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Future To-Do .... **Error! Bookmark not defined.**

May 2022

Wednesday, May 3, 2023

To Do:

1. ~~Colony PCR for ΔFTL_1753~~
2. ~~Run gel~~
3. ~~Streak out potential mutants~~

Results and Data:

PCR

Total reaction volume	20
Total number of reactions	16
Number of reactions plus error	17.6

Component	Stock concentration	Final concentration	1 rxn volume	Master Mix
ddiH2O			11.8	207.68
PrimeSTAR GXL Buffer	5x	1x	4	70.4
dNTPs	2.5 mM	0.2 mM	1.6	28.16
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	7.04
KROL658	10 uM	0.3 uM	0.6	10.56
KROL659	10 uM	0.3 uM	0.6	10.56
template	100 ng/ul	2 ng/ul	1	indiv
		Total volume	20	334.4

			Growth	
Sample Number	**Sample**	**Expected size**	CHA	CHA-Kan
1	Potential ΔFTL_1753 1	1851 / 1753	good	good
2	Potential ΔFTL_1753 2	1851 / 1753	good	good
3	Potential ΔFTL_1753 3	1851 / 1753	**poor**	**poor**
4	Potential ΔFTL_1753 4	1851 / 1753	good	good
5	Potential ΔFTL_1753 5	1851 / 1753	good	good
6	Potential ΔFTL_1753 6	1851 / 1753	good	good
7	Potential ΔFTL_1753 7	1851 / 1753	good	**poor**
8	Potential ΔFTL_1753 8	1851 / 1753	good	good
9	Potential ΔFTL_1753 9	1851 / 1753	good	good
10	Potential ΔFTL_1753 10	1851 / 1753	good	good
11	Potential ΔFTL_1753 11	-	**None**	**None**
12	Potential ΔFTL_1753 12	1851 / 1753	good	good
13	LVS from patch	1851		
14	LVS gDNA	1851		
15	pKR188 (0.5 uL in 9.5 uL)	-		
16	-DNA	-		

Use STN1 program for PCR:

1. 94°C 2'
2. 94°C 20"
3. 50°C 30"
4. 68°C **2 minutes**
5. Go to step 2, rep 32x
6. 68°C 5'
7. Hold 12°C

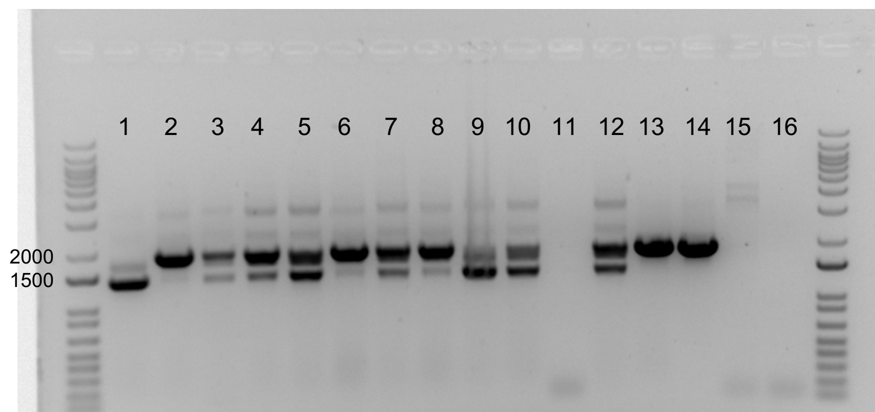


Figure 1. Colony PCR to check for FTL_1753 deletion in sucrose-resistant cells.
Aisling has been using pKR188 and allelic exchange protocol. Screening of sucrose-resistant colonies AM patched out on 5/1. Lots of potentially mixed populations. Interpretation is that upper band is ~1851bp indicating WT and lower band is ~1752 bp, indicating ∆FTL_1753. The most promising patch is #1, and #9 could also be promising. Streak both to single colony and label 2-1 and 2-9, because these are the second sets of patches that Aisling struck out.

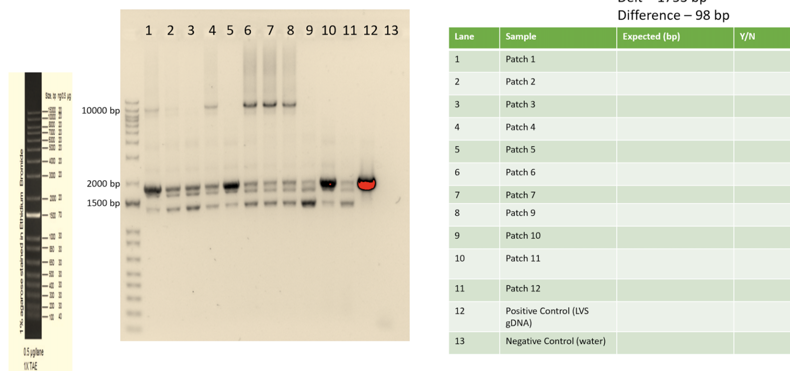
Thursday, May 4, 2023

To Do:

1. ~~Patch out colonies from potential mutants AM struck out on 5/2~~

Results and Data:

Info from Aisling on Monday:



Lane	Sample	Expected (bp)	Y/N
1	Patch 1		
2	Patch 2		
3	Patch 3		
4	Patch 4		
5	Patch 5		
6	Patch 6		
7	Patch 7		
8	Patch 9		
9	Patch 10		
10	Patch 11		
11	Patch 12		
12	Positive Control (LVS gDNA)		
13	Negative Control (water)		

Figure 2. Aisling's initial screen for ∆FTL_1752 from 5/1.
On Tuesday, Aisling struck out three potential mutants (2, 3, and 9) to single colony. There are very small single colonies today. We are limited on the number of CHA plates we have, so I patched out 6 colonies from one, Mutant 2 (now I've labeled 1-2) onto CHA and CHA-Kan.

Friday, May 5, 2023

To Do:

1. ~~Check patches from yesterday~~
2. ~~Check on single colonies from second set of patches~~
3. ~~Streak out *ppk* mutants for comparison~~

4. ~~Check on Oli's transformations~~
5. ~~Start O/N cultures for Oli's plasmids~~

<u>Results and Data:</u>

The patches from potential mutant 1-2 have grown up well on CHA, but it looks like there is growth on CHA-Kan for all of them as well. Not promising, not going to move forward with them. Patch out 3 from each 1-3 and 1-9 onto CHA and CHA-Kan to check and see if they are Kan sensitive.

Plates with potential mutants 2-1 and 2-9 (PCR from 5/3) are growing but not single colonies yet. Will have to check tomorrow.

ppk test

During Ellen's rotation, she identified a ΔrpsU1 ΔrpsU2 mutant that grows faster than standard ΔrpsU1ΔrpsU2.

Streak to single colony:

LVS	from EM glycerol stocks
Δppk	from EM glycerol stocks
ΔrpsU1 ΔrpsU2	from EM glycerol stocks
Δppk	ARLVS17

Around 4, was looking at Oli's transformation plates with Kira. Colonies have popped up on a few!

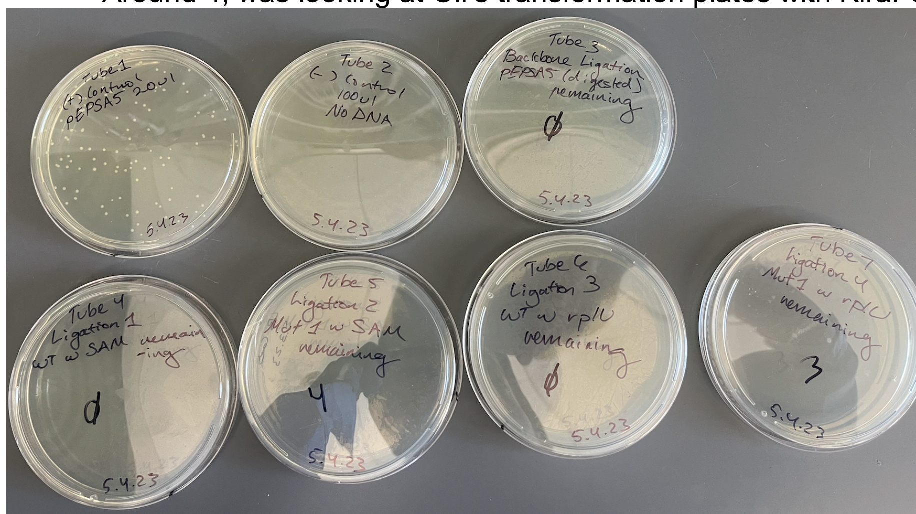


Figure 3. Plates from Oli's transformation

Oli transformed XL1-blue cells with ligations yesterday. Earlier in the day, colonies were not visible but there are a few now!

Observations:
Tube 1, positive control: lots of colonies (all plates)
Tube 2, negative control: no colonies
Tube 3, backbone only: no colonies
Tube 4, ligation 1, WT methyltransferase: no colonies
Tube 5, ligation 2, mutant methyltransferase: 4 colonies on remaining plate
Tube 6, ligation 3, WT rplU: no colonies
Tube 7, ligation 4, mutant rplU: 3 colonies on remaining plate

Note that none of the WT genes are cloned yet- a bit concerned about toxicity, but this plasmid uses a xylose-inducible promoter. I hope it's off in E. coli!

Start O/N cultures for Oli to miniprep:

Tube	Contents
1	Ligation 2 colony 1
2	Ligation 2 colony 2
3	Ligation 2 colony 3
4	Ligation 2 colony 4
5	Ligation 4 colony 1
6	Ligation 4 colony 2
7	Ligation 4 colony 3
8	plasmid pEPSA5

Saturday, May 6, 2023

To Do:

1. ~~Check ppk comparison plates growth~~
2. ~~Check on ΔFTL_1753 strain construction~~
3. ~~Spin down cultures for Oli's minipreps~~
4. ~~Pour more LB-carb plates~~
5. ~~Find NEB DH5alpha comp cells and protocol for OH~~
6. ~~Test pH-ed minimal media~~

Results and Data:

Strain comparison with ppk

Plates are growing, no obvious contamination, no single colonies yet (as expected)

Oli's plasmid construction

Spun down cultures grown O/N. No obvious toxicity (cultures turbid without apparent lysis). Put pellets at -20°C until Oli can miniprep.

Make more LB-carb plates. Melt 500 mL LB agar, cool to 56°C, add 500 uL 100 mg/mL carbenicillin, pour plates.

NEB DH5alpha competent cells are in -80°C freezer rack 5E. There are only 4 tubes.

NEB DH5alpha transformation protocol (C2987H):

1. Thaw a tube of NEB 5-alpha Competent *E. coli* cells on ice for 10 minutes.
2. Add 1-5 μl containing 1 pg-100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. **Do not vortex.**
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
5. Place on ice for 5 minutes. Do not mix.
6. Pipette 950 μl of room temperature SOC into the mixture.
7. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in SOC.
10. Spread 50-100 μl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours.

NEB DH5alpha transformation protocol (C2987H) for Oli:

Reaction table

Tube	Purpose	DNA	Volume of DNA	Volume of transformation to plate	# of LB-carb plates
1	(+) control	pEPSA5	1 uL	20 ul, 100 ul	2
2	Backbone Ligation	Digested pEPSA5	10 uL	100 uL, remaining	2
3	Ligation 1	WT methyltransferase + pEPSA5	10 uL	100 uL, remaining	2
4	Ligation 3	WT *rplU* + pEPSA5	10 uL	100 uL, remaining	2
				Total number of plates	**8**

1. Thaw **4 tubes** of NEB 5-alpha Competent *E. coli* cells on ice for 10 minutes. While thawing, label the tops 1 – 4.
2. Add indicated volume of DNA to appropriate tube, as in table above. Carefully flick the tube 4-5 times to mix cells and DNA. **Do not vortex.**
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
5. Place on ice for 5 minutes. Do not mix.
6. Pipette 950 µl of room temperature SOC into each tube.
7. Tape tubes to bottom of at 37°C shaking incubator and shake for 60 minutes.
8. While waiting, be sure labeled LB-carb plates are warming at 37°C.
9. Be sure cells have been mixed thoroughly and do a quick spin to get all liquid to bottom of tube.
10. Plate each reaction as indicated above (either 20 uL and 100 uL or 100 ul and remaining).
11. Leave plates on benchtop for ~48 hours (Saturday – Monday).

FTL_1753 deletion strain construction:

Check on patches of 1-3 and 1-9 (from colonies AM screened on 5/1). Yesterday I had patched single colonies per isolate to CHA and CHA-Kan. They all seem to have grown on both plates – do not pursue.

Single colonies of 2-1 and 2-9 (I screened by PCR on 5/3) are present. Patch 6 of each to CHA and CHA-Kan.

Minimal media

Ben pH'ed the minimal media to 7.0. See if we can get O/N growth of single colonies. Inoculate 5 mL with pEPSA5 colony from OH's 5/4 plate, both + carb and – carb.

Sunday, May 7, 2023

To Do:

1. ~~Check ppk comparison plates growth~~
2. ~~Check on ΔFTL_1753 strain construction~~
3. ~~Assess pH-ed minimal media~~

Results and Data:

Strain comparison with ppk

Plates are growing, no obvious contamination

Single colonies on LVS, ΔppK, and very tiny colonies on suppressor. No single colonies on ΔrpsU1ΔrpsU2. The Δppk colonies don't seem to be bigger, but not easy to tell yet. Incubate until tomorrow.

Minimal media

No growth in either tube (~4pm). Adjusting pH is not sufficient to allow single colonies to grow.

FTL_1753 deletion strain construction:

Check patches of 2-1 and 2-9. Of the 12, 11 have growth on the Kan plate. Only 1, 2-1-2, has no growth on Kan but growth on CHA. Check by PCR!

			Growth on CHA	
Sample Number	**Sample**	**Expected size**	CHA	CHA-Kan
1	Potential ΔFTL_1753 2-1-2	1851 / 1753	good	none
2	Potential ΔFTL_1753 2-1-3	1851 / 1753	good	good
3	LVS	1851		
4	LVS gDNA	1851		
5	pKR188	-		
6	-DNA	-		

Total reaction volume	20
Total number of reactions	6
Number of reactions plus error	6.9

Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	**Master Mix**
ddiH2O			11.8	81.42
PrimeSTAR GXL Buffer	5x	1x	4	27.6
dNTPs	2.5 mM	0.2 mM	1.6	11.04
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2.76
KROL658	10 uM	0.3 uM	0.6	4.14
KROL659	10 uM	0.3 uM	0.6	4.14
template	100 ng/ul	2 ng/ul	1	indiv
		Total volume	20	131.1

Use STN1 program for PCR:

1. 94°C 2'
2. 94°C 20"
3. 50°C 30"
4. 68°C **2 minutes**
5. Go to step 2, rep 32x
6. 68°C 5'
7. Hold 12°C

Pour gel and leave at 4°C overnight. Leave PCR overnight. Leave plates at RT.

Just in case, patch out 6 more colonies from 2-1 (7 – 12) onto CHA and CHA-Kan and put at 37°C overnight.

Monday, May 8, 2023

To Do:

1. ~~Check ppk comparison plates, image~~
2. ~~Check on ΔFTL_1753 strain construction~~
3. ~~Check on Oli's transformations~~

Results and Data:

FTL_1753 deletion strain construction

Run gel of PCR from yesterday.

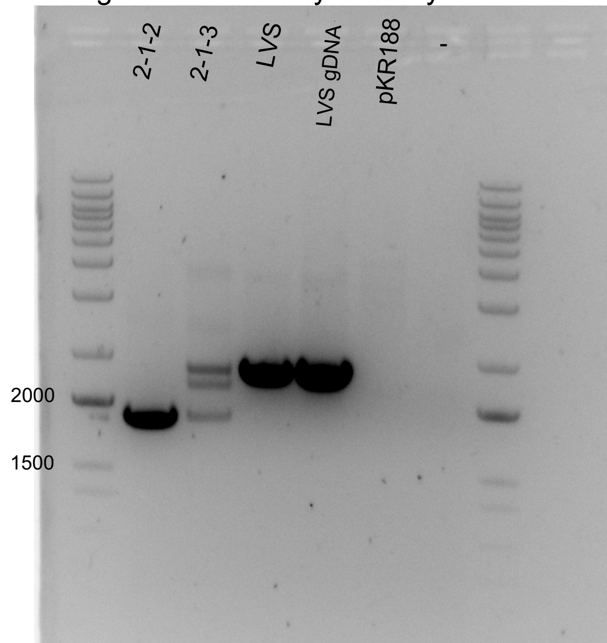


Figure 4. Colony PCR to check for FTL_1753 deletion in isolated single colonies

Again, had struck patch 2-1 to single colony then patched 6 of those single colonies to CHA and CHA-Kan. Only patch 2-1-2 did not grow on Kan (patch 2-1-3 did). This is a colony PCR from those patches and reveals that 2-1-2 is the strain we are looking for, ΔFTL_1753, while I suspect 2-1-3 probably still has the plasmid integrated into the chromosome (and similarly for the other 4 patches I didn't test that grew on Kan).

Note that the patch 2-1 cells came originally from the 1° integrant Aisling labeled 1B-A (integrated pKR188).

Aisling will freeze down the primary integrant (1B-A) as KRLVS273 and the ΔFTL_1753 cells as KRLVS274.

Oli's transformations

~12pm: Pinpricks visible on + control plates, no colonies visible on ligation plates yet.
~3pm looks like there might be some colonies on the methyltransferase ligation plate! Will need to see tomorrow.

Growth rate comparison with LVS vs Δppk

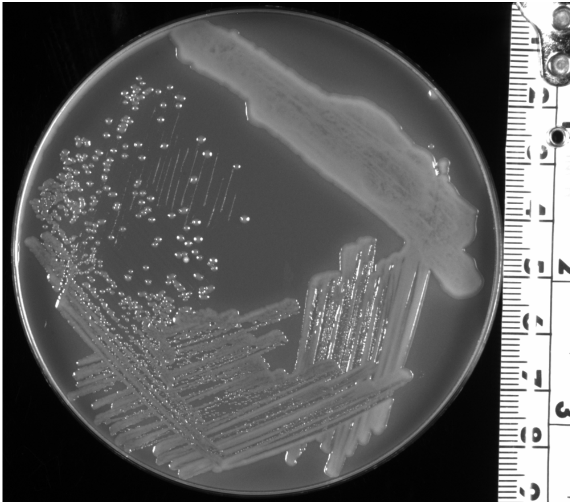


Figure 5. Plate of LVS single colonies

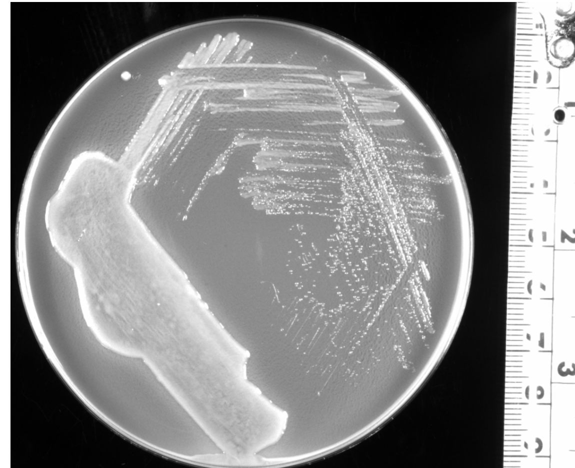


Figure 7. Plate of LVS ΔrpsU1 ΔrpsU2 single colonies

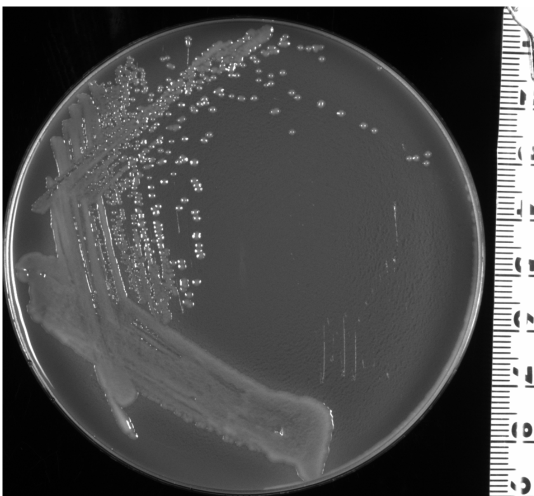


Figure 6. Plate of LVS Δppk single colonies

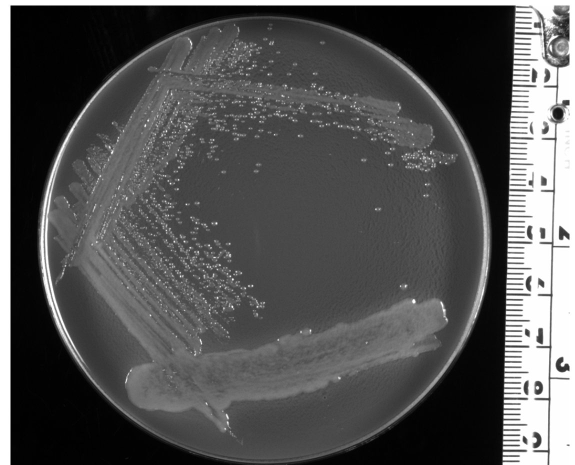


Figure 8. Plate of LVS ΔrpsU1 ΔrpsU2 suppressor single colonies

Analyzed colony size using ImageJ. Approximately 50 colonies per strain.

			T-tests		# of colonies counted
	Average (mm)	stdev	vs WT	vs Δ1Δ2	
LVS	1.17	0.228			53
Δppk	1.25	0.180	4.85E-02		50
ΔrpsU1ΔrpsU2	0.38	0.086	3.58E-41		49
ΔrpsU1ΔrpsU2 suppressor	0.84	0.158	9.94E-15	1.33E-34	60

Results Table 1. Colony sizes for LVS and mutants.

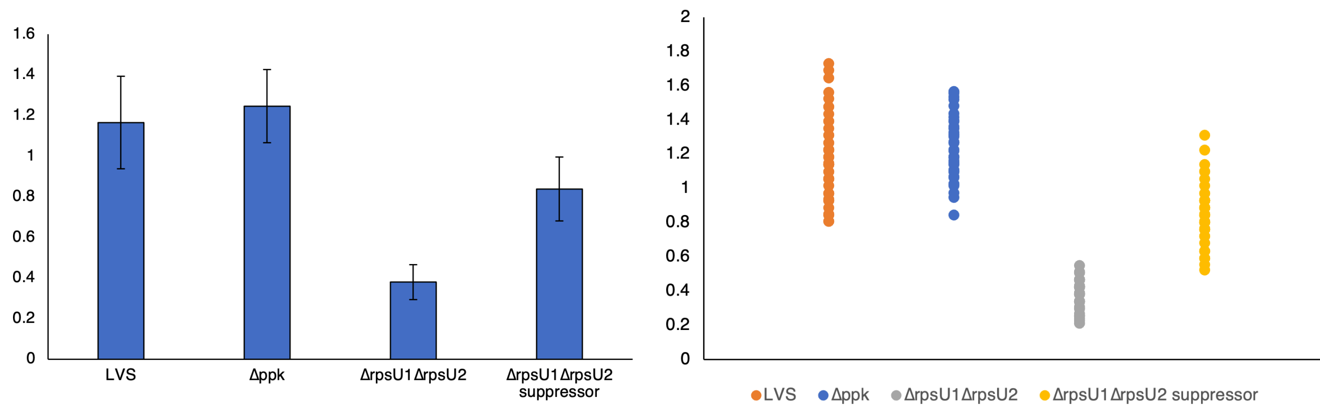


Figure 9. Graphs with colony size data for LVS and mutants.

Note that ppk mutant doesn't have a siginficant growth advantage in WT cells (aka in the absence of the ΔrpsU1ΔrpsU2 mutation)!

Reminder: Ellen found the ΔrpsU1ΔrpsU2 suppressor and it has a mutation in ppk.

Mutations found in ΔrpsU1ΔrpsU2:

Predicted mutations					
evidence	position	mutation	annotation	gene	description
RA	432,166	T→G	A63A (GCA→GCC)	*rpsU* ←	30S ribosomal protein S21
MC JC	432,169	177 bp→6 bp	coding (10-186/198 nt)	*rpsU* ←	30S ribosomal protein S21
RA	717,695	T→G	intergenic (+13/-9)	*FTL_0727* → / → *FTL_0728*	monooxygenase family protein/hypothetical protein
MC JC	1,002,968	178 bp→7 bp	coding (11-188/198 nt)	*rpsU* →	30S ribosomal protein S21
RA	1,447,131	Δ2 bp	coding (590-591/594 nt)	*isftu2* →	transposase
RA	1,713,576	G→T	G410G (GGC→GGA)	*FTL_1783* ←	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex

Mutations found in ΔrpsU1ΔrpsU2 suppressor:

Predicted mutations					
evidence	position	mutation	annotation	gene	description
RA	432,166	T→G	A63A (GCA→GCC)	*rpsU* ←	30S ribosomal protein S21
MC JC	432,169	177 bp→6 bp	coding (10-186/198 nt)	*rpsU* ←	30S ribosomal protein S21
RA	527,861	$(T)_{5\to4}$	coding (29/810 nt)	*FTL_0544* ←	hypothetical protein
RA	717,695	T→G	intergenic (+13/-9)	*FTL_0727* → / → *FTL_0728*	monooxygenase family protein/hypothetical protein
MC JC	1,002,968	178 bp→7 bp	coding (11-188/198 nt)	*rpsU* →	30S ribosomal protein S21
RA	1,447,131	Δ2 bp	coding (590-591/594 nt)	*isftu2* →	transposase
RA	1,713,576	G→T	G410G (GGC→GGA)	*FTL_1783* ←	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex

The ppk mutation in the suppressor results in a frameshift and premature truncation of the coding sequence.

WT ppk (269 aa)
MKVLSQEERQKLFLENIFPYKHKIPRNVYEKQKHYLQIELLKFQKWVKENNKKVLIIFEGRDAAGKGGTIK
RMMEHLNPRGAKVIALEKPSEQERNQWYFQRYIEHLPSGGEIVLFDRSWYNRAGVERVMGFCTEREYF
LFLEQAPQLEKMLVDSGTMIIKFWFSVSQQEQKNRFAARESHPLKQWKLSPIDKASLDKWDDYTEAKER
MFIYTDKPYAPWVIVKSDDKKRARLNAIRYILNNVDYDNKDHEVAIPPDPLIVGTSSKIYK*

Mutant ppk (13 aa)
MKVLSQEERQ**NSF***

In E. coli, polyphosphate stimulates Lon protease to degrade proteins, including ribosomal proteins. Without ppk, there would be no polyphosphate, so less r-protein degradation? (Kuroda 2001, Kornberg paper). Note interpretation by Downey 2019 that "PolyP binds the ATP-dependent Lon protease with

high affinity and promotes its activity toward select ribosomal proteins to slow translation and cell growth" – read carefully at the earlier papers – general translation slowing?

Polyphosphate prevents errors (McInerney et al., 2006), so more mis-translation?