Enhanced Arginase production: *rocF*

Purpose and Justification:

Bacillus subtilis produces urease, which catalyses the hydrolysis of urea into ammonium and carbonate. Since the cell wall of the bacteria is negatively charged, the bacteria draw cations from the environment, including Ca^{2+}, to deposit on their cell surface. The Ca^{2+} ions subsequently react with the CO_3^{2-} ions, leading to the precipitation of $CaCO_3$ at the cell surface.

In order for *B. subtilis* to fill up cracks in concrete, enhanced production of calcium carbonate must be achieved.

Previous experiments involving up-regulating *ureA*, *ureB* and *ureC* in *B. subtilis* have not lead to an increase in urease production. This could be due to yet unidentified genes that are involved in the process. Therefore we are looking at another strategy, of increasing urea hydrolysis indirectly by increasing arginine and arginase production. Arginase breaks down arginine to urea and ornithine, leading to an increase of urea inside the cell. We believe that in turn the urea itself will increase urease production. By targeting this pathway, it will hopefully be able to activate additional unknown pathways and enzymes that are involve in calcium carbonate production.

We plan to produce two BioBricks, *SR1*, which will enhance arginine production (please see the *SR1* BioBrick cloning strategy), and *rocF*, which will enhance arginase production.

The naturally occurring gene *rocF* codes for the protein arginase.

Modelling:

We have done computational modelling of our system. The graph below shows that the final output of the pathway, carbonate, increases as time progresses.

Details of this model can be found on the wiki.

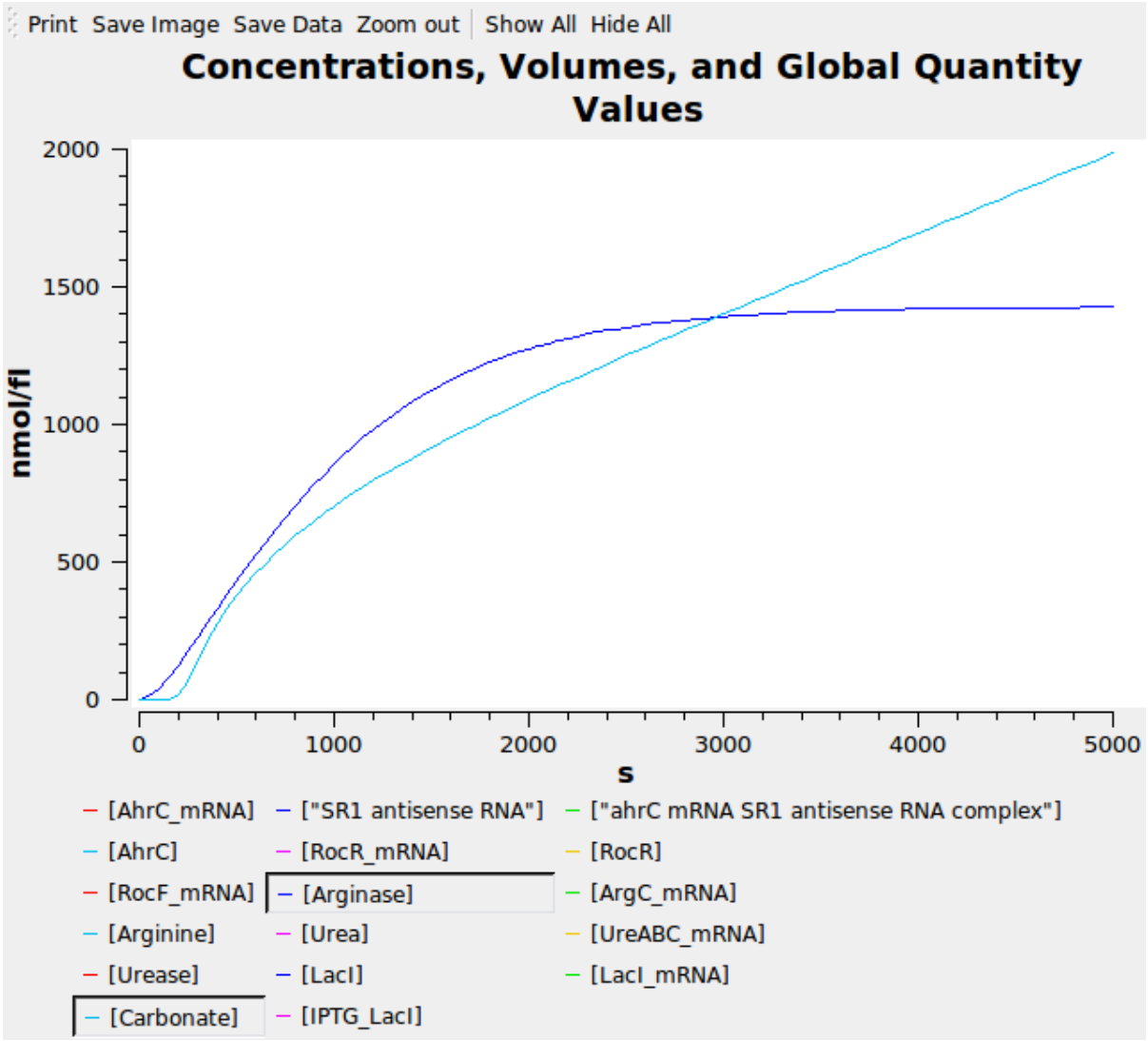


Fig 1. A graph showing the final output of the biochemical pathway, carbonate, increases over time

Construction:

By Gibson method

Parts:

1. 01bbprefix - The standard BioBrick prefix.
2. 03pspac_oid – The Pspacoid promoter including the oid operator, repressed by *lacI*. Allows us to test the brick by adding IPTG. This sequence is from part Bba_K174004 on the Parts Registry.
3. 05rocF_cds - The coding sequence for *rocF*. This coding sequence is from GenBank (X81802.1).
4. 06dbTerm09 - A double terminator. This is part BBa_B0014 on the parts registry.
5. 07bb_suffix – The standard BioBrick suffix.

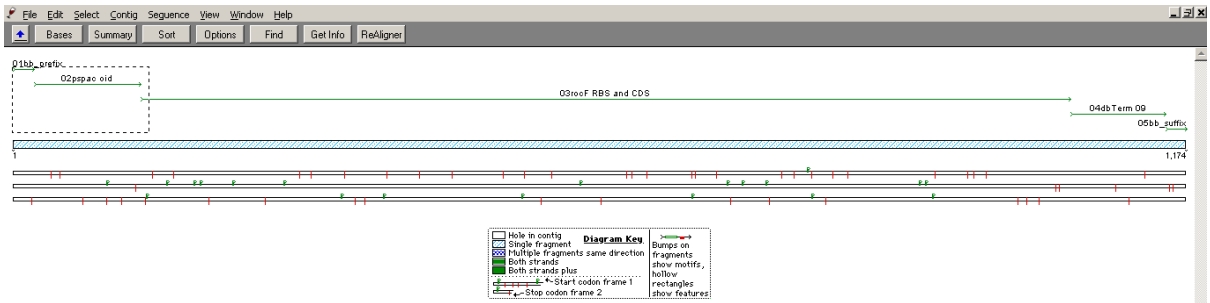


Fig 2. Screenshot of the *rocF* BioBrick components in Sequencher

The BioBrick is going to be 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 *rocF* coding sequence has two EcoR1 restriction sites which must be removed if the construct is to be BioBrick compatible (see Fig 3.). In order to do this, we will PCR the *rocF* coding sequence as three overlapping products and join them using the Gibson method. Our primers will have single base changes which preserve the amino acid sequence but make the coding sequence BioBrick-compatible (see Fig 4. , 5. and 6.).

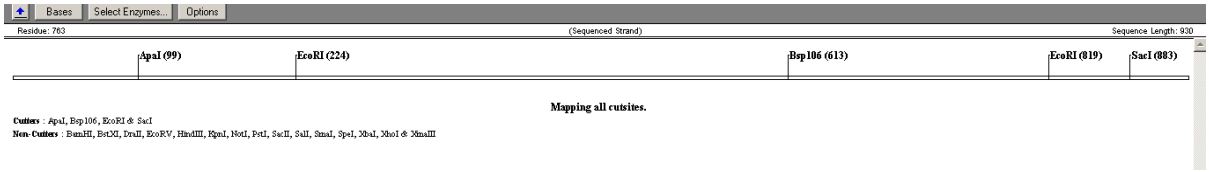
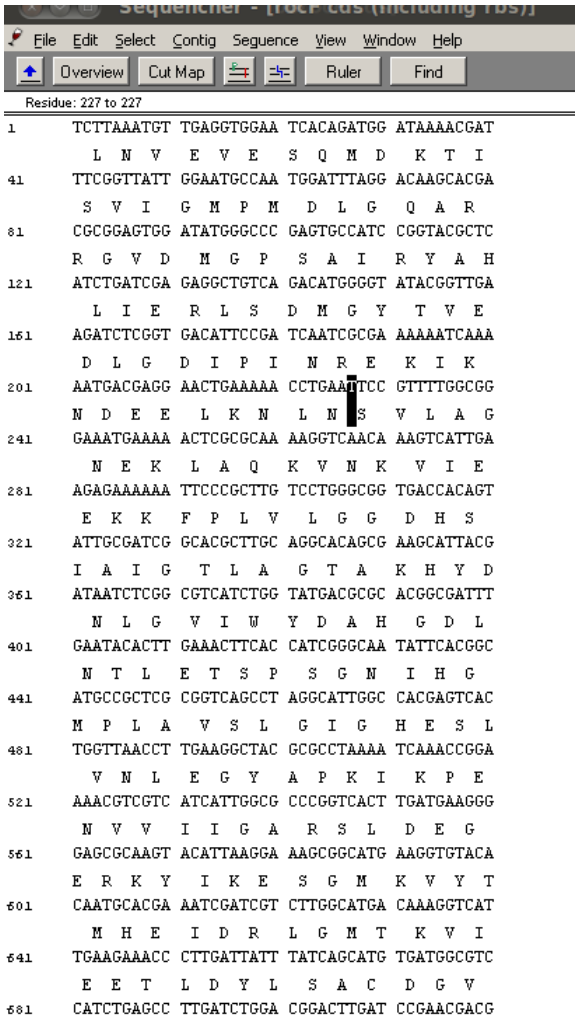


Fig 3. A restriction map of the *rocF* coding sequence, showing two EcoR1 sites we must remove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L N V E V E S Q M D K T I
41 TTCGGTTATT GGAATGCCAA TGGATTTAGG ACAAGCACGA
S V I G M P M D L G Q A R
81 CGCGGAGTGG ATATGGGCCC GAGTGCCATC CGGTACGCTC
R G V D M G P S A I R Y A H
121 ATCTGATCGA GAGGCTGTCA GACATGGGGT ATACGGTTGA
L I E R L S D M G Y T V E
161 AGATCTCGGT GACATTCCGA TCAATCGCGA AAAAATCAAA
D L G D I P I N R E K I K
201 AATGACGAGG AACTGAAAAA CCTGAATCC GTTTTGGCGG
N D E E L K N L N S V L A G
241 GAAATGAAAA ACTCGCGCAA AAGGTCAACA AAGTCATTGA
N E K L A Q K V N K V I E
281 AGAGAAAAAA TTCCCGCTTG TCCTGGGCGG TGACCACAGT
E K K F P L V L G G D H S
321 ATTGCGATCG GCACGCTTGC AGGCACAGCG AAGCATTACG
I A I G T L A G T A K H Y D
361 ATAATCTCGG CGTCATCTGG TATGACGCGC ACGGCGATTT
N L G V I W Y D A H G D L
401 GAATACACTT GAAACTTCAC CATCGGGCAA TATTCACGGC
N T L E T S P S G N I H G
441 ATGCCGCTCG CGGTCAGCCT AGGCATTGGC CACGAGTCAC
M P L A V S L G I G H E S L
481 TGGTTAACCT TGAAGGCTAC GCGCCTAAAA TCAAACCGGA
V N L E G Y A P K I K P E
521 AAACGTCGTC ATCATTGGCG CCCGGTCACT TGATGAAGGG
N V V I I G A R S L D E G
561 GAGCGCAAGT ACATTAAGGA AAGCGGCATG AAGGTGTACA
E R K Y I K E S G M K V Y T
601 CAATGCACGA AATCGATCGT CTTGGCATGA CAAAGGTCAT
M H E I D R L G M T K V I
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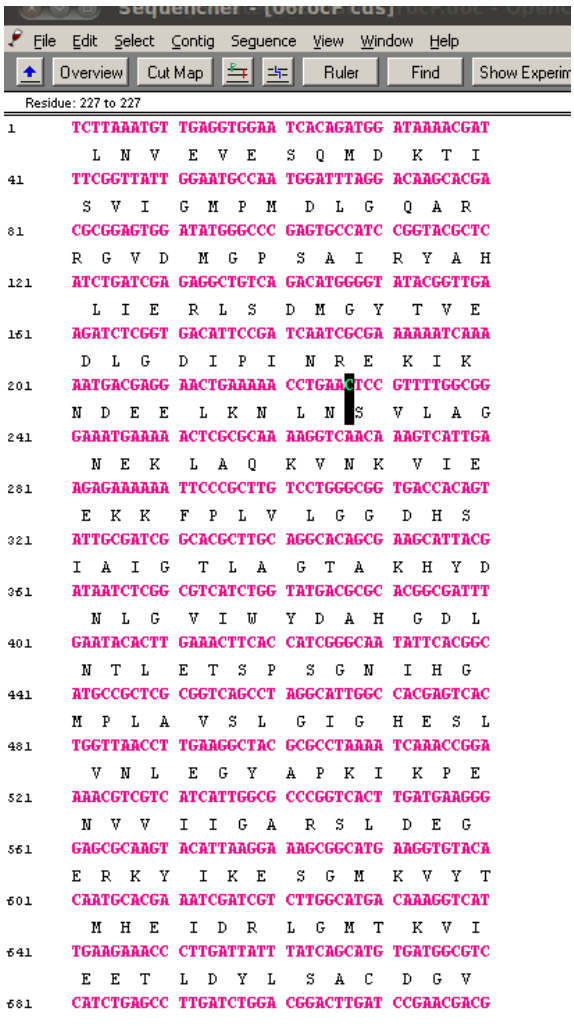
681 CATCTGAGCC TTGATCTGGA CGGACTTGAT CCGAACGACG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Residue: 227 to 227
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L N V E V E S Q M D K T I
41 TTCGGTTATT GGAATGCCAA TGGATTTAGG ACAAGCACGA
S V I G M P M D L G Q A R
81 CGCGGAGTGG ATATGGGCCC GAGTGCCATC CGGTACGCTC
R G V D M G P S A I R Y A H
121 ATCTGATCGA GAGGCTGTCA GACATGGGGT ATACGGTTGA
L I E R L S D M G Y T V E
161 AGATCTCGGT GACATTCCGA TCAATCGCGA AAAAATCAAA
D L G D I P I N R E K I K
201 AATGACGAGG AACTGAAAAA CCTGAACTCC GTTTTGGCGG
N D E E L K N L N S V L A G
241 GAAATGAAAA ACTCGCGCAA AAGGTCAACA AAGTCATTGA
N E K L A Q K V N K V I E
281 AGAGAAAAAA TTCCCGCTTG TCCTGGGCGG TGACCACAGT
E K K F P L V L G G D H S
321 ATTGCGATCG GCACGCTTGC AGGCACAGCG AAGCATTACG
I A I G T L A G T A K H Y D
361 ATAATCTCGG CGTCATCTGG TATGACGCGC ACGGCGATTT
N L G V I W Y D A H G D L
401 GAATACACTT GAAACTTCAC CATCGGGCAA TATTCACGGC
N T L E T S P S G N I H G
441 ATGCCGCTCG CGGTCAGCCT AGGCATTGGC CACGAGTCAC
M P L A V S L G I G H E S L
481 TGGTTAACCT TGAAGGCTAC GCGCCTAAAA TCAAACCGGA
V N L E G Y A P K I K P E
521 AAACGTCGTC ATCATTGGCG CCCGGTCACT TGATGAAGGG
N V V I I G A R S L D E G
561 GAGCGCAAGT ACATTAAGGA AAGCGGCATG AAGGTGTACA
E R K Y I K E S G M K V Y T
601 CAATGCACGA AATCGATCGT CTTGGCATGA CAAAGGTCAT
M H E I D R L G M T K V I
641 TGAAGAAACC CTTGATTATT TATCAGCATG TGATGGCGTC
E E T L D Y L S A C D G V
681 CATCTGAGCC TTGATCTGGA CGGACTTGAT CCGAACGACG

Fig 4. Removal of the first EcoR1 restriction site by a single base change

Fig 5. Removal of the second EcoR1 restriction site by a single base change

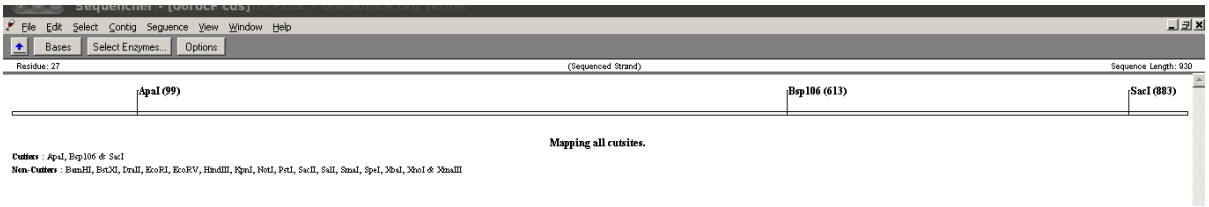


Fig 6. A restriction map of the *rocF* coding sequence with the two single base changes

Below (Fig 7.), the primers we are using are shown. In total there are 6 pairs:

1. Primer 1-P1 and Primer 2-P1 – Promoter PCR product. Primer 1-P1 includes standard BioBrick prefix on 5' end. Product overlaps with product 2, below.

2. Primer 1-S1 and Primer 2-S1 – First fragment of the *rocF* RBS and coding sequence. This product overlaps with product 1 above, and product 3, below.

3. Primer 3-S2 and Primer 4-S2 – Second fragment of the *rocF* coding sequence. This product overlaps with product 2 above, and product 4, below.

4. Primer 5-S3 and Primer 6-S3 – Third and final fragment of the *rocF* coding sequence. This product overlaps with product 3 above, and product 5, below.

5. Primer 1-T1 and Primer 2-T1 – Double terminator PCR product. Primer 2-T1 includes standard BioBrick suffix on 5' end. Product overlaps with product 4, above.

6. 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.

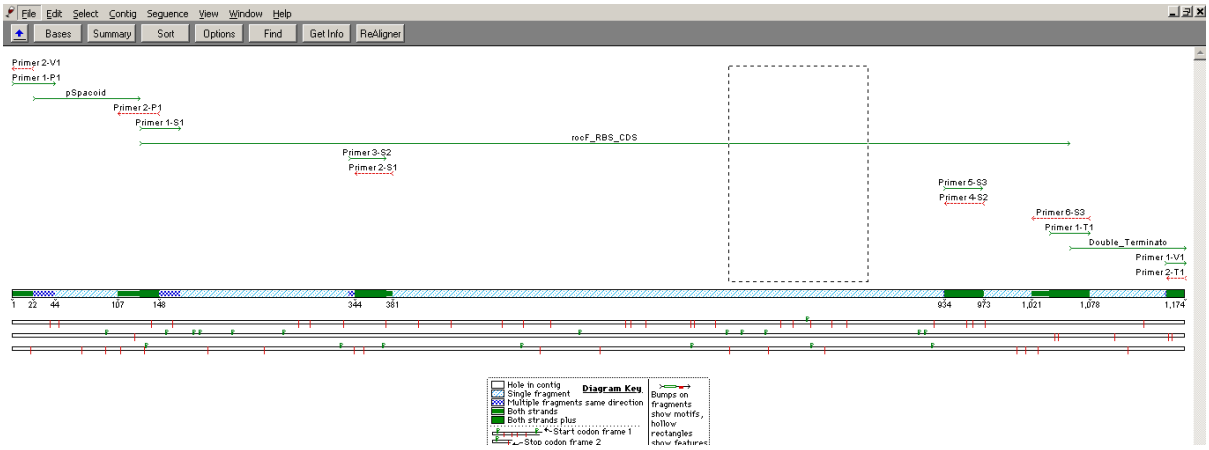


Fig 7. The primers used to PCR the overlapping promoter, *rocF* coding sequence and RBS, and double terminator products

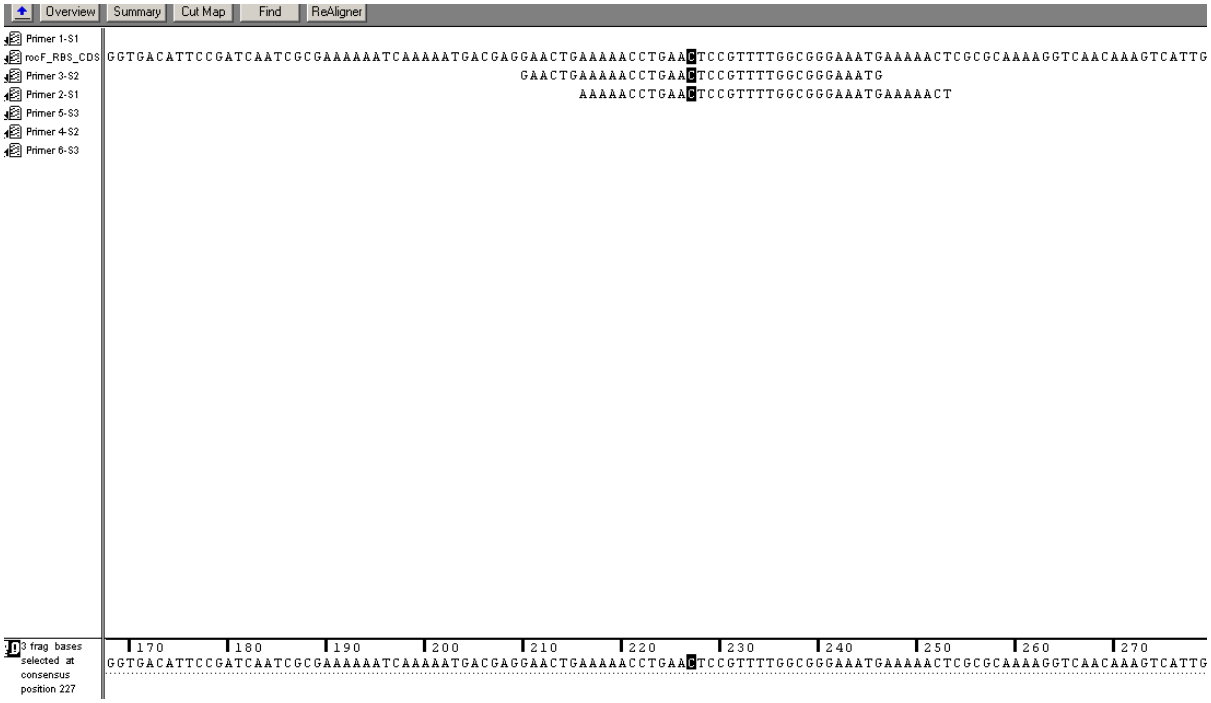


Fig 8. Removal of the first EcoR1 restriction site in the product by single base change in Primer 2-S1 and Primer 3-S2

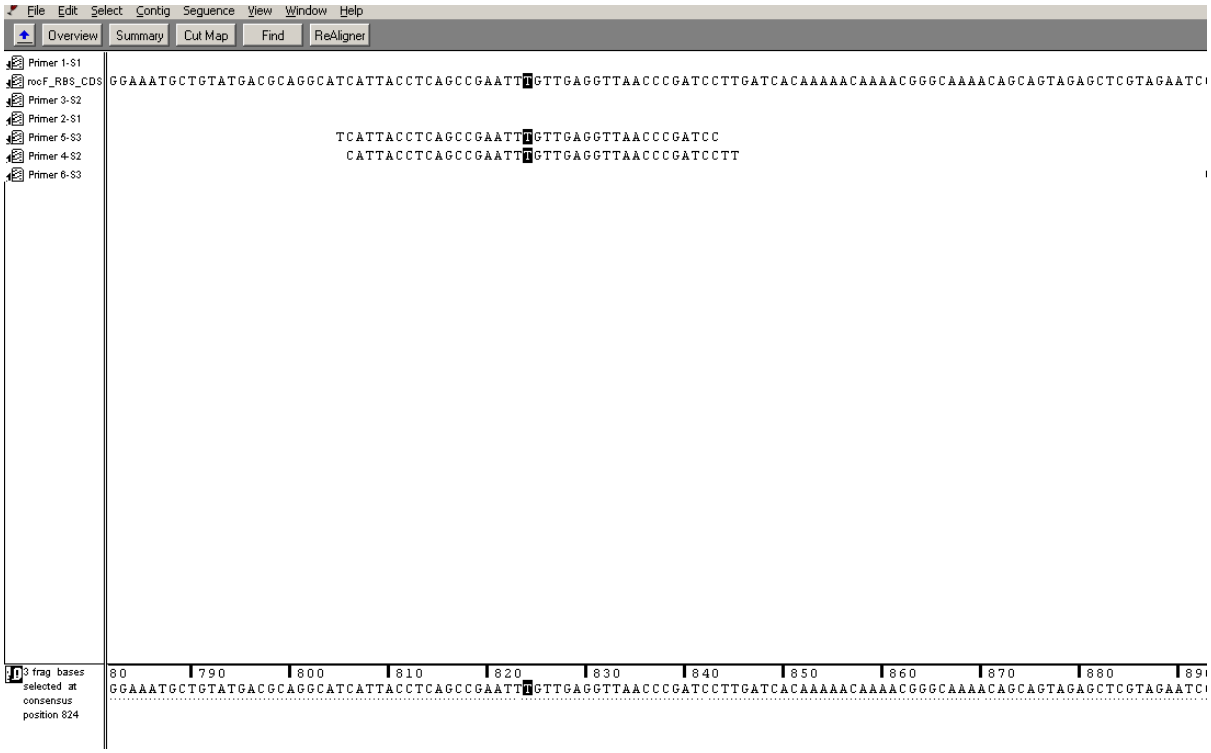
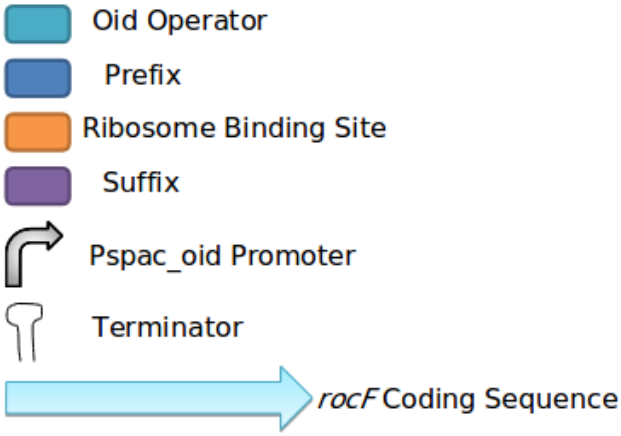


Fig 9. Removal of the second EcoR1 restriction site in the product by single base change in Primer 4-S2 and Primer 5-S3

Map of the *rocF* Biobrick



Procedure:

1. Take 6 PCR tubes and label them 1-6.

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

Tube	Part to be amplified
1	BioBrick-compatible Plasmid vector
2	Pspacoid Promoter
3	1st fragment of *rocF* CDS
4	2nd fragment of *rocF* CDS
5	3rd fragment of *rocF* CDS
6	Double terminator

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:

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

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

Tube	Part to be amplified	Forward Primer	Reverse Primer
1	Plasmid vector	Primer 1- V1	Primer 2-V1
2	Pspacoid Promoter	Primer 1- P1	Primer 2- P1
3	1st fragment of *rocF* CDS	Primer 1- S1	Primer 2- S1
4	2nd fragment of *rocF* CDS	Primer 3- S2	Primer 4- S2
5	3rd fragment of *rocF* CDS	Primer 5- S3	Primer 6- S3
6	Double terminator	Primer 1- T1	Primer 2- T1

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 size of the DNA	20 seconds
Extension*	72°C	30 seconds
Extension finish	72°C	5-10 minutes
Hold	4°C	N/A

***** Remember to set these steps for 30 cycles.

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
1	Plasmid vector	2072 bp
2	Pspacoid Promoter	+106 bp
3	1st fragment of *rocF* CDS	+ 246 bp
4	2nd fragment of *rocF* CDS	+597 bp
5	3rd fragment of *rocF* CDS	+125 bp
6	Double terminator	+116 bp

8. Set up a single PCR tube for the final step for the Gibson cloning. This tube would contain the 6 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) <u>1.33X Master Mix</u>

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.

(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

(C) Final Volume in the PCR Tube

Reagents	Volumes
Template DNA (consisting of all 6 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.

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.

Characterisation of *rocF*

The aim of this experiment is to determine whether the *rocF* BioBrick increases arginase production.

Materials Required

- Plate consisting of *Bacillus subtilis* 168 colonies.
- Flame (streaking) Loop
- LB media consisting arginine and ampicillin
- Auto pipette
- Bursen Burner
- Universal Tube

Procedure

- Perform the experiment using aseptic technique.
- Transfer *B. subtilis* 168 colonies into universal tubes containing 5 ml of LB media and allowed to grow overnight at 37° C.
- Transfer 1 ml of the overnight culture to another universal tube containing 4 ml of the following media:

1. Control (1) - LB media
2. Control (2) - LB media with 10 mM of arginine
3. Control (3) - LB media plus *B. subtilis* 168
4. Test (1) - LB media with 10 mM of arginine plus *B. subtilis* 168
5. Test (2) - LB media with 10 mM of arginine plus *B. subtilis* 168

- Incubate the culture at 37° C with shaking.
- Record the pH at every 30 min interval. Use 20 ul of the culture and measure the pH.

Expected results

Arginase breaks down arginine to orthinine. Increasing production of arginase in the cells will cause a faster breakdown of arginine, thus faster changes in pH level.

1. Control (1) - No change in pH
2. Control (2) - No change in pH
3. Control (3) - Increase in pH, however will be lower than test 1 and test 2.
4. Test (1) - Increase in pH
5. Test (2) - Increase in pH