FOOD SAFETY LABORATORY
CORNELL UNIVERSITY

Making a Mutant in *Listeria monocytogenes*

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Authored by: Barbara Bowen & Angela Roberts
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Approved by: Martin Wiedmann

EFFECTIVE DATE:

APPROVED BY:

_____ _____
Dr. Martin Wiedmann (date)

AUTHORED BY:

_____ _____
(Name) (date)

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose is to produce a nonpolar, null mutant of a gene. This means you want to knock out the gene without affecting transcription of any genes downstream. You will make your mutant by removing a piece of the gene. The procedure you will use is called SOEing PCR. SOE stands for Splicing by Overlap Extension. This is a technique which removes a chunk of sequence between two flanking sequences, then splices the two flanking parts together. The product of the SOEing PCR, the gene sequence with a deletion, will then be ligated into a suicide shuttle vector, pKSV7. This is a plasmid that can be grown in both *E. coli* and *L. mono*. The vector with your gene inserted is first grown in *E. coli* in order to make large amounts of the plasmid, then introduced into *L. mono*. Once electroporated into *L. mono*, the insert carried by pKSV7 will homologously recombine with the cell's wild type gene in two stages: integration and excision. The excision will remove the plasmid and leave behind either the wild type, or the mutant allele.

This protocol is broken down into four parts. Part I describes designing your primers. Part II concerns the PCR reactions, purification and ligation into pKSV7. Part III describes making competent *L. mono* cells and electroporating the vector into them. Part IV describes how to passage or "cure" your transformed cells to achieve the desired mutant.

1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases.

1.3 Definitions

nonpolar: a mutant which does not interfere with surrounding genes/operons.

SOEing PCR: splicing by overlap extension. PCR reactions done to create a construct which will delete a given region of DNA.

suicide shuttle vector: a vector which can be grown in both gram negative bacteria to make ample amounts, and then transformed into gram positive bacteria to be used for homologous recombination. pKSV7 is one; it contains a temperature sensitive origin of replication which at temperatures (39°C+) forces it to be integrated into the cell chromosome at the target gene in order to survive; it also carries a chloramphenicol resistance gene which forces the cell to take the plasmid up in order to survive in the antibiotic. When the temperature is dropped and the antibiotic is removed the plasmid is excised, leaving behind either the wild type or mutant gene.

electroporation: a means of transformation by passing a brief electric current through chemically prepared “competent” cells.

homologous recombination: Genetic recombination between similar (homologous) DNA sequences. This “DNA crossover” occurs during meiosis when the homologous

sequences align and exchange DNA. Used by the cell to repair DNA and to produce genetic variation, it is a technique in Molecular Biology to create targeted mutations.

passaging: continuous inoculation of fresh culture with bacteria, grown to a determined number of hours, and used to inoculate another fresh culture; repeated 8 to 10 times over. Used to help the bacteria with the plasmid construct lose (or excise) the integrated plasmid vector, thus leaving behind the wild type or mutant gene.

1.4 Safety

L. monocytogenes is a BSL-2 pathogen. Appropriate protective measures need to be taken when working with L. monocytogenes. All waste from these experiments needs to be treated as BSL-2 waste. DMF should be opened in the chemical fume hood.

SECTION 2 MATERIALS

- **NEB Q5 Pol: -20ºC in 354 -20°C freezer**
- **Taq Gold -20ºC in 352**
- **primers and dNTPs**
- **pKSV7 plasmid**
- **NEB restriction enzymes and buffers in -20ºC in 352**
- **NEB T4 DNA Ligase -20ºC 415**
- **Xgal:** Xgal is kept in -20ºC freezer near PFGE). Resuspend a 40mg/ml solution in Dimethyl Formamide, and spread 40 µl on each plate, then give the solution 30 min. to soak into the plate. **It is light sensitive!**
- **LB plus ampicillin @ 100 µg/ml, both broth and plates, recipe in media room**
- **BHI plus chloramphenicol @10 µg/ml, both broth and plates**
- **BHI broth + 0.5M sucrose:** add 17.1g sucrose to autoclaved 100 ml bottle of BHI, stir and heat briefly to dissolve sugar, & filter sterilize.
- **1mM Hepes pH7/0.5M sucrose/10% glycerol:** Make 1 mM Hepes pH 7/0.5M sucrose & filter sterilize. Add sterile glyercol to 10%.
- **BioRad electroporator**
- **0.1cm gap cuvettes for electroporator**
- **Go Taq Green polymerase -20ºC kept in 354 -20°C.**
- **Penicillin G: kept in dessicator in room ?. Make 10µg/ml solution in water and store at -20ºC.**

SECTION 3 PROCEDURES

3.1. Part I: Designing Primers

Using a *Listeria* genome sequence most closely related to the strain you are using, find your gene. The databases to use are the Broad Institute's Listeria monocytogenes, or RAST. Copy the gene sequence plus about 500 to 600 bases upstream and downstream into a DNA sequence file in the Lasergene Program, in SeqBuilder. This program will help you visualize both strands, and align the reading frame. If you are unfamiliar with SeqBuilder, sit down with someone who can show you how to use it. You should put a hard copy of your sequence in your notebook, with all the relevent regions carefully marked: start and stop codons, ribosome binding site (rbs), promoters, transcriptional terminators, and your chosen primers. Look at the gene located 5' of your gene and the gene located 3' of your gene for any overlap, and map where their regulatory regions, including transcriptional terminators for the upstream gene, and promoters, etc. for the downstream gene, so that you do not inadvertently affect these surrounding genes. The neighboring gene and its regulatory regions **must not** be changed. You should also make a literature search of the NCBI Pubmed website http://www.ncbi.nlm.nih.gov/ for your gene, and check any relevant papers, to make sure there are no regulatory regions which would be affected by the design of your mutant. Remember, you want a nonpolar mutant.

This map is also helpful in establishing the mutant allele's codon sequence, which you must work out to be sure it remains "in frame".

The two PCR reactions and the primers:

1.

A —▶ C —▶

——————————region to be excised——————————

◀—B ◀—D

2.

A —▶

——————————~~~——————————

◀——D

SOEing refers to the B and C primers, which will end up being spliced together. This is achieved by adding sequence complementary to the B primer to the 5' end of the C primer. The first PCR creates the templates used in the second PCR. The complementary sequence between the B and C ends of the PCR products will bridge the gap between them and "sew" the gene back together, creating a single template out of two.

Designing your primers

1. Make sure the A-B product is the same size as the C-D product (+ or - 5 bp). Aim to make the A-B and C-D products about 400 bases long; no larger than 500 bp nor smaller than 300 bp.

2. Remember to stay "in frame". Check the amino acid sequence to make sure your deletion will not throw it out of frame. This is done by aligning your B and C primers with the start and stop codons, respectively. You should leave 3 to 15 amino acids at each end (see illustration). Be careful not to disturb Ribosome binding sites, promoters or transcription terminators. **For example**:

The mutant allele:

SoeC tail, complement to SoeB SoeC proper

```
          5′  TTT AAA ACC CCC GGG GGA  ATT TTT GGG GGC CCC CCC 3′
Start, 6 a.a.,Phe Lys Thr Pro Gly Gly  Ile Phe Gly Gly Pro Pro 6 a.a.,stop

SoeB      3′  AAA TTT TGG GGG CCC CCT 5′
```

3. The Tm's of both primers in a set have to be similar and at 58°C or higher. The A and D primers must also have a similar Tm. To find your Tm, go to https://www.neb.com/tools-and-resources/interactive-tools/tm-calculator and enter each of the primers (without the added restriction sites or clamps, or for C the complement to B) for the Q5 enzyme. Run off a copy of the result for your notebook. Then go to http://www.idtdna.com , click on SciTools, then click on Oligo Analyzer. Here you can check the self-priming and the heteropriming analyses of the primers against themselves and the primers they will be paired with. These checks are important to avoid death by primer dimer.

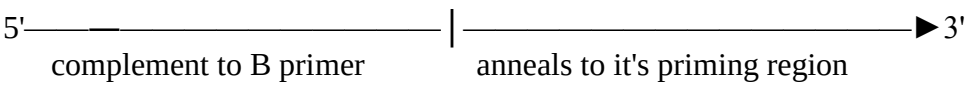
4. Primers should have sticky 3' ends (have G/C in two of the last six bases), and have no complementarity between their 3' ends (which would lead to primer dimers, check on IDT).

Look at the attached map of pKSV7 and its polylinker site. Compare the restriction sites there to a restriction map of the region you are cloning. You need to be sure no sites exist within the A-B or C-D sequence for the enzymes you choose. One enzyme each will be attached to the 5' ends of primers A and D to be used to ligate into the same site within the polylinker site of pKSV7. Go to www.neb.com to find out how many random bases each restriction site will need after it for a "clamp", and add those to the very 5' most end, just before the restriction site.

A and D primers look like this:

5' GG/GGTACC/18-30 bp annealing region► 3'
clamp/ restriction site/ rest of primer

C primers look like this:



XF and XR primer design

In addition to these four primers, you will want to design primers outside the A and D primers, called XF and XR. These do not have as many limits on their design as the others. The only rules are:

1. XF must be 50 bp or more upstream of SoeA
2. XR must be 50 bp or more downstream of SoeD
3. XF and XR must have approximately the same Tm
4. They must follow the same rules for 3' end as all primers (i.e., must be G/C rich and not primer-dimer forming)

These primers are then used to confirm that you have a mutant at the end of the process.

Write out all six primers and have two other people in the lab double-check your primers before ordering.
These people must be recorded in your notebook thusly:

Primers ordered (date)
Double-checked by: names of the two other people.

Order from the list on the page that was double-checked; do **not** recopy them, since this would re-introduce the possibility of error.

Checklist for primers:

1. Make sure that primers are in the correct orientation 5' to 3', **and** correspond to the correct strand.

2. Make sure the tail of B (or C) is the reverse complement of C (or B).

3. Check that the restriction sites are correct and have the right number of bases as "clamp". Check that there are no restriction sites for these enzymes that will form the mutant allele (inside the region to be excised is acceptable).

4. Check that 3 out of the last five or six bases at the 3' end are G or C.

5. Check that there are no significant hairpins.

6. The melting temperature must be higher than 52°C.

7. The B and C primers must be 'in frame'. The codons and their amino acids must be written out for both primers, from the start codon upstream of the B primer, to the stop codon downstream of the C primer, which will give the mutant allele.

8. All known regulatory elements gleaned from the Broad institute website and from PubMed publications, must be marked on the map.

9. Recalculate the distances from the 5' ends of A and B, and from C to D, to confirm that they are within 5 bp of each other in size.

10. Check that the primers as written for ordering are identical to the primers as outlined on the gene map. Also make sure that the A and D primers include their restriction sites and clamps, and that the "tail" is included in the B or C Soeing primer.

3.2 Making a Mutant in *Listeria monocytogenes:* Part II

A. The First PCR

Purpose:
The first PCR generates the two fragments, A-B and C-D, which will be "sewn" together to make our mutant in the second PCR. This first PCR should be done with Vent, a high-fidelity, proof-reading polymerase.

Procedure: PCR using Q5
Q5 comes from NEB with 5X buffer, which already contains Mg++. * (hence you will not need to add MgCl2 to the reaction below). Your template should be a lysate of 10403S. (If you need to make a lysate, see the protocol on the Laboratory website.) You can also use genomic DNA, but be sure to dilute it out to 1 to 10ng/µl.

Set up two PCRS: one with undiluted lysate and one with a 1:10 diluted lysate. Run negative controls on both sets of primers in a third tube.

1. Gather your reagents and let them thaw. Once they are thawed, place them on ice. Keep the lysates separate from your other reagents. **Do not bring the lysates into the PCR prep area**. Wear gloves. Take your PCR reagents on ice into the PCR prep alcove, where you will find a PCR rack. Make the mixes detailed below. For each mix, multiply the amounts of each reagent by the number of your samples, including controls, and for each 10 tubes, add one for error. In this case, you have 2 samples, times 2 for each dilution of lysate, plus a negative control = 6. Since you have more than one set of

primers, you must make a separate master mix for each set of primers or one master mix without primers, split in half, and then add primers.

Rxn	Sample	Primers
1	With undiluted template	BB500geneXSoeA,BB501geneX SoeB
2	With 1/10 dilution	Same primers (SoeA and SoeB)
3	Negative control	Same primers (SoeA SoeB)
4	With undiluted template	BB502geneXSoeC, BB503geneX SoeD
5	With 1/10 dilution	Same primers (SoeC and SoeD)
6	Negative control	SoeC SoeD

	uL per reaction	cocktail ____ X rxns
diH2O	31.75	
5 X Buffer	10	
25 mM MgCl2	N/A *	
dNTP (10 mM)	1	
Primer F: 10 µM	2.5	
Primer R: 10 µM	2.5	
Polymerase: Q5	0.25	

Template: 1 µl **DNA**
Total volume per reaction: 50µl

PCR in (name of Thermal cycler, i.e., Bubba, Belle)	
Initial hold	98ºC 30 sec.
30 cycles	98ºC 10sec, (xx)ºC 20 sec., 72ºC 20 sec.
cycles	
Final extension	72ºC 2 min.
Final hold	4ºC ∞

2.) Aliquot 49µl Master mix per PCR tube.

3.) Prepare template on your benchtop: dilute lysate 1:10 in water. Use 1µl each dilution as template.

4.) Run a gel on your PCR using 6 to 10 µl of your reaction per well, depending on the size of your wells. You can choose to gel extract these bands immediately, or if you are pressed for time, run an analytical gel first and a preparative gel later. Gel extract your bands using whatever good kit is available. I have used Qiagen, as well as SNAP and PureLink from Invitrogen. The gel extraction is important to get rid of the B and C primers. **Be careful to minimize u.v. exposure to your gel, as this can harm the DNA and interfere with your next PCR**. Run 5 µl of your gel extracted bands on another gel to be sure you recovered your DNA.

If you encounter difficulty with this first PCR ,i.e., extraneous bands, try a **Touchdown PCR** using Q5 polymerase. If you do not know how to set up a Touchdown, consult one of the Technicians. With Touchdown, your annealing temperature drops 0.5ºC per cycle for the first twenty cycles, then another twenty cycles is done at 10ºC below the starting annealing temperature. So, for instance, if you have Tm's of 60º and 62º for your primers, with a recommended annealing temperature for your Q5 polymerase of 63ºC, begin your Touchdown PCR at 68ºC and let it drop after twenty cycles to 58ºC. This will finesse your amplification, since by beginning at a higher temperature and then dropping, fewer extraneous bands will result, and fewer primer dimers. The total of 40 cycles will also give you a robust yield.

Touchdown PCR in (name of Thermal cycler, i.e., Bubba, Belle)	
Initial hold	98ºC 30 sec.
20 cycles	98ºC 10sec, 68ºC*↓58ºC, 20 sec., 72ºC 20 sec.
20 cycles	98ºC 10 sec, 58ºC, 20 sec., 72ºC 20 sec.
Final extension	72ºC 2 min.
Final hold	4ºC ∞

B.The Second PCR

Purpose: Using the A-B and C-D products as your template, run a PCR using the A and D primers. This will yield your gene sequence with the deletion.

Procedure: See protocol above for Q5 polymerase.

In an Eppendorf tube, combine your A-B and C-D gel extracted products.
Set up two PCR's, one with 1µl of each A-B and C-D (adjusted to achieve equimolar concentrations), and one with 1 µl of a 1:10 dilution of each A-B and C-D. Using A and D primers and Q5, do a PCR with annealing temperatures for the A and D primers together.

Run 8 µl of the A-D PCR on a gel to confirm you have a single band of the correct size. You can clone this PCR band directly into pKSV7, or you can clone it into an intermediate vector. Try cloning directly into pKSV7 first, and resort to the intermediate vector only as a last resort.

C. Cloning into pKSV7

1.) Pass your successful A-D PCR products over a Qiagen PCR Purification column, following Qiagen's protocol. This prepares the amplicon for digestion by getting rid of primers, polymerase and especially by changing the buffer.

2.) Meanwhile, if you have not already, prepare a large plasmid prep of pKSV7. This is a big, low copy number plasmid, so grow and prep 2 x 5 mls. Use the Qiagen miniprep kit and combine the pellets of three 1.5 ml aliquots of saturated culture in one tube, i.e., spin down 3 x 1.5 mls o/n culture, discard supernatant, and using 250 µl of P1 buffer, resuspend first one pellet, then transfer the cell suspension to the second tube, resuspend that pellet, and repeat for the third pellet. This will give you a concentrated amount to prep over one column, improving your yield.

3.) **Digest** 2 - 3 µg of pKSV7, and, in a separate tube at the same time, digest most of your insert with the two chosen restriction enzymes. Two to four hour digests are best. If your enzymes require incompatible buffers (check the NEB catalog appendix, or www.neb.com "Double Digestion" table), digest first with the enzyme that requires lower salt, pass that completed digest over a PCR purification column in order to change buffers, and proceed to your second digest

Restriction digests: Set up the digests to add the enzymes last, and keep the enzymes on ice while you are working. Tap and invert to mix. *Never vortex enzymes.*

1.) **Digest of pKSV7**	**Digest of PCR product**
1-3 µg of pKSV7	purified A-D PCR product
5 µl 10X NEB buffer	5 µl 10X NEB buffer
0.5 µl BSA	0.5 µl BSA
10-20 units of enzyme A	10-20 units of enzyme A (typically, 1-2 µl)
10-20 units of enzyme B	10-20 units of enzyme B
dH2O to bring volume of each digest to 50 µl	

2.) digest two hours in the 37ºC waterbath, or in 0.2 ml tubes in a thermal cycler.

2.) Purification: **NOT OPTIONAL** Purification now is important, as it gets rid of both the restriction enzymes and the salt from their buffers, as well as the small piece of DNA that has been digested out of your vector and off the ends of the amplicons. You can pass both the vector and the insert over Qiagen PCR purification columns, or use gel extraction as before. **Gel extraction will be necessary if you see smaller bands under your A to D band, or if upon ligating and transforming, you find only small inserts in your plasmid**. I highly recommend gel extraction. I routinely gel extract both my plasmid and insert. Gel extraction gives fewer colonies, but a greater percentage of correct inserts.

3.) After purification, run an analytical gel on 5 to 8 µl of your yield of pKSV7 vector and insert to confirm how much you have of both. You can gauge how much you have of each purified DNA by examining your analytical gel and estimating relative amounts.

Ligation:
1.) Now you must establish the ratio of vector to insert. For your ligation, you need an approximately 3:1 (insert:vector) concentration of ENDS. Since it is smaller, your insert will have many more ends **per nanogram** than the vector. To help you visualize this, let's say these

lines below represent the approximate lengths of the individual DNA molecules: the insert and vector may contain the same amount of DNA, but the shorter molecules of the insert have many more ends, and the ends are what we're trying to ligate together.

insert:	vector:
_____	_____
_____	_____
_____	
_____	
_____	
_____	

You can calculate the number of your ends by dividing the number of bp of pKSV7 (6900 bp long) by the number of bp in your insert. For example, an insert of 700 bp will have almost 10 x more ends **per nanogram** than the vector (6900/700). So, for an equimolar ratio, you would need 10 ng of insert for every 100 nanogram of vector. For a 3: 1 ratio, you would need 20 ng of insert for every 50 ng of vector

2.)Be sure to thaw the 10 X ligation buffer completely, at room temperature. (Remember, the ATP in the buffer is labile. Handle with care and do not freeze/thaw too many times.) For a negative control, leave out the insert and substitute water. This control will tell you how much background of undigested and incompletely digested vector you have. Prepare on ice and add the ligase last. Have your thermal cycler at 22ºC before putting in the tubes.

3.) x µl vector (your calculated amount)
x µl insert (your calculated amount)
2 µl 10x ligase buffer
1 µl ligase
dH2O to bring volume to 20 µl

4.) 22 ºC for one hour. (It can be done from 20 minutes to two hours but I find one hour works extremely well.) Store at 4ºC if necessary before proceeding.

Transform 2- 4 µl of your ligation and your negative control into E. coli TOP 10 competent cells, or into NEB's DH5alpha cells.

Transformation:
1.) Thaw the competent cells E. coli TOP 10 cells on ice. These cells are kept in the –80 freezer and should be taken out and placed very quickly on ice. Do not **ever** take the box itself out of the –80. **Keep these cells cold** until time for the heat shock.

2.) To the tube of cells, add: 2-4 µl of your ligation.

3.) Hold on ice for 15 min (Top 10) or 30 min (DH5alpha).

4.) Heat shock at 42ºC for 30 seconds.

5.) Replace on ice

6.) Add: 250 µl R. T. (room temperature) S.O.C. media (Top 10), or 950 µl (DH5 alpha).

7.) Parafilm the top of the tube to prevent leakage, and shake at 37ºC 1 hr.

8.) Spread aliquots of 20, 50 and 100 µl on three LB/amp(100 ug/ml) plates to which Xgal has been added for blue/white color selection, for Top 20. For DH5 alpha, make dilutions of 1:10, and spread plate 100 µl of each.

9.) Grow O/N at 37ºC.

10.) Pick about 10 colonies and perform colony PCR on them, using M13 forward(–20) and M13 revmut primers (vector specific). As you pick each colony with a sterile toothpick, streak it out on LB/amp100 plates immediately after touching it to the PCR tube. (Alternatively, you can use a "dirty lysate" by placing your colony in 100µl of dH2O, and microwaving for 30 seconds (for E. coli). If choosing this, touch the plate with the colony on a stick BEFORE swishing it in the tube of water. Incubate the plates overnight at 37.

11.) Of the plasmids that have the correct size insert, choose 2 to prep: inoculate 5 mls LB/amp100 with the colony from the PCR streak plate and grow overnight. Perform plasmid preps and send the plasmids to be sequenced at Biotech, using the M13 primers. You can request that the forward sequence be performed with BRC's own M13 Forward primer, but you will need to send the reverse sequence with our M13 mut rev primer.

As you pick and grow them, give names to your plasmids: p for plasmid, your initials, and a number. So, Jane Mary Doe's first plasmid is pJMD1.

12.) Analyze the sequence, following the lab protocol, to be sure you have the correct sequence with no errors. Be sure to confirm the mutant allele by examining the amino acid sequence in lasergene 8. Run the sequence against the wild type sequence in MegAlign using "Align", "One Pair" and choosing Martinez NW. Place the print-out in your notebook. Indicate on this aligned sequence where your A, B, C and D primers fall.

13.) Grow your plasmid in LB/amp100 and freeze it down at –80ºC, following the lab protocol for freezing down strains. **Do not put this off! Freeze it down as soon as you know you have the insert with correct sequence**.

When you know you have the correct insert, proceed with your electroporation into the target Listeria, Part III of **Making a mutant in *Listeria monocytogenes*.**

3.3 Making a mutant in *Listeria monocytogenes* Part III: Electroporation

Making Competent Cells:

1) Make 100ml BHI/0.5M sucrose by adding 17.1g sucrose to autoclaved bottle of BHI, stir and heat briefly to dissolve sugar & filter sterilize.
Make 1 mM Hepes pH 7/0.5M sucrose/10% glycerol:
- Make 10 mM Hepes pH7.0 and dilute to 1mM using diH2O, and dissolving 17.1g sucrose 0.5M sucrose.
- Filter sterilize.
- Add sterile glyercol to 10%.

Prepare 10 mg/ml penicillin G in dH2O. This can be stored at -20ºC and reused.

2) inoculate L. monocytogenes strain in 5 mls BHI
- grow at 37°C with shaking overnight

3) inoculate 50 ml BHI/0.5M sucrose with 0.5ml of the overnight (use a 300 ml sidearm flask)
- 37° + shaking until OD_{600} = 0.2 (0.22 – 0.27 is best)
  for 10403S strains, check OD between 3-5 hrs (usually at OD by 4- 4 1/2 hrs).
- if overgrown by more than 0.27, start over

3) add 50 µl 10mg/ml penicillin G to culture (therefore 10µg/ml final conc.)
- 37° + shaking 2 hrs
- do not grow longer than 2 hrs

*** KEEP LISTERIA AT 0-4 DEG. FOR REMAINDER ***
OF THIS PROTOCOL
chill your pipets in the refrigerator, keep Hepes/sucrose/glycerol on ice, etc.

4) centrifuge = 7000rpm for 10 min. @ 0-4°
- resuspend pellet in 45 mls1mM HEPES (pH 7) + 0.5M sucrose + glycerol
- centrifuge = 7000rpm for 10 min. @ 0-4°
- resuspend pellet in 22.5ml 1mM HEPES (pH 7) + 0.5M sucrose + glycerol
- centrifuge = 7000rpm for 10 min. @ 0-4°
  resuspend pellet in 0.2-0.4ml 1mM HEPES (pH 7) + 0.5M sucrose + glycerol

7) electroporate cells immediately or save at -80°
- to save cells, immediately place in a freezer box pre-chilled to -80º C.
  Handled correctly, frozen competent cells are good for at least 6 weeks.

Electroporating Competent Cells:

1) thaw competent Listeria <u>on ice</u> (if frozen @ -80ºC) & prechill cuvette on ice

2) Add1-3µg plasmid to competent Listeria and mix (in no more than 10% volume of cells)
   - for a 0.1cm cuvette, use 100µl *Listeria* , <10µl plasmid

3) Using the time constant protocol (#2 on the menu), set electroporator to yield:

   voltage = 1400
   time constant= 5
   cuvette mm= 1

   The machine will display the actual voltage and the time constant. If the machine is working properly, they should be very close to these numbers. Resistance and capacitance should be 100 and 50, respectively. Record these in your notebook.

4.)Electorporate at above settings, and add 1ml BHI + 0.5M sucrose <u>immediately</u>
   - Place on ice for 30 sec.
   - Incubate for 1-2 hours without shaking @30° C

5) Plate 100 µl on each of four to ten selection plates (BHI /10 µg/ml Chloramphenicol)
   - incubate at 30° for 2 days to see colonies; at 48 hrs you should have a good count. Rarely, some colonies take longer to grow.
   - streak out colonies on BHI/10 µg/ml Chloramphenicol & incubate @ 37º overnight.

3.4 <u>Making a mutant in Listeria monocytogenes Part IV</u>

Homologous Recombination in *L. monocytogenes* using Temperature-Sensitive Suicide Shuttle Vector, pKSV7

Purpose: To describe a procedure for achieving homologous recombination in *L. monocytogenes* by use of the temperature-sensitive suicide shuttle vector, pKSV7. This procedure is useful in making deletion mutants or swapping gene alleles by allelic exchange.

You will do two sets of passages. The first one, performed at 40ºC with Chloramphenicol, forces the vector to integrate. (The vector has a temperature sensitive origin of replication and cannot survive extrachromasomally. The cells need the vector to give them chloramphenicol resistance.) The second, permissive set of passages, is performed at 30ºC with no antibiotic and enables the second part of recombination which will either revert the cells to wild type, or create your mutant.

Procedure:

1. Following electroporation, pick several colonies from BHI + cm_{10} plates and inoculate into 5mls of BHI + cm_{10}. and grow at 40°C with **gentle** shaking (<100 rpm).

2. To select for vector insertion, passage transformed cells 4 to 5 times (once per day) at 40°C in BHI + cm_{10} using a 1:100 dilution (50µl culture per 5mls BHI) per passage. Incubate overnight with gentle shaking. (If you are unable to obtain growth with a 1:100 dilution, try a 1:50 dilution and passage 5 or 6 times.) You may have to let the culture grow 48 hours at one point, and you may lose one or more tubes, so begin with at least four different colonies from your electroporation plate. If you fail to obtain growth, try over again at 39.5°C.

3. Streak out last passage on BHI + cm_{10} plates. Incubate plates at 40°C overnight or longer. It often takes 36-48 hours.

4. Grow up and freeze down a colony from this plate. (This is insurance in case you have to return to this stage.)

5. Pick at least two colonies and begin selection for vector excision by passaging cells in BHI **without** chloramphenicol at 30°C with shaking using a 1:1000 dilution per passage. Passage 2 times per day (3 times a day to stay in log phase for ΔSigB's and related stress genes -- i.e. anything required for stationary phase survival). If you do the 3 times a day, or 8 hour passages, give the first passage, inoculated from a single colony, 10 to 12 hours to grow, before initiating the 8 hour passages.

6. Starting with the third passage, plate out some of **each** passage's culture. You may replica plate, or "patch". For patches, which we do in this lab, make serial dilutions in PBS and plate 100 µl of 10^{-6} for 12 hour passages, 10^{-5} for 8 hour passages. Incubate plates at 37°C 24 to 36 hours. At the same time, keep the passages going. Typically, 8 or 10 passages are enough to generate your mutant.

7. Screen for vector excision: for patches, draw numbered grids on two plates, a pair with and without chloramphenicol. For each colony from a plate: touch a sterile toothpick to the colony, touch the toothpick to the drug plate, then touch it to the plain plate. Incubate overnight at 30°C. Those colonies which show growth on the plain plate but not on the chloramphenicol plate are "chloramphenicol sensitive" and possibly your mutant. You will want to screen at least 200 colonies per passage, preferably more.

8. To confirm chloramphenicol sensitivity, pick putative chloramphenicol-sensitive colonies and sub-streak onto BHI and BHI + cm_{10} plates. (Continue passaging broth cultures in BHI without chloramphenicol at 30°C and repeat replica plating or patching for each passage.)

9. Confirm by colony PCR that homologous recombination has occurred in chloramphenicol sensitive colonies. Screen using a set of primers outside the set used to make the mutant, ie., your XF and XR primers.
10. Send your amplicons to be sequenced with the XF and XR primers to confirm that you have the gene excised.

11. Give your mutant an FLS number, and freeze down two tubes in your box, and a third tube in the Mutants box.

SECTION 4 TROUBLESHOOTING

Part II Potential problems encountered in creating the plasmid construct: 1.) Many white colonies, no inserts. The solution to this problem is to gel extract both the plasmid and the insert. 2.)Many blue colonies, few or no white colonies. The solution is first of all, if you have any white colonies, screen them (you only need one). If you still have no inserts, and your negative control has a near equal number of colonies, one of your restriction enzymes is not working. Check the conditions of the digest by looking up www.neb.com . Use more enzyme; digest longer. Gel extract both insert and plasmid as above. 3.) If problems persist, consider using an intermediate vector, such as the pCR2.1 that comes with Invitrogen's kit for cloning PCR products. Your insert can then be digested out of this vector in large, pure amounts and then ligated into pKSV7.

Part III Potential problems in electroporation: 1.) Arcing. This is caused by too much salt in the competent cells/plasmid, or air bubbles. Try again with fresh competent cells, making sure they are made properly, and there are no air bubbles. Check to be sure the machine is functioning well. 2.) No transformants. The best results are obtained with 2 or more µg of plasmid in cells that were well over OD600 = 0.2 when the Penn G was added to them (up to OD600 = 0.27). These two factors are the most important for good yield after the proper functioning of the machine.

Part IV Potential problems in passaging and finding the mutant: 1.) Transformants will not grow at 40ºC. Start with at least four tubes from four different colonies, and let them grow at least 2 or 3 days before giving up on them. If they still will not grow at 40 ºC, try them at 39.5 or 39ºC. 2.) Many wild type but no mutants. The mutant is probably too weak to survive the competition of stationary phase. Go to 8 hour passages. 3.) No chloramphenicol resistant colonies. Keep passaging! If by ten passages you are still seeing very few, start the 30ºC passages over again. 4.) Nothing works, you've gone back and done everything over and over again, six different times. Sometimes (very rarely), a mutant is lethal. Search the literature and see if anyone else has made the mutant. If not, there may be a reason. This is not a conclusion to come to quickly or lightly, however, so do persist. This lab has succeeded in making mutants that others concluded were "lethal".

References

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