Table of Contents

November 2019

Thursday, November 8, 2019

To Do:

1. ~~Pour plates for Fno~~
2. ~~Streak out Fno~~
3. ~~Streak out LVS, ΔchaC, ΔchaC_S for viability assay~~

Results and Data:

Culturing Fno

Modified Thayer-Martin Agar Plates (mTM)

Combine 7.2 g GC agar base and 100 mL ddiH2O in flask, mix completely
Autoclave at 121°C for 15 min
Warm 100 mL 2% hemoglobin to 55°C
Cool GC base media to 55°C
Add 10 mL iso-vitalex and hemoglobin to GC agar base
Pour plates

When I made these plates in the Dove lab, I used GC agar base (purchased or found?). I forgot to order this media type, so in searching to borrow some, I found some GC medium base.

GC agar base recipe (Remel):

REAGENTS (CLASSICAL FORMULAE)*

Casein Peptone	7.5 g	Corn Starch	1.0 g
Meat Peptone	7.5 g	Monopotassium Phosphate	1.0 g
Sodium Chloride	5.0 g	Agar	10.0 g
Dipotassium Phosphate	4.0 g	Demineralized Water	10000.0 ml

pH 7.2 ± 0.2 @ 25°C

*Adjusted as required to meet performance standards.

GC medium base (Difco):

Difco™ GC Medium Base

Approximate Formula* Per Liter

Proteose Peptone No. 3	15.0	g
Corn Starch	1.0	g
Dipotassium Phosphate	4.0	g
Monopotassium Phosphate	1.0	g
Sodium Chloride	5.0	g
Agar	10.0	g

*Adjusted and/or supplemented as required to meet performance criteria.

These medias are similar and ultimately I need to add a vial of Iso-vitalext to either, so move forward with the GC medium base we have on hand.

Note that according to a pdf from Difco found at this site:
http://legacy.bd.com/europe/regulatory/Assets/IFU/Difco_BBL/228950.pdf
the media we are making can be described as chocolate agar:

Chocolate Agar

1. Suspend 7.2 g of GC base medium in 100 mL of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes. Cool to 45-50°C.
4. Aseptically add 100 mL Hemoglobin Solution 2%.
5. Aseptically add 2 mL Supplement B or Supplement VX or **IsoVitaleX** Enrichment.
6. Dispense into sterile Petri dishes or tubes as desired.
7. Test samples of the finished product for performance using stable, typical control cultures.

Poured mTM plates. Note that the GC agar base was extremely cloudy even after coming out of the autoclave. I didn't boil the media pre-autoclaving, so maybe I should try next time.
Struck out one plate with our wild-type *F. noatunensis* strain (LADL 07-285A) and one plate with *F. noatunensis* Δ*dotU*, put in Nelson lab incubator at 27°C.

chaC
Struck out new ΔchaC strain (KRLVS42.1), LVS, and ΔchaC suppressor (8-3) for viability assay

Friday, November 8, 2019

To Do:

1. ~~Electroporations~~
2. ~~Check on Fno plates~~
3. ~~Cell viability experiment~~
4. ~~Plate electroporations~~

Results and Data:

Strain construction: electroporations

Electroporation	Strain	EC cells	Plasmid	Volume	Time constant
72	KRLVS26.1 LVS *katG* mod RBS	HT 6.30.19	pKR11	5 uL	4.5
73	KRLVS26.1 LVS *katG* mod RBS	HT 6.30.19	pKR11	5 uL	4.5
74	KRLVS40.1 Δ*pmrA*	JW 10.29.19	pKL75	5 uL	Arc
75	KRLVS40.1 Δ*pmrA*	JW 10.29.19	pKL75	5 uL	4.8
76	LVS	KMR 8.16.19	pKL02	5 uL	4.1
77	LVS	KMR 8.16.19	pKL02	5 uL	arc

Plasmid sources: pKL02 – from my old stocks, others from lab working stocks

Use new EP cuvette for even-numbered electroporations
Recovered in 4 mL MHB at 9:10am - ~3:45 (~6.5 hours), plated each on 2 CHA-Kan(50) plates.

chaC
Check viable CFU per OD600:

Number	Strain	Measured OD600	Actual OD600	Volume for 0.03 in 1 mL (uL)	Final OD600
1	LVS		1.45	20.69	0.030
2	LVS ΔchaC		3.48	8.62	0.030
3	LVS ΔchaC_S		1.94	15.46	0.032

All cells resuspended in RPMI with sodium bicarb, no glucose (from MMR lab), diluted serially to 10^{-5} in 1X PBS, plated 10 uL undiluted plus all 5 dilutions on track plates, put at 37°C.

Fno: some growth visible, still early.

Saturday, November 9, 2019

To Do:

1. Check on Fno plates
2. ~~Design and order primers to confirm ΔchaC_S SNP~~

Results and Data:

Primer Design
KROL278 Ck_capA_SNP_F
5' taaatgataaattgaatcataaatcaaagc 3'

KROL279 Ck_capA_SNP_R
5' cggttaattctgttgctgttgatttc 3'

Together will amplify 299 bp, SNP is 113 bp from F primer and 129 bp from R primer

Tuesday, November 12, 2019

To Do:

1. ~~Count colonies for viability assay~~
2. ~~Check on electroporation plates~~
3. ~~Check on Fno plates~~
4. ~~Start ΔpmrA strains for RNA~~
5. ~~Start Fno strains for RNA~~

Results and Data:

chaC

On Monday, (JW or HT?) put CFU plates at 4°C. Count today:

		Track Plate 1		Track Plate 2						
Number		5	6	5	6	Dilution factor counted	Average Cells	CFU per mL	OD600	CFU per mL per OD600
1	LVS	TMTC	14	TMTC	16	0.00001	15	1.50E+08	0.03	5.00E+09
2	LVS ΔchaC	TMTC	6	TMTC	10	0.00001	8	8.00E+07	0.03	2.67E+09
3	LVS ΔchaC_S	27	-	22	-	0.0001	25	2.45E+07	0.032	7.66E+08

The colonies on plate 3 looked smaller and more variable than on 1 and 2. Colonies on plates 2 looked like those on plates 1, larger. Did I screw up the strains? Or is the RPMI media I used very different from the DMEM? Will need to re-do, again.

Strain construction: electroporation results

Electroporation	Strain	Plasmid	Colonies (plate 1, plate 2)
72	KRLVS26.1 LVS *katG* mod RBS	pKR11 pEXΔrpsU2	4, 3
73	KRLVS26.1 LVS *katG* mod RBS	pKR11 pEXΔrpsU2	1, contaminated
74	KRLVS40.1 Δ*pmrA*	pKL75 pEX_FTL_0702-V	0, contaminated
75	KRLVS40.1 Δ*pmrA*	pKL75 pEX_FTL_0702-V	1, 0
76	LVS	pKL02 pEX_FTL_1743-VSVG	1 small plus contaminated, contaminated
77	LVS	pKL02 pEX_FTL_1743-VSVG	Contaminated, tiny?

Patched onto CHA-Kan
KRLVS26.1 pKR11 EP72-1 -> EP72-6
KRLVS26.1 pKR11 EP73-1
KRLVS40.1 pKL75 EP75-1 (struck out to single colony bc worried about carrying over contamination from plate)
LVS pKL02 EP76-1, EP77-1 -> EP77-3

RNA sample preparation

Mueller-Hinton (II) cation-adjusted supplementation:

Per 500 mL:
Add 5 mL of 10% glucose
Add 10 mL Iso-vitalex

Jamie struck out ΔpmrA (old) and ΔpmrA(new) for me yesterday. Started Fno cultures from plates struck out on Thursday (!). Dilute cells into 25 mL of media, aliquot 6 mL per tube.

Number	Strain	Measured OD600	Actual OD600	Volume for 0.005 or 0.05 in 25 mL (uL)	Measured OD600
1	LVS ΔpmrA (old)	0.182	1.82	69	-
2	LVS ΔpmrA (new)	0.154	1.54	81	-
3	F. noatunensis WT	0.363	3.63	344	0.053
4	F. noatunensis ΔdotU	0.36	3.6	347	0.051

Put LVS at 37°C at 7:50, Fno at 27°C at 8:05

Wednesday, November 13, 2019

To Do:

1. ~~Count colonies for viability assay~~
2. ~~Re-streak strains~~
    a. ~~Fno~~
    b. ~~LVS ΔpmrAs (JW)~~
3. ~~Check on strains patched yesterday,~~
4. ~~Freeze down potential 1° integrants~~
    a. ~~Plate on sucrose~~

Results and Data:

Checked on LVS strains first: grew WAY too fast! Must be contaminated. Discard. Make new MH broth. Note that the Fno strains did not grow too fast, suggesting that media is fine; this indicates that the glucose is not the contaminated reagent.

			10:30AM
Number	Strain	Measured OD600 (1:2 or undiluted)	Actual OD600
1A	LVS ΔpmrA (old)	0.672	1.344
1B		0.655	1.31
1C		0.635	1.27
2A	LVS ΔpmrA (new)	0.675	1.35
2B		0.653	1.306
2C		0.599	1.198
3A	F. noatunensis WT	0.139	0.139
3B		0.166	0.166
3C		0.145	0.145
4A	F. noatunensis ΔdotU	0.141	0.141
4B		0.135	0.135
4C		0.142	0.142

Not sure the Fno are growing ideally. Check again in a few hours and in the meantime, streak out more Fno strains.

			10:30AM	2:10PM
Number	**Strain**	**Measured OD600 (1:2)**	**Actual OD600**	
3A	F. noatunensis WT	0.139	0.139	0.241
3B		0.166	0.166	
3C		0.145	0.145	
4A	F. noatunensis ΔdotU	0.141	0.141	0.244
4B		0.135	0.135	
4C		0.142	0.142	

Time elapsed between Fno measurements: 220 minutes
Calculated doubling time for
3A: 279 minutes
4A: 280 minutes

While I haven't done a great growth curve for Fno before, I'm pretty sure a ~4.5 hr doubling is a bit too long. ☹ Discard cultures and try to grow overnight tomorrow.

Strain construction: LVS katG_modRBS Δ*rpsU2*
Patches from EP 72 and 73 grew well (KRLVS26.1 LVS katG_modRBS with pKR11, pEXΔ*rpsU2*)
Freeze down 72-1, 72-2, 72-3, 73-1

Sucrose selection
Resuspend small amount of 72-1, 72-2, 73-1 in 300 uL 1X PBS, dilute 1:100 then serially 1:10 until 10^{-6}. Plate 50 uL of 10^{-2} – 10^{-6} on sucrose, plus 50 uL 10^{-6} on CHA. Put plates at 37°C

Strain construction: Δ*pmrA priM*-V and LVS pKL02 control
Had struck out single potential ΔpmrA priM-V colony to single colony yesterday. Patch did not grow very densely, but maybe because I struck out to single colony and the first streaks were bigger and thinner? Not sure, keep incubating.

None of the potential LVS pKL02 colonies patched well- probably not real. ☹

chaC
PCR to check for capA SNP:

RxnA: KROL278 & KROL279

Sample Number	Sample	Expected size
1	1: LVS	299
2	2: ΔchaC	299
3	3: ΔchaC_S	299
4	LVS gDNA	299
5	-DNA	no product

RxnB: P794 & P795

Sample Number	Sample	Expected size
1	1: LVS	2243
2	2: ΔchaC	1460
3	3: ΔchaC_S	1460
4	LVS gDNA	2243
5	-DNA	no product

				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	6
ddiH2O			12.4	74.4
PrimeSTAR GXL Buffer	5x	1x	4	24
dNTPs	2.5 mM	0.2 mM	1.6	9.6
F	10 uM	0.3 uM	0.6	3.6
R	10 uM	0.3 uM	0.6	3.6
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2.4
		Total volume	20	117.6

Set up 2X reaction A, so enough to purify, run on gel, and send for sequence confirmation.
Use program STN1 with 2:30 extension, run overnight

Thursday, November 14, 2019

To Do:

1. ~~Check on ΔpmrA priM-V patch~~
2. ~~Start ΔpmrA strains for RNA~~
3. ~~Isolate RNA from ΔpmrA strains~~
4. ~~Check PCR on gel~~
    a. ~~PCR purify~~
    b. ~~Send to sequence~~
5. ~~Start Fno strains O/N for RNA~~

Results and Data:

RNA

Start cultures of Δ*pmrA* old and new. Plate with Δ*pmrA*(old) didn't have many cells- scraped *everything*, had slightly less than 900 uL total, added it all to the 21 mL. Looks good to me!

					9:30AM
Number	**Strain**	**Measured OD600 (1:50)**	**Actual OD600**	**Volume for 0.08 in 21 mL (uL)**	**Measured OD600**
1	LVS ΔpmrA (old)	0.037	1.85	908	0.073
2	LVS ΔpmrA (new)	0.106	5.3	317	0.072

Check OD at 12:15PM and at 2:PM:

Elapsed time		165	105
	9:30AM	12:15	2PM
1A	0.073	0.258	0.711
1B	0.073	0.253	0.703
1C	0.073	0.252	0.711
2A	0.072	0.107	0.139
2B	0.072	0.107	0.143
2C	0.072	0.108	0.14

Average	1	92.2473882	71.5318976
Doubling times	2	288.38858	271.649926

Crap. There's no way that those ΔpmrA(old) samples are really LVS- the doubling time is waaay too fast!

Not sure where contamination would have come from. Worried it might have been on the plate. Try to start O/N cultures from plates struck out on 11/11, and Jamie will streak out more cells just in case.

chaC

Don't have time to check PCR on gel before sending to sequence. PCR-purify 40 uL of samples 1A, 2A, 3A, elute in 35 uL 0.1x EB. Nanodrop results:

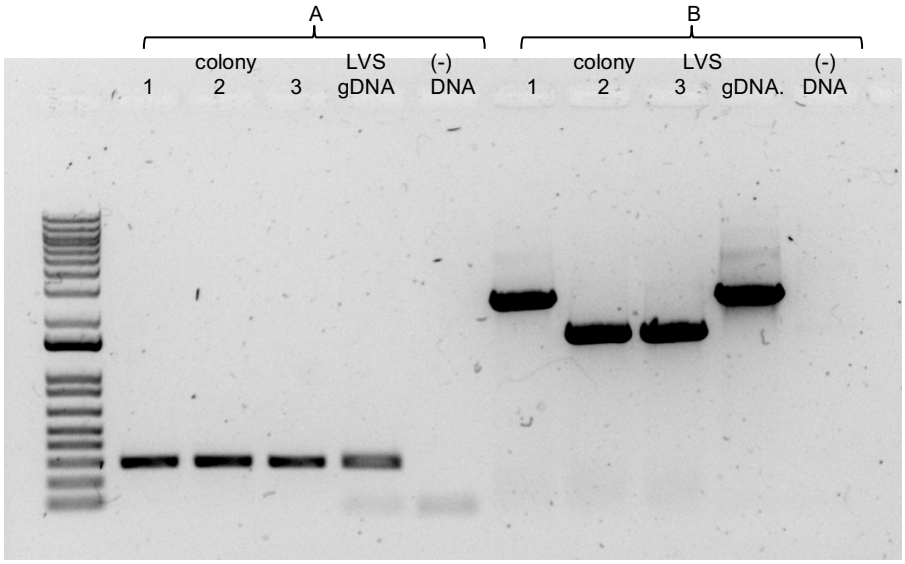
Sample ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.
1A	11/14/19	9:19 AM	40.6	0.812	0.436	1.86	2.86	50	230	0.284
2A	11/14/19	9:20 AM	37.68	0.754	0.391	1.93	2.87	50	230	0.263
3A	11/14/19	9:21 AM	41.27	0.825	0.444	1.86	2.79	50	230	0.295

Looks good! Have to dilute 1:10 for sequencing, just adjust to use 2 uL of 1:10 for all the rxns

Sample number	Template Type	Template Name	Primer Name[a]	Template Size (bases)	Template Stock Conc (ng/µl)	**PCR** template:	**PCR** template:	Volume **H_2O needed**
						ng needed =	Volume =	
						(A ÷ 100) × 2.5	**(C ÷ B)µl**	**(12 less D or E - 2.56)µl**
KMR1	PCR	capA-1	KROL278	300	3.768	7.50	2.00	7.44
KMR2	PCR	capA-2	KROL278	300	3.768	7.50	2.00	7.44
KMR3	PCR	capA-3	KROL278	300	3.768	7.50	2.00	7.44

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

Also run 8ul of purified (A 1-3) and non-purified reactions (A 4,5, B 1-5) on a gel:



Looks great! Hopefully the sequencing turns out well.

Strain construction

ΔpmrA PriM-V

Patch looks like it's grown up! Certainly more than the LVS pKL02 patches, so let's check by PCR.

When I made this strain previously, I used the following setup:
P439 & P35
25 uL reaction with 2x OneTaq, 0.75 uL each 10 uM primer, 2 uL lysate
Product size = 688 bp

Try this reaction with OneTaq and with PrimeStar

Don't have P439! ☹ Try using P697 instead. Product size should be 906 bp

Samples:

Sample Number	Sample	Expected size
1	LVS patch	none
2	EP 75-1 ΔpmrA PriM-V patch	906
3	LVS gDNA	none
4	-DNA	no product

PrimeStar reactions

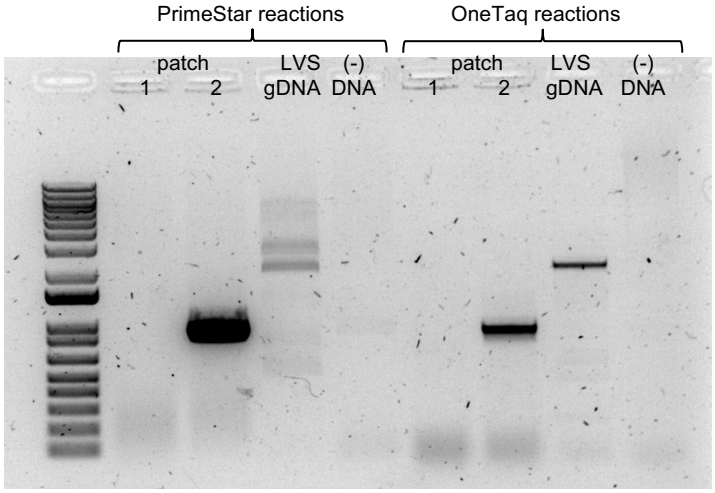
Total reaction volume	25
Total number of reactions	4

Component	Stock concentration	Final concentration	1 rxn volume	Factor 5
ddiH2O			15.5	77.5
PrimeSTAR GXL Buffer	5x	1x	5	25
dNTPs	2.5 mM	0.2 mM	2	10
P439	10 uM	0.3 uM	0.75	3.75
P35	10 uM	0.3 uM	0.75	3.75
template	100 ng/ul	2 ng/ul	0.5	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.5	2.5
		Total volume	25	122.5

OneTaq reactions

Total number of reactions	4

Component	Stock concentration	Final concentration	1 rxn volume	Factor 5
ddiH2O			17.375	86.875
OneTaq Quick-load buffer	5x	1x	5	25
dNTPs	10 mM	0.2 mM	0.5	2.5
P439	10 uM	0.3 uM	0.75	3.75
P35	10 uM	0.3 uM	0.75	3.75
template	100 ng/ul	2 ng/ul	0.5	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.125	0.625
		Total volume	25	122.5



Looks great! See correct product from patch 75-1, but not from LVS.
Freeze down two vials of patch- KRLVS56.1 = ΔpmrA FTL_0702-V (75-1)

RNA

Okay, try *again*.

F. noatunensis

Scrape up Fno cells and resuspend in MHB(II). Adjust to 0.05 and aliquot 3 x 6 mL for each strain. Then aliquot 600 uL into 5.4 mL MHB(II) for a final OD of 0.005. Incubate O/N at 27°C.

Number	Strain	Measured OD600	Actual OD600	Volume for 0.05 in 21 mL (uL)	Measured OD600
3 A-C	F. noatunensis WT	0.249	4.98	211	0.042
	adjustment		4.98	21	0.047
4 A-C	F. noatunensis ΔdotU	0.333	6.66	158	0.047

Tubes A-C had starting OD of 0.047, tubes D-F should have 0.0047.
Put in incubator at 4:15PM

LVS

Start at OD600 of 0.004 and leave O/N. Worried b/c these cultures are from old plates- asked JW to re-streak strains for me again, just in case I need to start them in the morning.

Number	Strain	Measured OD600	Actual OD600	Volume for 0.4 in 1.4 mL (uL)	Volume MHB	Measured OD600	Volume for 0.004 in 21 mL (uL)
1	LVS ΔpmrA (old), plated 11/11	0.098	0.98	571	829	0.398	211
2	LVS ΔpmrA (new), plated 11/11	0.178	1.78	315	1085	0.37	227
LVS	LVS, plated 11/13	0.15	1.5	373	1027	0.369	228

Included LVS as growth control.
Put at 37°C shaking at 4:50PM.

Friday, November 15, 2019

To Do:

1. ~~Isolate RNA from ΔpmrA strains~~
2. ~~Isolate RNA from Fno strains~~
3. ~~Check on sucrose plates (ΔrpsU2)~~
4. ~~Check chaC_S sequencing results~~

Results and Data:

RNA: LVS

WT LVS has grown quite well overnight, but both ΔpmrA strains look like they have barely grown at all- poor growth from old plates. ☹
Set up new cultures (AGAIN) from plates Jamie struck out last night at a starting OD of 0.08 to isolate RNA today. Started at 7:15 AM.

Number	Strain	Measured OD600	Actual OD600	Volume for 0.08 in 21 mL (uL)	Measured OD600
1	LVS ΔpmrA (old), plated 11/14	0.242	4.84	347	0.079
2	LVS ΔpmrA (new), plated 11/14	0.275	5.5	305	0.08

RNA: *F. noatunensis*

The cultures started at 0.05 last night have lots of growth- probably too much? Check ODs.

	6:55AM (diluted 1:2)
3A	0.393
3B	0.397
3C	0.399
4A	0.355
4B	0.349
4C	0.386

Yup. Those cultures are at around 0.8- too dense!

Check on the Fno cultures started at 0.005:

	7:20AM
3D	0.113
3E	0.112
3F	0.112
4D	0.09
4E	0.093
4F	0.094

LVS data

Minutes elapsed	0	145			145			95			20	80	
Time	7:15AM	9:40AM			12:05PM			1:40PM			2PM	3PM	
		Measured OD	Actual OD	doubling time	Measured OD	Actual OD	doubling time	Measured OD	Actual OD	doubling time	Actual OD	Actual OD	doubling time
1A	0.079	0.024	0.096	519.1	0.061	0.122	422.1	0.072	0.144	399.8		0.175	286.3
1B	0.079	0.025	0.1	429.2	0.06	0.12	554.9	0.072	0.144	363.6		0.175	286.3
1C	0.079	0.025	0.1	429.2	0.063	0.126	437.8	0.076	0.152	353.3		0.174	412.9
2A	0.08	0.031	0.124	230.9	0.109	0.218	179.3	0.147	0.294	221.6	0.315		202.3
2B	0.08	0.031	0.124	230.9	0.108	0.216	182.3	0.15	0.3	201.8	0.338		117.0
2C	0.08	0.03	0.12	249.5	0.106	0.212	177.8	0.149	0.298	194.7	0.32		195.9
Averages													
ΔpmrA (old)	0.079		0.099	459.2		0.123	471.6		0.147	372.2		0.175	286.3
ΔpmrA (new)	0.08		0.123	237.1		0.215	179.8		0.297	206.0	0.324		171.7

Isolated RNA at 2PM for samples 2A-2C, at 3PM for 1A-1C

***F. noatunensis* data**

Minutes elapsed	0	130			150			110		
Time	7:20AM	9:30 AM			12PM			1:50PM		
	measured OD	measured OD	Actual OD	Doubling time	measured OD	Actual OD	Doubling time	measured OD	Actual OD	Doubling time
3D	0.113	0.041	0.164	243.5	0.128	0.256	235.0	0.176	0.352	241.0
3E	0.112	0.042	0.168	223.7	0.129	0.258	244.0	0.176	0.352	247.1
3F	0.112	0.042	0.168	223.7	0.129	0.258	244.0	0.18	0.36	230.4
4D	0.09	0.037	0.148	182.4	0.106	0.212	291.2	0.15	0.3	221.1
4E	0.093	0.033	0.132	259.0	0.108	0.216	212.5	0.157	0.314	205.2
4F	0.094	0.035	0.14	227.7	0.11	0.22	231.6	0.157	0.314	215.7
Averages										
WT	0.112		0.167	230.3		0.257	241.0		0.355	239.5
ΔdotU	0.092		0.140	223.0		0.216	245.1		0.309	214.0

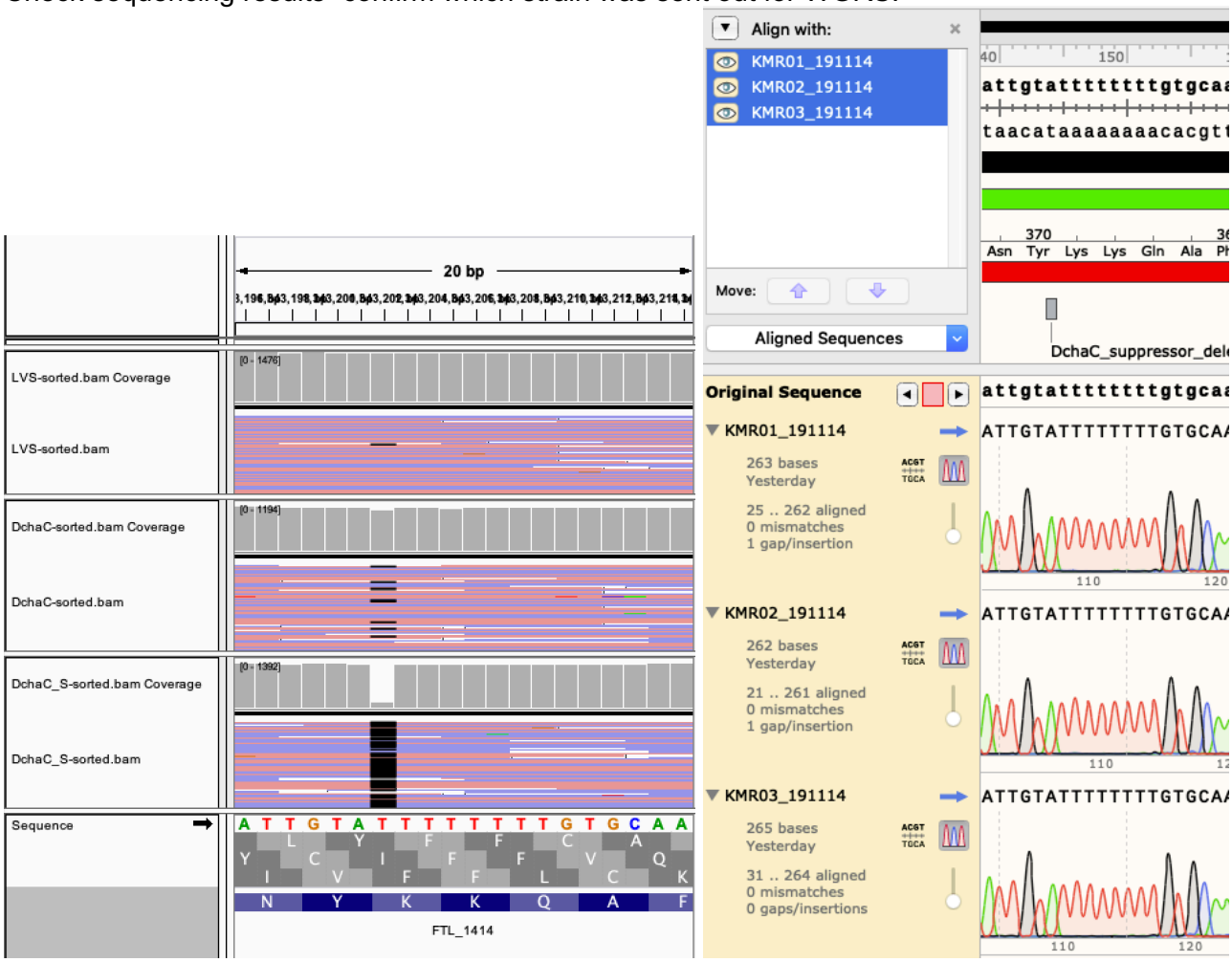
Isolated RNA at 1:50 PM for 3D-3F, 4D-4F

RNA isolation (day 1)

Harvest 3.4 mL cells by spinning max speed 3 minutes (room temp)
    2x 800 uL in 1.5 mL tubes, 2x 900 uL in 2 mL tubes
Resuspend pellets in 1 mL Trizol
Incubate 60°C for 10 minutes
Centrifuge 10 minutes max speed at 4°C
Transfer supernant to new tube
Add 200 uL chloroform and shake 15 seconds
Let sit 5 min at room temp
    Note: could see phase separation in F. noatunensis samples but not LVS after the 5' incubation
Spin at 12,000 x g at 4°C for 15 min
Transfer 500 uL aqueous phase (upper layer) to RNase-free new tube
Add 1 mL 100% ethanol
Put at -20°C to precipitate O/N (over weekend)

chaC

Check sequencing results- confirm which strain was sent out for WGRS!



Note that I clearly annotated the suppressor deletion spot in SnapGene incorrectly.
1 = LVS
2 = ΔchaC
3 = ΔchaC_S 8-3

Regardless, this "suppressor" strain does not have the mutation revealed by WGRS. Will need to check the other suppressor strains I froze down.

Monday, November 18, 2019

To Do:

1. ~~RNA~~
    a. ~~Resuspend RNA~~
    b. ~~DNase-treat~~
    c. ~~Re-purify and precipitate O/N~~
2. ~~Streak cells from sucrose plates (ΔrpsU2)~~
3. ~~Streak out other chaC_S strains~~

Results and Data:

Strain construction: LVS katG_modRBS Δ*rpsU2*
Checked on sucrose plates- larger and smaller colonies present on sucrose plates (-2 -> -4 for each 1° integrant), colonies on CHA only (-6) are quite large and overgrown. No colonies on -6 sucrose plates, so selection worked quite well.
Patched out 12 potential ΔrpsU colonies from each 1° integrant (72-1, 72-2, 73-1) to CHA, cross-patched to CHA-Kan. Check by PCR tomorrow.

RNA

RNA isolation (day 2)

Spin tubes 4°C for 30 mins, max speed to pellet nucleic acid
Pipet off the supernatant and wash pellet with 1 mL 75% EtOH
Centrifuge samples max-g x 5 min @ 4°C
Pipet off the supernatant and wash pellet with 1 mL 75% EtOH (total 2 washes)
Centrifuge samples max-g x 5 min @ 4°C
Pipet off 900uL of supernatant and briefly spin to collect residual EtOH from sides of tube.
Remove remaining EtOH and let pellet air dry for about 5 min or until it becomes slightly glassy.
Resuspend in 80 uL RNase-free water.
Check purity of nucleic acids on Nanodrop*
Add 10ul 10X DNase I buffer and 10ul of RNase free DNase I. Mix well.
Digest at 37 degrees for 1 hour.
Add 1ml Trizol, mix.
Add 200ul chloroform, mix.
Incubate 5 minutes at room temp.

> **As during initial purification, after the 5' incubation I can see separation of the two layers in the Fno samples but not the LVS samples**

Spin at 12,000 x g at 4°C for 15 min
Transfer ~500 uL aqueous phase (upper layer) to RNase-free new tube

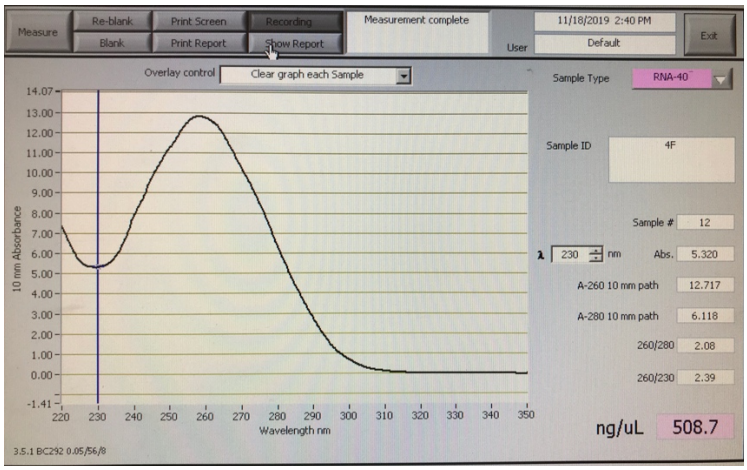
> **There is a significant amount of white interface in the LVS samples but not the Fno samples at this step. Recovered 500 uL of the LVS samples, but only 450 of the Fno samples because I was worried that it would be difficult to avoid pipetting up the organic layer. Turns out it probably would have been fine (the aqueous layer seems to have more volume in the Fno samples) but I opted to remain consistent.**

Add 1 mL 100% ethanol
Put at -20°C to precipitate O/N

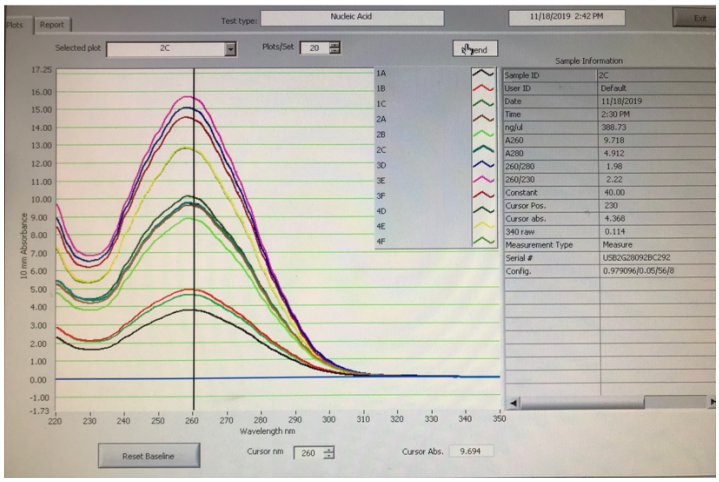
*After first precipitation, check quality / quantity before DNase treatment:

Sample ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1A	11/18/19	2:24 PM	150.96	3.774	1.922	1.96	2.34	40	230	1.612	0.044
1B	11/18/19	2:25 PM	196.01	4.9	2.467	1.99	2.31	40	230	2.118	0.06
1C	11/18/19	2:26 PM	185.08	4.627	2.338	1.98	2.24	40	230	2.066	0.059
2A	11/18/19	2:27 PM	383.44	9.586	4.839	1.98	2.29	40	230	4.195	0.097
2B	11/18/19	2:28 PM	353.3	8.833	4.454	1.98	2.33	40	230	3.797	0.091
2C	11/18/19	2:30 PM	388.73	9.718	4.912	1.98	2.22	40	230	4.368	0.114
3D	11/18/19	2:31 PM	599.81	14.995	7.231	2.07	2.3	40	230	6.52	0.19
3E	11/18/19	2:33 PM	624.84	15.621	7.484	2.09	2.29	40	230	6.827	0.2
3F	11/18/19	2:34 PM	577.34	14.434	6.922	2.09	2.33	40	230	6.192	0.206
4D	11/18/19	2:35 PM	402.88	10.072	5.019	2.01	2.28	40	230	4.416	0.127
4E	11/18/19	2:36 PM	510.27	12.757	6.168	2.07	2.37	40	230	5.384	0.206
4F	11/18/19	2:37 PM	508.7	12.717	6.118	2.08	2.39	40	230	5.32	0.161

Looks great! Note that the ΔpmrA (old) samples (1A – 1C) were the lowest OD600 and have the least RNA.
Example (sample 4F) screenshot:



Ah, on this screen I can see all the samples together!



Tuesday, November 19, 2019

To Do:

1. RNA
    a. ~~Resuspend RNA~~
    b. ~~Check on Nanodrop~~
    c. ~~Check on gel~~
    d. Give to MRamsey lab
2. ~~Check for ΔrpsU by PCR~~
3. Check for ΔchaC by PCR, purification, and sending to sequence

Results and Data:

Strain construction: LVS katG_modRBS Δ*rpsU2*

Check on plates- looks good, only 1 patch grew on Kan (72-1-7). 72-2-5 grew near some contamination, so skip, and 72-2-8 didn't grow.. Check first 8 of each set of patches by PCR (skipping 72-2-7, 72-2-5, 72-2-8).

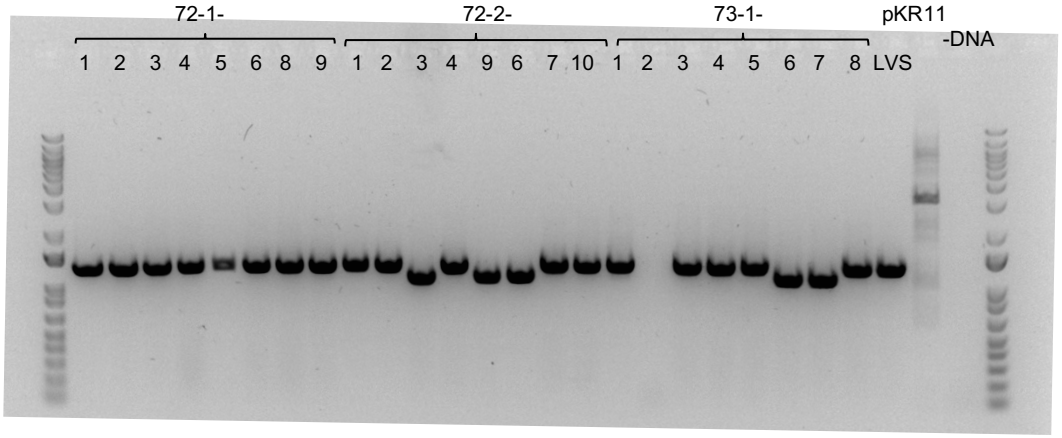
KROL147 & KROL148 = 1275 bp WT, 1095 bp = ΔrpsU2

Sample Number	Sample	Expected size	Sample Number	Sample	Expected size
1	patch 72-1-1	1275 or 1095	15	patch 72-2-7	1275 or 1095
2	patch 72-1-2	1275 or 1095	16	patch 72-2-10	1275 or 1095
3	patch 72-1-3	1275 or 1095	17	patch 73-1-1	1275 or 1095
4	patch 72-1-4	1275 or 1095	18	patch 73-1-2	1275 or 1095
5	patch 72-1-5	1275 or 1095	19	patch 73-1-3	1275 or 1095
6	patch 72-1-6	1275 or 1095	20	patch 73-1-4	1275 or 1095
7	patch 72-1-8	1275 or 1095	21	patch 73-1-5	1275 or 1095
8	patch 72-1-9	1275 or 1095	22	patch 73-1-6	1275 or 1095
9	patch 72-2-1	1275 or 1095	23	patch 73-1-7	1275 or 1095
10	patch 72-2-2	1275 or 1095	24	patch 73-1-8	1275 or 1095
11	patch 72-2-3	1275 or 1095	25	LVS gDNA	1275
12	patch 72-2-4	1275 or 1095	26	pKR11 (1:10)	no product
13	patch 72-2-9	1275 or 1095	27	-DNA	no product
14	patch 72-2-6	1275 or 1095			

Total reaction volume	20
Total number of reactions	27

Component	Stock concentration	Final concentration	1 rxn volume	Factor 29
ddiH2O			12.4	359.6
PrimeSTAR GXL Buffer	5x	1x	4	116
dNTPs	2.5 mM	0.2 mM	1.6	46.4
F	10 uM	0.3 uM	0.6	17.4
R	10 uM	0.3 uM	0.6	17.4
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	11.6
		Total volume	20	568.4

STN1 with 1:30 extension



Great, I've got some ΔrpsU strains! Streak out to single colony:
72-2-3, 72-2-6, 73-1-6, 73-1-7

RNA

RNA isolation (day 3)

Spin tubes 4°C for 30 mins, max speed to pellet nucleic acid
Pipet off the supernatant and wash pellet with 1 mL 75% EtOH
Centrifuge samples max-g x 5 min @ 4°C
Pipet off the supernatant and wash pellet with 1 mL 75% EtOH (total 2 washes)
Centrifuge samples max-g x 5 min @ 4°C
Pipet off 900uL of supernatant and briefly spin to collect residual EtOH from sides of tube.

Remove remaining EtOH and let pellet air dry for about 5 min or until it becomes slightly glassy. Resuspend in 100 uL RNase-free water.

Concerned about low RNA concentrations for LVS samples, so use 75 uL

Check for concentration and potential contamination of RNA by Nanodrop and integrity by agarose gel

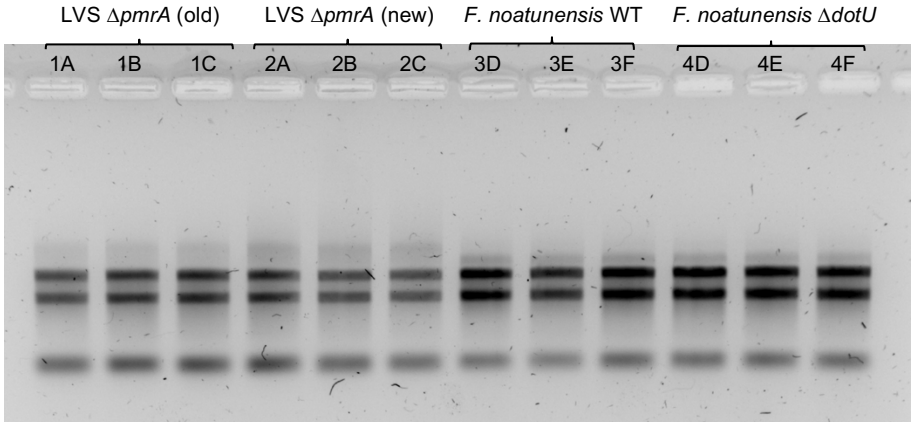
RNA still looks good by Nanodrop!

Sample ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1A	11/19/19	10:51 AM	119.75	2.994	1.574	1.9	2.52	40	230	1.186	0.052
1B	11/19/19	10:52 AM	109.36	2.734	1.446	1.89	2.52	40	230	1.086	0.028
1C	11/19/19	10:53 AM	97.92	2.448	1.292	1.89	2.49	40	230	0.982	0.023
2A	11/19/19	10:54 AM	236.65	5.916	3.009	1.97	2.48	40	230	2.387	0.044
2B	11/19/19	10:55 AM	256.49	6.412	3.28	1.95	2.53	40	230	2.53	0.045
2C	11/19/19	10:56 AM	290.7	7.267	3.709	1.96	2.46	40	230	2.956	0.063
3D	11/19/19	10:57 AM	344.58	8.615	4.409	1.95	2.38	40	230	3.613	0.104
3E	11/19/19	10:58 AM	350.47	8.762	4.496	1.95	2.42	40	230	3.62	0.098
3F	11/19/19	10:59 AM	324.83	8.121	4.141	1.96	2.42	40	230	3.352	0.098
4D	11/19/19	11:00 AM	274.48	6.862	3.528	1.95	2.45	40	230	2.796	0.29
4E	11/19/19	11:01 AM	289.85	7.246	3.72	1.95	2.45	40	230	2.96	0.081
4F	11/19/19	11:02 AM	284.46	7.111	3.629	1.96	2.43	40	230	2.93	0.09

Aliquot 1.5 ug to check integrity on agarose gel. Adjust so all samples have the same volume (15.3 uL) and add 2.5 uL of 6x purple dye from NEB (less likely to have nuclease contamination). Run on 1% agarose gel (standard agarose, TAE, etc).

Sample ID	Volume for 1.5 ug	water
1A	12.5	2.8
1B	13.7	1.6
1C	15.3	0.0
2A	6.3	9.0
2B	5.8	9.5
2C	5.2	10.1
3D	4.4	10.9
3E	4.3	11.0
3F	4.6	10.7
4D	5.5	9.8
4E	5.2	10.1
4F	5.3	10.0

Load on gel, run at ~80V.



Pick samples for RNA-Seq. Need to provide 100 ng in technical triplicate and extra for mixing in with 7 other strains for a mixed experiment (presumably a total of 100 ng in the entire mix)- about 350 ng total.

Provide 17.5 uL total (report 15 uL):

Tube label	Contents	[RNA] (ng/uL)	Volume (uL)	Total RNA (ng)	KMR sample number

KMR1	*Francisella tularensis* LVS Δ*pmrA* suppressor	109.4	15	1640	1B
KMR2	*Francisella tularensis* LVS Δ*pmrA*	256.5	15	3847	2B
KMR3	*Francisella noatunensis* LADL 07-285A	350.5	15	5257	3E
KMR4	*Francisella noatunensis* LADL 07-285A Δ*dotU*	289.9	15	4348	4E

chaC

PCR to check frozen chaC suppressor strains

RxnA: KROL278 & KROL279

Sample Number	Sample	Expected size
1	LVS	299
2	ΔchaC	299
3	ΔchaC_S 8-3	299
4	ΔchaC_S 8-8	299
5	ΔchaC_S 8-9	299
6	chaC supp gDNA	299
7	LVS gDNA	299
8	-DNA	no product

Total reaction volume	20
Total number of reactions	7

RxnB: P794 & P795

Sample Number	Sample	Expected size
1	LVS	2243
2	ΔchaC	1460
3	ΔchaC_S 8-3	1460
4	ΔchaC_S 8-8	1460
5	ΔchaC_S 8-9	1460
6	LVS gDNA	2243
7	-DNA	no product

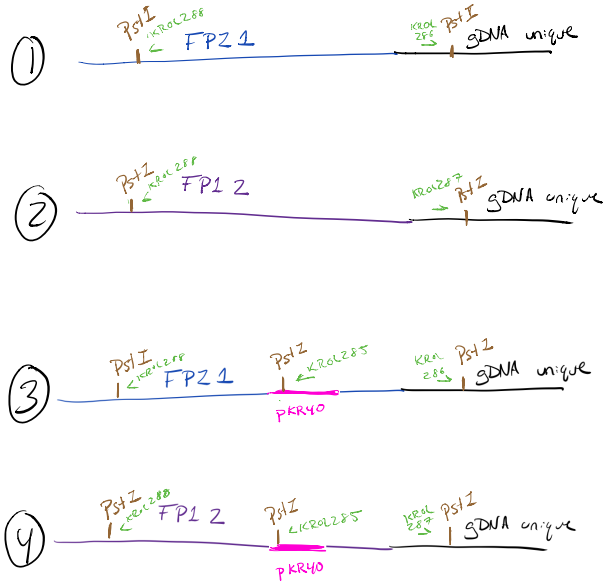
Component	Stock concentration	Final concentration	1 rxn volume	Factor 8
ddiH2O			12.4	99.2
PrimeSTAR GXL Buffer	5x	1x	4	32
dNTPs	2.5 mM	0.2 mM	1.6	12.8
F	10 uM	0.3 uM	0.6	4.8
R	10 uM	0.3 uM	0.6	4.8
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	3.2
		Total volume	20	156.8

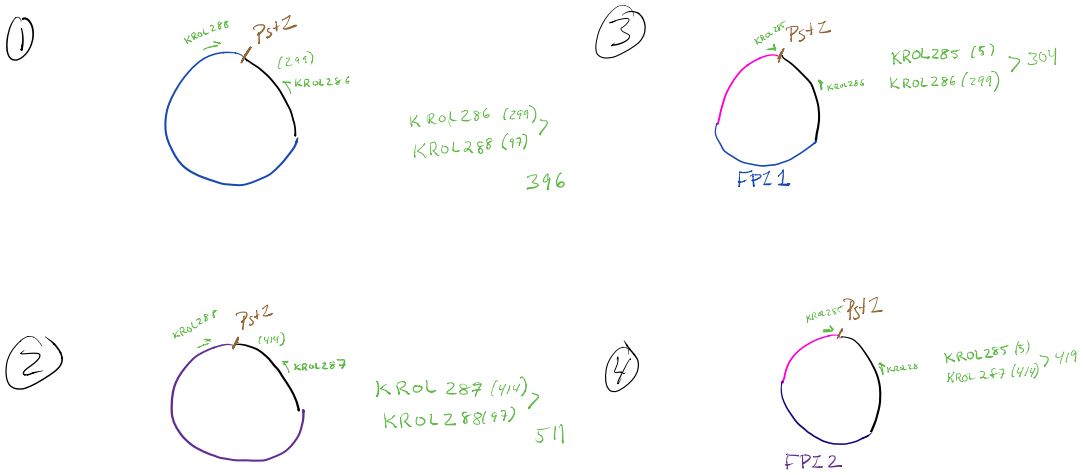
STN1 with 2:30 extension, run O/N

Inverse PCR plan and primer design

Digest gDNA with PstI. This will create a fragment that encompasses from FTL_0108/FTL_1154 (encompassing pdpB) to outside the FPI/ rRNA operon. For FPI1, the PstI site is in FTL_0131 and for FPI2, the PstI site is in trmE/FTL_1178.

Set up ligations so that intramolecular ligations are preferred. Then PCR with primers designed to amplify across ligation junctions such that they can be diagnostic for an insertion.





Thursday, November 21, 2019

To Do:

1. ~~Check on ΔrpsU patches~~
2. Check for ΔchaC by ~~PCR, purification~~, and sending to sequence

Results and Data:

Strain construction: LVS katG_modRBS Δ*rpsU2*
Check on single colonies. Still small- patch out tomorrow

chaC
PCR-purify rxns A1 – A7, elute in 35 uL 0.1x EB. Run on gel tomorrow.

Friday, November 22, 2019

To Do:

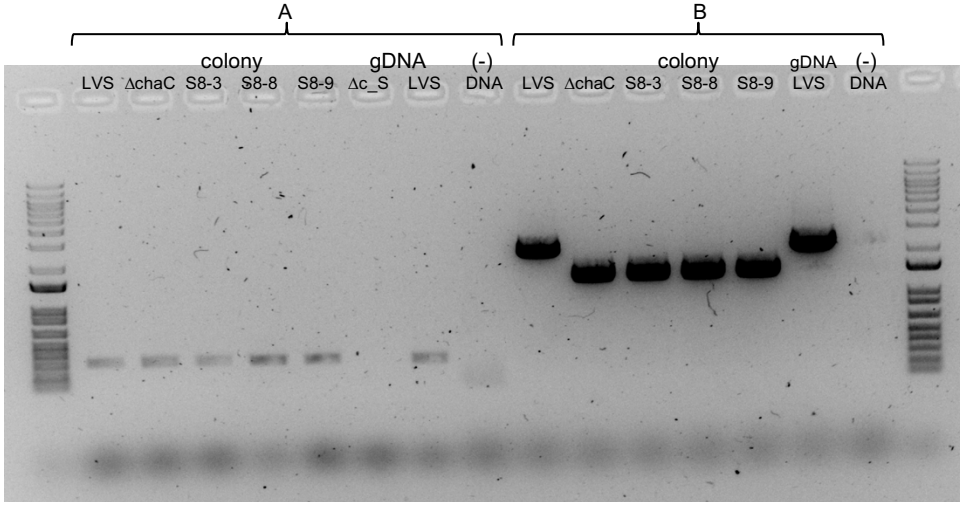
1. ~~Check on ΔrpsU patches~~
2. ~~Check for ΔchaC by PCR, purification, and sending to sequence~~

Results and Data:

Strain construction: LVS katG_modRBS Δ*rpsU2*
Patched 3 colonies (A-C) from each potential strain, 72-2-3, 72-2-6, 73-1-6, 73-1-7, to CHA

chaC

Run PCR from 11/19 on gel



Strange- looks like there wasn't amplification from the suppressor gDNA! Try sending to sequence anyway. All the chaC phenotypes are as expected, which is good.

Check concentrations by Nanodrop:

Sample ID	Contents	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1	LVS patch	**17.68**	0.354	0.18	1.97	1.8	50	230	0.196	0.043
2	ΔchaC patch	**17.87**	0.357	0.179	1.99	1.89	50	230	0.189	0.024
3	ΔchaC_S 8-3 patch	**13.39**	0.268	0.15	1.78	1.81	50	230	0.148	0.015
4	ΔchaC_S 8-8 patch	**20.11**	0.402	0.21	1.91	1.66	50	230	0.242	0.018
5	ΔchaC_S 8-9 patch	**17.13**	0.343	0.194	1.77	1.83	50	230	0.187	0.022
6	chaC supp gDNA	**4.22**	0.084	0.055	1.54	0.86	50	230	0.098	0.034
7	LVS gDNA	**14.88**	0.298	0.158	1.88	1.75	50	230	0.17	0.027

Use a 1:5 dilution to send for sequencing, for all samples except #6.

Sample number	Template Type	Template Name	Primer Name[a]	Template Size (bp)	Template Stock Conc (ng/µl)	PCR template:	PCR template:	Volume H_2O needed
						ng needed =	Volume =	
						(A ÷ 100) × 2.5	**(C ÷ B)µl**	**(12 less D or E - 2.56)µl**
KMR1	PCR	capA-1	KROL278	300	3.536	7.5	2.1	7.3
KMR2	PCR	capA-2	KROL278	300	3.574	7.5	2.1	7.3
KMR3	PCR	capA-3	KROL278	300	2.678	7.5	2.8	6.6
KMR4	PCR	capA-4	KROL278	300	4.022	7.5	1.9	7.6
KMR5	PCR	capA-5	KROL278	300	3.426	7.5	2.2	7.3
KMR6	PCR	capA-6	KROL278	300	4.22	7.5	1.8	7.7
KMR7	PCR	capA-7	KROL278	300	2.976	7.5	2.5	6.9

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

Monday, November 25, 2019

To Do:

1. ~~Check ΔrpsU patches by PCR~~
2. ~~Run gel of PCR~~
3. ~~Freeze down ΔrpsU strain~~

Results and Data:

Strain construction: LVS katG_modRBS Δ*rpsU2*

Patched 3 colonies (A-C) from each potential strain, 72-2-3, 72-2-6, 73-1-6, 73-1-7, to CHA

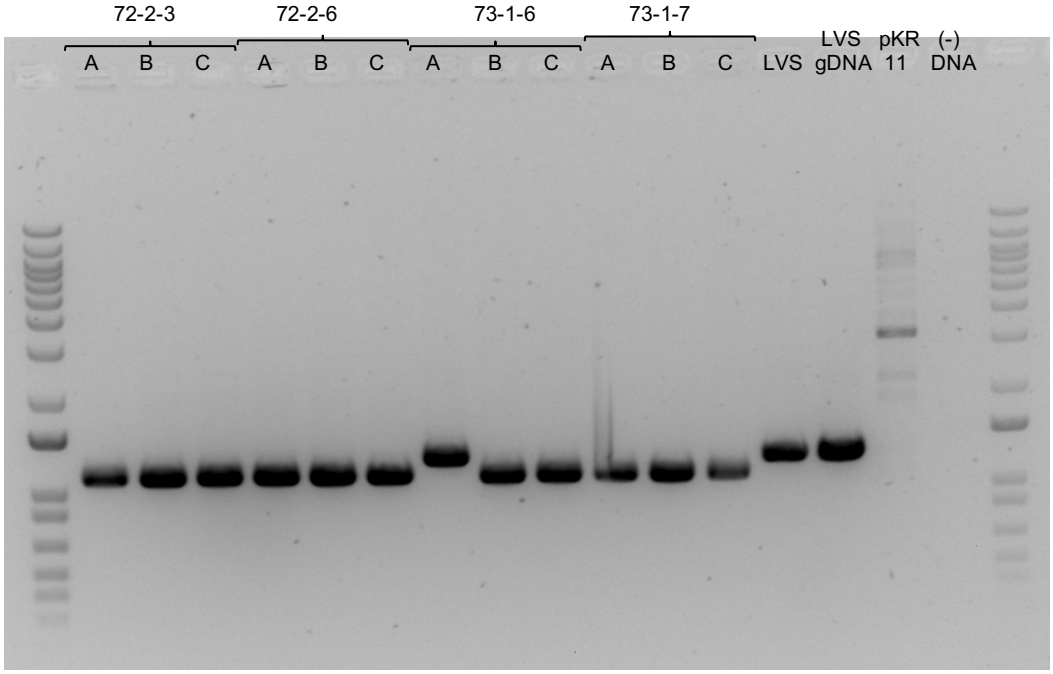
Check patches by PCR for ΔrpsU:

Sample Number	Sample	Expected size	Sample Number	Sample	Expected size
1	patch 72-2-3A	1275 or 1095	9	patch 73-1-6C	1275 or 1095
2	patch 72-2-3B	1275 or 1095	10	patch 73-1-7A	1275 or 1095
3	patch 72-2-3C	1275 or 1095	11	patch 73-1-7B	1275 or 1095
4	patch 72-2-6A	1275 or 1095	12	patch 73-1-7C	1275 or 1095
5	patch 72-2-6B	1275 or 1095	13	patch LVS	1276
6	patch 72-2-6C	1275 or 1095	14	LVS gDNA	1275
7	patch 73-1-6A	1275 or 1095	15	pKR11 (1:10)	no product
8	patch 73-1-6B	1275 or 1095	16	-DNA	no product

Total reaction volume	20
Total number of reactions	15

Component	Stock concentration	Final concentration	1 rxn volume	Factor 17
ddiH2O			12.4	210.8
PrimeSTAR GXL Buffer	5x	1x	4	68
dNTPs	2.5 mM	0.2 mM	1.6	27.2
KROL147	10 uM	0.3 uM	0.6	10.2
KROL148	10 uM	0.3 uM	0.6	10.2
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	6.8
		Total volume	20	333.2

STN with 1:30” extension, run on gel:



Great, lots of ΔrpsU mutants!
Freeze down:
KRLVS60.1 = 72-2-3A
KRLVS60.2 = 73-1-6B
Re-name frozen stocks:
KRLVS59.1 = KRLVS26.1 with pKR11 72-2
KRLVS59.2 = KRLVS26.1 with pKR11 73-1

Wednesday, November 27, 2019

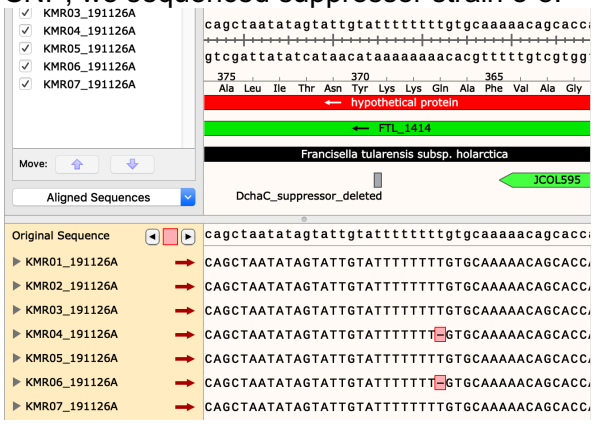
To Do:

1. ~~Check chaC sequencing results~~

Results and Data:

chaC

Have sequencing results back. The suppressor strain gDNA is sample 6, and sample 4 has the same SNP; we sequenced suppressor strain 8-8!



Future To-Do

Inverse PCR:
Digest gDNA with PstI-HF
Purify digests
Set up ligations O/N:

Tuesday
chaC experiment:
    Resuspend cells to same OD600, dilute and plate for CFUs
Reporter strains:
    If significantly different, freeze down Tn insertions.
    Plan on gDNA isolations.

Move 1° LVS pKR10-1 into strain box

Bibliography