ABSTRACT

Antimicrobial resistance has become a growing threat worldwide. Using CRISPR-Cas9 (cluster regularly interspaced short palindromic repeats) gene editing technology, it may be possible to eliminate these resistant microorganisms with extreme precision. The goal of this study was to kill an ESBL (extended-spectrum beta-lactamase) ATCC 700603 strain of Klebsiella pneumoniae using CRISPR-Cas9 technology. Continuing their promising research done with a CRISPR-Cas9 system in methicillin resistant Staphylococcus aureus, another microbial research group designed plasmids for a new bacterial model. ESBL K. pneumoniae, which can cause the lung infection pneumonia. These plasmids targeted a housekeeping gene, DeaD, needed for survival and the bla(SHV-1) gene coding for beta-lactam resistance. Klebsiella pneumoniae was transfected with these CRISPR-Cas9 plasmids through electroporation. The CRISPR-Cas9 plasmids, effective in targeting these genes, caused double-stranded DNA breakage resulting in cell death (as demonstrated in other model systems). A non-specific GFP plasmid was transfected into K. pneumoniae to act as a positive control. Two plasmids targeting DeaD and bla(SHV-1) respectively, were transfected into K. pneumoniae to observe the targeted genes effect on colony count. Cultures were grown on Thermostrphtin antibiotic selective media. Successful transfection of the CRISPR-Cas9 plasmids led to decreased colony count of K. pneumoniae when plated on selective media. Ultimately, these CRISPR-Cas9 plasmids can be fused with bacteriophage DNA to form a phagemid, which can then be tested as a novel therapeutic for treating antimicrobial resistant organism.

RESULTS

Table 1. Transformation efficiencies in gray and yellow Klebsiella pneumoniae

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<thead>
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<th>Gray</th>
<th>Yellow</th>
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<tr>
<td>Run 1</td>
<td>1.07 X 10⁵</td>
<td>1.60 X 10⁵</td>
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<tr>
<td>Run 2</td>
<td>2.79 X 10⁵</td>
<td>1.06 X 10⁵</td>
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Two separate runs were performed for both colony types with the 1µL of blank (sterile water), GFP, DeaD, or bla(SHV-1) plasmid electroporated with 50µL of colony specific K. pneumoniae after which 10µL, 30µL, and 50µL aliquots were plated. Interpretation of colony counts for plates occurred at 24 hrs. The concentration below GFP indicates concentration CRISPR-Cas9 DNA in that plasmid. This shows how successful transformation was after electroporation.

Targeting and removal of the DeaD housekeeping gene successfully reduced growth in both colonies showing that is crucial for the survival of both organisms. The yellow colony up-took the bla(SHV-1) plasmid but did not show significantly reduced growth compared to the the positive control GFP, which means the bla(SHV-1) is not necessary for the survival of the yellow organism.

Figure 2: Mean CFU (colony forming units) per µL K. pneumoniae

Each target is shown. The negative blank control (blue). The positive GFP control (gray), The Bla1 (green) and the Dead (purple).

*p-value of 0.496
**p-value of 0.000983
***p-value of 0.00100
Error bars indicate standard deviation from the mean.

Figure 3: Mean CFU per µL in yellow K. pneumoniae

Each target is shown. The negative blank control (blue). The positive GFP control (gray), The Bla1 (green) and the Dead (purple).

*p-value of 0.000182
**p-value of 0.135
***p-value of 0.0142
Error bars indicate standard deviation from the mean.

METHODS

CRISPR plasmids specific to K. pneumoniae (ATCC 700603) were created by the University of Utah CORE lab and were designed to target specific sequences in the housekeeping DeaD gene, beta-lactam resistance bla(SHV-1) gene, and a non-specific positive control GFP. Each plasmid also contained a Thermostrphtin resistance gene which helped to select for positive transforming colonies grown on 30 µg/mL Thermostrptin agar. For the “blank” sterile water was added instead of plasmid which acted as a negative control when plated on the Thermostrphtin media.

Two separate distinct colonies grew from the ATCC 700603 K. pneumoniae strain. One colony was noted “gray” and the other “yellow”. A Mic (minimal inhibitory concentration) panel was done, confirming that the colonies were K. pneumoniae. The gray colony was found to be resistant to Thermostrptin and the yellow one susceptible.

Electrocompetent gray and yellow K. pneumoniae each had a blank, GFP, DeaD, and bla(SHV-1) cuvette electrooporated, each cuvette was plated on 30 µg/mL Thermostrphtin media plates (warmed for 1 hour in air incubator at 47°C) in 10µL, 50µL, and 5µL amounts.

Klebsiella pneumoniae was made electrocompetent by taking a colony less than 48 hours old and growing it up to 15 mL of SDB (super optimal broth) for at least 18 hours on a rotating air incubator at 37°C with speed set to 250 rpm. With an optical density of 0.50 – 0.70, cells were spun down at room temperature on a Thermo Scientific ST16 Bircloph Centrifuge at 3800 rpm for 10 minutes. After decanting, cells were resuspended in ice at 1:10 increased, 2 mL were then placed into four 2 mL aliquots. Aliquots were spun on a Sarvell Legendi Micro 21r microcentrifuge (4700 rpm, 10 min, 4°C). Cells were decanted and re-suspended in 1 mL ice cold 10% glycerol and aliquots were combined in pairs. Aliquots were spun down at the same speed, time, and cold conditions; after each centrifugation step, aliquots were combined in pairs after decanting and re-suspending in 5 mL ice cold 10% glycerol. On the final resuspension of the last aliquot, 100 µL of bacterial suspension was pipetted into 10 separate fresh aliquots that were stored at -80°C until used.

For transformation using electroporation, 50 µL of competent cells and 1µL of the specific CRISPR-Cas9 plasmid were combined, placed in a Gene Pulser/MicroPulse cuvet, and electricity shocked (1,700 V, 25 µF, 200 Ω, time interval 4.0-4.4) using a Bio-Rad MicroPulse electroporation machine. The suspension was added to 500 µL of SOC (super optimal broth with catalase) recovery broth and plated in an air incubator at 47°C for 1 hour before being plated. Plates were placed in an air incubator at 47°C to grow for 24 hours at which time the plates were read.

DISCUSSION

The yellow strain may have not had the bla(SHV-1) incorporated into it’s genome as a crucial gene for survival like the gray strain did. This could be due the yellow strain harboring this gene on a pathogenicity island. The yellow strain could have also reverted back to a wild-type strain rendering the ATCC 700603 type specific CRISPR-Cas9 system ineffective.

Further testing can be run to determine if resistance to beta-lactam drugs have been reduced. For future studies a bacteriophage could be fused with these CRISPR-Cas9 plasmids that target these resistance gene sequences to form a phagemid which can then be tested as a novel therapeutic for treating antimicrobial resistant organisms.

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Elimination of Antibiotic Resistant Klebsiella pneumoniae Using CRISPR-Cas9 System

Figure 4: pCas9 Plasmid Design

Sequence specific to the targeting gene used in this research.

Figure 5: Two Sample t Test

Assuming normal distribution, independent of variables, the ANOVA test done for each group had a p-value less than the alpha level 0.05, thus we concluded that the target plasmids were not the same.