Development of a Novel System for Recombineering in *Mycoplasma*

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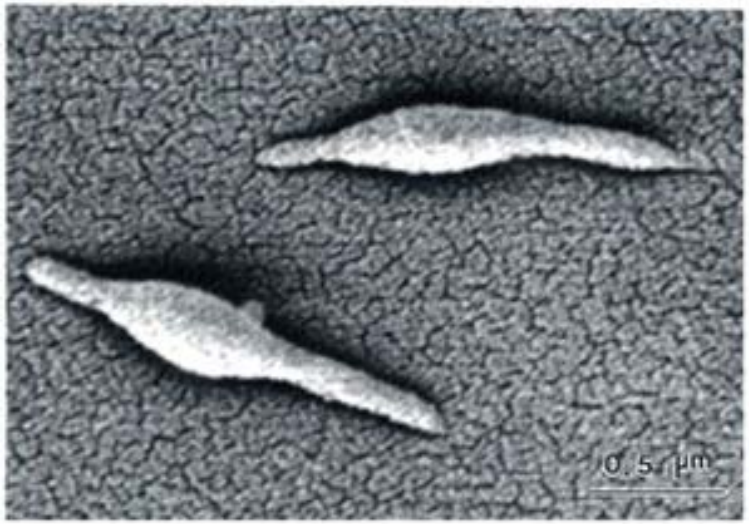
Overview

- *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

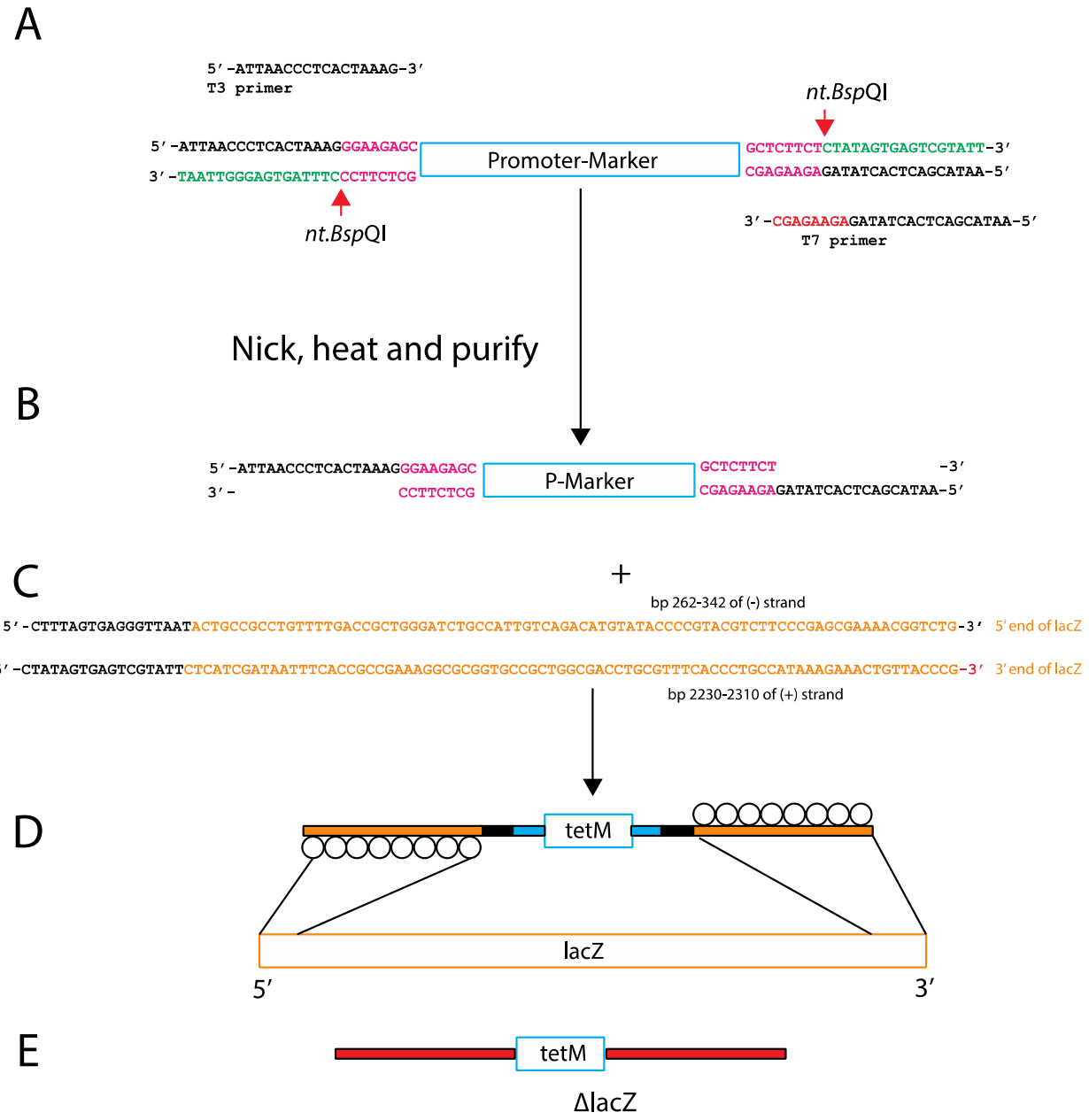
- Colonizes mucosal surfaces
- Important diseases:
  - Atypical pneumonia (humans)
  - Contagious Bovine Pleuropneumonia (cattle)
  - Porcine Respiratory Disease Complex (pigs)
- Resistant toβ-lactam antibiotics
  - Difficult to treat due to lack of cell wall
- Vaccines are partially effective in production 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 Tn*4001*) 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


A
5′-ATTAACCCTCACTAAAG-3′
T3 primer
nt.BspQI
5′-ATTAACCCTCACTAAAGGGAAGAGC
3′-TAATTGGGAGTGATTTCCCTTCTCG
Promoter-Marker
GCTCTTCTCTATAGTGAGTCGTATT-3′
CGAGAAGAGATATCACTCAGCATAA-5′
nt.BspQI
3′-CGAGAAGAGATATCACTCAGCATAA-5′
T7 primer
Nick, heat and purify
B
5′-ATTAACCCTCACTAAAGGGAAGAGC
3′-
CCTTCTCG
P-Marker
GCTCTTCT
-3′
CGAGAAGAGATATCACTCAGCATAA-5′
C
+
bp 262-342 of (-) strand
5′-CTTTAGTGAGGGTTAATACTGCCGCCTGTTTTGACCGCTGGGATCTGCCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTG-3′
5′ end of lacZ
5′-CTATAGTGAGTCGTATTCTCATCGATAATTTCACCGCCGAAAGGCGCGGTGCCGCTGGCGACCTGCGTTTCACCCTGCCATAAAGAAACTGTTACCCG-3′
3′ end of lacZ
bp 2230-2310 of (+) strand
D
tetM
lacZ
5′
3′
E
tetM
ΔlacZ

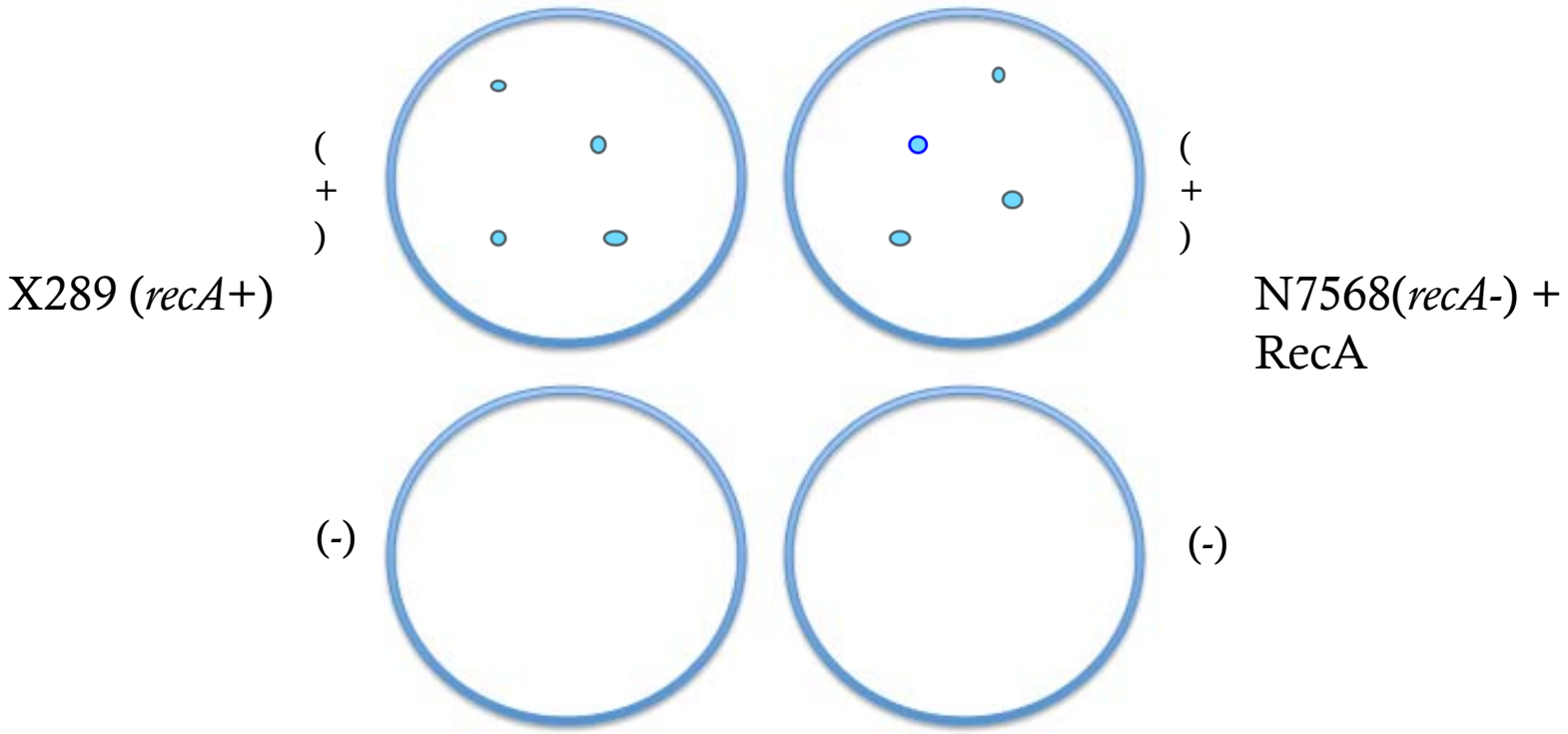
Results – Experiment 1

- 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

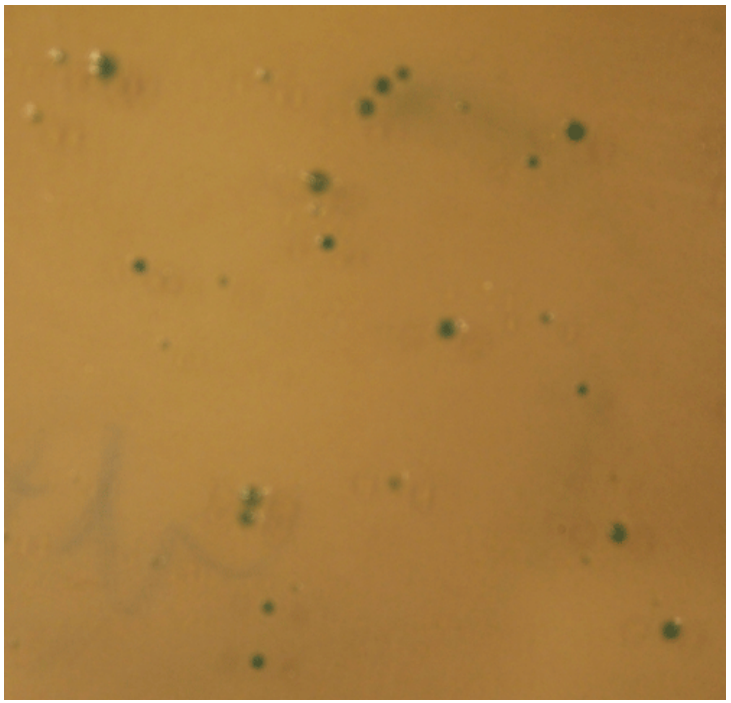
- Generated fragment, added oligos and exogenous RecA protein; transformed mixture directly into RecA+ and RecA- *E. coli* strains using electroporation
- Result:
  - Repeatedly arced
  - Reason: high salt concentration in RecA buffer
- Solution:
  - Transform the strains using $CaCl_2$ competent cells
  - Selected for tetracycline resistant colonies
  - Screened for β-galactosidase activity

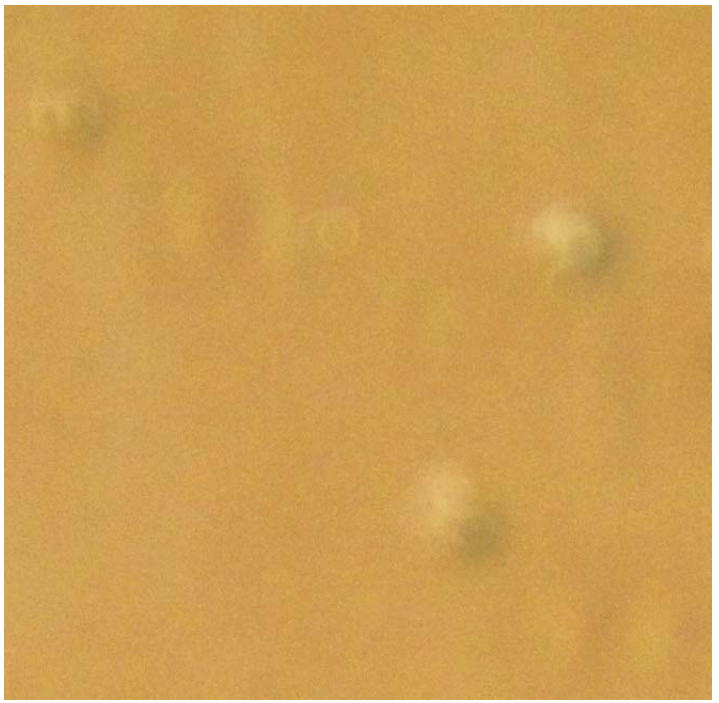
- $CaCl_2$ transformation results – Tet^R colonies with both Rec+ and Rec- cells with RecA protein added exogenously



Experiment 3

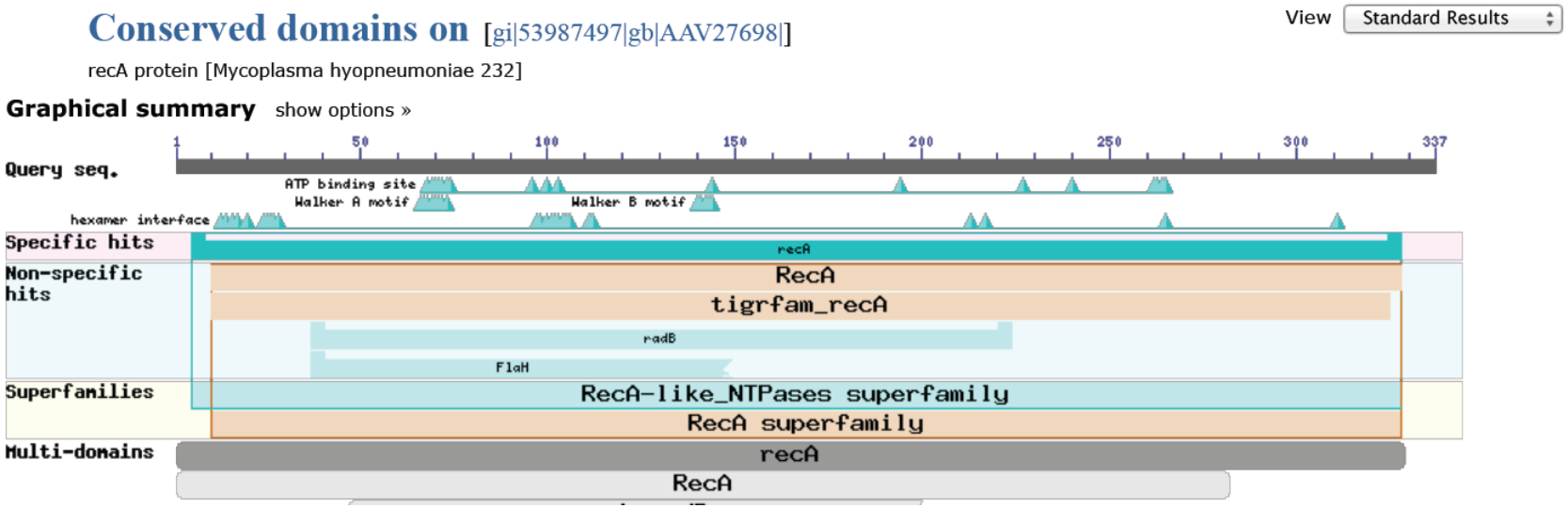
- Repeat Exp 2 but with phosphorylated oligos, added ligase, purified product
- Transformed into X289 (*recA*+)
- Results:
  - Successful transformation
  - Slow growing colonies
  - Low β-galactosidase activity with Tet^R





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

Acknowledgements

- Dr. F. Chris Minion
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