Swarming: *sfp* and *swrA*

Purpose and Justification:

It is known that *Bacillus subtilis* 168 is unable to swarm on a solid surface e.g., agar plate or concrete. This is due to two issues:

1. Strain 168 is unable to produce surfactin, a natural surfactant which helps in reducing surface tension and aids bacteria to swarm on a solid or semi solid medium. This is because the 168 chromosome contains a frameshift mutation in the gene *sfp*. In *Bacillus subtilis* 3610 the *sfp* gene is intact.

2. Strain 168 is also unable to bio-synthesize flagella. In the undomesticated strain 3610, the gene *swrA* helps in the bio-synthesis of flagella. The laboratory strain 168 again contains a fameshift mutation in the gene *swrA* and thus the stain moves on the surface at a very slow pace.

We plan to produce a BioBrick with these two genes.

Construction:

By Gibson method

Parts:

1. Pveg_SpoVG – Pveg promotor and spoVG RBS BioBrick including its prefix. 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. Post_RBS_spacer1 – TACTAG spacer between the RBS and *sfp* coding sequence.

3. sfp – *sfp* coding sequence. This is from GenBank, accession number EU146075.1.

4. SpoVG - SpoVG is a strong RBS. This is BioBrick Bba_K143021 on the parts registry.

5. Post-RBS-spacer2 – TACTAG spacer between the RBS and *swrA* coding sequence.

6. swrA – *swrA* coding sequence. This is from GenBank, accession number EU146080.1.

7. Double_Terminator – A double terminator BioBrick including its suffix. This is BioBrick BBa_B0014 on the parts registry.

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

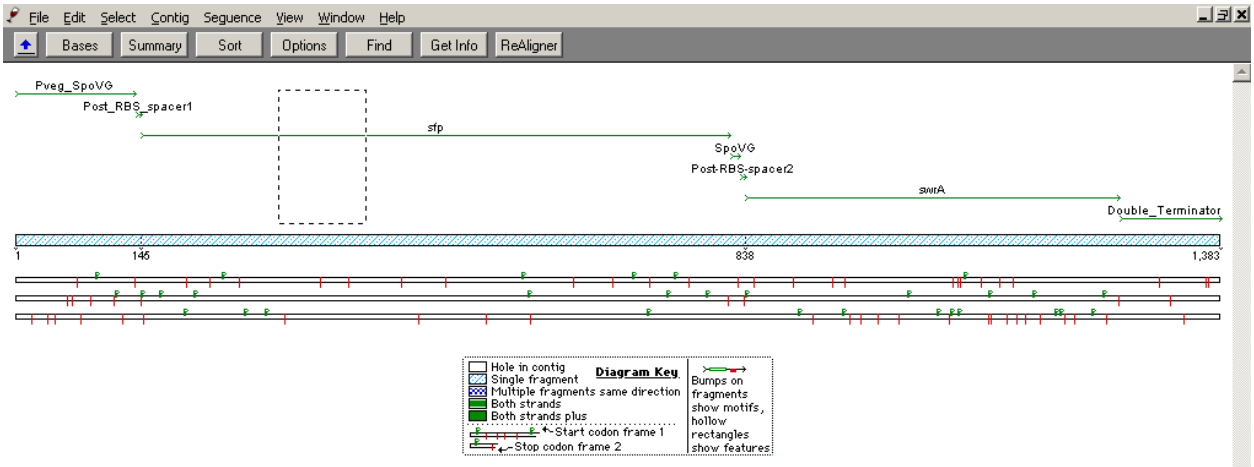

Fig 1. The Subtilin Immunity BioBrick components

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 2.

The *sfp* and *swrA* genes do not contain any BioBrick restriction sites (see Fig 2.), making the above construct BioBrick-compatible.

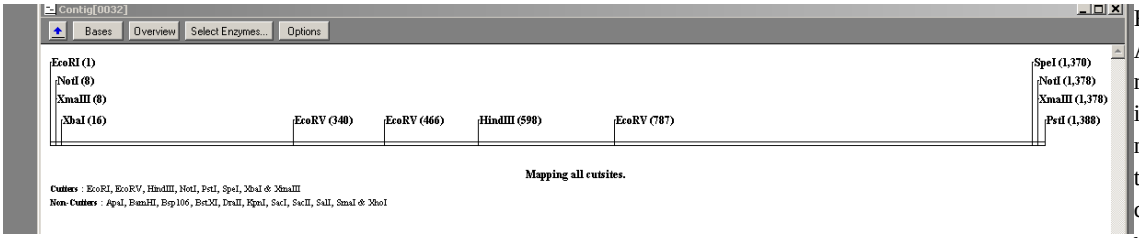

Fig 2. A restriction map of the construct, showing that the BioBrick restriction sites are only present in the prefix and suffix.

Below (Fig 3.), the primers we are using are shown. Including vector primers there are five pairs:

1. Pveg_SpoVG forward and reverse – Promoter and RBS PCR product. Reverse primer includes as an extension the TACTAG post RBS spacer and the beginning of the *sfp* coding sequence. This product overlaps with product 2, below.

2. *sfp* forward and reverse – *sfp* coding sequence PCR product. Reverse primer includes as an overlap the SpoVG RBS and post RBS spacer. This product overlaps with product 1 above, and product 3, below.

3. *swrA* forward and reverse – *swrA* coding sequence PCR product. Overlaps with product 2 above, and product 4, below.

4. Term forward and reverse – Double terminator PCR product. Overlaps with product 3, above.

5. Vector primers. Used for cloning into a BioBrick-compatible plasmid, again using the Gibson method (see *Procedure*, below).

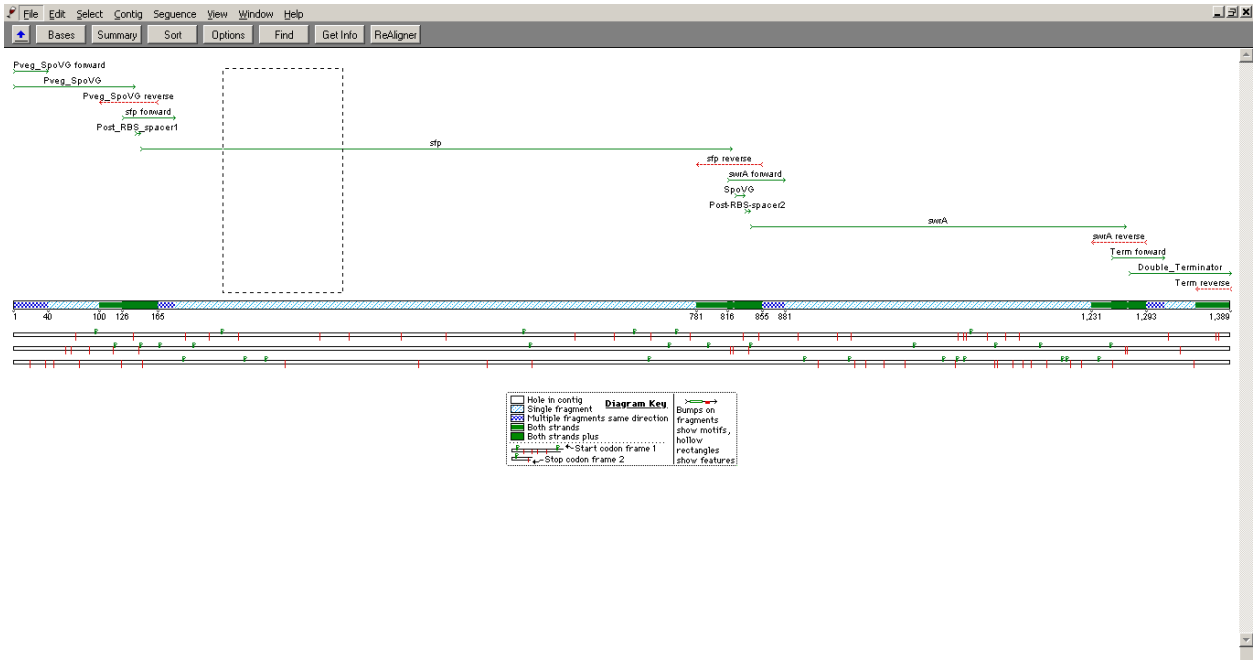

Fig 3. Primers shown to be complementary to the Swarming BioBrick

Procedure:

1. Take 5 PCR tubes and label them 1-5.
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 (Pveg_SpoVG)
3	*sfp* gene
4	*swrA* gene
5	Double terminator

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

3. Add 1 µl of the plasmid (template DNA) extracted from *E. coli* DH5α into all the tubes.
4. 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

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	Plasmid pSB1C3	P1 V1 forward	P2 V1 reverse	
2	Promoter and RBS	BioBrick Bba_K143053 (on parts registry)	Pveg_SpoVG forward	Pveg_SpoVG reverse	
3	*sfp* gene	*B. subtilis* 3610	*sfp* forward	*sfp* reverse	
4	*swrA* gene	*B. subtilis* 3610	*swrA* forward	*swrA* reverse	
5	Double terminator	pSB1AK3 consisting BBa_B0014	Term forward	Term reverse	

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.

5. 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 (1% 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		
2	Promoter and RBS (Pveg_SpoVG)		
3	*sfp* gene		
4	*swrA* gene		
5	Double terminator		

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	Stock
25% PEG-8000	0.75 g	Powder
500 mM Tris-HCl pH 7.5	1.5 ml	1M
50 mM $MgCl_2$	75 µl	2M
50 mM DTT	150 µl	1M
1 mM dATP	30 µl	100mM
1 mM dTTP	30 µl	100mM
1 mM dCTP	30 µl	100mM
1 mM dGTP	30 µl	100mM
5 mM NAD	300 µl	50mM

H_2O	105 µl	N/A
Total Volume	3 ml	N/A

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.

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.