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October 2020

Tuesday, October 6, 2020

To Do:

1. Streak out cells to test SDS sensitivity
2. Make plates for SDS sensitivity test

Results and Data:

Streak out:

Number	Description	Strain number
1	LVS	0
2	LVS Δ*pmrA*	KRLVS40
3	LVS Δ*pmrA* Δ*priM*	KMLFT49
4	LVS Δ*priM*	KMLFT47

Hannah autoclaved CHA and hemoglobin so I poured plates

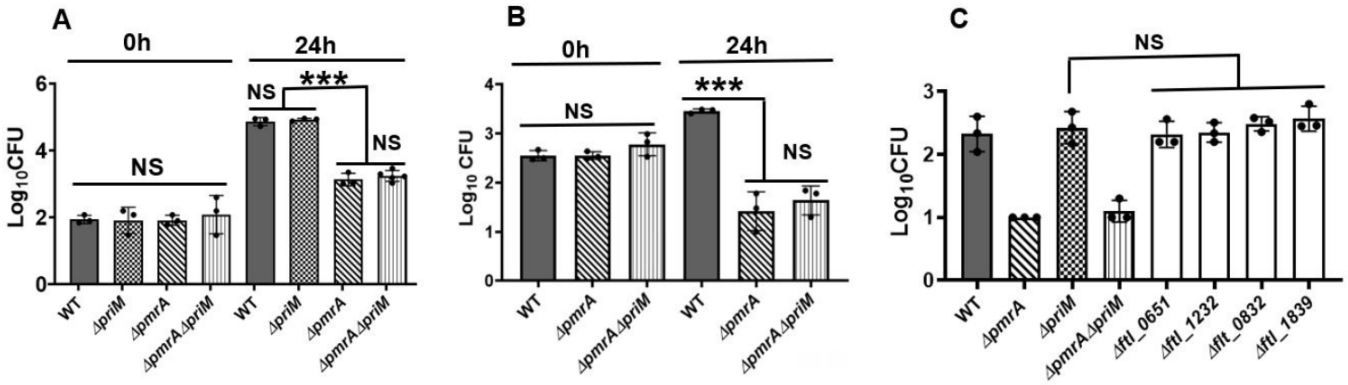
Wednesday, October 7, 2020

To Do:

1. Test sensitivity to SDS
2. Make EC LVS
3. Make stock of ΔpriM
4. Make single-use stocks of strains

Results and Data:

We're interested in seeing if we can replicate these results, from Hoang et al., 2020, Figure 5:



Note that Δ*pmrA* cells do not replicate in their assay, but neither do Δ*pmrA* Δ*priM* (unlike in Ramsey et al., 2016). We suspect this might be due to differential sensitivity to the detergent used to lyse macrophage, 0.1% SDS.

The description of lysing the macrophage is as follows:

"The macrophages were lysed with 0.1% sodium dodecyl sulfate (SDS) at the indicated time points post infection, and the lysates were serially diluted and plated on mTSB agar plates for determination of viable bacteria."

There is no indication if the SDS was diluted in water or PBS or how long they incubated to lyse macrophage. Assume SDS diluted in water and try a 5 minute incubation.

Prepare 0.1% SDS solution:
(20 mL * 0.1%) / 20% = 100 uL 20% SDS
Use 1% saponin in PBS used in macrophage assays

Scrape up cells
Resuspend to specific OD in DMEM (0.04)

	Strain	Resuspended to	For 1.3 mL at 0.04	Final OD600
1	LVS	1.86	28.0	0.035
2	ΔpmrA	4.39	11.8	0.036
3	ΔpmrA ΔpriM	4.63	11.2	0.036
4	ΔpriM	2.52	20.6	0.033

Diluted 60 uL into 6 mL DMEM. Spun down 1 mL each, max speed for 2.5 minutes. No visible cell pellet- too few cells!

Will just have to dilute into final solution- use 5 uL in 995 uL of given solution, so concentration of DMEM is 0.5% of the final conc.

For each dilutant, do the following:
-Dilute 5 uL of each strain in DMEM into 995 uL dilutant
-Wait 5 minutes
-During 5' incubation, transfer ~200 uL into 96-well plate 2x for each sample.
-Perform 10-fold serial dilutions in 1x PBS, 3x.
-Plate all 8 samples 2x using track plates: undiluted on one plate, 1:10 on one plate, 1:100, 1:1000, etc.

Then do the same for the next dilutant, then the third.

Dilutants:
A. 0.1% SDS
B. PBS plus saponin
C. DMEM

96-well plate layout: (note the mixup in samples 1 and 2 in dilutant A.

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

Friday, October 9, 2020

To Do:

1. ~~Electroporate LVS with pF, pKR72~~
2. ~~Check SDS sensitivity phenotypes~~

Results and Data:

Electroporate EC LVS cells from 8/16/19
3 uL pF or 3 uL pKR72 pF-rpsU2-TAP, 50 uL cells

Reaction	Plasmid	Time constant
1	pF	4.2
2	pF	4.3
3	pKR72	4.2
4	pKR72	4.2

Recover at 37°C shaking around 3:25pm for ~ 1hour, 15'.Plate 20 uL and 200 uL on CHA-Kan, move to 37°C over the weekend.

Check sensitivity to dilutants.

Colony counts:

	A: 0.1% SDS				
	Dilution	1	0.1	0.01	0.001
1	LVS	0	0	0	0
1		0	0	0	0
1		0	0	0	0
1		0	0	0	0
2	*ΔpmrA*	0	0	0	0
2		0	0	0	0
2		0	0	0	0
2		0	0	0	0
3	*ΔpmrA ΔpriM*	0	0	0	0
3		0	0	0	0
3		0	0	0	0
3		0	0	0	0
4	*ΔpriM*	0	0	0	0
4		0	0	0	0
4		0	0	0	0
4		0	0	0	0

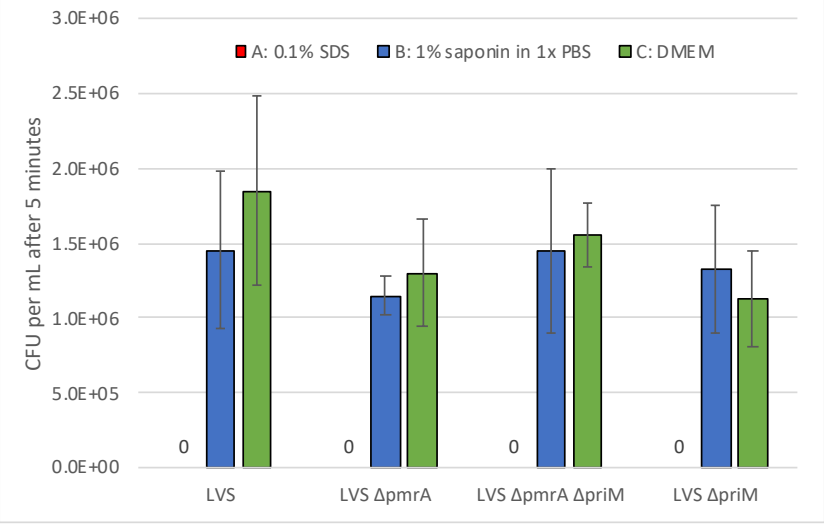
	B: 1% saponin in 1x PBS				
	Dilution	1	0.1	0.01	0.001
1	LVS	TMTC	TMTC	TMTC	15
1		TMTC	TMTC	TMTC	19
1		TMTC	TMTC	TMTC	17
1		TMTC	TMTC	TMTC	7
2	*ΔpmrA*	TMTC	TMTC	TMTC	12
2		TMTC	TMTC	TMTC	11
2		TMTC	TMTC	TMTC	13
2		TMTC	TMTC	TMTC	10
3	*ΔpmrA ΔpriM*	TMTC	TMTC	TMTC	16
3		TMTC	TMTC	TMTC	20
3		TMTC	TMTC	TMTC	7
3		TMTC	TMTC	TMTC	15
4	*ΔpriM*	TMTC	TMTC	TMTC	15
4		TMTC	TMTC	TMTC	16
4		TMTC	TMTC	TMTC	7
4		TMTC	TMTC	TMTC	15

	C: DMEM				
	Dilution	1	0.1	0.01	0.001
1	LVS	TMTC	TMTC	TMTC	23
1		TMTC	TMTC	TMTC	14
1		TMTC	TMTC	TMTC	
1		TMTC	TMTC	TMTC	
2	*ΔpmrA*	TMTC	TMTC	TMTC	18
2		TMTC	TMTC	TMTC	10
2		TMTC	TMTC	TMTC	13
2		TMTC	TMTC	TMTC	11
3	*ΔpmrA ΔpriM*	TMTC	TMTC	TMTC	17
3		TMTC	TMTC	TMTC	14
3		TMTC	TMTC	TMTC	
3		TMTC	TMTC	TMTC	
4	*ΔpriM*	TMTC	TMTC	TMTC	10
4		TMTC	TMTC	TMTC	9
4		TMTC	TMTC	TMTC	16
4		TMTC	TMTC	TMTC	10

Analysis:

	A: 0.1% SDS				
	Average	St Dev	Dilution	CFU / mL	Stdev
LVS	0	0.00	0.001	0.00E+00	0.00E+00
LVS *ΔpmrA*	0	0.00	0.001	0.00E+00	0.00E+00
LVS *ΔpmrA ΔpriM*	0	0.00	0.001	0.00E+00	0.00E+00
LVS *ΔpriM*	0	0.00	0.001	0.00E+00	0.00E+00
	B: 1% saponin in 1x PBS				
	Average	St Dev	Dilution	CFU / mL	Stdev
LVS	14.5	5.26	0.001	1.45E+06	5.26E+05
LVS *ΔpmrA*	11.5	1.29	0.001	1.15E+06	1.29E+05
LVS *ΔpmrA ΔpriM*	14.5	5.45	0.001	1.45E+06	5.45E+05
LVS *ΔpriM*	13.25	4.19	0.001	1.33E+06	4.19E+05
	C: DMEM				
	Average	St Dev	Dilution	CFU / mL	Stdev
LVS	18.5	6.36	0.001	1.85E+06	6.36E+05
LVS *ΔpmrA*	13	3.56	0.001	1.30E+06	3.56E+05
LVS *ΔpmrA ΔpriM*	15.5	2.12	0.001	1.55E+06	2.12E+05
LVS *ΔpriM*	11.25	3.20	0.001	1.13E+06	3.20E+05

Detergent sensitivity assay 10/06/20 results



Thoughts

All the plates using dilutant A were clear- no colonies at all. None of the Gunn papers report details beyond using 0.1% SDS to lyse (i.e., no incubation times or indication that the SDS was made in PBS). The paper that reports differences in the sensitivity of *F. tularensis* to detergents (*Chalabaev* et al., 2011) reports:

"LVS sensitivity to SDS in PBS exhibited some variability: after a 5 min exposure, the number of CFU was sometimes the same as in PBS control (Fig. 1A), sometimes drastically reduced by 2 logs (Fig. 1B). Therefore we recommend to avoid the use of SDS as a lysis reagent."

But they don't assess SDS made in water. The issue is that I don't want to do a macrophage assay with SDS made in water and not recover any bacteria at all; I wouldn't be surprised if the Gunn lab made the SDS in PBS and didn't report it. I should check the sensitivity of these strains to SDS made in PBS before trying the macrophage assay.

Tuesday, October 13, 2020

To Do:

1. Patch cells from electroporation, plate out rpoC-TAP cells

Results and Data:

Electroporations worked well, patched out 2 plates each pF, pF-rpsU2-TAP and streak out rpoC-TAP.
Make more 2.5M glycine (MW=75.07)
(75.07 g / 1 M)(2.5 moles / L)(0.2 L) = 37.535 g

Wednesday, October 14, 2020

To Do:

1. ~~Grow up cultures for TAP purification (6x 500 mL)~~
2. ~~Pellet cells, store at -80°C~~
3. ~~Patch out cells for SDS sensitivity test repeat~~
4. ~~Pour plates for SDS sensitivity test~~

Results and Data:

Try 3 cultures with crosslinking and 3 cultures without.
Have 500 mL flasks of MHB that Hannah prepared
Will need to add 13.7 mL formaldehyde to get 1% formaldehyde and will need to quench with 57.5 mL 2.5 M glycine.
Supplement flasks of MHB, scrape up cells (spilled almost all sample 1! Forgot to streak out 2 plates for sample 3!)

Sample #	Strain	Measured OD	Actual OD	OD aiming for	Total volume (mL)	Volume cells to add (mL)
1	LVS pF	0.792	15.84	0.04	500	1.26
2	LVS pF-rpsu2-TAP	0.692	13.84	0.04	500	1.45
3	LVS rpoC-TAP	0.279	5.58	0.04	500	3.58

Really not enough cells to get to a reasonable starting OD. Just move forward, splitting the resuspended cells equally between flasks, and pellet late!

Started 6x 500 mL cultures growing at 37°C but had to reduce shaking speed down to 200 rpm- shaker not robust enough.

Sample #	Strain	11:00 AM	1:55 PM	6:20 PM	8:00 PM
1A	LVS pF	0.011	0.038	0.142	**0.207**
1B	LVS pF	0.01	0.039	0.151	**0.225**
2A	LVS pF-rpsU2-TAP	0.04	0.108	**0.318**	-
2B	LVS pF-rpsU2-TAP	0.042	0.114	**0.333**	-
3A	LVS rpoC-TAP	0.016	0.052	0.201	**0.291**
3B	LVS rpoC-TAP	0.016	0.052	0.193	**0.282**

At the 6:20 pm timepoint, stopped the LVS pF-rpsU2-TAP cultures.
Started crosslinking culture 2B in the flask on the rocker by adding 13.7mL formaldehyde, rock for 30' at room temp.
Transferred culture 2A to large centrifuge tubes (balanced). Someone is using the INBRE core centrifuge- will have to wait 16' (!), so put the cells at 4°C. Not sure what that will do to ribosomes, but they were going to be pelleted anyway.
Add the 57.5 mL glycine to 2B (2x 28.75 mL), incubate 15' rocking at room temp.

Spin 2A for 10' at 4°C at 8,000 rcf in two bottles (large bottles, rotor JA-10). Remove most of the MHB, but keep less than 50 mL. Resuspend cell pellets in remaining MHB, transfer all the cells from the two large bottles to one 50 mL conical. Spin 10' at 4°C at 8,000 rcf in conical (balanced, rotor JA-20). Remove media, store cell pellet at -80°C.

By the time the spins for 2A are over, time to spin down 2B (x-linked sample) in large bottles then in 50 mL conical as for 2B. Store cell pellet in 50 mL conical at -80°C.

Time to check on the other cultures- now 8pm, this takes a lot of time! Both cultures over 0.2- not ideal, but fine for the controls. Treat both as did culture 2 (pellet and freeze 1A and 3A immediately, x-link and quench 1B and 3B before pelleting).

Wednesday, October 21, 2020

To Do:

1. ~~TAP purification day 1~~
2. ~~Patch out cells for SDS sensitivity test repeat~~

Results and Data:

TAP purification day 1.

Sample#	Date prepared	Labeled	Volume of cells	OD600 at harvest
1A	10/14/2020	1A	500 mL	0.207
2A	10/14/2020	2A	500 mL	0.318
3A	10/14/2020	3A	500 mL	0.291

Resuspend each sample in 5mL in Buffer 1 + Bugbuster + PI from 5/19 (probably an aliquot from JW, had been stored at -20°C). Buffer was cloudy when thawed, had to let come to room temp to re-dissolve detergents.

Add 10 uL DNase I, pipet up and down, move to 37°C incubator for 30'
Spin in 50 mL conicals, 8,000xg in Beckman JA-20 rotor for 20' at 4°C.
Transfer supernatant into 30 mL syringe with 0.22 um filter (PES, 0.22µm 30mm diameter), filter supernatant. Needed to use two filters for samples 2A and 3A- either left in tube too long (pellet started to resuspend) or should have used a larger volume of lysis buffer (definitely next time) for 500 mL cells.

Adjust buffer concentration:

Sample	Recovered volume (mL)	Add Tris pH8 and NP-40 (ul)	Add NaCl (ul)
1A	4.4	44 ul	88 ul
2A	3.3 mL	33 ul	66 ul
3A	3.1	31 ul	62 ul

Save 50 uL lysate for future analysis.

Had prepped columns with IgG beads:

1. Cut tip of 1mL pipette tip slightly
2. Resuspend IgG Sepharose beads completely
3. 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
4. Wash beads with 10 mL of IPP150 buffer (put in fridge for this step)
5. When all the IPP150 buffer has passed through the column, cap the bottom of the column

Add samples to column. No decent rocker in the cold room, so bring over to rocker in CBLS- rock for 2 hours.

Prepare TEV Cleavage Buffer for this set of purifications, ~13 mL per purification = 40 mL

Composition	Stock solutions	For 40 mL
10 mM Tris-HCl pH 8	1 M	400 uL
150 mM NaCl	5 M	1.2 mL
0.1% NP40	10.00%	400 uL
0.5 mM EDTA	0.5 M	40 uL
1 mM DTT	0.5 M	80 uL
Water		37.88 mL

1. After 2 hour incubation, open columns and let lysate pass through column.
2. Save 50 uL aliquot of flow through in microfuge tube (FT1), discard remaining.
3. Wash columns 3x with 10 mL IPP150 buffer, discarding flow through
4. Wash columns with 10 mL TEV Cleavage buffer, discarding flow through
5. Cap column bottoms, add 1 mL TEV Cleavage buffer and 10 uL AcTEV Protease to each.
6. Incubate rocking at 4°C overnight in CBLS 3rd floor cold room (started at about 2:15pm)

Thursday, October 22, 2020

To Do:

1. ~~TAP purification day 2~~
2. ~~SDS sensitivity assay~~
3. Run gel of TAP purifications

Results and Data:

1. Open columns incubated O/N and place on new clean 50 mL conical tubes, letting liquid elute out
2. Wash columns with 300 uL TEV cleavage buffer, eluting into same tube.
6. KEEP eluted material, transfer 10 uL aliquot of elution in microfuge tube (E1).
7. Add 3.3 uL of 1 M $CaCl_2$ to each 1.1 mL sample
   Samples -> 1.2 mL, so add 3.6 uL.

Prepare buffer:
IPP150 Calmodulin Binding Buffer (no detergent), ~45 mL per purification = 140 mL
Need ~45 mL per sample

Composition	Stock solutions	For 150 mL
10 mM β–mercapto ethanol	14.3 M	104.9 uL
10 mM KHEPES pH 7.9	1 M	1.5 mL
150 mM NaCl	5 M	4.5 mL
1 mM Mg-acetate	1 M	150 uL
1 mM Imidazole	5 M	30 uL
2 mM $CaCl_2$	1 M	300 uL
Water		to 150 mL

1. Completely resuspend Calmodulin beads by vortexing
2. Cut tip of 1 mL tip and use to add 200 uL calmodulin beads per column
3. Wash calmodulin beads with 10 mL calmodulin binding buffer
4. After wash liquid is through columns, plug bottom of columns closed.
5. Add samples to columns, add 3 mL IPP150 Calmodulin Binding Buffer to each column
6. Add top cap to close columns (make sure columns are closed completely and are not dripping liquid)
7. Incubate rocking at 4°C- 1 hour plus 20' or so.
8. Unplug columns, let liquid flow through
9. Save 50 uL aliquot of flow through in microfuge tube (FT2), discard remaining.
10. Wash 3 times with 10 mL IPP150 Calmodulin Binding Buffer, discarding flow-through

Prepare buffer:

IPP150 Calmodulin Elution Buffer (no detergent), ~1.5 mL per purification = 5 mL
Need ~1.5 mL per sample

Composition	Stock solutions	For 5 mL
10 mM β–mercapto ethanol	14.3 M	3.5 uL
10 mM KHEPES pH 7.9	1 M	50 uL
150 mM NaCl	5 M	150 ul
1 mM Mg-acetate	1 M	5 uL
1 mM Imidazole	5 M	1 uL
2 mM EGTA	0.5 M	20 uL
Water		4,770.5 uL

1. Wash columns once with 200 uL IPP150 Calmodulin Elution Buffer, discarding flow-through.

2. Place columns on clean new 50 mL conical.
3. Elute protein by running 500 uL IPP150 Calmodulin Elution Buffer over column twice (final protein volume ~ 1mL)

No time to run gel- would like to compare this purification with a ribosome purification from Hannah (bS21-2 if possible).
Gel plan

SDS sensitivity assay
Based on previous results, no difference in sensitivity to SDS between cells with and without *pmrA*- it was toxic to all of them. Check the sensitivity of these cells to 0.1% SDS made in 1x PBS.

Prepare 0.1% SDS solution in 1x PBS
(10 mL * 0.1%) / 20% = 50 uL 20% SDS into 10 mL 1x PBS

Use 1% saponin in PBS used in macrophage assays

Scrape up cells
Resuspend to specific OD in DMEM (0.04)

	Strain	Resuspended to	For 1.3 mL at 0.04	Final OD600	Volume to add for each dilution
1	LVS	4.49	11.58	0.045	5 ul
2	ΔpmrA	4.13	12.59	0.045	5 ul
3	ΔpmrA ΔpriM	2.82	18.44	0.05	4.5 ul
4	ΔpriM	3.78	13.76	0.043	5 ul

Dilute 1:200 into the final solution- use ~5 uL in 995 uL of given solution, so concentration of DMEM is 0.5% of the final conc.

For each dilutant, do the following:
-Dilute 5 uL of each strain in DMEM into 995 uL dilutant: 10' sample
-Wait 5 minutes
-During 5' incubation, transfer ~200 uL into 96-well plate 2x for each sample.
-Dilute 5 uL of each strain in DMEM into 995 uL dilutant: 5' sample
-Wait 5 minutes
-During 5' incubation, transfer ~200 uL into 96-well plate 2x for each sample.
-Perform 10-fold serial dilutions in 1x PBS, 3x.
-Plate all 8 samples 2x using track plates: undiluted on one plate, 1:10 on one plate, 1:100, 1:1000, etc.

Then do the same for the next dilutant, then the third.

Dilutants:
A. 0.1% SDS
B. PBS plus saponin
C. DMEM

	5' timepoint				10' timepoint			
	A	B	C	D	E	F	G	H
1	1A	2A	3A	4A	1A	2A	3A	4A
2								
3								
4								
5	1B	2B	3B	4B	1B	2B	3B	4B
6								
7								
8								
9	1C	2C	3C	4C	1C	2C	3C	4C
10								
11								
12								

Saturday, October 24, 2020

To Do:

1. ~~Check SDS sensitivity plates~~
2. ~~Thaw macrophage~~

Results and Data:

Thawed 1 vial P4 macrophage into 10 mL DMEM-F, using Clements lab hood and incubator.

Count colonies on detergent sensitivity plates

Colony Counts from 10/22/20 assay

		A: 0.1% SDS in 1X PBS			
Time	Strain	1	0.1	0.01	0.001*
5'	LVS	TMTC	TMTC	76	0
		TMTC	TMTC	68	0
	ΔpmrA	TMTC	TMTC	57	0
		TMTC	TMTC	58	0
	ΔpmrA ΔpriM	TMTC	TMTC	73	0
		TMTC	TMTC	68	0
	ΔpriM	TMTC	TMTC	39	0
		TMTC	TMTC	42	0
10'	LVS	TMTC	TMTC	30	0
		TMTC	TMTC	36	0
	ΔpmrA	TMTC	TMTC	18	0
		TMTC	TMTC	10	0
	ΔpmrA ΔpriM	TMTC	TMTC	26	0
		TMTC	TMTC	19	0
	ΔpriM	TMTC	TMTC	37	0
		TMTC	TMTC	29	0

*Note that I didn't actually bother counting the 0.001 dilution tracks

		B: 1% saponin in 1x PBS			
Time	Strain	1	0.1	0.01	0.001
5'	LVS	TMTC	TMTC	113	8
		TMTC	TMTC	115	18
	ΔpmrA	TMTC	TMTC	TMTC	10
		TMTC	TMTC	TMTC	8
	ΔpmrA ΔpriM	TMTC	TMTC	TMTC	14
		TMTC	TMTC	TMTC	14
	ΔpriM	TMTC	TMTC	TMTC	18
		TMTC	TMTC	TMTC	22
10'	LVS	TMTC	TMTC	TMTC	14
		TMTC	TMTC	TMTC	15
	ΔpmrA	TMTC	TMTC	TMTC	12
		TMTC	TMTC	85	5
	ΔpmrA ΔpriM	TMTC	TMTC	TMTC	12
		TMTC	TMTC	TMTC	13
	ΔpriM	TMTC	TMTC	TMTC	18
		TMTC	TMTC	TMTC	26

		C: DMEM			
Time	Strain	1	0.1	0.01	0.001
5'	LVS	TMTC	TMTC	TMTC	19
		TMTC	TMTC	TMTC	20
	ΔpmrA	TMTC	TMTC	TMTC	6
		TMTC	TMTC	TMTC	14
	ΔpmrA ΔpriM	TMTC	TMTC	TMTC	10
		TMTC	TMTC	TMTC	19
	ΔpriM	TMTC	TMTC	TMTC	17
		TMTC	TMTC	TMTC	16
10'	LVS	TMTC	TMTC	TMTC	37
		TMTC	TMTC	TMTC	16
	ΔpmrA	TMTC	TMTC	TMTC	16
		TMTC	TMTC	TMTC	9
	ΔpmrA ΔpriM	TMTC	TMTC	TMTC	19
		TMTC	TMTC	TMTC	12
	ΔpriM	TMTC	TMTC	TMTC	16
		TMTC	TMTC	TMTC	21

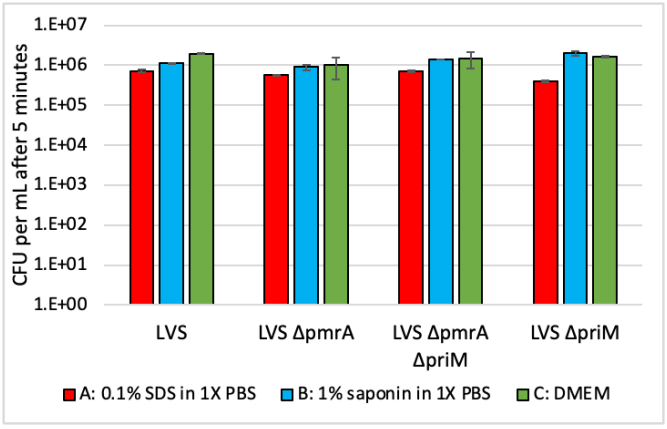
Analysis of 10/22/20 detergent sensitivity assay

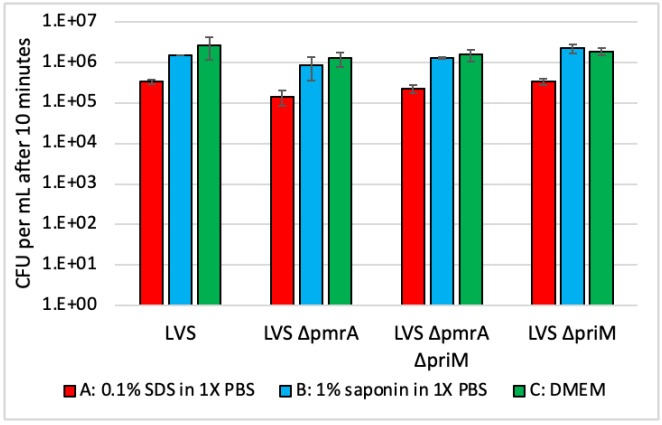
		A: 0.1% SDS in 1X PBS				
		Average	St Dev	Dilution	CFU / mL	Stdev
5'	LVS	72	5.7	0.01	7.20E+05	5.66E+04
	LVS *ΔpmrA*	57.5	0.7	0.01	5.75E+05	7.07E+03
	LVS *ΔpmrA ΔpriM*	70.5	3.5	0.01	7.05E+05	3.54E+04
	LVS *ΔpriM*	40.5	2.1	0.01	4.05E+05	2.12E+04
10'	LVS	33	4.2	0.01	3.30E+05	4.24E+04
	LVS *ΔpmrA*	14	5.7	0.01	1.40E+05	5.66E+04
	LVS *ΔpmrA ΔpriM*	22.5	4.9	0.01	2.25E+05	4.95E+04
	LVS *ΔpriM*	33	5.7	0.01	3.30E+05	5.66E+04

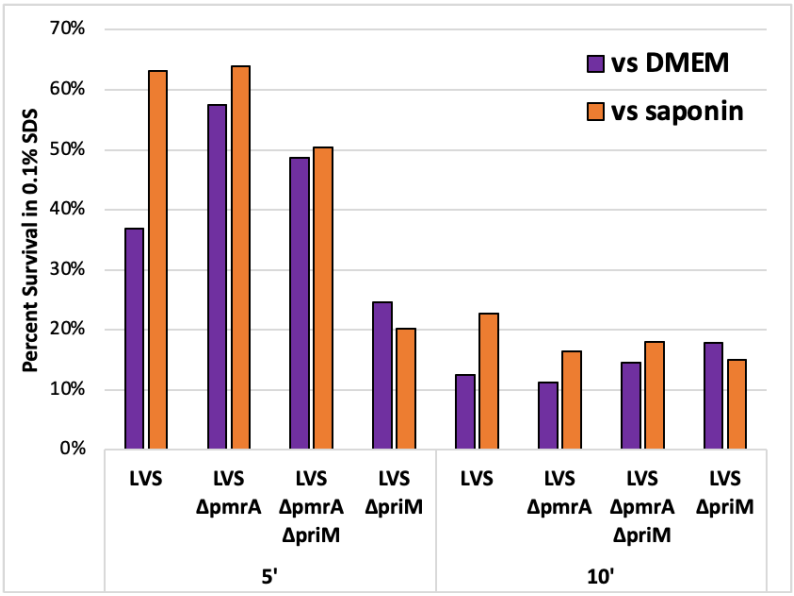
		B: 1% saponin in 1X PBS				
		Average	St Dev	Dilution	CFU / mL	Stdev
5'	LVS	114	1.4	0.01	1.14E+06	1.41E+04
	LVS *ΔpmrA*	9	1.4	0.001	9.00E+05	1.41E+05
	LVS *ΔpmrA ΔpriM*	14	0.0	0.001	1.40E+06	0.00E+00
	LVS *ΔpriM*	20	2.8	0.001	2.00E+06	2.83E+05
10'	LVS	14.5	#DIV/0!	0.001	1.45E+06	#DIV/0!
	LVS *ΔpmrA*	8.5	4.9	0.001	8.50E+05	4.95E+05
	LVS *ΔpmrA ΔpriM*	12.5	0.7	0.001	1.25E+06	7.07E+04
	LVS *ΔpriM*	22	5.7	0.001	2.20E+06	5.66E+05

		C: DMEM				
		Average	St Dev	Dilution	CFU / mL	Stdev
5'	LVS	19.5	0.7	0.001	1.95E+06	7.07E+04
	LVS *ΔpmrA*	10	5.7	0.001	1.00E+06	5.66E+05
	LVS *ΔpmrA ΔpriM*	14.5	6.4	0.001	1.45E+06	6.36E+05
	LVS *ΔpriM*	16.5	0.7	0.001	1.65E+06	7.07E+04
10'	LVS	26.5	14.8	0.001	2.65E+06	1.48E+06
	LVS *ΔpmrA*	12.5	4.9	0.001	1.25E+06	4.95E+05
	LVS *ΔpmrA ΔpriM*	15.5	4.9	0.001	1.55E+06	4.95E+05
	LVS *ΔpriM*	18.5	3.5	0.001	1.85E+06	3.54E+05

Detergent sensitivity assay 10/06/20 results







Thoughts

All the cells survive in 0.1% SDS made in 1X PBS much better, although there is clear loss of viability starting at 5' and increasing by 10'.

There doesn't seems to be a significant decrease in viability of the ΔpmrA cells- almost the opposite trend!

The questions is how to proceed with the macrophage assay. I cannot replicate the Gunn paper exactly due to the lack of detail provided in their paper. These in vitro assays may not replicate the results when lysing bacteria in macrophage. Rather than try to replicate the Gunn results, perhaps the best method is to try lysing with different detergents, to show the same results, as well as an alternate method (perform qPCR to detect LVS abundance)?

Monday, October 26, 2020

To Do:

1. ~~Run gel of TAP purifications~~
2. ~~Stain TAP purification gel~~

~~3. Feed macrophage~~
~~4. Pour plates for mac experiment~~

Results and Data:

Gel to assess TAP purification:

4-12% MES, 15 well

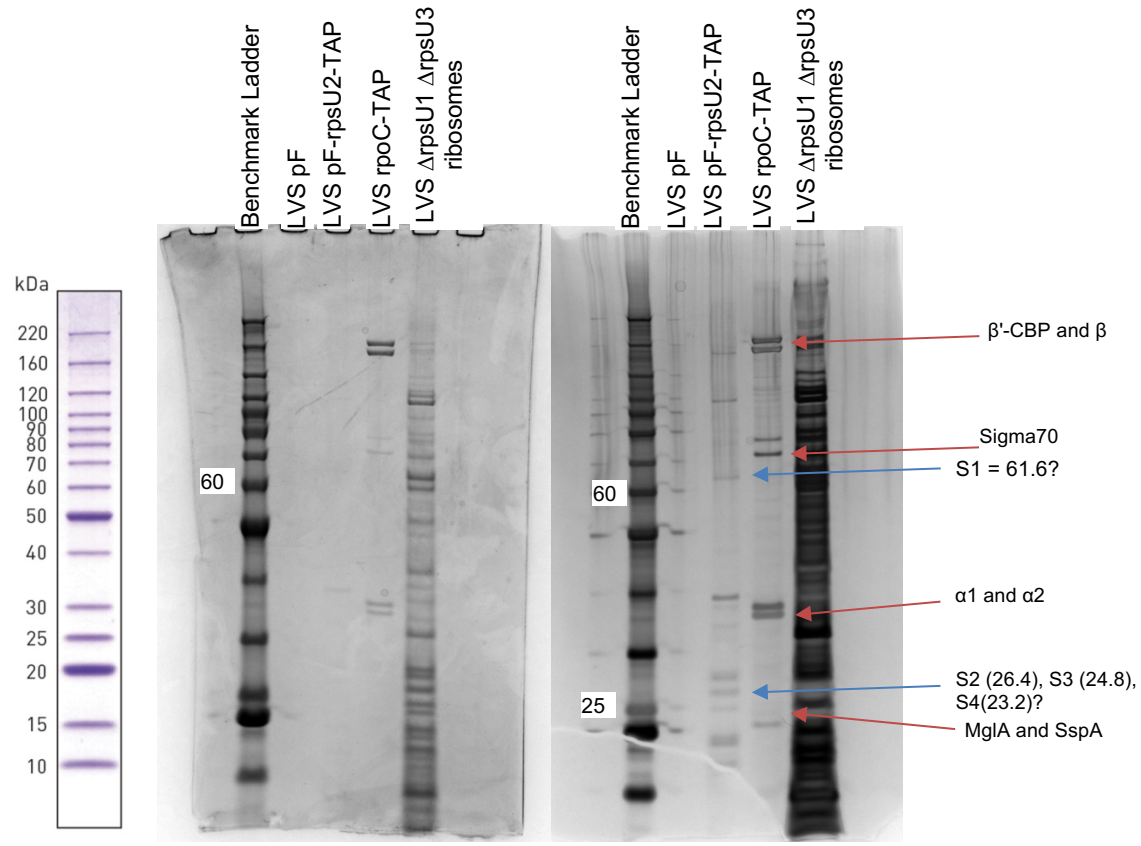
Lane	Volume	Contents	Processing
1	10 uL	1x SLB	Silver stain
2	10 uL	Benchmark ladder 1:10	
3	10 uL	LVS pF	
4	10 uL	LVS pF-rpsU-TAP	
5	10 uL	LVS rpoC-TAP	
6	10 uL	ribosomes	
7	10 uL	1x SLB	
8	10 uL	1x SLB	
9	10 uL	1x SLB	Coomassie stain
10	10 uL	Ladder	
11	10 uL	LVS pF	
12	10 uL	LVS pF-rpsU-TAP	
13	10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

Ribosome from 8/2019: Hannah previously diluted 10 uL in 32.2uL 2x SLB and ran 5 ul
For today, dilute 2 ul into 22 ul and load 10 ul

Run at about 146 V for about 1 hour- yellow dye is *just* visible at the bottom of the gel. Use Colloidal blue staining kit, following protocol and staining for ~3.5 hours. Destain in water.

Plan mac experiment:
LVS, ΔpmrA, ΔpmrA ΔpriM, ΔpriM x4 plates:
2 hours lyse with saponin vs SDS in PBS

bS21-2-TAP IP Coomassie Stain and Silver Stain



Thoughts

The β'-TAP purification worked and it looks like some proteins purified with the bS21-2-TAP purification, but not the entire 70S ribosome. In fact, I can't even quite tell if the bS21-2 protein purified- the gel ran too long! ☹
Looked at the MW for other 30S proteins and some of the bands might correspond to them…. but I'd certainly have to send the samples out for mass spec to check. Re-run the gel to see how much bS21-2 even purified and consider next steps. May be worth optimizing buffer conditions for ribosome purification?

Tuesday, October 27, 2020

To Do:

1. ~~Patch out cells for macrophage experiment~~
2. ~~Seed macrophage for mac experiment~~

Results and Data:

Wednesday, October 28, 2020

To Do:

1. ~~Infect macrophage~~
2. ~~Wash macrophage~~
3. ~~Prep 0.1% SDS in 1x PBS~~

4. ~~T=2~~
5. ~~Re-run protein gel~~
6. ~~Silver stain protein gel~~
7. Bag CHA

Results and Data:

Infect at 11:00am; add

Prep 15 mL DMEM-F with gent:
Gentamicin stock is 50 mg/mL, need 10 ug/mL
(15,000 uL * 10) / 50,000 = 3 uL

-12% MES, 15 well

Lane	Volume	Contents	Processing
1	10 uL	1x SLB	Silver stain
2	10 uL	1x SLB	
3	10 uL	1x SLB	
4	13 uL	LVS pF	
5	13 uL	LVS pF-rpsU-TAP	
6	10 uL	1x SLB	
7	10 uL	Ribosomes (1ul)	
8	10 uL	1x SLB	
9	13 uL	LVS rpoC-TAP	Coomassie stain
10	10 uL	1x SLB	
11	10 uL	Benchmark ladder 1:10	
12	10 uL	1x SLB	
13	10 uL	1x SLB	
14	10 uL	1x SLB	
15	10 uL	1x SLB	

Run at approx. 130V for 40ish minutes, then turn down voltage for another 30ish.

Prep 0.1% SDS in 1X PBS:
(30 mL * 0.1%) / 20% = 150 uL 20% SDS
3 mL 10 X PBS
Water to 30 mL, filter-sterilize

1pm: Wash wells 2x with PBS, add back 200 uL DMEM-F with 10 ug/mL gentamicin

3 pm: Remove media from one plate, wash 2x with PBS. Add 200 uL 1% saponin in 1X PBS to bottom wells and incubate for 30'. Add 200 uL 0.1% SDS in 1X PBS to top wells and incubate 5'. Plate 60 uL from each well 2x, with the exception of the LVS only and macrophage only wells – plate 100 uL each once. Plate sequentially.

Thursday, October 29, 2020

To Do:

1. ~~T=24~~
2. Bag CHA
3. Feed macrophage

Results and Data:

Lyse cells for 24 hour timepoint at 1pm:

Remove media. Wash wells twice with sterile 1x PBS.

Add 200 uL 1% saponin in PBS to appropriate wells, start timer for 30 minutes.
Add 200 uL 0.1% SDS to appropriate wells, start timer for 5 minutes.

After 5 minutes, remove SDS samples 1, 3, 4 to top of new 96-well plate. Perform 10-fold serial dilutions.
Plate 60 uL of samples 2-1, 2-2, 2-3 on round plates (use sterile glass beads).
Plate 10 uL samples and serial dilutions on square plates: 1-1, 1-2, 1-3, 3-1, 3-2, 3-3, 4-1, 4-2, 4-3

After 30', remove saponin samples 1, 3, 4 to top of new 96-well plate. Perform 10-fold serial dilutions.
Plate 60 uL of samples 2-1, 2-2, 2-3 on round plates (use sterile glass beads).
Plate samples: 1-1, 1-2, 1-3, 3-1, 3-2, 3-3, 4-1, 4-2, 4-3
Plate 10 uL samples and serial dilutions on square plates: 1-1, 1-2, 1-3, 3-1, 3-2, 3-3, 4-1, 4-2, 4-3

Put plates in 37°C incubator.

Friday, October 30, 2020

To Do:

1. ~~Bag CHA~~
2. ~~Split macrophage~~
3. ~~Check inoculum plates~~

Results and Data:

Split macrophage- pretty dense, so split 1:5 into one new dish.

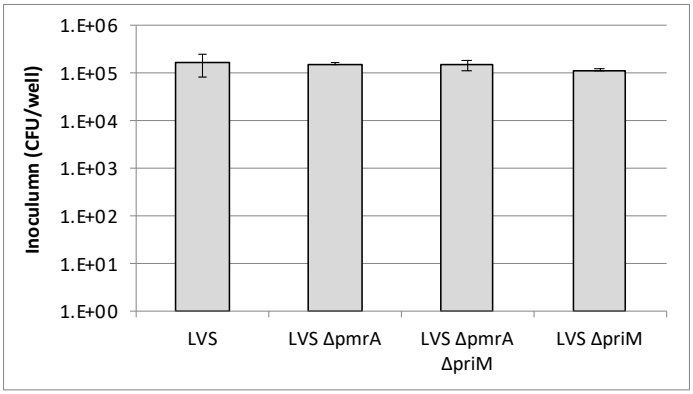
Count inoculum plates:

Inoculum										
	Replicate	3	4	Dilution factor counted	Cells / mL	Average Cells / mL	St dev	CFU/well	St dev	MOI
LVS	1A	TMTC	21	0.001	2.10E+06	3.25E+06	1.63E+06	1.63E+05	8.13E+04	10.1
	1B	TMTC	44	0.001	4.40E+06					
Δ*pmrA*	2A	TMTC	32	0.001	3.20E+06	3.10E+06	1.41E+05	1.55E+05	7.07E+03	9.6
	2B	TMTC	30	0.001	3.00E+06					
Δ*pmrA* Δ*priM*	3A	TMTC	24	0.001	2.40E+06	2.90E+06	7.07E+05	1.45E+05	3.54E+04	9.0
	3B	TMTC	34	0.001	3.40E+06					
Δ*priM*	4A	TMTC	24	0.001	2.40E+06	2.30E+06	1.41E+05	1.15E+05	7.07E+03	7.1
	4B	TMTC	22	0.001	2.20E+06					
Dilution Factor		0.01	0.001							

Macrophage assay 10/28/20 inoculum

Strain	CFU/well	St dev
LVS	1.63E+05	8.13E+04
LVS Δ*pmrA*	1.55E+05	7.07E+03
LVS Δ*pmrA* Δ*priM*	1.45E+05	3.54E+04
LVS Δ*priM*	1.15E+05	7.07E+03

Inoculums look nice and even!



Monday, November 2, 2020

To Do:

1. Count plates

Results and Data:

Can count plates from 2 hour timepoint, but 24 hour timepoint colonies are still too small. But it looks like Δ*pmrA* grew?!?!?

Looking back at my notes- I used a single-use aliquot of Δ*pmrA* I'd frozen down. I believe it was from cells struck out on 7/28- would have been a single-use Δ*pmrA* aliquot from JW?

Going back to my qRT-PCR results from those cells (9/12/20)- I think these are the suppressor cells after all! They had the same amount of *priM* as the Δ*pmrA*(sup) cells, which was only about 100x, not 300x- that would be consistent with Jamie's results if there were cells that lacked *pmrA* that produced more *priM* transcript.

Get rid of all my KRLVS40 stocks and re-make before doing more experiments. ☹

Future To-Do

Move 1° LVS pKR10-1 into strain box

Bibliography

Chalabaev, S., Anderson, C., Onderdonk, A., Kasper, D. (2011). **Sensitivity of Francisella tularensis to ultrapure water and deoxycholate: implications for bacterial intracellular growth assay in macrophages.** Journal of microbiological methods 85(3), 230 - 232. https://dx.doi.org/10.1016/j.mimet.2011.03.006

Hoang, K., Fitch, J., White, P., Mohapatra, N., Gunn, J. (2020). **The sensor kinase QseC regulates the unlinked PmrA response regulator and downstream gene expression in Francisella** Journal of Bacteriology https://dx.doi.org/10.1128/jb.00321-20