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Dual-Plasmid Technology for Precision Genome Editing in *Staphylococcus aureus*

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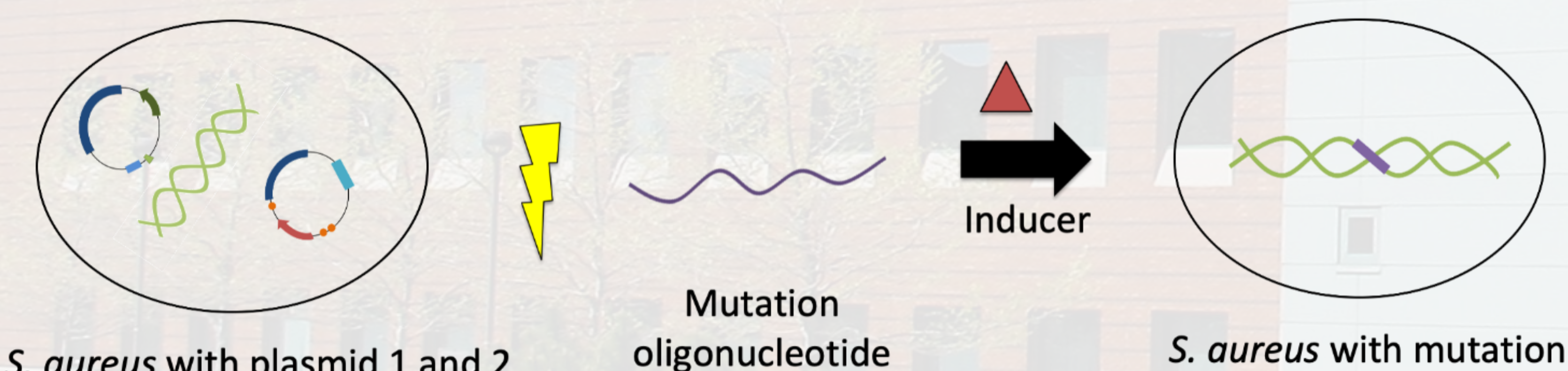
ABSTRACT

Background
Genetic manipulation of *Staphylococcus aureus* (both methicillin sensitive *S. aureus*, MSSA, and methicillin resistant *S. aureus*, MRSA) poses a technical challenge due to poor transformation efficiency, limited endogenous DNA repair activity, lineage-specific methylation patterns and intrinsic resistance to common selectable markers.

Methods
To address transformation efficiency we have optimized electrocompetent cell preparation and electroporation protocols for staphylococci. Further, we have improved a CRISPR counterselection platform that delivers a heterologous ssDNA recombinase and an inducible Cas9 endonuclease. When used for recombineering and counterselection, this strategy allows minimization of the number of elements necessary to transform in a single electroporation event. The Cas9 delivery platform has been modified to include a range of selectable markers including resistance to apramycin, erythromycin, kanamycin, nourseothricin, spectinomycin or trimethoprim.

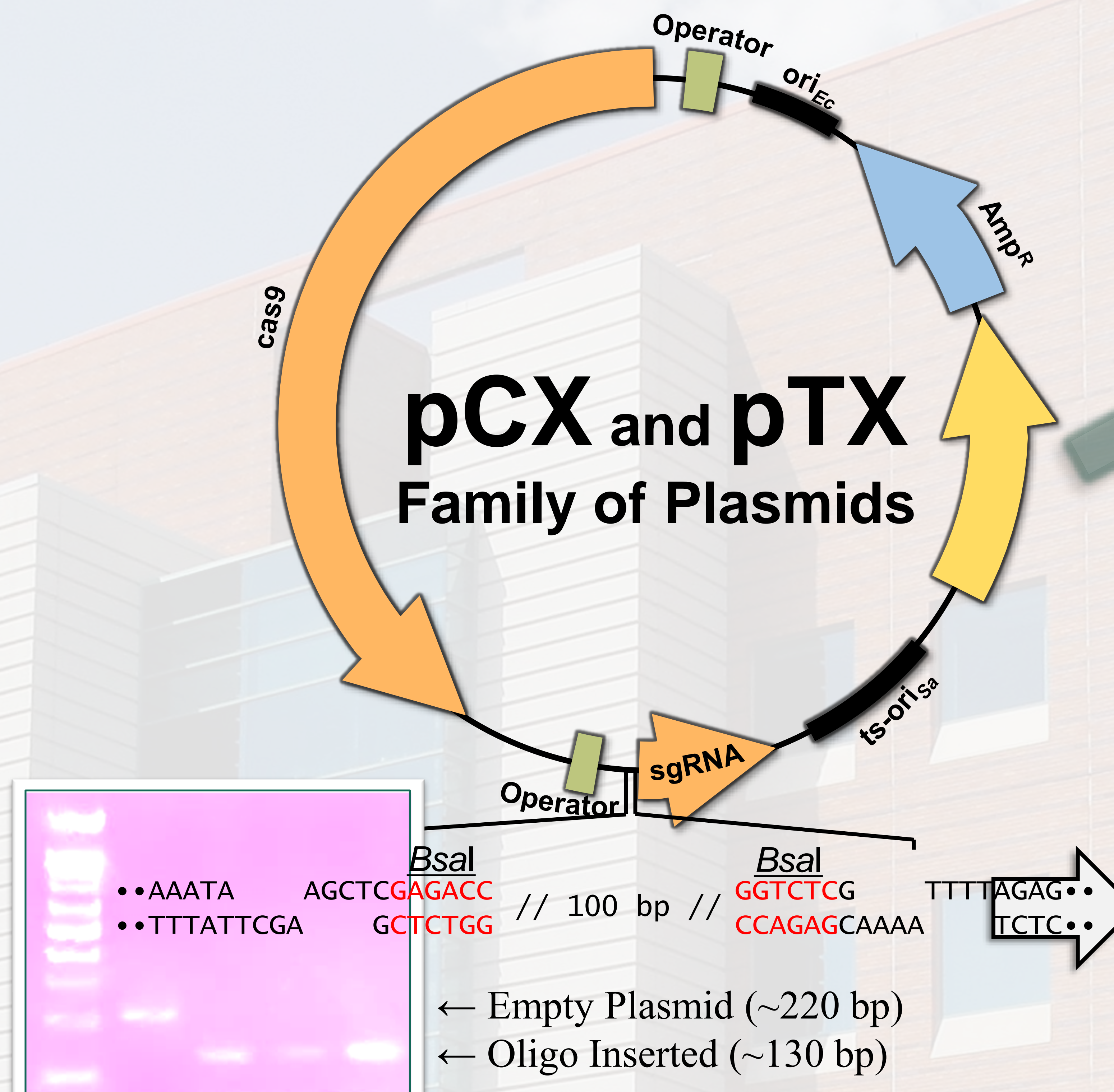
Results
Overall electroporation efficiency increased by multiple orders of magnitude (> 100 ×) using the optimized cell preparation protocol. The CRISPR delivery platform can be stably maintained in a repressed state for multiple generations and induced with anhydrotetracycline. We have introduced targeted mutations in *rpoB* using this system with an average turnaround time of 12 days.

Conclusion
This improved dual-plasmid CRISPR platform is robust and allows the investigator to rapidly and specifically alter the genomes of staphylococci. These tools will facilitate the study of how specific genetic polymorphisms contribute to various phenotypes in *S. aureus*, including the virulence of MRSA.



- Repressor on pTetRec allows propagation of pTX
- Addition of inducer turns on Cas9 selecting for mutant formation
- Both plasmids are lost following incubation above 35° C

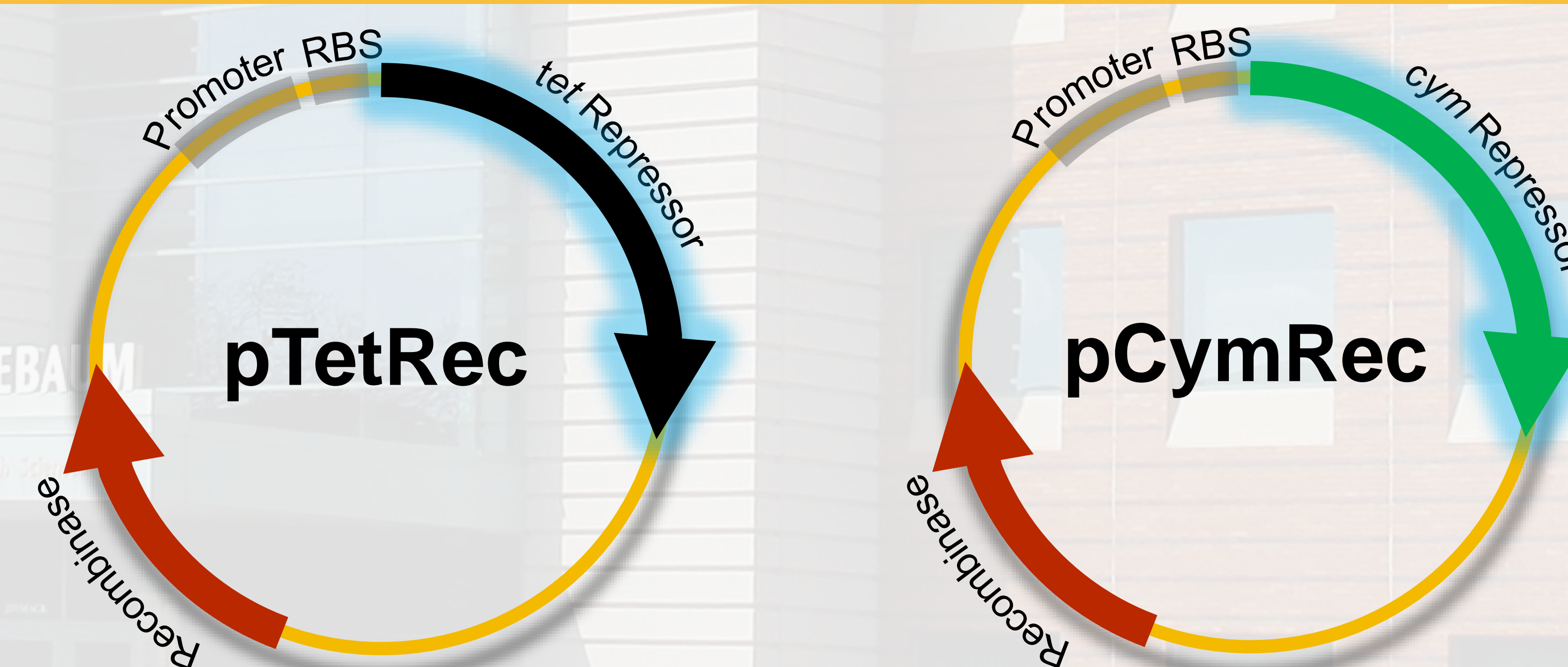
CRISPR PLASMIDS



← Empty Plasmid (~220 bp)
← Oligo Inserted (~130 bp)

- Facilitates rapid insertion and screening of target site-specific oligonucleotides

RECOMBINASE PLASMIDS



- Facilitates CRISPR-Cas9 induction with either anhydrotetracycline or cumate

SELECTABLE MARKERS

Antibiotic	Gene	Plasmid Name	Marker Source
Apramycin	*apmA*	pCA, pTA	*S. aureus* str Rd 11
Erythromycin	*ermC*	pCE, pTE	*S. aureus* str SK1396
Kanamycin	*aph(3')-IIIa*	pCK, pTK	*S. aureus* str J01
Nourseothricin	*sat4*	pCN, pTN	*S. aureus* str J01
Spectinomycin	*aad(9)*	pCS, pTS	*S. aureus* str N315
Trimethoprim	*dfrG*	pCT, pTT	*S. aureus* str J01

- Facilitates recombineering in clinical isolates with diverse resistance profiles

RESULTS

- Generated H481Y mutation in *rpoB* conferring a rifampin resistance trait in *S. aureus* str J01 using pTS-*rpoB* and pTetRec, **validating** the mutagenesis system.
- Identified need for anhydrotetracycline as a **barrier to broad applicability** of system
- Diversified pTX family of plasmids to incorporate CuO operators
- Modified pTetRec plasmid to incorporate CymR repressor
- Cumate induction system is currently undergoing validation to ensure continuous repression of Cas9 in the absence of cumate

REFERENCES

1. Ballal A, Manna AC. Regulation of superoxide dismutase (sod) genes by SarA in Staphylococcus aureus. *J Bacteriol*. 2009;191(10):3301–3310.
2. Penewit K, Holmes EA, McLean K, Ren M, Waalkes A, Salipante SJ. Efficient and Scalable Precision Genome Editing in *Staphylococcus aureus* through Conditional Recombineering and CRISPR/Cas9-Mediated Counterselection. *MBio*. 2018;9(1):e00067-18.

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