Project Title: Construct a Phage-Mediated System to Deliver CRISPR-Cas9 Antimicrobials for Sequence-Specific Elimination of Foodborne Pathogens in Beef Production

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Background

In a previous checkoff-funded study (Yang et al., 2017), proof-of-concept work demonstrated that the CRISPR- Cas9 system with guide RNA (gRNA) could selectively kill Shiga toxin-producing *Escherichia coli* (STEC) cells by targeting their Shiga toxin genes, *stx*1 and/or *stx*2. Furthermore, the killing efficiency of the CRISPR-Cas9 system against STEC cells was improved by optimizing the design of, and using multiple, gRNAs. Specifically, researchers constructed a pCRISPR with two gRNAs that can selectively kill STEC cells containing the *stx*1 and/or *stx*2 genes. Because of the simultaneous cleavage of the chromosome at two (rather than one) locations, this pCRISPR cloned with two gRNAs achieved significantly greater reductions of *E. coli* O157:H7 cells than pCRISPRs cloned with only a single gRNA.

The CRISPER-Cas9 mediated killing system changes the current understanding about how to reduce or eliminate pathogen contamination on meat products. This is important for the meat industry because the CRISPR-Cas9 system could serve as a novel antimicrobial intervention for the control of foodborne pathogens. For example, gRNAs can be designed to target (i) virulence genes for sequence-specific removal of foodborne pathogens other than STEC (e.g., *Salmonella* and *Listeria monocytogenes*), (ii) antibiotic resistance genes for killing antibiotic-resistant bacteria, and (iii) genes involved in biofilm development and formation of bacterial persister cells in biofilms to prevent biofilm formation, and to improve sanitizer efficiency against biofilms in meat processing environments.

Currently, the main obstacle for the application of this technology in meat production and processing environments is an efficient delivery mechanism for the CRISPR-Cas9 system into bacterial cells. Recently, use of bacteriophage has been shown to be a promising delivery vehicle for introducing CRISPR-Cas9 antimicrobials into living cells (Luo et al., 2016; de la Fuente-Núñez et al., 2017). For example, Bikard et al. (2014) used a phage system to deliver the Cas9 gene and its gRNA into *Staphylococcus aureus* cells for sequence-specific killing of kanamycin-resistant cells.

Yosef et al. (2015) engineered a prophage to deliver the type I-E CRISPR-Cas system targeting plasmid-borne ß-lactamase genes to sensitize and kill antibiotic-resistant bacteria. In this study, researchers focused on developing a phage- mediated delivery system that would allow the delivery of the CRISPR-Cas9-based antimicrobials into target STEC cells.

The overall goal of the project was to construct a phage-mediated system for the efficient delivery of the CRISPR- Cas9 antimicrobials into bacterial cells, and the specific objectives

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were to:

a. Synthesize a DNA fragment with sequences encoding Cas9 proteins and guide RNAs that target Shiga toxin 1 and 2 genes.
b. Clone this DNA fragment into a phagemid vector so that the resulting phagemid vector can successfully express Cas9 proteins and the guide RNAs.
c. Package the phagemid with the CRISPR-Cas9 system targeting Shiga toxin 1 and 2 genes into a helper phage.
d. Infect *E. coli* O157:H7 cells with the phage stock that carries the CRISPR-Cas9 system targeting Shiga toxin 1 and 2 genes and evaluate kill efficiency.

Methodology

To develop a phagemid system suitable for *E. coli* O157:H7, a ~5-kb fragment that contains tracrRNA, Cas9, crRNA and two 20-bp spacers (one spacer targeting the *stx*1 gene and the other spacer targeting the *stx*2 gene) was synthesized. The synthesized fragment was mainly based on the genomic sequence of *Streptococcus pyogenes* M1GAS with some modifications (Bikard et al., 2014) (Figure 1). The synthesized fragment was cloned into the phagemid vector, pBluescript KS (+) (Agilent Technologies, Santa Clara, CA), to construct a phagemid that carries the CRISPR-Cas9 system targeting the *stx*1 and *stx*2 genes (Figure 2). A control phagemid that carried tracrRNA, Cas9, and crRNA but without any spacers was also constructed (Figure 2).

The constructed phagemids were separately introduced into four *E. coli* O157:H7 strains to evaluate their cell reductions. These four *E. coli* O157:H7 strains had different Shiga toxin gene profiles: the Sakai strain has both the *stx*1 and *stx*2 genes; the C1-158 strain has only the *stx*1 gene; the C1-010 strain has only the *stx*2 gene, and the C1-057 strain has neither the *stx*1 nor the *stx*2 gene. Phagemids that carried the CRISPR-Cas9 system targeting the *stx*1 and *stx*2 genes were packaged into M13KO7 helper phages (Figure 4). Additionally, control phagemids without any gRNAs were packaged into M13KO7 phages as controls (Figure 4).

Other than the pure culture of *E. coli* O157:H7 in 2-YT broth, the efficacy of the antimicrobial CRISPR-Cas9 system was also evaluated in an ex vivo model rumen system. Fresh cattle rumen fluid containing 107 CFU/reaction of natural microflora was inoculated with *E. coli* O157:H7 (F') cells at the concentration of 106 CFU/reaction. The inoculated fresh cattle rumen fluid was infected with the M13KO7 helper phages packaged with the CRISPR-Cas9 system targeting the *stx*1 and *stx*2 genes.

Findings

Introduction of the phagemids into the *stx*1- and/or *stx*2-containing *E. coli* O157:H7 strains resulted in significant ($P < 0.05$) cell reductions and the reductions were in the order of: Sakai > C1-158 = C1-010 (Figure 3). No significant ($P > 0.05$) reductions were observed for the C1-057 strain, which lacked both Shiga toxin genes (Figure 3). The significantly higher reductions of *E. coli* O157:H7 cells containing both the *stx*1 and *stx*2 genes could be explained by the simultaneous cleavage of the chromosome at two locations rather than just one location.

Overall, the results obtained for the *E. coli* O157:H7 strains with different Shiga toxin gene profiles indicated that the constructed phagemid is highly specific to the *stx*1 and *stx*2 genes. The constructed phagemid that carries the CRISPR-Cas9 antimicrobials will only efficiently kill Shiga toxin-producing *Escherichia coli* (STEC) cells by specific recognition and

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cleavage of the bacterial chromosome at the sites of the *stx*1 and *stx*2 genes, not anywhere else. A significant ($P < 0.05$) reduction of *E. coli* O157:H7 cells, from 6.96±0.10 to 4.44±0.10 log CFU/reaction, was achieved at a MOI of 25 (Table 2). At a MOI of 0.25, a significant ($P < 0.05$) reduction of *E. coli* O157:H7 cells, from 5.44±0.12 to 2.70±0.12 log CFU/reaction, was achieved as well (Table 1). These results suggested that the complex microbial communities present in cattle rumen fluid did not interfere with the CRISPR-Cas9 antimicrobial efficacy of the developed phages. The kill efficiencies were well retained when the phages that carry the CRISPR-Cas9 antimicrobials were used to reduce *E. coli* O157:H7 cells inoculated in cattle rumen fluid. Therefore, it is promising that the developed phages could be applied as a means of reducing the carriage of STEC cells in ruminant animals.

Implications

The CRISPR-Cas9-based antimicrobial system constructed in the current work will potentially be applied in food animal production environments. Currently, the biosafety aspects of the CRISPR-based technique are still under international debate. To prepare for the potential biosafety concerns that may arise when applying the CRISPR- Cas9 antimicrobials to food animals, the design of the gRNAs was modified by extending the length of the spacers to avoid off-target effects. In addition to phagemids containing two 20-bp spacers, new phagemids containing two 60-bp spacers targeting the same *stx*1 and *stx*2 gene areas were constructed. Phagemids containing two 60-bp spacers killed significantly more ($P < 0.05$) *E. coli* O157:H7 cells (from 4.21±0.13 to 0.50±0.13 log CFU/reaction) than phagemids containing two 20-bp spacers (from 4.21±0.13 to 1.86±0.13 log CFU/reaction) when they were introduced into *E. coli* O157:H7 cells.

The development of phages that can successfully deliver the CRISPR-Cas9-mediated antimicrobials into bacterial cells offers a solution to overcome the first obstacle incurred during the application of the CRISPR-Cas9 technique in actual meat production and processing environments. The developed CRISPR-Cas9-carrying phages could potentially serve as a novel antimicrobial intervention and/or an alternative to antibiotics for selective killing of bacterial pathogens in food animals in the pre-harvest environment. Results from the study indicate that the complex microbial community present in cattle rumen fluid will not interfere with the antimicrobial efficacy of the developed phages. Furthermore, this research explored the strategy of designing longer gRNA to achieve enhanced specificity and efficacy. This approach offers another opportunity to overcome a second potential obstacle, i.e. biosafety concerns, that may be encountered when applying the CRISPR-Cas9 technique *in vivo* in the future.

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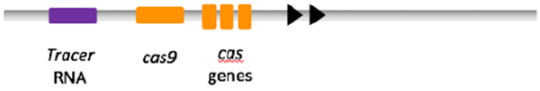
2a



2b

Figure 1. The ~5-kb fragment that contains tracrRNA, Cas9, crRNA and two 20-bp spacers targeting the *stx1* and *stx2* genes (2a) and the control fragment that contains tracrRNA, Cas9, and crRNA but without spacers (2b).

Figure 2. The constructed phagemid that contains the full CRISPR-Cas9 system (left) and the control phagemid that contains the Cas9 gene but does not produce any gRNAs (right).

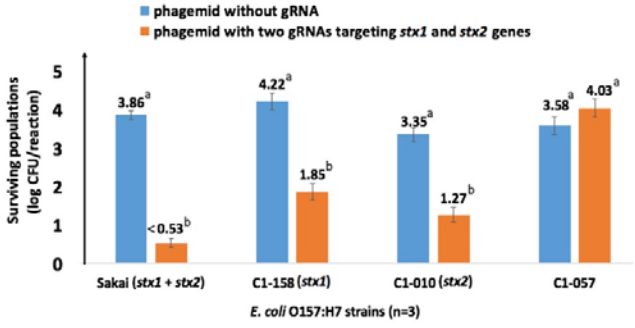


Figure 3. Surviving *E. coli* O157:H7 cell populations (± SE). Differing superscript letters within each strain are significantly different ($P < 0.05$). Detection limit is 1 CFU/ reaction. Four *E. coli* O157:H7 strains with different Shiga toxin gene profiles were used: the Sakai strain has both the *stx1* and *stx2* genes; the C1-158 strain has only the *stx1* gene; the C1-010 strain has only the *stx2* gene, and the C1-057 strain has neither the *stx1* nor the *stx2* gene.

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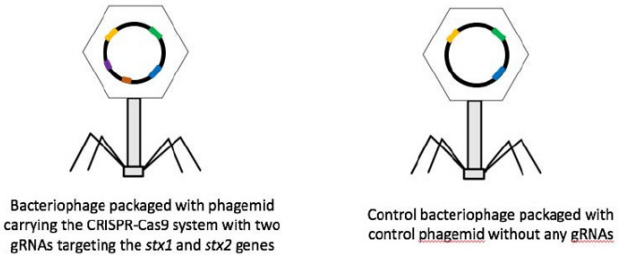

Figure 4. The bacteriophages that carry the phagemid containing the full CRISPR-Cas9 system (left) and the control bacteriophages that carry the control phagemid and do not produce any gRNAs (right).

Table 1. Surviving bacterial populations (log CFU/reaction) after infection of *E. coli* O157:H7 strain Sakai (F') cells, in cattle rumen fluid containing 10^7 log CFU/reaction natural microflora, with control phages or phages carrying the CRISPR-Cas9 system, at two levels of multiplicity of infection (MOI).

		Log CFU/reaction			
Phage	Phage MOI	Trial 1	Trial 2	Trial 3	Mean±SE
Control phages[a]	25	7.09	7.10	6.68	6.96±0.10A[c]
Phages (*stx1*::*stx2*)[b]	25	4.39	4.47	4.46	4.44±0.10B
Control phages	0.25	5.17	5.45	5.71	5.44±0.12a[d]
Phages (*stx1*::*stx2*)	0.25	2.74	2.77	2.60	2.70±0.12b

[a] Phages that carry the control phagemids do not produce any gRNAs (Figure 4).
[b] Phages that carry the phagemids that contain the CRISPR-Cas9 system targeting the *stx1* and *stx2* genes (Figure 5).
[c] Differing uppercase letters are significantly different ($P < 0.05$).
[d] Differing lowercase letters are significantly different ($P < 0.05$).

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