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Sunday, May 16, 2021

To Do:

1. ~~Thaw macrophage~~

Results and Data:

Thawed 1 vial of P9 cells I froze down on 4/28/21, plated in 100mm dish. Checked after about an hour, they seemed to adhere well.

Monday, May 17, 2021

To Do:

1. ~~Streak strains~~
2. ~~Seed macrophage~~

Results and Data:

Patch out cells for macrophage experiment:

1	LVS	LVS
2	Δ*rpsU2* pF	KRLVS121
3	Δ*rpsU2* pF-*rpsU1*-V	KRLVS122
4	Δ*rpsU2* pF-*rpsU2*-V	KRLVS123
5	Δ*rpsU2* pF-*rpsU3*-V	KRLVS124
6	Δ*pigR*	JCLVS106

Put at 37°C around 12:30 pm

Seed macrophage. Remove media from plate, add ~5 mL media (DMEM + 10% FBS) to wash, remove. Add 10 mL media, scrape up all cells. Transfer to 15 mL tube, spin for 5' at 900xg.

While spinning, prepare 96-well plate (mark off wells to seed), new 100 mm tissue culture dish, 1.5mL tubes to take samples to mix with Trypan blue, etc.

After spin, remove supernatant, resuspend in ~4 mL media. Take 50 ul, mix with 50 ul trypan blue, check density using new disposable hemocytometer.

Density is about 5.1×10^5. Dilute 3 mL cells in 9 mL media for final goal density of ~1.25×10^5. Transfer diluted cells into a sterile reservoir, use multichannel to add cells to 96 well plates for setup:

	1	2	3	4	5	6	7	8	9	10	11	12
A	LVS	LVS	LVS		ΔrpsU pF	ΔrpsU pF	ΔrpsU pF		ΔrpsU pF-rpsU1	ΔrpsU pF-rpsU1	ΔrpsU pF-rpsU1	
B												
C												
D	ΔrpsU pF-rpsU2	ΔrpsU pF-rpsU2	ΔrpsU pF-rpsU2		ΔrpsU pF-rpsU3	ΔrpsU pF-rpsU3	ΔrpsU pF-rpsU3		ΔpigR	ΔpigR	ΔpigR	
E												
F												
G												
H												

Mix 50 ul with 50 ul trypan blue. Remove 8 mL media with 10 mL pipet, then aspirate remaining undiluted cells (~0.8 mL?), then aspirate enough of the diluted cells to reach 10 mL, transfer to 100 mm tissue culture dish (P10). Move with 96-well plates to 37°C CO_2 incubator at around 8:30PM.

Check final density: 1.43×10^5, very close to the target of 1.25×10^5!

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	5.10E+05
Volume stock needed (mL)	3.0
Volume media for dilution	9.0
Measured cells per ml, seeded	1.43E+05
Measured cells per well	2.87E+04

1st measurement				
42	22	20	18	
Average				25.5
Undiluted				51
Density				5.10E+05

2nd measurement				
6	5	9	5	
12	10	7	12	
7	1	7	5	
Average				7.16667
Undiluted				14.3333
Density				1.43E+05

Tuesday, May 18, 2021

To Do:

1. ~~Infect macrophage~~
2. Wash
3. T=24

Results and Data:

Prepared cells to infect macrophage:

Bacterial Calculations	Actual
MOI	5
Macrophage cells per well	2.87E+04
Volume bacteria to add (mL)	0.05
Bacterial density needed (cells/mL)	2.87E+06
Cells/mL per OD600	5.81E+09
OD needed for given density	0.00049
Resuspend to	0.050
Final MOI 5, dilute 1:100	0.00050

*Note that LVS and ΔpigR grew really poorly- because I plated them on Kan plates! Derf!! I should have struck out the LVS pF cells (KRLVS120.1)! Need to re-plan plates- don't use Kan plates for samples 1 and 6.

Opt to make inoculum in 3-well reservoirs, so I can add cells with multichannel. Increase inoculum volume to 3 mL.

			For final vol 1.3 mL at 0.05		For final vol 3 mL at 0.0005	
Number	Strain	Resuspend cells to (OD600)	Cells (uL)	OD600	Cells (uL)	Volume media (mL)
1	LVS	1.16	56.0	0.054	30	2.97
2	ΔrpsU2 pF	2.37	27.4	0.053	30	2.97
3	ΔrpsU2 pF-rpsU1	2.48	26.2	0.054	30	2.97
4	ΔrpsU2 pF-rpsU2	2.42	26.9	0.053	30	2.97
5	ΔrpsU2 pF-rpsU3	2.04	31.9	0.054	30	2.97
6	ΔpigR	1.25	52.0	0.053	30	2.97

Note made a mistake- added 2.7 mL media to wells instead of 2.97 mL. Inoculum will be a bit higher than planned.

Add 50 uL of each inoculum per well, as indicated. Could have gotten away with 2 ml inoculum.

Transfer 200 ul inoculum to 96-well plate, serially dilute 1:10 to 10^{-5}. Plate 10 ul each dilution on track plates 2x.

Infected cells at 12:20 PM.

At 2:20 PM, wash wells 2x with 1x PBS. Then add 200 uL of DMEMF with 10 ug/mL gentamycin (made stock of 10 mL DMEMF with 2 ul of 50 mg/mL gentamycin). Put plates back in the incubator.

At 4:20 PM, wash wells 2x with 1x PBS. Add 200 uL 1% saponin in 1x PBS incubate 30'.

Fed P10 macrophage.
Made single-use aliquots of

Saturday, April 24, 2021

To Do:

1. ~~T=24~~
2. ~~Feed macrophage~~

Results and Data:

Washed 2x with PBS, lysed in 200 uL 1% saponin in 1x PBS for 30'. Mixed and transferred samples with strains 1 – 7 to top of new 96-well plate. Serially diluted 1:10 to 10^{-5}. Mixed wells with strain 8 in original plate, plated 50 uL twice on round plates. Go back to serially-diluted samples and plate 10 ul each on track plates 2x. Put plates @37°C

Sunday, April 25, 2021

To Do:

1. ~~Pull inoculum plates~~
2. ~~Feed macrophage~~

Results and Data:

Macrophage seem quite dense, should be able to survive until splitting tomorrow. Remove most of the inoculum plates; some have very small colonies so leave at 37°C until tomorrow.

Monday, April 26, 2021

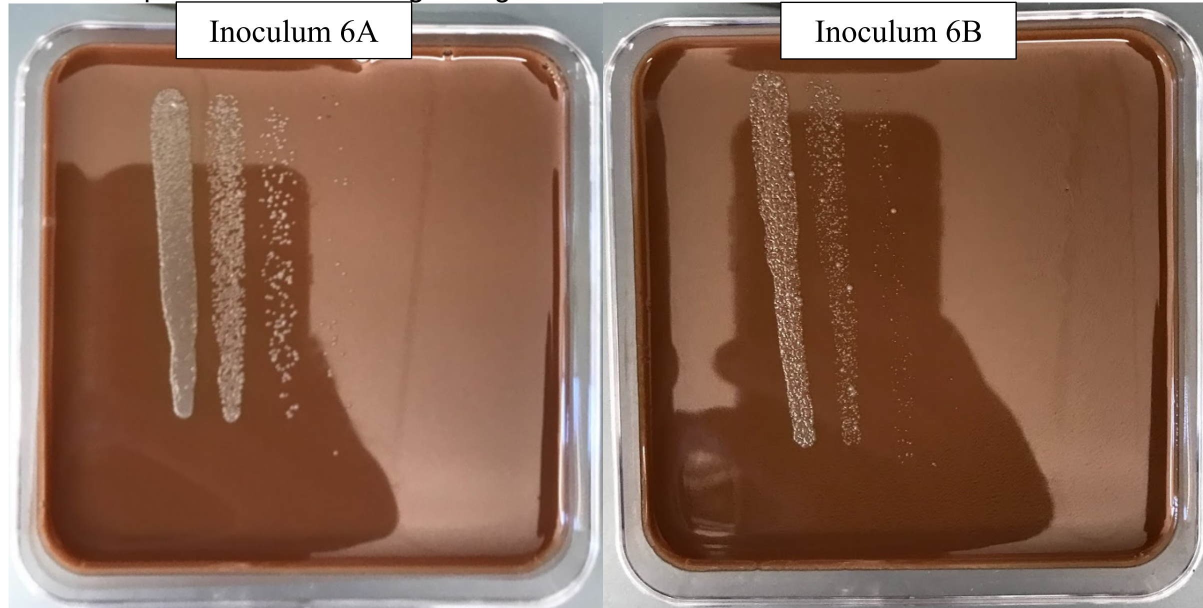
To Do:

1. Count inoculum plates
2. Count T=24 plates
3. Set up another macrophage experiment
    a. Streak strains
    b. Seed macrophage
    c. Continue macrophage propagation

Results and Data:

Macrophage assay results seem inconsistent. This is frustrating.

Specific examples:
The same samples plated on two different plates have different appearances. This is quite clear in the case of the inoculum. For example, see the plates below, which represent inoculums from sample 6. On one plate, the colonies have grown to a clearly visible size and are fairly homogenous in appearance. Counting individual colonies is straightforward. However, the colonies on the other plate have grown to varying sizes; most are quite small still but there are a few large outliers. These cells came from the same samples and have been growing the same amount of time in the incubator.



Given that it's the exactly the same sample it seems like it must have something to do with the plate??

Note that one of the sample 6 and one of the sample 5 inoculums have this issue- maybe one of the inoculums was contaminated and I switched the plates? This would be consistent with the appearance of these plates and a simple explanation.

However, with respect to the T=24 plates, there are similar issues. For example, plates with sample 2-2 (∆pmrA∆priM) have 56 clearly countable colonies in the second dilution on one plate, but no visible colonies on the second plate at all! Some hazes sometimes.

Finally, there seems to be growth of all the ∆pmrA cells from the original KMLFT37.1 and KMLFT37.2 stocks- at least to some extent. (Note that all the cells from sample 4, KMLFT37.2 from KR lab, are too small to count but there appears to be growth).

Yet there does not appear to be any growth on the ∆pmrA cells that JW made (KRLVS40). Is this real or due to some odd plate issue??

Inoculum (not likely to change, plates had 3 days at 37°C)

Inoculum				
	Replicate	2	3	4
LVS	1A	TMTC	TMTC	29
	1B	TMTC	TMTC	12
KMLFT49.1	2A	TMTC	TMTC	22
	2B	TMTC	TMTC	30
KMLFT37.1 from KR lab	3A	TMTC	TMTC	19
	3B	TMTC	TMTC	17
KMLFT37.2 from KR lab	4A	TMTC	TMTC	23
	4B	TMTC	TMTC	20
KMLFT37.1 from SD lab	5A	TMTC	TMTC	26
	5B	TMTC	TMTC	?
KMLFT37.2 from SD lab	6A	TMTC	TMTC	23
	6B	TMTC	TMTC	?
KRLVS40.1	7A	TMTC	TMTC	27
	7B	TMTC	TMTC	32
JCLVS106.1	8A	TMTC	TMTC	39
	8B	TMTC	TMTC	19
Dilution Factor		0.1	0.01	0.001

Strain	CFU/well	St dev	MOI
LVS (master stock)	1.03E+05	6.01E+04	4.9
KMLFT49.1	1.30E+05	2.83E+04	6.2
KMLFT37.1 from KR lab	9.00E+04	7.07E+03	4.3
KMLFT37.2 from KR lab	1.08E+05	1.06E+04	5.1
KMLFT37.1 from SD lab	1.30E+05	#DIV/0!	6.2
KMLFT37.2 from SD lab	1.15E+05	#DIV/0!	5.5
KRLVS40.1	1.48E+05	1.77E+04	7.0
JCLVS106.1	1.45E+05	7.07E+04	6.9

MOIs all very close- good!

T=24. will need to check this again tomorrow before numbers are final:

		Track Plate 1			Track Plate 2					
	Plate	1	2	3	1	2	3	Dilution factor counted	Average Cells	CFU per well
LVS	1A	TMTC	TMTC	**30**	TMTC	TMTC	**14**	0.01	22	4.40E+04
	1B	TMTC	TMTC	**25**	TMTC	TMTC	**20**	0.01	23	4.50E+04
KMLFT49.1	2A	TMTC	**49**		TMTC	**48**		0.1	49	9.70E+03
	2B	**0**			TMTC	**56**		0.1	56	1.12E+04
KMLFT37.1 from KR lab	3A	TMTC	**52**		TMTC	**50**		0.1	51	1.02E+04
	3B	TMTC	**62**		TMTC	**59**		0.1	61	1.21E+04
KMLFT37.2 from KR lab	4A	**0**			**0**			1	0	0.00E+00
	4B	**0**			**0**			1	0	0.00E+00
KMLFT37.1 from SD lab	5A	TMTC	**88**		TMTC	**100**		0.1	94	1.88E+04
	5B	**0**			**0**			1	0	0.00E+00
KMLFT37.2 from SD lab	6A	**0**			**0**			1	0	0.00E+00
	6B	TMTC	**42**		TMTC	**58**		0.1	50	1.00E+04
KRLVS40.1	7A	**0**			**0**			1	0	0.00E+00
	7B	**0**			**0**			1	0	0.00E+00
JCLVS106.1*	8A	**0**			**0**			1	0	0.00E+00
	8B	**0**			**0**			1	0	0.00E+00
Dilution Factor		1	0.1	0.01	1	0.1	0.01			
*Plated 50 ul on circular plate										

Next experiment- test:
LVS
ΔpmrA KMLFT37.1 (KR lab)
ΔpmrA KRLVS40.1
ΔpigR

Plate T=24 (all the Δ*pmrA* strains) on standard CHA and BD CHA.

This experiment will:
-address reproducibility
-determine if one or the other ΔpmrA strain is attenuated
-determine if the plate composition (salt conc?) influences growth after macrophage infection

Steps for after this experiment:
-WGRS for ΔpmrA strains that are attenuated vs grow in macrophage
-Plan an experiment to assess intramacrophage growth without cell lysis/ growth on plates.

Seed macrophage:

	1	2	3	4	5	6	7	8	9	10	11	12
A	LVS	LVS	LVS		KMLFT37.1	KMLFT37.1	KMLFT37.1		mac only			
B												
C												
D	KRLVS40	KRLVS40	KRLVS40		ΔpigR	ΔpigR	ΔpigR		LVS only			
E												
F												

G													
H													

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	9.95E+05
Volume stock needed (mL)	1.5
Volume media for dilution	10.5
Measured cells per ml, seeded	1.53E+05
Measured cells per well	2.30E+04

1st measurement				
52	65	36	46	
Average				49.75
Undiluted				99.5
Density				9.95E+05

2nd measurement				
9	6	5	7	
7	10	7	12	
12	8	5	4	
Average				7.66667
Undiluted				15.3333
Density				1.53E+05

Seed macrophage. Remove media from plate, add 10 mL media (DMEM + 10% FBS) to wash, remove. Add 10 mL media, scrape up all cells (P8). Transfer to 15 mL tube, spin for 5' at 900xg. While spinning, prepare 2x 96-well plate (mark off wells to seed), new 100 mm tissue culture dish, 1.5mL tubes to take samples to mix with Trypan blue, etc.

After spin, remove supernatant- this time, a much larger pellet! Resuspend in ~5 mL media with 5 mL pipet, then add ~10 ml more for ~15 mL total. Take 50 ul, mix with 50 ul trypan blue, check density using disposable hemocytometer. A good number of cells- make more diluted cells than necessary to make life easier. Mix 1.5 mL resuspended cells and 10.5 mL media for a total of 12 mL cells. Take 50 ul, mix with 50 ul trypan blue, then add 200 uL cells to each well indicated on the plates. Move 96-well plates to incubator and check actual density. A little high, but pretty close, $2.3x10^4$ cells per well.

To continue propagating J774A.1 cells, mix 2.5 mL of the original resuspension with 7.5 mL media and plate in 5 x 100 mm dishes for P9 cells.

Tuesday, April 27, 2021

To Do:

1. ~~Count T=24 plates~~
2. ~~Infect macrophage~~
3. ~~Wash macrophage~~
4. ~~T=2~~
5. ~~Feed macrophage~~

Results and Data:

Prepared cells to infect macrophage:

Bacterial Calculations	Actual
MOI	5
Macrophage cells per well	2.30E+04
Volume bacteria to add (mL)	0.05
Bacterial density needed (cells/mL)	2.30E+06
Cells/mL per OD600	5.81E+09
OD needed for given density	0.00040
Resuspend to	0.040
Final MOI 5, dilute 1:100	0.00040

			For final vol 1.3 mL at 0.04			
Number	Strain	Resuspend cells to (OD600)	Cells (uL)	Media (uL)	OD600	Volume to dilute in 1 mL (usually 1:100)
1	LVS	1.52	34.2	1265.8	0.048	9.38
2	KMLFT37.1 from KR lab	2.27	22.9	1277.1	0.053	8.49

3	KRLVS40.1	1.93	26.9	1273.1	0.051	8.82
4	JCLVS106.1	2.00	26.0	1274.0	0.045	10.00

Repeat replacement of media in macrophage plates with fresh media, as last expt.
Add 50 uL of each inoculum.
Transfer 200 ul inoculum to 96-well plate, serially dilute 1:10 to 10^{-4}. Plate 10 ul each dilution on track plates 2x.

Infected cells at ~10:15 AM.

Wash: At 12:15 PM, wash wells 2x with 1x PBS. Then add 200 uL of DMEMF with 10 ug/mL gentamycin (made stock of 8 mL DMEMF with 1.6 ul of 50 mg/mL gentamycin). Put plate back in the incubator until tomorrow.

T=2: At 2:15 PM, remove one 96-well plate. Wash wells 2x with 1x PBS, add 200 uL 1% saponin in 1x PBS for 30'. Plate 50 uL each well 2x on round plates.

Look back at plates from 4/22 assay:
Inoculum plates
One from 5- maybe contaminated? Put back at 37° and see if it looks like FT
One from 6 (the one with 15 colonies)- looks mixed. Take with grain of salt.

T=24 plates
Sample 2-2- one plate has 55 colonies at dilution 2, the other has 0 (mixup??) (ΔpmrAΔpriM)
Sample 3-1 – one plate has really smallish colonies (the one with 56 colonies)
Sample 4-1 – one plate has really small colonies (the one with 34 colonies), while 4-2 doesn't have any colonies at all.
Colonies on plate 5-2 very small.
On plates from KRLVS40, only one has any colonies at all, on the first dilution track: 37 small colonies. No colonies on the other plates (??!)

Started a 5 mL O/N culture of XL1 Blue cells.

Wednesday, April 28, 2021

To Do:

1. ~~T=24 (12:15 PM)~~
2. ~~Feed macrophage / freeze macrophage?~~
3. ~~Check on plates from last mac experiment~~

Results and Data:

Bad headache in morning, can't do *E. coli* experiment.

At 12:15 PM, washed cells 2x with PBS, lysed in 200 uL 1% saponin in 1x PBS for 30'. Mixed and transferred samples with strains 1 – 3 to top of new 96-well plate. Serially diluted 1:10 to 10^{-5}. Mixed wells with strain 4 in original plate, plated 50 uL twice on round plates. Go back to serially-diluted samples and plate 10 ul each on track plates 2x on CHA from Alpha Biosciences and 2x on CHA from BD. Put plates @37°C

Have 5 plates of very dense P9 macrophage. Scrape them all up, check density, passage some, freeze the remainder. Specifically, removed spent media, washed with 5 mL fresh media, added 5 mL fresh media, scraped up cells and combined in two 15 mL conicals. Spin down 5' at 900xg in Clements lab

centrifuge. Remove media, resuspend in ~10 mL each conical (there was a clump I couldn't quite get resuspended- spilled some cells and just discarded the clump!), combined the two tubes into one 50 mL conical, checked density. One hemocytometer count yielded: 98, 82, 78, 77 -> 83.75 *2 = 167.5x10^4 cells / mL = 1.68x10^6 cells / mL. Use 3 mL to seed 3 new 100 mm dishes - combined with 7 mL DMEMF- and put the P10 cells in TC incubator. Have 9 mL cells left over. Add 1 mL DMSO, mix, and aliquot into 10 cryotubes. Put tubes in freezing container and moved to -80°C O/N.

Checked on last macrophage experiment plates (last time!). New notes:
On plate 2-2 (ΔpmrAΔpriM) plate that seemed to have zero colonies, 5 colonies apparenet in dilution 1 (the other plate of well 2-2 had 55 colonies in dilution 2!)

Still no visible colonies from either 4-2 plate (KMLFT37.2 from KR lab).
Still no visible colonies from either 6-1 plate (KMLFT37.2 from SD lab).
Still no visible colonies on the one 7-1 plate with no colonies (KRLVS40).
On 7-2 plates, one still has no visible colonies but the other has 31 in dilution 1 that were too small to see until today (sooo small before!)

Could the issue be that the gentamicin is not effective???

Thursday, April 29, 2021

To Do:

1. ~~Pull inoculum plates (4/27 plates)~~
2. Move P9 cells from -80°C to liquid nitrogen
3. ~~Seed plates for next macrophage experiment~~

Results and Data:

Seed macrophage. Have 3 plates of reasonably dense macrophage. Remove media from plates, add ~6 mL media (DMEM + 10% FBS) to wash, remove. Add 10 mL media, scrape up all cells (P10). Transfer to 2x 15 mL tube, spin for 5' at 900xg.

While spinning, prepare 2x 12-well plate (will seed all wells), new 100 mm tissue culture dish, 1.5mL tubes to take samples to mix with Trypan blue, etc.

After spin, remove supernatant (decent pellets). Resuspend both with 5 mL pipet, then more media. Take 50 ul, mix with 50 ul trypan blue, check density using disposable hemocytometer.

A good number of cells- make more diluted cells than necessary to make life easier. Need 24 ml of cells, make up a total of 35 in a 50 mL conical tube. Mix 5.2 mL resuspended cells and 29.8 mL media for a total of 35 mL cells. Take 50 ul, mix with 50 ul trypan blue, check one grid on hemocytometer. Based on that one measurement, there are about 3.25x10^5 cells / mL, which is close to the 2.97x10^5 desired. Aliquot cells to all the wells (1 mL each), transfer plate to 37°C incubator, and then take more measurements to assess actual cell density. Pretty close, 2.95x10^5 cells per ml (and also per well!).

Plated some P10 cells into new dish just in case, but did not continue with maintaining them.

12-well plate setup:

	1	2	3	4
A	LVS	ΔpmrA	ΔpmrAΔpriM	ΔpigR
B	LVS	ΔpmrA	ΔpmrAΔpriM	ΔpigR
C	LVS	ΔpmrA	ΔpmrAΔpriM	ΔpigR

All mixed 1:1 with LVS Tn7::GFP

Macrophage Calculations	12 well
Surface area (cm^2)	3.8
Cells per well	2.97E+05

Volume to plate (mL)	1
Density needed (cells/mL)	2.97E+05
Total volume needed (mL)	35

Measured cells per ml	2.01E+06
Volume stock needed (mL)	5.2
Volume media for dilution	29.8
Measured cells per ml, seeded	2.95E+05
Measured cells per well	2.95E+05

1st measurement				
112	103	87	99	
Average				100.25
Undiluted				200.5
Density				2.01E+06

2nd measurement				
17	22	10	16	
13	12	17	6	
11	21	13	19	
Average				14.75
Undiluted				29.5
Density				2.95E+05

Keep inoculum plates in incubator one more day- the colonies are still pretty small.

Friday, April 30, 2021

To Do:

1. ~~Infect macrophage~~
2. ~~Wash macrophage~~
3. ~~T=2~~

Results and Data:

Prepared cells to infect macrophage. Note that this is a bit of a different setup, because the goal is to mix LVS Tn7::GFP at a 1:1 ratio with each strain. So the final MOI for each strain will be ~5, with a total MOI of ~10, still in a 50 uL volume. Resuspend all 5 strains and prepare 5 dilutions but mix the strain of interest with the GFP strain when making the final inoculum dilutions.

Bacterial Calculations

MOI (combined strains)	10
Macrophage cells per well	2.95E+05
Volume bacteria to add (mL)	0.05
Bacterial density needed (cells/mL)	5.90E+07
Cells/mL per OD600	5.81E+09
OD needed for given density	0.01015
Resuspend to	0.2
Final MOI **each strain 5**, dilute 1:40	0.005

			For final vol 1.4 mL at 0.2			
Number	Strain	Resuspend cells to (OD600)	Cells (uL)	Media (uL)	Actual OD600	Need 0.005 of 2 strains in 1.3 mL
1	LVS	3.94	71.1	1328.9	0.184	35.3
2	LVS Δ*pmrA* KRLVS40	5.05	55.4	1344.6	0.221	29.4
3	LVS Δ*pmrA* Δ*priM* KMLFT49	2.93	95.6	1304.4	0.191	34.0
4	LVS Δ*pigR* JCVLS106	3.99	70.2	1329.8	0.211	30.8
5	LVS Tn7::GFP KMR113	5.41	51.8	1348.2	0.193	33.7

To prep the final inoculum dilution, first remove 33.7 uL from 1.3 mL media from all 4 tubes, then remove indicated amount for test strain (e.g., remove 35.3 from tube 1), then add the appropriate test strain, then add 33.7 ul of the Tn7::GFP strain to every tube.

Infect wells with 50 ul of the mixed inoculum. Dilute inoculums serially 1:10 in 96-well plate to 10^{-6} in 1x PBS and plate 2x each on CHA and CHA-Kan square plates. Spin remaining inoculum, remove supernatant, and freeze for later gDNA extraction (I hope there is enough- tiiinnny pellet!)

Infected cells at ~10:10 AM.

Wash: At 12:10 PM, wash wells 2x with 1.5 mL 1x PBS. Removed media with 1 mL pipette tip, then added PBS with 10 mL pipet, dripping into each well (not touching). Remove PBS from each column (each column has same strain) using 5 mL pipet. Drip in another ~1.5 mL 1x PBS using 10 mL pipet. Remove PBS from each column with 5 mL pipet again. Have 25 mL DMEMF + 10 ug/mL gentamicin (5 ul of the 50 mg/mL stock) prepared, add 1 mL to each well using 1 mL pipette tip. Put plates back in the incubator until tomorrow.

T=2: At 2:10 PM, remove one 12-well plate. Wash wells 2x with 1x PBS (similar to above, only washing with 1 mL). Then add 1 mL TrypLE Express (Invitrogen) to try to dislodge the macrophage from the plate. Incubate 45 minutes in 37°C incubator. Checked adhesion after ~10 min, ~30 mins, and ~45 minutes. No apparent lifting of cells- not strong enough to dissociate J774A.1 cells from plate!?
Remove TrypLE Express to sterile 1.5 mL tubes. Add 0.8 mL 1% saponin in 1x PBS to each well. Spin down any dislodge cells in microfuge tubes (3' x max speed), add 200 uL saponin, transfer to appropriate well. Incubate plates with saponin for 30 minutes. Remove 50 uL x 4 to plate 2 hour timepoint – 2x on round CHA plates and 2x on round CHA-Kan plates. Transfer remaining material (about 800 uL) to sterile 1.5 mL tubes, spin, discard supernatant and freeze at -20°C to isolate gDNA later (pellets are visible).

Plate counting from 4/27 experiment:
T=2 plates:
All LVS plates (1-1 – 1-3) are countable. Some ΔpmrA KRLVS37.1 (2-1 – 2-3) are countable, but not all. All the ΔpmrA KRLVS40 cells (3-1 – 3-3) are too small. The ΔpigR JCLVS106 cells (4-1 – 4-3)are mostly countable.

Saturday, May 1, 2021

To Do:

1. ~~T=24~~
2. ~~Move P9 cells from -80°C to liquid nitrogen~~

Results and Data:

T=24: At 12:10 PM, remove last 12-well plate. Wash wells 2x with 1x PBS (as above). Add 1 mL 1% saponin in 1x PBS to each well. Incubate plates with saponin for 30 minutes. Remove 100 uL and transfer to 96-well plate. Dilute serially 1:10 in 96-well plate to 10^{-7} in 1x PBS. Transfer remaining material from 12-well plate (about 900 uL) to sterile 1.5 mL tubes, spin, discard supernatant and freeze at -20°C to isolate gDNA later (reasonable pellets!). Plate dilutions 2x each on CHA and CHA-Kan square plates.

Plate counting from 4/27 experiment:

Count LVS T=24 plates- the LVS were a reasonable size yesterday, left them out. Note that the cells grew significantly better on the BD plates (could have counted those yesterday, but not the Alpha Biosciences CHA). The others look like they can be counted, but leave them at room temp and count tomorrow.

Inoculum plates: sample 3 (KMRLVS40) have a mixed phenotype on one dilution plate (63 colonies on dilution 3; a few large colonies and many small ones- contamination??) and they are too small to count on the second plate. Not great.
On T=2 plates (from 4/27) that were left in the incubator another day
 can count cells on 3-1 plate (14, 17).
 2-1 = 0; 2-2 = 1; 2-3 = 0, 3; 4-3 = 0

Monday, May 3, 2021

To Do:

1. ~~Count plates~~

Results and Data:

Pull plates from 4/30 12-well plate experiment. Count inoculums- all look reasonable to count. Pull T=2 but leave at room temp- there are so many colonies! Should have used track plating. Will attempt to count tomorrow.

Inoculum: CHA plates										
	Replicate	**4**	**5**	**Dilution factor counted**	**Cells / mL**	**Average Cells / mL**	**St dev**	**CFU/well**	**St dev**	**MOI (based on # of seeded macrophage)**
LVS	1A	TMTC	**56**	0.0001	5.60E+07	5.90E+07	4.24E+06	2.95E+06	2.12E+05	10.0
	1B	TMTC	**62**	0.0001	6.20E+07					
ΔpmrA	2A	TMTC	**40**	0.0001	4.00E+07	2.90E+07	1.56E+07	1.45E+06	7.78E+05	4.9
	2B	TMTC	**18**	0.0001	1.80E+07					
ΔpmrA ΔpriM	3A	TMTC	**63**	0.0001	6.30E+07	5.75E+07	7.78E+06	2.88E+06	3.89E+05	9.7
	3B	TMTC	**52**	0.0001	5.20E+07					
ΔpigR	4A	TMTC	**52**	0.0001	5.20E+07	4.35E+07	1.20E+07	2.18E+06	6.01E+05	7.4
	4B	TMTC	**35**	0.0001	3.50E+07					
Dilution Factor		0.001	1E-04							

Inoculum: CHA-Kan plates										
	Replicate	**4**	**5**	**Dilution factor counted**	**Cells / mL**	**Average Cells / mL**	**St dev**	**CFU/well**	**St dev**	**MOI (based on # of seeded macrophage)**
LVS	1A	TMTC	**20**	0.0001	2.00E+07	2.05E+07	7.07E+05	1.03E+06	3.54E+04	3.5
	1B	TMTC	**21**	0.0001	2.10E+07					
ΔpmrA	2A	TMTC	**26**	0.0001	2.60E+07	2.30E+07	4.24E+06	1.15E+06	2.12E+05	3.9
	2B	TMTC	**20**	0.0001	2.00E+07					

ΔpmrA ΔpriM	3A	TMTC	**24**	0.0001	2.40E+07	2.10E+07	4.24E+06	1.05E+06	2.12E+05	3.6
	3B	TMTC	**18**	0.0001	1.80E+07					
ΔpigR	4A	TMTC	**28**	0.0001	2.80E+07	2.75E+07	7.07E+05	1.38E+06	3.54E+04	4.7
	4B	TMTC	**27**	0.0001	2.70E+07					
Dilution Factor		0.001	1E-04							

ALL CELLS

Strain	CFU/well	St dev
LVS	2.95E+06	2.12E+05
LVS Δ*pmrA*	1.45E+06	7.78E+05
LVS Δ*pmrA* Δ*priM*	2.88E+06	3.89E+05
LVS Δ*pigR*	2.18E+06	6.01E+05

KanR CELLS

Strain	CFU/well	St dev
LVS	1.03E+06	3.54E+04
LVS Δ*pmrA*	1.15E+06	2.12E+05
LVS Δ*pmrA* Δ*priM*	1.05E+06	2.12E+05
LVS Δ*pigR*	1.38E+06	3.54E+04

Ratio KanS to KanR

Strain	CFU/well
LVS	2.9
LVS Δ*pmrA*	1.3
LVS Δ*pmrA* Δ*priM*	2.7
LVS Δ*pigR*	1.6

Ratios are not perfect but for cells that grow like wild-type (all but ΔpmrA) they are pretty good. Will be interested to see how the qPCR looks.

Tuesday, May 4, 2021

To Do:

1. ~~Count plates~~

Results and Data:

Count T=24 plates from 4/30 12 well plate experiment and start on the 2 hour timepoint plates.

Future To-Do

Move 1° LVS pKR10-1 into strain box

Bibliography

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