5P Seq - Vaya gabiome 1

Materials and Methods Continue

Bacterial strains used in this Study (Ref strains) add other ID#

ATCC 8014 Lactobacillus plantarum

ATCC 6633 Bacillus subtilis

DSM 17938 Lactobacillus reuteri

DH5α Invitrogen subcloning efficiency comp. cells Cat# 18265017

Note: A 25% Glycerol stock was prepared, for all the bacterial strains listed above.

New box of bacterial strains Pakchung lab was established

Labeling was done the following bVP# 1 b = bacteria
2 VP = Vaya Pakchung

Growth conditions Lactobacillus sp (gram +)

Lactobacillus strains ATCC 8014 and DSM 17938 were grown @ 37°C in MRS Broth. O/N cultures (1 colonie) not exceeding OD 1 were used to start 10 ml (MRS Broth) Cultures OD_{600} 0.05 - 0.07

Bacteria was grown to log / mid-log phase ~ 5h for ATCC 8014
~ 7h for DSM 17938

OD_{600} for 3 biological replicates

	(OD_{600})		(OD_{600})
ATCC 8014 - 1	0.49	DSM 17938 - 1	0.34
ATCC 8014 - 2	0.47	DSM 17938 - 2	0.56
ATCC 8014 - 3	0.55	DSM 17938 - 3	0.376

Note: DSM 17938 needed ~ 7h to reach OD_{600} log-phase
ATCC 8014 needed ~ 5h to reach OD_{600} log phase

Growth conditions Bacillus subtilis (gram +) and DH5α (gram −) (Control Strains)

Bacillus subtilis ATCC 6633 and DH5α were grown in LB @ 37°C O/N Cultures were used to start 60 ml (LB) Cultures to OD_{600} 0.05 - 0.07

Bacteria were grown to mid-log phase ~ 3h

OD600 for three biological replicates of Control Strains

	OD600		OD600
ATCC 6633-1	0.8	DH5α-1	0.88
ATCC 6633-2	0.78	DH5α-2	0.89
ATCC 6633-3	0.98	DH5α-3	0.7

Note ATCC 6633 B Subtilis exhibited flocculent growth. Possibly because it was grown @ 37°C. (rec. @ 30°C)
Both Strains needed ~3h to reach mid-log phase

Culture Conditions Continued

20mL of Culture was harvested for each treatment and pellet was frozen in EtOH dry ice bath for RNA analysis

Experiments were done in three biological replicates (See page 4, 5) OD600

For Strains ATCC 8014 and DSM 1938 I had total of 100mL for each replicate (in TRS Broth) 5 conditions
2x Control (untreated) 1x CAM 1x SHX and 1x HUP
For Control Strains ATCC 6633 and DH5α I had total of 60mL Culture for each replicate in CB 3 Conditions
2x Control (untreated) 1x CAM

Chloramphenicol (CAM) treatments

CAM was added to final concentration of 100μg/mL (20mL Culture + 67μl of 30mg/mL stock in EtOH) incubated for 5' @ 37C shaker and harvested On ice (50mL Conical filled with ice) containing 67μl CAM in table top centrifuge for 2' full speed Pellet was Shock frozen and stored for RNA analysis

DL-Serine hydroxamate treatment (SHX)

SHX was added to final concentration of 1.25mg/mL
(20mL Culture + 500µL of 50mg/mL SHX Stock in H_2O)
10' @ 37°C, harvested in table-top centrifuge full speed
2'. Pellet was shock frozen and stored @ -20°C for
RNA analysis

Nisin (NIS) treatments

NIS was added to final concentration of 6µg/mL
(130µL of NIS 10mg/mL Stock in DH5O to 20mL Culture)

Stationary grown bacteria Cultures

For stationary phase Cultures Strain ATCC8014 was
grown in MRS Broth for 27h @37°C
OD_{600} for 3 biological replicates: ATCC 8014-S1 4.3
ATCC 8014-S2 4.5
ATCC 8014-S3 4.65

2mL Culture was harvested and stored for RNA analysis

Note: Prior I setup Cultures for stationary phase
and grew them 72h for strain ATCC8014 and DSM17938
RNA of these samples was degraded. Therefore I decided
to only use ATCC8014 and left out DSM17938 due to
slow growth.

Heat shock and nutrient deprivation Culture conditions

Lactobacillus plantarum ATCC8014 was grown in MRS Broth
@37°C. I used the 27h grown stationary phase
Cultures as precultures (as above) and inoculated in
40mL MRS Broth to OD_{600} 0.05. Bacteria was grown to
Log-phase (OD_{600}) ~ 4.5h

	OD_{600}
ATCC 8014-4	0.2
ATCC 8014-5	0.31
ATCC 8014-6	0.39

10mL of each replicate for untreated control was harvested and Pellet was Stored for RNA analysis.
15mL of each replicate was harvested after corresponding Stress condition

Heat Shock treatments HS

15mL Culture was harvested in table-top centrifuge @ full speed for 2' ATCC 8014-HS1(4) ATCC 8014-HS2(5) ATCC 8014-HS3(6)

Nok: 1mL TSB Broth was preheated in Thermomixer to 60°C in 2mL Epi tube

Pellet was resuspended in preheated Media and cells were incubated for 15' @ 60°C Light Shaking in Thermo Mixer

Pellet was Stored for RNA analysis

Nutrien Deprivation treatments (ND) 15' in 0.5xLB

15mL Culture was harvested in table-top centrifuge full speed 2'. Pellet was washed excessively with (50mL) 0.5xLB

→Start timer

Spin 2' full speed. Sup was discarded pellet was resuspended in 20mL 0.5xLB Media

Culture was incubated @ 37°C Shaking for remaining time (~10 min remain) (15' total start of timer @ wash)

Culture was harvested and Pellet Stored for RNA analysis.

ATCC 8014-ND1 (4) # corresponds to biological replicate control sample
ATCC 8014-ND2 (5)
ATCC 8014-ND3 (6)

Nok: I called it Nutrien deprivation, however I am not sure this is valid because LB is rich media. Talk to Rachel he recomends to call it unfavorable culture media condition

RNA Isolation (Cotter Protocol Book pg 63 glass bead RNA iso)

RNA was isolated as described in Cotter Protocol Book (see attached PDF) with minor modifications)

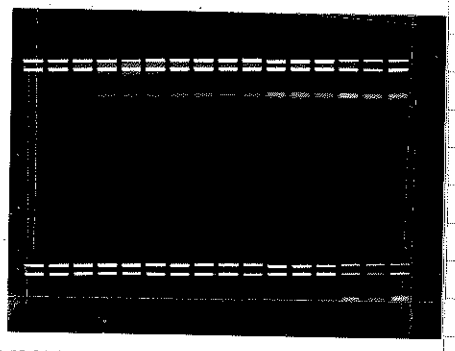
- Step 4 Vortex in multimixer for 90"
- Step 6 Vortex for 90" and Spin for 2' full speed
- Step 7 Vortex for 30" Spin for 1' full speed
- Step 8 Vortex for 30" Spin for 1' full speed
- Step 10 Centrifuge for 20' full speed 4°C Carefully
- Step 11 Resuspend pellet/RNA in 47µl of nuclease-free H_2O

Quantification of RNA was done by Nanodrop * (Used the 1-10 variations to determine final conc)
↳ See attachments, Vagayabiome_1_RNAquantification

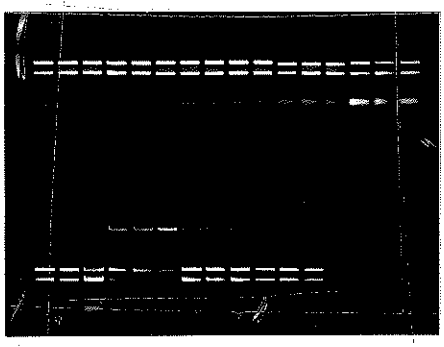
57 Seq (20µl)

- A 1µg/µl working RNA Stock was prepared for Library prep of all the RNA samples. Original RNA Stock is stored @ -80
- ~1µg of total RNA was run on 1.2% Agarose gel in TAE to assess quality of RNA preps total of 60 RNA preps

1A top RNAPreps 1-1 - 1-16



2A top RNAPreps 3-1 - 3-16



1B bottom RNAPreps 2-1 - 2-16

2B bottom RNAPreps 4-1 - 4-12

Note: 1µg of total RNA separates roughly to: 600ng bacteria 23S
300ng 16S
100ng tRNA

Note: Quality of RNA was OK
- little smear/degradation of sample 1-5
- for samples 4-3, 1-14, 1-16 23S/16S Ratio not great
- Samples 4-4, 4-5, 4-6 low yield of RNA (see Nanodrop concentrations) PDF

Legend for RNA quality check for biological replicates #1, #2, #3 + #4 Stool

1A. E#1			1B E#2			2A E#3		
1-1	ATCC 8014 - 1	Ctr.	2-1	ATCC 8014 - 2	Ctr.	3-1	ATCC 8014 - 3	Ctr.
1-2	ATCC 8014 - 1	CHX	2-2	ATCC 8014 - 2	CHX	3-2	ATCC 8014 - 3	CHX
1-3	ATCC 8014 - 1	CAM	2-3	ATCC 8014 - 2	CAM	3-3	ATCC 8014 - 3	CAM
1-4	ATCC 8014 - 1	SHX	2-4	ATCC 8014 - 2	SHX	3-4	ATCC 8014 - 3	SHX
1-5	ATCC 8014 - 1	MUP	2-5	ATCC 8014 - 2	MUP	3-5	ATCC 8014 - 3	MUP
1-6	DSM 17938 - 1	Ctr.	2-6	DSM 17938 - 2	Ctr.	3-6	DSM 17938 - 3	Ctr.
1-7	DSM 17938 - 1	CHX	2-7	DSM 17938 - 2	CHX	3-7	DSM 17938 - 3	CHX
1-8	DSM 17938 - 1	CAM	2-8	DSM 17938 - 2	CAM	3-8	DSM 17938 - 3	CAM
1-9	DSM 17938 - 1	SHX	2-9	DSM 17938 - 2	SHX	3-9	DSM 17938 - 3	SHX
1-10	DSM 17938 - 1	MUP	2-10	DSM 17938 - 2	MUP	3-10	DSM 17938 - 3	MUP
1-11	ATCC 6633 - 1	Ctr.	2-11	ATCC 6633 - 2	Ctr.	3-11	ATCC 6633 - 3	Ctr.
1-12	ATCC 6633 - 1	CHX	2-12	ATCC 6633 - 2	CHX	3-12	ATCC 6633 - 3	CHX
1-13	ATCC 6633 - 1	CAM	2-13	ATCC 6633 - 2	CAM	3-13	ATCC 6633 - 3	CAM
1-14	DHS2 - 1	Ctr.	2-14	DHS2 - 2	Ctr.	3-14	DHS2 - 3	Ctr.
1-15	DHS2 - 1	CHX	2-15	DHS2 - 2	CHX	3-15	DHS2 - 3	CHX
1-16	DHS2 - 1	CAM	2-16	DHS2 - 2	CAM	3-16	DHS2 - 3	CAM

2B E#4 Stool sample

4-1	ATCC 8014 - 4	Ctr
4-2	ATCC 8014 - 5	CHX
4-3	ATCC 8014 - 6	CHX
4-4	ATCC 8014 - HS1 (4)	
4-5	ATCC 8014 - HS2 (5)	
4-6	ATCC 8014 - HS3 (6)	
4-7	ATCC 8014 - ND1 (4)	
4-8	ATCC 8014 - ND2 (5)	
4-9	ATCC 8014 - ND3 (6)	
4-10	ATCC 8014 - S1 (4)	
4-11	ATCC 8014 - S2 (5)	
4-12	ATCC 8014 - S3 (6)	

Note:
Ctr = Control (untreated)
CAM = Chloramphenicol @ 100µg/mL
SHX = DL Serine hydroxamate 125µg/mL
MUP = Mupirocin 65µg/mL
HS = HEAT SHOCK 15' @ 60°C
ND = Nutrient deprivation (5' 0.5X PB)
S = Stationary-phase 27h OD ~ 4

Additional Libraries to prepare for MIXED POPULATIONS FS

5-1	= 2-6 + 2-1	1:1		
5-2	= 2-6 + 2-3	1:1	5µL + 5µL	1µg/µL
5-3	= 2-6 + 2-4	1:1		
5-4	= 2-6 + 2-5	1:1		
5-5	= 2-6 + 2-1	1:10		
5-6	= 2-6 + 2-3	1:10	9µL + 1µL	100ng/µL
5-7	= 2-6 + 2-4	1:10		
5-8	= 2-6 + 2-5	1:10		
5-9	= 2-6 + 2-1	1:100		
5-10	= 2-6 + 2-3	1:100	9.9 + 0.1µL	10ng/µL
5-11	= 2-6 + 2-4	1:100		
5-12	= 2-6 + 2-5	1:100		

5P Seq Library preparation

- See Modified 5PSeq Protocol Sus PDF

Note: total of 72 Libraries; 60 x Standard 5PSeq
12 x random frag controls

Random frag Control samples

E1 1-2, 1-7, 1-12, 1-15
E2 2-2, 2-7, 2-12, 2-15
E3 3-2, 3-7, 3-12, 3-15

- DNase treatment as described in Protocol.

First round was E1, E2, E3, E4 (except random frag Control samples)

Left the samples on @ -20

next day I did E5 and the random frag controls (Purification)
RNA
- Ligation was done for all of them on @ 16°C (1h)

next day
- Ethno precipitation and eluted in 16µl nuclease free water

Qubit RNA

- for rRNA depletion RNA concentration cannot exceed 1µg total Conc. but do not have enough RNA Qubit reagent

Therefore I can only measure in couple and dilute then 1:4 as I do.

Qubit Measurements

sample	ng/µl	ng/µl	total in 16µl
1-1	0.186	146	1971
1-6	0.06	60	810
1-11	0.19	190	2565
1-14	0.07	70	945
2-1	0.1	100	1350
2-6	0.07	70	945
2-11	0.19	190	2565
2-14	0.06	60	810
3-1	0.07	70	945
3-6	0.17	170	2295
3-11	0.03	30	405
3-14	0.03	30	405
4-1	0.17	170	2295
4-2	0.12	120	1620
4-3	0.18	180	2430
5-1	0.19	190	2565
5-2	0.19	190	2565
5-3	0.18	180	2430

- I did compare 1 measure ~~Some~~ Some samples with nanodrop but the values were all over the place

Conf. I cannot do it like that

	nanodrop	Qubit
e.g 1-1	277 ng/µl	146
1-6	180 ng/µl	60
1-11	400 ng/µl	190

given that the Nanodrop values and Qubit values do not correlate and the RiboZero Kit guide specifically says that RNA concentrations should be measured with Qubit ~~we~~ have a problem: we do not have enough RNA Qubit solution

I decided that I will take ½ V for rRNA depletion as it is stated in my protocol. For the reason that highest concentration is 2.5 µg and lowest 400 ng in 13.5 µl

The rRNA depletion kit can go as low as 100 ng.

Start with E1 and E2 total of 32 samples

For bead washing I need 45 µl/sample x 33 1.5 mL beads

batch
~~After~~ Wash resuspend in ~~5~~ ~~00~~ µl resusp. Buffer + (16.5 µl Ribo Ero.)

Do in 2 batches 750 µl beads, wash, wash

resuspend in 200 µl resp. Buff + ~~8.25 µl~~ pull them and add 16.5

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mix 6 µl nuclease free H_2O

+ 7.5 µl RNA
+ 4.5 removal
+ 2 µl 10x react. Buffer

] MM for 33 s — 6 µl 148.5, 66

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next set E3, E4, E5 total 40 / 41 sam

7.5 µl
4.5 rem 187
2 µl 10x 83

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batch wash strip beads 45 x 41 1845
922,5 each

res
492 susp.
246 each
20 µl RiboEr

mRNA frag ✓

For RT I have to recalculate the amount for random Hexamer we have 2µg/µl therefore I will use 1.5µl

After RT I will do Ampure bead purification and elute In 10µl of nuclease free H_2O. The reason for that is we are almost out of BioNotI-P5-Oligo (17µl)

~~10~~ 9.5µl Sample
10µl ~~of~~ Phusion HM
0.25µl 5µM BioNotI-P5-PET Oligo

20µl - 5µM
0.23 - x

Then I will have final concentration of 57.5pM

- Binding to Strep Beads: M280 hydrophobic, M270 hydrophilic

- O/N Common adaptor Ligation Start @ 8:45
Jupe took my stuff out @ 10am (~13h total t. of L.)

Ask why important → - wash after ligation and I did let the beads settle as written.

~~F~~ For the PCR Amplification I did total of 50µl

- Stored the beads with 10µl EB in 4C 22.06.18

- PCR @ -20. Will do Ampure Purification on Monday 25.

Index and Multiplexbarcode for Library amplification

	NEBL	PE2_MPX
1-1	503	04
1-2	503	05
1-3	503	06
1-4	503	07
1-5	503	08
1-6	503	09
1-7	503	10
1-8	503	11
1-9	503	12
1-10	503	13
1-11	503	14
1-12	503	15
1-13	503	16
1-14	503	17
1-15	503	18
1-16	503	19

	NEBL	PE2_MPX
2-1	504	04
2-2	504	05
2-3	504	06
2-4	504	07
2-5	504	08
2-6	504	09
2-7	504	10
2-8	504	11
2-9	504	12
2-10	504	13
2-11	504	14
2-12	504	15
2-13	504	16
2-14	504	17
2-15	504	18
2-16	504	19

	NEBL	PE2_MPX
3-1	505	04
3-2	505	05
3-3	505	06
3-4	505	07
3-5	505	08
3-6	505	09
3-7	505	10
3-8	505	11
3-9	505	12
3-10	505	13
3-11	505	14
3-12	505	15
3-13	505	16
3-14	505	17
3-15	505	18
3-16	505	19

	NEBL	PE2_MPX
4-1	506	04
4-2	506	05
4-3	506	06
4-4	506	07
4-5	506	08
4-6	506	09
4-7	506	10
4-8	506	11
4-9	506	12
4-10	506	13
4-11	506	14
4-12	506	15

	NEBL	PE2_MPX
5-1	503	20
5-2	503	21
5-3	503	22
5-4	504	20
5-5	504	21
5-6	504	22
5-7	505	20
5-8	505	21
5-9	505	22
5-10	506	20
5-11	506	21
5-12	506	22