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January 2021

Sunday, January 17, 2020

To Do:

1. Prepare BHIc media +/- KCl
2. ~~Patch strains for T6SS assay~~
3. ~~Pull Hannah's plates from incubator~~

Results and Data:

Prepare BHIc:

Want to use Instant Pot, so make 250 mL BHIc (in 500 mL bottle) and 125 mL BHIc + 5% KCl (in 250 mL bottle)

250 mL BHIc:

- 9.25 g Brain Heart Infusion
- 0.25 g L-cysteine hydrochloride

125 mL BHIc + 5% KCl

- 4.63 g BHI
- 0.13 g L-cysteine hydrochloride
- 6.25 g KCl

Sterilize in Instant Pot for 30'

Streak out strains:

From Kira's plates struck to single colony on Friday 1/15: LVS, Tn7::rpsU1, Tn7::rpsU2, Tn7::rpsU3
Patched 3 colonies each

From single-use aliquot from JW, labeled 9-5: Δ pigR
Put at 37°C O/N (~1:30pm)

Monday, January 18, 2021

To Do:

1. Grow cells for T6SS assay
2. Harvest pellets and supernatant for T6SS assay

Results and Data:

Supplement BHIc (per 500mL): 5 mL 1 mg/mL β -NAD, 5 mL 1 mg/mL heme-histidine, 12.5 mL 20% glucose

250 mL:

- a. 2.5 mL 1 mg/mL β -NAD
- b. 2.5 mL 1 mg/mL heme-histidine
- c. 6.25 mL 20% glucose

125 mL:

- a. 1.25 mL 1 mg/mL β -NAD
- b. 1.25 mL 1 mg/mL heme-histidine
- c. 3.13 mL 20% glucose

Incubator was shut off overnight (!!! ☹), so patches are pitiful. Turn on incubator, wait 2 hours, try anyway.

Scrape up cells, resuspend in 350 μ L sBHlc:

Number	Strain	Measured OD (25 μ L in 475 μ L)	Resuspend in BHlc to:	For 8 mL at 0.1
1	LVS A	0.059	1.18	254.2
2	LVS B	0.074	1.48	202.7
3	LVS Tn7::rpsU1 A	0.031	0.62	483.9
4	LVS Tn7::rpsU1 B	0.097	1.94	154.6
5	LVS Tn7::rpsU2 A	0.036	0.72	416.7
6	LVS Tn7::rpsU2 B	0.093	1.86	161.3
7	LVS Tn7::rpsU3 A	0.077	1.54	194.8
8	LVS Tn7::rpsU3 B	0.03	0.6	500.0
9	LVS Δ pigR A	0.173	3.46	86.7
10	LVS Δ pigR B	0.173	3.46	86.7

Not enough cells by a long shot! Need to prep 2 tubes for each. Lower the volume (to 5mL), and the starting OD (to 0.07), combine the two replicates (so the experiment will have singletons rather than duplicates).

Number	Strain	Calculated OD600	For 5 mL at 0.07
1	LVS	1.33	263.2
2	LVS Tn7::rpsU1	1.28	273.4
3	LVS Tn7::rpsU2	1.29	271.3
4	LVS Tn7::rpsU3	1.07	327.1
5	LVS Δ pigR	3.46	101.2

Not great, but should work.

Media	Number	11:15am
sBHlc	1A	0.079
	2A	0.097
	3A	0.079
	4A	0.098
	5A	0.108
sBHlc + 5% KCl	1B	0.092
	2B	0.102
	3B	0.09
	4B	0.069
	5B	0.096

Seems like my calculated OD600s were off. At least most of the ODs are higher than expected!

Media	Number	11:15am	4:30pm
sBHlc	1A	0.079	0.666
	2A	0.097	0.555
	3A	0.079	0.6
	4A	0.098	0.557
	5A	0.108	0.498
sBHlc + 5% KCl	1B	0.092	0.168
	2B	0.102	0.13
	3B	0.09	0.223
	4B	0.069	0.113
	5B	0.096	0.143

Darn it! Came back too late. After ~ 5 hours, ODs are way too high- there would be too much lysis in the control samples to really be able to assess secretion. ☹ Will need to repeat. Put plates with pitiful patches back in the incubator.

Tuesday, January 19, 2021

To Do:

1. ~~Try re-growing cultures for T6SS assay with Tn7 strains~~
2. ~~Run old samples on gel for WB~~
3. ~~Transfer to nitrocellulose, block O/N~~

Results and Data:

Perform WB on T6SS samples from last March (200312).

Run 2x 4-12% gels, 1x MOPs buffer, ~115 V for ~70 minutes (a little under-run, but better than too far!)

Lane	volume	Contents			
1	10	prestained ladder			
2	-	IR blot: 2 uL 1:10 Biorad; Chemi: 10 uL 1:10 Magic Mark			
3	10	1x SLB			
		Sample #	Strain	Media	Component
4	10	1	LVS	sBHlc	pellet
5	10	2	$\Delta pigR$		
6	10	3	$\Delta rpsU2$		
7	10	1	LVS		sup
8	10	2	$\Delta pigR$		
9	10	3	$\Delta rpsU2$		
10	10	4	LVS	sBHlc + 5% KCl	pellet
11	10	5	$\Delta pigR$		
12	10	6	$\Delta rpsU2$		
13	10	4	LVS		sup
14	10	5	$\Delta pigR$		
15	10	6	$\Delta rpsU2$		

Transfer to nitrocellulose membrane, 60 minutes at 20 V.

Note that Hannah cuts off the prestained ladder after cutting membrane post-transfer.

Put

Blocking

Block the membrane with Li-Cor Odyssey Blocking Buffer (PBS) diluted 1:5 in PBS. Use ~25 mL or enough to cover the membrane and rock for 1 hour **or overnight** at room temperature. DO NOT add any detergents (Surfact-Amps, Tween, SDS) to the blocking buffer.

Store diluted blocking buffer at 4C.

Planning for next steps:

Will take ~4-5 hours before cells are ready (want OD600 ~0.3). Expect to pellet around 3:45. Check OD600s around 2pm to be sure cells are growing appropriately.

1. Transfer 1 mL of each culture to 1 mL tube
2. Transfer ~1.8 mL of each culture into 2 mL tube
3. Pellet cells by centrifuging 6 min at max speed
4. Remove 900 uL of supernatant from 1 mL tube with 1 mL pipette, transfer to syringe with 2 micron filter. Remove last ~100 uL with 200 uL pipette and discard.
5. Remove ~1.7 mL supernatant from 2 mL tube, add to syringe with filter
6. Filter-sterilize supernatant, keeping as much will fit in 2 mL tube

7. Add 1x SLB to cell pellets in 1 mL tube, , using the formula below to calculate the volume SLB for each pellet. Mix by pipetting when adding SLB.

$$\text{OD600} \times \text{volume cells (uL)} = \text{volume SLB to add (uL)}$$
 Example: $0.2 \times 1000 \text{ uL} = 200 \text{ uL SLB}$
8. Heat pellet samples for 5-10' at 95°C, store at -20°C
9. Transfer 1.7 mL of sterilized supernatant to new 2 mL tube.
10. Add 170 uL 100% TCA (for 1/10 volume), mix by inversion, put at 4°C to precipitate

Thursday, January 21, 2021

To Do:

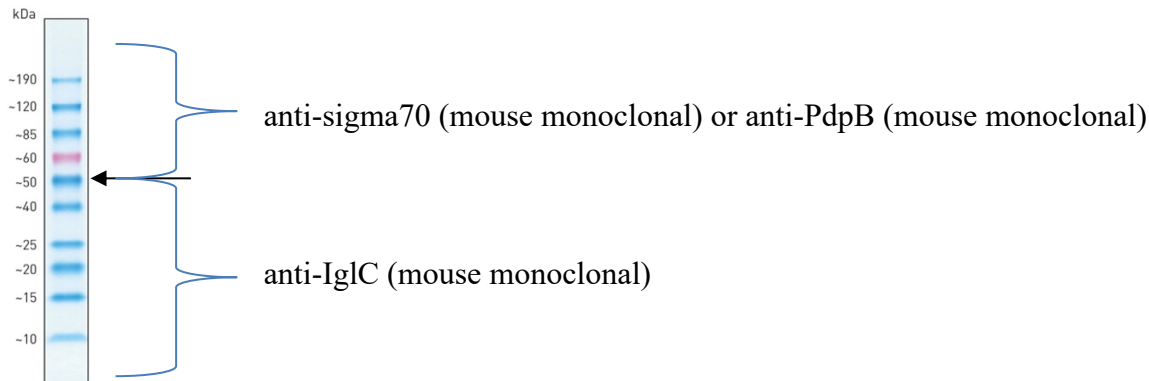
1. ~~Run WB on T6SS samples from March 2020~~
2. Finish precipitating supernatant samples from 1/19
3. Decide how to move forward with T6SS WBs (IR or chemi?)

Results and Data:

Perform WB

Probing

Will be probing for sigma70 (67.7 kDa) and IgIC (22 kDa). Cut membranes at ~ fifth blue band from top (one below the pink band).



IR blot:

Make more 1:5 diluted Blocking Buffer in PBS
 200 mL Odyssey blocking buffer (927-40000)
 100 mL 10x PBS
 700 mL ddiH2O
 Store at 4°C

Incubate in 15 mL blocking buffer (no detergent):
~~Top~~ ~~anti-sigma70~~ 1:500 x uL in 15 mL

** Essentially no anti-sigma70 antibody!!!

For this experiment, try anti-PdpB

Top	anti-PdpB	127.5 kDa	1:250 HT dilution from 210112, use for 3 rd time
Bottom	anti-IgIC	22 kDa	1:100 HT dilution from 200929, use for 5 th time

Chemiluminescent blot:

Incubate in 15 mL blocking buffer (with detergent):

Top	anti-sigma70	67.7 kDa	1:250	HT dilution from 200115
Bottom	anti-IgC	22 kDa	1:750	HT dilution from 200115

Incubate chemi blot for ~2 hours at room temp

Incubate IR blot for ~ 1.5 hours at room temp

Save 1° antibodies, store at -20°C

Wash blots:

IR:	PBS-T	1x PBS with (2.5 mL)(20%)/(500mL) = 0.1% NP-40
Chemi:	TBS-T	1x TBS with 0.1% NP-40

4 x 10' each

Re-block:

IR	Use 25 mL diluted blocking buffer
Chemi	Use 10 mL blocking buffer

Both incubated about 15 minutes before 2° antibody addition.

Incubate in 2° antibodies. All 1° antibodies are mouse monoclonal, so use anti-mouse.

IR: 1:10,000 anti-mouse 800CW (1.5 uL in 15 mL PBS-T)

Chemi 1:5,000 anti-mouse (2 ul in 10 mL blocking buffer with NP-40, same buffer used for re-block)

Incubated about 1 hr 15 minutes in 2° antibodies. Note that the smaller portion of the blot seemed to be on the side of the IR blot box- may not have been rocking in antibody!!!! ☹ We'll see how it goes...

Wash both blots 4x10' in their respective wash buffers

Keep chemi blot in wash buffer while washing IR blot 2x 10' more in 1x PBS *without* detergent.

Go to darkroom in CBLS to image chemi blot. Needed to use femto kit to image the sigma70 signal well. Use Odyssey in INBRE core to image IR blot.

!!! Should have cut the pre-stained ladder off before incubating in 1°! Much of the signal is from that ladder.

Results:

Chemi: good detection of IgC and decent detection of sigma70 in pellets from cells grown without KCl, less great from cells grown with KCl. No detection of secretion.

Plan: Try to re-do, with new BHI supplemented with fresh supplements. Test:

Expt

A	β-NAD fresh	Heme-His fresh	glucose fresh
B	β-NAD old	Heme-His fresh	glucose fresh
C	β-NAD fresh	Heme-His old	glucose fresh

Only with LVS and ΔpigR, check by chemi WB

Prep 40 mL each media. Make 125 mL more BH1c, BH1c + 5% KCl

125 mL BHlc + / - 5% KCl

- 4.63 g BHI
- 0.13 g L-cysteine hydrochloride
- + / - 6.25 g KCl

Supplement (per 40 mL media):

- 400 uL 1 mg/mL β-NAD
- 400 uL 1 mg/mL heme-histidine
- 1 mL 20% glucose

Friday, January 29, 2021

To Do:

1. Start WB on T6SS samples from HT

Results and Data:

Hannah grew cells to test the influence of media components on T6SS activity. 😊

A	b-NAD fresh	Heme-His fresh	glucose fresh
B	b-NAD old	Heme-His fresh	glucose fresh
C	b-NAD fresh	Heme-His old	glucose fresh

Tube	Strain	KCl?	Media (A,B,C)
1	LVS	No	A
2	<i>ΔpigR</i>	No	A
3	LVS	No	B
4	<i>ΔpigR</i>	No	B
5	LVS	No	C
6	<i>ΔpigR</i>	No	C
7	LVS	Yes	A
8	<i>ΔpigR</i>	Yes	A
9	LVS	Yes	B
10	<i>ΔpigR</i>	Yes	B
11	LVS	Yes	C
12	<i>ΔpigR</i>	Yes	C

Run 2x 4-12% gels, 1x MOPs buffer, ~150 V for ~45 minutes (a bit short again- increased to 200V at the end, but should be fine).

Gel 1:

Lane	volume	Contents			
1	10	prestained ladder			
2	-	10 uL 1:10 Magic Mark			
3	10	1x SLB			
		Sample #	Strain	Media	Component
4	10	1	LVS	sBHlc vA	pellet
5	10	2	<i>ΔpigR</i>	vA	
6	10	3	LVS	sBHlc vB	
7	10	4	<i>ΔpigR</i>	vB	
8	10	5	LVS	sBHlc vC	
9	10	6	<i>ΔpigR</i>	vC	
					Component
10	10	1	LVS	sBHlc vA	supernatant
11	10	2	<i>ΔpigR</i>	vA	
12	10	3	LVS	sBHlc vB	
13	10	4	<i>ΔpigR</i>	vB	
14	10	5	LVS	sBHlc vC	
15	10	6	<i>ΔpigR</i>	vC	

Gel 2:

Lane	volume	Contents			
1	10	prestained ladder			
2	-	10 uL 1:10 Magic Mark			
3	10	1x SLB			
		Sample #	Strain	Media	Component
4	10	7	LVS	sBHlc vA	pellet
5	10	8	<i>ΔpigR</i>	+ 5% KCl	
6	10	9	LVS	sBHlc vB	
7	10	10	<i>ΔpigR</i>	+ 5% KCl	
8	10	11	LVS	sBHlc vC	
9	10	12	<i>ΔpigR</i>	+ 5% KCl	
					Component
10	10	7	LVS	sBHlc vA	supernatant
11	10	8	<i>ΔpigR</i>	+ 5% KCl	
12	10	9	LVS	sBHlc vB	
13	10	10	<i>ΔpigR</i>	+ 5% KCl	
14	10	11	LVS	sBHlc vC	
15	10	12	<i>ΔpigR</i>	+ 5% KCl	

**The supernatant samples for gel 2 were floating up out of the wells! Did the best I could, but had to load some more than once and definitely didn't get all the sample in. ☹️ Next time, resuspend the sup samples in 2x SLB.

Transfer to nitrocellulose membrane, 60 minutes at 20 V.
Mark blot one with diagonal cut at corner, blot 2 with square cut

Wednesday, February 3, 2021

To Do:

1. Finish WB on HT's T6SS samples

Results and Data:

Cut membrane and start incubating in 1° antibodies- reuse from last time.

Top	anti-sigma70	67.7 kDa	1:250	HT dilution from 200115	Third use?
Bottom	anti-IgIC	22 kDa	1:750	HT dilution from 200115	Third use?

Incubate for ~1 hour

1. Pellet supernatant samples by centrifuging at 4°C for 30' at max speed
2. Remove supernatant (pellet will not be very visible)
3. Add 1 mL 100% ice-cold ethanol
4. Spin at 4°C for 30' at max speed
5. Remove ethanol, let pellets air-dry completely
6. Add 1x SLB, normalizing for cell density in original sample. Use the following formula:
 - a. $(35 \text{ uL}) / (\text{lowest density sample OD600}) \times (\text{current sample OD600}) = \text{uL 1x SLB to add}$
7. Heat pellet samples for 5-10' at 95°C. Store samples at -20°C if not using immediately.

Plan for WB:

4-12% gel with MOPS buffer

Check IgIC (22 kDa) and sigma70 (67.7 kDa) (tul4 similar to IgIC in size?).