this page should go to: http://2017.igem.org/Team:Edinburgh_OG/Notebook

Yuri's lab work
Engineering the P1 phage with CRISPR SaCas9

<Designing of *E.coli* testing platform>

May

12th: Decide target regions to be taregeted by the CRISPR Cs9 system and be inserted into the *E. coli* testing platform
17th: Continue designing
25th: Make competent cells from *E. coli* TOP10
30th: Design primers
Decide the strategy to construct the phagemid with SaCas9 and spacers

< Amplification of plasmids>

June

1st: Order IDT to synthesise the CRISPR and spacer cassettes
6th: Amplify the phagemid (Transformation, overnight culture)
7th: Make competent cells from *E. coli* C600 (Lysogen of P1 phage)
8th: Mini-prep phagemid
Digestion to check the Mini-prep product
14th:Maxi-prep phagemid
29th: PCR to amplify the DNA parts for the KPC spacers

< Inserting SaCas9 Part2 into the phagemid-SaCas9 Part1>

July

4th: Digestion of phagemid-SaCas9 Part1
Gel extraction
5th: Ligation of Phagemid-SaCas9 Part1 and SaCas9 Part2
Transformation
7th: Mini-prep phagemid with complete SaCas9
Digestion to check the construct

So as to check whether these colonies contain the desired construct, the phagemids were digested with Hind III (**Figure 1**). As can be seen, two bands with the expected sizes were obtained. Therefore, it can be tentatively concluded that the insertion of SaCas9 Part 2 was successful. The insertion was later verified by sequencing.

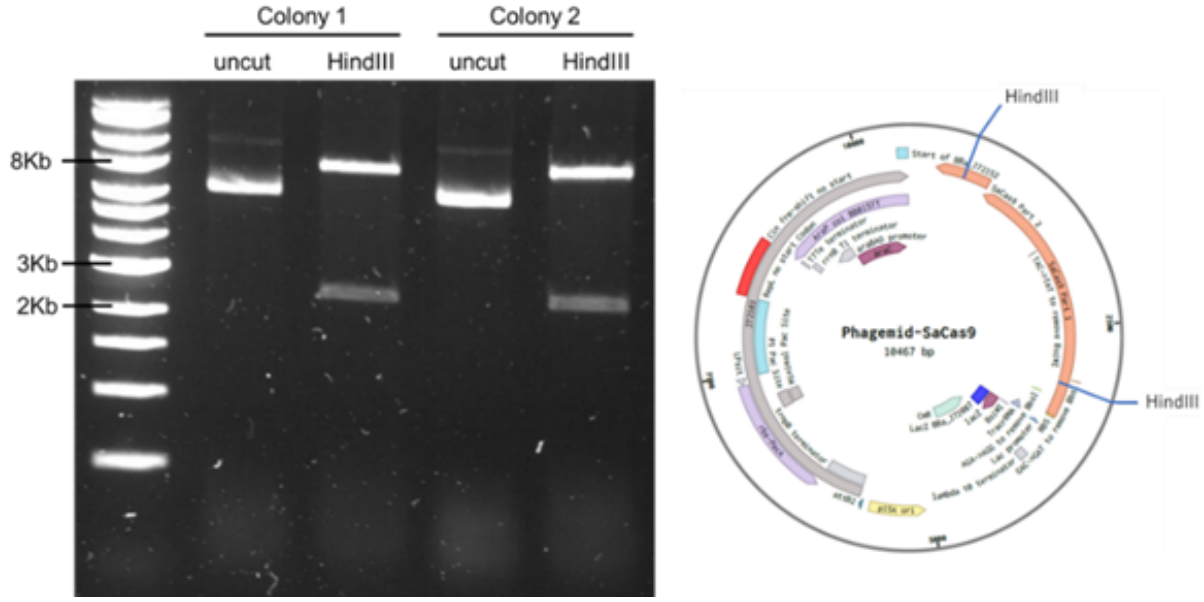


Figure 1: Digestion of the phagemid- SaCas9. There are two HindIII restriction sites, one in SaCas9 Part 1, the other in SaCas9 Part 2. This digestion is expected to yield bands of 2.5Kb and 7.9Kb.

<Inserting KPC spacers>

10th: PCR: amplify the KPC spacer Part A and B
Digestion of phagemid-SaCas9, KPC spacer PartA , B
Ligation (Fail)

The KPC spacer cassette Part A and B were amplified by PCR. For KPC Part A, “For universal spacer” was used as a forward primer. While, two primers were used for reverse; one was “Rev universal spacer”, which was intended to yield Part A connectable to KPC Part B, and the other was “KPC- SaCas9-rev” primer, for single use in Part A. On the other hand, for part B, “For universal spacer” and “Rev universal spacer” were used. Electrophoresis was conducted to check the PCR products (Figure 2), and the products were verified to be the correct size. Expected sizes for PartA and PartB were 121bp and 118bp, respectively.

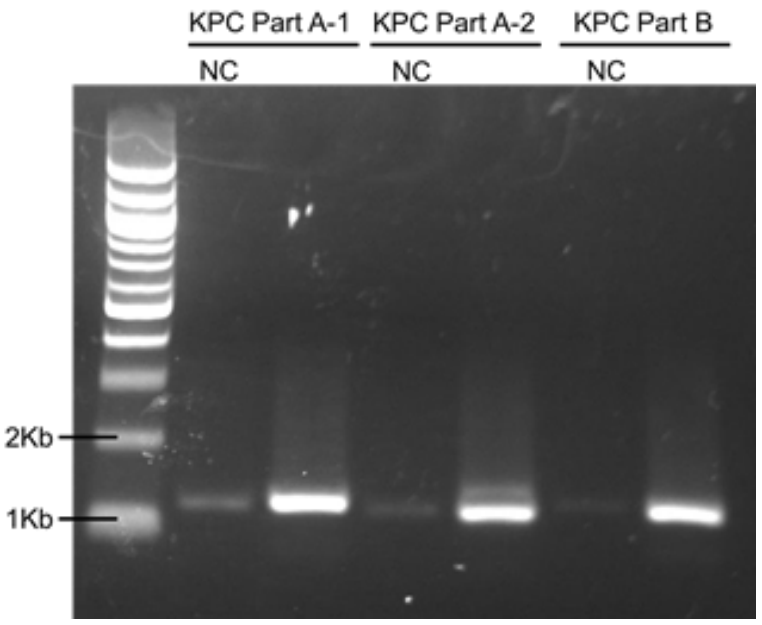


Figure 2: PCR for the amplification of the KPC spacers. For the forward primer, the "For universal primer" was used for all the reaction. While for the reverse primer, the "Rev universal primer" was used for KPC Parts A-1 and B, and the "KPC-SaCas9 rev" primer was used for the KPC Part A-2. NC: a negative control without any template DNA.

11th:Digestion of phagemid-SaCas9, KPC spacer Part A
PCR: amplify the KPC spacer Part A and B
12th: Mini-prep phagemid-SaCas9
Digestion of phagemid-SaCas9, KPC spacer Part A and B,
SaCas9 Part2
Gel extraction: phagemid-SaCas9
13th: Ligation
KPC Part A+ B (Fail)
phagemid-SaCas9+ KPC PartA >> Transformation
SaCas9 Part 2 + KPC Part A(Fail)

14th: Colony PCR1(Pick 4 colonies for KPC, pick 4 for VanA)
17th: Colony PCR2
Gel running: Check the result of colony PCR1 (Fail)
18th: Gel running: Check the result of colony PCR1, 2 (Fail)
Mini-prep from colony cultures (4 samples)
Digestion to check the construct
(None of them was desired construct)
19th: Pick another 20 colonies and culture them overnight
2nd Ligation of phagemid-SaCas9 and KPC spacer Part 1
Transformation

One-pot digestion and ligation: Inserting the spacer cassettes into phagemid-SaCas9. Following SaCas9 Part 2, the KPC spacer was also inserted into the construct, by means of one pot digestion and ligation. For this, only KPC Part A was inserted into phagemid-SaCas9, becase we experienced failure of inserting KPC Part B (Data not

shown). The ligated construct was transformed into *E. coli* TOP10 cells (**Figure 3**). As a result, seven colonies were gained from plate (F). Colonies in plate (B) indicated the high frequency of self-ligation of BsaI- cut phagemid-SaCas9. Unexpected results were seen for plates (C) and (E). Even though plate (C) and (E) were expected to yield no colonies and numerous colonies, respectively, both plates gave rise to several dozens of colonies. In search of the colonies with a desired construct, all the colonies from plate (F) were subjected to further investigation.

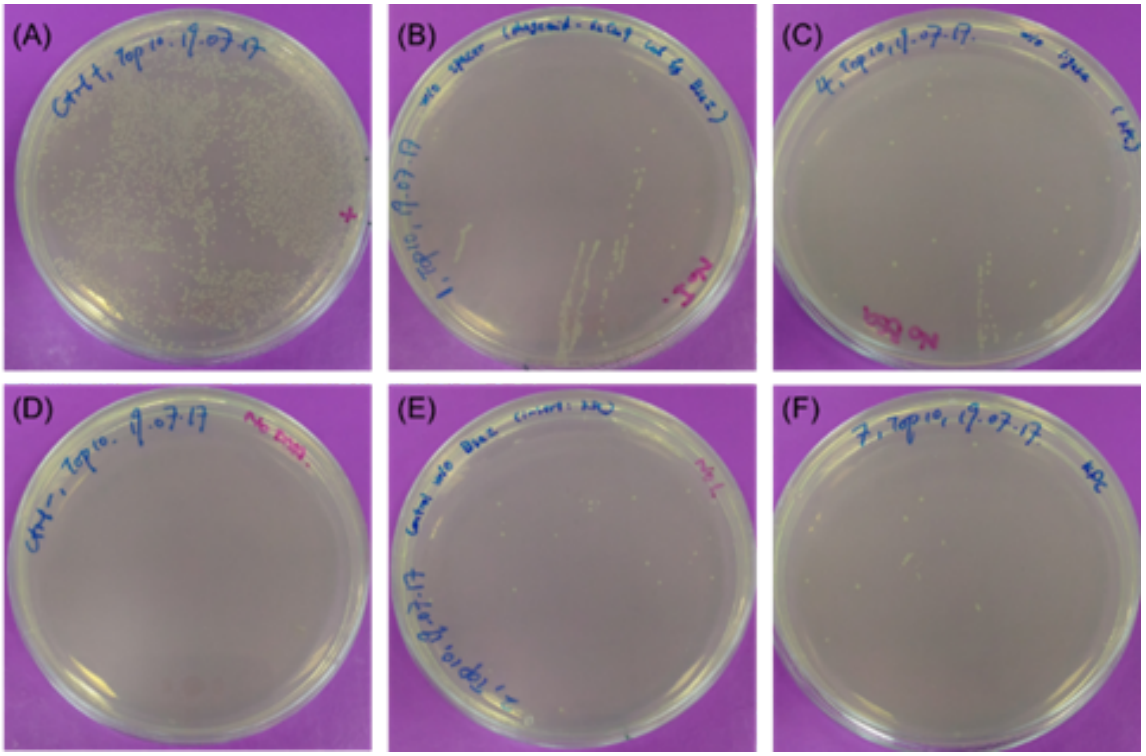


Figure 3: Transformation of the construct from one pot digestion and ligation. (A): positive control, for which phagemid-SaCas9 was transformed. Plates (B)- (E) are negative controls. (B): Phagemid-SaCas9 digested by BsaI was transformed. (C): No ligase. (D) : No DNA. (E): No BsaI. Finally, (F) is the sample with all the reagents, including the DNA construct generated from one pot digestion and ligation.

20th: Pick 9 colonies and culture them overnight
21st: Colony PCR (with a new primer) (Fail)
Mini-prep 29 colonies
24th: Digestion to check the construct
Successfully got several samples of phagemid-SaCas9 with spacers

Phagemid DNA from each colony which had been purified by the use of mini-prep, were digested with BsaWI and BsaI. This method clearly indicates the result of ligation, because ones without the spacer cassette will be digested by both BsaI and BsiWI, yielding two bands (3.4kb and 7.0kb), whereas ones with the spacer will only be cut by BsiWI, as there will be no BsaI sites after the successful ligation. As shown in Figure 4, colonies 3-7 yielded two bands, suggesting that the KPC spacer Part A was successfully inserted into phagemid-SaCas9.

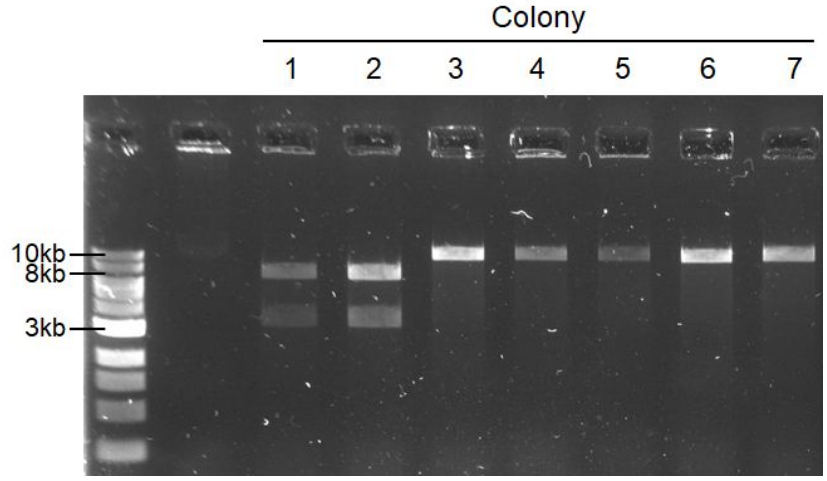


Figure 4: Checking the phagemid DNA by digestion with BsiWI and BsaI.

<Producing P1 phage>

24th: Transformation of *E. coli* C600 (Lysogen of P1 phage) using phagemid-SaCas9 with spacers
25th: Harvest colonies and culture them over night
26th: Add arabinose to the culture to induce lytic cycle, and harvest engineered phage

<Infection of the *E. coli* with P1 phage>

26th: Mix phages and *E.coli* testing platform, and plate them onto LB plates (with no antibiotics/Chloramphenicol /Kanamycin)
27th:Verification of efficiency of the SaCas9 system and phage infection
28th:Observation of GFP from *E. coli* to check the cleavage of target

Infection of the *E. coli* with P1 phage

MoClo cells (bla_{kpc} / *vanA*) and Phages (Ones for targeting *vanA*, ones for bla_{kpc}, and the negative control without any spacer cassette) were mixed together and plated on LB plates (with no antibiotics/Kanamycin/Chloramphenicol). As a result, colonies were obtained as summarised in **Table 1**. The pictures of plates are available in **Appendix 1**.

For both KPC and VanA phages, four kinds of phages from the different colonies did not yield differences in the number of colonies. Regardless of the types of phages, the number of colonies decreased, as the amount of phage increased. Infected KPC MoClo cells grew on all three kinds of LB plates. No significant difference in the number of colonies was observed between those infected by the control phage and those by KPC phage.

In contrast, for VanA MoClo cells, a decrease in survival of the cells was evident when they are infected by phage, even on LB plates without any antibiotics. In addition, the number of VanA MoClo colonies appeared to be slightly less than that for KPC, even in control without phage infection. A reason for the compromised growth might be because the cells were not healthy, or expression of VanA MoClo plasmid might have a negative effect on the cell

growth, both of which could have made cells vulnerable to phage infection. Further experiments are needed to verify the cause.

Table 1: Infection of MoClo cells with P1 phage. MoClo KPC/VanA denotes the host E.coli testing platform with the target sequence either from KPC or VanA. The column for "Phage" indicates the type of phage. (-): no phage, (control): phage with SaCas9 system without spacer, (K3-1-K6-2): phages with SaCas9 system targeting the *blaKPC* gene, which were harvested from four different colonies. (V1-1-V2-2): phages with SaCas9 system targeting the *vanA* gene, from four different colonies. Ratio indicates the volume ratio MoClo cells: phage, from 1:1 to 1:4. The number of colonies from each plate was categorised from level 0 (no growth) to level 5 (confluent).

Plate No.	Moclo	Phage	Ratio	Plate	Clonies
1	KPC	-	-	LB	5
2				Kan	5
3				Chl	0
1	VanA	-	-	LB	5
2				Kan	5
3				Chl	0
4	KPC	control	1:1	LB	5
5				Kan	5
6				Chl	4
7			1:2	LB	5
8				Kan	5
9				Chl	4
10			1:4	LB	4
11				Kan	2
12				Chl	2
4	VanA	control	1:1	LB	3
5				Kan	1
6				Chl	1
7			1:2	LB	2
8				Kan	1
9				Chl	1
10			1:4	LB	1
11				Kan	almost 0
12				Chl	0

Plate No.	Moclo	Phage	Ratio	Plate	Clonies
13	KPC	K3-1 K3-2 K6-1 K6-2	1:1	LB	5
14				Kan	5
15				Chl	5
16			1:2	LB	5
17				Kan	5
18				Chl	4
19			1:4	LB	4
20				Kan	3
21				Chl	2
22	VanA	V1-1 V1-2 V2-1 V2-2	1:1	LB	4
23				Kan	2
24				Chl	1
25			1:2	LB	1
26				Kan	1
27				Chl	1
28			1:4	LB	1
29				Kan	almost 0
30				Chl	almost 0

Verification of efficiency of the SaCas9 system and phage infection

To check the efficiency of this SaCas9 system and phage infection, we took advantage of different antibiotic resistant genes (**Figure 5**).The MoClo cells possess plasmid which contains Kanamycin resistant gene and target sequence of the SaCas9 system. On the contrary, the phagemid, which P1 phage carries, has the SaCas9 system and Chloramphenicol resistant gene. Therefore, If cleavage by SaCas9 system occurs, it results in the loss of Kanamycin resistance gene in the host cell, while acquiring the Chloramphenicol resistance. On the other hand, if Sa Cas9 system doesn't work, the host cells still displays Kanamycin resistance. (These cells should also have Chloramphenicol resistance, as the result of the phage infection, even if the cleavage by SaCas9 is not successful.) Likewise, if infection by phage is successful, the cells should have conferred Chloramphenicol resistance.

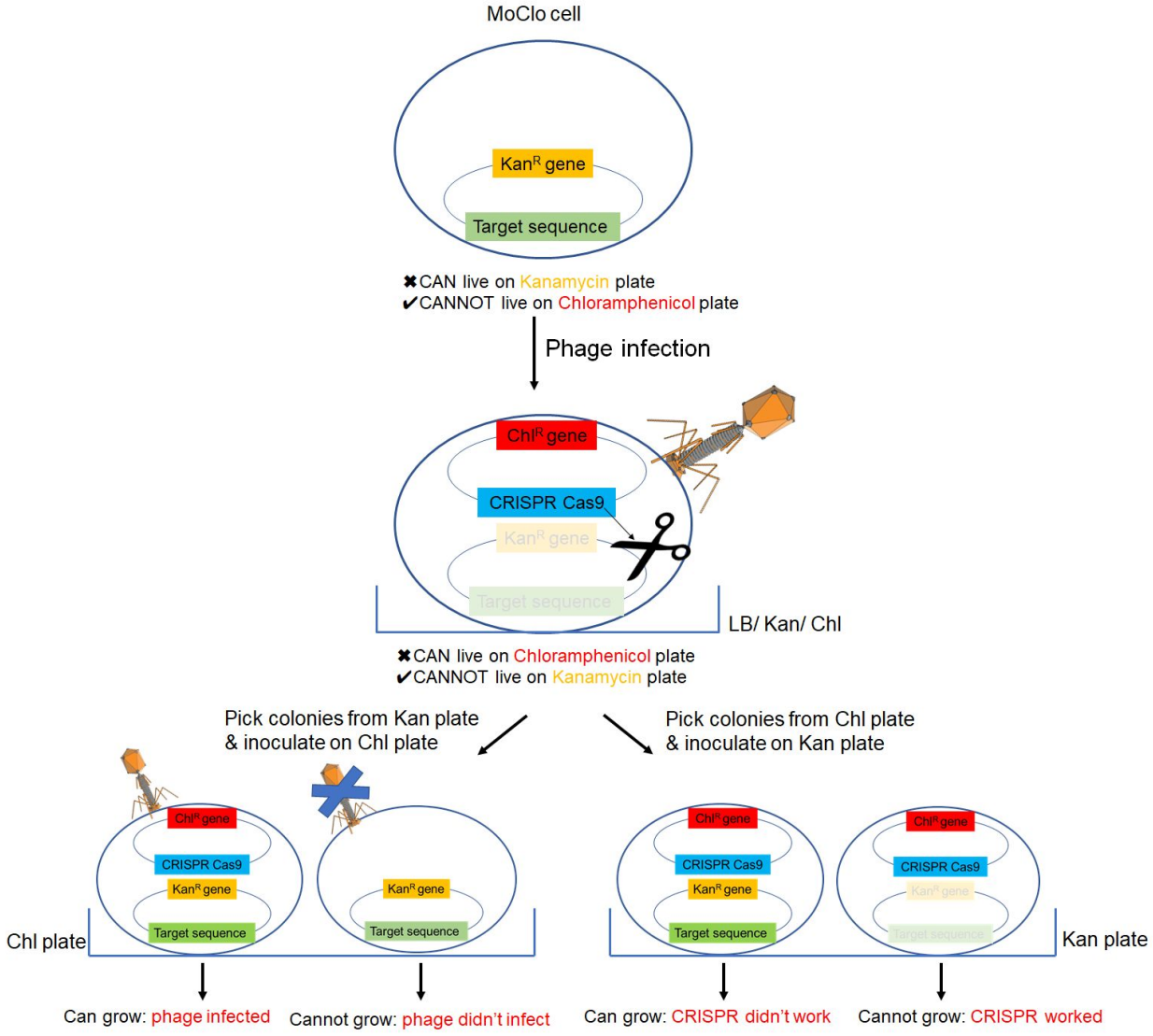


Figure 5: Strategy to verify the efficiency of the SaCas9 system and phage infection.

Efficiency of SaCas9 system

To investigate the efficiency of the SaCas9 system to cleave the target sequence, colonies were picked from plates No. 9, 18, 27 (**Table 2**). Colonies 1-4 came from KPC MoClo cells infected by phage with KPC spacer, while colonies 5-8 wereVanA MoClo cells infected with one with VanA spacer. In addition, colonies from KPC and VanA MoClo cells infected by negative control phage without any spacer were also picked (A-D for KPC MoClo cells, E-H for VanA MoClo cells) .They were inoculated onto Kanamycin plates, including ones with no antibiotics and with Chloramphenicol. The result is shown in **Table 2**. The pictures of plates are available in **Appendix 2**.

Table 2: Verification of efficiency of the CRISPR SaCas9 system and phage infection. Vertical axis shows original plates from **Table 1**. Horizontal axis shows the new plates which were inoculated colonies from the original plate (**Table 1**).

		Newly inoculated plate								
		Colony	LB	Kan	Chl		Colony	LB	Kan	Chl
Original plate	KPC MoClo cells infecgted by control phage on Chl plate (Plate No.9)	A	+	+	+	KPC MoClo cells infecgted by KPC phage on Chl plate (Plate No.18)	1	+	+	+
		B	+	-	+		2	+	+	+
		C	+	-	+		3	+	+	+
		D	+	+	+		4	+	+	+
	VanA MoClo cells infecgted by control phage on Chl plate (Plate No.9)	E	+	+	+	VanA MoClo cells infecgted by vanA phage on Chl plate (Plate No.27)	5	+	+	+
		F	+	-	+		6	+	-	+
		G	+	+	+		7	+	+	+
		H	+	+	+		8	+	+	+
	KPC MoClo cells infecgted by control phage on Kan plate (Plate No.8)	I	+	+	-	KPC MoClo cells infecgted by kpc phage on Kan plate (Plate No.17)	9	+	+	-
		J	+	+	-		10	+	+	-
		K	+	+	-		11	+	+	-
		L	+	+	-		12	+	+	-
	VanA MoClo cells infecgted by control phage on Kan plate (Plate No.8)	M	+	+	-	VanA MoClo cells infecgted by vanA phage on Kan plate (Plate No.26)	13	+	+	-
		N	+	+	-		14	+	+	-
		O	+	+	-		15	+	+	-
		P	+	+	-		16	+	+	-

→ Can check efficiency of the SaCas9

→ Can check efficiency of phage infection

The SaCas9 system targeting the Kan^R plasmid within VanA MoClo cells showed promising - albeit not consistent - results. Among colonies 5-8 from the Chloramphenicol plate, colony 6 did not grow on the Kanamycin plate indicating that the SaCas9 might have cleaved the target Kan^R plasmid. However, the experiment was performed only once and further replicates to confirm the efficiency of the SaCas9 should be tested.

On the contrary, survival of the KPC MoClo cells on the Kanamycin plates implied that none of the bla_{kpc} target sequences and the Kanamycin resistance gene were cleaved. This may be due to an error in the spacer design, as SaCas9 cassettes are identical between ones targeting KPC and ones for VanA.

Despite these results, three colonies which was infected by negative control phage did not grow on Kanamycin plates. This might imply that KanR plasmid has been lost from the MoClo cells due to unknown mechanism. Further repetitive experiments are needed, to make sure that our SaCas9 is functional.

Efficiency of phage infection

The efficiency of phage infection suggested to be low (**Table 2**). This is because no growth of colonies 9-16 on Kanamycin plates meant they were not conferred Chloramphenicol resistant gene via phage infection. Quantitative analysis on the number of phage in solution could have been done for more precise result.

Observing GFP expression within the MoClo cells

As described in page “Experiments”, the GFP gene is incorporated into the region just downstream of the spacer cassettes. To test the cleavage of the target sequence, expression of GFP was observed through a fluorescence microscope. Unexpectedly, fluorescence from GFP was not detectable, even from the MoClo cells before infection by P1 phage. Even though very weak fluorescence was observed, its level was equivalent to that from auto- fluorescence (Data not shown).

<Making Biobricks>

September

12th: Extract the SaCas9 gene from the phagemid-SaCas9 by PCR, with primers which can attach prefix and suffix to the gene.

13th: Digestion of the PCR products SaCas9 and backbone (pSB1C3)

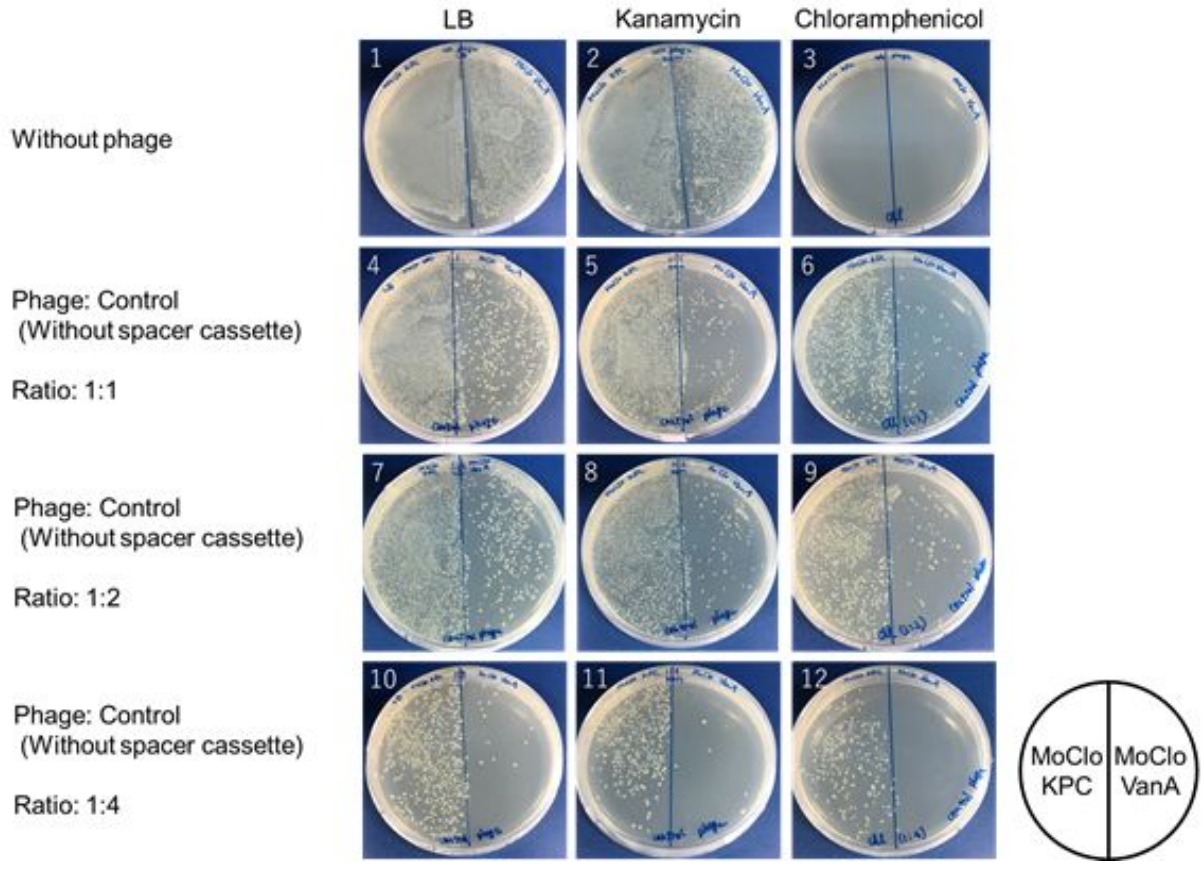
14th: Ligation and transformation

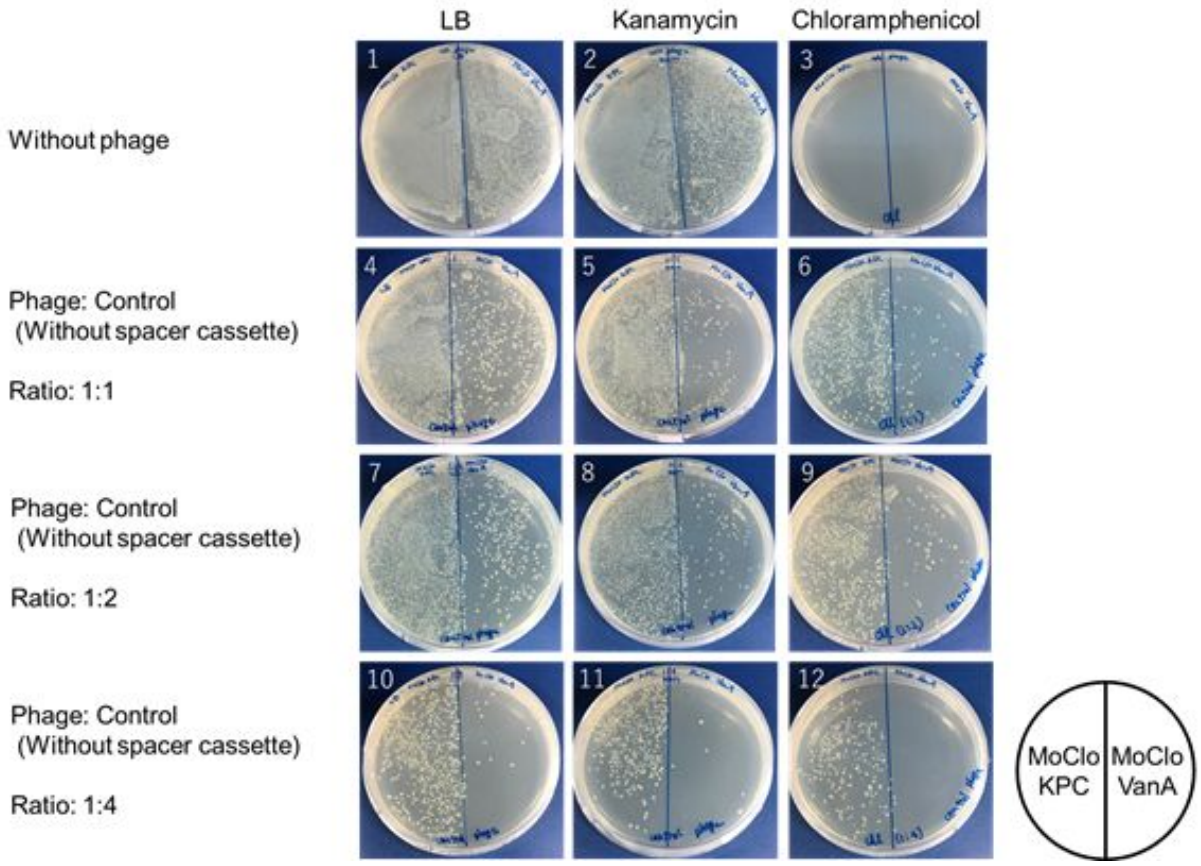
15th: Colony PCR

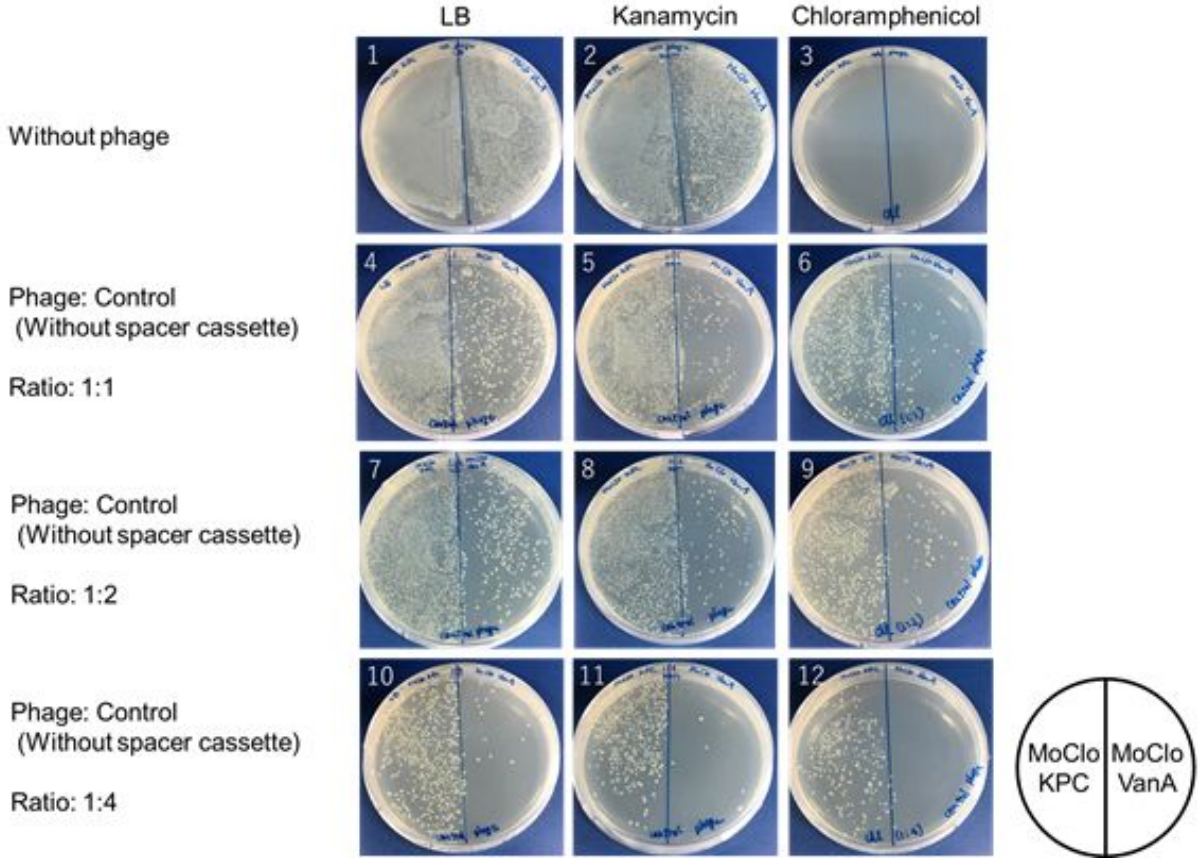
16th: Do overnight culture and Maxi-prep

20th: Digestion to check the construct

Appendix 1: Infecting the MoClo cells with P1 phages.




LB
Kanamycin
Chloramphenicol
Without phage
Phage: Control
(Without spacer cassette)
Ratio: 1:1
Phage: Control
(Without spacer cassette)
Ratio: 1:2
Phage: Control
(Without spacer cassette)
Ratio: 1:4
1
2
3
4
5
6
7
8
9
10
11
12
MoClo
KPC
MoClo
VanA


LB
Kanamycin
Chloramphenicol
Without phage
Phage: Control
(Without spacer cassette)
Ratio: 1:1
Phage: Control
(Without spacer cassette)
Ratio: 1:2
Phage: Control
(Without spacer cassette)
Ratio: 1:4
1
2
3
4
5
6
7
8
9
10
11
12
MoClo
KPC
MoClo
VanA

Appendix 2: Verification of efficiencies of the SaCas9 system and phage infection