Lysostaphin

Week 9

Summarized below are the experiments conducted this week in chronological order. Click on the experiment name to view it. To go back to this summary, click **Summary** in the footer.

Summary

1 Colony PCR of BL21 (DE3) cells containing pSB1C3-T7-Lys-LT

Responsible

Sigrun Stulz

Protocols used

- Colony PCR
- Gel electrophoresis

Modifications and comments to protocols

Instead of performing a PCR on the colony directly, 1 μl of the purified plasmid was used as template.

Experimental Set Up

The PCR was done with verification primers (VF2 and VR). Plasmids from the overnight cultures were extracted using a Mini prep prior to PCR. Table 1 shows the composition used for the PCR and Table 2 shows the PCR conditions used in the thermocycler. The PCR reaction used for each sample was 25 μl.

Table 1: PCR Reaction

Master Mix	Component	Volume [μl]
1	PCR Grade Nucleotide mix	2
	R_Primer (VR)	10
	F_primer (VF2)	10
	Template DNA	1
	Sterile water	27
	Total Volume	50
2	PCR Reaction buffer 10 x	10
	Taq DNA Polymerase	2.5
	Sterile water	37.5
	Total Volume	50

Table 2: PCR condition using Taq polymerase

Step	Cycles	Temperature {°C}	Time
Initial Denaturation	1	94	2 min
Denaturation	30	94	30 secs
Annealing		52	1 min
Extension		72	1 min
Final Extension	1	72	7 min
Hold	indefinitely	4	-

Sample Calculation

No further calculations were done.

Results and Conclusions

The result of the PCR with the verification primers show bands of the desired size. The amplified fragment be 1147 bp in size and this is supported by the fact that the DNA bands are located slightly lower than that of the 1200 bp band on the ladder (Figure 1).

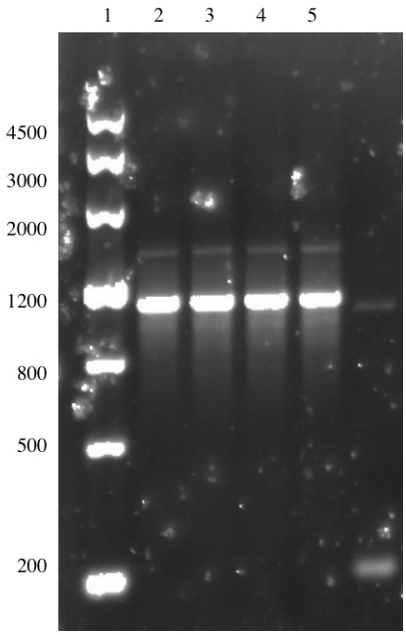

Figure 1: PCR of pSB1C3-T7-Lys-LT. (1) Ladder (2) Sample pSB1C3-T7-Lys1-LT (3) Sample pSB1C3-T7-Lys2-LT (4) Sample pSB1C3-T7-Lys3-LT (5) pSB1C3-T7-Lys4-LT

Discussion and Troubleshooting

The pSB1C3-T7-Lys-LT construct can now be expressed in BL21 (DE3) cells and further analysed by performing a SDS Page analysis.

2 Kirby Bauer Test on Cell Lysates of BL21 (DE3) containing pSB1C3-T7-Lys

Responsible

Oscar Ohman, Maren Maanja and Sigrun Stulz

Protocols used

- Protein Expression
- Sonication
- Kirby Bauer Test

Modifications and comment to protocol

Inoculation of plates: 400 μl of *E. coli* TOB1 culture was used instead of 200 μl

Experimental Set Up

Liquid culture of *E. coli* TOB1 was first spread on to Endo and McConkey agar plates. Following this, sterile filter paper disks were soaked with cell lysates that have been sonicated beforehand. The soaked filter paper disks were placed onto the *E. coli* TOB1 culture to determine antagonistic activities of the cell lysate. The different lysostaphin samples used on *E. coli* TOB1 are listed in the table below.

Table 3: Samples to be tested on the Kirby Bauer Test

Sample	Description
Tested Sample	Lys 0.5 μl IPTG insoluble
	Lys 0.5 μl IPTG soluble
	Lys 1 μl IPTG insoluble
	Lys 1 μl IPTG soluble
Negative Control	Untransformed cells insoluble
Negative Control	Untransformed cells soluble
Positive Control	Kanamycin

Sample Calculation

The concentration of the cell lysates were adjusted to the concentration of kanamycin used, which was 25 mg/ml.

Results and Conclusions

The Kirby Bauer result on both Endo and McConkey agar is shown in the two figures below.

Figure 2: Kirby Bauer test of lysostaphin cell lysates on McConkey agar

Figure 3: Kirby Bauer test of lysostaphin cell lysates on Endo agar

Discussion and Troubleshooting

Figure 2 shows the Kirby Bauer test that was done on McConkey agar. McConkey agar is a differential medium used for isolating Gram Negative bacteria. On this medium, *E. coli* displays a pink to rose colour colony, this medium was used to improve visualization of plated *E. coli* TOB1. As observed, a portion of the plate has turned yellow in colour and this is most likely due to a pH drop as a result of bacterial growth.
Regarding the zone of inhibition observed, kanamycin which served as a positive control, displayed the largest and most clear zone compared to the cell lysates. Induced cells from both the pellet and supernatant sample with 0.5 mM IPTG and 1.0 mM IPTG also showed zones of inhibition. The zones from induced samples were larger than that of uninduced samples. This indicates that even though bacteriolytic substances or proteins could be present in the cell lysate (based on the zone of inhibition from unstransformed samples), the samples containing lysostaphin showed a greater lytic activity.
Figure 3 pictures the same Kirby Bauer test, however instead of using the McConkey agar, we opted for Endo agar. Here, the results were similar to that of the McCOnkey agar where kanamycin displayed the largest zone of inhibition. Zones of inhibition were also observed on all samples. However, here it was difficult to differentiate the difference in size of the zone of

inhibition between the samples. Thus, this could either mean that the expression of Lys was insignificant compared to other bacteriolytic substances or proteins present in the cell lysate. Or the use of Endo agar was unbeneficial in providing us reassuring results.
Hence, as a conclusion from the Kirby Bauer tests we believe that lysostaphin has been expressed and it is capable of displaying bacteriolytic activity towards *E. coli* TOB1. The mechanism behind the eradication of *E. coli* TOB1 is however unknown, as *E. coli* is Gram negative bacteria and lysostaphin disrupts the peptidoglycan layers which are more prominent in Gram positive bacteria, like *S. aureus* Thus, to ensure that we are expressing lysostaphin, we would need to perform an SDS Page to confirm its size and a antimicrobial assay towards *S. aureus* .

3 Expression of pSB1C3-T7-Lys in E. coli BL21 (DE3)

Responsible

Sigrun Stulz and Shuangjia Xue

Protocols used

- Protein Expression
- Sonication
- SDS Page

Experimental Set Up

The cells were first induced with either 0.5 mM and 1 mM IPTG in LB medium containing chloramphenicol 20 μM/ml. Induced cells were then disintegrated with sonication and visualized using SDS Page.

Table 4: Lysostaphin samples to be expressed

Sample	Description
Lys 1	Induced with 0.5 mM and 1 mM IPTG
Lys 2	Induced with 0.5 mM and 1 mM IPTG
Lys 3	Induced with 0.5 mM and 1 mM IPTG

Results and Conclusions

Table 5 shows the OD measurement of the BL21 (DE3) culture before induction. Each Lysostaphin sample was induced with both 0.5 mM and 1 mM IPTG for 4 hours.

Table 5: OD measurements before induction

Sample	OD
Lys 1	0.524
Lys 2	0.53
Lys 3	0.55

The induced cells were then sonicated and separated into pellets and supernatant. This separation serves to identify the solubility of lysostaphin. It is considered soluble if it is found in the supernantant or insoluble if it is bound to cellular compartments and found in the pellet. Thus the separated samples with its protein concentrations are listed in Table 6.
The samples were then subjected to SDS Page using 12 percent separating gel, as shown below. The description for each well is stated in Table 7.

Table 6: Protein concentration of different Lys samples after induction

Sample	Concentration mg/ml
pSB1C3-T7-Lys uninduced soluble	2.75
pSB1C3-T7-Lys 1mM IPTG soluble	2.70
pSB1C3-T7-Lys 0.5 mM IPTG soluble	2.39
pSB1C3-T7-Lys uninduced insoluble	7.00
pSB1C3-T7-Lys 1mM IPTG insoluble	6.22
pSB1C3-T7-Lys 0.5 mM IPTG insoluble	6.33

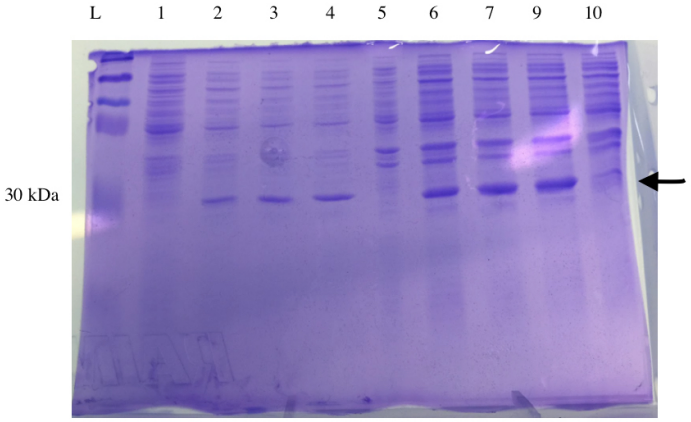

Figure 4: SDS Page of Expressed pSB1C3-T7-Lys

Table 7: Description of SDS Page (Figure 4)

Lane	Description
1	Ladder
2	Untransformed BL21 cells soluble
3	pSB1C3-T7-Ly uninduced soluble
4	pSB1C3-T7-Ly 1mM IPTG soluble
5	pSB1C3-T7-Ly 0.5mM IPTG soluble
6	Untransformed BL21 cells insoluble
7	pSB1C3-T7-Ly uninduced insoluble
8	pSB1C3-T7-Ly 1mM IPTG insoluble
9	pSB1C3-T7-Ly 0.5mM IPTG insoluble
10	pSB1C3-T7-Ly 1mM IPTG insoluble (2)

Discussion and Troubleshooting

Based on the open reading frame from the construct T7-Lys (741 bp) a fully expressed protein would be around 27 kDa in size. On the protein ladder, 30 kDa corresponds to the fifth band from the top. The result of the SDS Page show bands of approximately 30 kDa on all the transformed samples including that from the supernatant and pellet. Whereas the untransformed samples do not display protein bands of this size (lane 2 and 6). However, we also see that the uninduced samples (lane 3 and 7) show protein bands at 30 kDa. We think that the emergence of these protein bands could possibly be due to a leaky promotor of the T7 DNA polymerase in the *E. coli* BL21 (DE3) system.

4 Re-Do: Kirby Bauer Test on Cell Lysates of BL21 (DE3) containing pSB1C3-T7-Lys

Responsible

Reskandi Rudjito and Sigrun Stulz

Protocols used

- Kirby Bauer Test

Experimental Set Up

As previously done, soluble and insoluble components of the cell lysates were tested on E. coli TOB1 strains on McConkey agar. The experiment was done in triplicates and the samples used are exactly the same as the samples written in Table 7. Kanamycin was used as a positive control and sterile deionized water was used as a negative control.

Results and Conclusions

The colony on the plate did not grow as much as expected and zone of inhibitions were not observed.

Discussion and Troubleshooting

We believe that the inoculum used to plate the McConkey agar was too dilluted therefore it did not grow as well as we expected it to.

5 Digestion of Overhang PCR Product of T7-Lys-BamHI

Responsible

Reskandi Rudjito and Maren Manjaa

Protocols used

- Digestion
- Gel electrophoresis

Experimental Set Up

The sequencing result of the construct pSB1C3-T7-Lys-LT showed that the linker tag (LT) had not been successfully assembled. Thus, we decided to re-do the the assembly of pSB1C3-T7-Lys-LT from the beginning. To achieve this we would start from digesting the overhang PCR product of T7-Lys-BamHI.
The overhang T7-Lys-BamHI is digested with EcoR1 and BamHI and the linker tags (LT) would then be digested with BamHI and Pst1. The backbone is linearized with EcoR1 and Pst1. The three fragments would then be ligated together and transformed into competent cells.

Table 8: Digestion reaction of Overhang Lysostaphin

Reaction	Volume [μl]
Overhang Lys (23.5 ng/μl)	10
Buffer SB 10 X	2.0
EcoR1	0.2
BamH1	0.2
Sterile water	7.6

Sample Calculation

1 μl of restriction enzyme is used for every 1 μg/μl of DNA.

Results and Conclusions

Discussion and Troubleshooting

The digestion of the PCR product should result in a sticky end product of 795 bp. The result on Figure 5 (lane 6) displays a DNA band of around this size. The digested product were then purified to obtain a solution of 4.6 ng μl.

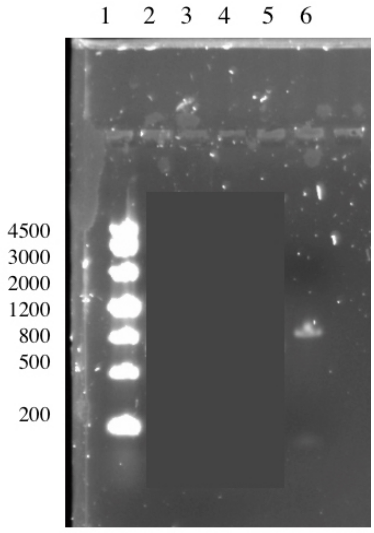

Figure 5: Digestion of Overhang PCR Product T7-Lys-BamHI. (1) Ladder (6) Digested T7-Lys-BamHI

6 Ligation and Transformation of pSB1C3-T7-Lys-LT

Responsible

Reskandi Rudjito

Protocols used

- Ligation
- Transformation

Experimental Set Up

To perform ligation procedure, all three DNA fragments consisting of the overhang Lys, linkertag and pSB1C3 backbone are digested and purified prior to ligation. The concentrations of each DNA fragment are stated in Table 9.

Table 9: Concentrations of DNA fragments for Ligation

Sample	Concentration [ng/μl]	Size [bp]
Digested Overhang Lys promoter (E and B)	4.6	795
Digested Linker Tags (B and P)	16	88
Digested pSB1C3 (E and P)	5.9	2029

To increase the chances of a positive transformation, both TOP10 and Bl21 cells were used. Positive controls plated onto LB plates without antibiotics were done to ensure that the cells were viable.

Table 10: Transformation of pSB1C3-T7-Lys-LT

Sample	Comment
pSB1C3-T7-Lys-LT Top10	Transformed into Top10 cells
pSB1C3-T7-Lys-LT BL21 (DE3)	Transformed into BL21 (DE3) cells
positive control Top 10	No antibiotics
positive control BL21	No antibiotics

Sample Calculation

The insert:vector molar ratio used in the ligation was 10:1. The calculations shown on the tables below are adjusted so that each reaction contains 50 ng of backbone and the inserts are adjusted according to the desired molar ratio. The final reaction volume was 25 μl.

Table 11: Ligation Reaction of pSB1C3-T7-Lys-LT

Component	Volume [μl]
Overhang Lys	17.79
Linker Tags	0.63
pSB1C3	4.24
T4 DNA Ligase	0.5
T4 DNA Ligase Buffer 10X	2.5
Sterile water	-0.65

Results and Conclusions

To follow the ligation process of pSB1C3-T7-Lys-LT, we decided to to run a gel on the ligated products. Figure 6 (Lane 8) shows a picture of this.
Only one colony from the TOP10 culture was observed the next day indicating that ligation and transformation were successful. The colony was cultivated and plasmids containing pSBC13-T7-Lys-LT were directly transformed into BL21 (DE3) cells.

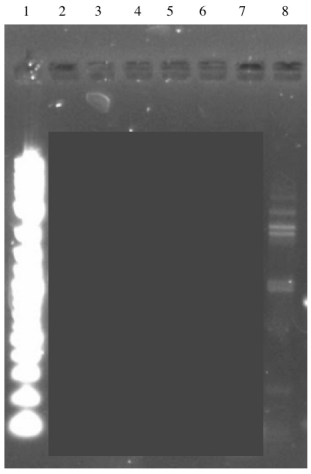

Figure 6: Ligated product of pSB1C3-T7-Lys-LT. (1) Ladder (2) Ligation product of pSB1C3-T7-Lys-LT

Discussion and Troubleshooting

The ladder on Figure 6 did not separate nicely, however we still saw bands at around 2-5 kb, and suspect that these bands could possibly be the backbone or ligated plasmids.

7 Re-Transformation of pSB1C3-T7-Lys-LT

Responsible

Reskandi Rudjito and Aman Mebrahtu

Protocols used

- Transformation
- Colony Picking
- Glycerol Stocks

Modifications to protocols

In order to improve the transformation efficiency, we decided to purify the ligated product before the transformation procedure.

Experimental Set Up

The experimental begins with a transformation and colony picking of successfully transformed cells. We used positive controls with no antibiotics to ensure that we have viable cells. Successfully transformed cells are then picked and analyzed by colony PCR. The colony PCR was done with verification primers (VF2 and VR). Plasmids from the overnight cultures were then extracted using a Mini prep and sent for sequencing. Table 13 shows the composition used for the colony PCR and Table 14 shows the PCR conditions used in the thermocycler.

Table 12: Transformation and Colony Picking of pSB1C3-T7-Lys-LT

Sample	Comment
pSB1C3-T7-Lys-LT Top10	Transformed into Top10 cells
pSB1C3-T7-Lys-LT BL21 (DE3)	Transformed into BL21 (DE3) cells
positive control Top 10	No antibiotics
positive control BL21	No antibiotics

Table 13: Colony PCR Reaction

Master Mix	Component	Volume [μl]
1	PCR Grade Nucleotide mix	2
	R_Primer (VR)	10
	F_primer (VF2)	10
	Template DNA	-
	Sterile water	28
	Total Volume	50
2	PCR Reaction buffer 10 x	10
	Taq DNA Polymerase	2.5
	Sterile water	37.5
	Total Volume	50

Table 14: Colony PCR condition using Taq polymerase

Step	Cycles	Temperature {°C}	Time
Initial Denaturation	1	94	2 min
Denaturation	30	94	30 secs
Annealing		52	1 min
Extension		72	1 min
Final Extension	1	72	7 min
Hold	indefinitely	4	-

Results and Conclusions

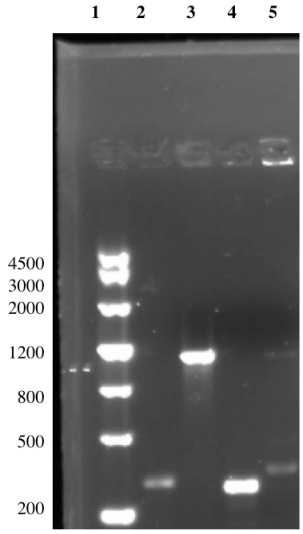

Figure 7: Colony picking of transformed TOP 10 cells containing pSB1C3-T7-Lys-LT. (1) Ladder (2) Colony 1 (3) Colony 2 (4) Colony 3 (5) Colony 4

Four colonies were observed from the TOP 10 culture and the colonies were picked and cultured. The result of the colony PCR is shown in the figure below.

Discussion and Troubleshooting

The result of the colony PCR (Figure 7) show that not all transformed colonies contain the right pSB1C3-T7-Lys-LT insert. The exact size of the amplified construct is 1093 bp, thus only colony 2 show positive result. The purified plasmid of colony 2 have a concentration of 176.1 ng μl and were sent for sequencing. While we wait for the sequencing results, we transformed the pSB1C3-T7-Lys-LT into BL21 (DE3) cells.