen
- Probiotic Expresses Toxin and Antitoxin
- Probiotic Secretes Toxin
The Type VI Secretion System punctures the cell wall of target Gram-negative cells and injects proteins.
The Type VI Secretion System punctures the cell wall of target Gram-negative cells and injects proteins.
Fosmid containing T6SS does not express in \textit{E. coli}

- **Operon 1**
  - Promoter Region
  - 50kb Type VI Secretion Fosmid

- **Operon 2**

**Type VI Protein**

- \textit{P. aeruginosa} High copy # Low copy #
- \textit{E. Coli}

**Recombineering**

- T6SS Original Promoter T6SS
- T6SS T7 promoter T6SS
Engineered fosmid directs expression of the T6SS in *E. coli*

*P. aeruginosa*  
*E. coli* Uninduced  
*E. coli* Induced

Type VI Protein

Positive Control protein
Tse2Tsi2 is a toxin/antitoxin system recognized by the T6SS
Toxin/Antitoxin is induced in response to an example bacterially-produced small molecule.
Toxin/Antitoxin is induced in response to an example bacterially-produced small molecule.
Engineered probiotic detects and destroys Gram-negative pathogens

Essential Probiotic Components
✓ Expressed Type VI secretion in *E. coli*, fosmid available
✓ Characterized, BioBricked and submitted Tse2/Tsi2
21st Century Antibiotics

Gram Negative Antibiotic
✓ Characterized, BioBricked, and submitted Tse2/Tsi2
✓ Submitted Fosmid containing T6SS
✓ Expressed Type VI secretion in E. Coli

Gram Positive Antibiotic

Plasmid Library

Software
No Anthrax (live or dead) was used in this project!
Removing *Bacillus anthracis* protective coat makes it vulnerable to immune system.

Protected Anthrax

Decapsulation

Vulnerable Anthrax
Redesign CapD to break down protective coat

Transpeptidation (Native)

Hydrolysis ( Desired)
CapD auto-cleaves to reveal active site

CapD

Self Cleavage

Inactive CapD

N  C

Active CapD

C'  N'

* : Key Catalytic Residue
Avoiding auto-cleavage: redesigning CapD to produce enzyme in active state

Circular Permutation

Inactive CapD

Active CapD_CP

Genetically encoded peptide linker

*: Key Catalytic Residue
CapDCP is easy to express and quantify!

* : Key Catalytic Residue
CapDCP shows enzymatic activity!

<table>
<thead>
<tr>
<th>Transpeptidation</th>
<th>Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>kcat (hr⁻¹)</td>
<td>kcat (hr⁻¹)</td>
</tr>
<tr>
<td>Km (nM)</td>
<td>Km (nM)</td>
</tr>
<tr>
<td>CapD</td>
<td>27.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>22.1 ± 2.9</td>
</tr>
<tr>
<td>CapD_CP</td>
<td>67.2 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>23.2 ± 5.4</td>
</tr>
</tbody>
</table>

* : Key Catalytic Residue
Using FoldIt to design a better anthrax destroyer

FoldIt: Developed by the Baker and Popović Labs at the University of Washington
Designed, built, & tested 87 variants
Succeeded in altering the reaction specificity

Transpeptidation Relative to CapD_CP

Hydrolysis Relative to CapD_CP

Improved Variant

More Potent Therapeutic
Michaelis-Menten profile shows 10 fold switch in reaction specificity
21st Century Antibiotics

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**Gram Positive Antibiotic**
- Created and characterized 87 CapD_CP mutants
- BioBricked and submitted CapD, CapD_CP, and best variant
- Collaboration to test best variant on live Anthrax

**Plasmid Library**

**Software**
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**Plasmid Library**

**Software**
Protein Expression Vectors

**Inducible**
- F1 Origin
- LacI
- RBS
- E
- X
- R0011
- T7
- Antibiotic Resistance
- Copy #

**Constitutive**
- F1 Origin
- RBS
- E
- X
- J23100
- J12114
- Antibiotic Resistance
- Copy #
Protein Expression Vectors

Inducible

Constitutive

pSB3K3

pSB3K3 + f1 origin

3kb
2 kb
1.5 kb
psb1A3 expression cassettes with gfp
WikiDust makes interactive diagrams that link directly to the parts registry.

TinkerCell developed by the Sauro Lab at the University of Washington.
PartsRobot simplifies the BioBrick submission process.
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**Plasmid Library**
- 3 New BioBricks (F1 origin, new Lacl, new T7 promoter)
- 4 New BioBrick protein expression cassettes
- Characterized existing Registry promoters

**Software**
- WikiDust Allows users to quickly generate diagrams that link to the parts registry – **BBF RFC 68**
- PartsRobot simplifies registry submission process
Faculty
• David Baker
• Joseph Mougous
• Herbert M Sauro
• Eric Klavins

Advisors
• Ingrid Swanson
• Michal Galdzicki
• Justin Siegel
• Josh Bishop
• Matthew Smith
• Rob Egbert
• Jeremy Mills
• Deepak Chandran
• Joe Harrison

Space Donations
• Alan Weiner
• Dominic Chung
• Ling Liu

UW Microbiology Department
UW Biochemistry Department
UW MCB Department
Arthur Friedlander at USAMRIID
Michael Jacobs at UW

Fisher Scientific
VWR International
Questions?

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Assay utilizes synthetic fluorescent peptide

Catalytic knockout confirms active site chemistry

![Graph showing fluorescence units over time for different knockout conditions](chart.png)
Expected weight of CapD_CP without Methionine=55285Da, with Methionine=55417Da. Our mass spec detected a peak at 55274.8Da (no Methionine) well within the 0.02% error limit for our mass spec.
Circular Permutation

Linker

~526

351

352

526

1

1

Linker
Notes

• Pixilate PacMan
• Switch focus to removing transpeptidation not increasing hydrolysis
• Get new protein expression efficiency
• Change anthrax slide to get rid of mariners and Yankees
Tse2 toxicity