Development of a Naturally Derived Mosquito Larvicide

3-phenylprop-2-enal (Cinnamaldehyde) + Escherichia coli

NEVADA iGEM 2009
Mosquitoes: A Pathogenic Vector

- Predominant disease harboring genus:
  - **Anopheles**
    - Malaria
  - **Aedes**
    - Dengue/Yellow Fever
  - **Culex & Culiseta**
    - West Nile

W.H.O., Jan 2009

*P. falciparum*
Mosquitoes: An Extremely Adaptive Vector

W.H.O., Jan 2009
Mosquitoes

- **Food sources**
  - **Adults**
    - nectar, blood meals
  - **Larva**
    - algae, bacteria, other micro-organisms, and plant debris
### Common Mosquito Pesticides

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>DDT</th>
<th><em>Bacillus thuringiensis</em> (BT Toxin)</th>
<th>Methoprene</th>
<th>Pyrethrins</th>
<th>Organo-phosphates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Health Concerns</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Environmental Consequences</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Requires Persistent Supplementation</td>
<td>-</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Susceptible to UV Degradation</td>
<td>-</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
Cinnamaldehyde
A Novel Alternative

- Biosynthetic intermediate in plant cell wall synthesis
- Primary flavor and aromatic component of cinnamon
- Comprises 40-50% of cinnamon bark dry weight composition
- LD$_{50}$
  - Mosquito Larvae - 29 ppm
- Humans consume an estimated 4900 ppm daily
ACUTE MAMMALIAN TOXICITIES COMPARISON

Rat Oral LD$_{50}$ (mg/kg)

- Cinnamaldehyde (3400)
- Sodium chloride (3000)
- Salicylic acid (1600)
- Pyrethrins (1500)
- Caffeine (355)
- DDT/Phosmet (113)
# Delivery Systems

<table>
<thead>
<tr>
<th>E. coli</th>
<th>Duckweed</th>
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</table>
| - Excellent model system to identify control points of the pathway  
- Provides an inexpensive way to produce mass amounts of cinnamaldehyde | - Duckweed development parallels mosquitoes life cycle  
- Food source for mosquito larvae  
- Easy to genetically engineer |

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*E. coli* has excellent model system to identify control points of the pathway and provides an inexpensive way to produce mass amounts of cinnamaldehyde.

*Duckweed* development parallels mosquitoes life cycle and is a food source for mosquito larvae. It is also easy to genetically engineer.
Cinnamaldehyde Synthesis

- **Deamination of phenylalanine into cinnamic acid**
  - Phenylalanine-ammonia lyase

- **Acid-thiol ligation of cinnamic acid into cinnamoyl-CoA**
  - 4-coumarate: CoA ligase

- **Reduction of cinnamoyl-CoA into cinnamaldehyde**
  - Cinnamoyl-CoA reductase
Project Aims

- Development of an *in silico* model of pathway flux
  - Obtain information to rationally augment endogenous plant pathway
- Convert each gene into a biobrick compatible submission
- Introduction of novel cinnamaldehyde synthetic pathway into *E.coli*
  - Assess enzymatic activity in engineered lines to validate model
Modeling Metabolic Flux

Phenylalanine \xrightarrow{\text{Lyase}} \text{Cinnamic Acid}

- $K_m = 0.33 \, \mu\text{M}$
- $V_{max} = 163 \, \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ enzyme}$

Cinnamic Acid \xrightarrow{\text{Ligase}} \text{Cinnamoyl-CoA}

- $K_m = 6642 \, \mu\text{M}$
- $V_{max} = 1.2 \, \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ enzyme}$

Cinnamoyl-CoA \xrightarrow{\text{Reductase}} \text{Cinnamaldehyde}

- $K_m = 0.42 \, \mu\text{M}$
- $V_{max} = 0.11 \, \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ enzyme}$
Wild Type Enzymes

Concentration (µM)

Time (hours)

- Phenylalanine
- Cinnamic Acid
- Cinnamoyl-CoA
- Cinnamaldehyde
- Lyase
- Ligase
- Reductase
Wild Type Enzymes with a High Concentration of Ligase

Concentration ($\mu$M)

Time (hours)

Phenylalanine
Cinnamic Acid
Cinnamoyl-CoA
Cinnamaldehyde
Lyase
Ligase
Reductase
Engineered enzymes


<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>Mutant (M293P + K320L + N256A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>6642</td>
<td>163</td>
</tr>
<tr>
<td>$V_{max}$ ($\mu$mol$\cdot$min$^{-1}$$\cdot$mg$^{-1}$$_{enzyme}$)</td>
<td>1.2</td>
<td>0.87</td>
</tr>
</tbody>
</table>
Favorable Enzymes with a High Concentration of Ligase

- Phenylalanine
- Cinnamic Acid
- Cinnamoyl-CoA
- Cinnamaldehyde

Time (hours) vs. Concentration (µM)
Parts: Initial Aim

- Complete IPTG-inducible polycistronic operon of all three genes in the pathway
Revised Aim

- Two separate expression vectors for 4-coumarate:CoA ligase and phenylalanine-ammonia lyase/cinnamoyl-CoA reductase
Future Experiments: *E. coli*

- Assemble dual expression system
- Complete IPTG-inducible polycistronic operon
- Contrast expression systems
- Characterize
  - Gene Expression
  - Product Formation
  - Larvicidal Activity
Enzyme Expression and Characterization

- Demonstrate functional protein expression of the three enzymes
- Develop assays for each enzyme
  - Progress
Expression of Mutant CoA Ligase in E. coli

KDa  Wild Type  Transformed  Purified  KDa

260  160  110  80  60  50  40  30  20  260  160  110  80  60  50  40  30

66 KDa
Cinnamoyl-CoA-Ligase assay

Cinnamic acid + CoASH + ATP → cinnamoyl CoA + ADP + Pi

Abs (311nm)

Time (seconds)

Transformed Mutant

Wild Type
Future Experiments: Plants

- Transform duckweed to augment endogenous cinnamaldehyde production
- Standardize Plant transformation by creating a Biobrick-compatible binary plant vector (pBI121)
- Investigate duckweed efficacy as a cinnamaldehyde vehicle
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- Alex Dussaq

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Questions?