iGEM 2016 – Microbiology – BMB – SDU

Project type: Bacteriocin **Project title:** IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag) **Sub project:** Purify bacteriocin Lacticin Q from *E. coli:*ER2566	**Creation date:** 2016.09.29 **Written by:** Brian Kenn Baltzar **Performed by:** Brian Kenn Baltzar & Cathrine Høyer Christensen

1. SOPs in use.

SOP number: SOP0007_v01 LA plates with antibiotic

SOP number: SOP0022_v01 Competent cell - freeze-stock

SOP number: SOP0023_v01 Ca^{++} transformation.

Plasmid purification kit

SOP number: SOP0001_v01 ON culture of *E.coli*

SOP number: SOP0004_v01 Bacterial freeze stock

SOP number: SOP0017_v01 Fast digest

SOP number: SOP0105_v01 Ligation

Gel purification kit

SOP number: SOP0021_v01 Colony PCR with MyTaq

phusion PCR

SOP number: SOP0009_v01 TSB transformation

iGEM 2016 SOP0028_v01 - Bradford Protein Concentration determination

iGEM 2016 SOP0025_v01 - Impact_Purification

iGEM 2016 SOP0027_v01 - MIC

2. Purpose.

To Purify bacteriocin Lacticin Q from *E. coli:*ER2566

3. Overview.

Day	SOPs	Experiments
1		Design primers with restriction sites for NdeI and SapI
2	SOP0010	Phusion PCR to give gene the correct restriction sites
2		Gel Electrophoresis to test if PCR were successful
2	Fermentas GeneJET Gel Extraction Kit	Purify pPCR product
3	SOP0025_v01	Impact purification, step. 8.1.1→ 8.1.4
4	SOP0021_v01	colony PCR to see which ligations were correct
4	SOP0025_v01	Impact purification, step. 8.1.5
5	SOP0025_v01	Impact purification, step. 8.1.6
6	SOP0025_v01	Impact purification, step. 8.1.7→ 8.1.8
7	SOP0025_v01	Impact purification, step. 8.2.1
8	SOP0025_v01	Impact purification, step. 8.2.2→ 8.2.3
9	SOP0025_v01	Impact purification, step. 8.2.4→ 8.3.9
10	SOP0025_v01	Impact purification, step. 8.4.1→ 8.4.2
10	SOP0028_v01	Bradford Protein Concentration Determination
10	SOP0025_v01	Impact purification, step. 8.4.3
11	SOP0027_v01	Determination of effect with MIC test.

4. Materials required.

Materials in use

Name	Components (Concentrations)	Manufacturer / Cat. #	Room	Safety considerations
Appropriate medium ex. LB	1% Tryptone 1% NaCl 0.5% Yeast extract	Oxoid Sigma-Aldrich Merck	Media lab or V18-40 5-0	
Glycerol	50 %	AppliChem	Anne Mette, RT	

LB		Anne-Mette		
LA	1% Tryptone 1% NaCl 0.5% Yeast extract 1.5% agar	Oxoid Sigma-Aldrich Merck Difco agar from BD	Anne-Mette Or V18-405-0	
Water	Demineralised milli-Q autoclaved water	Milli-Q water purification system (Millipore)	RT	Water
MyTaq™HS Red Mix	http://www.bioline.com/documents/product_inserts/MyTaq%E2%84%A2%20HS%20Red%20Mix.pdf#zoom=130	Bioline	V18-405a-2	
Reverse primer	Made specific to the template	Sigma-Aldrich		
Forward primer	Made specific to the template	Sigma-Aldrich		
Ligasebuffer		Agilent Technologies	Freezer at 1. Floor	
Ligase			Freezer 1. Floor	Ligase
FastDigest enzyme		Agilent Technologies	Freezer at 1. Floor	
Fast digest green / 10 x FastDigest Buffer		Agilent Technologies	Freezer at 1.	
CaCl,	0.1M		Chem room	
MgCl,	0.1M		Chem room	MgCl,
liquid nitrogen	liquid nitrogen	liquid nitrogen	liquid nitrogen	
Fast digest green		Agilent Technologies	Freezer at 1.	
6x DNA Loading Dye		GeneRuler	fridge floor 1	
Fort. LB		the new Anne-Mette	Autoclave room	

Polyethylene glycol (PEG) 3.350		Sigma Aldrich	Micro Chemical room
Dimethylsulfoxid (DMSO)		Sigma Aldrich	Micro Chemical room
Magnesiumchloride (MgCl2) 1M	1M	The New Anne-Mette	Autoclave
Microtiter plates			Chem Room
Tris-base			Chem Room
dH_2O			Chem Room
Tris-HCl 1M			Chem Room
NaCl 5M			Chem Room
EDTA 0,5M			Chem Room
Tween 20			Chem Room
DTT 1M			Chem Room
Bio-Rad Filter Columns			Chem Room
Bio-Rad Econo-Pac 10DG columns			Chem Room
Chitin Beads			Chem Room
Bio-Rad protein Assay solution			Special Buy
BSA 1 mg/ml			Chem Room

5. Other

As competent cells, LB and LA media was used by all parts of our project and not just this protocol the dates for use of these SOPs are not added. This comment deal with SOP number: SOP0007_v01 and SOP0022_v01

Gel Electrophoresis is set at 75 V for 30-45 minutes, dependent on the gel percentages.

6. Experiment history.

<table>
<tr><th>Date (YY.MM.DD)</th><th>SOPs</th><th>Alterations to SOPs and remarks to experiments</th></tr>
<tr><td>16.08.25</td><td>SOP0010_v01</td><td>Phusion PCR (pPCR) using designed primers with NdeI and SapI restriction sites. Total Volume: 50 µl

Following program was used:

<table>
<tr><td>Cycle</td><td>ºC</td><td>Time (min)</td></tr>
<tr><td>Initial denature</td><td>98</td><td>00:30</td></tr>
<tr><td>Denature</td><td>98</td><td>00:05</td></tr>
<tr><td>Anneal</td><td>69</td><td>00:20</td></tr>
<tr><td>Extention</td><td>72</td><td>00:15</td></tr>
<tr><td>Final extention</td><td>70</td><td>10:00</td></tr>
<tr><td>Hold</td><td>4</td><td></td></tr>
</table>
</td></tr>
<tr><td>16.08.25</td><td>Gel Electrophoresis</td><td>To see if pPCR was successful</td></tr>
<tr><td>16.08.25</td><td>Plasmid purification</td><td>Plasmid purification of pTXB1 our vector.</td></tr>
<tr><td>16.08.29</td><td>Gel purification</td><td>The pPCR product is purified using Fermentas GeneJET Gel Extraction Kit</td></tr>
<tr><td>16.09.02</td><td>SOP0025_v01</td><td>Impact_Purification step. 8.1.1
<table>
<tr><td></td><td>Insert (purified pPCR product)</td><td>pTXB1(Vector)</td></tr>
<tr><td>H2O</td><td>15.5µl</td><td>9.5µl</td></tr>
</table>
</td></tr>
</table>

Buffer	2µl	2µl
DNA	10µl	5µl
NdeI	1.5µl	1.5µl
SapI	1µl	1µl
FastAP	---	1µl

The reaction stood for 7 hours at 37ºC

16.09.03	SOP0025_v01	Impact_Purification step. 8.1.3 → 8.1.4 *Ligation alterations:* T7 ligase buffer: 10µl T7 ligase: 1µl DNA insert: 1, 2, 20, 0µl Vector: 4µl H2O: 14, 13, 15, 0 µl
16.09.08	SOP0025_v01	Impact_Purification step. 8.1.5 *Transformation deviations:* 5µl ligation mixture were used.
16.09.09	SOP0025_v01	Impact_Purification step. 8.1.6→ 8.1.7
16.09.12	SOP0025_v01	Impact_Purification step. 8.1.8→ 8.1.9
16.09.12	SOP0025_v01	Impact_Purification step. 8.2.1 1 ml. cell culture *E. Coli*:ER2566 and 2µl were used
16.09.21	SOP0025_v01	Impact_Purification step. 8.2.2 → 8.2.3
16.09.15	SOP0025_v01	Impact_Purification step. 8.2.4 → 8.2.7
16.09.23	SOP0025_v01	Impact_Purification step. 8.2.8 → 8.3.9
16.09.24	SOP0025_v01	Impact_Purification step. 8.4.1
16.09.25	SOP0028_v01	Bradford Protein Concentration determination
16.09.25	SOP0025_v01	Impact_Purification step. 8.4.3 *Aliquots #1, #2, #4, #5 and #6 were pooled*
16.09.27	SOP0027_v01 - MIC	MIC step. 7.2
16.09.28	SOP0027_v01 - MIC	MIC step. 7.3 → 7.9 *The bacteriocin were diluted 2 fold from row A G, starting with a concentration of 22,2* µg/ml *and ending at 0* µg/µl in row G The layout of the microtiter plates were as follows:

Column:	
1	Mueller-Hinton broth
2	Mueller-Hinton broth
3	*S. aureus*:CC398
4	*S. aureus*:CC398
5	*S. aureus*:USA300
6	*S. aureus*:USA300
7	*S. aureus*:hVISA
8	*S. aureus*:hVISA
9	*P. aeruginosa:*PAO1
10	*P. aeruginosa:*PAO1

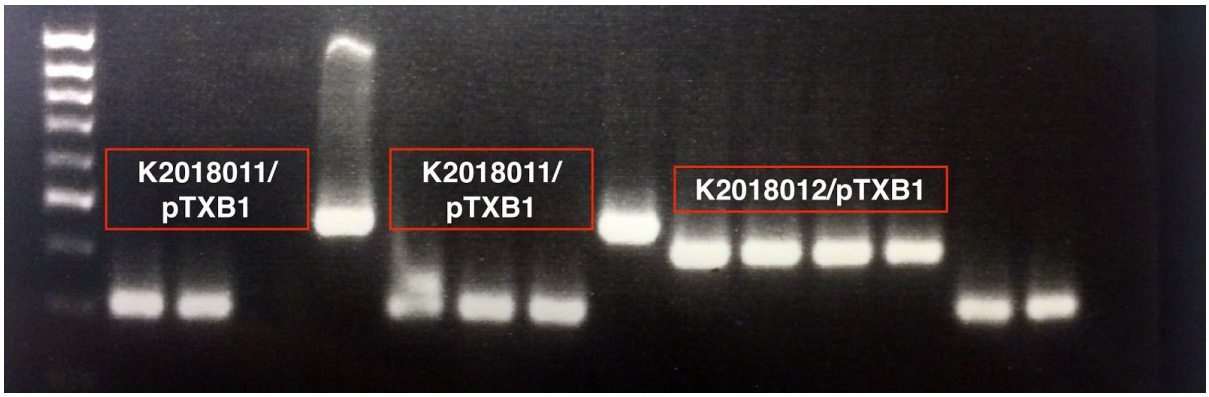
7. Sample specification.

Sample name	Sample content	From	Used for / Saved where
BR81	pSB1C3:k2018012	freeze stock	
BR174	pTXB1/K2018012		
BR126	*E. Coli*:K12/pTXB1	MGJ strains freezer	IMPACT vector
BY153	K2018012 with IMPACT overhangs		IMPACT purification

8. Remarks on setup.

9. Results and conclusions.

- The following gel image were used to verify that K2018012 were successfully cloned into pTXB1 (the IMPACT vector). Lane 9-12 is K2018012/pTXB1 and they all showed the correct sized band.



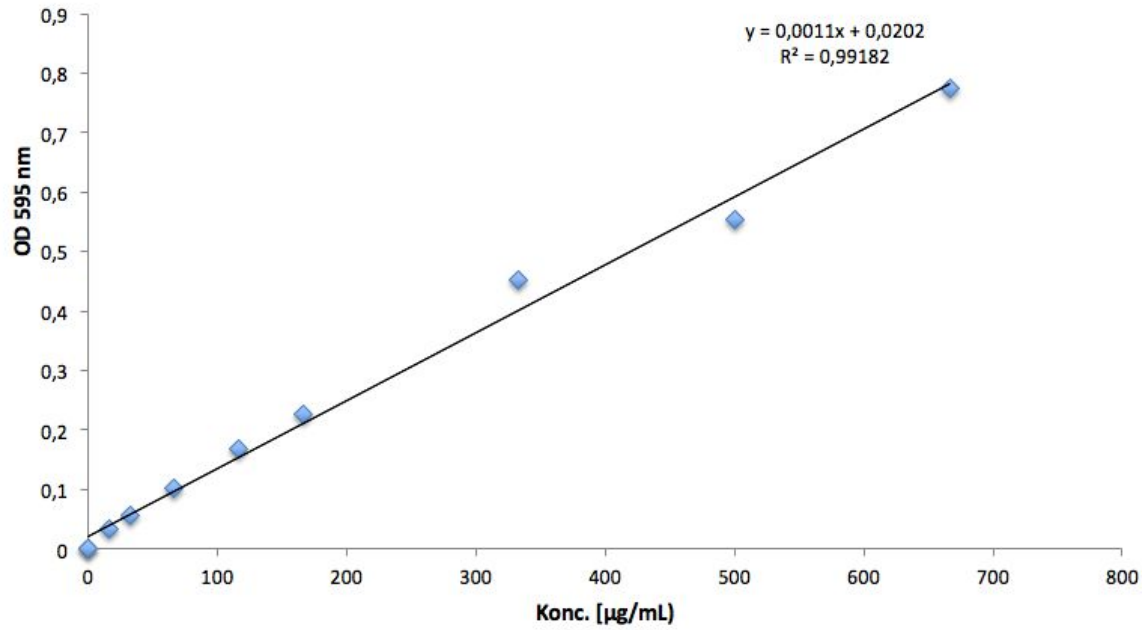
- The Bradford assay: We measured OD_{595}= 0,06900, together with the BSA standard curve (see appendix) we calculated a final protein concentration as being 44,4 µg/ml

- The MIC assay gave the following MIC values:

Strain	MIC (µg/ml)
S. aureus:CC398	22,2
S. aureus:USA300	22,2
S. aureus:hVISA	22,2
*P. aeruginosa:*PAO1	>22,2

Based on the above we concluded that our bacteriocin (Lacticin Q) were successfully expressed and purified using the IMPACT system.
Furthermore we showed that our recombinant bacteriocin had antimicrobial effect on three multiresistant *S. aureus* strains.
We also made a control MIC where we treated the same strains with ampicillin (2000µg/ml → 0µg/ml) and chloramphenicol (5000µg/ml → 0µg/ml), no strains were inhibited which confirms that the strains is resistant.

10. Appendixes



Bradford Assay standard BSA protein curve.