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August 2022

Monday, August 8, 2022

To Do:

1. ~~Plan~~
2. ~~Submit data to GEO~~

Results and Data:

PmrA

After discussion with Simon about PmrA project, need to assess how lysis conditions (5 minutes vs 30 minutes) impacts recovery of ∆pmrA cells.
Test:

WT	
KMLFT37	∆pmrA
KMLFT69	∆pmrA mre(S)
KRLVS40	∆pmrA

Need to plan.
Thursday: pour plates
Friday: thaw macrophage
Monday: Seed
Tuesday: infect
Wednesday: plate

Design:

-rpsU1-GFP
-rpsU3-GFP
Replace rpsU gene with GFP? In that case, the bS21 homolog wouldn't repress its own production… but we might not see the same phenotypes in cells with rpsU genes, which is what we are interested in. Keep it a translational fusion. And double check that GFP is bright, versus the other proteins that Sierra tested.

IP bS21-ribosomes

Have samples after TEV cleavage from bS21 TAP purification (201022: E1). Run on gel to see if ribosome is associated.

Grow more cells with pKR72 pF-rpsU2-TAP. Try purification with buffers containing Mg2+.
Thursday: electroporation
Monday: patch cells
Tuesday: grow cells, harvest
Wednesday: IP

Tuesday, August 9, 2022

To Do:

1. ~~Run gel of old protein samples~~
2. ~~silver stain gel~~
3. ~~Coomassie stain gel~~
4. ~~Check for Kan plates for transformation~~

5. ~~Lab meeting~~
6. ~~Check with Sierra about fluorescent proteins (GFP is brightest!)~~
7. Design translational GFP fusions, order oligos

Results and Data:

Gel to assess TAP purification:
4-12% MES, 15 well

Lane	Volume	Contents	Processing
1	10 uL	1x SLB	Silver stain
2	10 uL	Benchmark ladder 1:10	
3	7 uL	E1A: LVS pF	
4	7 uL	E2A: LVS pF-rpsU-TAP	
5	7 uL	E3A: LVS rpoC-TAP	
6	7 uL	ribosomes	
7	10 uL	1x SLB	
8	10 uL	1x SLB	
9	10 uL	1x SLB	Coomassie stain
10	10 uL	Ladder	
11	7 uL	E1A: LVS pF	
12	7 uL	E2A: LVS pF-rpsU-TAP	
13	7 uL	E3A: LVS rpoC-TAP	
14	7 uL	ribosomes	
15	10 uL	1x SLB	

Ribosome from 3/22/2019 (26.7 ug/ul): For today, dilute 2 ul into 23 ul 1xSLB and load 7 ul

Run at 140 V for 55 min. Yellow dye and blue dye are visible at bottom of gel.
Cut in half and stain with silver and Coomassie as indicated.
Incubate in Coomassie stain from ~1:40 – after 4:40 (Sierra will remove the stain and add water for me).

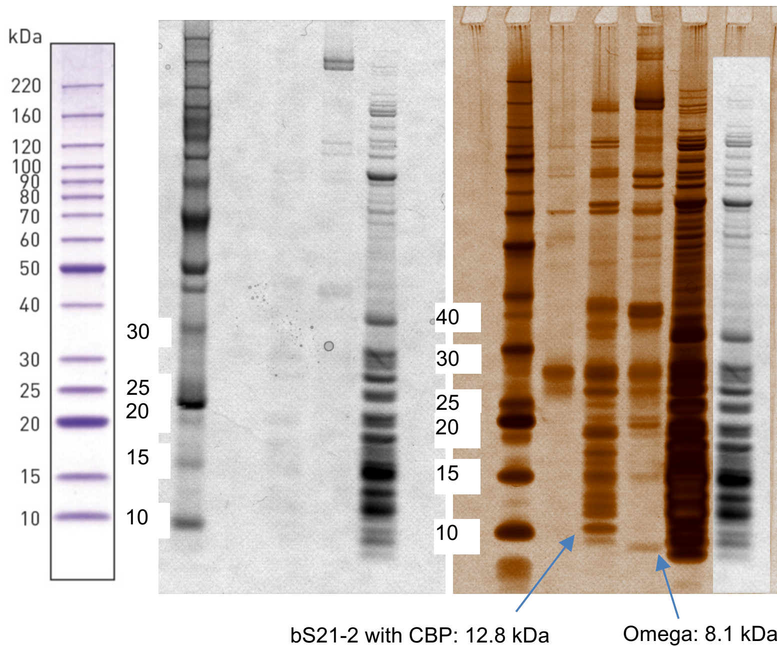

Figure 1. bS21-2-TAP purification from 10/22/20.

The RNAP purification isn't so clean, but that makes sense- this is only the first IP step. Can see the omega subunit, which is 8.1 kDa.
bS21-2 with the CBP moiety should be 12.76 kDa.

For electroporations on Thursday:

-Hannah is making more CHA-Kan plates tomorrow

-I have one vial of EC LVS

Wednesday, August 10, 2022

To Do:

1. ~~Spend time with K. Palmer and family~~

Thursday, August 11, 2022

To Do:

1. ~~Pour macrophage plates~~
2. ~~Transform LVS with pF-rpsU2-TAP~~
3. ~~Plate electroporations~~
4. ~~Prep fresh DMEM-F~~

Results and Data:

Plan for macrophage experiment next week:

Expectations:

WT		~ equal amounts recovery
KMLFT37	ΔpmrA	less at 5’, more at 30’
KMLFT69	ΔpmrA mre(S)	unknown?
KRLVS40	ΔpmrA	unknown?

Testing recovery after 5’ vs 30’ lysis. Rationale: potential that KMLFT37 will have less recovery after 5’ and more after 30’ compared to WT. If this is due to priM production, then we expect to see that KRLVS40 and KMLFT69 will have the same phenotype. If this is due to the SNP in FTL_0146, then we expect that KMLFT37 and KMLFT69 will have the same phenotype but KRLVS40 will have a different phenotype.

Potentially, it has nothing to do with lysis times. In that case, we expect less recovery of all bacteria at 5’ and more at 30’.

Plan:

Infect macrophage, wash after 2 hours (no 2 hour timepoint)

Lyse 2 plates of bacteria after 24 hours. Plate one set of wells after 5 minutes of lysis. Plate the same set after 25 more minutes, and plate from another set of wells after 30 mins.

Plate needs:

3 wells * 2 plates * 4 strains = 24 plates per timepoint

3 timepoints = 72 square plates

	Number of plates- CHA		
Use	Round	Square	Notes
Patch strains for infections	5		2x each, each 1/2 plate
Plate inoculumns	0	10	5 strains in duplicate
Timepoint 2 hours	0	0	Round: 5 strains x triplicate wells x duplicate
Timepoint 24 hours	6	72	Square: 4 strains x 3 wells x 2 plates x 3 timepoints; round: ΔpigR x 3 wells x 2 plates
Total plates	11	82	
Flasks of 600 mL CHA	0.44	4.1	Round plates: 24 mL, 25 per flask; Square plates: 30 mL, 20 per flask
Total number of CHA flasks	4.54		

Electroporate LVS with pKR72 pF-rpsU2-TAP

Electroporation	Cells	Plasmid	vol DNA	time constant
1	LVS	pKR72	3	didn't note
1	LVS	pF	3	4.5

Electrocomp LVS from 8-16-19
10:37 - ~2:15pm (~3.75 hrs). Plated 20 uL and 200 uL.

Friday, August 12, 2022

To Do:

1. ~~Joint lab meeting~~
2. ~~Thaw macrophage~~
3. ~~Bring nieces to NYC~~

Results and Data:

Thawed P5 J774.1 stock, in DMEMF supplemented yesterday (8/11/22).

Monday, August 15, 2022

To Do:

1. ~~Streak cells~~
2. ~~Seed macrophage~~
3. ~~Patch LVS cells with pF or pKR72~~

Results and Data:

Remove media, wash with 5 mL media.
Add back ~8 mL media.
Scrape cells off plate.
Transfer cells to 15 mL conical
Spin **750**xg for 5 minutes
Remove media, resuspend cell pellet in 7 mL using 5 mL pipet.
Check cell density:

combine 50 ul of cells with 50 ul of trypan blue.
Pipet 10 uL into single use hemocytometer
Count cells

1st measurement				
68	36	40	45	
Average				47.25
Undiluted				94.5
Density				9.45E+05

Dubious that the first measurement is representative. Should be 1.6 mL resuspended cells plus 10.4 mL media. Adjust calculations so that I'm adding 2 mL resuspended cells to 10 mL media.

Macrophage Calculations

Cells per well	2.50E+04
Volume to plate (mL)	0.2
Density needed (cells/mL)	1.25E+05
Total volume needed (mL)	12
Measured cells per ml	9.45E+05
Volume stock needed (mL)	2.0
Volume media for dilution	10.0

Make appropriate dilution in sterile reservoir

Use multichannel to pipet 200 ul cells into each well, as indicated, for **2 x 96 well plates**

	1	2	3	4	5	6	7	8	9	10	11	12
A	LVS	LVS	LVS		KMLFT37	KMLFT37	KMLFT37		ΔpigR	ΔpigR	ΔpigR	
B												
C												
D	ΔpmrA mre(S)	ΔpmrA mre(S)	ΔpmrA mre(S)		KRLVS40	KRLVS40	KRLVS40					
E												
F												
G												
H												

1	LVS	-
2	KMLFT37.1 (KR lab fine)	KMLFT37
3	KRLVS40.1	KRLVS40
4	ΔpmrA mre(S)	KMLFT69
5	ΔpigR	JCLVS106

Plate 1mL of responded cells with 9mL media into 100 mm TC dish (P6).
Place all cells back in incubator, 37°C 5% CO_2 **at ~5pm.**
Check actual density of resuspension:

2nd measurement				
4	5	10	9	
10	7	19	9	
9	17	9	16	
Average				10.33
Undiluted				20.66666667
Density				2.07E+05
Measured cells per ml, seeded				2.07E+05
Measured cells per well				4.13E+04

Higher than I'd like (aim for 2.5×10^5)! So glad to have the ΔpigR control. If the macrophage don't behave properly, then the ΔpigR cells won't be killed.

Check electroporations:
All worked well, too many cells on the 200 ul plate to get good single colonies. Even the 20ul plates are crowded. Patch 2 colonies from each electroporation onto a CHA plate.

Tuesday, August 16, 2022

To Do:
1. ~~Infect macrophage~~
2. ~~Wash macrophage~~
3. Grow cultures for TAP purification
4. ~~Freeze down patches of cells for future TAP purification~~

Results and Data:

Checked macrophage plates under microscope, look good. Forgot to put CHA plates with bacteria at 37°C last night! Grew at room temp, all the cells started from single-use aliquots (all except ΔpmrA mre(S)) obviously grew well enough for at least the mac assay. Try to scrape up what's there and move forward.
Prep 96-well plate with wells B1 – F5 with 180 ul sterile 1x PBS (multichannel).

Scrape up bacteria and resuspend in 400 uL DMEMF. Add 450 uL more. Check OD600 (100 uL in 900 uL DMEMF), dilute to OD600 of 0.04 in 1 mL, check OD600 again with 600 uL. Dilute cells to OD600 of 0.00075.

For final vol 1 mL at 0.04	For final vol 1 mL at 0.00075

Number	Strain	Resuspend cells to (OD600)	Cells (uL)	OD600	Cells (uL)	Volume media (uL)
1	LVS	0.91	44.0	0.039	19.2	981
2	KMLFT37	1.42	28.2	0.042	17.9	982
3	KRLVS40	2.06	19.4	0.034	22.1	978
4	KMLFT69		-	-	-	-
5	JCLVS106	1.77	22.6	0.06	12.5	988

Adjust setup: no KMLFT69 ∆pmrA mre(S), now strain 4 = ∆pigR!

Add 50 uL of appropriate cells to each well, as indicated, in each 96-well plate (2x).

	1	2	3	4	5	6	7	8	9	10	11	12
A	LVS	LVS	LVS		KMLFT37	KMLFT37	KMLFT37					
B												
C												
D	KRLVS40	KRLVS40	KRLVS40		∆pigR	∆pigR	∆pigR					
E												
F												
G												
H												

Put infected macrophage back at 37°C with 5% CO_2 at 9:00am.

Transfer 200 ul of each inoculum to wells A1 – A5, one per well.
Perform 10x serial dilutions down plate.
Plate each dilution 2x on square CHA plates using drip plate method, 10 uL at top. Put plates at 37°C.

At ~11:00am, wash macrophage twice with sterile 1x PBS and replace media with media containing 10 ug/mL gentamicin.
(14 mL)(10 ug/mL)/(50,000 ug/mL gent) = 2.8 uL

NO T=2 timepoint.

Check on tissue culture. P6 macrophage look good and media is still pretty pink- no need to feed yet.

Shaking incubator is at 30°C overnight, so plan on growing cells for TAP purification on another night.
Freeze down cells in the meantime.
Froze down 2 cryovials each of:
LVS pKR72 pF-rpsU2-TAP patch A
LVS pKR72 pF-rpsU2-TAP patch B
LVS pF patch A
LVS pF patch B
Put in "KMR working" box in -80°C.

Wednesday, August 17, 2022

To Do:

1. ~~Macrophage T=24~~

Results and Data:

At ~11:00 am, lyse macrophage and plate T=24 timepoint. Process 2 plates separately.

First wash 1 plate twice with 1x PBS and add 200 ul 1% saponin in 1x PBS. Incubate 5' and plate out LVS, KMLFT37, KRLVS40 (don't bother with ∆pigR). For this plating step, remove only 20 uL and perform serial dilutions, leaving remainder of liquid in wells. When plating, plate out diluted material and manually add 10 uL of undiluted for the first track. Leave saponin in plate to plate more after 30'.

While first plate continues to incubate, wash second plate twice with 1x PBS and add 200 ul 1% saponin in 1x PBS. Incubate for 30'.

While second plate incubates, after 1st plate has incubated for 30', plate out LVS, KMLFT37, and KRLVS40 again, plus ∆pigR.

After 2nd plate has incubated for 30', plate out LVS, KMLFT37, KRLVS40, and ∆pigR.

For each plating step, transfer 190ul of wells with samples 1 – 3 to top of one 96-well plate, samples 4 to top of second 96 well plate. Perform 10x serial dilutions. Plate undiluted plus 5 dilutions (dilution factor 1 – 0.00001) for each well 2x using track plate method (10 ul). Plate 50 uL twice on round plates for all samples from ∆pigR (5). Put plates at 37°C.

Check on P6 macrophage. Look good, should deal with tomorrow if I want to continue culturing. Not sure it's worth it- I need to see results from this experiment before planning the next one, and I expect it will be Saturday or Sunday before all the colonies from the T=24 plates are ready to count. Not really soon enough to have another experiment planned for early next week.

Thursday, August 18, 2022

To Do:

1. ~~Count inoculum plates~~

Results and Data:

Check on track plates with inoculums. Can clearly count plates from samples 1 (LVS) and 2 (KMLFT37). Not surprising that KRLVS40 still has pretty small colonies, so not worth trying to count today- this growth defect is expected based on past experiments. However, sample 4, which is ∆pigR, also has tiny colonies! That's not expected, ∆pigR typically grows like WT. Keep an eye on this.
Current MOIs calculated:
LVS: 4.7
KMLFT37: 3.2

These are lower than I'd like, but there were more macrophage plated that I usually use. Hopefully it all turns out okay.

Friday, August 19, 2022

To Do:

1. ~~Count remaining inoculum plates~~
2. ~~Count T=24 plates for faster growers~~
3. ~~Autoclave media to grow TAP strains next week~~

Results and Data:

Checked on plates from macrophage assay.
From the inoculum plates, can count KRLVS40 and ∆pigR. Note that the ∆pigR colonies are a bit strange, as there seem to be large and small sizes. Not typical, should have grown like WT and be consistently big by today. Also fewer cells than expected. Not sure what's happening there.

Inoculum

	Replicate	3	4	Dilution factor counted	Cells / mL	Average Cells / mL	St dev	CFU/well	St dev	MOI (based on number of seeded macrophage- see setup)
LVS	1A	TMTC	**41**	0.001	4.10E+06	3.90E+06	2.83E+05	1.95E+05	1.41E+04	4.7
	1B	TMTC	**37**	0.001	3.70E+06					
LVS Δ*pmrA* KMLFTS37	2A	TMTC	**35**	0.001	3.50E+06	2.64E+06	1.22E+06	1.32E+05	6.12E+04	3.2
	2B	177	**37**	0.01	1.77E+06					
LVS Δ*pmrA* KRLVS40	3A	TMTC	**52**	0.001	5.20E+06	5.00E+06	2.83E+05	2.50E+05	1.41E+04	6.0
	3B	TMTC	**48**	0.001	4.80E+06					
LVS Δ*pigR*	5A	TMTC	**17**	0.001	1.70E+06	1.35E+06	4.95E+05	6.75E+04	2.47E+04	1.6
	5B	TMTC	**10**	0.001	1.00E+06					
Dilution Factor		0.01	0.001							

Strain	CFU/well	St dev	MOI
LVS	1.95E+05	1.41E+04	4.7
LVS Δ*pmrA* KMLFT37	1.32E+05	6.12E+04	3.2
LVS Δ*pmrA* KRLVS40	2.50E+05	1.41E+04	6.0
LVS Δ*pigR*	6.75E+04	2.47E+04	1.6

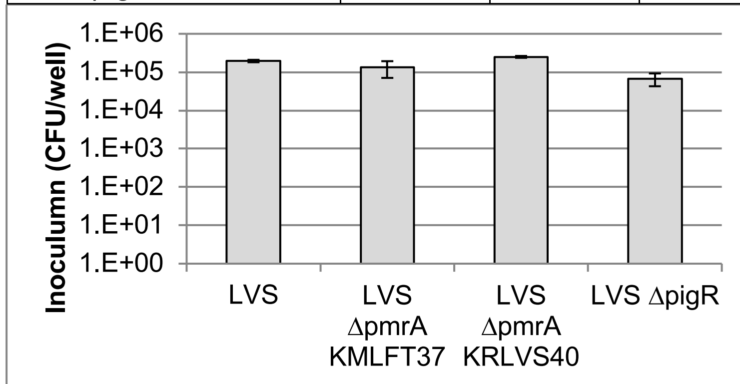

Figure 2. Quantification of inoculums from 8/16/22 macrophage assay.

They look pretty even! MOIs are a bit lower than I'd like, particularly ΔpigR, but should be fine.

The only 24 hour plates countable today are the LVS plates (check on others Sat or Sunday). Results so far:

Plating after 5'

		Average CFU per well	St Dev	Original MOI	T-test (vs LVS)	Fold change from WT
1	LVS	1.02E+05	2.76E+04	4.7		1.00

Plating after 30' (2nd plating)

		Average CFU per well	St Dev	Original MOI	T-test (vs LVS 5')	Fold change from WT 5'
1	LVS	1.87E+05	6.45E+04	4.7	0.103671	1.84

Plating after 30' (1st plating)

		Average CFU per well	St Dev	Original MOI	T-test (vs LVS)	Fold change from WT 5'
1	LVS	2.24E+05	8.35E+04	4.7	0.074226	2.20

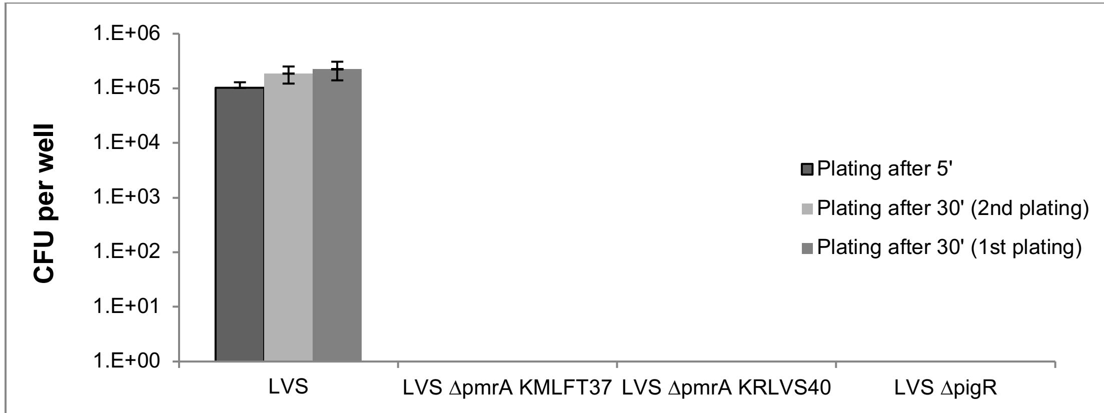

Figure 3. Recovery of LVS from 8/16/22 macrophage assay.

As expected, recovered more LVS after 30’ incubation with saponin than after a 5’ incubation. Not really sure how to interpret the second plating after 30’, since lysis must be uneven and the volume is reduced. Probably disregard this timepoint.

Sunday, August 21, 2022

To Do:

1. ~~Check on plates, remove if necessary~~

Results and Data:

Removed plates, some colonies look quite big. Leave on bench at RT.

Monday, August 22, 2022

To Do:

1. ~~Wrap up family visit~~

Results and Data:

Family still in town, take day off.

Tuesday, August 23, 2022

To Do:

1. ~~Finish counting macrophage assay plates~~
2. ~~Patch out LVS pF and LVS pKR72 cells~~

Results and Data:

Count all remaining macrophage assay plates:

5 min												
		Track Plate 1				Track Plate 2						
	Plate	1	2	3	4	1	2	3	4	Dilution factor counted	Average Cells	CFU per well
LVS	1A	TMTC	TMTC	**66**	6	TMTC	TMTC	**67**	13	0.01	66.5	1.33E+05
	1B	TMTC	TMTC	**50**	6	TMTC	TMTC	**41**	6	0.01	45.5	9.10E+04
	1C	TMTC	TMTC	**33**	6	TMTC	TMTC	**48**	17	0.01	40.5	8.10E+04
LVS ΔpmrA KMLFT37	2A		**12**				**10**			0.1	11	2.20E+03
	2B		**13**				**9**			0.1	11	2.20E+03
	2C		**14**				**12**			0.1	13	2.60E+03
LVS ΔpmrA KRLVS40	3A	**3**				**6**				1	4.5	9.00E+01
	3B	**3**				**3**				1	3	6.00E+01
	3C	**12**				**5**				1	8.5	1.70E+02
Dilution Factor		1	0.1	0.01	0.001	1	0.1	0.01	0.001			

30 min (2nd plating)

		Track Plate 1				Track Plate 2						
	Plate	1	2	3	4	1	2	3	4	Dilution factor counted	Average Cells	CFU per well
LVS	1A	TMTC	TMTC	TMTC	**14**	TMTC	TMTC	TMTC	**12**	0.001	13	2.60E+05
	1B	TMTC	TMTC	**80**	8	TMTC	TMTC	**81**	10	0.01	80.5	1.61E+05
	1C	TMTC	TMTC	**70**		TMTC	TMTC	**69**		0.01	69.5	1.39E+05
LVS ΔpmrA KMLFT37	2A		**12**							0.1	12	2.40E+03
	2B		**17**				**22**			0.1	20	3.90E+03
	2C		**13**				**17**			0.1	15	3.00E+03
LVS ΔpmrA KRLVS40	3A	**5**				**5**				1	5	1.00E+02
	3B	**6**				**4**				1	5	1.00E+02
	3C	**12**				**10**				1	11	2.20E+02
LVS Δ*pigR**	5A	**1**								1	1	1.00E+01
	5B	**3**								1	3	3.00E+01
	5C	**4**				**0**				1	2	2.00E+01
Dilution Factor		1	0.1	0.01	0.001	1	0.1	0.01	0.001			

*Plated 50 ul on circular plate

30 min (1st plating)

		Track Plate 1				Track Plate 2						
	Plate	1	2	3	4	1	2	3	4	Dilution factor counted	Average Cells	CFU per well
LVS	1A	TMTC	TMTC	**94**	10	TMTC	TMTC	**77**	9	0.01	85.5	1.71E+05
	1B	TMTC	TMTC	TMTC	**15**	TMTC	TMTC	TMTC	**17**	0.001	16	3.20E+05
	1C	TMTC	TMTC	TMTC	**11**	TMTC	TMTC	TMTC	**7**	0.001	9	1.80E+05
LVS ΔpmrA KMLFT37	2A		**53**				**46**			0.1	50	9.90E+03
	2B		**58**				**64**			0.1	61	1.22E+04
	2C		**80**	**7**				**7**		0.1	80	1.60E+04
LVS ΔpmrA KRLVS40	3A	**27**				**21**				1	24	4.80E+02
	3B	**33**				**31**				1	32	6.40E+02
	3C	**5**				**3**				1	4	8.00E+01
LVS Δ*pigR**	5A	**2**				**4**				1	3	3.00E+01
	5B	**3**				**5**				1	4	4.00E+01
	5C	**4**				**2**				1	3	3.00E+01
Dilution Factor		1	0.1	0.01	0.001	1	0.1	0.01	0.001			

*Plated 50 ul on circular plate

Plating after 5'

	Average CFU per well	St Dev	Original MOI	T-test (vs LVS)	Fold change from WT 5'
LVS	1.02E+05	2.76E+04	4.7		1.00
LVS Δ*pmrA* KMLFT37	2.33E+03	2.31E+02	3.2	0.003	-43.57
LVS Δ*pmrA* KRLVS40	1.07E+02	5.69E+01	6.0	0.003	-953.13

Plating after 30' (2nd plating)

	Average CFU per well	St Dev	Original MOI	T-test (vs LVS 5')	Fold change from WT 5'
LVS	1.87E+05	6.45E+04	4.7	0.103671	1.84
LVS Δ*pmrA* KMLFT37	3.10E+03	7.55E+02	3.2		-32.80
LVS Δ*pmrA* KRLVS40	1.40E+02	6.93E+01	6.0		-726.19
LVS Δ*pigR*	2.00E+01	1.00E+01	1.6		-5083.33

Plating after 30' (1st plating)

	Average CFU per well	St Dev	Original MOI	T-test (vs LVS)	Fold change from WT 5'	Fold change from WT 30'
LVS	2.24E+05	8.35E+04	4.7	0.074226	2.20	1.00
LVS Δ*pmrA* KMLFT37	1.27E+04	3.08E+03	3.2		-8.01	-17.61
LVS Δ*pmrA* KRLVS40	4.00E+02	2.88E+02	6.0		-254.17	-559.17
LVS Δ*pigR*	3.33E+01	5.77E+00	1.6		-3050.00	-6710.00

I'm not really sure how to interpret the results from "Plating after 30' (second plating)" because there has been some volume loss and there is the potential for heterogeneity. I think I should just focus on the two independent plates, which were plated after either 5 or 30 minutes.

	Fold Change (30' vs 5')	St dev	T-test (30' vs 5')
LVS	2.20	1.02	0.0742
LVS Δ*pmrA* KMLFT37	5.44	1.43	0.0044
LVS Δ*pmrA* KRLVS40	3.75	3.36	0.159

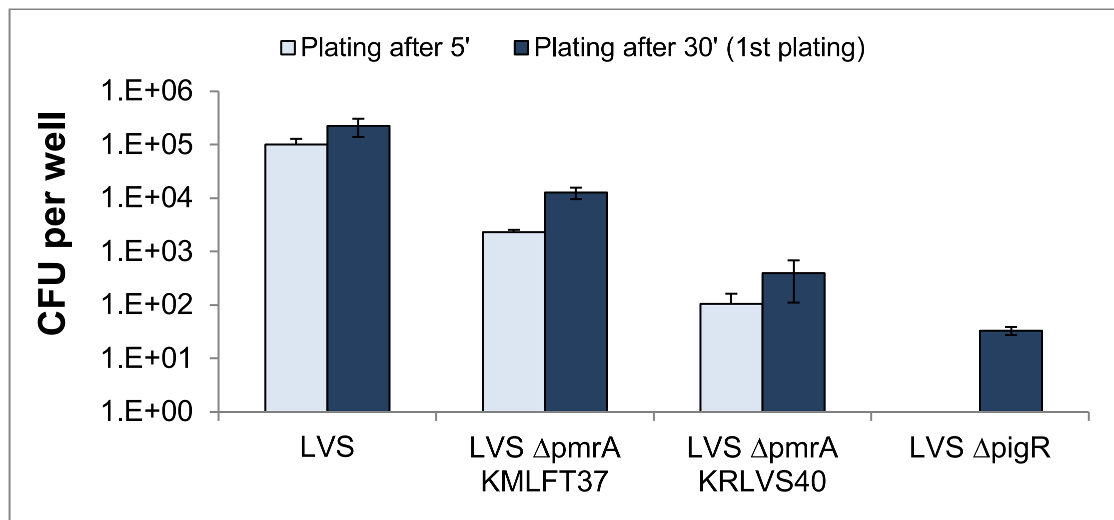

Figure 4. Recovery of all cells from 8/16/22 macrophage assay.

Important points:

- Seeing more LVS recovered in this experiment, likely due to higher numbers of initial macrophage. Note that I didn't do a 2 hr timepoint so unclear how replication rate compares to previous experiments.
- In previous experiments, saw ~10x reduction in KMLFT37 recovered and ~100x reduction in KRLVS40 recovered. After 5' lysis, we see 50x reduction in KMLFT37 and almost 1,000x reduction in KRLVS40.

Wednesday, August 24, 2022

To Do:

1. ~~PCR for GFP reporter plasmids (pKR153 and pKR154)~~
2. ~~Clean up PCR~~
3. ~~Run on gel~~
4. ~~Supplement media to grow 2x500 mL cultures O/N~~
5. ~~Start growing LVS pKR72 (bS21-TAP) cells overnight~~

Results and Data:

PCR for pKR153 and pKR154. Resuspend primers (KROL551 – KROL554) and making working stocks.

Reaction	Locus	Primers	DNA	Expected size
1	*PrpsU1*	KROL551, KROL552	LVS gDNA	250
2	*PrpsU2*	KROL553, KROL554	LVS gDNA	384
3	*PrpsU2*	KROL553, KROL554	-	-

Total reaction volume	100
Total number of reactions	3

Component	Stock concentration	Final concentration	1 rxn volume	Master Mix
ddiH2O			62	186
PrimeSTAR GXL Buffer	5x	1x	20	60
dNTPs	2.5 mM	0.2 mM	8	24
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	6
Primer 1	10 uM	0.3 uM	3	indiv
Primer 2	10 uM	0.3 uM	3	indiv
template	100 ng/ul	2 ng/ul	2	indiv
		Total volume	100	276

Use STN1 with a 30” extension time.
Run 5 uL of each PCR on a gel.

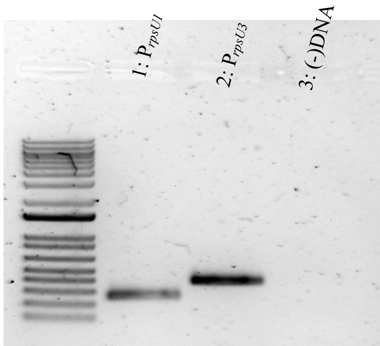

Figure 5. PCR of rpsU promoters for GFP fusion plasmids.

Use PCR cleanup kit, purify PCRs 1 and 2, elute each in 35 uL 0.1x EB. Want to check on the Nanodrop, so check concentrations.

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
PCR1 PrpsU1	131.2	ng/µl	2.624	1.38	1.9	27.99	DNA	50
PCR2 PrpsU3	184.1	ng/µl	3.682	1.961	1.88	7.32	DNA	50

Looks good, no issues with Nanodrop today. Freeze purified PCRs and set up digest tomorrow.

Start cells O/N for future TAP purification. Supplement 500 mL of MH broth in baffled flasks.

Number	Cells	OD600	vol cells	vol media	dilution factor	Actual OD600	For 0.003 in 500 mL
1	LVS pKR72	0.196	25	975	40	7.84	191.3
2	LVS pF	0.247	25	975	40	9.88	151.8

Started cells at ~3pm.

Thursday, August 25, 2022

To Do:

1. ~~Harvest LVS pKR72 (bS21-TAP) cells~~
2. ~~Digest PCR products for GFP reporter plasmids (pKR153 and pKR154)~~
3. ~~Run gel of digests for GFP reporter plasmids (pKR153 and pKR154)~~
4. ~~Gel-purify for GFP reporter plasmids (pKR153 and pKR154)~~
5. ~~Ligate for GFP reporter plasmids (pKR153 and pKR154)~~

Results and Data:

bS21-TAP

Check on O/N cultures for TAP purification at ~9:15 am.

Number	Cells	OD600
1	LVS pKR72	0.373
2	LVS pF	0.481

Spin down cells in TX-1000 rotor, 1 L bottles at 3108rpm, 2257xg for 20 mins, after splitting each sample into two bottles. Should have split into two in 250 mL tubes and used fixed-angle rotor so could spin faster. OOPS! It was just on the cool-down run. Cells look mostly pelleted, so give them 5' more spin at max speed. Remove supernatants, leave a bit, transfer to 50ml conical. Spin down 5' at max speed in rotor for conical tubes. Discard supernatants, freeze pellets at -80°C.

Cloning pKR153 and pKR154

Set up digests of PCR products to make pKR153 and pKR154.

	1 PCR 1 P_{rpsU1}	2 PCR 2 P_{rpsU3}	pKR145 backbone
H_2O	10.8	10.8	22.8
10x CSB	3.0	3.0	3.0
DNA	15.0	15.0	3.0
KpnI-HF	0.6	0.6	0.6
NotI-HF	0.6	0.6	0.6
Total	30.0	30.0	30.0

Incubate in thermocycler for 1 hour at 37°C.
CIP backbone with new Quick CIP – add 1 uL, mix, incubate for 10 mins at 37°C, then put at 80°C for 2 minutes to inactivate the CIP.

Run digests (entire volume) on 1% agarose gel.

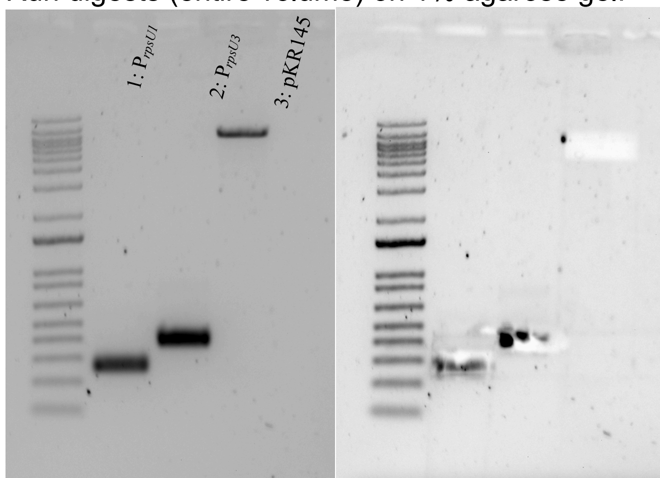

Figure 6. Digest and gel purification of *rpsU* promoters for GFP fusion plasmids.

Cutting was tough, it's not dark in the lab! Move forward with what I've got. Gel-purify slices, elute in 35 uL 0.1x EB.

Plan ligations:

Tube	Insert	Backbone
1	NotI, KpnI digested, purified PCR1	NotI, KpnI digested, purified pKR145
2	NotI, KpnI digested, purified PCR2	NotI, KpnI digested, purified pKR145
3	-	NotI, KpnI digested, purified pKR145

Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Reaction 3 (uL)	Master Mix (3.5x)
H_2O	11.5	11.5	15.5	40.25
10x ligase buffer	2	2	2	7
Insert	4	4	-	-
Backbone	2	2	2	7
Ligase	0.5	0.5	0.5	1.75
TOTAL	20	20	20	-

Add 4 uL of either DNA (insert, reactions 1&2) or water (reaction 3) to 16 uL of master mix in 0.2 mL PCR strip tubes. Incubate at 16°C O/N in thermocycler.

Friday, August 26, 2022

To Do:

1. ~~Transform XL1 Blue E. coli with ligations for GFP reporter plasmids (pKR153 and pKR154)~~
2. Start TAP purification?

Results and Data:

Transform ligations

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates
1	pKR153	Ligation 1	8 uL	100 uL, remaining	2
2	pKR154	Ligation 2	8 uL	100 uL, remaining	2
3	backbone control	Ligation 3 (backbone only)	8 uL	remaining	1
4	(+) control	pKR145	1 uL	20 ul, 100 ul	2
Total number of plates					7

Add DNA to sterile tubes for transformation as indicated above. Thaw chemically competent XL1 blue cells on ice. Transfer 100 uL of cells to each transformation tube. Incubate on ice for 20’. Heatshock at 42°C for 30”. Put tubes on ice, add 1 mL LB, recover in shaking incubator (set at 30°C today) for ~1 hour. Plate as indicated above. Put all plates at 37°C O/N at 1pm, except for pKR145 20 uL- put that at 30°C to see if there are single colonies by Sunday night at 30°C.

Saturday, August 27, 2022

To Do:

1. ~~Pull transformation plates from 37°C incubator~~

Sunday, August 28, 2022

To Do:

1. ~~Pick colonies for GFP reporter plasmids (pKR153 and pKR154)~~

Results and Data:

Transformation results (~4pm)

Tube number	Purpose	DNA	20 uL	100 uL	Remaining
1	pKR153	Ligation 1	-	0	3
2	pKR154	Ligation 2	-	0	1
3	backbone control	Ligation 3 (backbone only)	-	-	0
4	(+) control	pKR145	2	14	-

Had the pKR145 remaining plate at 30°C since Friday at ~2pm. Single colonies present. Had other plates at 37°C O/N, left on bench until today. Start O/N cultures of all colonies from ligations 1 and 2, plus two of pKR145.

Monday, August 29, 2022

To Do:

1. ~~Miniprep for GFP reporter plasmids (pKR153 and pKR154)~~
2. ~~Send miniprep plasmids to sequence~~

Results and Data:

Pellet cells to miniprep. Check out pellet colors! Looks like I probably have at least pKR154!

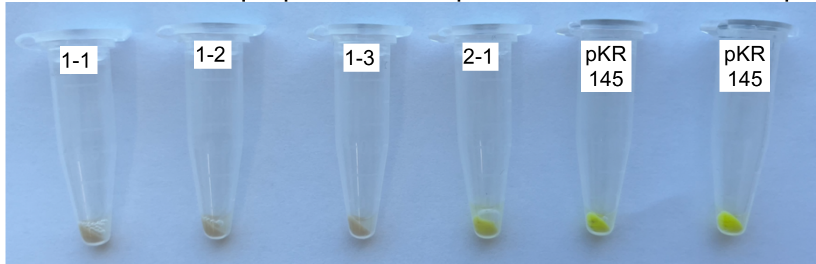

Figure 7. Pellets of cells to miniprep for potential GFP fusion plasmids.

Finish miniprep, elute in 50 uL 0.1x EB.

Check concentration by Nanodrop:

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR153-1	426.7	ng/µl	8.534	4.543	1.88	2.45
pKR153-2	345	ng/µl	6.899	3.677	1.88	2.5
pKR153-3	365.8	ng/µl	7.317	3.914	1.87	2.48
pKR154-1	362.9	ng/µl	7.258	3.937	1.84	2.33
pKR145a	265.2	ng/µl	5.304	2.846	1.86	2.52
pKR145b	333.3	ng/µl	6.666	3.563	1.87	2.49

All look good! Send to sequence with KROL525 (HT used this to sequence pKR145 recently). Delivered samples to Janet in INBRE.

Sample number	Template Type	Template Name	Primer Name[a]	A.	B.	E.	F.
	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/µl)	PLASMID template:	Volume H_2O needed
						Volume =	
						2x(~200 ÷ B)µl	(12 less E - 2.56)µl
KR1	Plasmid	153-1	KROL525	7800	426.7	0.94	8.50
KR2	Plasmid	153-2	KROL525	7800	345	1.16	8.28
KR3	Plasmid	153-3	KROL525	7800	365.8	1.09	8.35
KR4	Plasmid	154-1	KROL525	7800	362.9	1.10	8.34

a.Add 2.56 µl of 2.5 µM stock to each reaction

Tuesday, August 30, 2022

To Do:

1. ~~COP retreat~~
2. ~~Sierra struck out LVS for me~~

Wednesday, August 31, 2022

To Do:

1. ~~Make EC LVS cells~~
2. ~~Electroporate LVS with pKR153 and pKR154~~
3. ~~Plate electroporations~~

Results and Data:

Sequencing results back- pKR153 confirmed, combine all three plasmid preps. The pKR154 isn't confirmed yet- Janet thinks there may have been a problem with the instrument and will re-run tomorrow. Be optimistic and move forward with it anyway.

Sierra plated two lawns for EC cells; one of them has a point of contamination and the other looks like it might. Nervous about using them, so loot from JW's old freezer boxes for now.

EP	Cells	Plasmid	vol DNA (uL)	time constant
1	LVS	pKR153	3	4.6
2	LVS	pKR154	3	4.4
3	LVS	pF	3	4.5
4	LVS	-	3ul 0.1xEB	4.3

Electrocomp LVS from Jamie W's electropcomp cells box, tubes labeled 8-13 (undocumented year) 1:30pm – 3pm (~1.5 hours). Plated 20 uL and 200 uL.

Thursday, September 1, 2022

To Do:

1. ~~Start TAP purification~~

Results and Data:

Purify bS21-2-TAP from samples harvested on 8/25, use modified TAP protocol to IP, capturing Protein A moiety on IGG beads. Buffers:

Buffer A + PI +BB (modified Buffer 1)

Final composition	Stock solutions	For 10 mL
20 mM KHEPES pH 7.9	1 M KHEPES	200 uL
10 mm $MgCl_2$	1 M $MgCl_2$	100 uL
10 mM NH_4Cl	1 M NH_4Cl	100 uL
0.5 mM DTT	0.5 M DTT	10 uL
1X Bug Buster	10X Bug Buster	1 mL
10% glycerol	75% glycerol	1.33 mL
Water		7.26 mL

+ 1 tablet protease inhibitor

Buffer B (modified buffer IPP150)

Final composition	Stock solutions	For 25 mL
20 mM KHEPES pH 7.9	1 M KHEPES	500 uL
10 mm $MgCl_2$	1 M $MgCl_2$	250 uL
50 mM NH_4Cl	1 M NH_4Cl	1250 ul
0.1% NP-40	10% NP-40	250 uL
Water		22.75

*needs to be compatible with TEV!

TEV Cleavage Buffer MOD

Composition	Stock solutions	For 30 mL
20 mM KHEPES pH 7.9	1 M KHEPES	600 uL
10 mm $MgCl_2$	1 M $MgCl_2$	300 uL
50 mM NH_4Cl	1 M NH_4Cl	1500 uL
0.1% NP40	10.00%	300 uL
1 mM DTT	0.5 M	60 uL
	Water	27.24 mL

1. Resuspend samples in 4.5 mL Buffer A + PI + BB by pipetting with 5 mL pipette. Be sure no clumps remain
2. Add 10 uL DNase I (Lucigen/Epiceter), mix by pipetting
3. Incubate at 37°C for 30’. Cool down centrifuge while waiting.
4. Spin 50 mL conical tubes at 4°C for 20’ at max speed (14635xg).
5. Transfer lysates into new 50 mL conical tubes, leaving behind insoluble material at bottom (okay to leave some behind; better than taking too much)
6. Filter-sterilize lysates using 30mL syringe and syringe filter into new 50mL conical tube. Specifically, pull plunger out from 30mL syringe, thread tip of syringe onto syringe use filter (use MillexGP 0.22 micrometer filter, has 33mm diameter), pour/pipette lysate into syringe, insert plunger and recover filtered lysate into new (nonsterile) 50mL conical tube
7. Obtain a good estimate of sample volume using pipette.
8. Adjust buffer concentration to buffer B by adding to each sample:
   50 uL 1M NH_4Cl / mL sample -> __ uL for final 50 mM NH_4Cl
   10 uL 10% NP-40 /mL sample -> __ uL for final 0.1% NP-40
   Sample 1: 4.5 mL
   225 uL for final 50 mM NH_4Cl
   45 uL for final 0.1% NP-40
   Sample 2: 4 mL
   200 uL for final 50 mM NH_4Cl
   40 uL for final 0.1% NP-40
9. Remove 50 uL into microfuge tube for control analysis (LYS).

In cold room

10. Cut tip of 1mL pipette tip slightly
11. Resuspend IgG Sepharose beads completely
12. Pipette 200 uL IgG Sepharose beads into each column, placing column on top of Qiagen tip holder placed on 50 mL conical for flow-through
13. Wash beads with 10 mL of buffer B
14. When all the buffer B has passed through the column, cap the bottom of the column
15. Add 1 sample per polyprep column, add top cap to close column (make sure column is closed completely and is not dripping liquid)
16. Incubate samples with beads, rocking at 4°C, for 2 hours (2:34pm)During incubation, prepare:
    a. TEV Cleavage Buffer MOD for this set of purifications, ~13 mL per purification
    b. Make more :

Buffer B (modified buffer IPP150)

Final composition	Stock solutions	For 150 mL
20 mM KHEPES pH 7.9	1 M KHEPES	3 mL
10 mm $MgCl_2$	1 M $MgCl_2$	1.5 mL
50 mM NH_4Cl	1 M NH_4Cl	7.5 mL
0.1% NP-40	10% NP-40	1.5 mL
Water		136.5 mL

18. After 2 hour incubation, open columns and let lysate pass through column.

19. Save 50 uL aliquot of flow through in microfuge tube (FT1), discard remaining.
20. Wash columns 3x with 10 mL buffer B, discarding flow through
21. Wash columns with 10 mL TEV Cleavage buffer MOD, discarding flow through
22. Cap column bottoms, add 1 mL TEV Cleavage buffer MOD and 10 uL AcTEV Protease to each.
23. Incubate rocking at 4°C overnight- make sure columns are closed completely and are not dripping liquid. **Remainder of protocol FOR TOMORROW:** Recover TEV eluates by gravity flow (1 ml)Rinse each column with additional 200 ul of TEV Cleavage Buffer MOD

Friday, September 2, 2022

To Do:

1. ~~Check on EP plates~~
2. ~~Finish TAP purification~~
3. ~~Check pKR154 sequencing results~~

Results and Data:

TAP purification continued: Recover TEV eluates by gravity flow (1 ml)Washed columns with 200 uL TEV Cleavage Buffer MOD to get beads to the bed.

3. Rinse each column with additional 200 ul of TEV Cleavage Buffer MOD.
4. Keep samples at 4°C.

EP plates look good- clearly see pinpoint colonies!

Check second run of pKR154 sequencing. Sequence is correct, have pKR154! Put in appropriate boxes.

Saturday, September 3, 2022

To Do:

1. ~~Patch out LVS pKR153 and LVS pKR154~~
2. ~~Run gel of purified proteins~~
3. Stain gel with purified proteins (silver)

Results and Data:

Gel to assess modified TAP purification. Use the remainder of the E2A purification from 10/22/20. Combine elutions 1:1 with 2x SLB.

4-12% MES, 15 well

Lane	Volume	Contents
1	10 uL	Benchmark ladder 1:10
2	10 uL	E2- LVS pF
3	10 uL	E1- LVS pF-rpsU2-TAP
4	10 uL	Ribosomes diluted 1:10
5	10 uL	Benchmark ladder 1:10
6	5 uL	E2- LVS pF
7	5 uL	E1- LVS pF-rpsU2-TAP
8	10 uL	Ribosomes diluted 1:100
9	5 uL	E2A: LVS pF-rpsU-TAP (10/22/20 purification)
10	5 uL	Ribosomes diluted 1:10

Ribosome from 3/22/2019 (26.7 ug/ul): For today, dilute 2 ul into 23 ul 1xSLB (~2.1 ug/ul) and dilute further as indicated.

Run at 120 V for 50 min. Yellow dye and blue dye are visible at bottom of gel.
Use SilverQuest kit to stain.

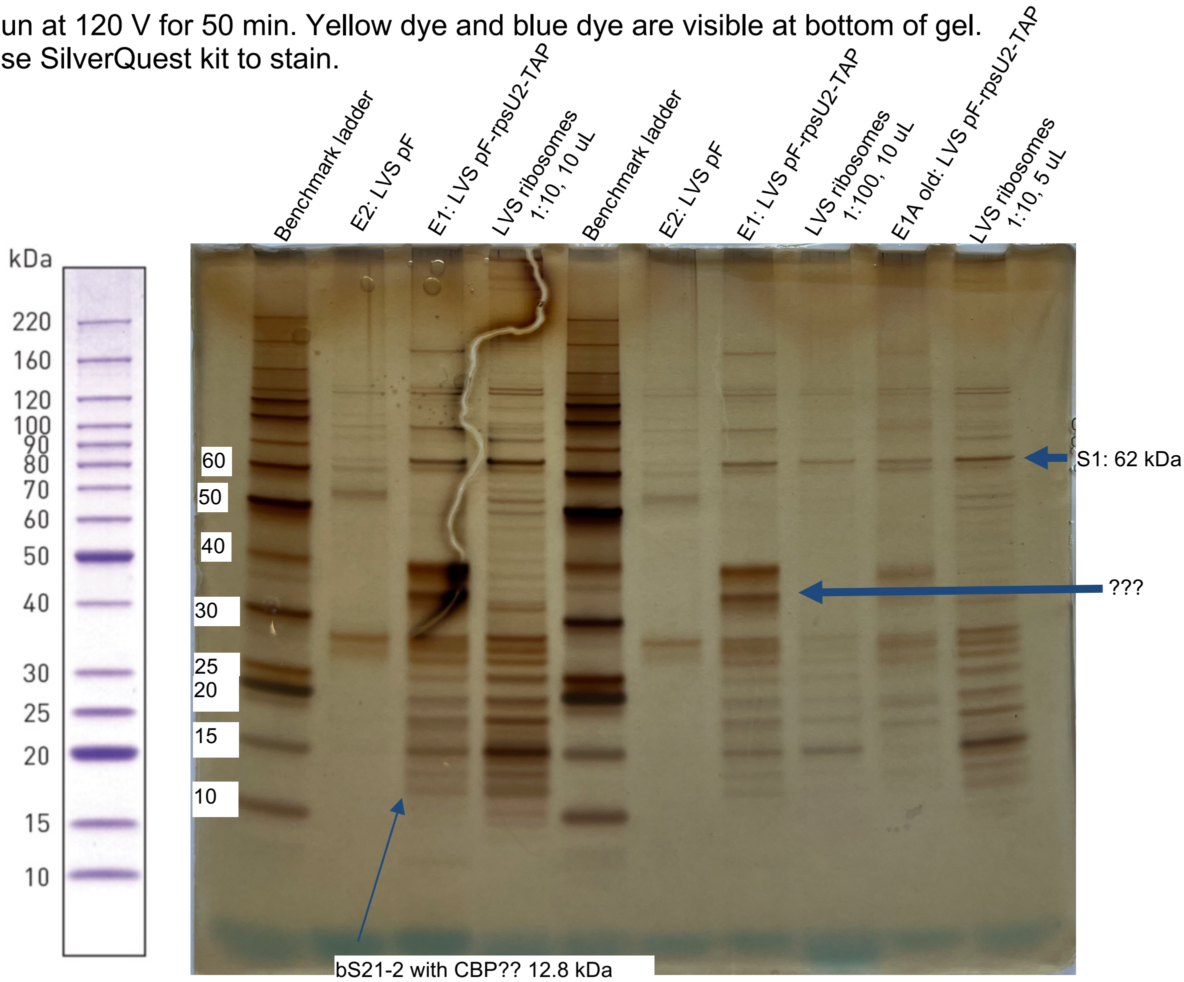

Figure 8. bS21-2-TAP purification from 9/2/22.

bS21-2 with the CBP moiety should be 12.76 kDa. Can see S1. There seem to be some lower bands missing- is this only the 30S and not the 50S? And what is between 30kDa and 40kDa? Not something from the purification, because it's not appearing in the LVS pF control.

Looks pretty good, but I should send to mass spec to see what is actually there. Where to send?

These are the ribosomal proteins purified from our previous mass spec, in ascending size order:

Molecular Weight (kDa)	Gene Locus	Gene Name	Protein
5	FTL_0175	*rpmH*	50S ribosomal protein L34
6	FTL_0521	*rpmG*	50S ribosomal protein L33
7	FTL_1143	*rpmF*	50S ribosomal protein L32
7	FTL_0254	*rpmD*	50S ribosomal protein L30
7	FTL_1405	*rpmI*	50S ribosomal protein L35
8	FTL_0244	-	50S ribosomal protein L29
8	FTL_1025	*rpsR*	30S ribosomal protein S18
8	FTL_1303	*rpmE*	50S ribosomal protein L31
8	FTL_1047	*rpsU2*	30S ribosomal protein S21-2
8	FTL_0456 / FTL_1360	*rpsU1 / rpsU3*	30S ribosomal protein S21
9	FTL_0522	*rpmB*	50S ribosomal protein L28
9	FTL_1738	*rpsP*	30S ribosomal protein S16
9	FTL_1452	*rpmA*	50S ribosomal protein L27
10	FTL_1538	*rpsO*	30S ribosomal protein S15
10	FTL_0240	*rpsS*	30S ribosomal protein S19
10	FTL_0245	*rpsQ*	30S ribosomal protein S17

10	FTL_0070	*rpsT*	30S ribosomal protein S20
11	FTL_0247	*rplX*	50S ribosomal protein L24
11	FTL_0950	-	50S ribosomal protein L25
11	FTL_0238	*rplW*	50S ribosomal protein L23
12	FTL_0249	*rpsN*	30S ribosomal protein S14
12	FTL_0235	*rpsJ*	30S ribosomal protein S10
12	FTL_0241	*rplV*	50S ribosomal protein L22
12	FTL_1453	*rplU*	50S ribosomal protein L21
13	FTL_1745	*rplL*	50S ribosomal protein L7/L12
13	FTL_0246	*rplN*	50S ribosomal protein L14
13	FTL_0252	*rplR*	50S ribosomal protein L18
13	FTL_1735	*rplS*	50S ribosomal protein L19
13	FTL_0258	*rpsM*	30S ribosomal protein S13
13	FTL_1404	*rplT*	50S ribosomal protein L20
13	FTL_1024	*rpsF*	30S ribosomal protein S6
14	FTL_0250	*rpsH*	30S ribosomal protein S8
14	FTL_0259	-	30S ribosomal protein S11
14	FTL_0232	*rpsL*	30S ribosomal protein S12
15	FTL_1186	*rpsI*	30S ribosomal protein S9
15	FTL_1748	*rplK*	50S ribosomal protein L11
15	FTL_0255	*rplO*	50S ribosomal protein L15
16	FTL_1026	*rplI*	50S ribosomal protein L9
16	FTL_1187	*rplM*	50S ribosomal protein L13
16	FTL_0243	*rplP*	50S ribosomal protein L16
17	FTL_0262	*rplQ*	50S ribosomal protein L17
18	FTL_0253	*rpsE*	30S ribosomal protein S5
18	FTL_0233	*rpsG*	30S ribosomal protein S7
19	FTL_1746	*rplJ*	50S ribosomal protein L10
19	FTL_0251	*rplF*	50S ribosomal protein L6
20	FTL_0248	*rplE*	50S ribosomal protein L5
22	FTL_0236	*rplC*	50S ribosomal protein L3
23	FTL_0260	*rpsD*	30S ribosomal protein S4
23	FTL_0237	*rplD*	50S ribosomal protein L4
25	FTL_0242	*rpsC*	30S ribosomal protein S3
25	FTL_1747	*rplA*	50S ribosomal protein L1
26	FTL_0224	*rpsB*	30S ribosomal protein S2
30	FTL_0239	*rplB*	50S ribosomal protein L2
62	FTL_1912	*rpsA*	30S ribosomal protein S1

Patch out LVS pF, LVS pKR153, LVS pKR154, four patches each (A-D) and put at 37°C O/N.
Electroporation plates look great:

EP	Cells	Plasmid	20 uL plate	200 ul plate	remaining plate
1	LVS	pKR153	22	>100	-
2	LVS	pKR154	17	>100	-
3	LVS	pF	~80	>100	-
4	LVS	-		~4 pinprick-sized	~50? Pinprick sized

Good reminder that Kan selection isn't perfect- LVS will start growing on kan plates if left long enough- not genetically resistant. Can see some pinprick-sized colonies on the transformations with plasmid DNA, probably background just like on negative control.

Patch out LVS pF, LVS pKR153, LVS pKR154, four patches each (A-D) and put at 37°C O/N.

Sunday, September 4, 2022

To Do:

1. Freeze down LVS pKR153 and LVS pKR154

Results and Data:

Words

Future To-Do

Check on plasmids that might restore growth to ∆rpsU1∆rpsU2 cells (Hannah's gDNA insert screen, Oli's Fall 2021 project).

Move 1° LVS pKR10-1 into strain box

Bibliography

Suh, Moo-Jin et al. "Extending ribosomal protein identifications to unsequenced bacterial strains using matrix-assisted laser desorption/ionization mass spectrometry." Proteomics vol. 5,18 (2005): 4818-31. doi:10.1002/pmic.200402111