A Biobrick toolkit for cyanobacteria

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Team: Hawaii 2008
Why *Synechocystis*?

*Synechocystis* sp. PCC 6803:
- Model organism for the study of photosynthesis
- Minimal nutrients required for growth
- Demonstrated bioproduction
Toolkit

1. Mobilizable broad-host-range BioBrick vectors derived from RSF1010

2. A cassette for protein secretion from *Synechocystis* sp. PCC 6803

3. A nitrate-inducible cyanobacterial promoter BioBrick
Tri-Parental Mating

Donor Strains

- Mobilizable plasmid
  - pRL1383a
  - 8 kb

- Self-transmissible plasmid
  - RP4
  - 60kB

Recipient Strain

Counter Selects for RP4 with antibiotic resistance
Combine Parts from 3 Plasmids

RSF1010 derived plasmid (pRL1383a) 9 kb

RP4 60 kb

BioBrick Base Vector 2348 bp

oriT
oriT(mob)
oriV
rep
MCS
aadA
mob

oriT

E X S P

N A N
Broad-Host-Range Mobilizable BioBrick Plasmid
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Broad-Host-Range Mobilizable BioBrick Plasmid

BBa_I52002

P(Lac) IQ/RBS
rep (RSF1010)
orN (RSF1010)
P(Lac) IQ/RBS
aadA
oriT (RP4)

7.3 kb
1) BioBrick Parts Construction

Synthesis of oriT

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer</th>
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<tbody>
<tr>
<td>oriT (RP4) 1</td>
<td>ctagaggaataagggacagtgaagaaggaacacccgctcg</td>
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<tr>
<td>oriT (RP4) 2</td>
<td>cgggtgggcctacttcacctatcctgccgctgacgccg</td>
</tr>
<tr>
<td>oriT (RP4) 3</td>
<td>ttggatacaccaaggaagtctacatactagtagccgctgca</td>
</tr>
<tr>
<td>oriT (RP4) 4</td>
<td>gcggccgctactagtagtgtagactttcccttggtg</td>
</tr>
<tr>
<td>oriT (RP4) 5</td>
<td>tatccacggcgtcagccggcaggataggtgaagtaggcc</td>
</tr>
<tr>
<td>oriT (RP4) 6</td>
<td>cacccgcggagcgggtgttccctttcactgtccctttattct</td>
</tr>
</tbody>
</table>
1) BioBrick parts construction

PCR amplification of rep, oriV, and aadA
2) Ligations Using BioBrick Methods

- *aadA*
- *rep (RSF1010)*
2) Ligations Using BioBrick Methods

- **aadA**
- **rep (RSF1010)**
- P(Lac)I/Q/RBS
2) Ligations Using BioBrick Methods

- $\text{aadA}$
- $\text{rep (RSF1010)}$
- $\text{P(Lac) IQ/RBS}$
- $\text{P(Lac) IQ/RBS}$
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- $\text{aadA}$
2) Ligations Using BioBrick Methods

- **aadA**
- **rep (RSF1010)**
- **P(Lac) IQ/RBS**
- **oriT**
- **oriV**
2) Ligations Using BioBrick Methods

- **aadA**
- **rep** (RSF1010)
2) Ligations Using BioBrick Methods

- **aadA**
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  - **rep** (RSF1010)

- **oriV**
  - **oriT**
  - **aadA**
  - **P(Lac) IQ/RBS**

- **rep** (RSF1010)

Diagram shows the process of ligations using BioBrick methods.
2) Ligations Using BioBrick Methods

- **aadA**
- **rep** (RSF1010)
- **P(Lac) IQ/RBS**
- **oriV**
- **oriT**
- **P(Lac) IQ/RBS**
- **rep** (RSF1010)
- **aadA**
2) Ligations Using BioBrick Methods
2) Ligations Using BioBrick Methods

- **aadA**
- **rep (RSF1010)**
- **P(Lac) IQ/RBS**
- **oriV**
- **rep (RSF1010)**
- **aadA**
- **oriT**

Broad-Host-Range Mobilizable BioBrick Plasmid 7.3 kb

Base Vector BBa_K1020
Characterization of the Plasmid

- Is it mobilizable?
  - Results suggest it is mobilizable to *E. coli*.
- Does it replicate autonomously in *Synechocystis* sp. PCC 6803?
- What is the copy # in *Synechocystis*?
**aadA works!**

The *aadA* construct has been shown to grow on *Sp*<sub>100</sub> as well as SmSp, as needed in *Synechocystis* sp. PCC 6803.
Does the oriT from RP4 work?

oriT: 371 bp
BBa_J01003

Origin of transfer available in the BioBrick Registry
Available in Registry
Has been shown to transfer conjugatively between E. coli

oriT: 99 bp

Origin of transfer designed by Team Hawaii
Designed to not contain extraneous DNA
Has not been shown to work
Inducible Secretion Device

• Clinical/industrial protein production performed by bacterial cell cultures

• Problems occur in recovering proteins in substantial yields
  – Large amount of biomass waste
  – Product isolation is time and resource intensive
The **nirA** promoter

- **nir operon**
  - *Synechococcus* sp. strain PCC 7942
  - Nitrate reductase promoter
  - Inhibited by ammonia/induced by nitrate

- “Off” in *E.coli*
Signal Sequences

• Derived from pilA and slr2016

• Naturally used by Synechocystis sp. PCC 6803

• Lichenase secretion
Constructs

Inducible GFP secretion construct:

Controls:
1) BioBrick parts construction

Synthesis of nir promoter, slr2016, pilA:

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer</th>
<th>Length</th>
<th>G/C content</th>
<th>Tm</th>
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</thead>
<tbody>
<tr>
<td>pni1_forward</td>
<td>CTAGAGCTAATGCGTAAACTGCAATATGCGCTCGAGGATGTAATTTACGGTTCA</td>
<td>55</td>
<td>40%</td>
<td>72.5C</td>
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<tr>
<td>pni1_reverse</td>
<td>GTAATTACACTCACTGCAAGCGATATGCGATTATACGCTTGT</td>
<td>45</td>
<td>40%</td>
<td>70.6C</td>
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<tr>
<td>pni2_forward</td>
<td>AATTTTAACGAAACGGAACCTATATTGATCTCTACTACATAGCGGGCCTGCA</td>
<td>57</td>
<td>43.90%</td>
<td>74.3C</td>
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<td>pni2_reverse</td>
<td>GCGGCCGCTAATGAGTATAGAGGTACCTAGTTCCGCTTCTCGTTAAATTTGTAAC</td>
<td>59</td>
<td>42.40%</td>
<td>73.0C</td>
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<tbody>
<tr>
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<td>CTAGATGCTAGTAGATTATAATTCCAAACTCCTCTCTCAAC</td>
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<tr>
<td>pilA1_reverse</td>
<td>AGAGGAGTGGATATTAAATTACTAGCCAT</td>
<td>31</td>
<td>29.00%</td>
<td>60.6C</td>
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<tr>
<td>pilA2_forward</td>
<td>TCTCTAAAAACGGCAGAAGGTTGACTAGTAGCGGGCCTGCA</td>
<td>45</td>
<td>55.60%</td>
<td>76.8C</td>
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<td>GCGGCCGCTAATGAGTACCTAGCCACCTCTCTGCGCCTTTTGAGATGCAG</td>
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<td>55.30%</td>
<td>75.5C</td>
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<tr>
<td>slr2016-1_forward</td>
<td>CTAGATGGCGAGCGAAACGACTATGGGAATTTCAATC</td>
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<tr>
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<td>59.00%</td>
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<tr>
<td>slr2016-2_reverse</td>
<td>GCGGCCGCTAATGAGTACCTAGCCACCTCTCATCG</td>
<td>29</td>
<td>62.10%</td>
<td>70.5C</td>
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</tbody>
</table>
1) BioBrick parts construction

Conversion of GFP (BBa_E0040) into a fusion brick:

- Regular protein-protein Biobrick ligations cause a translational frameshift
1) BioBrick parts construction

Conversion of GFP (BBa_E0040) into a fusion brick:

– Site-directed mutagenesis to remove the first 2 nucleotides of the ORF to restore reading frame
2) Device assembly

Planned assembly schematic of GFP devices
2) Device assembly

promoter

rbs

signal sequence

GFP fusion

txn. term.

Planned assembly schematic of GFP devices
2) Device assembly

Planned assembly schematic of GFP devices
2) Device assembly

Planned assembly schematic of GFP devices
2) Device assembly

Planned assembly schematic of GFP devices
2) GFP secretion device

1) Transformed DH5α containing the constitutive GFP secretion device

2) Transformed DH5α without GFP device

3) Untransformed DH5α
3) pRL1383a as a BioBrick vector

Conversion of pRL1383a into a Biobrick vector:
3) pRL1383a as a BioBrick vector

Conversion of pRL1383a into a Biobrick vector:
3) pRL1383a as a BioBrick vector

Conversion of pRL1383a into a Biobrick vector:
3) Replacement of pRL1383a MCS

- PCR of lac promoter-lacZ BioBrick (J33207)
3) Replacement of pRL1383a MCS

- PCR of lac promoter-lacZ BioBrick (J33207)
- Restriction digest of PCR product and pRL1383a with HindIII and BamHI
3) Replacement of pRL1383a MCS

- PCR of lac promoter-lacZ BioBrick (J33207)
- Restriction digest of PCR product and pRL1383a with HindIII and BamHI
- Ligation of H/B flanked J33207 with pRL1383a
3) Replacement of pRL1383a MCS

- PCR of lac promoter-lacZ BioBrick (J33207)
- Restriction digest of PCR product and pRL1383a with HindIII and BamHI
- Ligation of H/B flanked J33207 with pRL1383a
- Transformation into DH5α
  - Plated on LB + sp100 + X-gal
  - Blue/white selection of transformants
3) Converted pRL1383a in E. coli
3) Converted pRL1383a in E. coli
3) Antibiotic test of pRL1383a
3) Blue/white screen

- Mutation in the lac promoter
- Unknown interactions between lacZ (J33207) and pRL1383a
3) Conjugation into *Synechocystis*

Conjugation of BioBrick plasmid pRL1383a w/ GFP secretion device:
3) Conjugation into Synechocystis

Triparental conjugation:

Day 1

5% LB + BG-11

Day 14

BG-11 + sp2.5 + sm2.5
## Accomplishments

<table>
<thead>
<tr>
<th>Description</th>
<th>Type</th>
<th>Designed</th>
<th>Constructed</th>
<th>Sequenced</th>
<th>Submitted</th>
<th>Tested</th>
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<tbody>
<tr>
<td>Broad host range plasmid derived from pRL1383a</td>
<td>Plasmid</td>
<td>✅</td>
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<td><em>rep/oriV/aadA/oriT</em></td>
<td>Plasmid intermediate</td>
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<td>nitrate inducible <em>nirA</em> promoter</td>
<td>regulator</td>
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<td>nitrate inducible <em>nirA</em> promoter with an rbs (BBa_B0030)</td>
<td>regulator</td>
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<td><em>pIIA</em> derived N-terminus protein secretion signal sequence</td>
<td>protein coding</td>
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<td>protein coding</td>
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<td><em>oriT</em> derived from RP4</td>
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<td>✅</td>
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<td><em>oriT</em> (BBa_J01003) attached to <em>lacZ</em> (BBa_J33207)</td>
<td>conjugation</td>
<td>✅</td>
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<td><em>oriT</em> (K125320) attached to <em>lacZ</em> (BBa_J33207)</td>
<td>conjugation</td>
<td>✅</td>
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<tr>
<td>GFP fusion protein</td>
<td>protein coding</td>
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<td>Constitutive <em>aadA</em> expression device</td>
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<td>N-terminal <em>slr2016</em> signal sequence attached to GFP fusion</td>
<td>protein coding</td>
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<tr>
<td>Constitutive GFP secretion device</td>
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3) Conjugation into *Synechocystis*

Conjugation of pRL1383a: