Week 2 Notes (6/June - 10/June/2016)

TUESDAY, 6/7/2016

- mob genes as mobile elements in transformation
- need to get gBLOCK sequence files for alignment with cloned samples
- codon optimized for cyano and/or rhizo
- citrine plasmids: verify in frame with another sequencing Rx
- + control → find plasmid(s) where fluorescence worked
- need strain and plasmid stock
- - controls already made (the LIC cassettes)
- exps. verified with sequence
- ready: entry plasmids for LICs (just need to make right insert); determine which ones to use.
- test that once citrine transformed into rhizobia, fluorescence still works
- then test for recombinases in MAGE in rhizobia
- *get pORTMAGE plasmid (plasmid or strain?) → what exactly is it
- need plasmid map, expected sequence file
- what is Ori, what is selectable marker
- 1. move plasmid into rhizobia strains
    - a. CIAT 899
    - b. S. meliloti
- rhizobia competent cells technique
- *what E. coli strain is pORTMAGE in → some do not give good DNA plasmid preps
- ensure rhizobia is sensitive to antibiotic selector (not resistant)
- make media and electroporate plasmids into strains
- construct plasmids and test/verify in E. coli → then, move finished plasmid into rhizobia
- verify protocol: point mutation in RNAPol
- search cyano transformation and linear vectors: Ori, rifampicin marker will work?
- bioinformatics BLAST search for homology/analog in rhizobia; get sequence to *make synthetic entry construct. Must have sequence similarity and functionality
    - ask Jaymin
- outreach with other organizations about iGEM
- construct submission, used with LIC instead of restriction?
    - how to make compliant, or get approval
    - preparation of biological sample
- 20kb gBLOCK sequence for construction once gene has been identified; need sequence.
    - legHb in R. tropici
    - cbb3 (or other) in R. tropici
- partition who will work with the 3 constitutive promoters of different strengths; 4 inducible promoters.
- blueprint of MAGE modifications in rhizobia

7/June/2016

- Promoter-citrine testing:
    - - control = cell with syn. gene but with dysfunctional citrine = entry vector (LIC cassette)
    - + control = not present
    - experimental = proposed cell with citrine gene in the interchanged LIC cassette
- fluorescence 96-plate reader has limit of detection; needs min.; avoid saturation
    - make serial dilution (8 dilutions per column)
- put plate in reader and do od600(nm) measure (how many cells per mL in each well)
- read at right nm excitation/emission level
    - control = curve of # cells-amount of fluorescence

    - exp = amount should be different in each sample, but relational in dilution.
- od.6 = actual number that 0-1 of absorbance/turbidity of bacterial activity
- need PBS, spectinomycin, LB, cell lines (plasmids), glycerol stocks for plasmid posterity, cultures
- reference sequences for all of citrine plasmids → how much sequence across plasmid was done? look for mutations (translation feature of lasergene)
- Database! For stock, oligos, primers,
- Stock = strain carrying plasmid
- Strain = the bacterial host itself
- Promoter inducibility (conditions and uninduced v. induced controls... leaky promoter? in absence of inducer, no citrine fluor. should be observed) vs. consitutiveness
- find primers (GA)
- read about the promoters (strength, how to induce)
- depending on protein, T, aggregation, → first trial use literature protocol
    - conc. range
- problem with gBLOCKS:
    - - recombinases found with data mining approach
    - find virus that infect rhizobia
    - bioinformatics approach to identify most-likely sequence for purpose
    - similar DNA sequence; similar protein function
    - checked for functional domains → 8 for rhizo, 8 for cyano
    - when making long (75 bp) oligo, accuracy of synthesis decreases
    - recode – to use tRNA most available in given organism; ran simulation for codon optimization
    - CTG start codons; single aa substitutions → should be there or not?
    - need to find files of what the company actually sequenced
- proof-of-principle that this method works: test rifampicin (efficiency of pORTMAGE) and select in rhizobia
- *pORTMAGE in rhizobia* --> publishable?

- evaluate recombinase with rifampicin
- ? rifampicin assay for pORTMAGE

WEDNESDAY, 6/8/2016

- objectives: to transform pORTMAGE in E. coli, R. tropici and plate on 3 media: streaked, 5 uL, and 50 uL cell vol.
    - to make two types of negative controls for R. tropici: LB untransformed CIAT 899 and LB-kan^{50} untransformed CIAT 899.
- When choosing the gene to colony PCR verify for pORTMAGE plasmid, choose 300-400 bp sequence (min. 200).
    - primer length: 15-18 nt
    - Tm around 60C
- to detect mutation frequency, screen with selections (such as another Ab) by integrating a reporter gene into the genome that is initially off until the oligo turns it on.
    - long homology arms help target oligo
- Test MAGE with and without the DNA/plasmid (controls)
- Can caluclate the efficiency of DNA incorporation of MAGE with pORTMAGE on selection and normal plates (numerator/denominator)
    - the ratio of how many cells are resistant to Ab/ total cells
- 1. + selection (colonies grown when gene turned on)
- 2. control selection (expect 100% growth)
    - ratio will determine recombination frequency
- Or, can use fluorescent protein (and erase the stop codon to turn it on); repeat with or without pORTMAGE.
- To see if our pORTMAGE is significant, look up literature of past efficiency.

- Example of mutation: target ribosomal binding site or rare codon (cell doesn't have many of that tRNA, but can change to mainstream codon so to increase translation) --> to upregulate protein.
- Once a cassette is put into rhizobia, can use MAGE to make protein more native.
    - Putting reporter gene into rhizobia difficult?
- knock-out mutS in MAGE with reporter off and use oligo that turns it on, plus one of each of the recombinases.
    - pORTMAGE might not work due to beta not working
- RT-PCR to ensure that recombinase is being transcribed, but is unnecessary considering the outcomes of MAGE.

THURSDAY, 6/9/2016

- Objectives: to design two primers for colony PCR to verify pORTMAGE in R. tropici; to create nodulation assay to ensure R. tropici can nodulate and fix N2 on clover; to design protocol for measuring pORTMAGE efficiency in R. tropici
- For tomorrow: run through MAGE in E. coli
- Need to:
    - [ ] verify pORTMAGE is in R. tropici by:
        - (E. coli) restriction site and run gel
        - (R. tropici) design primers and run colony PCR on gam protein
    - [ ] test if pORTMAGE works in R. tropoci by:
        - selection assay with another Ab (such as rafamcyin) after oligo design and electroporation
            - design and order oligos
    - [ ] nodulation assay to verify that R. tropici can nodulate
    - [ ] finalize 10-week plans
        - 5 slide presentation on legHb
    - [x] make glycerol stocks, plate cultures, of R. tropici

SATURDAY, 6/11/2016

- Notes on mutS:
    - when designing targetter oligos for MAGE, create long homology arms (2kb)
        - flanking mutS if excising
    - pORTMAGE transiently inhibits mutS using mutL
- No need to knock out mutS if pORTMAGE is sufficient.
- CaCl used when plasmid is less than 8kb
    - if larger, use electroporation

Week 3 Notes (13/June - 17/June/2016)

MONDAY, 6/13/2016

- [ ] Selection assay for pORTMAGE effciency in R. tropici:
  - insert Ab^R marker in R. tropici that is "off" by point mutation
  - create oligos with long homology arms (45 nt ea. flank) that can "turn on" Ab resistance
  - electroporate pORTMAGE-transformed R. tropici with oligos and plate on (+) selection plates (with antibiotic) and (-) plates (LB only--expect all colonies to grow)
    - ratio = (# colonies on +)/(# colonies on -) = mutated cells/normal cells
- [x] Once primers designed and ordered for pORTMAGE identification/verification in R. tropici:
  - run colony PCR to identify gam protein in R. tropici; then sequence
  - repeat with E. coli
- [x] Work on nodulation assay:
  - obtain and grow cowpea plants
- [ ] Work on project idea:
  - identify best high affinity terminal oxidase (HATO) for R. tropici
    - identify small molecules/proteins that upregulate HATO
    - identify site on small molecule/protein gene to use MAGE to upregulate it
      - or, identify site on HATO itself for upregulation with MAGE
- [x] test pORTMAGE in E. coli as well
  - 0.1-30% efficiency is reported

- if very rare transformation, do one plate of dilution and one plate of spin/pellet/suspend in small volume/plate everything
- optimal/max temperature for rhizobia = 28C
- *bring E. coli and rhizobia glycerol stocks to WC; change incubator to 28C
- along with doing restriction enzymes with E. coli and sequencing, can do colony PCR validation of a few loci across E. coli plasmid to amplify (like what is done in R. tropici).
- when interpreting data from MAGE in pORTMAGE-R. tropici, need to troubleshoot
  - rifampicin mutations without mutS occur naturally
- create mutS knock-out in rhizobia
  - identify other targets in rhizobia that can help with MAGE
- with 10-week project proposal:
  - instead of point mutations (which can be done with other methods), look at doing several sites to ensure MAGE is most appropriate.
  - identify the best assay for measuring respiration
  - look into the necessity of respiration in rhizobia (before/after nodulation and differentiation).
    - how relevant/significant is this?

TUESDAY, 6/14/2016

- To do for today:
  - [x] streak pORTMAGE-transformed E. coli plates and bring to WC
  - [x] transfer electroporation/rhizobia transformation protocol online
  - [x] make competent cells of S. meliloti
  - [x] order primers for colony PCR of R. tropici
  - [x] create and order oligos for pORTMAGE assay in E. coli and rhizobia
  - [x] Half of isolated plasmid DNA (pORTMAGE-3) will be taken to WC.
- From Jaymin's email: (Underlined = bring to WC)
  - [x] Someone needs to check in on the portmage transformations.
    - [x] growth of transformed S. meliloti

- [x] growth of transformed R. tropici
- [x] We need to begin **transforming the citrine constructs into rhizo**. This may involve **making comp cells out of meliloti.** Neg. control = E. coli.
- [x] The citrine constructs in e coli need to be brought to west campus for testing
- [x] We will be testing portmage in e coli tomorrow at WC.
- [x] Someone needs to **make some rifampicin and LB plates**.
- [x] Of the messed up recombinase constructs, someone needs to **pick additional colonies for sequencing**.
- [x] Perform and take notes on nodulation assays.

- When using antibiotics: (use exact protocols)
  - if salt: dilute to water in desired conc. (1000X); filter, sterilize, and aliquot for freezing at 1mL each.
  - if not: find adequate solvent (DMSO). Once in soln, take up to final conc. with water.
    - EtOH and chlor.
- Transformed cells must be stored at 4C, not -20C.

WEDNESDAY, 6/15/2016

- Jaymin has oligo for CIAT for MAGE, a "H536Y mutation in the gene rpoB"
  - "we knew what the mutation was in E coli that led to rif resistance (H526Y), so we aligned the CIAT version of the gene to E coli allowing us to determine that H526 in E coli is equivalent to H536 in CIAT."
- He also sent the rhizobia antibiotic resistance data for CIAT and meliloti: MIC (ug/mL of Ab)
  - "in Meliloti, you cannot use Streptomycin, and need to use a higher concentration of Kanamycin (using neomycin instead might improve results). In CIAT, you cannot use spectinomycin, streptomycin, or zeocin."

Table1

	A	B	C	D	E
1	Antibiotic	MIC Meliloti	Recommended Concentration Meliloti	MIC CIAT	Recommended Concentration CIAT
2	Carbenicillin	<2.5	50	10	50
3	Kanamycin	50	100	<2.5	35
4	Spectinomycin	25	100	>100	Do Not Use
5	Streptomycin	>100	Do Not Use	>100	Do Not Use
6	Tetracycline	<2.5	7.5	<2.5	7.5
7	Rifampicin	<2.5	100	<2.5	100
8	Gentamycin	10	50	<2.5	10
9	Zeocin	<2.5	7.5	>100	Do Not Use
10	Hygromycin	5	25	<2.5	25
11	Erythromycin	<2.5	25	<2.5	25
12	Chloramphenicol	<2.5	12.5	25	100

- A separate oligo will be designed for S. meliloti for MAGE of transformation with pORTMAGE-3.
- Notes on oligo design from Koen:
  - "The first thing you'll need to find out is what exact mutation you want to insert. Doesn't rifampicin resistance come from an alteration of an amino acid in a certain gene?

    - ecocyc.org (https://urldefense.proofpoint.com/v2/url?u=http-3A__ecocyc.org&d=AwMFaQ&c=-dg2m7zWuuDZ0MUcV7Sdqw&r=mrVg8_jY8xE9g19E-wzn2RR_e4ijyO30vXw7q00gFBU&m=Y8hA7T3XfJwImUessA3sJlm61y3lfB5urdr8Khza8ew&s=QnhAfRKx5t_w3zdZ2sn5xfMA98B7erduxNs7nZxJzsA&e=): It's a database where you can find the sequences and function of genes. Note, ecocyc will by default show you E.coli K12, but under the search bar you can change the organism to Rhizobia. This website will tell you: location of the gene on the genome, orientation of the gene, sequence
    - Based on this information you can identify the bases surrounding your desired mutation (40-45 on each side), to give you the 90bp oligo.
    - Here comes the challenging part. If you recall, MAGE oligos are most efficient if they can replace an Okazaki fragment during genome duplication. Bacterial genomes are circular and have one or two origins of replication.
    - You'll have to find out: Where is the ori of the Rhizobia genome? What is the position and orientation of your gene in relation to the ori? With help from the nature protocols paper, and the answers to the last two questions, you should be able to find the correct orientation for your oligo.
    - If you get stuck on these questions for more than a day, or if you feel really unsure, you could also order the oligo in both directions, and you can try them both.
    - Lastly, before you order, make sure the MAGE oligo doesn't fold back on itself. You can use the website **Mfold**, and select DNA folding. You just feed in the sequence of your oligo (keep everything on default settings) and find out if it has strong secondary structures. If it does, you will have to redesign the oligo, meaning having your mutation more to one side or the other of the oligo.
- Before testing MAGE in meliloti-pORTMAGE plate cultures, want to run colony PCR to ensure pORTMAGE is in the transformed meliloti.
    - then proceed to performing MAGE with deisnged oligo

SATURDAY, 6/18/2016

Updated:

- Did test run of PCR and AGE of pORTMAGE and piG 302
- Did colony PCR for both strains of rhizobia, and ran AGE.

Week 4 Notes (20/June - 24/June/2016)

MONDAY, 6/20

- Objectives for this week:
  - [ ] to make competent cells of rhizobia (CIAT 899 and S. meliloti) and have successfully transformations of pORTMAGE-3 in these colonies.
  - [ ] verify pORTMAGE in rhizobia using the 8 sequencing reactions (4 per strain)
  - [ ] do MAGE on the pORTMAGE-transformed rhizobia with rifampicin assay.
  - [ ] continue working on project idea
  - [ ] continue working on nodulation assay protocol, once transformed rhizobia colonies have been grown
- pORTMAGE-3 was successfully transformed in E. coli
  - in the AGE picture of pORTMAGE plasmid only (template) and isolated/extracted transformation DNA from E. coli, two bands were shown for the plasmid only sample (from Jaymin), and one band was shown for our extracted DNA (experimental).
  - objective: to amplify section of isolated DNA from E. coli
  - from the kb marker (NEB), our band was roughly 800 bp. The two most prominent bands from the plasmid only sample, there were 800 bp and 400 bp bands.
  - reduce template (5 ng) and re-run AGE
    - problem: small (400bp) band is amplified in plasmid only, but not in our DNA
    - sequence 3 fragments: 2 of the plasmid only ones, 1 of ours

      need to know if multiple priming sites = messy sequencing Rx.

      make second seqeuncing Rx (Keck) of plasmid
  - supercoiled excess plasmid may be the larger ghost bands
- Goals for today:
  - [x] run AGE of both rhizobia colony PCR results
  - [x] run AGE of isolated/original pORTMAGE plasmids to further purify the three bands
  - [x] sequence the pORTMAGE-3 plasmid with the gam primers to determine if there are more sites of elongation.
- When doing a sequencing reaction at Keck facility, use the instructions: https://medicine.yale.edu/keck/dna/protocols/tube/ (https://medicine.yale.edu/keck/dna/protocols/tube/)

TUESDAY, 6/21

- Goals for today:
  - [x] Analyze results of gam sequencing to see if there are multiple sites of priming.
    - found that forward primers were unspecific, but reverse primer was okay for PiG302.
  - [x] Write protocol for resuspending primers and making both master and working mixes.
  - [x] Submit 2 sequencing reactions with the known pORTMAGE-3 plasmid.
  - [x] Run PCR of known pORTMAGE-3 plasmid with new primers (365 and 366)
- gam sequence from database vs. our isolated pORTMAGE from E. coli: 94% match
  - when tried to compare with known plasmid (from Jaymin), sequence was too short to match.

WEDNESDAY, 6/22

- For today:
  - [x] AGE of PCR products from yesterday--the known plasmid and PriG 365 and 366 (for the 901bp fragment of pORTMAGE).
  - [x] PCR and AGE of both rhizobia and E. coli (3 total subjects) with Prig 365 and 366 (901 bp) and Prig 367 and 368 (603 bp) to verify transformation.
    - Both strains of rhizobia gave expected bands.
    - E. coli

- [x] Make 50mL kan stocks of transformed E. coli.

- Will test pORTMAGE and recombinases in rhizobia.
- When performing MAGE, know how competent cells are, number of cells recovered.
- Needed to repeat PCR and AGE of rhizobia with Prig 365+366 and 367+368 because bands may have been contaminated with too many cells.

THURSDAY, 6/23

- For today:
  - [x] Inoculate the 50-mL transformed E. coli liquid stock into 500 mL.
  - [x] Make more LB-kan plates.
  - [x] Repeat colony PCR and AGE of PriG 365+366 and 367+368 with both rhizobia.
- When repeating colony PCR of both rhizobia, will streak 8 NEW colonies as well.

FRIDAY, 6/24

- For today:
  - [x] For colonies 1, 3, and 10 of rhizobia, gel extract and purify plasmid for sequencing.
    - [ ] Submit sequencing reactions:
  - [ ] QIAGEN Midi Prep --> Concentrate and extract plasmid DNA from E. coli liquid cultures for more pORTMAGE-3 plasmid.
  - [x] Run PCR and AGE of known plasmid with all 4 primers at once.
    - possible problems with different Tms of both sets of primers, and extension time/temp.
    - it worked!
- When possible:
  - [ ] **Find paper on protocol of MAGE** and determine order of introducing recombinases and oligos (together or separate).
  - [ ] Restriction digest of plasmid for final verification. **Find suitable restriction sites**.
  - [ ] When recombinases are ready, start rhizobia cultures and streak kan plates with recombinases to transform.
- Clones 10 of R. tropici and 1, 3 of S. meliloti worked best with the re-attempt of colony PCR with both sets of pORTMAGE primers.
- Received nodulation assay kit today. Protocol is uploaded.
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Week 4 AGE Data

FRIDAY, 6/24

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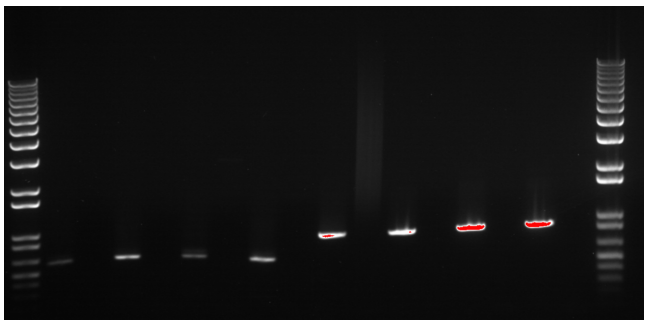

Figure 1. AGE of **known pORTMAGE-3 plasmid** (PiG 319) with primers PriG 367+368 (GW, expected 600 bp, bmutL-F) and PriG 365+366 (ML, expected 900 bp, cI857-F). Products are as expected.

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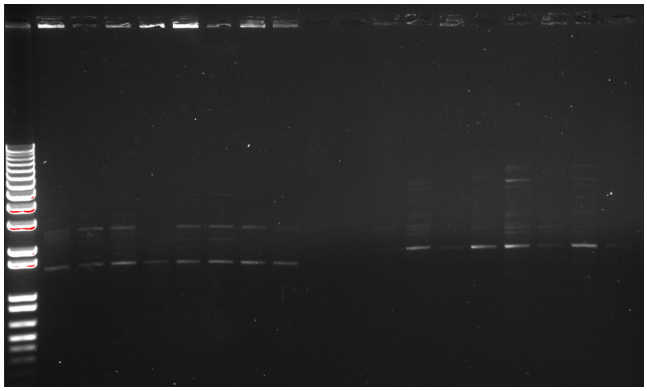

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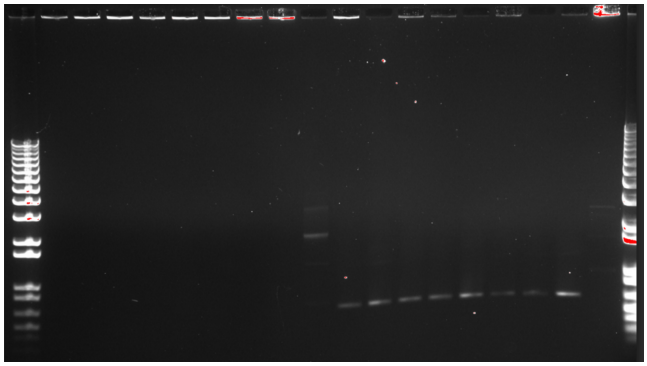

Figure 2. AGE of **transformed rhizobia** (right: R. tropici, left: S. meliloti) with PriG 365+366 (top) and PriG 367+368 (bottom). Products may be contaminated by cell debris.

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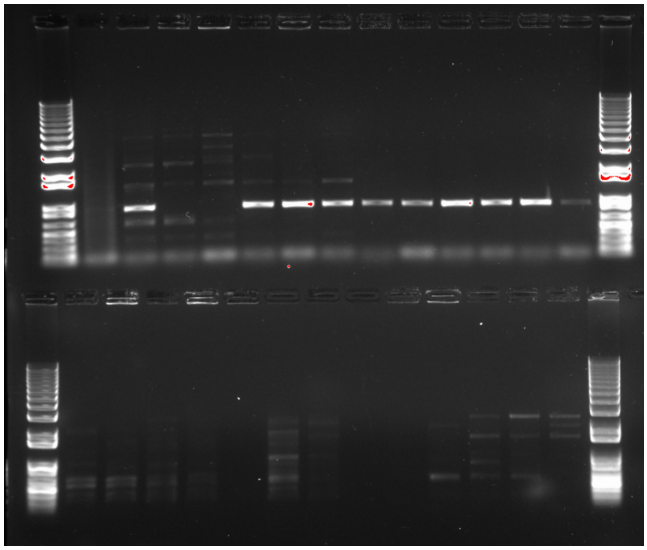

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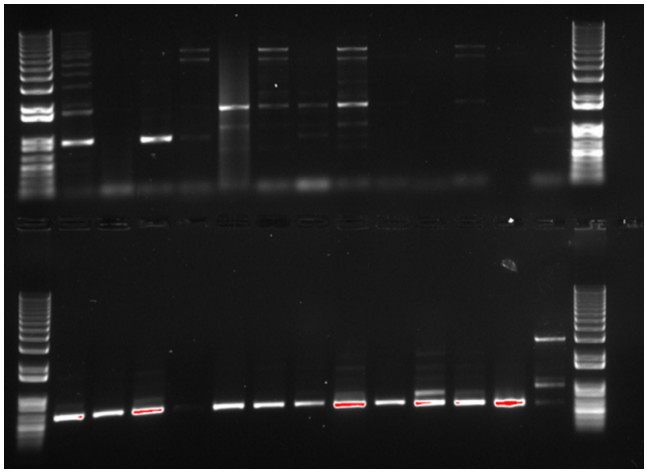

Figure 3. **Re-attempt** of *Figure 2* experiment, with R. tropici (above) PriG 365+366 (top) and PriG 267+268 (bottom). S. meliloti (below) PriG 365+366 (top) and PriG 267+268 (bottom). R. tropici colony 10 seemed best to work with both sets of primers. S. meliloti colonies 1 and 3 seemed best with both sets of primers.

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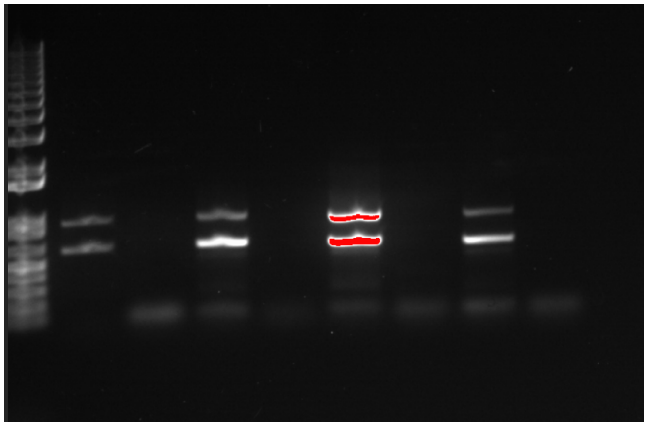

Figure 4. AGE of PiG 319 with two sets of primers: PriG 365+366 and 367+368. PCR conditions were modified to accommodate both primer set conditions.

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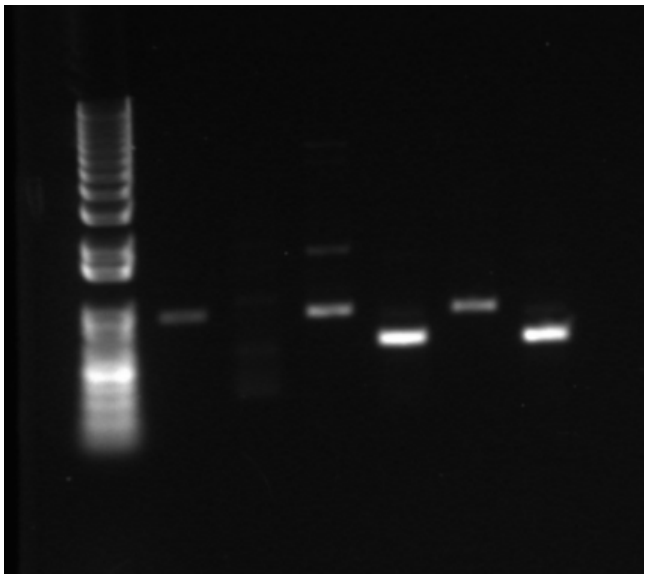

Figure 5. AGE of chosen rhizobia colonies for sequencing. PriG 365+366 and 367+368. Lanes 1+2 are of R. tropici colony 10. Lanes 3+4 and 5+6 are of S. meliloti colonies 1 and 3.

Week 5 Notes (27/June - 1/July/2016)

MONDAY, 6/27

- Objectives for this week:
  - [ ] (Restriction digest of pORTMAGE-3 plasmid.)
  - [ ] Perform MAGE with transformed rhizobia.
    - neg. control: test MAGE in untransformed rhizobia; without recombinases.
    - [ ] Obtain oligos from Jaymin.
  - [ ] Make competent cells to transform pORTMAGE.
  - [ ] Start nodulation assay with transformed rhizobia.
    - Both rhizobia untransformed, both rhizobia transformed (4 groups).

TUESDAY, 6/28

- Need to start the nodulation of rhizobia.
- Move R. tropici into 50 mL --> make competent cells of transformed rhizobia
  - [x] 2L of 10% glycerol by volume (100 mL) with miliQ water, autoclave --> put into cold room (of transformed rhizobia; glycerol will mix when autoclaved (loose caps, autoclave tape)
    - [x] move 1 culture today; the other 2 tomorrow
    - glycerol has high heat capactiance (do not put in microwave)
- MAGE results with E. coli, obtained from Ying:

MAGE results from last Friday

After doing MAGE, you plated serial dilutions on plates with and without rifampicin to get colony counts (CFUs). The ratio of CFUs between the two plates gives you a conversion frequency.
Ryan's strain 1561 (EcNR2 enhanced Beta expression) was grown to OD 0.8, including a 90 minute heat induction at 42C.
Per reaction, 1mL of cells were washed 2x with cold sterile water and resuspended to a final volume of 50uL. 1uL of oligonucleotide (100uM stock concentration) was added and the cells were electroporated. After a 3 hour recovery, cells were serially diluted and 50uL were plated on LB and LB+100ug/mL rifampicin for CFU counts.
The oligonucleotide made a H526Y mutation in RNA polymerase

Table1

	A	B	C	D
1	**Sample**	**CFUs on LB**	**CFUs on LB + Rif**	**Conversion Rate**
2	Jinny	26x106	0.88x106	3.4%
3	Ying	22x106	2.10x106	9.5%
4	Zach	23x106	2.48x106	10.8%
5	No oligo control	168x106	33	1.9x10-7%

Table 1. MAGE data with E. coli; oligos used for rifampicin assay. Plated with serial dilution (26 x 106 = 26 colonies on a 106 dilution).

- Heat-shocking rhizobia (pORTMAGE is heat-activated) can lose plasmids (both nodulation and pORTMAGE possibly).
- Nodulation with varying amount of heat-shock to see if rhizobia lose nod plasmid.
- Inducible recombinases stress cells out
  - pORTMAGE uses temp. so no need to add small molecules.

WEDNESDAY, 6/29

- iGEM Meeting on Project Applications:
  - [ ] design oligos for increasing N2-fixation in R. tropici and S. meliloti
    - the terminal oxidase chain
    - the fix genes
    - nitrogenase
    - what should these oligos target? RBS... etc.
      - RBS calculator, Salis Lab.
  - [x] work on project description (due F)
  - [ ] plan for outreach goals
  - [ ] work on HTML
- Goals for today:
  - [x] make competent rhizobia cells (R. tropici colony 10) for the constructs to be transformed

THURSDAY, 6/30

- Goals for today:
  - [x] plate for competency of R. tropici cells using 7 serial dilutions.
- To perform MAGE with pORTMAGE: https://www.addgene.org/static/data/plasmids/72/72679/72679-attachment_SIjmYayfdiir.pdf (https://www.addgene.org/static/data/plasmids/72/72679/72679-attachment_SIjmYayfdiir.pdf)
  - Note that one must induce cells at 42C for 15 mins. before freezing down the cells.
- iGEM Wiki Page Meeting 1: http://2016.igem.org/Team:Yale (http://2016.igem.org/Team:Yale)
  - [ ] look at iGEM website to see what the Wiki requirements/rules are.
  - Team distribution:
    - Wiki regulations: Mindy
    - Wiki design/builders: Zach
  - always upload files on wiki server
  - Keep consolidated document of all text files
  - For next week:
    - basics of design
    - information to put on webstie
    - mock-up design pages (from other wikis)

FRIDAY, 7/1

- For today:
  - [x] made serial dilutions to test competency of transformed S. meliloti colony 1.
- When sterilizing seeds, need to remove fungi during germination; use sterile water (autoclaved milliQ or distilled H2O; dispense in laminar flow food) for any step when necessary.
  - use 5% hypochlorite solution (diluted Clorox) for 30 mins.
  - do in flow hood
  - Clorox has surface tension so need detergent (Tween-20, one drop with safety glasses and gloves) to wet surface of seeds.
  - Place in beaker with stirbar.
- Do 1 experiment where wash seeds with water (neg. control) to see if fungi grows.
  - Fungi gives most trouble because can feed from endosperm, esp. when tissue softens.
- To check if laminar flow hoods are good, test 30 mins. or 1 hr. with open LB
-

Week 6 Notes (5/July - 8/July/2016)

MONDAY, 7/4

- [x] test pORTMAGE in R. tropici (untransformed R. tropici, transformed R. tropici)
  - use protocol provided by Jaymin
- [ ] to make plate cultures for nodulation assay, take 5uL aliquot of competent cells and swab plates. Also swab plates of pORTMAGE rhizobia colonies.
- [ ] make liquid rhizobia colonies for nodulation
  - competent transformed R. tropici, transformed R. tropici, re-do untransformed R. tropici.

TUESDAY, 7/5

- Need to revise making stock of competent cells--heat shock at 42C for 15 min. before starting protocol.
- [x] Started nodulation assay
- [x] Counted colonies for R. tropici serial dilutions with PZP200 to ensure competency--successful growth on spectinomycin plates.

WEDNESDAY, 7/6

- Salis Lab RBS Calculator: https://www.denovodna.com/software/ (https://urldefense.proofpoint.com/v2/url?u=https-3A__www.denovodna.com_software_&d=CwMFaQ&c=-dg2m7zWuuDZ0MUcV7Sdqw&r=mrVg8_jY8xE9g19E-wzn2RR_e4ijyO30vXw7q00gFBU&m=y7eYIMyuE-uynAxA51LDGWdfTu_3OySwBNmRt4U7h30&s=xVzg5ywyIib35iBy5CWqTukGf2zMhvslHv3sLgo-zLU&e=)
  - From Jaymin: "You can use it to predict RBS strength or make new RBSs. But for you, you can use the "Optimize: Genome Editing" mode to automatically design oligos that mutate genomic RBSs to span a wide range of predicted strengths."
- When plating MAGE transformations, use LB; LB-kan; and LB-kan^{50}rif^{100}.
- [x] Made LB-kan^{50}rif^{100} plates for MAGE protocol

THURSDAY, 7/7

- rifampicin plates are marked with brown
- NO colony PCR of PZP200 to test competency of rhizobia cells--instead, will plate on sucrose-spec-kan plates b/c the PZP200 contains a LIC sites with sacB gene for sucrose fatality.
  - Expect no cell growth on sucrose plates if successful transformation.
- For MAGE, there will be four groups for both R. tropici and S. meliloti each:
  - 1. heat induce straight from freezer
  - 2. heat induce after transformation
  - 3. group 1 without oligos (neg. control)
  - 4. group 2 without oligos (neg. control)
- MAGE rifampicin oligos for CIAT 899: "P-THIO rpoB-CIAT 90mer 40 nm"; for S. meliloti: "P-THIO rpoB-Mel 92mer 40 nm"
- Wiki meeting 2:
  - [x] color scheme and mock-ups--Gloria.
  - [x] get bios and pictures for wiki and experiment.com page (next Monday)
  - [ ] finish workflow
  - [ ] MAKE ABSTRACT OF PROJECT DESCRIPTION

FRIDAY, 7/8

- Goals for today:
  - [x] Finish MAGE of transformed rhizobia

Week 7 Notes (11/July - 15/July 2016)

MONDAY, 7/11

- Update to nodulation assay:
  - Over the weekend, paper dried out, so unsure if beans will continue growing (roots were observed for some of each bean--cowpea/black-eyed and pink--but not all).
  - Revised protocol:
    I. Make 5% NaClO4 (bleach) sterilizing soln, with a few drops of Tween-20.
    II. Soak beans in beaker with sterilizing soln for 15 mins. on table-top shaker.
    III. Wash beans several times with dH2O, then soak in dH2O overnight to imbibe seeds.
    IV. The following day, place beans on nodulation apparatus (4-5 per bag) using forceps.
- Results of competency test using PZP200:
  - negative control (spec+kan): expected growth of all colonies
  - **experimental** (spec+kan, +sucrose): unexpected growth of all colonies (S. meliloti had less growth, but still present)--sacB gene in PZP200 should have induced sucrose fatality.
  - possible problems: promoter for sacB gene does not work with rhizobia
- Results of rifampicin assay with pORTMAGE-3 transformed R. tropici and S. meliloti:
  - negative controls (streaked, not dilution): all as expected; growth on kan, no growth on kan+rif.
  - R. tropici:
    - #1: control--all growth (10^{-1} plate had more colonies than orig.). experimental--no growth
    - #2: control (kan)--all growth, with dilutions at a good rate. experimental (kan+rif)--no growth (problem with cell clump?), but contamination on original conc. plate
  - S. meliloti:
    - #1: control--all growth, with contamination on plate 10^{-5}. experimental--growth on orig. conc. plate; contamination on plates $10^{-1\ -4\ -5}$.
    - #2: control--most growth except $10^{-5\ -6}$ (too dilute?), contamination on 10^{-6}. experimental--nothing.
  - Possible problems:
    - Plating clump of cells
    - contamination on rifampicin and kanamycin plate
    - heat inducing pORTMAGE? **what does the heat induce?**
  - implications:
    - rhizobia does not lose pORTMAGE-3 plasmid during MAGE/heat inducing
    - one growth on S. meliloti experimental is promising... how to determine if from oligos or from random mutation.
- Next steps:
  - re-try with modifications
    - heat-shock before freezing--or, no freezing --> straight to MAGE
  - attempt to optimize recombinases in separate vector, b/c recombinase in pORTMAGE-3 is beta for E. coli
- Goals for today:
  - [x] re-streak transformed rhizobia colonies on fresh plate (1 of R. tropici, 1 of S. meliloti)
  - [x] pour LB-kan^{50} and LB-$kan^{50}rif^{100}$ plates for further MAGE testing.
- iGEM meeting week 7:
  - knock-out mutS and mutL in rhizobia
  - test MAGE with recombinases
  - follow-up sequencing of 4 colonies for point mutation in MAGE
  - follow-up PCR of PZP200 fragment in competency test
  - shaking water bath--temp. shift to help conducing, plus aeration/stimulation of cells
  - ***show work-flow of method
  - validate other pORTMAGE plasmids for different Ab markers
  - research mismatch repair system in rhizobia

TUESDAY, 7/12

- Goals for today:
  - [x] check in on nodulation: water bags
- Issues to focus on:
  - make plates on MC and bring to WC; also with transformed strains
  - we have stocks of competent, untransformed rhizobia--for if we don't use pORTMAGE
    - each has EC and piG number--label plates as such
  - 1. EC POM1 and POM2 = verified rhizobia to have plasmid
    - on WC: streak stocks for single colonies; pick 1 colony and make induced comp. cells (iCC1 and iCC2) to pre-heat induce for rifampicin assay
      - 50-mL preps of comp. cells with separate starter culture
        - 50-mL because of 50-mL LB, on shaker --> heat induce --> 50-mL Falcon tubes to spin down at 0C --> test comp. cells
      - leftover cells: freeze down in glycerol in -80C and see if after freeze, MAGE still works?
    - control: without oligos (don't plate without rifampicin)
    - experimental: with oligos
    - OD reader to calculate number of cells
  - 2. test competent cells first with PZP200 in untransformed comp. cells.
    - also make plates there and do parallel testing
  - 3. transform CCps with the recombinases
  - EC can have multiple pIGs if multiple plasmids
  - sequence of mutS and mutL? to knock out both
- when streaking plate from glycerol stock, do not let glycerol thaw (scrape from top), and use sterile wood sticks under sterile conditions to streak. Then pick single colony for growing liquid culture.
- For future:
  - [ ] obtain recombinases from Jinny and transform into rhizobia.

WEDNESDAY, 7/13

- To do today:
  - [x] primer design and order for rifampicin assay
  - [x] PCR and AGE of PZP200 fragment to verify competency and successful transformation in rhizobia
    - pYU 3154-19 with PriG 3381 and

THURSDAY, 7/14

- To do today:
  - [ ] grow rhizobia transformant plates
    - [ ] make new glycerol stocks
  - [ ] once rifampicin primers (4) arrive, make sequencing reactions and do colony PCR, to ensure the rif colonies for S. meliloti (4 total) have the correct point mutation.
    - [ ] also, will re-patch plate of the colonies grown on the rif plates (and negative control)
    - [ ] pick up wild type allele with colony from no oligo (control)
  - [x] made colony 10 (R. tropici) liquid culture (2X, one for MC and one for WC). Waiting on colonies 1 and 3 (S. meliloti)
- For near future:
    - [ ] test rhizobia transformation on WC first, with the PZP200 vector (cells on dry ice)

- [ ] once liquid cultures of transformed rhizobia are grown, make correct heat-induced, competent rhizobia cells (50 mL sample).
    - [ ] WC: perform MAGE again with corrected heat-induce (must stir for the 15 min. water bath) in rhizobia and proper electroporator
- [ ] make WC box of both strains: untransformed, untrans-comp, transformed, trans-comp (8 total, dry ice)
- [ ] drop liquid culture of rhizobia on seed of nodulation, with H2O if needed, and it will seep down to roots

Week 8 Notes (18/July - 22/July/2016)

MONDAY, 7/18

- Goals for today/this week:
  - [x] Re-setup nodulation apparatus with larger perforations and 3 seeds per bag.
    - [ ] Grow liquid cultures of untransformed, pORTMAGE-transformed, and pORTMAGE/MAGE-transformed R. tropici AND S. meliloti--for future nodulation.
      - [x] untransformed rhizobia (started)
      - [x] transformed rhizobia
      - [ ] 2X transformed rhizobia
  - [x] Continue growing liquid cultures of pORTMAGE-transformed R. tropici and S. meliloti to prepare for MAGE (induce competency, heat-induce, test MAGE)

TUESDAY, 7/19

- Goals for today/this week:
  - [x] Make glycerol stocks (2X per each stock) of transformed cells; do untransformed cells tomorrow.
    - R. tropici colony 10; S. meliloti colony 1; S. meliloti colony 3. ALL pORTMAGE-transformed.
  - [ ] Set up **sequencing reactions** for both sets of the two primers for rifampicin assay (R. tropici and S. meliloti colonies)--using the PCR products from last week's AGE.
  - [x] Re-attempt **colony PCR** on R. tropici and S. meliloti rifampicin assay (Gloria).
  - [ ] **Re-perform MAGE** at WC with correct heat induction of competent, transformed cells. (Bring glycerol stocks).
    - [ ] Induce competency before taking cells to WC.
    - [ ] Bring both LB-kan^{50} and LB-kan^{50}rif^{100} plates.
      - For 1X experiment: 2X (1 kan, 1 kan-rif glass beads) of 7 dilutions (orig. conc. to 10^{-6}) plus 2 neg. no-oligo controls (1 kan, 1 kan-rif streak) = 16 plates (8 of each) per experiment.
      - Will do 1X experiment per strain --> 2X experiments total per group with 2 groups --> 4X total (untransformed control; pORTMAGE- and MAGE-transformed)
      - Total of 2X, 32 = 64 plates for pORTMAGE-transformed rhizobia
        - Do not plate dilutions on untransformed cells, because expect such low transformation efficiency. **Just plate original cell conc.?**
- Things done today:
  - Transferred untransformed, electrocompetent R. tropici and S. meliloti (2X each) AND S. meliloti #3B pORTMAGE-transformed 3-mL stocks to 50-mL LB.
  - Transferred pORTMAGE-transformed R. tropici and S. meliloti (2X tropici and 1X meliloti) 50-mL stocks to NEW 50-mL stocks (took 2 mL of old stock).
  - Made 2X (OML and WC) glycerol stocks of transformed rhizobia: 2X (A and B) of each colony
    - Colony #1 and #3 for S. meliloti
    - Colony #10 for R. tropici
    - Need to do glycerol stock of #3B for S. meliloti
  - Also streaked plates for continued growth of these colonies (2X: WC and OML).

WEDNESDAY, 7/20

- Things done today:
  - Made 2X glycerol stocks of untransformed, electrocompetent R. tropici and S. meliloti (2X each, #1 and #2) AND S. meliloti #3B pORTMAGE-transformed 3-mL stocks.
  - Streaked plates of these rhizobia.
  - Gloria--ran AGE of the PCR products from yesterday.
    - PCR conditions:

95C	5 min.	
98C	2 min.	2X
64C	15 sec.	
72C	30 sec.	
98C	30 sec.	33X
64C	15 sec.	
72C	30 sec.	
72C	2 min.	
4C	hold	

- To plan for outreach meeting this afternoon:

"Building with Biology (BwB) activity leaders

· Planning and organizing BwB activity

· Coordination with Peabody museum

· Plan and lead BwB volunteer orientation"

  - Will contact Peabody Museum.
  - Will organize/plan BwB volunteer orientation.

- Outreach meeting notes:
  - ask Natalie for Peabody Museum contact--**when and where** (at museum)
  - pick one (or more) of the six activities, depending on demographics, for activity
  - develop library idea for older audience
- Experiment.com notes:
  - making a video
  - revise Methods section

THURSDAY, 7/21

- Things done today:
  - .
- Wiki meeting notes:
  - .
-

Week 9 Notes (25/July - 29/July/2016)

MONDAY, 7/25

- To do:
  - [ ] re-streak needed plates
  - [ ] grow 1-mL culture of rhizobia for repeat of colony PCR for rifampicin fragment
  - [ ] start nodulation set-up for chemical mutagenesis
- Notes on MAGE trial 1 plates (8/July/2016):
  - No growth observed on negative control plates on rif^{100}: "MAGE R. trop #3, #4 (-)" and "MAGE S. mel #3, #4 (-)"
  - No growth on S. mel experimental plates (rif^{100}) from 10^{-2} to 10^{-6}
  - Growth observed on positive control plates (kan^{50} only): R. trop (#1 and #2) and S. mel (#1 and #2)
  - No growth on R. trop experimental plates from #2 10^{-4} to 10^{-6} or #1 10^{-1} to 10^{-6}
- iGEM Meeting Notes:
  - work on 2-week chemical mutagnesis and time nodulation growth.
  - make workflow of MAGE protocol
  - Knock-out mutL in rhizobia to compare to mutL-DN insert in pORTMAGE
  - **make scheme** for transforming recombinase cassettes into pORTMAGE-transformed rhizobia, or modifying pORTMAGE itself.
  - use primers that have previously worked with R. trop to ensure working with colony PCR
    - or, design new primers
  - write goals for what to present at iGEM Competition
    - conservative vs. ideal/reach (3 mo.)

THURSDAY, 8/4

WEEK 10: N/A

Week 11 Notes (8/August - 12/August/2016)

MONDAY, 8/8

- Notes from Gloria (Week 10):
  - attempted to optimize colony PCR in R. tropici: pre-lysing the cells in 0.1% Triton X in pH 8.0 TE buffer at 100C for 10 minutes; centrifuge to pellet the cell debris and only take the supernatant for PCR; use 0.5 uL of template.
  - conducted gradient touchdown PCR (doing 5 cycles at (X)C, 5 cycles at (X-1)C and 25 cycles at (X-2)C) to identify the best PCR conditions (to reduce problems with non-specific priming): annealint temps. of 68.2, 67.2, and 66.2C
  - then conducted pre-lysing and colony PCR on putative positive colonies; desired band present but faint. Non-specific priming still present. **PCR products need to be gel-purified before submitting for sequencing**.
  - to **verify rifampicin assay**: insert a mutant copy of the gene into a vector that we use to transform *S. meliloti/R. tropici*. Do this by amplifying the gene in 2 fragments with the mutation (introduced using the primers) contained in the overlap between the two.
    - Gloria designed the 4 primers; double-check on 4th (reverse of 2nd fragment).
    - before ordering, check with Dr. Moreno on how to add the LIC overhangs (on the 1st and 4th primers).
- Objectives for the next 2 weeks:
  - [ ] Conduct a gel purification of *R. tropici* PCR + submit for sequencing
    - run AGE of good PCR templates and gel-purify for sequencing.
  - [ ] Set up chemical mutagenesis experiment
  - [ ] Verify the rifampicin resistance assay
    - Use LIC to insert mutated gene into PZP200
    - Transform *S. meliloti* and *R. tropici* and test for rifampicin resistance
    - [ ] re-check 4th primer and read how to do LIC overhang.
  - [ ] **Title and Abstract**
  - [ ] finish nodulation assay (correct heat-induced cells)--grow roots, inoculate with heat-induced untransformed, and transformed cells.
  - [ ] optimize pORTMAGE system in rhizobia.

TUESDAY, 8/9

- Done today:
  - [x] Start nodulation assay with high-contrast (blue) nodulation apparatus for: untransformed (-) and pORTMAGE-transformed (E) R. tropici and S. meliloti after heat-shock (no need to do after MAGE, as well).

WEDNESDAY, 8/10

- To design primers for rifampicin verification (designed by GW; notes from Dr. Moreno):
  - use LIC to incorporate the predicted rifampicin resistance gene into a vector for transforming rhizobia in order to confirm that the SNP does indeed cause rifampicin resistance in rhizobia (and not just E. coli).
  - do a PCR of the gene in two fragments that overlap in the spot containing the mutation. GW designed the primers, but was wondering how to add the LIC overhang.

  First segment:
  F-GGTCTCGGCCTCAAGGAA
  R-GGCGCTTGT**a**GGTGATTTCCG

  Second segment:
  F-TCGGAAATCACC**t**ACAAGCG
  R-GGACGCGGCCGAAtaa
  - MM: if you clone the gene in to the LIC vector, you need to make sure you have a promoter and a terminator. Not just the coding sequence.

- Are you planning to amplify the complete gene from Rhizobium and introduce the mutation? If so, generate your insert using overlapping PCR in order to introduce the mutation you postulate will confer rifampicin resistance.
- We have a vector that is PZP200b-with just the LIC cassette. This would be perfect for this experiment. I can't remember the pYU number of the top of my head. Look in the database.

The LIC 5' extension should be: 5'-TACTTCCAATCC-3'
The LIC 3' extension should be: 5'=TATCCACCTTTACTG-3'

- This means, the 5'end of the target gene needs to have the 5'LIC sequence added to the primer. The 3'end of the target gene also needs to have the LIC 3' sequence added.
- Since you need to amplify the target gene with the mutation that confers resistance to rifampicin, make sure the primer pair that you generate to introduce the mutation is at least 23-25 bp long so you have enough homology between the two fragments you need to join to make the complete gene.