Development of a Novel System for Recombineering in *Mycoplasma*

Clair Mitzelfeldt
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Minion Laboratories
Overview

- *Mycoplasma* background
- Project description
- Methods
- Results
- Future Studies
Mycoplasma

- Smallest self-replicating organisms
- Evolved from Gram-positive bacteria
- Lack a cell wall
- Minimal genomes
- Strict nutrient requirements
- Reliant on host macro-molecules
**Pathogenesis**

- Colonizes mucosal surfaces
- Important diseases:
  - Atypical pneumonia (humans)
  - Contagious Bovine Pleuropneumonia (cattle)
  - Porcine Respiratory Disease Complex (pigs)
- Resistant to β-lactam antibiotics
  - Difficult to treat due to lack of cell wall
- Vaccines are partially effective in production animals
Project Goal

Develop gene-specific mutagenesis in *Mycoplasma hyopneumoniae* to better understand pathogenic mechanisms that lead to chronic infections in pigs.
Experimental Design

1. Synthesize an antibiotic marker with a promoter that will function in both *E. coli* and mycoplasmas
   - Fragment also must have unique flanking sequences
   - *tetM* and *pur* genes will be used along with promoters from *aac-aph* (gentamycin marker of Tn4001) and Spirilin (*Spiroplasma*)

2. Generate PCR products of the construct
   - Purify, digest with nt.*Bsp*QI enzyme, ΔT, repurify
   - Add gene-specific oligos, ligate, repurify to remove salts

3. Transform into *E. coli*
   - Antibiotic resistance requires RecA-catalyzed recombination of fragment into gene specified by oligos (*lacZ* for this project)

4. For the purposes of this study, selection of transformants occurred via tetracycline resistance and the colonies were scored for β-galactosidase activity
Results – Experiment 1

• Designed plasmid with tetracycline resistance cassette (tetM) with aac-aph promoter

• PCR Result:
  • Primers were not specific enough
  • Second, unexpected fragment was generated in PCR (2kb) and concentration was low
  • Found a second site for one PCR primer in the cloning vector

• Solution:
  • Redesigned PCR primer – yields were consistent and only one fragment was generated
Experiment 2

• Generated fragment, added oligos and exogenous RecA protein; transformed mixture directly into RecA+ and RecA- E. coli strains using electroporation

• Result:
  • Repeatedly arced
  • Reason: high salt concentration in RecA buffer

• Solution:
  • Transform the strains using CaCl₂ competent cells
  • Selected for tetracycline resistant colonies
  • Screened for β-galactosidase activity
• CaCl₂ transformation results – Tet⁺ colonies with both Rec+ and Rec- cells with RecA protein added exogenously

X289 (recA+)  
( + )  
N7568(recA-) + RecA
Experiment 3

- Repeat Exp 2 but with phosphorylated oligos, added ligase, purified product
- Transformed into X289 (recA+)
- Results:
  - Successful transformation
  - Slow growing colonies
  - Low β-galactosidase activity with TetR
Future Studies

• Does *M. hyopneumoniae* have recombination activity?
  • RecA homolog should be
Future Studies (Continued)

- Test puromycin resistant determinant with *Spiroplasma* spiralin promoter in *E. coli*
- Prepare fragment with ligated oligos for transformation into *M. hyopneumoniae*
  - Target is P97, the ciliary adhesin
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