Enhanced Arginine production: SR1

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, and *rocF*, which will enhance arginase production (please see the *rocF* BioBrick cloning strategy).

The naturally occurring antisense RNA, *SR1*, suppresses translation of the arginine repressor *ahrC.* An increase of *SR1* results in an increase of arginine.

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.

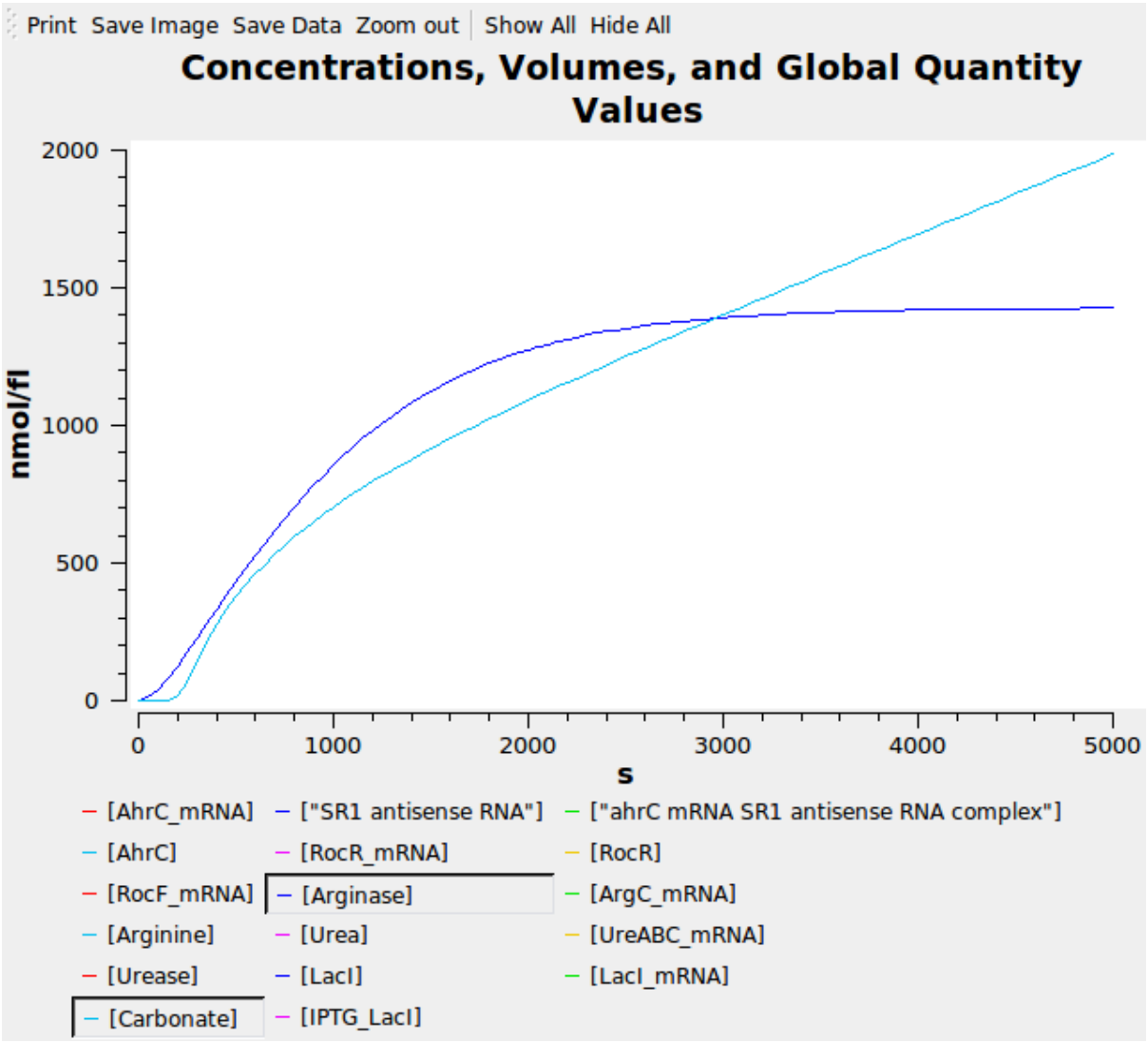


Fig 1. A graph showing the final output of the biochemical pathway, carbonate, increases over time

Construction:

Synthesised by Mr Gene, fragment: 466bp

Parts:

1. 00Clamp - A clamp to allow restriction enzymes to bind to the DNA.
2. 01bbprefix - The standard BioBrick prefix.
3. 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.
4. 05SR1 cds - The coding sequence for the SR1 antisense mRNA. This coding sequence is from *The small untranslated RNA SR1 from the Bacillus subtilis genome is involved in the regulation of arginine catabolism*, Heidrich, et al. (2006)
5. 06dbTerm09 - A double terminator BioBrick, minus its BioBrick prefix. This is BioBrick BBa_B0014 on the parts registry.
6. 07bb_suffix – The standard BioBrick suffix.
7. 09Clamp02 - A clamp to allow restriction enzymes to bind to the DNA.

Fig 2. Screenshot of the *SR1* BioBrick components in Sequencher

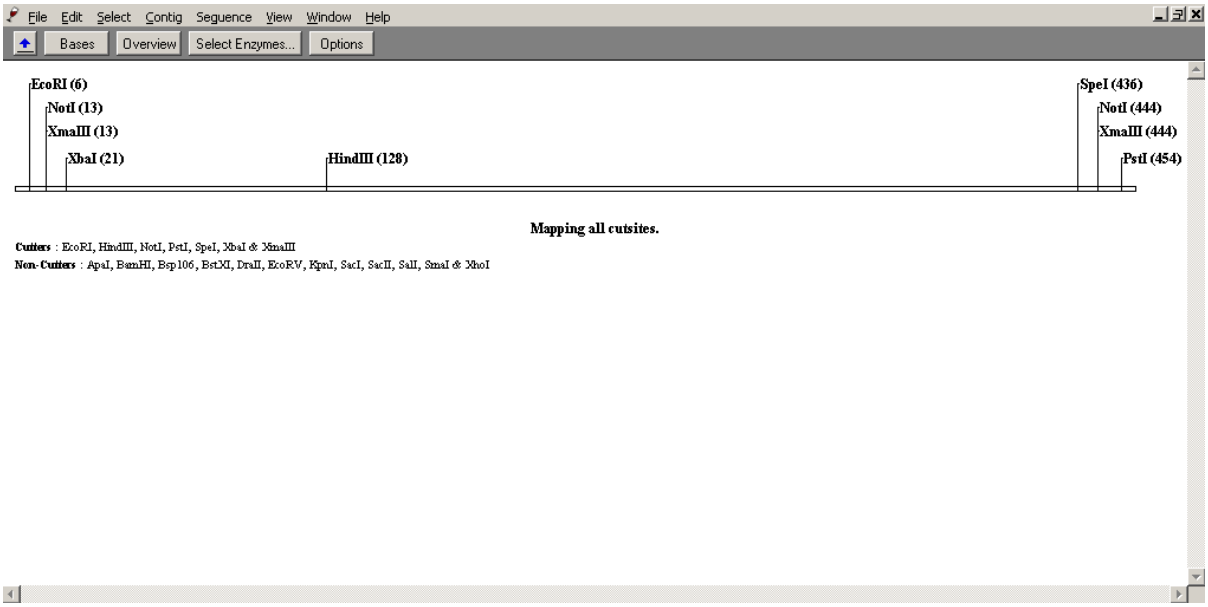


Fig 3. A restriction map of the construct, showing that the BioBrick restriction sites are only present in the prefix and suffix

The SR1 antisense bound to ahrC mRNA.

The extra nucleotides will be present on the 5’ end which will not interfere with the binding ability of *SR1* with *ahrC*. In the endogenous *SR1* mRNA found in the strain 168, there are already a small number of nucleotides found on the 5’ end which do not bind to the *ahrC* mRNA.

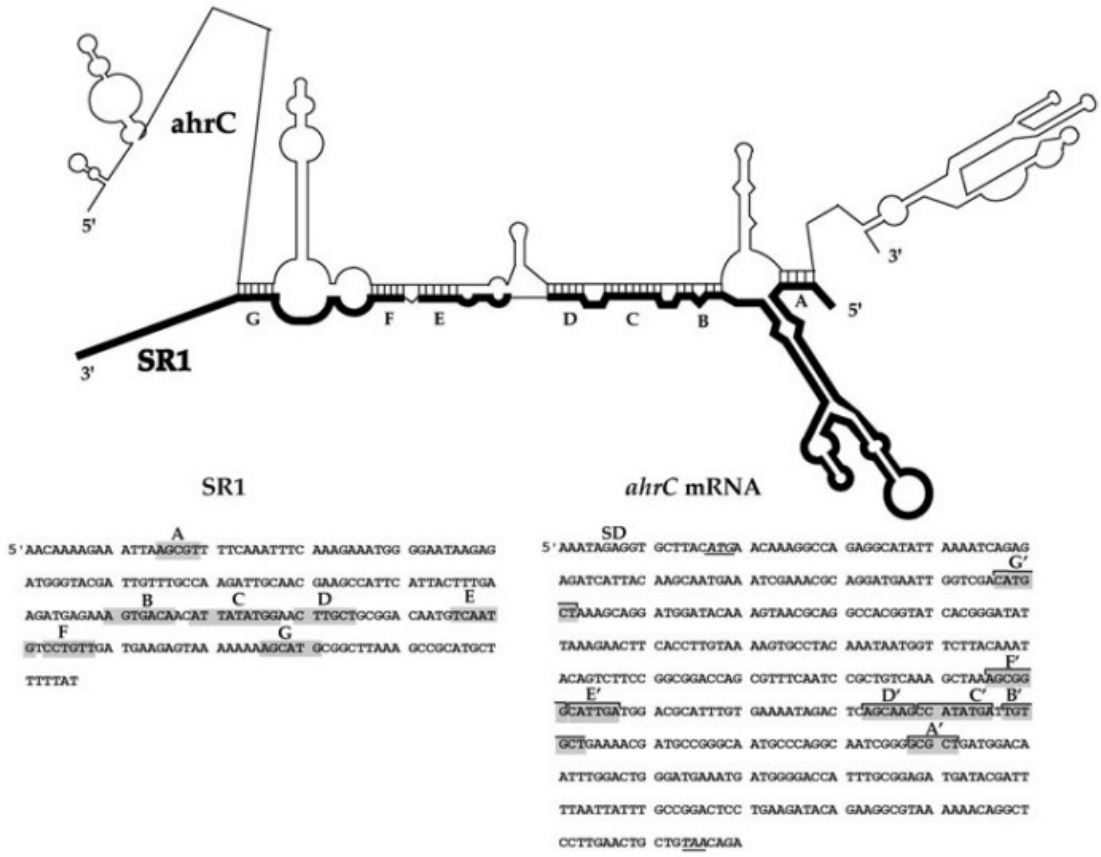


Fig 4. SR1 antisense mRNA bound to ahrC mRNA

Cloning and Integration:

The fragment is to be cloned into pGFPrrnB and integrated into the *Bacillus subtilis* 168 chromosome at *amyE*.

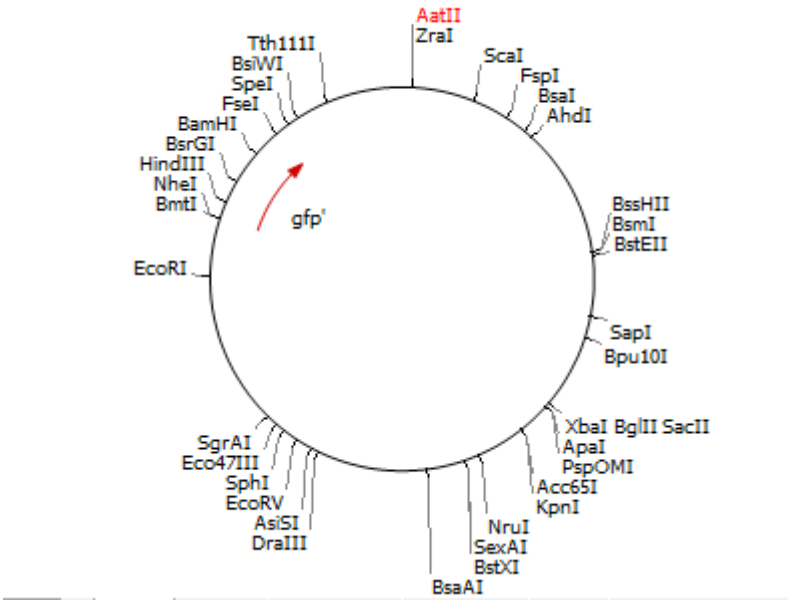


Fig 5. pGFPrrnB plasmid

Procedure:

1. Cut SR1 BioBrick suffix with Spe1 restriction enzyme in the 1st eppendorf tube.
2. Cut the *rocF* BioBrick (refer to the *rocF* BioBrick cloning strategy) with Xba1 restriction enzyme, removing both EcoR1 and Not1 from the prefix in the 2nd eppendorf tube.
3. Now join both *SR1* and *rocF* in the 3rd eppendorf tube with the help of ligase enzyme. The enzyme will ligate the 2 biobricks together because Spe1 (found on SR1 Biobrick suffix) and Xba1 (found on *rocF* Biobrick prefix) contains the same restriction sequence but after ligation, the restriction site becomes inactive (refer to ligation protocol).
4. Run the ligated fragments on agarose gel and extract the fragments will the help of gel extraction (refer gel extraction protocol).
5. Cut the ligated fragments with the restriction enzyme Xba1 (found on the prefix of *SR1* Biobrick).
6. Cut the *lacI* BioBrick (please refer to the *lacI* BioBrick cloning strategy) from the pSB1AT3 plasmid by using EcoR1 (this restriction site is found on prefix) and Spe1 (this restriction site is found on suffix) restriction enzyme in the 4th eppendorf tube. Do gel extraction to purify the *lacI* fragments.

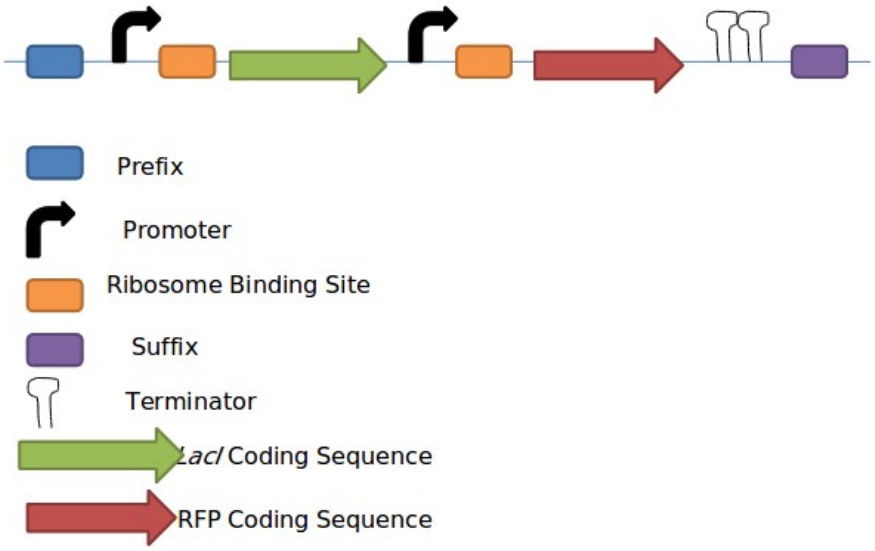
7. Now ligate the *lacI* BioBrick with the fused *SR1* and *rocF* BioBricks in the 5th eppendorf tube by adding ligase (refer to ligation protocol). *lacI* will be able to get ligated to the fused biobricks because Spe1 (found on SR1 Biobrick suffix) and Xba1 (found on *rocF* Biobrick prefix) contains the same restriction sequence but after ligation, the restriction site becomes inactive (refer to ligation protocol). After this, add Nhe1 restriction enzyme to the eppendorf tube and it will cut the fused Biobrick sequence at the suffix of *rocF.*

8. Cut the pGFPrrnB plasmid with EcoR1 and Nhe1 in the 6th eppendorf tube and do gel extraction so as to get the cut plasmid and to purify the cut fragment (refer gel extraction protocol).

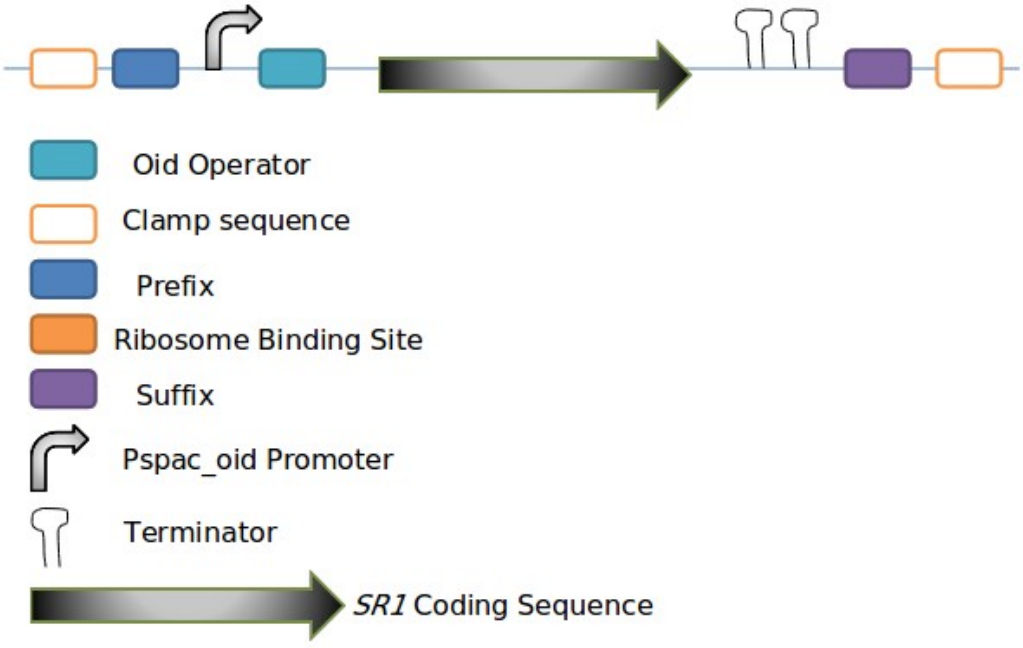
9. Now we ligate the fused *lacI*, *SR1* and *rocF* BioBricks with the plasmid pGFP-rrnB with the help of ligase enzyme in the 7th eppendorf tube (refer ligation protocol).

10. The plasmid in the 8th eppendorf tube is then transformed into *Bacillus subtilis* 168.

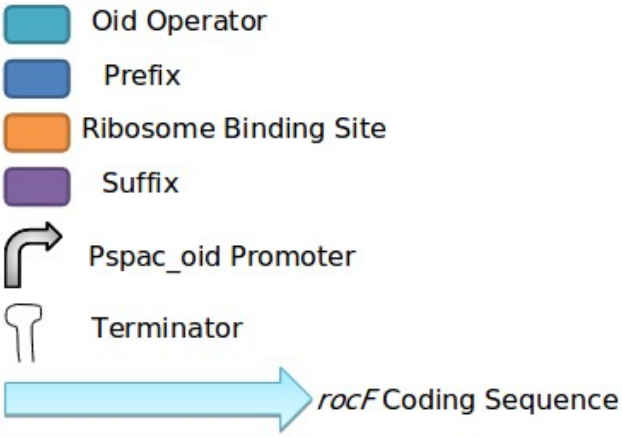
Map of the *LacI* Biobrick



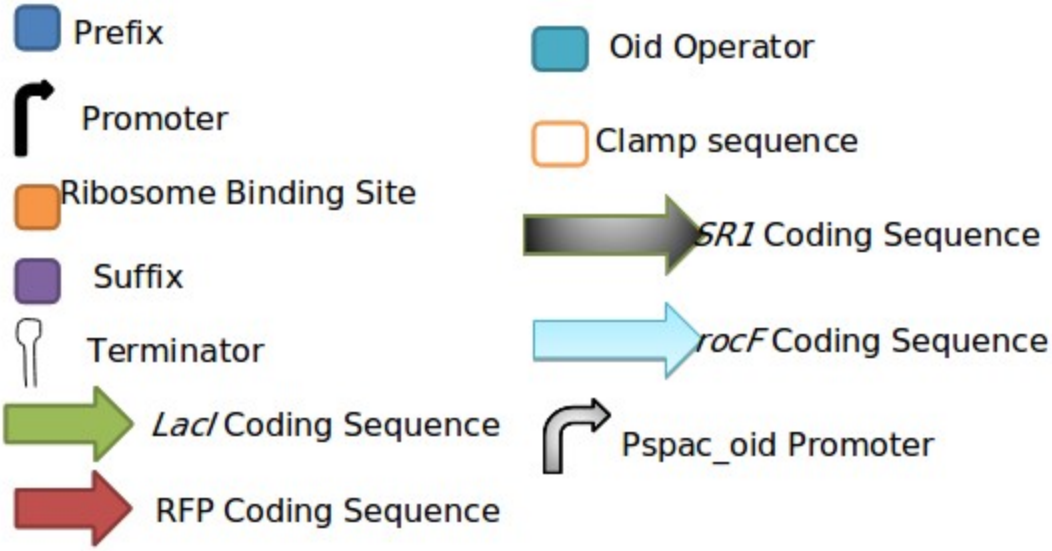
Map of the *SR1* Biobrick



Map of the *rocF* Biobrick



Map of the Final Integrated Biobrick



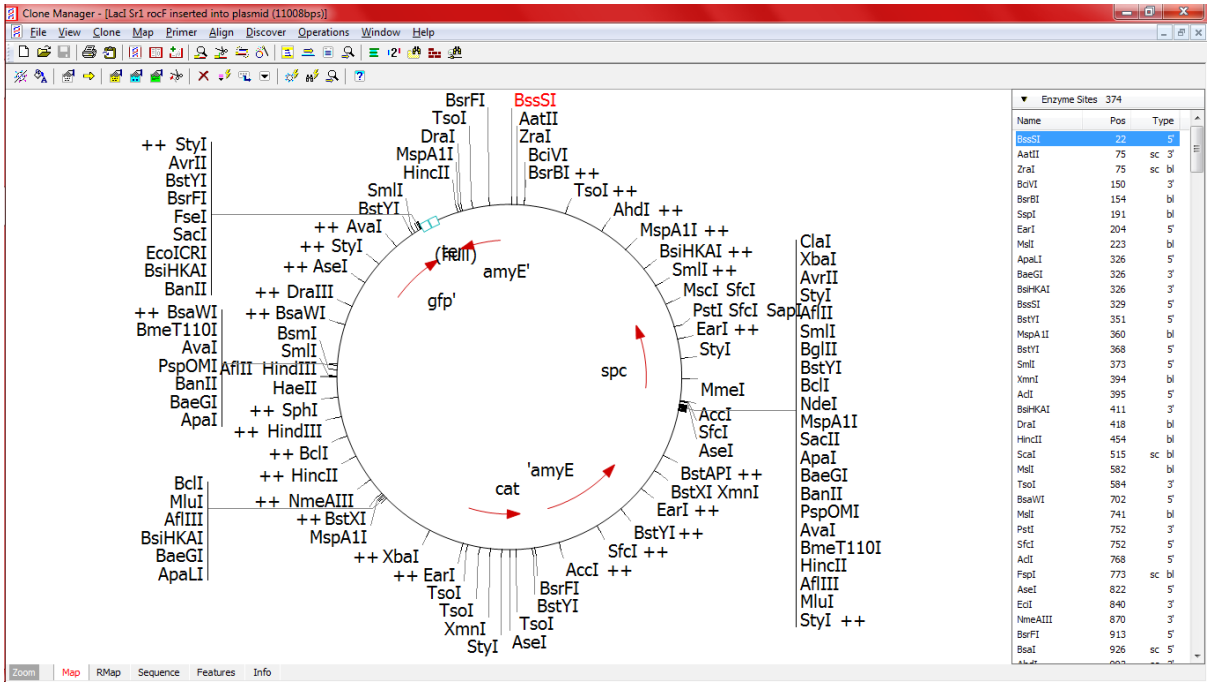


Fig 6. After integrating the BioBrick into the pGFPrrnB plasmid

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.

Calcium carbonate production

Plate the transformed *Bacillus subtilis* 168 onto precipitation agar containing (per litre) 3g of nutrient broth, 10g of NH_4Cl, and 30mM CaCl2. $2H_2O$.

Incubate at 37°C. Colonies should be assessed every day with a microscope, and positive colonies selected based on visual crystal formation within 10 days.

Protocol adapted from *Strain-Specific Ureolytic Microbial Calcium Carbonate Precipitation*, Hammes et al. 2003.