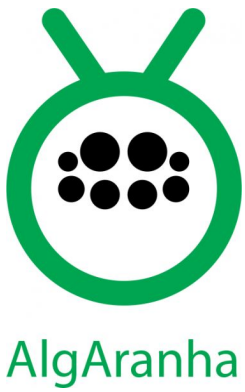
Expression of Lysostaphin in *Escherichia coli*



Summary

- We were able to clone the IDT fragment in the pSB1C3 vector and decide to try and express the peptide in either *E. coli* or *Chlamydomonas reinhardti.*

- We were not able to detect successful cloning of the lysostaphin in our microalgae vector (pJP22) neither in the pET-DUET plasmid for expression in *E. coli* yet.

----------08/17----------

Preparation of pETDuet-1 expression plasmid (1)

Edmar and Mireia

3

08/17 → BL21DE3 pETDuet-1 cells were inoculated in 3 mL LB with ampicillin (100 ug/mL). The cultures were incubated at 37°C and 150pm ON for further plasmid extraction.

08/19 → The plasmid pETDuet was extracted from BL21DE3 cells with the extraction kit SD Wizard (Promega). It was obtained at a concentration of 180 ng/μL and stored at -20°C.

----------08/20----------

Digestion of enzybiotic sequences and/or pET-DUET (1)

Edmar and Mireia

Item	Volume
H_2O	2.7 μL
Buffer H	1 μL
LysK (8 ng/μL)	5 μL
pETDuet (180 ng/μL)	0.3 μL
EcoRI	0.5 μL
PstI	0.5 μL
Total	10 μL

Item	Volume
H_2O	6.6 μL
Buffer H	1 μL
lysostaphin (22.7 ng/μL)	1.1 μL
pETDuet (180 ng/μL)	0.3 μL
EcoRI	0.5 μL
PstI	0.5 μL
Total	10 μL

Reaction conditions: 37°C 16h, 80°C 20min, 4°C hold. (The probes were kept about 2h at 4°C).

----------08/21----------

Ligation and transformation of cells with of lysostaphin and pETDuet (1)

Mireia

Why is this document in the USER folder?

Item	Volume
Digestion reaction	10 µL
ATP (10 mM)	2 µL
DTT (100 mM)	2 µL
H_2O	4,5 µL
T4 Ligase (5U)	1 µL
Volumen total	20 µL

Item	Volume
Digestion reaction	10 µL
ATP (10 mM)	2 µL
DTT (100 mM)	2 µL
H_2O	4,5 µL
T4 Ligase (5U)	1 µL
Volumen total	20 µL

Conditions

Temperature °C	Time
25	1 h
On ice	30 min

Electroporation:

2 uL ligation reaction was pipetted into the electroporation cuvette. 100 uL electrocompetent cells were added. The cuvette was shaken horizontally.
Conditions: 1 mm cuvette (-20°C for 30 min before use), 1800 V.
After electroporation cells were incubated for 1h in LB at 37°C and 150 rpm and plated on LB Amp agar plates.

----------08/22----------

Colony PCR of pET-Duet + lysostaphin transformed *E. coli* (1)

Mireia & Karent :)

Step	Temperature °C	Time
Initial Denaturation	98	30 seconds
35 Cycles	98 60 72	10 seconds 30 seconds 1 min
Final Extension	72	5 min
Hold	4	

Component	1 reaction *(L)*	21 reaction (*L*)
Nuclease-Free water	6,6	138
5X phusion HF or GC Buffer	4	84
10 mM dNTPs	0,4	8,5
10 M Forward Primer	1	21
10 M Reverse Primer	1	21
Template DNA	1	---
X7 DNA- Polymerase 100X stock	2	42

Betaine 5M	4	84
Volumen total	20	398,5

Several electroporated cells were obtained on the ampicillin LB agar plates of both LysK and lysostaphin transformations.

Colony PCR of pET-Duet + lysostaphin transformed *E. coli* (1)

Mireia

This time taq master mix was used. Colonies were further diluted 10x.

Reaction composition:

Item	Volume for 1 reaction	Volume for 25 reactions
Taq Master Mix (2x)	5 uL	125 uL
Template	1 uL	25 uL
GP0001	1 uL	25 uL
GP0002	1 uL	25 uL
Betaine 5M	1 uL (0.5M)	25 uL
DMSO 50%	1 uL (10%)	25 uL
Total	10 uL	250 uL

Reaction conditions:

	Temperature	Time
Initial denaturation	95°C	1 min
35 cycles	95°C	15 sec
	60°C	30 sec
	72°C	45 sec
Final extension	72°C	5 min

Hold	4°C	Hold

Result: No amplification.

----------08/24----------

Digestion of enzybiotic sequences and/or pET-DUET (2)

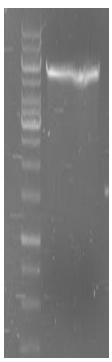
Mireia

Reaction composition:

Item	Volume for 1 reaction	Volume for 3 reactions
pETDuet (180 ng/uL)	6 uL	18 uL
EcoRI	0.5 uL	1.5 uL
PstI	0.5 uL	1.5 uL
Buffer H	2 uL	6 uL
H_2O	11 uL	33 uL
Total	20 uL	60 uL

Reaction conditions:
37°C (agar plate incubator) for 16 h.

08/25 → The total 60 uL digestion were loaded on a 40 mL, 0.8% agarose gel. It was run at 90 V 45 min. The 5 kb band was purified using Wizard SV Cleaner (Promega). It was obtained 50 uL of 18.0 ng/uL double digested pETDuet.



----------08/29----------

Digestion of enzybiotic sequences and/or pET-DUET (3)

Mireia

Item	Volume for 1 reaction	Volume for 3 reactions
pSB1C3-Lysostaphine (165,5 ng/uL)	6 uL	18 uL
EcoRI	0.5 uL	1.5 uL
PstI	0.5 uL	1.5 uL
Buffer H	2 uL	6 uL
H_2O	11 uL	33 uL
Total	20 uL	60 uL

Reaction conditions:

37°C (agar plate incubator) for 19

----------09/01----------

The total 60 uL digestion were loaded on a 25 mL, 0.75% agarose gel. It was run at 70 V 30 min. The 0.8 kb band was purified using Wizard SV kit (promega). It was obtained 50 uL of 18 ng/uL double digested Lysostaphine

----------09/27----------

Transformation of pET Duet and pSB1C3 Lysostaphin in *E.coli* DH5α strain

Sair

Heat shock protocol:

- The cells were thawned on ice for 15 minutes. 100-200 ng of plasmid were added and mixed in the competent cells tube. After that, the cells were put on ice for 30 minutes.
- The heat shock was performed putting the cells in a 42ºC water bath for 55 seconds and putting the cells back on ice for 5 minutes.

- 200 µL of LB media were added and the mix was incubated at 37ºC for 45 minutes before plating in pre-warmed LB agar plates with ampicillin. The plates were incubated at 37ºC overnight.

09/28 → 3 colonies were taken from each plate and inoculated in 10 mL of LB medium with 100 µg/mL of ampicillin. The inoculums were grown overnight at 37ºC and 200 rpm.
09/29 → The inoculums were pelleted by centrifugation at 5000 rpm for 10 minutes. The supernatant was discarded and the pellets were stored at -20ºC until plasmid purification.
09/30 → The plasmid purifications were done using the GeneJET Plasmid Miniprep Kit of Thermo Scientific.

----------09/30----------

Digestion of pET Duet and pSB1C3 Lysostaphin

Sair

Once the plasmids were ready, the digestion reactions were set as specified in the following tables.

Buffer R (Thermo Scientific)	2,0 µL
pSB1C3 Lysostaphin	8 µL
PstI	1 µL
EcoRI	1 µL
Water	8 µL

10/04 → The pET duet digestion was repeated following the conditions specified in the next table.

Buffer R (Thermo Scientific)	2,0 µL
pET Duet	16 µL
PstI	0,8 µL
EcoRI	0,8 µL
FastAP Alkaline Phosphatase	0,4 µL

----------10/08----------

Ligation of Lysostaphin in pET Duet

Sair

The ligation reactions were performed at 16ºC overnight following the conditions specified in the following table.

	Ligation 1	Ligation 2
Digested Lysostaphin (30 ng/μL)	4,5 μL	4 μL
Digested pET Duet (60 ng/μL)	2 μL	2,5 μL
Ligase buffer 10X	1 μL	1 μL
ATP 10mM	1 μL	1 μL
T4 ligase (NEB)	1 μL	1 μL
Water	0,5 μL	0,5 μL

10/10 → The ligation products were transformed in *E.coli* DH5α strain by heat shock. Seven transformations were made including controls as specified in the following table.

Control 1	Transformation with undigested pET Duet
Control 2	Transformation with digested pET Duet
Control 3	No transformation made
Ligation 1-1	Plating with 1X of the transformation inoculum
Ligation 1-2	Plating with 2X of the transformation inoculum
Ligation 2-1	Plating with 1X of the transformation inoculum
Ligation 2-2	Plating with 2X of the transformation inoculum

----------10/11----------

11/10 → It was observed growth in the plates as specified in the following table.

Control 1	+
Control 2	-
Control 3	-
Ligation 1-1	+
Ligation 1-2	++

Ligation 2-1	+
Ligation 2-2	++

Four colonies were taken from Ligation 1-2 and other four from Ligation 2-2 plates to make 10 mL inoculums. The inoculums were incubated at 37ºC and 200 rpm overnight. The next day, the inoculums were centrifuged and the pellets were stored at -20ºC until the Colony PCR was done.

----------10/13----------

Confirmation of the ligation reaction: Colony PCR

Sair

	1 reaction	10 reactions
10 X NEB Standard Taq Buffer	2,5 µL	25 µL
dNTPs 10mM	0,5 µL	5 µL
T7 promotor primer 10 µM	0,5 µL	5 µL
T7 terminator primer 10 µM	0,5 µL	5 µL
Taq Polymerase	0,125 µL	1,25 µL
Template	1 µL	-
DMSO	19,125 µL	191,25 µL
Water	0,75 µL	7,5 µL

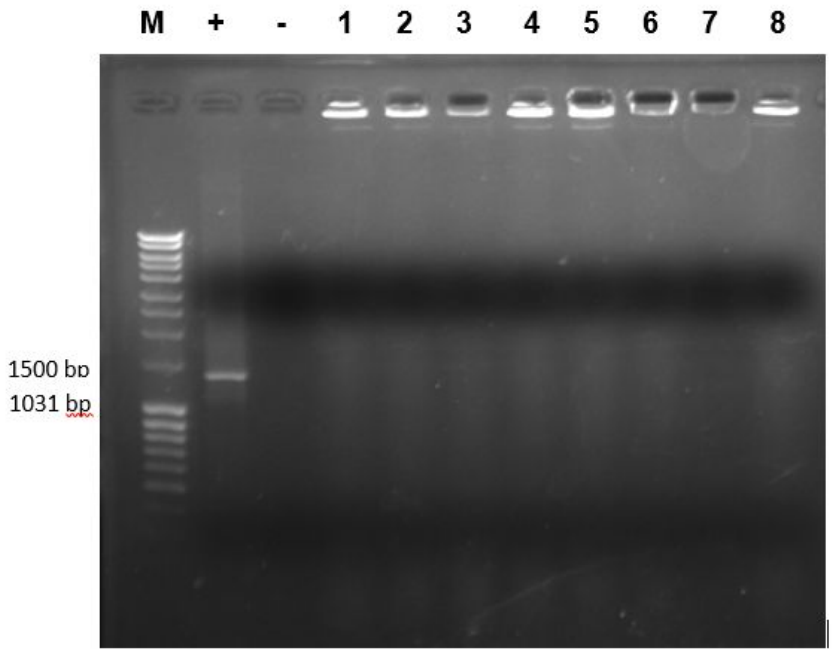
- 1 reaction for positive control (another template with known amplification)
- 1 reaction for negative control (no template)
- 8 reactions for each colony taken

PCR cycle

	95ºC x 5 min
30X	95ºC x 30 sec
	58ºC x 45 sec
	68ºC x 1 min 30 sec
	68ºC x 5 min

	4ºC forever

10/14 → Agarose gel electrophoresis of Colony PCR amplicons



Result: No amplification was observed.

Expression in microalgae

----------08/18----------

Digestion of enzybiotic sequences for cloning with *Chlamydomonas* expression vector (1)

Tiago

LysK, Lysostaphin and MV-L IDT sequences and Lysostaphin PCR product were digested overnight (10 hours) with XhoI and BamHI for cloning in the lab's *Chlamydomonas* expression vector.

BamHI-HF and XhoI from NEB, lab stock,
4U for 100ng of LysK (LK), Lysostaphin (LS) and MVL sequences(10 microliters)
4U for 330ng of Lysostaphin PCR product (L*, 20 microliters)
CutSmart Buffer

----------08/19----------

Ligation of enzybiotic sequences with *Chlamydomonas* expression vector (1)
Tiago

The enzybiotics digested in 08/18 were ligated directly (without purification) with XhoI/BamHI digested pJP22 (a expression plasmid for microalgae with Amp resistance and E. coli ori).

Ligation reaction:
9,5µL H2O
1,5µL CutSmart Buffer
2µL ATP 10mM
1µL (50ng) DNA vector (pJP22)
1µL T4 Ligase (NEB)
5µL digested inserts

Insert preparation:
5µL (50ng) of LysK and MV-L (separately), 30ng (3µL of IDT Lysostaphin) and ~ 40 ng (3µL of Lysostaphin PCR product) were used.
Times:
1 hour and 40 minutes at RT before transformation (and left for several hours at RT after, before freezing).

Transformation of ligation products of enzybiotic sequences with *Chlamydomonas* expression vector (1)

Tiago

5-DHalpha cells were transformed by the standard heat-shock protocol. T

----------08/25----------

Analytic digestion of putative clones with enzybiotic sequences with *Chlamydomonas* expression vector (1)

Tiago

Standard digestion protocol. About 600ng of DNA, EcoRI, PstI 2U each/reaction and Buffer H:

pJP22+LysK 2

pJP22+LysK 1

Or

pJP22+LysK 2 (3uL) with 3 U of XhoI and 3 U of BamHI, CS buffer, total 10 uL

pJP22+LysK 1 (3uL) with 3U of XhoI and 3 U of BamHI, CS buffer, total 10 uL

Resuls: a lot of smear and no detectabl bands in either.