Journal of Visualized Experiments

Generation of In-frame gene deletion mutants in Pseudomonas aeruginosa and testing for virulence attenuation in a simple mouse model of infection

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60297R1
Full Title:	Generation of In-frame gene deletion mutants in Pseudomonas aeruginosa and testing for virulence attenuation in a simple mouse model of infection
Section/Category:	JoVE Immunology and Infection
Keywords:	Pseudomonas aeruginosa, genetic engineering, multiple gene deletion, marker-free, virulence attenuation, strain validation, safety evaluation, mouse model of infection, reproducibility
Corresponding Author:	Hongwei D Yu, Ph.D. Joan C. Edwards School of Medicine at Marshall University Huntington, WV UNITED STATES
Corresponding Author's Institution:	Joan C. Edwards School of Medicine at Marshall University
Corresponding Author E-Mail:	yuh@marshall.edu
Order of Authors:	Meagan E. Valentine
	Brandon D. Kirby
	Hongwei D Yu, Ph.D.
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Huntington, West Virginia, United States

Dear Editor,

I would like to submit our revised manuscript to be considered for publication in JOVE. The title of the manuscript is as follows: “Generation of in-frame gene deletion mutants in *Pseudomonas aeruginosa* and testing for virulence attenuation in a simple, reproducible mouse model of infection.”

A point by point response to editor’s and reviewers’ comments is attached. The title has been modified to reflect the two separate protocols described in this paper The highlighted portion of the manuscript is recommended for video script production. Thank you so much for consideration.

Sincerely,

Hongwei D. Yu, Ph.D.

Professor, Joan C Edwards School of Medicine at Marshall University

Tel: 304-696-7356

E-mail:yuh@marshall.edu

TITLE:

Generation of In-Frame Gene Deletion Mutants in *Pseudomonas aeruginosa* and Testing for Virulence Attenuation in a Simple Mouse Model of Infection

AUTHORS AND AFFILIATIONS:

Meagan E. Valentine[1], Brandon D. Kirby[1], Hongwei D. Yu[1,2]

[1]Progenesis Technologies, LLC, Huntington, WV
[2]Department of Biomedical Sciences, Pediatrics, Joan C. Edwards School of Medicine at Marshall University, Huntington, WV

Email addresses of co-authors:
Meagan E. Valentine (lester64@live.marshall.edu)
Kirby, Brandon D. (kirbyb@marshall.edu)

Corresponding author:
Hongwei D. Yu (yuh@marshall.edu)

KEYWORDS:

Pseudomonas aeruginosa, genetic engineering, multiple gene deletion, marker-free, strain validation, safety evaluation, mouse model of infection, reproducibility

SUMMARY:

Here, we describe a simple and reproducible protocol of mouse model of infection to evaluate the attenuation of the genetically modified strains of *Pseudomonas aeruginosa* in comparison to the United States Food and Drug Administration (FDA)-approved *Escherichia coli* for commercial applications.

ABSTRACT:

Microorganisms are genetically versatile and diverse and have become a major source of many commercial products and biopharmaceuticals. Though some of these products are naturally produced by the organisms, other products require genetic engineering of the organism to increase the yields of production. Avirulent strains of *Escherichia coli* have traditionally been the preferred bacterial species for producing biopharmaceuticals; however, some products are difficult for *E. coli* to produce. Thus, avirulent strains of other bacterial species could provide useful alternatives for production of some commercial products. *Pseudomonas aeruginosa* is a common and well-studied Gram-negative bacterium that could provide a suitable alternative to *E. coli*. However, *P. aeruginosa* is an opportunistic human pathogen. Here, we detail a procedure that can be used to generate nonpathogenic strains of *P. aeruginosa* through sequential genomic deletions using the pEX100T-NotI plasmid. The main advantage of this method is to produce a marker-free strain. This method may be used to generate highly attenuated *P. aeruginosa* strains for the production of commercial products, or to design strains for other specific uses. We also describe a simple and reproducible mouse model of bacterial systemic infection via

intraperitoneal injection of validated test strains to test the attenuation of the genetically engineered strain in comparison to the FDA-approved BL21 strain of *E. coli*.

INTRODUCTION:

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that can cause life-threatening diseases in humans, especially in the immunocompromised. The pathogenicity of *P. aeruginosa* is due to the expression of many virulence factors, including proteases and lipopolysaccharide, as well as its ability to form a protective biofilm[1]. Because of its ability to produce virulence factors and cause disease in humans, using *P. aeruginosa* to make commercial products presents safety concerns. Nonpathogenic strains of *E. coli* have traditionally been used to bioengineer medical and commercial products for human use. However, some products are difficult for *E. coli* to make, and many are packaged in inclusion bodies, making extraction laborious. Engineered bacterial strains with the ability to make and secrete specific products is highly desirable, as secretion would likely increase yield and ease purification processes. Thus, nonpathogenic strains of other species of bacteria (e.g., species that utilize more secretion pathways) may provide useful alternatives to *E. coli*. We recently reported the development of a strain of *P. aeruginosa*, PGN5, in which the pathogenicity and toxicity of the organism is highly attenuated[2]. Importantly, this strain still produces large quantities of the polysaccharide alginate, a commercially interesting component of the *P. aeruginosa* biofilm.

The PGN5 strain was generated using a two-step allelic exchange procedure with the pEX100T-NotI plasmid to sequentially delete five genes (*toxA*, *plcH*, *phzM*, *wapR*, *aroA*) known to contribute to the pathogenicity of the organism. pEX100T-NotI was generated by changing the SmaI to a NotI restriction enzyme recognition site within the multiple cloning site of the plasmid pEX100T, which was developed in Herbert Schweizer's lab[3,4]. The recognition site for the restriction enzyme NotI is a rarer DNA sequence compared to SmaI and less likely to be present in sequences being cloned, thus it is more convenient for cloning purposes. The plasmid carries genes that allow for selection, including the *bla* gene, which encodes β-lactamase and confers resistance to carbenicillin, and the *B. subtilis sacB* gene, which confers sensitivity to sucrose (**Figure 1A**). The plasmid also carries an origin of replication (*ori*) compatible with *E. coli*, and an origin of transfer (*oriT*) that allows for plasmid transfer from *E. coli* to *Pseudomonas* species via conjugation. However, the plasmid lacks an origin of replication compatible with *Pseudomonas*, and thus cannot replicate within *Pseudomonas* species (i.e., it is a *Pseudomonas* suicide vector). These characteristics make pEX100T-NotI ideal for targeting genetic deletions from the *Pseudomonas* chromosome. Plasmid cloning steps are carried out using *E. coli* and the resultant plasmid is transferred to *Pseudomonas* by transformation or conjugation. Then, through homologous recombination events and selective steps, the targeted in-frame deletion is generated, marker-free. This method of sequentially deleting genomic regions from the chromosome of *P. aeruginosa* could be used to generate highly attenuated *Pseudomonas* strains, like PGN5, or to design strains for other specific uses (e.g., strains deficient in endonucleases for plasmid propagation or strains deficient in proteases for production of proteins of interest).

The overall virulence of strains of bacteria is affected by growth conditions and phases, during which mutations occur frequently. Therefore, measuring the safety of genetically-engineered

strains can be challenging. To evaluate bacterial isolates for systemic virulence, we adapted a previously published protocol of infection by intraperitoneal injection of C57BL/6 mice[5]. We modified this procedure to use frozen bacterial stocks for injection, which allowed for precise dosing and easy validation of the strains used. In this model, the *E. coli* strain BL21, which has been FDA-approved for production of biopharmaceuticals, was used as a control safety standard for determining the relative pathogenesis of the strain[6-8]. The main advantage to using this method is that it is reproducible and minimizes sources of variation, as infecting strains are validated for bacterial cell number, phenotype, and genetic markers both before and after infection. With these controlled steps, the number of animals required is reduced. In this model, *P. aeruginosa* strains that result in C57BL/6 murine mortality rates equal to or less than *E. coli* BL21 when injected intraperitoneally may be considered attenuated. This simple mouse model of infection may also be used to assess the attenuated pathogenicity of genetically engineered strains from other species using the FDA-approved *E. coli* strain as the reference. Steps 1-7 detail the generation of sequential genomic deletions in *P. aeruginosa* (**Figure 1**) and steps 8-12 detail the use of a mouse model to test the pathogenicity of *P. aeruginosa* strains.

PROTOCOL:

Before beginning animal experiments, the protocol to be used must be approved by the Institutional Animal Care and Use Committee (IACUC). Approval for the protocol described was obtained through the IACUC at Marshall University (Huntington, WV, USA).

1. Plasmid design

1.1. To generate a genetic deletion using the pEX100T-NotI plasmid, clone the regions of DNA flanking the desired deletion sequence and insert into the NotI restriction site of the plasmid. The plasmid insert should contain about 500 nucleotides upstream of the target sequence directly adjacent to about 500 nucleotides downstream of the target deletion sequence. Additionally, the insert should contain the NotI recognition sequence (GCGGCCGC) at its 5' and 3' ends (**Figure 1B**).

2. Plasmid preparation

2.1. Option 1: Utilize traditional cloning procedures. Use PCR to amplify genomic regions upstream and downstream of the gene of interest, followed by crossover PCR[9,10] to join the generated fragments, restriction endonuclease digestion of the PCR product and plasmid, and ligation[11] (**Figure 1B**,**C**).

2.2. Option 2: After designing the deletion sequence in silico, contract a company that de novo synthesizes it to insert into the plasmid pEX100T-NotI. Many companies have streamlined the process of cloning to quickly and efficiently generate the plasmid of interest. Additionally, sequence verify the plasmids to be mutation-free prior to delivery.

3. *E. coli* transformation

3.1. Transform electrocompetent *E. coli* with the plasmid according to the manufacturer's recommendations. Using a sterile inoculating loop, streak 10 µL of the transformation reaction for isolated colonies onto a pre-warmed Luria Broth (LB) agar plate supplemented with 100 µg/mL of carbenicillin and incubate overnight at 37 °C.

NOTE: All equipment and media used to culture bacteria should be treated according to the institution's safety guidelines.

3.2. Passage twice.

3.2.1. Remove the plate from the incubator and identify an isolated colony. Using a sterile inoculating loop, pick up the colony and streak a pre-warmed LB agar plate supplemented with 100 µg/mL of carbenicillin for isolated colonies. Incubate overnight at 37 °C. Repeat this step once more to generate a pure culture.

3.3. Using a sterile inoculating loop, inoculate 5 mL of LB with a single colony from the final agar plate. Place the culture in a shaking incubator at 37 °C overnight. The next day, mix 1 mL of this culture with 1 mL of 5% in a cryovial and store at -80 °C to generate a frozen stock of the strain.

4. Bacterial strain preparation and triparental conjugation

4.1. Use a single isolated colony from agar plates of the following strains to inoculate broth cultures and place in a shaking incubator overnight at 37 °C.

4.1.1. Add *E. coli* pEX100T-NotI into 5 mL of LB supplemented with 100 µg/mL of carbenicillin.

4.1.2. Add *P. aeruginosa* strain PAO1 into 5 mL of *Pseudomonas* Isolation Broth (PIB).

4.1.3. Add *E. coli* prk2013 into 5 mL of LB supplemented with 50 µg/mL of kanamycin.

NOTE: The prk2013 plasmid is a helper plasmid that replicates in *E. coli* but not *P. aeruginosa*; it carries the trans-acting transfer genes that mobilize the pEX100T-NotI plasmid from the *E. coli* donor to the *P. aeruginosa* recipient[12]. *P. aeruginosa* is a Biosafety Level 2 (BSL-2) pathogen. Please follow the institution's guidelines for safety when working with BSL-2 organisms.

4.2. The next day, remove overnight cultures from the incubator and add 0.5 mL of each culture to a 1.5 mL microcentrifuge tube. Centrifuge at 6,000 x *g* for 5 min. Discard the supernatant and suspend the cell pellet in 50–100 µL of LB.

4.3. Pipette the entire cell suspension in one droplet onto a pre-warmed LB agar plate. Allow the droplet to dry. Then invert the plate and incubate at 37 °C for 4–6 h.

4.4. After the incubation, use a sterile inoculation loop to collect the cells into 1 mL of LB in a microcentrifuge tube. Pipette up and down to mix the cells.

4.5. Using a cell spreader, streak cells evenly onto a dry pre-warmed *Pseudomonas* Isolation Agar (PIA) plate supplemented with 300 µg/mL of carbenicillin. Streak multiple plates with increasing volumes of the cell mixture (e.g., 10 µL, 100 µL, 500 µL). Incubate overnight at 37 °C.

5. Detection of single-crossover recombinants of *P. aeruginosa*

5.1. Remove plates from the incubator and inspect for isolated carbenicillin-resistant colonies. Because the pEX100T-NotI plasmid cannot replicate in *P. aeruginosa*, colonies that grew on carbenicillin-supplemented plates should have arisen from cells in which the plasmid was integrated into the chromosome.

5.1.1. Choose at least 4 of these colonies and streak for isolation onto pre-warmed plates of PIA supplemented with 300 µg/mL of carbenicillin. Incubate plates overnight at 37 °C.

5.2. Remove plates from the incubator and inspect for growth. Carbenicillin-resistant colonies should be single-crossover recombinants (i.e., they have incorporated the plasmid into the chromosome via a recombination event between a homologous region of the plasmid insert and the chromosome of *P. aeruginosa*).

5.2.1. Patch 8 or more colonies with sterile toothpicks onto pre-warmed plates of: 1) PIA supplemented with 300 µg/mL of carbenicillin and 2) PIA supplemented with 300 µg/mL of carbenicillin and 10% sucrose (without glycerol).

5.2.2. If no colony growth was obtained from step 5.1, repeat the conjugation and increase the volume of the cell mixture streaked in step 4.5. If too much growth occurred, repeat conjugation and decrease the volume streaked.

5.2.3. If the conjugation repeatedly fails, prepare electrocompetent cells of the *P. aeruginosa* strain and transform directly with the pEX100T-NotI plasmid. Detailed protocols for preparation of electrocompetent *P. aeruginosa* and transformation are available elsewhere[13,14].

5.3. Incubate plates at 37 °C overnight.

5.4. Remove plates from incubator and inspect for growth. True single-crossover recombinants will be carbenicillin-resistant and sucrose-sensitive (i.e., colonies that grew on PIA supplemented with carbenicillin, but did not grow on PIA supplemented with carbenicillin and sucrose).

5.4.1. Choose 4 or more true single-crossover recombinants and inoculate each into 5 mL of LB without selection. Incubate in a shaking incubator at 37 °C overnight.

5.4.2. If no single-crossover recombinants were detected, repeat the conjugation.

6. Detection of double-crossover recombinants of *P. aeruginosa*

6.1. For each broth culture, inoculate 10 µL of culture onto a pre-warmed plate of PIA supplemented with 10% sucrose (without glycerol) and streak for isolated colonies. Incubate plates overnight at 37 °C.

6.2. The next day, remove plates from the incubator and inspect them for growth. Sucrose-resistant colonies should be double-crossover recombinants (i.e., have removed the plasmid from the chromosome via a recombination event between the other homologous region of the plasmid insert and the *P. aeruginosa* chromosome).

6.2.1. Patch at least 20 colonies with sterile toothpicks onto pre-warmed plates of: 1) PIA, 2) PIA supplemented with 10% sucrose (without glycerol), and 3) PIA supplemented with 300 µg/mL of carbenicillin.

6.3. Incubate plates overnight at 37 °C.

6.4. Remove plates from the incubator and examine them for growth. True double-crossover recombinants will be carbenicillin-sensitive and sucrose-resistant (i.e., colonies that grew on PIA and PIA supplemented with sucrose, but did not grow on PIA supplemented with carbenicillin).

7. Gene deletion confirmation via colony PCR

7.1. Prepare 10-20 colonies for a deletion screen with PCR.

7.1.1. Pick up the growth from a suspected double-crossover recombinant with a sterile toothpick and suspend cells in 50 µL of 1x phosphate buffered saline (PBS). Boil suspension at 100 °C for 10 min, centrifuge for 3 min at 13,000 x *g*, and then place on ice.

7.2. Perform PCR to screen colonies for the targeted deletion.

7.2.1. Use 1 µL of the supernatant as the template in a 25 µL PCR reaction to confirm deletion of the gene of interest.

7.2.2. Use gene-specific primers that amplify the region of the genomic deletion. Use primers that amplify the region of the genomic deletion plus 100–200 bp of flanking upstream and downstream sequences.

7.2.3. Prepare a separate control PCR reaction with the parent strain (e.g., PAO1).

NOTE: Thermocycler conditions will vary depending on the optimal annealing temperature for primer pairs, the polymerase cocktail used, and the length of the region to be amplified.

7.3. Perform agarose gel electrophoresis on the PCR products. Colonies in which the region of interest has been deleted yield smaller amplification products than colonies that lack the deletion (**Figure 2**).

7.4. Choose one or more colonies with the PCR-confirmed deletion. Streak for isolated colonies onto a pre-warmed PIA plate(s) and incubate at 37 °C overnight.

7.5. Passage at least one more time. Remove plate(s) from the incubator and identify an isolated colony. Using a sterile inoculating loop, pick up the colony and streak a pre-warmed PIA agar plate for isolated colonies. Incubate overnight at 37 °C.

7.6. Choose a colony from each final plate and use to inoculate 5 mL of PIB. Place in a shaking incubator at 37 °C overnight.

7.6.1. Mix 1 mL of this culture with 1 mL of 5% skim milk in a cryovial and store at -80 °C to generate a stock of the strain.

7.6.2. Using this culture, prepare genomic DNA from the strain (e.g., using a DNA purification kit). Amplify the genomic deletion region using PCR and primers specific to the region of interest.

7.6.2.1. Purify these PCR products (e.g., with a DNA purification kit, or phenol-chloroform extraction) and either sequence directly with the gene-specific primers or ligated into a vector for sequencing with plasmid-specific primers.

7.7. After the gene deletion is confirmed through sequencing, repeat this procedure with the new deletion strain to sequentially generate numerous marker-free genomic deletions. When the desired strain is generated, use whole genome sequencing to verify the targeted deletions and to detect other changes to the genome (compared to the reference strain, e.g., PAO1) that occurred throughout the process. After annotating the genes, deposit the sequence to GenBank and record accession numbers.

8. Preparation of bacterial strain for animal testing

8.1. To test for attenuated pathogenicity of *P. aeruginosa* strains, first prepare validated cultures and stocks. Prepare the *P. aeruginosa* strains of interest, a wild-type strain of *P. aeruginosa* (virulent), and an FDA-approved strain of *E. coli* (e.g., BL21) to serve as a nonpathogenic safety control.

8.2. Streak the strains of the bacteria being tested onto selective agar from sequenced and validated frozen stocks. Incubate at 37 °C overnight.

8.3. With a sterile inoculating loop, pick up a single colony from each strain and streak for isolated colonies onto selective media again. Incubate at 37 °C overnight.

8.4. Remove plates from the incubator. For each strain, choose a single colony and streak for isolation onto LB plates.

8.5. After 24 h of growth at 37 °C, inoculate a 500 mL flask containing 250 mL of LB with a single colony isolate from each strain.

8.5.1. Validation step: using the remnants of the same colony, validate the strain using PCR and strain-specific primers, and/or primers to verify the presence of genetic modifications made to the strain. Use the primers below for verification of strains in the example presented:

E. coli BL21:	T7 polymerase F: TGGCTATCGCTAATGGTCTTACG
	T7 polymerase R: TTACGCGAACGCGAAGTCC
VE2 and PGN5:	*aroA* F: GCGAACGCCAACAGCCGATAAAGC
	aroA R: ATCTGGCTCGCGATGCCGGTCC

8.6. Incubate the cultures in a shaking incubator at 160 rpm and 37 °C until they reach log phase growth (i.e., OD_{600} measurement of 0.4–0.6 on a spectrophotometer).

8.7. Using the OD_{600} value obtained when log phase was achieved, calculate the volume of broth required to yield 2.5 x 10^9 colony forming units (CFU) per mL. Pellet the volume of broth calculated in 50 mL tubes at 4,500 x *g* for 10 min.

8.8. Discard the supernatant and resuspend the pellet in one tube using 50 mL of 1x PBS to wash the cells. Centrifuge again at 4,500 x *g* for 10 min.

8.9. Discard the supernatant and resuspend the pellet in 25 mL of 5% skim milk in 1x PBS.

8.9.1. Validation step: Use a sample of the 25 mL resuspension to perform viable plate counts to determine the number of CFU/mL.

8.10. Aliquot the 25 mL skim milk culture resuspension into 2 mL culture stocks in 2 mL cryovials. Flash freeze in liquid nitrogen and store at -80 °C at least overnight before use.

9. Validation of growth and strain of stocks stored for animal testing.

9.1. For each strain to be tested, remove at least 3 cryovials of frozen stocks from -80 °C storage and thaw at 4 °C for 2–4 h. If any frozen stock remains, briefly warm at 37 °C.

9.2. Take small samples from each cryovial to validate each strain.

9.2.1. Perform viable plate counts to determine the number of CFU/mL. It is normal to have fewer CFU/mL after freezing, due to death of some bacterial cells.

9.2.2. Use PCR and strain-specific primers to validate each strain.

9.2.3. Streak each strain onto selective media to verify the phenotype.

9.3. After confirming that strains are of the correct genotype and phenotype, and validating CFU/mL, proceed to animal testing.

10. Inoculation of animals with bacterial strains by injection

10.1. On the morning of injections, remove cryovials of the bacterial strains being tested and thaw at 4 °C for 3–4 h. Thaw 0.5 mL for each mouse that will be injected. Keep vials on ice after thawing and inject mice within 2 h.

10.2. After thawing, transfer each cryovial to a new 2 mL tube and centrifuge at 4,500 x *g* for 10 min, discard the supernatant, and resuspend the cell pellet in 1 mL of 1x PBS.

10.3. Centrifuge again at 4,500 x *g* for 10 m. Discard supernatant and resuspend pellet in 1x PBS to a final concentration 2.5 x 10^9 CFU/mL. To determine the amount of 1x PBS needed for resuspension, use the CFU/mL data obtained from viable plate counts on frozen stocks in step 2.2.1. The exact amount of 1x PBS used will vary slightly between strains.

10.3.1. Take 3 samples from final suspension of each strain to validate CFU/mL, genotype, and phenotype as described above.

10.4. For each strain, aliquot 1.5 mL of PBS/cell suspension into one 2 mL tube per 5 mice to limit the number of times the tube is entered. Also, prepare tubes of 1x PBS for control injections.

10.5. Gather mice (10 male and 10 female C57BL/6 per group for this experiment) and materials needed for injections (syringes, needles, sharps containers, markers, pen and paper, etc.). Move to the sterile animal surgical room. Wipe all surfaces with sanitizing wipes.

10.5.1. To eliminate distress and risk of injury to experimenter, only bring one sex and experimental group of mice to the surgical room at a time (e.g., a group of 10 male mice to be infected with a particular strain). Wear two pairs of latex gloves to eliminate puncture of gloves if bitten. Wear lab coat, safety glasses, and face mask to avoid contamination.

10.6. Begin injections of the control group with 1x PBS. This will ascertain whether any adverse effects result from injection alone.

10.6.1. Remove a mouse from the cage. Only remove one mouse at a time.

10.6.2. Weigh the mouse and mark its tail with permanent marker to track for weight loss post-injection.

10.6.3. Open a new 1 mL syringe and 27 G needle (use a new syringe and needle for each mouse to eliminate contamination) and inject 200 µL of sterile 1x PBS.

10.6.3.1. Grab the mouse behind its ears using the thumb and forefinger. Pinch to create skin fold at nape of neck to hold onto – a tighter fold reduces neck movement and risk of being bitten during injection. Secure tail into the palm using the pinky to hold mouse flat with little movement.

10.6.3.2. Turn the mouse over and insert needle at 30° angle into the peritoneal cavity to the left or right side of midline. Lift the needle slightly once inserted to ensure it was inserted into the intraperitoneal area and not into organs. Slowly inject the PBS and then withdraw the needle.

10.6.3.3. Place the used needle in a designated biohazard sharps container. Do not re-use the syringe or needle. A bolus at the site of injection is typical.

10.6.4. After injection, move mouse to a separate cage.

10.6.5. Repeat the procedure with the next mouse. After all the mice of one cage are injected, return them to their original cage immediately.

10.7. After injecting the control group, begin injecting suspensions of strains to be tested following same procedure.

10.7.1. Inject 200 µL of the cell suspension. When beginning with cell suspensions of 2.5 x 10^9 CFU/mL, each mouse receives 5 x 10^8 CFU.

NOTE: These concentrations were optimized with preliminary animal research to determine lethal doses of each strain. The dosing may need to be adjusted for other strains/species.

10.8. Once all injections are complete, return mice to the housing room to alleviate distress. Clean work area with sanitizing wipes.

10.9. Monitor the animals for mortality following injection by checking cages for dead mice every 3 h for 72 h and every 12 h for 10 days. Record the weight of mice every 12 h to determine weight loss due to illness.

10.10. Record adverse behavior in the hours following injection, such as difference in posture, lack of grooming or burrowing, immobility, or changes in breathing. Mice that decease from injury associated with injection will exhibit adverse behavior and die quickly following injection. On the other hand, mice that decease from infection will not start to exhibit adverse behavior or death until after 18 h.

10.11. Following the 10-day monitoring period, allow the animals remaining to recover fully and become clear of any infection administered during the testing. Euthanize animals following IACUC procedure.

11. Statistical analysis of animal mortality

11.1. Perform statistical analysis using graphing software. Any software capable of producing graphs and performing statistical analysis is suitable.

11.2. To plot the mortality data, use the X column to represent time (h) and Y column to represent the groups tested.

11.3. Represent each mouse or subject in the study using code = 0 (zero) or code = 1, indicating survival or death, respectively.

11.3.1. For each animal that dies, place a 1 in the Y column of that group at the time of death on the X column. If there are multiple deaths at a single time point within a group, a copy of that time point can be placed in the X column. For example, if three subjects within a group die at 3 h, the 3 h time point will appear three times in the X column.

11.3.2. For all surviving animals within a group, place a zero in the Y column at the final time point measured. For example, if four mice survive, place four ending time points in the X column marked with four zeros in the Y column.

11.4. After animal data is entered for all groups, use a survival graph template to produce a survival graph.

11.4.1. Leave default coding as 0 and 1.

11.4.2. Set the parameters for the graph as percentage.

11.5. Once parameters for survival curve are selected, perform statistical analysis using a Mantel-Cox (log rank) test.

11.6. Format Kaplan-Meier plots using statistical data.

NOTE: Strains that exhibit mortality rates that are less than or equal to the parent strain and the FDA-approved strain (e.g., *E. coli* BL21) may be considered attenuated.

12. Visualization of the infection with bioluminescence

12.1. To visualize the progress of the infection, insert a chromosomal bioluminescent operon (*luxCDABE*) into the PGN5 and VE2 strain tested. The plasmids and protocol used to label these strains were developed in the Schweizer lab and may not be compatible with all species/strains of bacteria[13]. Importantly, visualization of the infection is optional; thus, genomic insertion of this operon is not necessary to perform the mortality study described above.

12.2. Prepare and validate strains using the method described above. Additionally, check for bioluminescence in labeled strains at each validation step.

12.3. After strains are prepared, inject the animals in groups of 10 with the bioluminescent strains following the steps above.

12.4. Image the animals every 3 h for 24 h using an animal imaging system capable of bioluminescence.

12.4.1. First prepare the imager by setting the camera parameters and heating the stage for the animals. Also set the oxygen flow to 1.5 L per min (or following manufacturer's recommendations).

12.4.2. After the imager and stage are stabilized, place one mouse into the anesthesia chamber immediately following injection and administer 3.5% isoflurane into the chamber with O_2 flow for about 4 min. The anesthesia methods may vary depending upon the chamber and/or anesthetic agent used; follow the manufacturer's recommendations. Determine proper anesthesia via withdrawal reflex test.

12.4.3. Move the mouse to the temperature stabilized stage. Position the mouse on its back with arms outstretched and fit the mouse with a nose cone for administration of 2.5% isoflurane throughout the imaging procedure.

12.4.4. Close the door and take bioluminescent images and X-rays of mouse.

12.4.5. When imaging is complete, return the mouse to its cage and monitor it. The mouse should regain consciousness within 3–5 min.

12.4.6. Continue to image mice every 3 h for 24 h, each time using a different mouse from each group. Do not reimage a mouse within 24 h due to the possibility of adverse effects due to re-exposure to anesthesia. A single mouse should only receive one dose of anesthesia every 24-36 h. Clean the imaging platform after each mouse is imaged. Turn off the imager between imaging time points.

NOTE: Bioluminescence will fade, regardless of the strain injected. The intensity and longevity of bioluminescence will vary depending on many factors, including the number of bacteria injected, mouse strain, bacterial strain, etc.

REPRESENTATIVE RESULTS:

As shown in **Figure 2**, the targeted genomic deletion can be confirmed using colony PCR with specific primers that amplify the region of interest. Colonies that carry a genomic deletion will yield a shorter PCR band size in comparison to wild-type colonies. A PCR-screen of 10-12 colonies is usually sufficient to detect at least one colony that carries the targeted deletion. If no deletions are detected after multiple rounds of screens, repeat the procedure beginning with the

conjugation. If the deletion still fails, the plasmid insert may need to be confirmed through sequencing, redesigned, or the deletion may be lethal. Upon the verification of a gene deletion via PCR, confirm the deletion through sequencing. The resulting strain may be subjected to the procedure repeatedly to generate sequential genomic modifications.

As shown in **Figure 3**, mortality associated with intraperitoneal injection of the attenuated strain of *P. aeruginosa* PGN5 (+mucE) was 0%, which was equivalent to mortality observed with *E. coli* BL21. On the other hand, intraperitoneal injection of the parent strain (VE2) was fatal to 80% of mice. These results were obtained with extensive steps to validate the strains injected. While the exact cause of death in these mice is unknown, it can at least in part be attributed to the expression of virulence factors in the parent strain that were deleted from the attenuated PGN5 strain. Differences in the infection progression was tracked using bioluminescence-marked parent and attenuated strains. The attenuated strain remained localized at the site of injection until bioluminescence faded (**Figure 4**). The clearance of the infection most likely coincided with the fading of the bioluminescence. Bioluminescence was not detected 24 h after injection and mice lived for weeks following injection until sacrificed, with no adverse effects observed.

FIGURE AND TABLE LEGENDS:

Figure 1. Generating gene deletions in *P. aeruginosa* with pEX100T-NotI. (**A**) Map of the pEX100T-NotI plasmid. (**B**) Generation of a construct composed of regions directly upstream (yellow) and downstream (blue) of the region of interest (ROI), flanked with NotI restriction enzyme recognition sites. First, PCR-amplify upstream and downstream regions independently with specific primers that add 5' NotI digestion sites (e.g., NotI-*aroA* F CGCGGCCGCTGAAGGTCCTGGGCTCCTATCCGAAAGCGGTGCTCT and NotI-*aroA* R GCGGCCGCAGTTGGGTTGTTCTGCGATGGCGCCAGGCA) and 3' overlapping homologous regions as shown (e.g., *aroA*-crossover F CTCCAGGCGCTGGGCAAGGTGCTGGCGCATGACTGAGGTCACGCCGGTCGCCGTGGAGAACA and *aroA*-crossover R TGTTCTCCACGGCGACCGGCGTGACCTCAGTCATGCGCCAGCACCTTGCCCAGCGCCTGGAG. Then, use PCR with NotI-containing primers to join the upstream and downstream products generated in the first PCR reaction. (**C**) The pEX100T-NotI plasmid, armed and ready. Ligate the NotI-digested cross-over PCR product into the NotI-digested plasmid. (**D**) Flow diagram of the process to delete genomic regions from the *P. aeruginosa* chromosome using the pEX100T-NotI plasmid. After the desired deletion has been confirmed and purified, the resultant strain can be taken through the procedure repeatedly to delete other genomic regions from the chromosome. When the desired strain is obtained, sequence the whole genome to confirm deletions and other changes to the chromosome. The pathogenicity of the strain can then be tested in mice using the procedure outlined in Part II of the Protocol.

Figure 2. Gel electrophoresis of colony PCR products from a screen for *aroA* deletion to generate the attenuated *P. aeruginosa* strain, PGN5. Colony PCR products run in lanes 2-5 and 8-11 indicate colonies with wild-type *aroA*. Colony PCR products run in lanes 6 and 7 carry the *aroA* gene deletion, indicated by the smaller PCR product (yellow asterisks). Primers used specifically amplified the genomic region containing the *aroA* gene: *aroA*-F:

GCGAACGCCAACAGCCGATAAAGC, and *aroA*-R: ATCTGGCTCGCGATGCCGGTCC. Expected PCR product size in wild-type colonies was 2,548 nucleotides (nt). Expected PCR product size in colonies with *aroA* deletion was 307 nt. A DNA ladder was run in lanes 1 and 12.

Figure 3. Overall mortality of mice injected with pathogenic *P. aeruginosa* strain (VE2), attenuated *P. aeruginosa* strain (PGN5+mucE), and FDA control *E. coli* strain (BL21). Only mice injected with pathogenic parent strain exhibited mortality at 80%. Attenuated *P. aeruginosa* strain and FDA control *E. coli* strain exhibited 0% mortality.

Figure 4. Image of mouse 3 h post-injection of attenuated strain of *P. aeruginosa* PGN5+mucE carrying a bioluminescent marker. The bioluminescent bacteria were detectable until 18-24 h following injection. During this period, the bioluminescence remained at the site of injection indicating the bacteria stayed localized to injection site. This mouse fully recovered with no adverse effects.

DISCUSSION:

The pEX100T-Not1 plasmid is an efficient mediator of sequential genomic deletions that are marker-free and in-frame. When engineering bacterial strains for attenuated virulence, deletion of entire gene sequences rather than generating point mutations decreases the likelihood of reversion to a virulent phenotype. Additionally, each pathogenicity gene deletion attenuates the pathogen further, reinforcing the stability of the attenuation.

This method can also be used to generate genomic modifications other than deletions, such as point mutations and insertions, simply by modifying the design of the plasmid insert. These types of modifications may be more useful than entire gene deletions for engineering bacteria with modified metabolism, for example. Sequential genomic modification has significant potential for generating designer bacterial strains to suit specific purposes in research and industry. Other methods of generating desired marker-free genomic modifications in bacteria have been described[15-18]. As with all genome-editing methods, attempted modifications to essential genomic regions may be lethal, and thus unsuccessful. In these cases, identification of different genetic modifications or other candidate genes is required to generate the bacterial strain of interest.

Given the numerous replication events and passages of each colony throughout this protocol, unintended changes to the genome will occur to the generated strain. The exact genomic changes can be identified through whole-genome sequencing. However, the impact of these changes is harder to determine. When engineering bacteria for a specific purpose, genomic changes that do not negatively affect the growth of the organism or the targeted pathway(s) are tolerable. Depending on the strain being generated, it may be possible to identify a “readout” to ensure that the strain is still useful for its intended purpose. For example, with PGN5, the goal was to create an attenuated strain that retained the ability to produce large amounts of alginate. After deletion of five pathogenicity genes, the amount and composition of alginate produced by PGN5 was measured and determined to be comparable to other alginate-producing strains. Thus,

alginate production was unaffected by the five gene deletions, nor by the unintended genomic changes that occurred during the development of PGN5.

A model of intraperitoneal mouse injection was used to determine whether an engineered strain was attenuated compared to the parent strain and *E. coli* BL21, a strain approved by the FDA for production of biopharmaceuticals. The most important steps taken during this animal testing procedure were preparation and validation of frozen bacterial stocks. Preparation and use of frozen bacterial cultures to inject mice is preferable to using continuous culture, as it reduces the number mutations that naturally occur in bacterial populations[19]. Additionally, frozen cultures should remain viable for years. Viable plate counts showed no significant difference between the CFU/mL directly after stocks were prepared and three months after preparation. The use of multiple validation steps throughout this procedure ensured that the method was reproducible, and the results were not skewed by contaminating bacteria. Additionally, with the number of precautionary steps taken to ensure reproducibility, fewer animals were needed. Using a bacterial strain that is FDA-approved for biopharmaceutical production as the control (such as *E. coli* strain BL21), this method could be used to test the attenuation of other genetically engineered strains of *P. aeruginosa*, or other species of bacteria.

Using bioluminescence as a marker provides additional validation of the bacterial strains injected, as the marker can be visualized at the injection site. Insertion of the bioluminescence marker into the bacterial chromosome is required for bioluminescence imaging but may not be possible if working with incompatible strains/species. However, marking strains with bioluminescence is not required to test for attenuation. The strains tested in this study were marked with bioluminescence, which allowed for visualization of localization differences between strains throughout the course of the infection. We observed that the pathogenic strain disseminated through the body of the mouse, but the non-pathogenic strain remained at the site of injection. While this experiment only tested two very closely related strains of *P. aeruginosa*, it suggests that bacterial dissemination is linked to virulence, at least in *P. aeruginosa*. Thus, this procedure of labeling with bioluminescence to visualize the progression of the infection could be used in the future to quickly evaluate the attenuation of engineered strains of bacteria.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (NIH) grants R44GM113545 and P20GM103434.

DISCLOSURES:

The author Hongwei D. Yu is the Chief Science Officer and Co-founder of Progenesis Technologies, LLC.

REFERENCES:

1 Gellatly, S. L., Hancock, R. E. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. *Pathogens and Disease.* **67** (3), 159-173 (2013).

2 Valentine, M. E. et al. Generation of a highly attenuated strain of Pseudomonas aeruginosa for commercial production of alginate. *Microbial Biotechnology.* (2019).

3 Schweizer, H. P., Hoang, T. T. An improved system for gene replacement and xylE fusion analysis in Pseudomonas aeruginosa. *Gene.* **158** (1), 15-22 (1995).

4 Damron, F. H., Qiu, D., Yu, H. D. The Pseudomonas aeruginosa sensor kinase KinB negatively controls alginate production through AlgW-dependent MucA proteolysis. *Journal of Bacteriology.* **191** (7), 2285-2295 (2009).

5 Yu, H., Boucher, J. C., Hibler, N. S., Deretic, V. Virulence properties of Pseudomonas aeruginosa lacking the extreme-stress sigma factor AlgU (sigmaE). *Infection and Immunity.* **64** (7), 2774-2781 (1996).

6 Baeshen, M. N. et al. Production of Biopharmaceuticals in E. coli: Current Scenario and Future Perspectives. *Journal of Microbiology and Biotechnology.* **25** (7), 953-962 (2015).

7 Marisch, K., Bayer, K., Cserjan-Puschmann, M., Luchner, M., Striedner, G. Evaluation of three industrial Escherichia coli strains in fed-batch cultivations during high-level SOD protein production. *Microbial Cell Factories.* **12**, 58 (2013).

8 Ferrer-Miralles, N., Domingo-Espin, J., Corchero, J. L., Vazquez, E., Villaverde, A. Microbial factories for recombinant pharmaceuticals. *Microbial Cell Factories.* **8**, 17 (2009).

9 Horton, R. M., Cai, Z. L., Ho, S. N., Pease, L. R. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques.* **8** (5), 528-535 (1990).

10 Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., Pease, L. R. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene.* **77** (1), 61-68, (1989).

11 Sambrook, J., Fritsch, E. F., Maniatis, T. in *Molecular cloning: a laboratory manual* (Cold Spring Harbor Laboratory Press, 1989).

12 Figurski, D. H., Helinski, D. R. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proceedings of the National Academy of Sciences of the United States of America.* **76** (4), 1648-1652 (1979).

13 Choi, K. H., Schweizer, H. P. mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa. *Nature Protocols.* **1** (1), 153-161 (2006).

14 Choi, K. H., Kumar, A., Schweizer, H. P. A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *Journal of Microbiological Methods.* **64** (3), 391-397 (2006).

15 Liang, R., Liu, J. Scarless and sequential gene modification in Pseudomonas using PCR product flanked by short homology regions. *BMC Microbiology.* **10**, 209 (2010).

16 Martinez-Garcia, E., de Lorenzo, V. Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of Pseudomonas putida KT2440. *Environmental Microbiology.* **13** (10), 2702-2716 (2011).

17 Song, C. W., Lee, S. Y. Rapid one-step inactivation of single or multiple genes in Escherichia coli. *Biotechnology Journal.* **8** (7), 776-784 (2013).

18 Yan, M. Y. et al. CRISPR-Cas12a-Assisted Recombineering in Bacteria. *Applied and Environmental Microbiology.* **83** (17) (2017).

19 Prakash, O., Nimonkar, Y., Shouche, Y. S. Practice and prospects of microbial preservation. *FEMS Microbiology Letters.* **339** (1), 1-9 (2013).

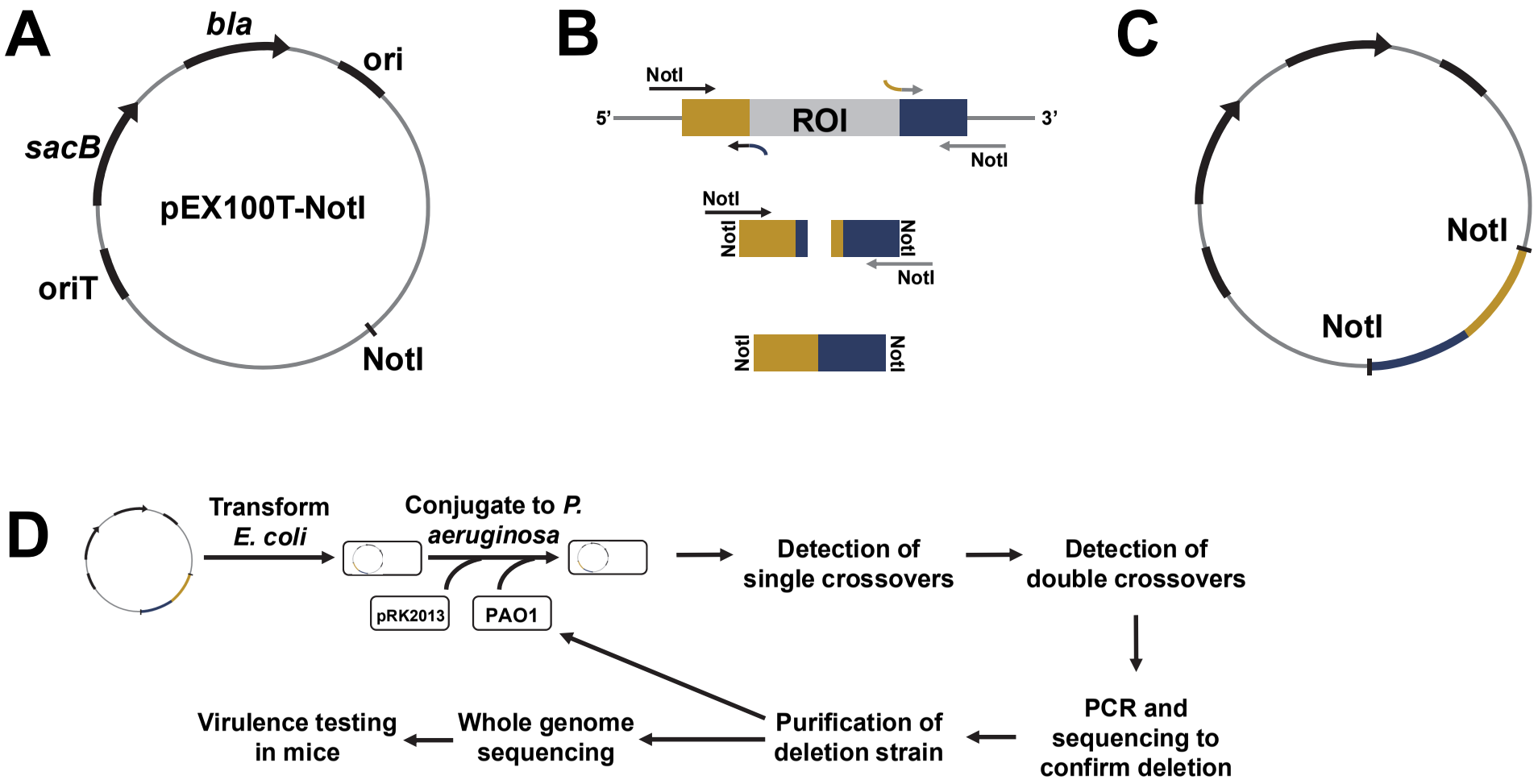

Figure 2

Click here to access/download;Figure;Figure 2.pdf

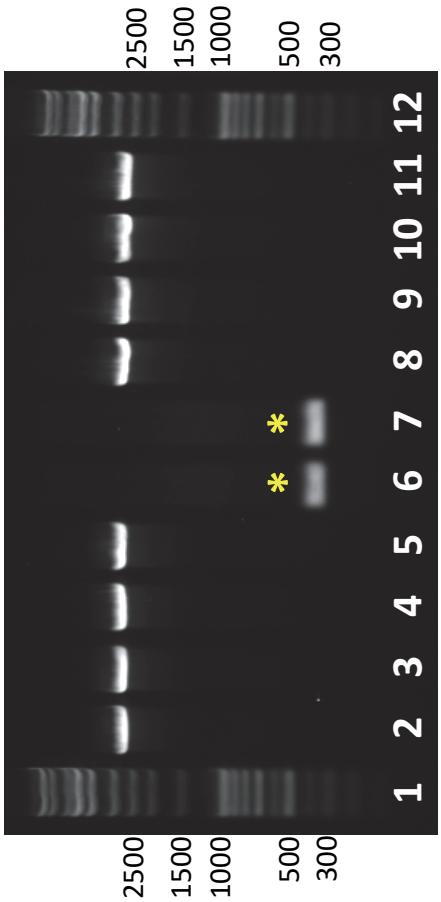

Figure 3

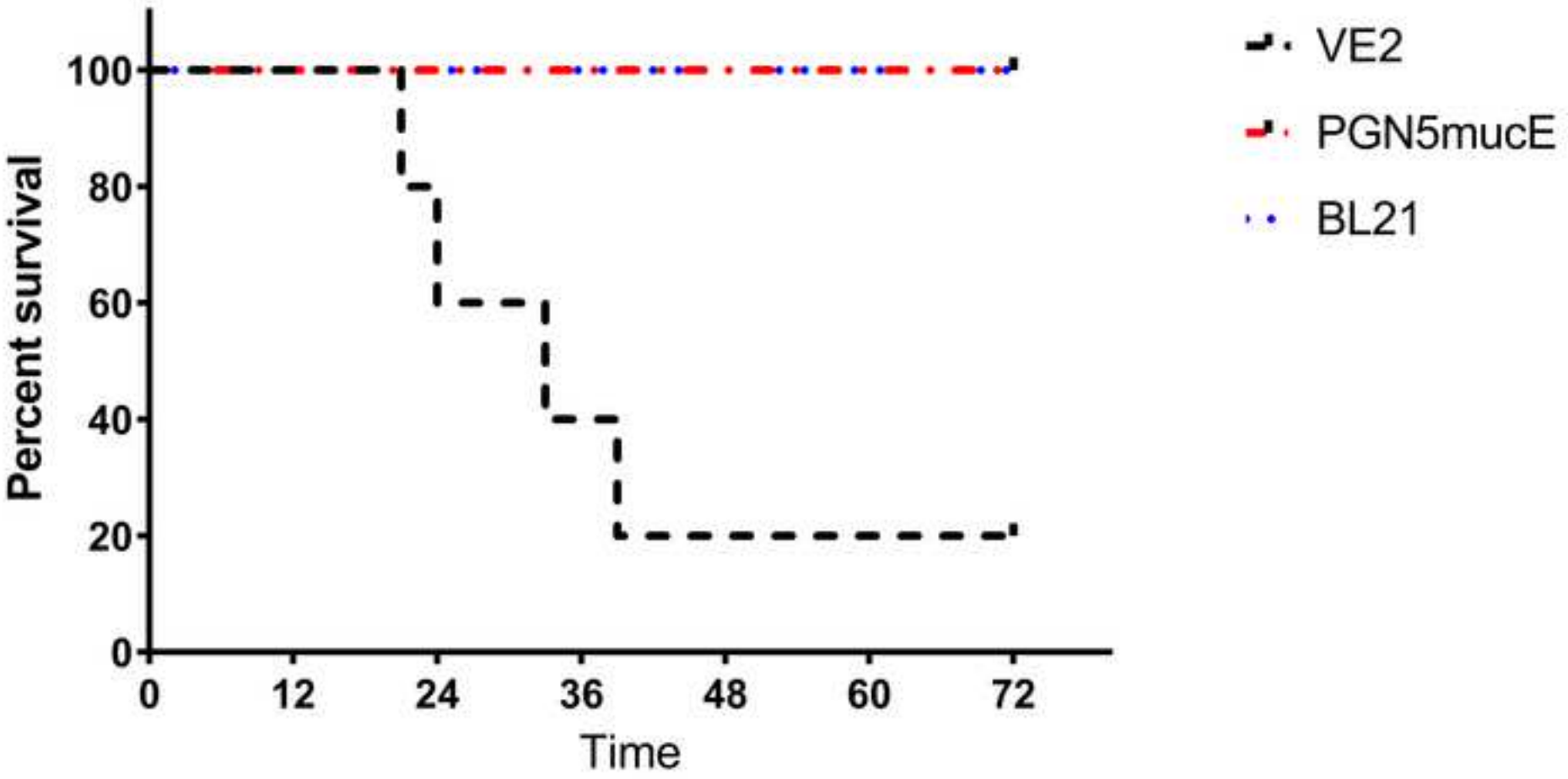

Figure 4

Click here to access/download;Figure;Figure 4.pdf

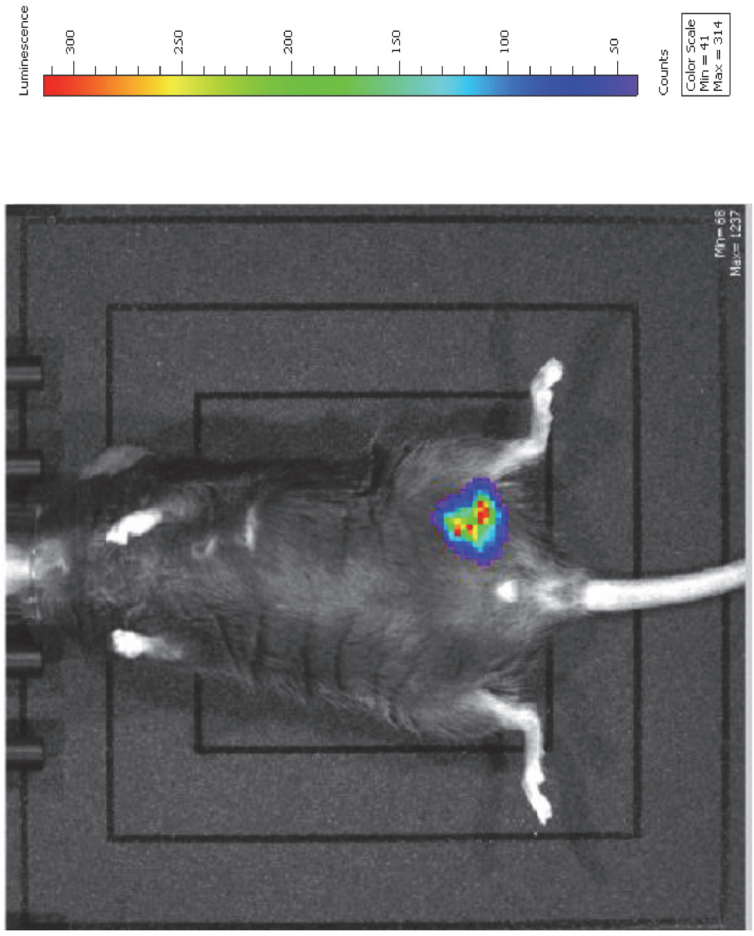

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2 mL tubes with flat caps	ThermoScientific	AB-0620	via Fisher Scientific
1 mL Syringe	BD	22-253-260	via Fisher Scientific
1.5 mL disposable polystyrene cuvette	Fisher Scientific	14955127	
1.5 mL Microcentrifuge Tubes	Fisher Scientific	05-408-129	
2.0 mL Cryogenic Vials	Corning	430659	via Fisher Scientific
27G needle	BD	14-821-13B	via Fisher Scientific
50 mL tubes	Fisher Scientific	05-539-13	via Fisher Scientific
Accu block Digital Dry Bath	Labnet	NC0205808	via Fisher Scientific
Benchtop Centrifuge 5804R	Eppendorf	04-987-372	via Fisher Scientific
Benchtop Microcentrifuge	Sorvall	75-003-287	via Fisher Scientific
Cabinet Incubator	VWR	1540	
Carbenicillin disodium salt	Fisher Scientific	BP2648250	
Culture Test Tube, Polystyrene	Fisher Scientific	14-956-6D	via Fisher Scientific
Diposable Inoculation Loops	Fisher Scientific	22-363-597	
Dneasy UltraClean Microbial Kit (50)	Qiagen	12224-50	or preferred method/vendor
E.Z.N.A. Cycle Pure Kit (50)	Omega bio-tek	D6493-01	or preferred method/vendor
EcoRI-HF, restriction endonuclease	New England BioLabs	R3101L	
Electroporation Cuvettes	Bulldog Bio	NC0492929	via Fisher Scientific
FastLink II DNA Ligation Kit	Epicentre Technologies	LK6201H	via Fisher Scientific
Gentamycin Sulfate	Fisher Scientific	BP918-1	
Glycerol	Fisher Scientific	BP229-4	
GoTaq G2 Colorless Master Mix	Promega	M7833	via Fisher Scientific
Isothesia Isoflurane	Henry Schein Animal Health	29405	
IVIS Lumina XRMS Series III In Vivo Imaging System	Perkins and Elmer	CLS136340	
Kanamycin monosulfate	Fisher Scientific	BP906-5	
LE agarose	Genemate	3120-500	via Fisher Scientific
Luria Broth	Difco	240230	via Fisher Scientific
MicroPulser Electroporator	BioRad	1652100	
Noble agar, ultrapure	Affymetris/USB	AAJ10907A1	via Fisher Scientific
NotI-HF, restriction endonuclease	New England BioLabs	R3189	
One Shot TOP10 Electrocomp *E. coli*	Invitrogen	C404052	via Fisher Scientific
Phosphate buffered saline powder	Sigma	P3813-10PAK	Sigma-Aldrich
Prism 7	GraphPad		https://www.graphpad.com/scientific-software/prism/
Pseudomonas isolation agar	Difco	292710	via Fisher Scientific
Pseudomonas isolation broth	Alpha Biosciences	P16-115	Custom made batch
QIAprep Spin Miniprep Kit (250)	Qiagen	27106	or preferred method/vendor
Shaking Incubator	New Brunswick Scientific	Innova 4080	shake at 200 rpm
SimpliAmp Thermal Cycler	Applied Biosystems	A24811	
Skim Milk	Difco	DF0032-17-3	via Fisher Scientific
Small Plates (100 O.D. x 10 mm)	Fisher Scientific	FB0875713	
SmartSpec Plus Spectrophotometer	Bio-Rad	170-2525	or preferred method/vendor
Sucrose	Fisher Scientific	S5-500	
Toothpicks, round	Diamond		Any brand of toothpicks, autoclaved
TOPO TA Cloning Kit, for seqeuncing	Invitrogen	45-0030	
XAF-8 Anesthesia System Filters	Perkins and Elmer	118999	
XGI 8 Gas Anesthesia System	Caliper Life Sciences/Xenogen		

jove VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	A simple, reproducible mouse model of infection for safety evaluation of genetically-engineered strains of Pseudomonas aeruginosa
Author(s):	Meagan E. Valentine, Brandon D. Kirby, and Hongwei D. Yu

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	**Hongwei Yu**
Department:	**Biomedical Sciences**
Institution:	**Joan C. Edwards School of Medicine at Marshall University**
Title:	**Professor**
Signature:	hwyu
Date:	**05/21/19**

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Yes.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: GraphPad Prism, IVIS Lumina.

We have updated the Table of Materials and attempted to remove all commercial language from the manuscript, e.g., removal of TOP10 from line 136, removal of GraphPad Prism from line 525, etc.

Protocol:
1. Please include all safety procedures used in the Protocol.

Added to the text:

Line 147-148 Note: All equipment and media used to culture bacteria should be treated according to your institution's safety guidelines.
Line 172-173: P. aeruginosa is a Biosafety Level 2 (BSL-2) pathogen. Please follow your institution's guidelines for safety when working with BSL-2 organisms.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible.

Text was updated to use imperative tense as much as possible, e.g., edits to lines 125 and 162, etc.

3. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We did this through the protocols.

Specific Protocol steps:
1. I.2: Please include references for less-common procedures used here (e.g., crossover PCR, design of the deletion sequence). It would be best to include specific primers that you have used as an example (also in II.1.5).

References for crossover PCR, specifically when designing sequences for deletion added (line 126). We used GenScript to generate the construct we used in this example, but we added

some examples of primers that could be used to generate the pEX100T-NotI plasmid to delete the *aroA* gene (lines 664-670). We also added primers we used to validate strains in part II (lines 338-344).

Figures and Tables:
1. Please remove 'Figure 1' etc. from the figures themselves. Please also remove unnecessary whitespace.

Fixed

2. Please remove the embedded table from the manuscript.

Removed embedded table.

References:
1. Please do not abbreviate journal titles.

Journal titles corrected.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

The Table of Materials has been updated to be more comprehensive for materials and equipment used.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Valentine and co-workers elegantly and exhaustively describe a very complete protocol from the starting point to the last result, intended to generate multiple marker-free knockout mutants in P. aeruginosa, and to validate the results of virulence attenuation (obviously caused by the mentioned genes deletions) in murine model. The authors validate the protocols by providing results regarding the virulence attenuation (in terms of mice survival after intraperitoneal inoculation) achieved in the previously constructed PGN5 mutant. The continuous steps of checkup stand out in this excellent paper. The paper is very detailed and well-written, and will become an excellent tool for the researchers interested in making P. aeruginosa knockout mutants, both related or not with virulence attenuation.

Major Concerns:
none

Minor Concerns:
The only concerns I have are:
i) since the paper starts with an exhaustive description of the protocol to generate the mutants, and finally describes the murine model of infection, it would be logical to change the title: I.e., when one reads the title, it seems that the paper deals only with the murine model.

Generation of in-frame gene deletion mutants in *Pseudomonas aeruginosa* and testing for virulence attenuation in a simple mouse model of infection

ii) Please explain the purpose of using the E. coli prk2013, and the features of this strain. In this regard, it could be of interest to add the strains needed for the whole process in the Table of Materials.

Added to the text: The prk2013 plasmid is a helper plasmid that replicates in E. coli but not P. aeruginosa; it carries the trans-acting transfer genes that mobilize the pEX100T-NotI plasmid from the E. coli donor to the P. aeruginosa recipient (lines 170-172).

iii) Could the authors please explain why the use of NotI site? why did they change the SmaI by the NotI restriction site?

Added to the text: The recognition site for the restriction enzyme NotI is a rarer DNA sequence and less likely to be present in sequences being cloned, thus it is more convenient for cloning purposes (lines 74-76).

iV) I am not sure that the last part of the paper, "5. Visualization of the infection" is necessary for this paper.

The reviewer is correct, visualization of the infection via bioluminescence isn't necessary for the paper. However, we think it is interesting and would like to keep it in the manuscript. We mentioned in the text that this is an optional step (lines 560-562, 748-751).

Reviewer #2:
Manuscript Summary:
This interesting manuscript describes 3 different techniques: gene deletion using double recombination, a murine model of intraperitoneal infection, and a method to visualize bacteria in vivo. These methods are interesting as they provide useful tools for the community and are described more precisely than most peer-reviewed publications.

Major Concerns:
1. There are 3 very different techniques. It seems that separating the molecular engineering from the animal models and publish them as two separate manuscripts would better serve these techniques and the reader. Based on the title, it sounds like the manuscript is exclusively about the mouse model. However, the most precise and well-detailed section in this manuscript covers the deletion of genes in P. aeruginosa. At minimum, the title should be modified to make sure that users looking for methods for gene deletion can actually find this paper, or the content of the paper should be split in two.

The title has been modified

2. Some concepts are brought up in the abstract but never described in the introduction or discussion, i.e.: why is P. aeruginosa a suitable alternative for biopharmaceutical production to E. coli (please discuss in the context of the virulence and intrinsic differences between P. aeruginosa and E. coli); what are the concerns associated with the use of this bacterium?

Added to the text: Because of its ability to produce virulence factors and cause disease in humans, using P. aeruginosa to make commercial products presents safety concerns. Nonpathogenic strains of E. coli have traditionally been used to bioengineer medical and commercial products for human use. However, some products are difficult for E. coli to make,

and many are packaged in inclusion bodies, making extraction laborious. Engineered bacterial strains with the ability to make and secrete specific products is highly desirable, as secretion would likely increase yield and ease purification processes. Thus, nonpathogenic strains of other species of bacteria, for example, species that utilize more secretion pathways, may provide useful alternatives to E. coli. (lines 57-65)

3. Safety evaluation: The authors claim that this method can be used to compare strain safety to the FDA-approved BL21 strain of E. coli. However, only the non-pathogenic strain was used and was not compared to a non-FDA approved strain. Adding this control is crucial as there is no indication in this manuscript (or literature cited) that this is a suitable model to evaluate the pathogenesis of E. coli. In addition, the word "safety" is very loosely defined. Here, the authors only examine dead/live events. There are a lot of other aspects of safety (toxicity, tumorigenicity, teratogenicity,...) that are not discussed here. The claim that this crude method can be used to determine strain safety is an over-interpretation of the data.

The BL21 strain of E. coli is approved by the FDA to be safe to use for the production of biopharmaceuticals, thus we used this strain as the “safety control strain” for these experiments. Our goal was to generate a strain of P. aeruginosa that was comparable in virulence to the BL21 strain, and so would also be suitable to use for production of commercial products or biopharmaceuticals (and hopefully for FDA approval). Rather than using a pathogenic E. coli strain (non-FDA approved) as a control in these experiments, we used the parent VE2 strain of P. aeruginosa (an alginate-producing version of PAO1). The strain VE2 strain of P. aeruginosa is the positive control strain from which PGN5 was derived. In addition, both genomes of VE2 and PGN5 were completely sequenced and deposited in NCBI GenBank (CP006831.1 for PAO1-VE2 and CP032541). Therefore, we think VE2 is the most suitable pathogenic control to test the attenuation of PGN5, as it is the parent strain.

We agree with the reviewer that we have only monitored lethality of test strains and have not explored other aspects of safety. To be clearer, we have tried to modify the text to refer specifically to strain lethality in mice in comparison with the BL21 strain, rather than strain safety in general.

4. Many of the techniques referred to in the text regarding the cloning section (crossover PCR, restriction digestion,...) are standard but references should be added for users who are not familiar with such techniques.

Added reference for crossover PCR (line 126), and restriction digests and ligations (line 128).

5. Please detail primer design considerations to verify the deletion (i.e. suggest designing primers that will amplify something, as a number of scientist erroneously use primers on the deleted region and negative amplification as a method to verity deletion)

Added to the text: Use primers that amplify the region of the genomic deletion plus 100-200 bp of flanking upstream and downstream sequences. (line 265-267)

6. Please discuss the potential for the apparition of additional genomic mutations on the genome given the large number of replication and passages of each colony and how this could impact the results. Also discuss this in relation to strain "authenticity" (loosely defined in 2.2)

Text added to discussion: Given the numerous replication events and passages of each colony throughout this protocol, unintended changes to the genome will occur to the generated strain of interest. The exact genomic changes can be identified through whole-genome sequencing. However, the impact of these changes is harder to delineate. Depending on the type of strain being generated, it may be possible to identify a "readout" to ensure that the strain is still useful for its intended purpose. For example. With PGN5, the goal was to create an attenuated strain that retained the ability to produce large amounts of alginate. After deletion of five pathogenicity genes, the amount and composition of alginate produced by PGN5 was measured and determined to be comparable to other alginate-producing strains. Thus, alginate production was unaffected by the five gene deletions, nor by the unintended genomic changes that occurred during the development of PGN5. (line 722-734)

Throughout the manuscript, rather than referring to strain authenticity, that as the reviewer pointed out is rather ambiguous, we tried to instead refer to validation of strains by ensuring the presence of the genetic modifications made to the strains.

7. The authors mention that growth conditions/time of growth may affect the virulence of strains in the murine model in the introduction. Please discuss the relevance of using frozen stocks and the viability of these stocks over time.

Added to the text: Preparation and use of frozen bacterial cultures to inject mice is preferable to using continuous culture, as it reduces the number mutations that naturally occur in bacterial populations[18]. Additionally, frozen cultures should remain viable for years. Viable plate counts showed no significant difference between the CFU/mL directly after stocks were prepared and three months after preparation. (lines 738-742)

8. Please discuss the time between bacterial thaw and injection. How long can the cultures be kept thawed before injection and in which conditions before seeing alterations in the phenotype observed in the mice?

Added to the text: On the morning of injections, remove cryovials of the bacterial strains being tested and thaw at 4 ^{o}C for 3-4 h. Thaw 0.5 mL for each mouse that will be injected. Keep vials on ice after thawing and inject mice within 2 h. (line 401-403)

9. Specify that the dose administered to the mice here was optimized based on the bacterial and murine strains recommended here and will likely need to be adjusted for other strains and/or pathogens. Also discuss the relevance of this model for other bacteria.

Added to the text:
Note: these concentrations were optimized with preliminary animal research to determine lethal doses of each strain. The dosing may need to be adjusted for other strains/species. (lines 458-460)

Using a bacterial strain that is FDA-approved for biopharmaceutical production as the control (such as E. coli strain BL21), this method could be used to test the attenuation of other genetically-engineered strains of P. aeruginosa, or other species of bacteria. (lines 745-748)

10. Please list alternative humane endpoints for the mice. Morbid mice should be euthanized instead of being left to die in the cage.

Added to the text: 3.11. Following the 10 day monitoring period, the animals remaining should recover fully and be clear of any infection administered during the testing. Animals should be humanely euthanized following IACUC procedure. (lines 473-475)

11. Soften the statement about bioluminescence fading after 24 hrs. This depends on the CFUs present at the site of infection and whether the lux construct is integrated on the chromosome, or present in single/multiple copies.

Modified the text: Bioluminescence will fade, regardless of the strain injected. The intensity and longevity of bioluminescence will vary depending on many factors, including the number of bacteria injected, mouse strain, bacterial strain, etc. (lines 624-626)

Minor Concerns:
- Please review the use of italics for gene names.

We italicized all gene names.

- Please capitalize Gram

Capitalized in the text.

- Please clarify the % milk used

5% skim milk – added to text.

- Fig 4 should at minimum include a negative control, as well as a scale for bioluminescence. Adding imaging points over time would also be helpful.

Bioluminescence scale was added to Fig. 4. The other time points were published in the original PGN5 paper.

TITLE:

Generation of in-frame gene deletion mutants in *Pseudomonas aeruginosa* and testing for virulence attenuation in a simple mouse model of infection~~A simple, reproducible mouse model of infection for safety evaluation of genetically-engineered strains of *Pseudomonas aeruginosa*~~

Formatted: Font: Italic

AUTHORS AND AFFILIATIONS:

Meagan E. Valentine[1], Brandon D. Kirby[1], and Hongwei D. Yu[1,2]

[1]Progenesis Technologies, LLC, One John Marshall Drive, Robert C. Byrd Biotechnology Science Center, Suite 314, Huntington, WV 25755

[2]Department of Biomedical Sciences, Pediatrics, Joan C. Edwards School of Medicine at Marshall University, Huntington, WV 25755-9320

Email addresses of co-authors:

Meagan E. Valentine (lester64@live.marshall.edu)

Kirby, Brandon D. (kirbyb@marshall.edu)

Corresponding author:

Hongwei D. Yu (yuh@marshall.edu)

KEYWORDS: *Pseudomonas aeruginosa,* genetic engineering, multiple gene deletion, marker-free, strain validation, safety evaluation, mouse model of infection, reproducibility

SUMMARY:

Here we describe a simple and reproducible protocol of mouse model of infection to evaluate the ~~safety~~ attenuation of the genetically-modified strains of *Pseudomonas aeruginosa* in comparison to the United States Food and Drug Administration (FDA)-approved *Escherichia coli* for commercial applications.

ABSTRACT:

Microorganisms are genetically versatile and diverse and have become a major source of many commercial products and biopharmaceuticals. Though some of these products are naturally produced by the organisms, other products require genetic engineering of the organism to increase the yields of production. Avirulent strains of *Escherichia coli* have traditionally been the preferred bacterial species for producing biopharmaceuticals; however, some products are difficult for *E. coli* to produce. Thus, avirulent strains of other bacterial species could provide useful alternatives for production of some commercial products. *Pseudomonas aeruginosa* is a common and well-studied G~~g~~ram-negative bacterium that could provide a suitable alternative to *E. coli*. However, *P. aeruginosa* is an opportunistic human pathogen. Here, we detail a procedure that can be used to generate nonpathogenic strains of *P. aeruginosa* through sequential genomic deletions using the pEX100T-NotI plasmid. The main advantage of this method is to produce a marker-free strain. This method may be used to generate highly attenuated *P. aeruginosa* strains for the production of commercial products, or to design strains for other specific uses. We also describe a simple and reproducible mouse model of

bacterial systemic infection via intraperitoneal injection of ~~confirmed and~~ validated test strains ~~to ensure the reproducibility and authenticity for safety evaluation~~ to test the attenuation of the genetically engineered strain in comparison to the FDA-approved BL21 strain of *E. coli*.

INTRODUCTION:

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that can cause life-threatening diseases in humans, especially in the immunocompromised. The pathogenicity of *P. aeruginosa* is due to the expression of many virulence factors, including proteases and lipopolysaccharide, as well as its ability to form a protective biofilm [1]. Because of its ability to produce virulence factors and cause disease in humans, using *P. aeruginosa* to make commercial products presents safety concerns. Nonpathogenic strains of *E. coli* have traditionally been used to bioengineer medical and commercial products for human use. However, some products are difficult for *E. coli* to make, and many are packaged in inclusion bodies, making extraction laborious. Engineered bacterial strains with the ability to make and secrete specific products is highly desirable, as secretion would likely increase yield and ease purification processes. Thus, nonpathogenic strains of other species of bacteria, for example, species that utilize more secretion pathways, may provide useful alternatives to *E. coli*. We recently reported the development of a strain of *P. aeruginosa*, PGN5, in which the pathogenicity and toxicity of the organism is highly attenuated [2]. Importantly, this strain still produces large quantities of the polysaccharide alginate, a commercially interesting component of the *P. aeruginosa* biofilm.

The PGN5 strain was generated using a two-step allelic exchange procedure with the pEX100T-NotI plasmid to sequentially delete five~~5~~ genes (*toxA, plcH, phzM, wapR, aroA*) known to contribute to the pathogenicity of the organism. pEX100T-NotI was generated by changing the SmaI to a NotI restriction enzyme recognition site within the multiple cloning site of the plasmid pEX100T, which was developed in Herbert Schweizer's lab [3,4]. The recognition site for the restriction enzyme NotI is a rarer DNA sequence compared to SmaI and less likely to be present in sequences being cloned, thus it is more convenient for cloning purposes. The plasmid carries genes that allow for selection, including the *bla* gene, which encodes β-lactamase and confers resistance to carbenicillin, and the *B. subtilis sacB* gene, which confers sensitivity to sucrose (Figure 1A). The plasmid also carries an origin of replication (*ori*) compatible with *E. coli*, and an origin of transfer (*oriT*) that allows for plasmid transfer from *E. coli* to *Pseudomonas* species via conjugation. However, the plasmid lacks an origin of replication compatible with *Pseudomonas*, and thus cannot replicate within *Pseudomonas* species (*i.e.*, it is a *Pseudomonas* suicide vector). These characteristics make pEX100T-NotI ideal for targeting genetic deletions from the *Pseudomonas* chromosome. Plasmid cloning steps are carried out using *E. coli* and the resultant plasmid is transferred to *Pseudomonas* by transformation or conjugation. Then, through homologous recombination events and selective steps, the targeted in-frame deletion is generated, marker-free. This method of sequentially deleting genomic regions from the chromosome of *P. aeruginosa* could be used to generate highly attenuated *Pseudomonas* strains, like PGN5, or to design strains for other specific uses, for example~~e.g.~~, strains deficient in endonucleases for plasmid propagation, or strains deficient in proteases for production of proteins of interest.

Formatted: Font: Italic

Formatted: Font: Italic

The overall virulence of strains of bacteria is affected by growth conditions and phases, during which mutations occur frequently. Therefore, measuring the safety of genetically-engineered strains can be challenging. To evaluate bacterial isolates for systemic virulence, we adapted a previously published protocol of infection by intraperitoneal injection of C57BL/6 mice [5]. We modified this procedure to use frozen bacterial stocks for injection, which allowed for precise dosing and easy validation of the strain ~~s used authenticity~~. In this model, the *E. coli* strain BL21, which has been FDA-approved for production of biopharmaceuticals, was used as a control safety standard for determining the relative pathogenesis of the strain [6-8]. The main advantage to using this method is that it is reproducible and minimizes sources of variation, as infecting strains are validated ~~as authentic~~ for bacterial cell number, phenotype, and genetic markers both before and after infection. With these controlled steps, the number of animals required is reduced. In this model, *P. aeruginosa* strains that result in C57BL/6 murine mortality rates equal to or less than *E. coli* BL21 when injected intraperitoneally may be considered attenuated. This simple mouse model of infection may also be used to assess the attenuated pathogenicity of genetically-engineered strains from other species using the FDA-approved *E. coli* strain as the reference.

PROTOCOL:

Part I. Generat~~e~~ing sequential genomic deletions in *P. aeruginosa* (Figure 1).

1. Design the plasmid.

1.1. To generate a genetic deletion using the pEX100T-NotI plasmid, the regions of DNA flanking the desired deletion sequence must first be cloned and inserted into the NotI restriction site of the plasmid. The plasmid insert should ~~consist of~~contain about 500 nucleotides upstream of the target sequence directly adjacent to about 500 nucleotides downstream of the target deletion sequence. Additionally, the insert should contain the NotI recognition sequence (GCGGCCGC) at its 5' and 3' ends (Figure 1B).

2. Prepare the plasmid.

2.1. Option 1: utilize traditional cloning procedures. Use PCR to ~~amplification~~ amplify of genomic regions upstream and downstream of the gene of interest, followed by crossover PCR [9,10] to join the generated fragments, restriction endonuclease digestion of the PCR product and plasmid, and ligation [11] (Figure 1B and 1C).

2.2. Option 2: after designing the deletion sequence *in silico*, contract a company that *de novo* synthesizes it to insert into the plasmid pEX100T-NotI (*e.g.*, GenScript, Piscataway, NJ, USA). Many companies have streamlined the process of cloning to quickly and efficiently generate the plasmid of interest. Additionally, the plasmids are sequence-verified to be mutation-free prior to delivery.

3. Transform ~~TOP10~~ electrocompetent *E. coli* with the plasmid according to the manufacturer's recommendations. Using a sterile inoculating loop, s~~S~~treak 10 µL of the transformation reaction

for isolated colonies onto a pre-warmed Luria Broth (LB) agar plate supplemented with 100 µg/mL of carbenicillin and incubate overnight at 37 °C. Note: All equipment and media used to culture bacteria should be treated according to your institution's safety guidelines.

3.1. Passage twice.

3.1.1. Remove the plate from the incubator and identify an isolated colony. Using a sterile inoculating loop, pick up the colony and streak a pre-warmed LB agar plate supplemented with 100 µg/mL of carbenicillin for isolated colonies. Incubate overnight at 37 °C. Repeat this step once more to generate a pure culture.

3.2. Using a sterile inoculating loop, inoculate 5 mL of LB with a single colony from the final agar plate. Place the culture in a shaking incubator at 37 °C overnight. The next day, mix 1 mL of this culture with 1 mL of 5%~~skim milk~~ in a cryovial and store at -80 °C to generate a frozen stock of the strain.

4. Prepare bacterial strains and perform triparental conjugation.

4.1. Use a single isolated colony from agar plates of the following strains to inoculate broth cultures and place in a shaking incubator overnight at 37 °C:

E. coli pEX100T-NotI into 5 mL of LB supplemented with 100 µg/mL of carbenicillin;
P. aeruginosa strain PAO1 into 5 mL *Pseudomonas* Isolation Broth (PIB), and
E. coli prk2013 into 5 mL of LB supplemented with 50 µg/mL of kanamycin.

Note: the prk2013 plasmid is a helper plasmid that replicates in *E. coli* but not *P. aeruginosa*; it carries the trans-acting transfer genes that mobilize the pEX100T-NotI plasmid from the *E. coli* donor to the *P. aeruginosa* recipient[12]. *P. aeruginosa* is a Biosafety Level 2 (BSL-2) pathogen. Please follow your institution's guidelines for safety when working with BSL-2 organisms.

Formatted: Font: Italic

4.2. The next day, remove overnight cultures from the incubator and add 0.5 mL of each culture to a 1.5 mL microcentrifuge tube. Centrifuge at 6000 *g* for 5 min. Discard supernatant and suspend cell pellet in 50 – 100 µL of LB.

4.3. Pipette the entire cell suspension in one droplet onto a pre-warmed LB agar plate. Allow droplet to dry and then invert plate and incubate at 37 °C for 4-6 h.

4.4. After the incubation, use a sterile inoculation loop to collect the cells into 1 mL of LB in a microcentrifuge tube. Pipette up and down to mix the cells.

4.5. Using a cell spreader, streak cells evenly onto a dry pre-warmed *Pseudomonas* Isolation Agar (PIA) plate supplemented with 300 µg/mL of carbenicillin. Streak multiple plates with increasing volumes of the cell mixture (*e.g.*, 10 µL, 100 µL, 500 µL). Incubate overnight at 37 °C.

5. Detect~~ion of~~ single-crossover recombinants of *P. aeruginosa*.

5.1. Remove plates from the incubator and inspect for isolated carbenicillin-resistant colonies. Because the pEX100T-NotI plasmid cannot replicate in *P. aeruginosa*, colonies that grew on carbenicillin-supplemented plates should have arisen from cells in which the plasmid was integrated into the chromosome. Choose at least 4 of these colonies and streak for isolation onto pre-warmed plates of PIA supplemented with 300 µg/mL of carbenicillin. Incubate plates overnight at 37 °C.

5.2. Remove plates from the incubator and inspect for growth. Carbenicillin-resistant colonies should be single-crossover recombinants (*i.e.*, they have incorporated the plasmid into the chromosome via a recombination event between a homologous region of the plasmid insert and the chromosome of *P. aeruginosa*). Patch 8 or more colonies with sterile toothpicks onto pre-warmed plates of:

PIA supplemented with 300 µg/mL of carbenicillin, and
PIA supplemented with 300 µg/mL of carbenicillin and 10% sucrose (without glycerol).

5.2.1. If no colony growth was obtained from step 5.1, repeat the conjugation and ~~try~~ increas~~eing~~ the volume of the cell mixture streaked in step 4.5. If too much growth occurred, repeat conjugation and decrease the volume streaked.

5.2.2. If the conjugation repeatedly fails, an alternative method is to prepare electrocompetent cells of the *P. aeruginosa* strain and transform directly with the pEX100T-NotI plasmid. Detailed protocols for preparation of electrocompetent *P. aeruginosa* and transformation are available elsewhere [13,14].

5.3. Incubate plates at 37 °C overnight.

5.4. Remove plates from incubator and inspect for growth. True single-crossover recombinants will be carbenicillin-resistant and sucrose-sensitive (*i.e.*, colonies that grew on PIA supplemented with carbenicillin, but did not grow on PIA supplemented with carbenicillin and sucrose). Choose 4 or more true single-crossover recombinants and inoculate each into 5 mL of LB without selection. Incubate in a shaking incubator at 37 °C overnight.

5.4.1. If no single-crossover recombinants were detected, repeat the conjugation.

6. Detect~~ion of~~ double-crossover recombinants of *P. aeruginosa*.

6.1. For each broth culture, inoculate 10 µL of culture onto a pre-warmed plate of PIA supplemented with 10% sucrose (without glycerol) and streak for isolated colonies. Incubate plates overnight at 37 °C.

6.2. The next day, remove plates from the incubator and inspect them for growth. Sucrose-resistant colonies should be double-crossover recombinants (*i.e.*, have removed the plasmid from the chromosome via a recombination event between the other homologous region of the plasmid insert and the *P. aeruginosa* chromosome). Patch at least 20 colonies with sterile toothpicks onto pre-warmed plates of:

PIA,
PIA supplemented with 10% sucrose (without glycerol), and
PIA supplemented with 300 µg/mL of carbenicillin.

6.3. Incubate plates overnight at 37 ºC.

6.4. Remove plates from the incubator and examine them for growth. True double-crossover recombinants will be carbenicillin-sensitive and sucrose-resistant (*i.e.*, colonies that grew on PIA and PIA supplemented with sucrose, but did not grow on PIA supplemented with carbenicillin).

7. Confirming gene deletion via colony PCR.

7.1. Prepare 10-20 colonies for a deletion screen with PCR.

7.1.1. Pick up growth from a suspected double-crossover recombinant with a sterile toothpick and suspend cells in 50 µmL of 1X phosphate buffered saline (PBS). Boil suspension at 100 ºC for 10 min, centrifuge for 3 min at 13000 *g*, and then place on ice.

7.2. Perform PCR to screen colonies for the targeted deletion.

7.2.1. Use 1 µL of the supernatant as the template in a 25 µL PCR reaction to confirm deletion of the gene of interest.

7.2.2. Use gene-specific primers that amplify the region of the genomic deletion. Use primers that amplify the region of the genomic deletion plus 100-200 bp of flanking upstream and downstream sequences.

7.2.3. Prepare a separate control PCR reaction with the parent strain (*e.g.*, PAO1), for comparison.

7.2.4. Thermocycler conditions will vary depending on the optimal annealing temperature for primer pairs, the polymerase cocktail used, and the length of the region to be amplified.

7.3. Perform agarose gel electrophoresis on the PCR products. Colonies in which the region of interest has been deleted yield smaller amplification products than colonies that lack the deletion (Figure 2).

7.4. Choose one or more colonies with the PCR-confirmed deletion. Streak for isolated colonies onto a pre-warmed PIA plate(s) and incubate at 37 ºC overnight.

7.5. Passage at least one more time: Remove plate(s) from the incubator and identify an isolated colony. Using a sterile inoculating loop, pick up the colony and streak a pre-warmed PIA agar plate for isolated colonies. Incubate overnight at 37 °C.

7.6. Choose a colony from each final plate and use to inoculate 5 mL of PIB. Place in a shaking incubator at 37 °C overnight.

7.6.1. Mix 1 mL of this culture with 1 mL of 5% skim milk in a cryovial and store at -80 °C to generate a stock of the strain.

7.6.2. Using this culture, prepare genomic DNA from the strain (*e.g.*, using a DNA purification kit). Amplify the genomic deletion region using PCR and primers specific to the region of interest.

Formatted: Font: Italic

7.6.2.1. These PCR products can be purified (*e.g.*, with a DNA purification kit, or phenol-chloroform extraction) and either: sequenced directly with the gene-specific primers; or ligated into ~~the pCR4~~a vector for sequencing with ~~M13~~ plasmid-specific primers.

7.7. After the gene deletion is confirmed through sequencing, this procedure can be repeated with the new deletion strain to sequentially generate numerous marker-free genomic deletions. When the desired strain is generated, ~~we recommend having the~~ whole genome sequencing can be used ~~ed~~ to verify the targeted deletions and to detect other changes to the genome (compared to the reference strain, *e.g.*, PAO1) that occurred throughout the process. After annotating the genes, deposit the sequence to GenBank and record accession numbers.

Part II. Use a~~A~~ mouse model to test the pathogenicity of *P. aeruginosa* strains.

1. Preparation of bacterial strain for animal testing

1.1. To test for attenuated pathogenicity of *P. aeruginosa* strains, validated cultures and stocks must first be prepared. ~~We recommend preparing~~Prepare the *P. aeruginosa* strains of interest, a wild-type strain of *P. aeruginosa* (virulent), and an FDA-approved strain of *E. coli* (*e.g.*, BL21) to serve as a nonpathogenic safety control.

1.2. Streak the strains of the bacteria being tested onto selective agar from sequenced and validated frozen stocks. Incubate at 37 °C overnight.

1.3. With a sterile inoculating loop, pick up a single colony from each strain and streak for isolated colonies onto selective media again. Incubate at 37 °C overnight.

1.4. Remove plates from the incubator. For each strain, choose a single colony and streak for isolation onto LB plates.

1.5. After 24 h of growth at 37 °C, inoculate a 500 mL flask containing 250 mL of LB with a single colony isolate from each strain.

1.5.1. Validation step: using the remnants of the same colony, validate the ~~authenticity of the~~ strain using PCR and strain-specific primers, and/or primers to verify the presence of genetic modifications made to the strain. The primers below were used for verification of strains in the example presented:

E. coli BL21:	T7 polymerase F: TGGCTATCGCTAATGGTCTTACG
	T7 polymerase R: TTACGCGAACGCGAAGTCC
VE2 and PGN5:	*aroA* F: GCGAACGCCAACAGCCGATAAAGC
	aroA R: ATCTGGCTCGCGATGCCGGTCC

1.6. Incubate the cultures in a shaking incubator at 160 rpm and 37 °C until ~~cultures~~ they reach log phase growth (*i.e.*, OD_{600} measurement of 0.4-0.6 on a spectrophotometer).

1.7. Using the OD_{600} value ~~once~~ obtained when log phase wa~~i~~s achieved, calculate the volume of broth required to yield 2.5 x 10^9 colony forming units (CFU) per mL. ~~After log phase is achieved,~~ P~~p~~ellet the volume of broth calculated in 50 mL tubes at 4500 *g* for 10 m~~in~~. ~~Add the volume of broth calculated to 50 mL tubes and centrifuge at 4500 *g* for 10 m.~~

1.8. Discard the supernatant and resuspend the pellet in one tube using 50 mL of 1X PBS to wash the cells. Centrifuge again at 4500 *g* for 10 m.

1.9. Discard the supernatant and resuspend the pellet in 25 mL of 5% skim milk in 1X PBS.

1.9.1. Validation step: use a sample of the 25 mL resuspension to perform viable plate counts to determine the number of CFU/mL.

1.10. Aliquot the 25 mL skim milk culture resuspension into 2 mL culture stocks in 2 mL cryovials. Flash freeze in liquid nitrogen and store at -80 °C at least overnight before use.

2. Validat~~e~~ion ~~of~~ growth and strain of stocks stored for animal testing.

2.1. For each strain to be tested, remove at least 3 cryovials of frozen stocks from -80 °C storage and thaw at 4 °C for 2-4 h. If any frozen stock remains, briefly warm at 37 °C.

2.2. T~~Once thawed, t~~ake small samples from each cryovial to validate ~~the authenticity of~~ each strain.

2.2.1. Perform viable plate counts to determine the number of CFU/mL. It is normal to have fewer CFU/mL after freezing, due to death of some bacterial cells.

2.2.2. Use PCR and strain-specific primers to validate ~~the identity of~~ each strain.

2.2.3. Streak each strain onto selective media to verify the phenotype.

2.~~3~~4. After confirming that strains are of the correct genotype and phenotype, and validating CFU/mL, proceed to animal testing.

3. Inoculate animals with bacterial strains by injection.~~Animal injection and inoculation~~

3.1. Before beginning animal experiments, the protocol to be used must be approved by the Institutional Animal Care and Use Committee (IACUC). Approval for the protocol described was obtained through the IACUC at Marshall University (Huntington, WV, USA).

3.2. On the morning of injections, remove cryovials of the bacterial strains being tested and thaw at 4 °C for 3-4 h. Thaw 0.5 mL for each mouse that will be injected. Keep vials on ice after thawing and inject mice within 2 h.

3.3. After thawing, transfer each cryovial to a new 2 mL tube and centrifuge at 4500 *g* for 10 m, discard supernatant, and resuspend cell pellet in 1 mL of 1X PBS.

3.4. Centrifuge again at 4500 *g* for 10 m. Discard supernatant and resuspend pellet in 1X PBS to a final concentration 2.5 x 10^9 CFU/mL. To determine the amount of 1X PBS needed for resuspension, use the CFU/mL data obtained from viable plate counts on frozen stocks in step 2.2.1. The exact amount of 1X PBS used will vary slightly between strains.

3.4.1. Take 3 samples from final suspension of each strain to validate CFU/mL, genotype, and phenotype as described above.

3.5. For each strain, aliquot 1.5 mL of PBS/cell suspension into one 2 mL tube per 5 mice to limit the number of times the tube is entered. Also prepare tubes of 1X PBS for control injections.

3.6. Gather mice (10 male and 10 female C57BL/6 per group for this experiment) and materials needed for injections: syringes, needles, sharps containers, markers, pen and paper, *etc.*, and move to sterile animal surgical room. Wipe all surfaces with sanitizing wipes.

3.6.1. To eliminate distress and risk of injury to experimenter, only bring one sex and experimental group of mice to the surgical room at a time (*e.g.*, a group of 10 male mice to be infected with a particular strain). Wear two pairs of latex gloves to eliminate puncture of gloves if bitten. Wear lab coat, safety glasses, and face mask to avoid contamination.

Formatted: Font: Italic

3.7. Begin injections of the control group with 1X PBS. This will ascertain whether any adverse effects result from injection alone.

3.7.1. Remove a mouse from the cage. Only remove one mouse at a time.

3.7.2. Weigh the mouse and mark its tail with permanent marker to track for weight loss post-injection.

3.7.3. Open a new 1 mL syringe and 27G needle (use a new syringe and needle for each mouse to eliminate contamination) and inject 200 µl of sterile 1X PBS.

3.7.4. To inject, grab the mouse behind its ears using the ~~with~~ thumb and forefinger. ~~and~~ P~~p~~inch to create skin fold at nape of neck to hold onto~~.~~ ~~with fingers~~ – a tighter fold reduces neck movement and risk of being bitten during injection. Secure tail into the palm using the pinky to ~~secure~~ hold mouse flat with little movement. Turn the mouse over and insert needle at 30-degree angle into the peritoneal cavity to the left or right side of midline. Lift the needle slightly once inserted to ensure ~~needle~~ it was inserted~~to~~ into the intraperitoneal area and not ~~inserted~~ into organs. Slowly inject the PBS and then withdraw the needle. Place the used needle in a designated biohazard sharps container. Do not re-use syringe or needle. A bolus at the site of injection is typical.

3.7.5. After injection, move mouse to a separate cage.

3.7.6. Repeat the procedure with the next mouse. ~~Once~~ After all the mice of one cage ~~is~~ are injected, return them ~~mice~~ to their original cage immediately.

3.8. After injecting ~~ons of~~ the control group, begin injectingons ~~of~~ suspensions ~~containing~~ of strains to be tested following same procedure.

3.8.1. Inject 200 µl of the cell suspension. When beginning with cell suspensions ~~with concentrations~~ of 2.5 x 10^9 CFU/mL, ~~this equates to~~ each mouse receivesing ~~an injection of~~ 5 x 10^8 CFU. Note: these concentrations were optimized with preliminary animal research to determine lethal doses of each strain. The dosing may need to be adjusted for other strains/species.

3.9. Once all injections are complete, return mice to the housing room to alleviate distress. Clean work area with sanitizing wipes.

3.10. Monitor the animals for mortality following injection by checking cages for dead mice every 3 h for 72 h and every 12 h for 10 days. Record the weight of mice every 12 h to determine weight loss due to illness. Also record adverse behavior in the hours following injection, such as difference in posture, lack of grooming or burrowing, immobility, or changes in breathing. Mice that decease from injury associated with injection will exhibit adverse

behavior and die quickly following injection. On the other hand, mice that decease from infection will not start to exhibit adverse behavior or death until after 18 h.

3.11. Following the 10 day monitoring period, the animals remaining should recover fully and be clear of any infection administered during the testing. Animals should be humanely euthanized following IACUC procedure.

4. ~~Statistical analysis~~Examine ~~of~~ animal mortality with statistical analysis.

4.1. Statistical analysis was performed using ~~GraphPad Prism. However, comparable software is suitable and available.~~ graphical software. Any software capable of producing graphs and performing statistical analysis is suitable.

4.2. To plot the mortality data, ~~select an XY table template in GraphPad with the~~use the X column to represent~~ing~~ time (h) and Y column~~s~~ to represent~~ing~~ the groups tested.

4.3. Each mouse~~,~~ or subject~~,~~ in the study will be represented using code = 0 (zero) or code = 1 meaning survival or death, respectively.

4.3.1. For each animal that dies, place a 1 in the Y column of that group at the time of death on the X column. If there are multiple deaths at a single time point within a group, a copy of that time point can be placed in the X column. For example, if three subjects within a group die at 3 h, the 3 h time point will appear three times in the X column.

4.3.2. For all surviving animals within a group, place a zero~~0~~ in the Y column at the final time point measured. For example, if four mice survive, ~~then~~ place four ending time points in the X column marked with four zero~~0~~s in the Y column.

4.4. After animal data is ~~plotted~~ entered for all groups, ~~click the Analyze button within GraphPad and select Survival Curve under Survival Analysis tab~~use a survival graph template to produce a survival graph.

4.4.1. Leave default coding as 0 and 1 will be the default codes for survival or death.

4.4.2. ~~Leave Curve Comparison section parameters selected for statistical analysis~~Set the parameters for the graph as percentage.

~~4.4.3. Select Percent Survival under Style section to display data as percent.~~

4.5. Once parameters for survival curve are selected, ~~the data can be viewed under Results as survival proportions, # of subjects at risk, curve comparison, and data summary. The statistical analysis using the Mantel-Cox (log rank) test is available under the Curve Comparison section.~~perform statistical analysis using a Mantel-Cox (log rank) test.

4.6. Kaplan-Meier plots ~~will be available in the Graphs section under Survival Proportions. Format as desired using the toolbar at the top~~can be formatted using statistical data.

4.7. Strains that exhibit mortality rates that are less than or equal to the parent strain and the ~~safety standard~~FDA-approved strain (*e.g.*, *E. coli* BL21) may be considered attenuated.

5. ~~Visualization~~ Visualize ~~of~~ the infection with bioluminescence.

5.1. To visualize the progress of the infection, a chromosomal bioluminescent operon (*luxCDABE*) was inserted into the PGN5 and VE2 strain tested. The plasmids and protocol used to label these strains were developed in the Schweizer lab and may not be compatible with all species/strains of bacteria [13]. Importantly, visualization of the infection is optional; thus, genomic insertion of this operon is not necessary to perform the mortality study described above.

5.2. Prepare and validate strains using the method described above. Additionally, check for bioluminescence in labeled strains at each validation step.

5.3. After strains are prepared, inject the animals in groups of 10 with the bioluminescent strains following the steps above.

5.4. Image the animals every 3 h for 24 h using an animal imaging system capable of bioluminescence.

5.4.1. First prepare the imager by setting the camera parameters and heating the stage for the animals. Also set the oxygen flow to 1.5 L per min (or following manufacturer's recommendations).

5.4.2. After the ~~temperature~~ imager and stage are~~is~~ stabilized, place one mouse into the anesthesia chamber immediately following injection and administer 3.5% isoflurane into the chamber with O_2 flow for about 4 min. The anesthesia methods may vary depending upon the chamber and/or anesthetic agent used; follow the manufacturer's recommendations. Determine proper anesthesia via withdrawal reflex test.

5.4.3. Move the mouse to the temperature stabilized stage. Position the mouse on its back with arms outstretched, and fit the mouse with a nose cone for administration of 2.5% isoflurane throughout the imaging procedure.

5.4.4. Close the door and take bioluminescent images and X-rays of mouse. ~~Exposure of bioluminescence is automatically controlled by the imager.~~

5.4.5. When imaging is complete, return the mouse to its cage and monitor it. The mouse should regain consciousness within 3-5 min.

5.4.6. Continue to image mice every 3 h for 24 h, each time using a different mouse from each group. Do not reimage a mouse within 24 h due to the possibility of adverse effects due to re-exposure to anesthesia. A single mouse should only receive one dose of anesthesia every 24-36 h. Clean the imaging platform after each mouse is imaged. Turn off the imager between imaging time points.

5.7. Bioluminescence will ~~likely~~ fade ~~around 24 h~~, regardless of the strain injected. The intensity and longevity of bioluminescence will vary depending on many factors, including the number of bacteria injected, mouse strain, bacterial strain, *etc.*

Formatted: Font: Italic

REPRESENTATIVE RESULTS:

As shown in Figure 2, the targeted genomic deletion can be confirmed using colony PCR with specific primers tha~~t~~ amplify the region of interest. Colonies that carry a genomic deletion will yield a shorter PCR band size in comparison to wild-type colonies. A PCR-screen of 10-12 colonies is usually sufficient to detect at least one colony that carries the targeted deletion. If no deletions are detected after multiple rounds of screens, repeat the procedure beginning with the conjugation. If the deletion still fails, the plasmid insert may need to be confirmed through sequencing, redesigned, or the deletion may be lethal. Upon the verification of a gene deletion via PCR, confirm the deletion through sequencing. The resulting strain may be subjected to the procedure repeatedly to generate sequential genomic modifications.

As shown in Figure 3, mortality associated with intraperitoneal injection of the attenuated strain of *P. aeruginosa* PGN5 (+mucE) was 0%, which was equivalent to mortality observed with *E. coli* BL21. On the other hand, intraperitoneal injection of the parent strain (VE2) was fatal to 80% of mice. These results were obtained with extensive ~~validation~~ steps to ~~ensure authentic~~ validate the strains ~~were~~ injected. While the exact cause of death in these mice is unknown, it can at least in part be attributed to the expression of virulence factors in the parent strain that were deleted from the attenuated PGN5 strain. Differences in the infection progression was tracked using bioluminescence-marked parent and attenuated strains. The attenuated strain remained localized at the site of injection until bioluminescence faded (Figure 4). The clearance of the infection most likely coincided with the fading of the bioluminescence. Bioluminescence was not detected 24 h after injection and mice lived for weeks following injection until sacrificed, with no adverse effects observed.

FIGURE AND TABLE LEGENDS:

Figure 1. Generating gene deletions in *P. aeruginosa* with pEX100T-NotI. (A) Map of the pEX100T-NotI plasmid. **(B)** Generation of a construct composed of regions directly upstream (yellow) and downstream (blue) of the region of interest (ROI), flanked with NotI restriction enzyme recognition sites. First, PCR-amplify upstream and downstream regions independently with specific primers that add 5' NotI digestion sites (*e.g.*, NotI-*aroA* F CGCGGCCGCTGAAGGTCCTGGGCTCCTATCCGAAAGCGGTGCTCT and NotI-*aroA* R GCGGCCGCAGTTGGGTTGTTCTGCGATGGCGCCAGGCA) and 3' overlapping homologous regions as shown (*e.g.*, *aroA*-crossover F CTCCAGGCGCTGGGCAAGGTGCTGGCGCATGACTGAGGTCACGCCGGTCGCCGTGGAGAACA and

aroA-crossover R
TGTTCTCCACGGCGACCGGCGTGACCTCAGTCATGCGCCAGCACCTTGCCCAGCGCCTGGAG. Then, use PCR with NotI-containing primers to join the upstream and downstream products generated in the first PCR reaction. **(C)** The pEX100T-NotI plasmid, armed and ready. Ligate the NotI-digested cross-over PCR product into the NotI-digested plasmid. **(D)** Flow diagram of the process to delete genomic regions from the *P. aeruginosa* chromosome using the pEX100T-NotI plasmid. After the desired deletion has been confirmed and purified, the resultant strain can be taken through the procedure repeatedly to delete other genomic regions from the chromosome. When the desired strain is obtained, sequence the whole genome to confirm deletions and other changes to the chromosome. The pathogenicity of the strain can then be tested in mice using the procedure outlined in Part II of the Protocol.

Figure 2. Gel electrophoresis of colony PCR products from a screen for *aroA* deletion to generate the attenuated *P. aeruginosa* strain, PGN5. Colony PCR products run in lanes 2-5 and 8-11 indicate colonies with wild-type *aroA*. Colony PCR products run in lanes 6 and 7 carry the *aroA* gene deletion, indicated by the smaller PCR product (yellow asterisks). Primers used specifically amplified the genomic region containing the *aroA* gene: *aroA*-F: GCGAACGCCAACAGCCGATAAAGC, and *aroA*-R: ATCTGGCTCGCGATGCCGGTCC. Expected PCR product size in wild-type colonies was 2548 nucleotides (nt). Expected PCR product size in colonies with *aroA* deletion was 307 nt. DNA ladder was run in lanes 1 and 12.

Formatted: Font: Italic

Formatted: Font: Italic

Figure 3. Overall mortality of mice injected with pathogenic *P. aeruginosa* strain (VE2), attenuated *P. aeruginosa* strain (PGN5+mucE), and FDA control *E. coli* strain (BL21). Only mice injected with pathogenic parent strain exhibited mortality at 80%. Attenuated *P. aeruginosa* strain and FDA control *E. coli* strain exhibited 0% mortality.

Figure 4. Image of mouse 3 h post-injection of attenuated strain of *P. aeruginosa* PGN5+mucE carrying a bioluminescent marker. The bioluminescent bacteria were detectable until 18-24 h following injection. During this period, the bioluminescence remained at the site of injection indicating the bacteria stayed localized to injection site. This mouse fully recovered with no adverse effects.

~~**TABLE OF MATERIALS:**~~

DISCUSSION:

The pEX100T-Not1 plasmid is an efficient mediator of sequential genomic deletions that are marker-free and in-frame. When engineering bacterial strains for attenuated virulence, deletion of entire gene sequences rather than generating point mutations decreases the likelihood of reversion to a virulent phenotype. Additionally, each pathogenicity gene deletion attenuates the pathogen further, reinforcing the stability of the attenuation.

This method can also be used to generate genomic modifications other than deletions, such as point mutations and insertions, simply by modifying the design of the plasmid insert. These types of modifications may be more useful than entire gene deletions for engineering bacteria

with modified metabolism, for example. Sequential genomic modification has significant potential for generating designer bacterial strains to suit specific purposes in research and industry. Other methods of generating desired marker-free genomic modifications in bacteria have been described [15-18]. As with all genome-editing methods, attempted modifications to essential genomic regions may be lethal, and thus unsuccessful. In these cases, identification of different genetic modifications or other candidate genes is required to generate the bacterial strain of interest.

Given the numerous replication events and passages of each colony throughout this protocol, unintended changes to the genome will occur to the generated strain. The exact genomic changes can be identified through whole-genome sequencing. However, the impact of these changes is harder to determine. When engineering bacteria for a specific purpose, genomic changes that do not negatively affect the growth of the organism or the targeted pathway(s) are tolerable. Depending on the strain being generated, it may be possible to identify a "readout" to ensure that the strain is still useful for its intended purpose. For example, with PGN5, the goal was to create an attenuated strain that retained the ability to produce large amounts of alginate. After deletion of five pathogenicity genes, the amount and composition of alginate produced by PGN5 was measured and determined to be comparable to other alginate-producing strains. Thus, alginate production was unaffected by the five gene deletions, nor by the unintended genomic changes that occurred during the development of PGN5.

A model of intraperitoneal mouse injection was used to determine whether ~~the~~ an engineered strain was attenuated compared to the parent strain and *E. coli* BL21, a strain approved by the FDA for production of biopharmaceuticals. ~~.~~The most important steps taken during this animal testing procedure were preparation and validation of frozen ~~the~~ bacterial stocks. ~~Spontaneous mutation is inevitable in bacterial populations.~~ Preparation and use of frozen bacterial cultures to inject mice is preferable to using continuous culture, as it reduces the number mutations that naturally occur in bacterial populations[19]. Additionally, frozen cultures should remain viable for years. Viable plate counts showed no significant difference between the CFU/mL directly after stocks were prepared and three months after preparation. The use of multiple validation steps throughout this procedure ensured that the method was reproducible, and the results were ~~authentic~~not skewed by contaminating bacteria. Additionally, with the number of precautionary steps taken to ensure reproducibility, fewer animals were needed. Using a bacterial strain that is FDA-approved for biopharmaceutical production as the control (such as *E. coli* strain BL21), this method could be used to test the attenuation of other genetically-engineered strains of *P. aeruginosa*, or other species of bacteria.

Using bioluminescence as a marker provides additional validation ~~that~~ of the bacterial strains injected ~~are authentic~~, as the marker can be visualized at the injection site. Insertion of the bioluminescence marker into the bacterial chromosome is required for bioluminescence imaging but may not be possible if working with incompatible strains/species. However, marking strains with bioluminescence is not required to test for attenuation. The strains tested in this study were marked with bioluminescence, which allowed for visualization of localization differences between strains throughout the course of the infection. We observed that the

Formatted: Font: Italic

Formatted: Font: Italic

Formatted: Font: Italic

pathogenic strain disseminated through the body of the mouse, but the non-pathogenic strain remained at the site of injection. While this experiment only tested two very closely related strains of *P. aeruginosa*, it suggests that bacterial dissemination is linked to virulence, at least in *P. aeruginosa*. Thus, this procedure of labeling with bioluminescence to visualize the progression of the infection could be used in the future to quickly evaluate the attenuation of engineered strains of bacteria.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (NIH) grants R44GM113545 and P20GM103434.

DISCLOSURES:

The author Hongwei D. Yu is the Chief Science Officer and Co-founder of Progenesis Technologies, LLC.

REFERENCES:

1 Gellatly, S. L. & Hancock, R. E. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. *Pathogens and Disease.* **67** (3), 159-173, (2013).

2 Valentine, M. E. *et al.* Generation of a highly attenuated strain of Pseudomonas aeruginosa for commercial production of alginate. *Microbial Biotechnology.* 10.1111/1751-7915.13411, (2019).

3 Schweizer, H. P. & Hoang, T. T. An improved system for gene replacement and xylE fusion analysis in Pseudomonas aeruginosa. *Gene.* **158** (1), 15-22, (1995).

4 Damron, F. H., Qiu, D. & Yu, H. D. The Pseudomonas aeruginosa sensor kinase KinB negatively controls alginate production through AlgW-dependent MucA proteolysis. *Journal of Bacteriology.* **191** (7), 2285-2295, (2009).

5 Yu, H., Boucher, J. C., Hibler, N. S. & Deretic, V. Virulence properties of Pseudomonas aeruginosa lacking the extreme-stress sigma factor AlgU (sigmaE). *Infection and Immunity.* **64** (7), 2774-2781, (1996).

6 Baeshen, M. N. *et al.* Production of Biopharmaceuticals in E. coli: Current Scenario and Future Perspectives. *Journal of Microbiology and Biotechnology.* **25** (7), 953-962, (2015).

7 Marisch, K., Bayer, K., Cserjan-Puschmann, M., Luchner, M. & Striedner, G. Evaluation of three industrial Escherichia coli strains in fed-batch cultivations during high-level SOD protein production. *Microbial Cell Factories.* **12** 58, (2013).

8 Ferrer-Miralles, N., Domingo-Espin, J., Corchero, J. L., Vazquez, E. & Villaverde, A. Microbial factories for recombinant pharmaceuticals. *Microbial Cell Factories.* **8** 17, (2009).

9 Horton, R. M., Cai, Z. L., Ho, S. N. & Pease, L. R. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques.* **8** (5), 528-535, (1990).

10 Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene.* **77** (1), 61-68, (1989).

11 Sambrook, J., Fritsch, E. F. & Maniatis, T. in *Molecular cloning: a laboratory manual* (Cold Spring Harbor Laboratory Press, 1989).

12 Figurski, D. H. & Helinski, D. R. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proceedings of the National Academy of Sciences of the United States of America.* **76** (4), 1648-1652, (1979).

13 Choi, K. H. & Schweizer, H. P. mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa. *Nature Protocols.* **1** (1), 153-161, (2006).

14 Choi, K. H., Kumar, A. & Schweizer, H. P. A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *Journal of Microbiological Methods.* **64** (3), 391-397, (2006).
15 Liang, R. & Liu, J. Scarless and sequential gene modification in Pseudomonas using PCR product flanked by short homology regions. *BMC Microbiology.* **10** 209, (2010).
16 Martinez-Garcia, E. & de Lorenzo, V. Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of Pseudomonas putida KT2440. *Environmental Microbiology.* **13** (10), 2702-2716, (2011).
17 Song, C. W. & Lee, S. Y. Rapid one-step inactivation of single or multiple genes in Escherichia coli. *Biotechnology Journal.* **8** (7), 776-784, (2013).
18 Yan, M. Y. *et al.* CRISPR-Cas12a-Assisted Recombineering in Bacteria. *Applied and Environmental Microbiology.* **83** (17), (2017).
19 Prakash, O., Nimonkar, Y. & Shouche, Y. S. Practice and prospects of microbial preservation. *FEMS Microbiology Letters.* **339** (1), 1-9, (2013).