PROJECT SUMMARY
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Expand the Capabilities of a CRISPR-CAS9 System for Sequence-Specific Elimination of Foodborne Pathogens in Beef Production

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Study Completed

October 2017

This project was funded by the Beef Checkoff.

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Background

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated proteins (Cas) together comprise the CRISPR-Cas system. In bacteria, this system confers an adaptive immunity against invading mobile elements like viruses and plasmids (Barrangou and Marraffini, 2014; Wiedenheft et al., 2012). The CRISPR-Cas9 system has emerged as a programmable and versatile tool for precise genome editing in a wide variety of organisms. In contrast, the current study focused on exploiting the CRISPR-Cas9 system for selective killing of a bacterial pathogen relevant to meat safety by targeting specific virulence genes.

The CRISPR-Cas9 system contains two key factors: the Cas9 protein, which can cleave double-stranded DNA, and guide RNA (gRNA) which is transcribed from the CRISPR sequence. The function of the Cas9 protein is like a pair of scissors. Guide RNA is complimentary to the target sequence and is able to guide the Cas9 protein to the sequence specific site of cleavage. The interaction between the Cas9 protein and the target DNA leads to the creation of a doublestranded DNA break. Such DNA cleavage can be used either to edit genes or to kill organisms. In recent studies, CRISPR-Cas9 systems have been explored for developing sequence-specific antimicrobials, which means that by targeting the cleavage of specific sites in a genome of interest, the kill should be limited to only those organisms containing that specific gene or genes (Bikard et al., 2014; Citorik et al., 2014).

Objectives

The project objectives were to provide proof-of-concept evidence that the CRISPR-Cas9 system with gRNA can selectively kill pathogenic bacteria by targeting a specific gene of choice.

Methods

In this study, gRNAs were designed and cloned targeting Shiga toxins (*stx1* or *stx2*) and used a two-plasmid platform to deliver this Shiga toxin specific CRISPR-Cas9 system into bacterial cells for specific killing of Shiga toxin-producing *Escherichia coli* (STEC). In this way, the CRISPRCas9 system could be programmed to selectively kill pathogens that harbor the Shiga toxin genes, while leaving those non-target bacterial populations unaffected.

Shiga toxin gRNAs were designed by screening Shiga toxin gene sequences for NGG on the 3' side. The designed 20-nucleotide gRNA was then cloned into a CRISPR plasmid (pCRISPR) and resulted in a CRISPR plasmid with a gRNA (pCRISPR w/gRNA) (Jiang et al., 2013). The successful cloning of gRNA was confirmed by Sanger Sequencing. A Cas9 plasmid (pCas9), which can express the Cas9 protein constitutively, and pCRISPR w/gRNA were used to introduce the two key factors (Cas9 protein and gRNA) into *E. coli* O157:H7 Sakai cells (Jiang et al., 2013). These two plasmids were introduced into *E. coli* O157:H7 Sakai cells sequentially by electroporation: first the pCas9 was introduced into *E. coli* O157:H7 cells and then the pCRISPR w/gRNA was introduced into the recipient *E. coli* O157:H7 cells containing the pCas9 plasmid. In addition, pCas9 and pCRISPR without a gRNA were introduced into *E. coli* O157:H7 Sakai cells as controls. After electroporation, transformed cells containing the different pCRISPRs (with and without gRNA) and pCas9 were plated onto appropriate culture media to enumerate surviving cells. When the pCRISPR w/gRNA was introduced into the recipient *E. coli* O157:H7 cells containing pCas9 plasmids, an approximately 2 log lower number of *E. coli* O157:H7 cells was obtained compared to that of the control pCRISPR without gRNA (Table 1). This result provided evidence that introduction of a CRISPR-Cas9 system targeting Shiga toxin genes can achieve sequence specific killing of STEC.

Killing of target cells using the CRISPR-Cas9 system could be highly related to its gRNA sequence. In the next step, three gRNAs were designed that targeted the *stx1* gene (stx1_1, stx1_2 and stx1_3) at different locations, and two gRNAs that targeted the *stx2* gene (stx2_1 and stx2_2) at different locations. We compared their efficiencies in killing *E.*

coli O157:H7 Sakai cells. For the *stx1* gene, we found no significant ($P > 0.05$) differences in cell reductions of cells that contained the stx1_1 (2.08 log reduction) and stx1_2 (1.53 log reduction) gRNAs (Figure 1). In comparison, however, significantly ($P < 0.05$) lower cell reductions were observed for cells containing the stx1_3 gRNA (0.38 log reduction) (Figure 1). For the *stx2* gene, no significant ($P > 0.05$) differences in cell reductions were obtained between cells that contained the stx2_1 (2.64 log reduction) and stx2_2 (2.23 log reduction) gRNAs (Figure 1). This result demonstrated varied reductions of *E. coli* O157:H7 Sakai cells when different gRNAs were used, suggesting that gRNAs are critical factors that determine the killing efficiencies of STEC.

Then, two gRNAs were cloned into a single pCRISPR resulting in a single plasmid containing two gRNAs named pCRISPR::stx1_1::stx2_2. This newly created pCRISPR::stx1_1::stx2_2 can target both Shiga toxin genes (*stx1* and *stx2*) at the same time and result in simultaneous cleavage of the *E. coli* O157:H7 chromosome at these two gene sites. The pCRISPR with two gRNAs achieved significantly greater ($P < 0.05$) reductions of *E. coli* O157:H7 cells (ca. 3.2 log reduction) compared to pCRISPRs with only a single gRNA (ca. 2.1 to 2.5 log reduction) (Table 2).

Important Findings

This study provides proof-of-concept evidence that the CRISPR-Cas9 system with gRNA can selectively kill pathogenic bacteria by targeting a specific gene of choice. Furthermore, killing efficiencies of the CRISPR-Cas9 system can be improved by optimizing the designs of gRNAs and the use of multiple gRNAs. The CRISPR-Cas9 system changes the way we traditionally think about reducing or eliminating pathogen contamination on meat products.

Industry Impact

This is important for the meat industry since the CRISPR-Cas9 system could serve as a novel antimicrobial intervention for the control of foodborne pathogens. For example, gRNAs can be designed to: (i) target virulence genes for sequence-specific removal of pathogenic bacteria, (ii) target antimicrobial resistance genes for killing of antibiotic resistant bacteria, and (iii) target genes involved in biofilm development and formation of bacterial persister cells in biofilms, and to improve sanitizer efficiency against biofilms in meat processing environments. Further research will focus on developing a delivery system that allows us to apply the CRISPR-Cas9 system in real meat producing and processing environments.

References

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Tables/Figures

Table 1: Viable cells (log CFU/reaction) after electroporation of pCRISPR, pCRISPR w/oligo, and pCRISPR w/stx into the recipient *E. coli* O157:H7 cells containing pCas9 plasmids.

	Viable cells: log CFU/reaction		
	pCRISPR	pCRISPR w/oligo	pCRISPR w/stx gRNA
Replication 1	4.20	4.64	2.14
Replication 2	4.05	3.84	2.05

Table 2: Viable cells (log CFU/reaction) after electroporation of pCRISPR with single and multiple gRNAs targeting *stx1* and *stx2* genes into the recipient *E. coli* O157:H7 cells containing pCas9 plasmids. Mean bacterial counts, in the last column, with different upper-case letters are significantly different ($P < 0.05$).

	Viable cells: log CFU/reaction				
Plasmids with different gRNA	Replication 1	Replication 2	Replication 3	Replication 4	Mean ± SE
pCRISPR (without gRNA)	5.18	4.39	4.79	4.47	4.71±0.19 A
pCRISPR::stx1_1	2.65	2.50	2.87	2.27	2.57±0.19 B
pCRISPR::stx2_2	2.63	2.20	2.40	1.53	2.19±0.19 B
pCRISPR::stx1_1::stx2_2	1.59	1.27	2.01	1.16	1.51±0.19 C

Figure 1: Log CFU reduction/reaction after electroporation of pCRISPR with different gRNAs into the recipient *E. coli* O157:H7 cells containing pCas9 plasmids.