Production of Filamentous cells: *yneA*

Purpose and justification:

Bacillus subtilis in response to stress such as DNA damage stops the cells from dividing. This is a part of the SOS response initiated by the accumulation of single stranded DNA from DNA damage or stalled replication. Two proteins are vital for this response: RecA and LexA. RecA forms filaments on ssDNA and promotes the autocleavage of LexA. LexA usually represses the SOS operon. *dinR* is homologous to *lexA* in *E. coli* and is transcribed in the opposite direction of *yneA*.

yneA stops the formation of *ftsZ* ring indirectly. When *ftsZ* forms a 30 subunit ring at the midpoint of the cell, it will contract and cause cell division. By expressing *yneA* and inhibiting *ftsZ* ring formation, the cells will grow filamentous.

By inhibiting cell division, *yneA* allows the DNA damage genes to repair the DNA damage before continuing with the cell division cycle. It is hypothesized that *yneA* acts through an unknown transmembrane protein to inhibit *ftsZ* ring formation; we call this/these unknown components “Blackbox proteins”. As the evidence shows expression of *yneA* leads to filamentation.

We are planning to induce expression of *yneA* under the control of a repressible promoter *lacI* (refer *lacI* cloning strategy) to demonstrate the effect of increased *yneA* expression on cell length.

Modelling:

We have done a computational model of our system showing an increase in cell length (via “blackbox proteins” which link *yneA* expression to reduced FtsZ ring polymerisation) in relation to increased *yneA* expression, induced by IPTG via a LacI repressed promoter.

Screen shot of the graphical model from Copasi:

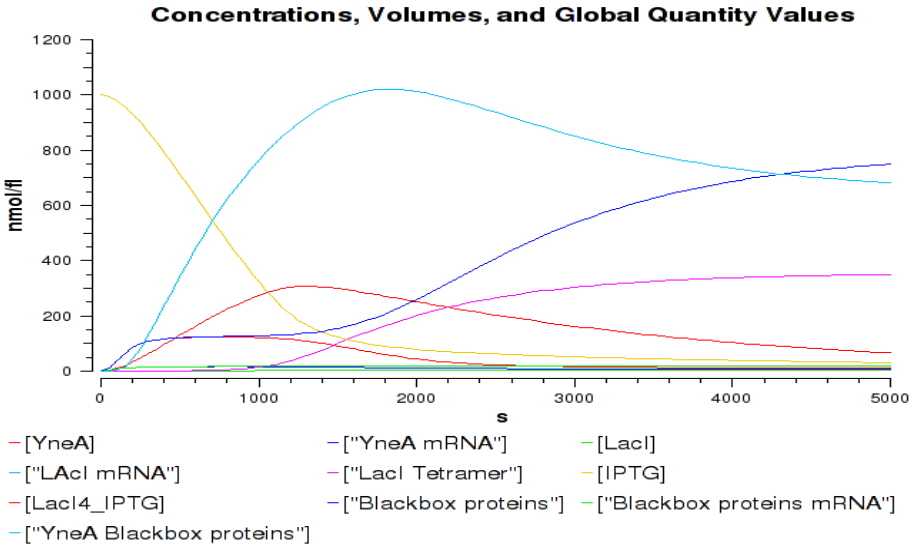

Figure 1: Copasi Model showing an increase in the "Blackbox proteins" with an increase in YneA. Graph developed on the software CopasiUI Version 4.5 (Build 30).

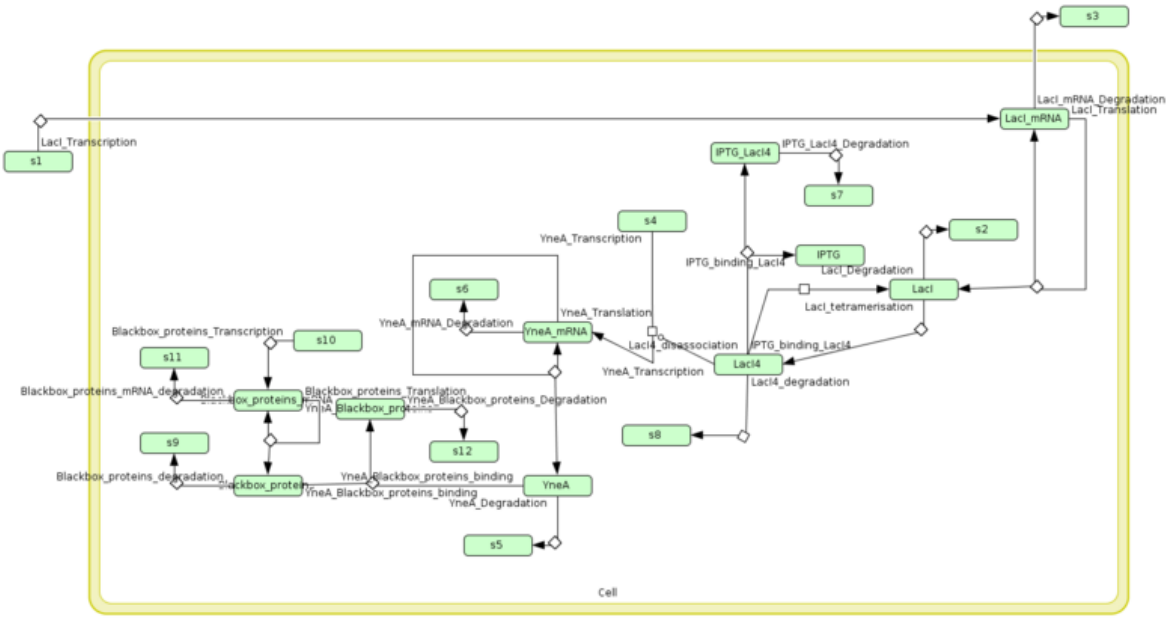

Figure 2: Cell designer Model showing all the species and compartments in the *yneA* pathway. Pathway designed in the software CellDesigner version 4.1.

More details of our model can be found on the wiki.

Construction:

Synthesized by MrGene, size 636bp.

Parts:

1. Clamp- allows restriction enzymes to bind the DNA
2. Bb prefix- Standard biobrick prefix
3. Pspankoid(hyper)- Novel Pspank promoter along with oid operator which is repressed by LacI protein.
4. Pre RBS- spacer
5. RBS- Ribosome binding site for *yneA*
6. Post RBS - spacer
7. *yneA* coding sequence- GenBank
8. Biobrick suffix –standard biobrick suffix
9. Clamp –allows restriction enzymes to bind the DNA
10. GFP RBS – marker RBS from GFPrrnb
11. Nhe1 cut site – for the integration vector

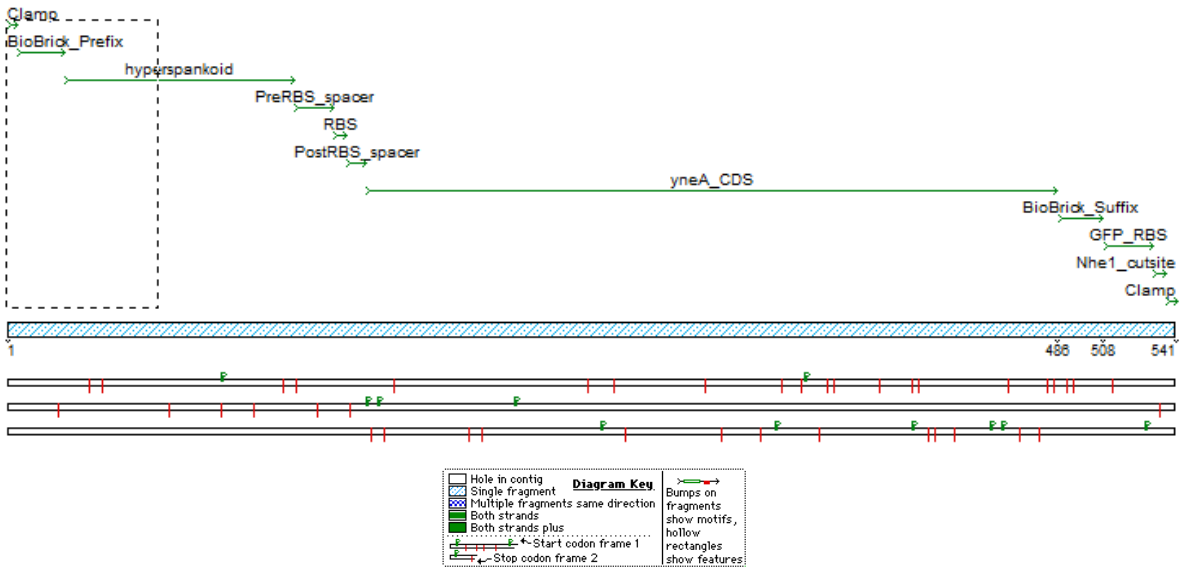

Figure 2: Sequencher screenshot showing all the parts for the yneA biobrick and the GFP RBS and Nhe1 cut site needed for Characterisation

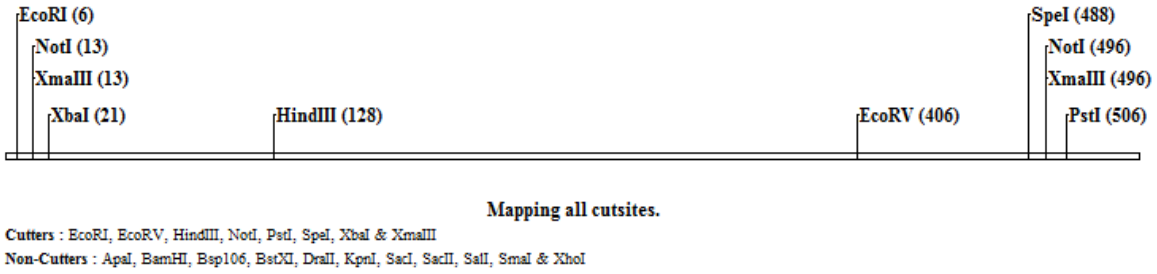

Figure 3: Sequencher screenshot cut site map showing no Biobrick restriction sites other than those in the prefix and suffix

Cloning:

The *yneA* gene fragment (636 bp) is going to be inserted into the plasmid vector pGFPrrnb which will in turn be inserted into *Bacillus subtilis* (strain 168) chromosomal DNA at the *amyE* locus. This plasmid will enable us to test for correct transformation by adding IPTG and testing for GFP expression.

pGFPrrnB plasmid:

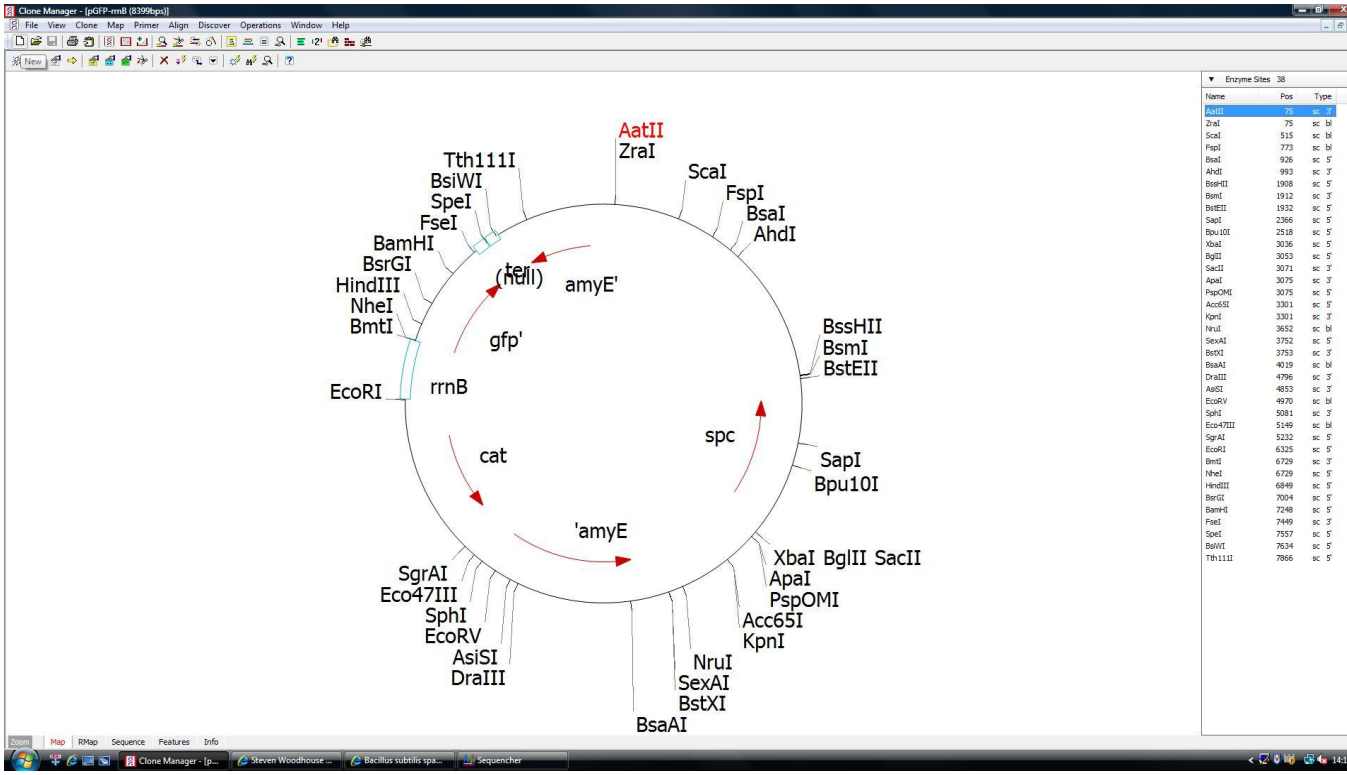

Figure 4: A screen shot showing pGFPrrnb plasmid vector before the integration of the insert. Screen shot taken from the software Clone Manager version 9.

After Inserts have been cloned in:

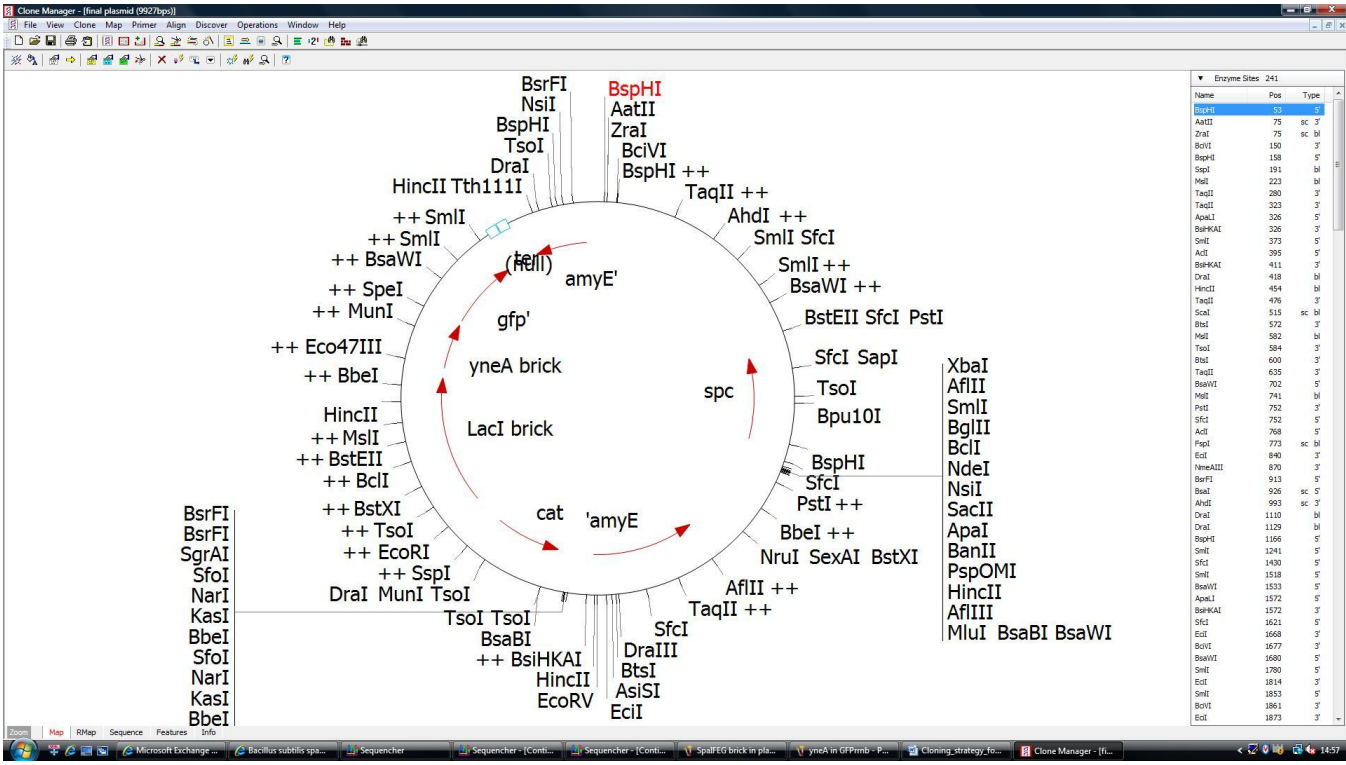

Figure 6: Screen shot from Clone manager: map of integrated *yneA* and *lacI*

Procedure:

1. Resuspend *yneA* (to a known concentration, refer synthesis report) (pGFPrrnb already at known concentration)
2. Transform *E.coli* DH5α (refer *E.coli* transformation protocol)
3. Plate out the *E.coli* DH5α cells and pick transformants (Ampicillin selection)
4. *yneA* plasmid extraction/purify making stock (refer plasmid extraction protocol)
5. Measure the weight of the plasmid DNA by nanodrop experiment (refer to nanodrop protocol).
6. Cut *yneA* in biobrick vector with EcoR1 and Nhe1
   General:

Reagents	Volumes
EcoR1	1 µl
Nhe1	1 µl
10X NEBuffer	3 µl
Plamid DNA	1 µl
Distill Water	22 µl
Total Volume	30 µl

Table 1: Table represents the materials and their concerntrations required for the digestion of the plasmid consisting *yneA* BioBrick. Table adapted from NEB website.

7. Run Gel electrophoresis (0.7 % agarose) to check the insert. (refer to gel electrophoresis protocol).
8. Extract the band (gel extraction protocol) of the right size after referring to the DNA ladder in the first lane. (the size of *yneA* BioBrick is approximately 636 bp)
9. Cut *yneA* Biobrick with Xba1
   General:

Reagents	Volumes
Xba1	1 µl
10X NEBuffer	3 µl
Template DNA	1 µl
Distill Water	25 µl
Total Volume	30 µl

Table 2: Table represents the materials and their volumes required for the digestion of the *yneA* BioBrick. Table adapted from NEB website.

10. Cut *lacI* with EcoR1 and Spe1 from the vector.
    General:

Reagents	Volumes
EcoR1	1 µl
Spe1	1 µl
10X NEBuffer	3 µl
Plamid DNA	1 µl
Distill Water	22 µl
Total Volume	30 µl

Table 3: Table represents the materials and their volumes required for the digestion of the plasmid consisting *lacI* Biobrick. Table adapted from NEB website.

11. Run Gel electrophoresis (0.7 % agarose) to check the insert. (refer to gel electrophoresis protocol).
12. Extract the band (gel extraction protocol) of the right size after referring to the DNA ladder in the first lane. (the size of *lacI* BioBrick is approximately ???bp)
13. Ligate *lacI* in front of *yneA*. Set up ligation (protocol) over night incubate at 4-16°C. (When Xba1 and Spe1 are ligated, the restriction site becomes inactive.)

Reagents	Concerntrations and Volumes
T4 DNA Ligase (Roche)	1 µl
10X Ligation Buffer	3 µl
Template DNA	1 µl
Distill Water	25 µl
Total Volume	30 µl

Table 4: Table represents the materials and their volumes required for the ligation of the *yneA* and *lacI* BioBrick. Table adapted from NEB website.

14. Run Gel electrophoresis (0.7 % agarose) to check the insert. (refer to gel electrophoresis protocol).
15. Extract the band (gel extraction protocol) of the right size after referring to the DNA ladder in the first lane. (the size of BioBrick is approximately ???bp)
16. Digest pGFPrrnb with EcoR1 and Nhe1.

Reagents	Concerntrations and Volumes
EcoR1	1 µl
Nhe1	1 µl
10X NEBuffer	3 µl
Plamid DNA	1 µl
Distill Water	22 µl
Total Volume	30 µl

Table 5: Table represents the materials and their volumes required for the digestion of the plasmid pGFPrrnB. Table adapted from NEB website.

17. Ligate *lacI* + *yneA* in vector pGFPrrnB. Set up ligation (protocol) over night incubate at 4-16°C.

Reagents	Concerntrations and Volumes
T4 DNA Ligase (Roche)	1 µl
10X Ligation Buffer	3 µl
Template DNA	1 µl
Distill Water	25 µl
Total Volume	30 µl

Table 6: Table represents the materials and their volumes required for the ligation of the *yneA+lacI* BioBrick in the vector pGFPrrnB. Table adapted from NEB website.

18. Run Gel electrophoresis (0.7 % agarose) to check the insert. (refer to gel electrophoresis protocol).
19. Extract the band (gel extraction protocol) of the right size after referring to the DNA ladder in the first lane. (the size of the final construct is approximately ???bp)
20. *E.coli* DH5α transformation with insert (*yneA*+ *lacI*) in pGFPrrnB vector (refer to transformation protocol).
21. Grow *E. coli* DH5α in 5ml of LB broth overnight.
22. Miniprep- plasmid extraction
23. Run on gel (Quantify – gel and nano drop protocol)
24. Gel purify (gel extraction protocol)protocol- *lacI*, *yneA* and vector GFPrrnb
25. *Bacillus subtilis* 168 Transformation Protocol . (refer to Wendy's transformation protocol for *Bacillus*)

For details of qiagen minipreps/protocols see Lab training/Lab notebook

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.

yneA - with insert with IPTG – cell growth /*yneA* fluorescence (gfp transcription follows yneA)(microscopy video?)

If the bricks work- cut *yneA* (from vector it arrived in) with EcoR1and Spe1 add double terminator to make it biobrick compatible ligate, run gel, gel extraction/purify. Cut with Pst1 and EcoR1 and ligate into Biobrick compatible vector to send to registry.

Send *lacI* in biobrick compatible vector to parts registry