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March 2022

Tuesday, March 1, 2022

To Do:

1. ~~Make and filter sterilize 2.5% iron pyrophosphate~~
2. ~~Supplement MHB~~
3. ~~Gel and transfer of KRLVS156~~

Results and Data:

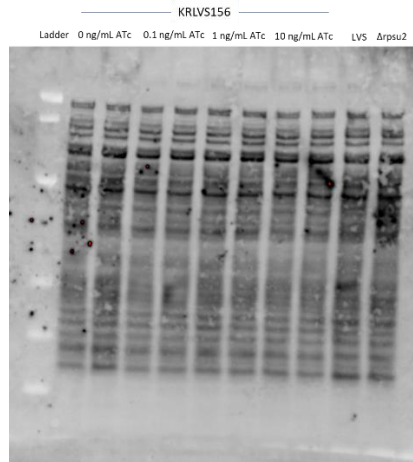
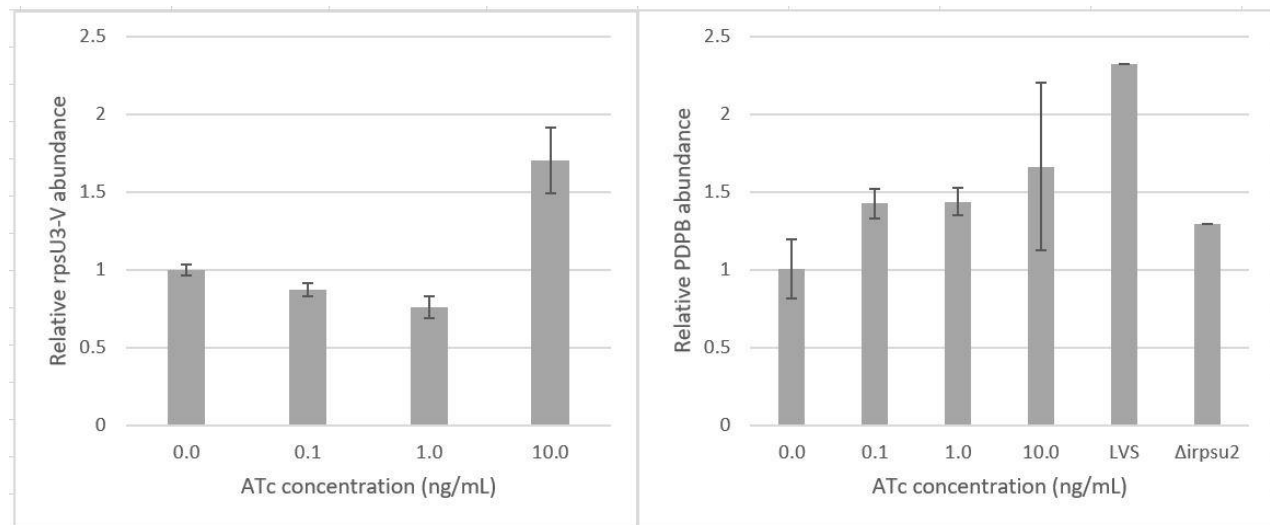


Fig. 1: Total Protein Quantification of KRLVS156 cells grown at 0, 0.1, 1, and 10 ng/mL Atc; in technical duplicate, as well as LVS and Δ rpsu2



Running Gel on ATc Titration Induction of KRLVS156 Samples

1. Heat samples for an additional 10 minutes.
2. Assemble gel chamber with 15-well pre-cast NuPAGE 4-12% Bis-Tris gel
3. Add Mes Running Buffer to gel chamber (front and back)
4. Use 200 uL pipet set to 200 to wash wells of gel
5. Loaded 9 uL of each sample
6. Used 5 uL of Western Sure ladder
7. Ran at 150V for 40-45 minutes.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15
SLB	WSL	1	2	3	4	5	6	7	8	LVS	Δrpsu2	SLB	SLB	SLB

Wet Transfer

1. Make transfer buffer and store in freezer to chill until the gel has stopped running.
2. Grab a bucket full of ice, then proceed to set up for the transfer.
 - a. Retrieve materials and cut filter paper and membrane.
3. Activate PVDF membrane (must use Millipore Immobilon-FL #IPFL00010) in ethanol from chemical storage cabinet
4. Remove transfer buffer from freezer and add 0.5 mL NuPAGE antioxidant
5. Presoak three sponges and filter paper in Pyrex dish with transfer buffer; roll air bubbles out of sponges, then presoak membrane in transfer buffer
6. Open gel case, cut off wells and bottom ridge on gel and place wet sheet of filter paper on the gel.
7. Peel gel and filter paper off and place wet membrane on gel.
8. Place other filter paper on membrane and roll out bubbles
9. Dunk the transfer cassette halves in the transfer buffer and begin assembling sandwich:
 - a. Thickest sponge
 - b. Filter/Membrane/Gel/Filter sandwich so that **the membrane is on top of the gel**
 - c. 2 more sponges
10. Close transfer apparatus and clamp into the gel box.
11. Place the gel rig into a large ice bucket. Fill the gel chamber with transfer buffer. Close the lid and pack ice around the sides of the active gel chamber.
12. Run at 20V for 1 hour.

Total Protein Quantification

1. Prepare No-Stain labeling buffer by diluting 20x stock to 1x (9.5mL ddiH₂O+0.5mL 20x buffer).
2. Thaw Activator and Derivatizer, add 20 uL of activator followed by 20 uL of derivatizer
3. Pre-wash the blot twice with water, for 2 minutes each, on a nutator.
4. Add 10 mL of No-stain labeling solution to the blot, and nutate for 10 minutes.
5. Post-wash with water for 2 minutes, three times.
6. Image using the Bio-Rad imager with protocol Blot->No Stain.
7. Quantify using LiCor Image Studio Lite.

Blocking and Probing

1. Block membrane with Li-Cor Odyssey Blocking Buffer (PBS) diluted 1:5 in PBS

Reagents

Running Buffer (400 mL | 1 gel)

Mix together:

- 1 x MOPS for large proteins
- 1x MES for <50 kD proteins
 - 380 mL ddiH₂O
 - 20 mL 20x MES
 - 1 mL NuPAGE antioxidant

Transfer Buffer (500 mL | 1 gel)

Mix together:

- 50 mL methanol
- 25 mL NuPAGE 20x transfer buffer

Water to 500 mL

No-Stain Labelling Buffer (10 mL)

Mix together:

- 9.5mL ddiH₂O
- 0.5mL 20x No-Stain Labelling buffer
- 20 uL Activator followed by 20 uL Derivatizer

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalex (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

2.5% Iron pyrophosphate0.75g iron pyrophosphate (cabinet) in 30mL of ddiH₂O (type 1), dissolved overnight, fresh solution every 2 weeks**Wednesday, March 2, 2022****To Do:**

1. Western of KRLVS156 samples
2. Imaging blot

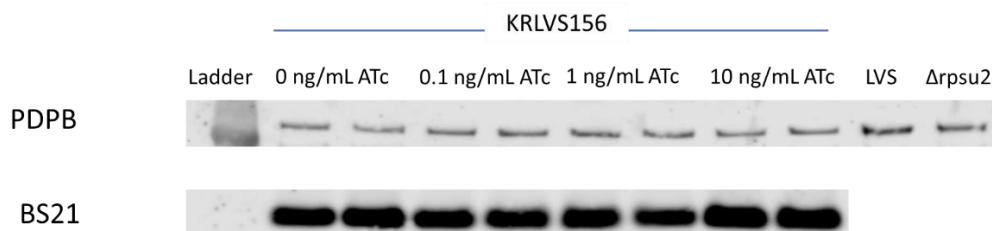
Results and Data:

Fig. 2: Western Blot of KRLVS156 cells grown at 0, 0.1, 1, and 10 ng/mL ATc; in technical duplicate.

Additionally, the PDPB of an LVS and $\Delta rpsu2$ sample is shown.

Blocking and Probing Pt. 2

1. Cut the membrane between the PDPB and BS21 bands (between fourth and fifth band of ladder)
2. Add 4.5 uL antibody (anti-VSVG and anti-PDPB) in 10 mL of blocking buffer. Rotate for 1 hour at room temperature.
3. Prepare 1X Wash Buffer
4. Wash (w/detergent) 4x on rotator for 10 minutes each..
5. Use diluted blocking buffer and block again, for 20 min.
6. Transfer membrane to black box and add 1 uL secondary antibody (anti-rabbit for VSVG, anti-mouse for PDPB) to 10 mL wash buffer (w/detergent). Also add 0.01% SDS to the wash buffer (10 uL of 10% SDS).
7. Label for 1 hour on rocker at room temperature.

8. Wash 4x on rotator for 10 minutes each.
9. Wash 2x on rotator for 10 minutes each with wash buffer WITHOUT detergent.
10. If faint bands are expected, do a quick methanol rinse by dunking the blot in methanol for 2 seconds then letting air dry for 2 minutes prior to imaging.

Imaging Blot

1. Leave the membrane in the box containing the final wash buffer. Bring the box, gloves, forceps, and a timer to the imager in the INBRE facility.
2. Open the Li-Cor and cleaned the surface of the scanning apparatus.
3. Place membrane face down in the bottom left hand corner. Note the size of the membrane using the grid (usually about 8x8 or smaller).
4. Set Image Quality to High and press 'Start'
5. Adjust the brightness and contrast on each channel until image appears the best. Using the black bands on white background setting is typically best.
6. Save image as Image Work File and export as Tiff
7. Remove your membrane and spray down the glass surface of the scanner with ethanol and kimwipes (provided there).
8. For quantification, click on Analyze. Either use "Add Rectangle" or "Draw Rectangle" features to instantly quantify the density of each band.

Reagents

1X Wash Buffer (Scaled down to 130 mL)

Mix together:

26 mL 10X PBS

234 mL diH₂O

Separate into 200 mL and 60 mL. To the 200 mL add 1 mL of NQ-40

Friday, March 4, 2022

To Do:

1. Check concentrations of KRLVS24 and KRLVS141 gDNA

Results and Data:

Sample	Nucleic Acid (ug/ul)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS141.2	227.2	4.544	2.625	1.73	1.32
KRLVS141.1	214.6	4.292	2.677	1.60	1.19
KRLVS24.1	275.2	5.504	3.050	1.80	1.15
KRLVS24.1 100ng/uL	118.4	2.369	1.427	1.66	1.12

Monday, March 7, 2022**To Do:**

- ~~1. Dilute gDNA according to needed concentrations and actual concentrations~~
- ~~2. Nanodrop final tubes for sequencing~~

Results and Data:

Sample	Nucleic Acid (ng/ul)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS24 60uL	261.4	5.228	2.764	1.89	1.46
KRLVS24 30uL	138.2	2.764	1.448	1.91	1.66
KRLVS141 60uL	135.9	2.719	1.464	1.86	1.43
KRLVS141 30uL	33.8	0.677	0.389	1.74	1.45
KRLVS156 60uL	207.5	4.151	2.316	1.79	1.09
KRLVS156 30uL	183.5	3.671	2.047	1.79	1.09
KRLVS157 60uL	111.5	2.229	1.268	1.76	1.14
KRLVS157 30uL	30.9	0.617	0.362	1.70	1.16

Tuesday, March 8, 2022**To Do:**

- ~~1. Prepare gDNA for shipping and take to loading dock to be picked up~~

Results and Data:

N/A.

Wednesday, March 9, 2022**To Do:**

- ~~1. Assist CR with writing up a protocol for agar overlay~~
- ~~2. Assist CR with plasmid mapping~~

Results and Data:

Note. Created pKR136 and pKR137 plasmid maps based on pF plasmid. iLOV-V and LanYFP-V will be ordered as gBlocks, so placed the gene for each respective fluorescent protein between NotI and BamHI. Included ribosomal binding site prior to gene.

Monday, March 14, 2022**To Do:**

- ~~1. Receive and dissolve primers~~

Results and Data:

N/A.

Receiving and Dissolving Primers

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated primers go to the bottom of the tube.
2. Add 0.1x EB to a final concentration of 100 uM. Calculate this by multiplying the reported nm by 10 and adding that volume in ul (i.e. 12.7 nmoles = add 127 uL of 0.1xEB).
3. Put on 42°C heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. Put Certificate of Analysis sheet in “Oligos Spec Sheet” binder and shipping sheet in Invoices box.

Tuesday, March 15, 2022**To Do:**

- ~~1. Put away caps and bottles from dishwasher~~
- ~~2. Begin looking at sequencing data~~

Thursday, March 17, 2022**To Do:**

- ~~1. Detect unexpected outcomes from triple mutant strain gDNA~~

Results and Data:

First passthrough of sequencing results. Compared each contig to pKR11 (the integrated plasmid, which should be present on the chromosome), pKR113 (the inducible plasmid), and pKR7 in the triple deletions as this was present in KRLVS24. Will not be able to align the chromosome with LVS chromosome as it is too big for SnapGene. There is not detection of any rpsu in the chromosomes of each triple deletion, however, part of the pKR113 plasmid does appear to have integrated into the chromosome of each. Additional document shows more detailed information.

Friday, March 18, 2022**To Do:**

- ~~1. Dilute primers~~
- ~~2. PCR and purification~~

Results and Data:

Note. Ran gel on 5/1/22; did not work. See Notebook 4 for more detail.

Diluting Primers

1. Make dilution for intended purpose of primer.
 - a. If a PCR primer, make a 10 uM stock by diluting 20 uL of the 100 uM stock into 180 uL of 0.1xEB. Label with purple sticker and put in appropriate freezer box.

PCR of KRLVS16 for fragment to be used in pKR135, EV

1. Acquire and label PCR tubes. Be sure to include at least 1 positive and 1 negative control for each PCR experiment.
2. Get a container of ice to keep the components on
3. Acquire the following components and put them on ice, labeling tubes if necessary:
 - Molecular grade H₂O in 1.5 mL microfuge tube
 - KOD/primestar buffer
 - dNTPs
 - oligo F (10uM)
 - oligo R (10uM)
 - template (eg. LVS gDNA, plasmid, colony, etc.)
 - Note: KOD/primestar enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - DO NOT vortex the enzyme itself or any solution with enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
 - The file is located in the Protocols folder
 - Also setup table below to specify which primers and source DNA will be used
7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
8. Add dszdi H₂O to negative control tube
 - Template volume for 1 reaction
9. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add KOD buffer
 - Add KOD enzyme
10. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
11. Add appropriate volume of master-mix to negative control PCR tube
12. Add template to Master Mix
 - Factor template volume minus 1 template reaction volume
13. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)
14. Close PCR Tubes until the caps are tight
15. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
 - Heat at 94 degrees for 2 minutes,

- 94 degrees C for 20 seconds
- 50 degrees C for 30 seconds
- 68 degrees C for 1 minute/kb (adjust based on expected size of product)
- Go back to step 2
- Repeat 32x
- 68 degrees C for 5 minutes
- 12 degrees C for infinity

PCR Purification

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 30uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Monday, March 21, 2022**To Do:**

- ~~1. Make and filter sterilize 2.5% iron pyrophosphate~~
- ~~2. Supplement MHB~~
- ~~3. Discuss CR fluorescent plasmids with Kathryn and make adjustments.~~

Results and Data:

Note. Adjust gene to be before NotI cut site, with VSVG after NotI cut site. Change codon that is creating a second BamHI cut site to the next best codon according to the codon optimization website. Confirm PCR and restriction digest using SnapGene. When designing primers, include four base pairs prior to the cut site.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalex (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH₂O (type 1), dissolved overnight, fresh solution every 2 weeks

Friday, March 25, 2022**To Do:**

- ~~1. Streak out LVS, KRLVS28, and KRLVS84 for agar overlay to single colony, one plate.~~

Results and Data:

Note. May not actually be to single colony, had a hard time streaking the strains on one plate. Will leave in the 37°C over the weekend.

Monday, March 28, 2022**To Do:**

1. ~~Assist CR with agar overlay~~
2. ~~Assist CR with designing and ordering primers~~

Results and Data:

N/A.

Tuesday, March 29, 2022**To Do:**

1. ~~Check agar overlay plates and leave out for CR to take images~~

Results and Data:

Note. 8 mL overlay worked the best. 5mL didn't quite cover everything, and 10mL was overkill.

Wednesday, March 30, 2022**To Do:**

1. ~~Assist CR with extracting colonies from agar overlay plate and streak out onto new plate.~~
2. ~~Assist CR with finalizing new protocol for agar overlay~~
3. ~~Assist CR with creating and ordering primers for fluorescent protein plasmids~~

Results and Data:

Note. Tried both a stick and a loop. Stick decimated more of the gel, and loop seemed to work better. However, since it is expected that with high levels of the beta-galactosidase from xgal that the cells will die, thought it would be useful to check both in case the loop doesn't pick up enough cells.

Thursday, March 31, 2022**To Do:**

1. ~~Check CHAH overlay plate for recovery~~

Results and Data:

Note. All strains patched out were recovered, whether using stick or loop. Yay!

April 2022

Monday, April 4, 2022

To Do:

- ~~1. Make and filter sterilize 2.5% iron pyrophosphate~~
- ~~2. Supplement MHB~~

Results and Data:

N/A.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH₂O (type 1), dissolved overnight, fresh solution every 2 weeks

Monday, April 11, 2022**To Do:**

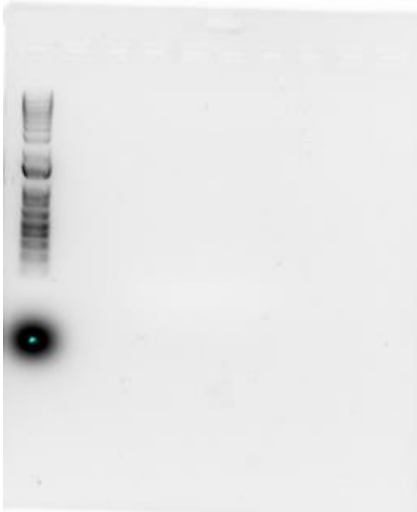
- ~~1. Assist CR with receiving and dissolving primers~~
- ~~2. Assist CR with PCR and running gel~~

Results and Data:

N/A.

Tuesday, April 12, 2022**To Do:**

- ~~1. Run gel on CR 20 uL PCR reaction to see if it worked.~~

Results and Data:

Note. Clearly did not work.

Wednesday, April 13, 2022**To Do:**

- ~~1. Run PCR with CR~~

Results and Data:

Note. PCR still did not work. May be the enzyme. CR will run PCR with Hannah on Monday, using her aliquots.

Monday, April 18, 2022**To Do:**

1. ~~Assist CR with PCR purification~~

Results and Data:

Note. CR ran PCR with Hannah today, using her aliquots of everything. It worked, and we deduced that it was a problem with the enzyme. Getting rid of all the Primestar enzymes and ordering new ones.

Wednesday, April 20, 2022**To Do:**

1. ~~Assist CR with restriction digest~~
2. ~~Assist CR with gel purification~~
3. ~~Assist CR with ligation~~

Results and Data:

N/A.

Thursday, April 21, 2022**To Do:**

1. ~~Make and sterile filter 2.5% iron pyrophosphate~~
2. ~~Supplement MHB~~
3. ~~Pull out CR ligations~~

Results and Data:

Note. Pulled out CR ligations and placed in my cloning box.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH₂O (type 1), dissolved overnight, fresh solution every 2 weeks

Monday, April 25, 2022**To Do:**

1. ~~Assist CR with *E. coli* chemical transformation~~

Tuesday, April 26, 2022**To Do:**

1. ~~Check CR transformation plates~~

Results and Data:

Note. Positive control did not grow, but backbone and iLOV + LanYFP transformants did. Hannah started overnight cultures (didn't know they shouldn't incubate for more than a day). She made 4 cultures of 5 mL + 50 ug/mL kan for each plasmids, for a total of 8 cultures.

Wednesday, April 27, 2022**To Do:**

1. ~~Help CR with miniprep~~
2. ~~Help CR with sequencing reaction set up.~~

Results and Data:

Note. Sequencing sheet in Sequencing>Orders

Thursday, April 28, 2022**To Do:**

1. ~~Patch out LVS from SUA~~

Friday, April 29, 2022**To Do:**

1. ~~Make electrocompetent LVS~~

Results and Data:

N/A.

Preparing Electrocompetent LVS Cells

1. Scrape up entire plate of cells into 400 uL of sterile 10% sucrose and resuspend (alternately, cells can be grown to mid-log in MHB)
2. Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL
3. Spin for 3 minutes at 10,000 rpm
4. Remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose
5. Repeat 3x-5x in 10% sucrose
6. After final spin, remove all supernatant.
7. Resuspend cells in 10% sucrose at high density (corresponding to $\sim 1 \times 10^{11}$ cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.
8. For any extra EC cells, aliquot ~ 110 uL / sterile tube (enough for 2 electroporations) and freeze at -80°C

Bibliography

Ramsey, K. M. and Dove, S. L. (2016) ‘ A response regulator promotes *Francisella tularensis* intramacrophage growth by repressing an anti-virulence factor ’, *Molecular Microbiology*. doi: 10.1111/mmi.13418.