Control Subgroup

In silico Lab book

Stage 1: Vector Design – Editing Templates

We sought to utilise the CRISPR-Cas9 genome editing vector pRECas1, previously shown to successfully edit the genome of *C. sporogenes* (Ines REF), to replace the native promoter sequences of four essential genes for sporulation, with an Anhydrotetracycline (aTc) inducible promoter. The four essential sporulation genes chosen are *spo0A*, *spoIID*, *spoIIIAA* and *spoIVA*. The pRECas1 vector contains; (i) *cas9* from Streptococcus pyogenes, (ii) a synthetic guide RNA (sgRNA) fragment including a protospacer sequence which recruits Cas9 to a specific DNA locus via complementary base pairing, and (iii) an editing template which features two regions of chromosomal DNA upstream and downstream of the intended target site allowing genome editing via homologous recombination.

First, we designed a vector for each of our four target genes containing the desired editing template sequence. This consisted of the first 500 bp of the sporulation gene targeted as the right homology arm (RHA) and a 500 bp region upstream of the intergenic region immediately preceding the sporulation gene as a left homology arm (LHA). In cases where the intergenic region was longer than 350 bp in length, only the initial 350 bp immediately upstream of the sporulation gene was omitted from the RHA. In between the LHA and RHA we inserted the Tet-inducible promoter cassette which comprises the *tetR* gene and its promoter P_{tet}, and a second promoter $P_{xyl/tet}$ which will control expression of our four sporulation gene targets. Therefore, after homologous recombination between this editing template and the *C. sporogenes* chromosome, we will have replaced the native sporulation gene promoter with an aTc-inducible promoter.

The vectors in this section were designed using the NEBuilder online tool accessible at https://nebuilder.neb.com and visualised using SnapGene software. The required fragments would be amplified by PCR using the primers listed in Tables 1-4 and assembled into AatII and AscI linearised pRECas1 fragments via HiFi Assembly.

Generation of pRECas1_spo0A

Gene Name / Fragment	Primer Name	Primer Sequence (5' – 3')	Template	Amplicon Size (bp)
spo0A upstream	spo0A_ups_F	gttttcgtcgacatagacgtcaagatttagttttaggcaatatagaaaataatac	*C. sporogenes* gDNA	531
	spo0A_ups_R	aagtgggtctagtttatattaaatattattaatagtttttaggttg		
TetR/Ptet	spo0A_TetR_Ptet_F	aatataaactagacccactttcacatttaag	pMTL_tet3nO_RFTetR	785
	spo0A_TetR_Ptet_R	tttcttccatagcttattttaattatactctatcaatg		
spo0A	spo0A_F	aaaataagctatggaagaaacaaagatcaatg	*C. sporogenes* gDNA	536
	spo0A_R	gttcaaaaaaataatggcggcgcgccataatatctgttatttcttgttctaaatc		

Table 1 Primers required to generate pRECas1_spo0A and the amplicon sizes of the PCR products.

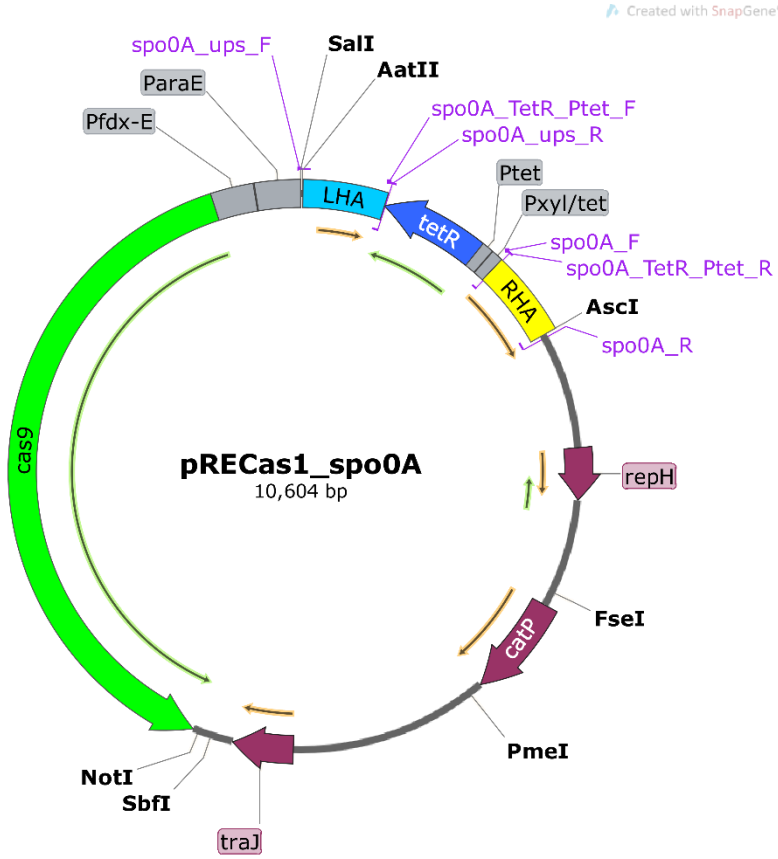

Figure 1 *Plasmid map of pRECas1_spo0A annotated with annealing sites of PCR primers (purple font).*

Generation of pRECas1_spoIID

Gene Name / Fragment	Primer Name	Primer Sequence (5' – 3')	Template	Amplicon Size (bp)
spoIID upstream	spoIID_ups_F	gttttcgtcgacatagacgtctaaatggtgttatagaagaacatttaaaac	*C. sporogenes* gDNA	531
	spoIID_ups_R	aagtgggtctttaatttactcgtataatatctgctc		
TetR/Ptet	spoIID_TetR_Ptet_F	agtaaattaaagacccactttcacatttaag	pMTL_tet3nO_RFTetR	785
	spoIID_TetR_Ptet_R	atcttctcatagcttattttaattatactctatcaatg		
spoIID	spoIID_F	aaaataagctatgagaagattaaataggtatactaatattaataag	*C. sporogenes* gDNA	536
	spoIID_R	gttcaaaaaaataatggcggcgcgcctattcatttctctttccttttg		

Table 2 *Primers required to generate pRECas1_spoIID and the amplicon sizes of the PCR products.*

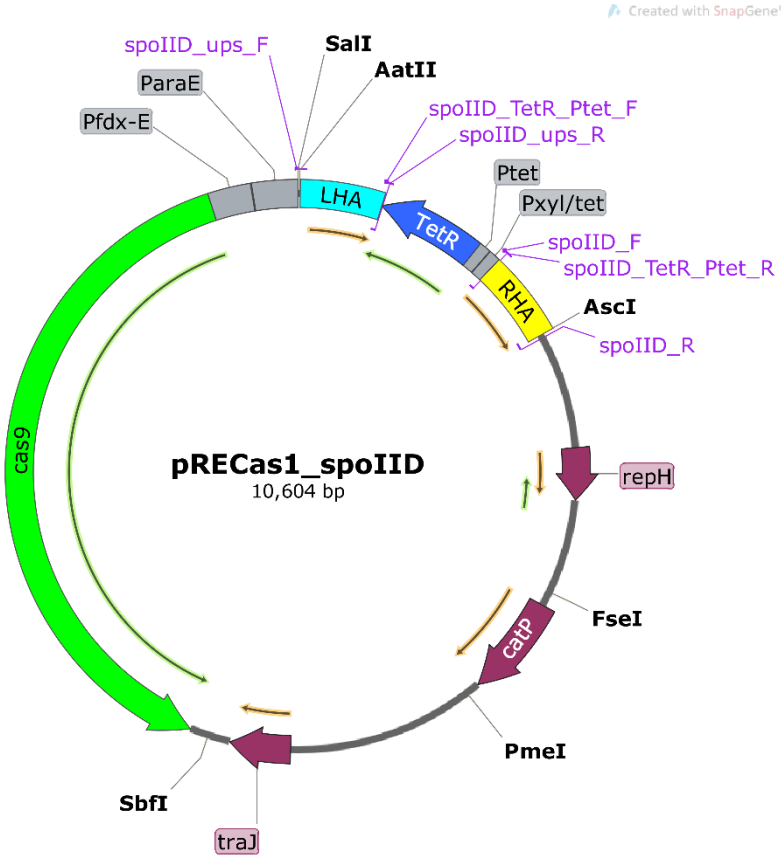

Figure 2 *Plasmid map of pRECas1_spoIID annotated with annealing sites of primers (purple font).*

Generation of pRECas1_spoIIIAA.

Gene Name / Fragment	Primer Name	Primer Sequence (5' – 3')	Template	Amplicon Size (bp)
spoIIIAA upstream	spoIIIAA _ups_F	gttttcgtcgacatagacgtcagtagatacaagaactggtg	*C. sporogenes* gDNA	531
	spoIIIAA _ups_R	aagtgggtctctaatcgtttttatttttttaattcttcac		
TetR/Ptet	spoIIIAA _TetR_Ptet_F	aaacgattagagacccactttcacatttaag	pMTL_tet3nO_RFTetR	785
	spoIIIAA _TetR_Ptet_R	ttgtatacaaagcttattttaattatactctatcaatg		
spoIIIAA	spoIIIAA _F	aaaataagctttgtatacaaaagaaattctaaatatacttcc	*C. sporogenes* gDNA	536
	spoIIIAA _R	gttcaaaaaaataatggcggcgcgccgaatccactccgtcagatatttttttag		

Table 3 *Primers required to generate pRECas1_spoIIIAA and the amplicon sizes of the PCR products.*

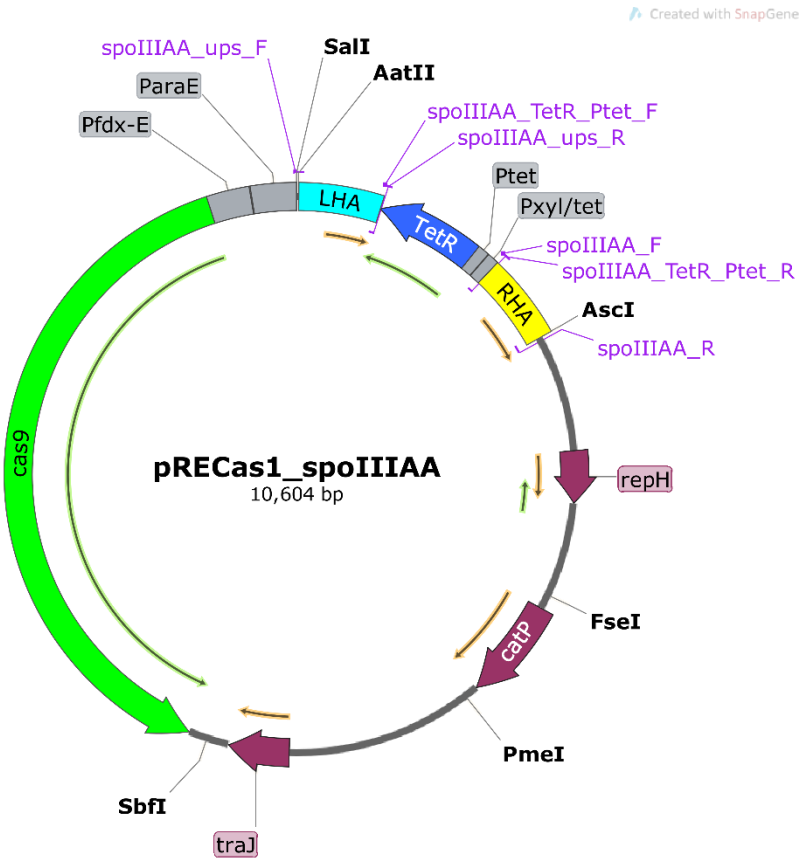

Figure 3 *Plasmid map of pRECas1_spoIIIAA annotated with annealing sites of primers (purple font).*

Generation of pRECas1_spoIVA

Gene Name / Fragment	Primer Name	Primer Sequence (5' – 3')	Template	Amplicon Size (bp)
spoIVA upstream	spoIVA_ups_F	gttttcgtcgacatagacgtcaaacttcagctattccattg	*C. sporogenes* gDNA	531
	spoIVA_ups_R	aagtgggtctttagaacagcagattaaattagtttttatatc		
TetR/Ptet	spoIVA_TetR_Ptet_F	gctgttctaaagacccactttcacatttaag	pMTL_tet3nO_RFTetR	785
	spoIVA_TetR_Ptet_R	aattatccaaagcttattttaattatactctatcaatg		
spoIVA	spoIVA_F	aaaataagctttggataattttaatatatacaaagatatcg	*C. sporogenes* gDNA	536
	spoIVA_R	gttcaaaaaaataatggcggcgcgccacatagctttctctatctatatc		

Table 4 *Primers required to generate pRECas1_spoIVA and the amplicon sizes of the PCR products.*

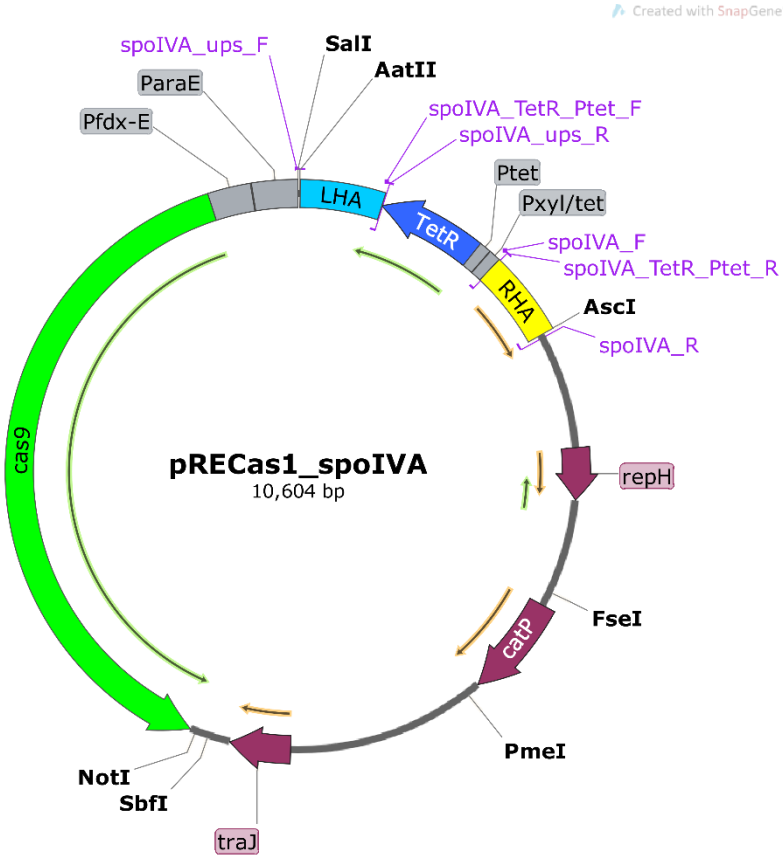

Figure 4 *Plasmid map of pRECas1_spoIVA annotated with annealing sites of primers (purple font).*

Stage 2: Identification of protospacers

Having designed the editing templates for each of our sporulation target genes, we next needed to design the sgRNA fragments which would guide Cas9 to cleave unedited chromosomal DNA thus allowing us to select for *C. sporogenes* cells which had successfully undergone the desired genome editing. To do this we searched for protospacer sequences within the intergenic regions immediately upstream of our four chosen sporulation target genes using the Design and Analyse Guides tool on the Benchling website, https://benchling.com. Protospacers are 20 bp sequences immediately adjacent to 5'-NGG-3' sequences, the protospacer adjacent motif (PAM) for Cas9.

As it can be difficult to reliably predict successful functioning of individual sgRNAs, we chose two protospacers for each of our target genes. These protospacers were then incorporated into overlapping primers which would be used to generate sgRNA components flanked by SalI and AatII restriction sites. The sgRNA components, along with the corresponding vector from the previous section, would then be digested with SalI and AatII and ligated together to generate our complete CRISPR vectors, shown in Figures 5-8 below.

Spo0A

Length of upstream intergenic region: 422 bp (350 bp to be deleted)

Number of protospacers identified: 11

Location	Strand	Protospacer sequence	PAM	On-target score	Off-target score
2072812	-	atatggtaattttatcaaaa	agg	18.0	49.7
2072829	-	ataaataatttgttaagata	tgg	11.5	49.2
2072872	**-**	**atatttttctcaaataagg**	**agg**	**37.9**	**49.8**
2072875	-	tatatatttttctcaaata	agg	1.4	48.6
2072933	**+**	**ctaatttcttacactttata**	**tgg**	**19.8**	**49.4**
2072961	+	catatattttattttgtcat	agg	6.9	49.7

2072980	-	tacaataattaacatataaa	agg	8.1	49.1
2073022	+	ctttcttactattaaattta	tgg	4.8	49.1
2073060	-	taagaatataaaatagcata	agg	3.1	74.2
2073117	-	taataaattagcaatttgaa	tgg	5.6	98.3
2073171	+	aaatattattaatagttttt	agg	2.6	91.8

Table 5 Protospacer and corresponding protospacer adjacent motif (PAM) sequences identified within the intergenic region upstream of spo0A *in* C. sporogenes.

Primer Name	Primer Sequence (5' – 3')
spo0A_sgRNA1_F	ttttcGTCGAC**atatttttttctcaaataagg**gttttagagctagaaat agcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcac cgagtcggtgct
spo0A_sgRNA2_F	ttttcGTCGAC**ctaatttcttacactttata**gttttagagctagaaat agcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcac cgagtcggtgct
sgRNA_R	ggccGACGTCataaaaataagaagcctgcaaatgcaggcttcttat ttttataaaaaaagcaccgactcggtgccacttttcaagttg

Table 6 Primer sequences to generate sgRNA components targeting the intergenic region upstream of spo0A.

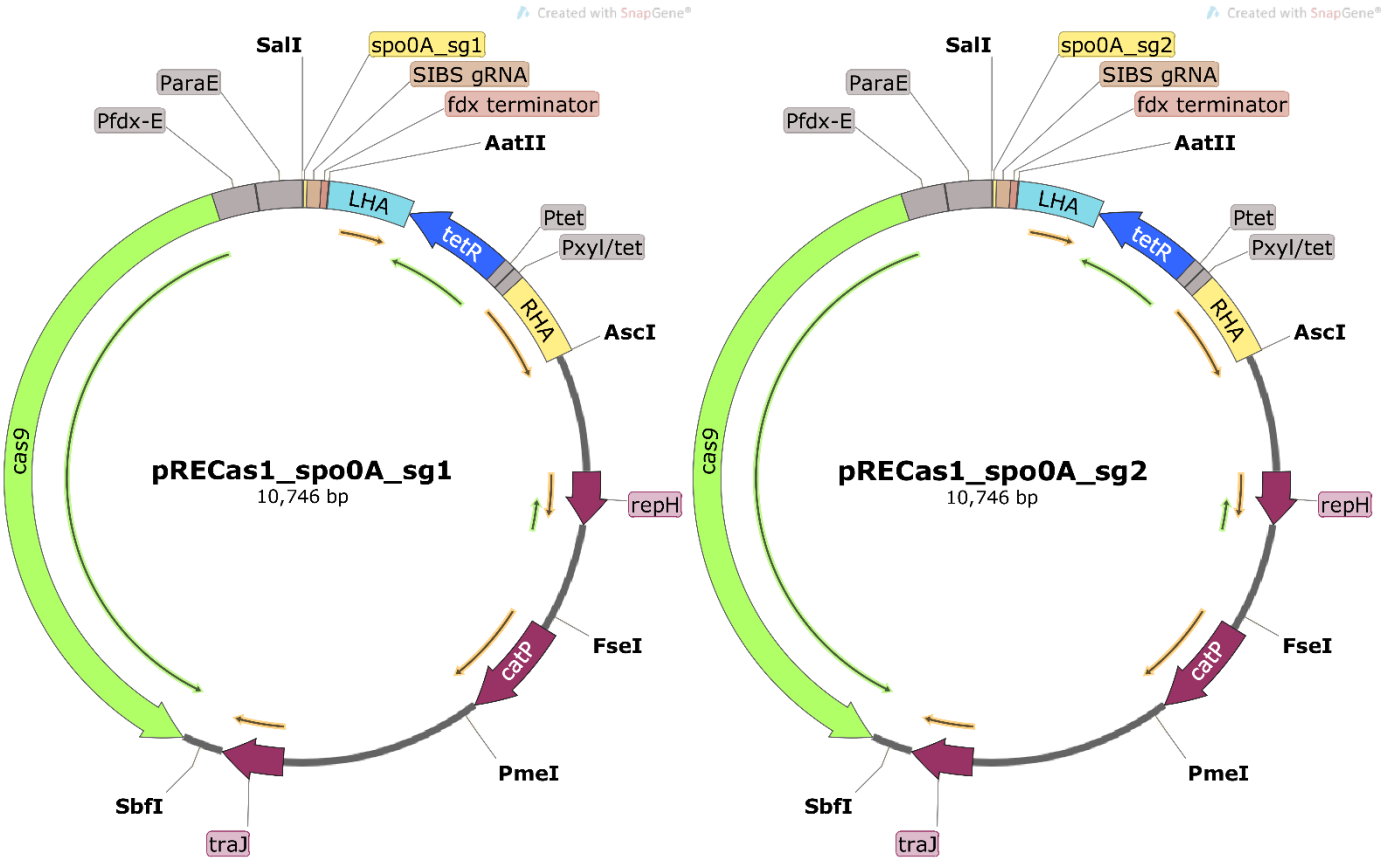

Figure 5 Plasmid maps of pRECas1_spo0A_sg1 (left) and pRECas1_spo0A_sg2 (right) generated following the ligation of spo0A_sg1 and spo0A_sg2 sgRNA components, respectively, between SalI and AatII sites of pRECas1_spo0A.

SpoIID

Length of upstream intergenic region: 272 bp

Number of protospacers identified: 4

Location	Strand	Protospacer sequence	PAM	On-target score	Off-target score
193531	**-**	**tacaattatatttttcatag**	**agg**	**11.0**	**68.0**
193455	+	aataatttgtgtatacatat	agg	10.3	49.8
193567	+	gaaaactttattatgaaaag	agg	7.5	49.5
193568	**+**	**aaaactttattatgaaaaga**	**ggg**	**17.3**	**49.1**

Table 7 Protospacer and corresponding protospacer adjacent motif (PAM) sequences identified within the intergenic region upstream of spoIID *in* C. sporogenes.

Primer Name	Primer Sequence (5' – 3')
spoIID_sgRNA1_F	ttttcGTCGAC**aaaactttattatgaaaaga**gttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgct
spoIID_sgRNA2_F	ttttcGTCGAC**tacaattatatttttcatag**gttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgct
sgRNA_R	ggccGACGTCataaaaataagaagcctgcaaatgcaggcttcttatttttataaaaaaagcaccgactcggtgccacttttcaagttg

Table 8 Primer sequences to generate sgRNA components targeting the intergenic region upstream of spoIID.

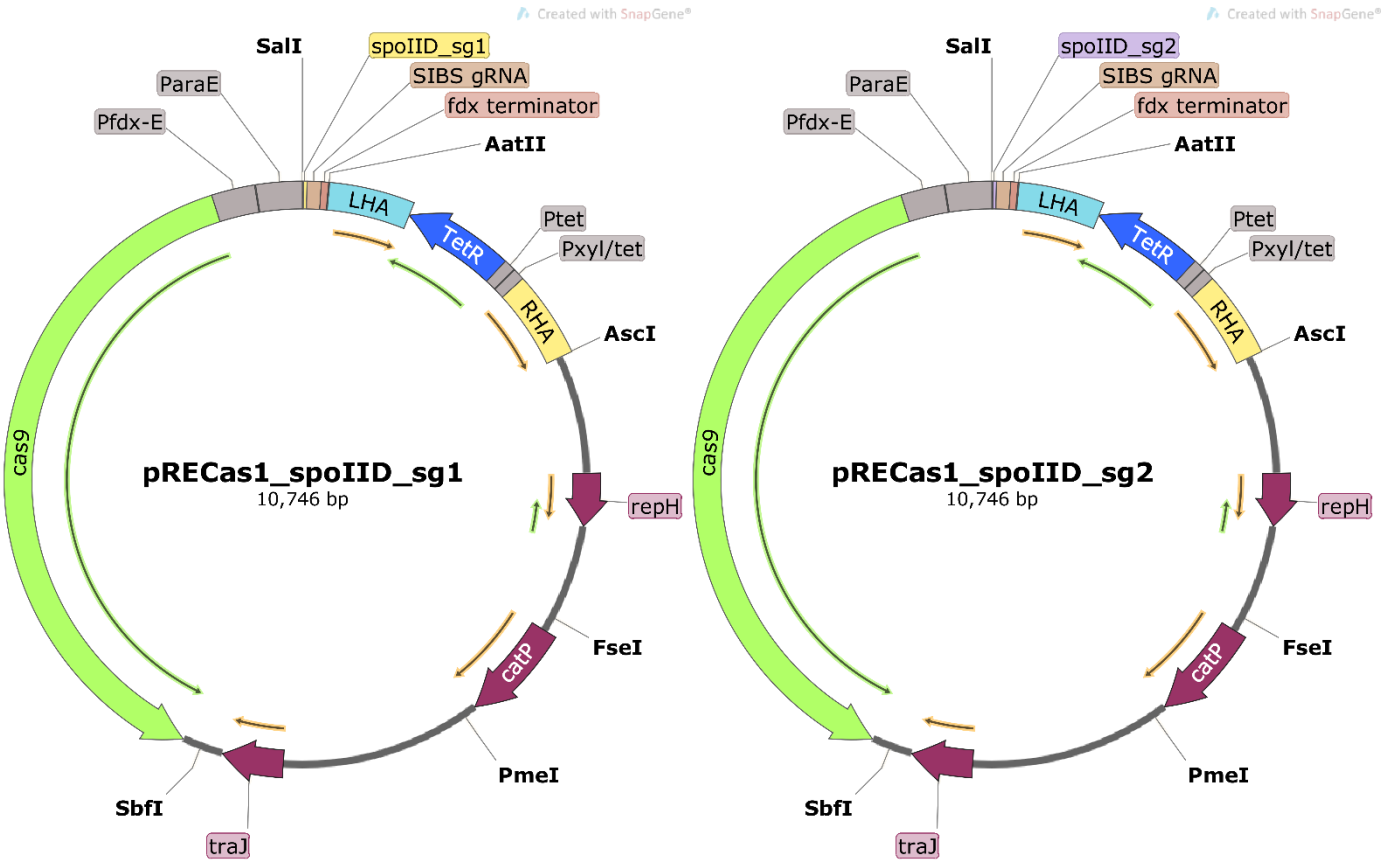

Figure 6 Plasmid maps of pRECas1_spoIID_sg1 (left) and pRECas1_spoIID_sg2 (right) generated following the ligation of spoIID_sg1 and spoIID_sg2 sgRNA components, respectively, between SalI and AatII sites of pRECas1_spoIID.

SpoIIIAA

Length of upstream intergenic region: 196 bp

Number of protospacers identified: 6

Location	Strand	Protospacer sequence	PAM	On-target score	Off-target score
2094537	**-**	**aatagatattttataaagaa**	**agg**	**10.4**	**47.6**
2094580	-	aattattttaataaagtttt	tgg	0.9	48.0
2094644	**+**	**agattattaatgatagtaat**	**agg**	**27.8**	**49.3**
2094661	-	tttttatacaaaaaattata	tgg	9.6	48.3
2094689	-	tataagattaaaatcttata	agg	4.9	48.1
2094703	+	tataagattttaatcttata	agg	4.5	48.5

Table 9 Protospacer and corresponding protospacer adjacent motif (PAM) sequences identified within the intergenic region upstream of spoIIIAA *in* C. sporogenes.

Primer Name	Primer Sequence (5' – 3')
spoIIIAA_sgRNA1_F	ttttcGTCGAC**agattattaatgatagtaat**gttttagagctagaaa tagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggca ccgagtcggtgct
spoIIIAA_sgRNA2_F	ttttcGTCGAC**aatagatattttataaagaa**gttttagagctagaaa tagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggca ccgagtcggtgct
sgRNA_R	ggccGACGTCataaaaataagaagcctgcaaatgcaggcttcttat ttttataaaaaaagcaccgactcggtgccactttttcaagttg

Table 10 Primer sequences to generate sgRNA components targeting the intergenic region upstream of spoIIIAA.

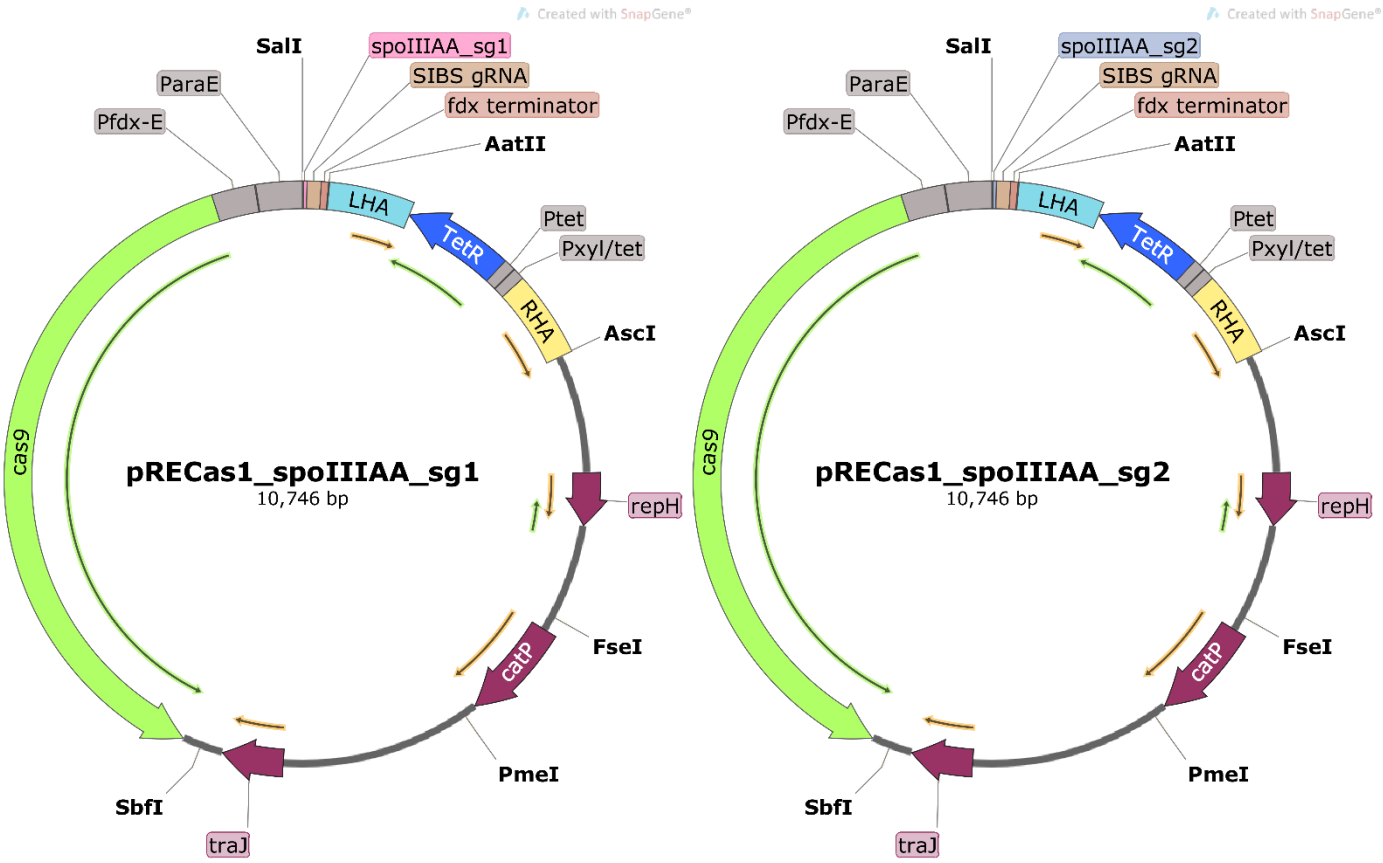

Figure 7 Plasmid maps of pRECas1_spoIIIAA_sg1 (left) and pRECas1_spoIIIAA_sg2 (right) generated following the ligation of spoIIIAA_sg1 and spoIIIAA_sg2 sgRNA components, respectively, between SalI and AatII sites of pRECas1_spoIIIAA.

SpoIVA

Length of upstream intergenic region: 726 bp (350 bp deleted)

Number of protospacers identified: 7

Location	Strand	Protospacer sequence	PAM	On-target score	Off-target score
2861115	**-**	**gttaatattataatattggg**	**ggg**	**42.1**	**49.8**
2861116	-	tgttaatattataatattgg	ggg	6.0	48.8
2861117	-	atgttaatattataatattg	ggg	11.1	48.8
2861217	-	atttataatatgaaagggta	agg	3.9	99.5
2861222	**-**	**atttaatttataatatgaaa**	**ggg**	**23.0**	**91.8**
2861223	-	aatttaatttataatatgaa	agg	3.8	95.7
2861265	-	ataaacttatattgttaata	agg	7.5	97.5

Table 11 Protospacer and corresponding protospacer adjacent motif (PAM) sequences identified within the intergenic region upstream of spoIVA *in* C. sporogenes.

Primer Name	Primer Sequence (5' – 3')
spoIVA_sgRNA1_F	ttttcGTCGAC**gttaatattataatattggg**gttttagagctagaaat agcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcac cgagtcggtgct
spoIVA_sgRNA2_F	ttttcGTCGAC**atttaatttataatatgaaa**gttttagagctagaaat agcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcac cgagtcggtgct
sgRNA_R	ggccGACGTCataaaaataagaagcctgcaaatgcaggcttcttat ttttataaaaaaagcaccgactcggtgccacttttcaagttg

Table 12 Primer sequences to generate sgRNA components targeting the intergenic region upstream of spoIVA.

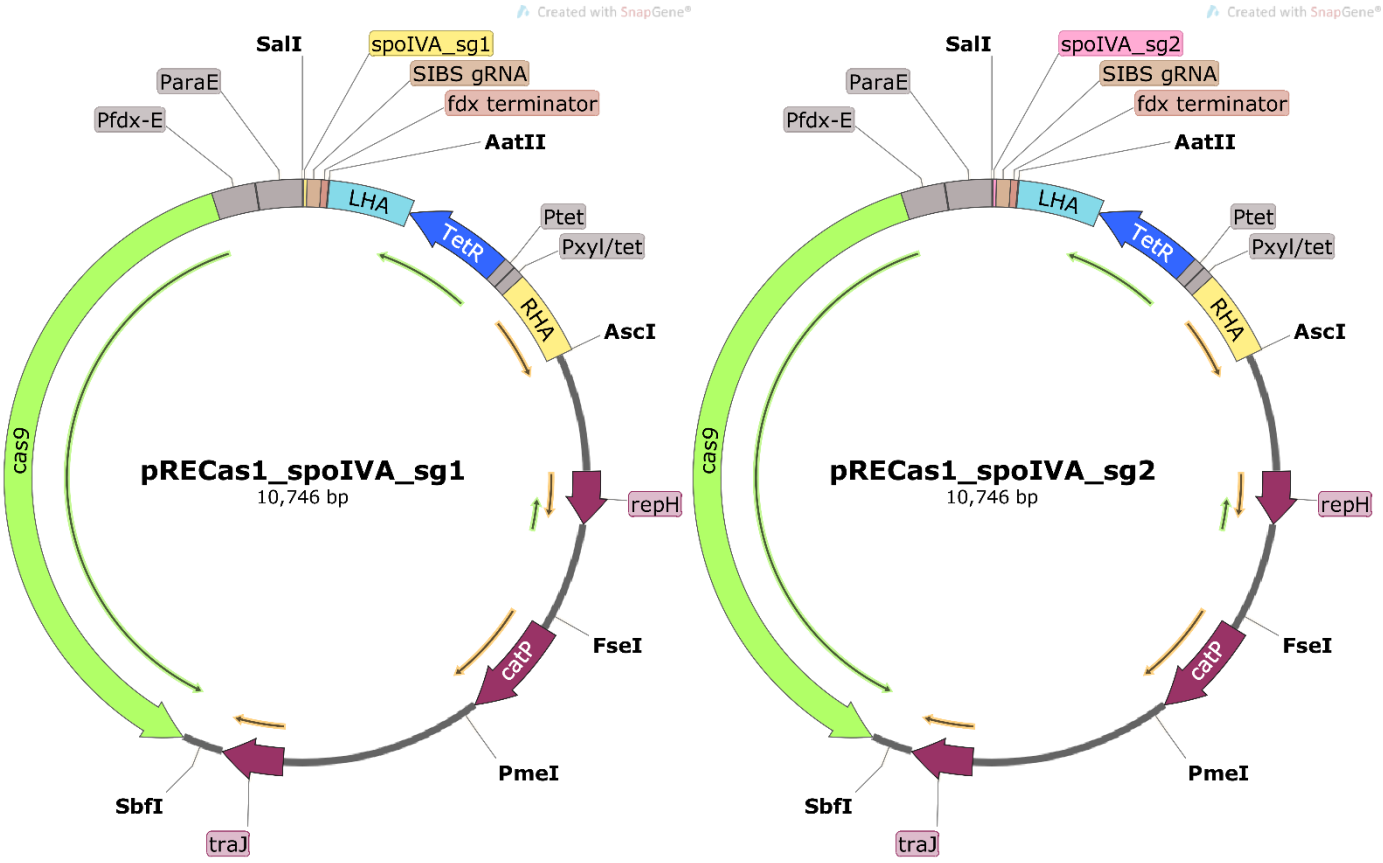

Figure 8 Plasmid maps of pRECas1_spoIVA_sg1 (left) and pRECas1_spoIVA_sg2 (right) generated following the ligation of spoIVA_sg1 and spoIVA_sg2 sgRNA components, respectively, between SalI and AatII sites of pRECas1_spoIVA.

Sequencing of constructed vectors

To confirm the successful construction of our 8 pRECas1 variant vectors we would purify each vector from the *E. coli* cloning host using a Plasmid Miniprep kit and send these for Sanger sequencing using the sequencing primers shown in Table 13. The annealing sites of these primers are shown in Figure 9. The resulting data from Sanger sequencing would be aligned to the respective plasmid template sequence to confirm successful generation of each vector.

Primer Name	Sequence (5'-3')
sgRNA_sF1	cctttcatttacaattcatacg
TetR_sF1	atccgcatatgatcaattcaagg
pCB102_R	ctgttatgccttttgactatc

Table 13 Primers to be used in Sanger sequencing reactions to confirm successful vector construction.

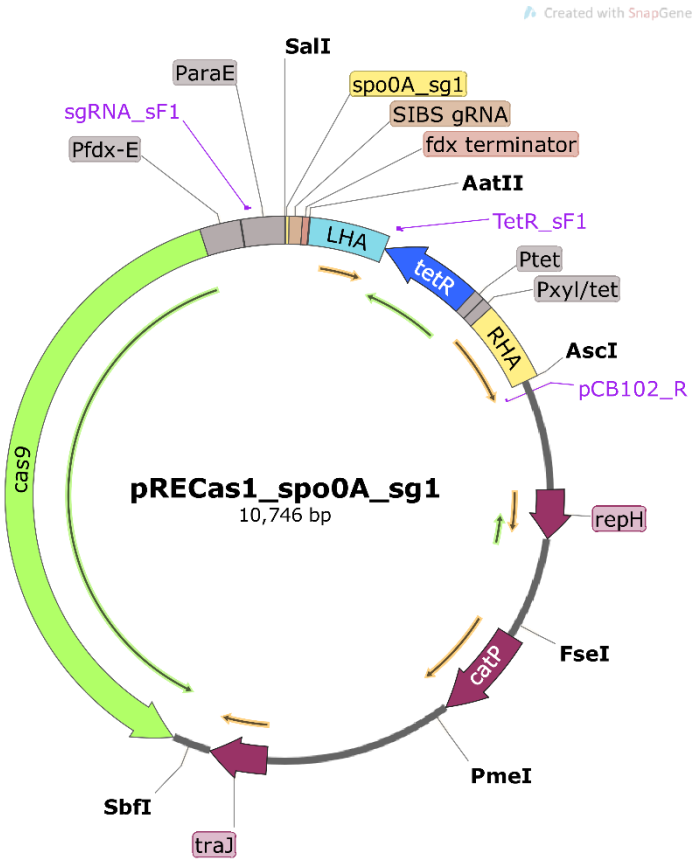

Figure 9 Plasmid map of pRECas1_spo0A_sg1 annotated with annealing sites of the sequencing primers (purple font).

Stage 3 – Confirmation of inducible sporulation strains

Having generated our final CRIPSR vectors, detailed in Figures 5-8 above, the next step would be to introduce these vectors into *C. sporogenes* via conjugative transfer. Successful conjugations would yield Thiamphenicol resistant (Tm^R) colonies due to the presence of the *catP* marker. To induce the expression of *cas9*, we would then streak several Tm^R colonies onto agar plates supplemented with Theophylline. Cas9 would be recruited to the intergenic regions upstream of our chosen sporulation genes by the sgRNA components and introduce lethal double strand breaks into the chromosome of wild-type *C. sporogenes* cells. Therefore, only cells which had undergone successful genome editing to replace the native promoter sequences with the aTc-inducible promoter should survive. We would then need to confirm this was the case by performing screening PCRs using primers which anneal to

chromosomal regions outside of the homology arms in each vector. Primers designed for this purpose and the expected results of such screening PCRs are detailed below.

spo0A

Primer name	Primer sequence (5'-3')	Amplicon size (bp)	
		Native promoter	$P_{xyl/tet}$
spo0A_scr_F	aggagaaatagtaccatcctctatagtatc	1521	2031
spo0A_scr_R	aagttccatgtcattaactacc		

Table 14 Screening primer sequences and expected amplicon sizes to determine the presence/absence of the aTc-inducible promoter upstream of spo0A *in* C. sporogenes.

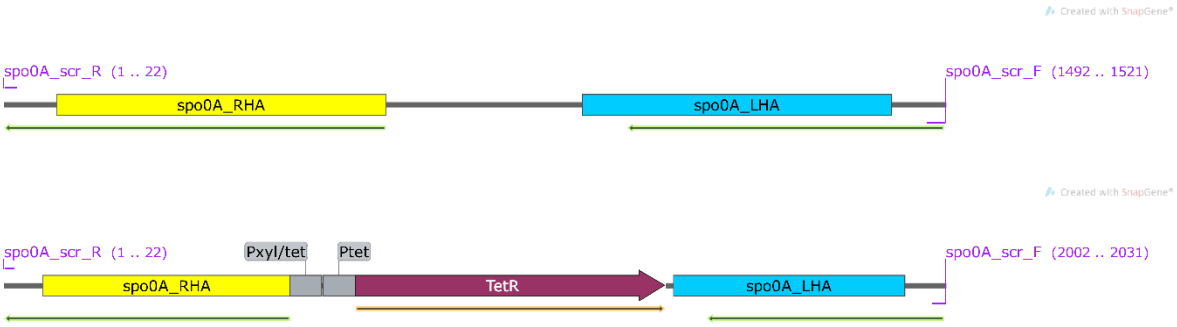

Figure 10 Primer annealing sites within C. sporogenes *chromosome to screen for replacement of the upstream region of* spo0A.

spoIID

Primer name	Primer sequence (5'-3')	Amplicon size (bp)	
		Native promoter	$P_{xyl/tet}$
spoIID_scr_F	atagaagcaggaacttttatgatagc	1379	1872
spoIID_scr_R	tacaagtacatttcccttagtgc		

Table 15 Screening primer sequences and expected amplicon sizes to determine the presence/absence of the aTc-inducible promoter upstream of spoIID *in* C. sporogenes.

spoIIIAA

Primer name	Primer sequence (5'-3')	Amplicon size (bp)	
		Native promoter	$P_{xyl/tet}$
spoIIIAA_scr_F	aactggagctacaattcaagttcc	1332	1901
spoIIIAA_scr_R	taactgaggaacaccattataactcc		

Table 16 Screening primer sequences and expected amplicon sizes to determine the presence/absence of the aTc-inducible promoter upstream of spoIIIAA *in* C. sporogenes.

spoIVA

Primer name	Primer sequence (5'-3')	Amplicon size (bp)	
		Native promoter	$P_{xyl/tet}$
spoIVA_scr_F	tatgagcagaacctggtacagg	1500	1915
spoIVA_scr_R	agactctttcttagctctatagtttcc		

Table 17 Screening primer sequences and expected amplicon sizes to determine the presence/absence of the aTc-inducible promoter upstream of spoIVA *in* C. sporogenes.

Stage 4 – Experimental Plan

Unfortunately, Covid-19 restrictions in our host laboratory prevented us from being able to conduct any wet-lab research. The following steps outline the experiments we would have conducted had the wet lab been available to us during our project. Protocol #s refer to protocols stated on the Experiments wiki page.

1. Extraction of required DNA templates. Genomic DNA (gDNA) from *C. sporogenes* would be extracted using a genomic DNA extraction kit (protocol #1 in Experiments page), whilst the extraction of the vectors pMTL_tet3nO_RFTetR and pRECas1 from *E. coli* would be performed using a Qiagen Miniprep kit (protocol #10).

2. Amplification of the DNA fragments required to generate vectors pRECas1_spo0A, pRECas1_spoIID, pRECas1_spoIIAA and pRECas1_spoIVA would be performed via Polymerase Chain Reaction (PCR) using KOD Hot Start Master Mix and the oligonucleotide primers listed in tables 1-4. Reaction set up and thermocycling conditions are detailed in protocol #2.

3. Resulting PCR products would be loaded onto a 1% Agarose gel and separated by gel electrophoresis (protocol #3). Bands with sizes corresponding to the expected values given in tables 1-4 would be excised from the Agarose gel and the DNA purified from gel slices using a DNA gel extraction kit (protocol #4). The concentration of the eluted DNA would be quantified using a Thermo Scientific Nanodrop (protocol #5).

4. Linearisation of vector pRECas1 would be performed via digestion with restriction enzymes AatII and AscI in a 20 µl reaction volume with 1 µg of pRECas1 plasmid DNA, 2 µl CutSmart buffer and 1 µl each of enzymes AatII and AscI. The reaction mix would be made up to a total volume of 20 µl with water and incubated at 37 ^{o}C for 2 hours. Digest reaction products would be loaded onto a 1 % Agarose gel and separated by gel electrophoresis. Successful linearisation of the pRECas1 vector would be indicated by the presence of a single 9 kilobase (kb) – sized band, which would be extracted from the Agarose gel and purified (protocol #4).

5. NEBuilder HiFi DNA assembly reactions with linearised pRECas1 vector backbone and purified PCR products would be conducted according to the manufacturer's instructions (protocol #6). HiFi reaction products would then be transformed into NEB 5-alpha competent *E. coli* cells (protocol #7). Selection for *E. coli* cells containing the desired vectors would be achieved via plating the cells on Luria Bertani (LB) agar plates supplemented with Chloramphenicol (25 µg.ml^{-1}; protocol #8).

6. Chloramphenicol (Cm) resistant (^{R}) *E. coli* colonies should appear within 24 – 48 hours. Individual Cm^{R} colonies would be grown overnight in LB broth supplemented with chloramphenicol (12.5 µg.ml^{-1}) at 30 ^{o}C with horizontal shaking (200 rpm). Plasmids would be extracted from the resulting turbid cultures (protocol #10) and restriction digests as detailed in Step #4 performed. These restriction digest products would be separated by gel electrophoresis (protocol #3). Successful assembly of vectors would be indicated by the presence of two bands, one 8.8 kb in size and another 1.8 kb in size.

7. Amplification of the sgRNA components would be performed via PCR using the overlapping primers detailed in tables 6, 8, 10 and 12 (Protocol #2). Amplified products would be purified using a PCR purification kit according to the manufacturer's instructions then digested, along with the corresponding vector into which they are to be inserted, with SalI-HF and AatII restriction enzymes, as outlined in Step 4. Purified, linearised vector and sgRNA DNA fragments would then be ligated together in 20 µl reaction volumes using T4 DNA Ligase according to manufacturer's instructions and left to incubate on ice water for 16 hours.

8. Ligation reaction products would then be transformed into competent *E. coli* NEB 5-alpha cells (Protocol #7) and the recovered cells plated onto LB agar plates supplemented with chloramphenicol (25 µg.mL^{-1}). Individual resulting Cm^{R} colonies would be grown overnight in LB broth supplemented with Cm (12.5 µg.mL^{-1}) and the plasmid extractions performed the next day (Protocol #10).

9. Confirmation of successful construction of the 8 different vectors would be achieved by sending the eluted plasmids for Sanger sequencing with the primers listed in table 13. Resulting sequencing data would be analysed via alignment with the expected DNA sequences of each vector.

10. Sequence-confirmed vectors would then be transformed into electrocompetent *E. coli* CA434 cells, the conjugal donor strain (Protocol #11). Individual transfer of these vectors from *E. coli* CA434 into *C. sporogenes* 10696 would occur via conjugative transfer (Protocol #12). Single subsequent Thimaphenicol-resistant (Tm^R) *C. sporogenes* colonies would be picked and re-streaked onto TYG plates supplemented with Thiamphenicol , D-Cylcoserine and Theophylline (final concentrations of 15 $\mu g.mL^{-1}$, 250 $\mu g.mL^{-1}$ and 15 $\mu g.mL^{-1}$ respectively). All resulting colonies should contain the desired genomic edits and therefore contain the Tetracycline inducible promoter system upstream of the given gene, essential for sporulation.

11. Colony PCRs would be performed to confirm successful genome editing (Protocol #9) using the primers listed in tables 14-17, depending on the vector transferred, and the PCR products resolved via Agarose electrophoresis (Protocol #3). Colonies which yielded PCR product sizes indicative of successful genome editing would then undergo plasmid loss (Protocol #13) and the subsequent Tm-sensitive, inducible sporulation mutant stored at -80^oC in a TYG-glycerol (10% v/v) stock.

12. Accordingly, we would hope to generate four *C. sporogenes* strains in which sporulation can occur only in the presence of the inducer molecule anhydroTetracycline (aTc). We would then need to test this by performing a rate of sporulation assay with each of our four mutant strains and wildtype *C. sporogenes* as a positive control, both with and without supplemental aTc. We would expect to see no heat-resistant CFU produced by our mutant strains in the absence of aTc and, in the presence of aTc we would expect levels of heat resistant CFU either at, or approaching that, produced by wildtype *C. sporogenes*.

13. Our next steps would be informed by the results of the above assay. Should all results be as expected, we would take the *C. sporogenes* $P_{xyl/tet}$:*spo0A* strain and incrementally repeat the above mutagenesis steps to develop a quadruple mutant in which all four target genes are regulated by a Tetracycline-inducible promoter. However, should we encounter an unexpected result we would need to employ the synthetic biology principles of learning and improving our designs, before rebuilding and retesting. Should we observe low levels of, or no, heat-resistant CFU produced by our mutant strains in the presence of aTc we may need to repeat the assay with an increased range of aTc concentrations to determine the amount of aTc required to effectively induce sporulation. Furthermore, whilst all our gene targets are known to be essential for sporulation in *C. sporogenes* we cannot rule out that they may have secondary functions within the vegetative cell. Consequently, when assaying our mutant strains for DBHB production, we must include the wildtype *C. sporogenes* strain as a control to ensure none of these biocontainment strategies have impacted on the metabolism of the cell.