Subtilin immunity: spaIFEG

Purpose and Justification:

We aim to use a subtilin-based cell signalling system to trigger calcium carbonate precipitation and filament formation once our bacteria have reached a sufficient density inside a microcrack.

Subtilin is a lantibiotic peptide produced naturally by *Bacillus subtilis* ATCC 6633 but not by *Bacillus subtilis* 168. We aim to produce two BioBricks: one for production of subtilin, and, since subtilin is a lantibiotic, one for subtilin immunity. A subtilin-sensing BioBrick was produced by Newcastle's 2008 iGEM team.

The *spaIFEG* gene cluster encodes two separate subtilin immunity systems, both present in ATCC 6633. Together they provide a high degree of subtilin resistance.

Construction:

By Gibson method

Parts:

1. pVeg_SpoVG – Pveg promotor and spoVG RBS BioBrick minus its suffix. Pveg is a constitutive promoter that contains binding sites for the *B. sutbilis* major sigma factor, Sigma factor A. SpoVG is a strong RBS. This is BioBrick Bba_K143053 on the parts registry.
2. spaIFEG – The subtilin immunity gene cluster. This sequence was taken from Genbank, accession number U09819.1 (7548-10299).
3. Double_Terminator - A double terminator BioBrick, minus its BioBrick prefix. This is BioBrick BBa_B0014 on the parts registry.

The BioBrick prefix from pVeg_SpoVG and the suffix of Double_Terminator are used.

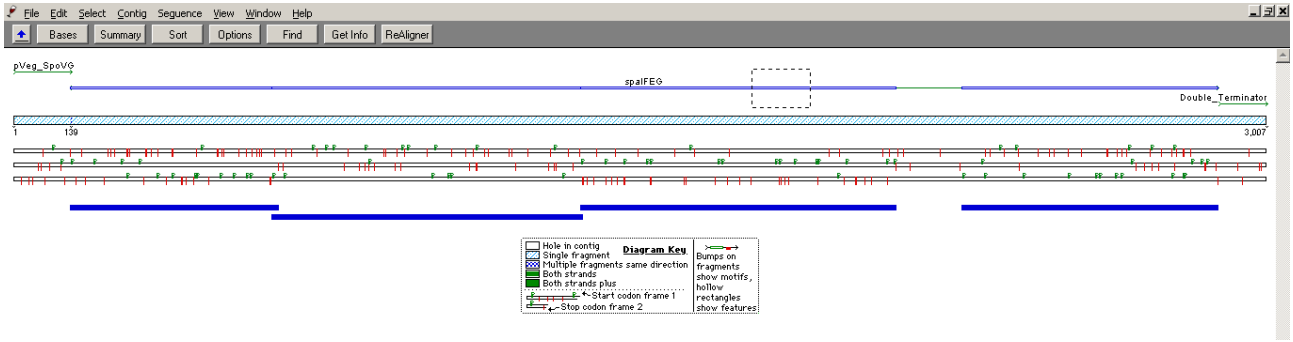

Figure 1: Screenshot of the Subtilin Immunity BioBrick components in Sequencher

The BioBrick is to constructed using the Gibson method (*Enzymatic assembly of DNA molecules up to several hundred kilobases*, Gibson et al.). Overlapping PCR products will be joined together to form the final construct shown in Fig 1.

The *spaIFEG* gene cluster does not contain any BioBrick restriction sites (see Fig 2.), making the above construct BioBrick-compatible.

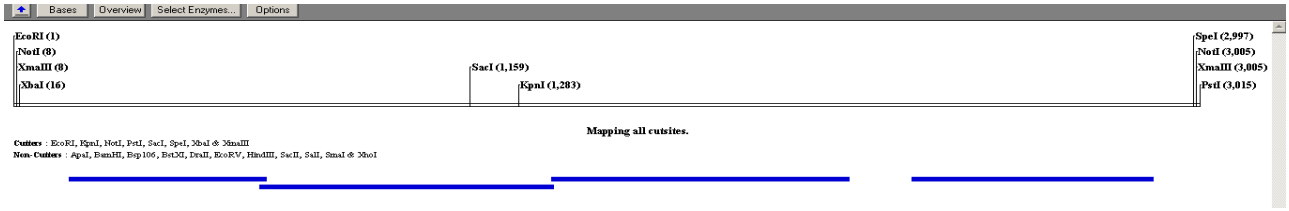

Figure 2: A restriction map of the construct, showing that the BioBrick restriction sites are only present in the prefix and suffix.

Below (Figure 3.), the primers we are using are shown. In total there are 4 pairs:

1. Primer 1-P2 and Primer 2-P2 – Promoter and RBS PCR product. Primer 1-P1 includes as an extension the standard BioBrick prefix. Primer 2-P2 includes as an extension the TACTAG post RBS spacer and 10bp of the start of the *spaIFEG* gene cluster. This product overlaps with product 2, below.

2. Primer 1-S1 and Primer 2-S1 – The *spaIFEG* gene cluster product. Primer 1-S1 includes as an extension 10bp of the end of promoter and RBS sequence. Primer 2-S1 includes as an extension 10bp of the start of the double terminator sequence. This product overlaps with product 1 above, and product 3, below.

3. Primer 1-T1 and Primer 2-T1 – Double terminator PCR product. Primer 1-T1 includes as an extension 10bp of the end of the *spaIFEG* gene cluster sequence. Primer 2-T1 includes as an extension the standard BioBrick suffix. This product overlaps with product 3, above.

4. Primer 1-V1 and Primer 2-V1 – Vector primers. Used for cloning into a BioBrick-compatible plasmid, again using the Gibson method (see *Procedure*, below). Primer 1-V1 is the standard BioBrick suffix, and Primer 2-V1 is the standard BioBrick prefix. By performing PCR on a BioBrick-compatible vector with these primers, you are left with a product which overlaps with the Subtilin Immunity BioBrick, allowing cloning by the Gibson method.

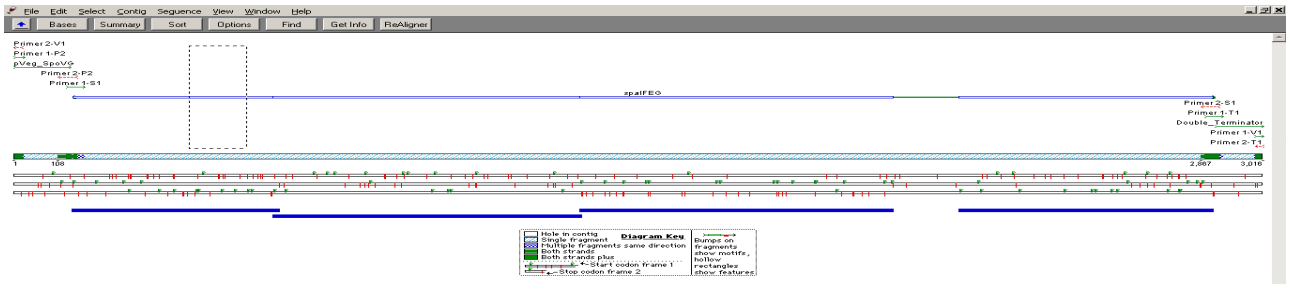

Figure 3: Primers shown to be complementary to the *spaIFEG* gene cluster

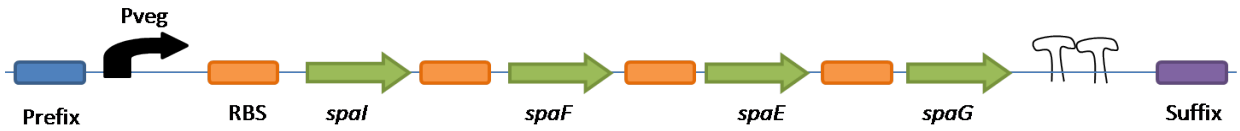

Figure 4: Map of the Subtilin Immunity BioBrick

Procedure:

1. Take 4 PCR tubes and label them 1-4.

2. The part which would be amplified in each tube are as following:

Tube	Part to be amplified
1	Plasmid vector
2	Promoter and RBS
3	*spaIFEG* gene cluster
4	Double terminator

Table 1: Table represents the tubes which would be containing the parts which are going to be amplified.

3. Add the PCR reagents as mentioned below in the tubes mentioned above:

Number	Reagents	Volume
1	Distilled H_2O	27.5 µl
2	5x PCR Buffer	10 µl
3	Nucleotide DNTPs	1 µl
4	Forward primer	5 µl
5	Backward primer	5 µl
6	Phusion Polymerase	0.5 µl
7	Template DNA*	1 µl
Total Volume	-	50 µl

* The source for the template DNA for the PCR reaction is mentioned in Table 3.

Table 2: Table represents the reagents which would be added in each tubes for the Phusion PCR reaction.

Now, the forward and reverse which should go into each PCR tube is:

Tube	Part to be amplified	DNA from which part is extracted	Forward Primer	Reverse Primer	Tm (in °C)
1	Plasmid vector	pSB1C3	Primer 1-V1	Primer 2-V1	58.3 and 58.6
2	Promoter and RBS	BioBrick Bba_K143053	Primer 1-P1	Primer 2-P2	59.9 and 56.7
3	*spaIFEG* gene cluster	*B. subtilis* ATCC 6633	Primer 1-S1	Primer 2-S1	51.1 and 56.2
4	Double terminator	BioBrick BBa_B0014	Primer 1-T1	Primer 2-T1	55.9 and 57.6

Table 3: Table represents a) Tubes containing fragments and the DNA source which is used for the amplification of the part. b) Primers which are to be used for each fragment and c) Melting temperatures for each PCR reaction.

4. Set the conditions as mentioned below for the thermocycler as following after putting the PCR tubes into the PCR wells present on the thermocycler:

Step	Condition	Time
Initialise	98°C	30 seconds
Denature*	98°C	10 seconds
Anneal*+	Depends on the melting temperature of the primers	20 seconds
Extension*$	72°C	Depends on the length of the fragment which is to be amplified and the rate of activity of the polymerase.
Extension finish	72°C	5-10 minutes
Hold	4°C	N/A

Table 4: Table represents the conditions in which the thermocycler should be set up for every reaction.
***** Remember to set these steps for 30 cycles.
+ Melting temperatures of all the primers are mentioned in Table 3.
$ Extension time is mentioned for all the 6 fragments in Table 5.

6. After the completion of the PCR reaction, run Gel electrophoresis (0.7 % agarose) to check whether the PCR reaction has worked.

7. Extract the band (Refer to the gel extraction protocol) of the right size after referring to the DNA ladder in the first lane.

Tube	Part to be amplified	Size of the fragment (in bp)	Extension Time (in seconds)*
1	Plasmid vector	2072	70
2	Promoter and RBS	155	15
3	*spaIFEG* gene cluster	2781	110
4	Double terminator	140	15

Table 5: Table represents the size of the fragments which would be achieved at the end of the PCR and the extension time required for each fragment during the reaction.

8. Set up a single PCR tube for the final step for the Gibson cloning. This tube would contain the 4 amplified DNA fragments from above and would be annealed in the subsequent steps.

9. For the Gibson cloning method, the following volumes of the chemicals are required:

(A) 1.33X Master Mix

Reagents	Volumes
5X isothermal buffer	100 µl
T5 exonuclease 1.0 U/ µl	2 µl
Phusion DNA pol 2 U/ µl	6.25 µl

Taq DNA ligase 40 U/ µl	50 µl
H_2O	216.75 µl
Total Volume*	375 µl

*This volume makes 25 aliquots of 15 µl each.

Table 6: Table represents the reagents and their concentrations required in the preparation of 1.33X Master Mix for the Gibson reaction.

(B) 5X Isothermal Reaction Buffer

Reagents	Volumes
25% PEG-8000	0.75 g
500 mM Tris-HCl pH 7.5	1.5 ml
50 mM $MgCl_2$	75 µl
50 mM DTT	150 µl
1 mM dATP	30 µl
1 mM dTTP	30 µl
1 mM dCTP	30 µl
1 mM dGTP	30 µl
5 mM NAD	300 µl
H_2O	105 µl
Total Volume	3 ml

Table 7: Table represents the reagents and their concentrations required in the preparation of 5X Isothermal Buffer for the Gibson reaction.

(C) Final Volume in the PCR Tube

Reagents	Volumes
Template DNA (consisting of all 4 parts which were amplified from the PCR reaction mentioned above)	5 µl
1.33 X Master Mix	15 µl
Total Volume	20 µl

* Remember to do this step very quickly as everything has to be done on ice.

Table 8: Table represents the reagents and their concentrations required in the preparation of the final volume in the PCR tube for the Gibson reaction.

10. Incubate the cells at 50°C for 60 minutes for the reaction to take place.

11. At the end, after the completion of the PCR reaction, run Gel electrophoresis (0.7 % agarose) to check whether the PCR reaction has worked. If the Gibson cloning has worked then you should get a band of approximately 5054 bp on the gel.

Integration:

The fragment is to be cloned into the BioBrick-compatible version of pGFPrrnB and integrated into the *Bacillus subtilis* 168 chromosome at *amyE*.

Testing and Characterisation:

Selection for integration

To select for integration of the plasmid into the chromosome, *B. subtilis* will be tested for the ability to hydrolyse starch. Integration of the BioBrick will be done by homologous recombination at the *amyE* gene, therefore destroying endogenous expression of amylase. Colonies that are not able to break down starch on agar plate will be selected and cultured for further test. Colonies that do not contain the integrated BioBrick will be able to hydrolyse starch, therefore forming a white halo around the colony as iodine interacts with starch to form blue colour.

Isolation of subtilin from *B. subtilis* ATCC6633

1. B. subtilis ATCC6633 were grown in 250 ml flasks containing 100 ml of BHI broth and incubate for 48 h at 32°C in shaker at 125 cycles/min.

2. Culture media were centrifuged at 10000g for 15 min and the supernatants were filtered through 0.22 µm membrane.

3. The pH of the filtrates has to be between 7.0 to 8.0 pH.

5. Store the filtrates at -20°C for further use.

Protocol adapted from: A.S. Motta, F. Cladera-Olivera and A. Brandelli (2004) Screen for antimicrobial activity among bacteria isolated from the amazon basin. *Brazilian Journal of Microbiology* 35, 307-310.

Characterisation of BioBrick

1. Make up 4 LB agar plates and inoculate with the following organism:
    1. Negative Control – *B. subtilis* 168
    2. Positive Control – *B. subtilis* ATCC6633
    3. Test (Duplicates) – *B. subtilis* 168 containing subtilin immunity BioBrick
2. Apply 20 µl of the cell free filtrate containing subtilin onto cellulose disks (6 mm) and place onto the LB agar plates.
3. Plates were incubated for 24 h at 37 °C.
4. Zone of inhibition were then measured.

Protocol adapted from: A.S. Motta and A. Brandelli. (2002) Characterization of an antibacterial peptide produced by *Brevibacterium linens*. *Journal of Applied Microbiology* 92, 63-70.

Expected results:

1. Negative control – Zone of inhibition to be observed
2. Positive control – Zone of inhibition not observed
3. Test (Duplicates) – Zone of inhibition not observed or a smaller zone of inhibition as compared to the negative control

Note: See figure 7 for comparison

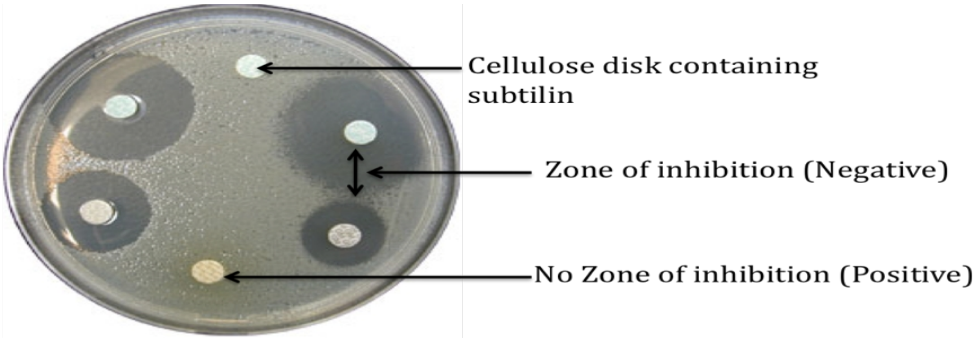

Figure 7: Sample picture showing the zone of inhibition.