Insilco Work Preformed

Stage 1 - Shuttle Vector Design

Shuttle vectors with based on the pMTL8000 modular vector series were designed to quickly test which ketone production operon (KPO) would be most suitable before stable integration into the *C. sporogenes* genome.

Shuttle Vectors designed for KPO $DBHB_A$

The following plasmid vectors variants in table 1 below have been designed for testing of the $DBHB_A$ operon.

Table 1 - List of pMTL8000 modular shuttle vectors designed to test KPO $DBHB_A$.

Vector	Description	Vector Map	Primer table (Primers to amplify fragments with overhangs suitable for HiFi assembly)	Gel of expected fragments needed to assemble vector (run on a 1% agarose gel)
pMTL82151-Px-$DBHB_A$	No promoter as a negative control - no expression of KPO genes.	Figure 1-A.	Table 2	Figure 1-B.
pMTL82151-Pntnh-$DBHB_A$	Expression of KPO genes dependent upon sigma factor *botR* expression.	Figure 2-A.	Table 3	Figure 2-B.
pMTL82151-Plac-$DBHB_A$	Expression of KPO genes dependent on presence of lactose (inducible system)	Figure 3-A.	Table 4	Figure 3-B.
pMTL82151-Pfdx-$DBHB_A$	Constitutive expression of KPO genes using the native *fdx* promoter as a positive control.	Figure 4-A.	Table 5	Figure 4-B.

DBHB$_A$ KPO vector maps, primer tables and agarose gel simulations

pMTL82151-Px-DBHB$_A$

Table 2 - Primer table to amplify fragments needed to assemble pMTL82151-pX-DBHB$_A$ by HiFi assembly

Gene Name/ Fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Thiolase *(thl)*	thl_pMTL_Px_F	*C.acetobutylicum* ATCC 824 gDNA	ctgtatccatatgaaagaag ttgtaatagctag	1199
	thl_pMTL_ (A)_R		ccttttaaatctagcacttttc tagcaatattg	
Acetoacetate CoA-transferase subunit A/B *(ctfA/ & ctfB)*	ctfA/B_pMTL_(A)_F	*C.acetobutylicum* ATCC 824 gDNA	aaagtgctagatttaaaagg agggattaaaatgaac	1363
	ctfA/B_pMTL_(A)_R		tcgtctttctctaaacagcca tgggtctaag	
3-hydroxubutanoate dehydrogenase *(bdhA)*	bdhA_pMTL_ (A)_F	*Streptococcus dysgalactiae subsp. equisimilis* AC2713 gDNA	ggctgtttagagaaagacg aggatagtatc	509
	bdhA_pMTL_ (A)_R		ttatttttatgctacaaataa gctgattttcc	
Vector Backbone	Vec_pMTL_ (A)_F	pMT82151	ttatttgtagcataaaaata agaagcctgcatt	5010
	Vec_pMTL_ Px_R		cttctttcatatggatacagc ggccgcg	

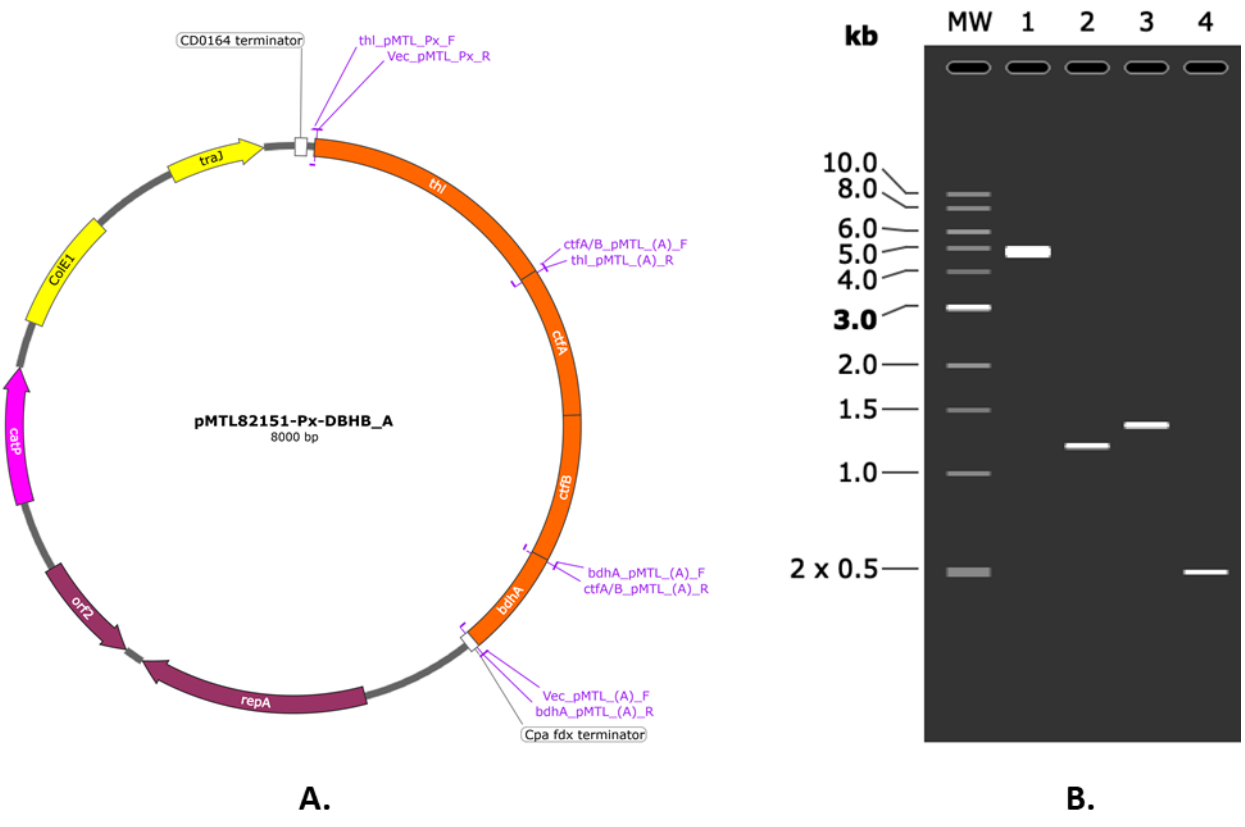

Figure 1 - pMTL82151-Px-DBHB$_A$ Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-thl; 3-ctfA/B; 4-bdhA.

pMTL82151-Pntnh-DBHB$_A$

Table 3 – Primer table to amplify fragments needed to assemble pMTL82151-pntnh-DBHB$_A$ by HiFi assembly.

Gene Name/ Fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Pntnh promoter	Pntnh_pMTL_F	*C. botulinum* ATCC 3502 gDNA	atgagtcgaccctaaaatttaaatatatcaaatttattagtatgtttac	873
	Pntnh _pMTL_R		cttctttcatatctaaccaccctcctaaatttatttc	
Thiolase *(thl)*	thl_pMTL_ Pntnh_F	*C.acetobutylicum* ATCC 824 gDNA	gtggttagatatgaaagaagttgtaatagctag	1199
	thl_pMTL_(A)_R		ccttttaaatctagcacttttctagcaatattg	
Acetoacetate CoA-transferase subunit A/B *(ctfA/ & ctfB)*	ctfA/B_pMTL_ (A)_F	*C.acetobutylicum* ATCC 824 gDNA	aaagtgctagatttaaaaggagggattaaaatgaac	1363
	ctfA/B_pMTL_(A)_R		tcgtctttctctaaacagccatgggtctaag	
3-hydroxubutanoate dehydrogenase *(bdhA)*	bdhA_pMTL_ (A)_F	*Streptococcus dysgalactiae subsp. equisimilis* AC2713 gDNA	ggctgtttagagaaagacgaggatagtatc	509
	bdhA_pMTL_(A)_R		ttatttttatgctacaaataagctgattttcc	
Vector Backbone	Vec_pMTL_(A)_F	pMTL82151	ttatttgtagcataaaaataagaagcctgcatt	4997
	Vec_pMTL_ Pntnh_R		aaattttagggtcgactcatagctgtttc	

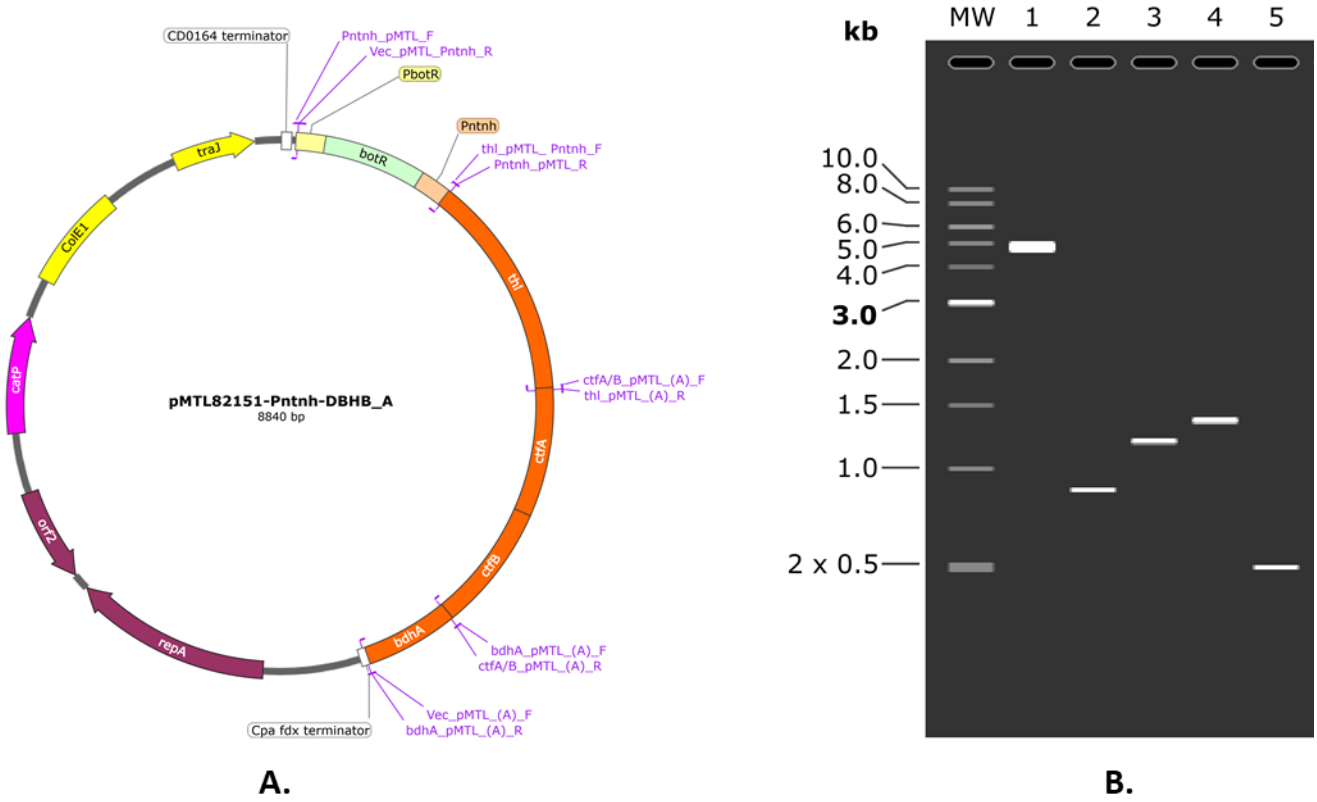

Figure 2 - pMTL82151-Pntnh-DBHB$_A$ Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Pntnh; 3--thl; 4-ctfA/B; 5-bdhA.

pMTL82151-Plac-DBHB$_A$

Table 4 - Primer table to amplify fragments needed to assemble pMTL82151-plac-DBHB$_A$ by HiFi assembly.

Gene Name/ Fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Plac promoter	Plac_pMTL_F	*C. perfringens* gDNA	atgagtcgactaatttagat attaattctaaattaagtgaa attaatatag	1421
	Plac_pMTL_R		cttctttcattttaccctccca atacatttaaaataattatg	
Thiolase *(thl)*	thl_pMTL_ Plac_F	*C.acetobutylicum* ATCC 824 gDNA	ggagggtaaaatgaaagaa gttgtaatagctag	1199
	thl_pMTL_(A)_R		ccttttaaatctagcacttttc tagcaatattg	
Acetoacetate CoA-transferase subunit A/B *(ctfA/ & ctfB)*	ctfA/B_pMTL_ (A)_F	*C.acetobutylicum* ATCC 824 gDNA	aaagtgctagatttaaaagg agggattaaaatgaac	1363
	ctfA/B_pMTL_ (A)_R		tcgtctttctctaaacagcca tgggtctaag	
3-hydroxubutanoate dehydrogenase *(bdhA)*	bdhA_pMTL_ (A)_F	*Streptococcus dysgalactiae subsp. equisimilis* AC2713 gDNA	ggctgtttagagaaagacga ggatagtatc	509
	bdhA_pMTL_ (A)_R		ttatttttatgctacaaataa gctgattttcc	
Vector Backbone	Vec_pMTL_ (A)_F	pMTL82151	ttatttgtagcataaaaataa gaagcctgcatt	4997
	Vec_pMTL_ Plac_R		atctaaattagtcgactcata gctgtttc	

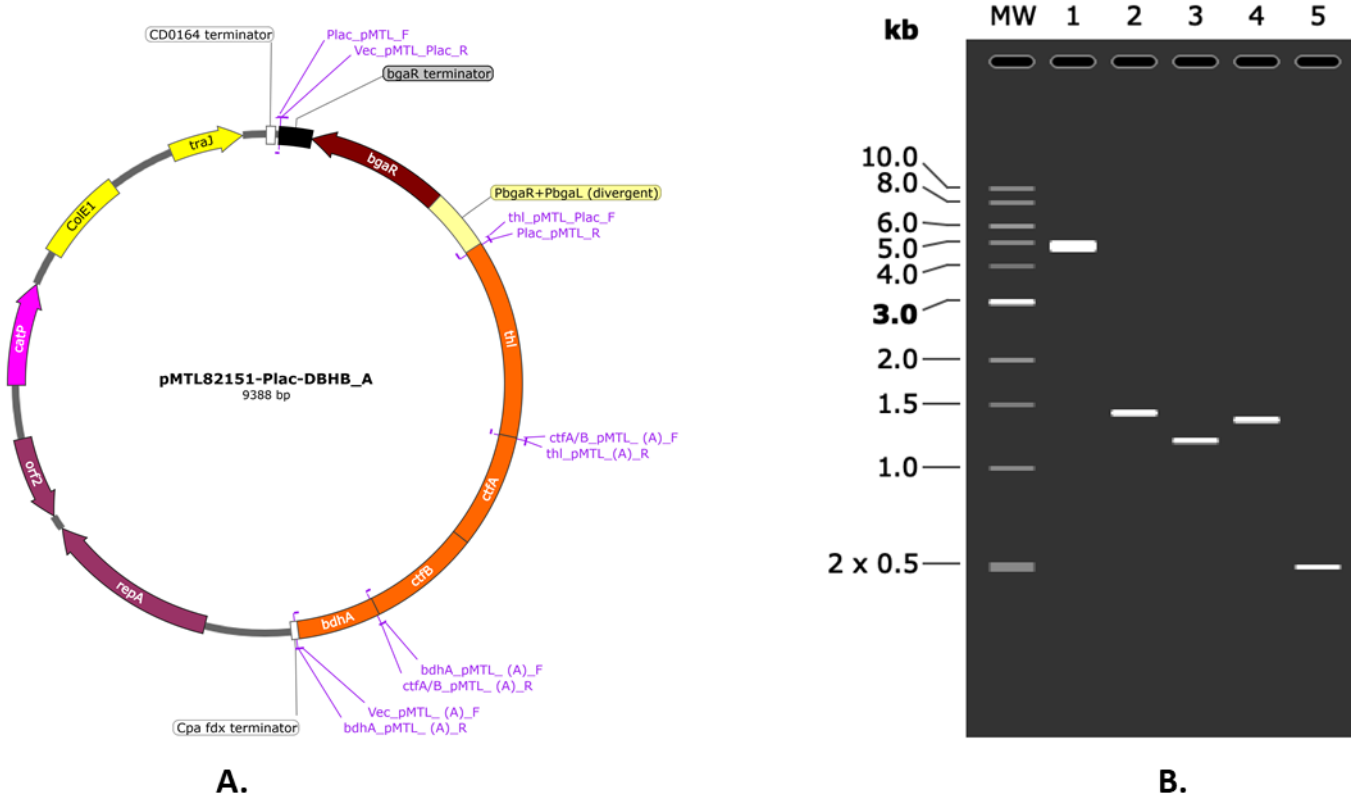

Figure 3 - pMTL82151-Plac-DBHBA Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Plac; 3--thl; 4-ctfA/B; 5-bdhA.

pMTL82151-Pfdx-DBHB$_A$

Table 5 - Primer table to amplify fragments needed to assemble pMTL82151-pfdx-DBHBA by HiFi assembly.

Gene Name/fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Pfdx promoter	Pfdx_pMTL_F	*C. sporogenes* 10696 gDNA	atgagtcgacgtgtagtagc ctgcgaaataag	223
	Pfdx_pMTL_R		cttctttcatatgtaacacac ctccttaaaaattac	
Thiolase *(thl)*	thl_pMTL_Pfdx_F	*C.acetobutylicum* ATCC 824 gDNA	tgtgttacatatgaaagaag ttgtaatagctag	1199
	thl_pMTL_ (A)_R		ccttttaaatctagcacttttc tagcaatattg	
Acetoacetate CoA-transferase subunit A/B *(ctfA/ & ctfB)*	ctfA/B_pMTL_ (A)_F	*C.acetobutylicum* ATCC 824 gDNA	aaagtgctagatttaaaagg agggattaaaatgaac	1363
	ctfA/B_pMTL_ (A)_R		tcgtctttctctaaacagcca tgggtctaag	
3-hydroxubutanoate dehydrogenase *(bdhA)*	bdhA_pMTL_ (A)_F	*Streptococcus dysgalactiae subsp. equisimilis* AC2713 gDNA	ggctgtttagagaaagacga ggatagtatc	509
	bdhA_pMTL_ (A)_R		ttatttttatgctacaaataa gctgattttcc	
Vector Backbone	Vec_pMTL_(A)_F	pMTL82151	ttatttgtagcataaaaataa gaagcctgcatt	4997
	Vec_pMTL_ Pfdx_R		gctactacacgtcgactcat agctgtttc	

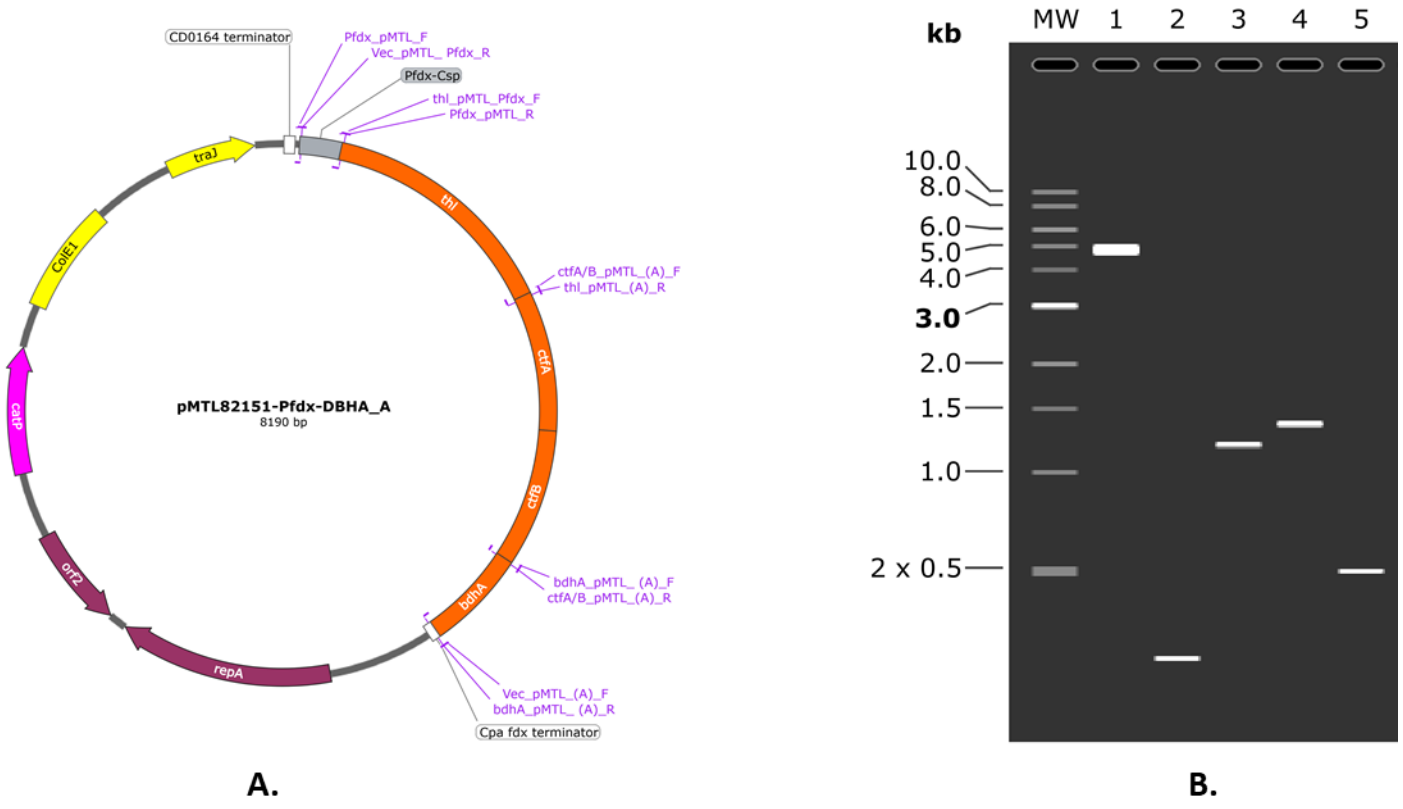

Figure 4 - pMTL82151-pfdx-DBHBA Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Pfdx; 3--thl; 4-ctfA/B; 5-bdhA.

Shuttle Vectors designed for KPO $DBHB_B$

The following plasmid vectors variants in table 6 below have been designed for testing of the $DBHB_B$ operon.

Table 6 - List of pMTL8000 modular shuttle vectors designed to test KPO $DBHB_B$.

Vector	Description	Vector Map	Primer table (Primers to amplify fragments with overhangs suitable for HiFi assembly)	Gel of expected fragments needed to assemble vector (run on a 1% agarose gel)
pMTL82151-Px-$DBHB_B$	No promoter as a negative control - no expression of KPO genes.	Figure 5-A.	Table 7	Figure 5-B.
pMTL82151-Pntnh-$DBHB_B$	Expression of KPO genes dependent upon sigma factor *botR* expression.	Figure 6-A.	Table 8	Figure 6-B.
pMTL82151-Plac-$DBHB_B$	Expression of KPO genes dependent on presence of lactose (inducible system)	Figure 7-A.	Table 9	Figure 7-B.
pMTL82151-Pfdx-$DBHB_B$	Constitutive expression of KPO genes using the native *fdx* promoter as a positive control.	Figure 8-A.	Table 10	Figure 8-B.

DBHB$_B$ KPO vector maps, primer tables and agarose gel simulations

pMTL82151-Px-DBHB$_B$

Table 7 - Primer table to amplify fragments needed to assemble pMTL82151-pX-DBHB$_B$ by HiFi assembly.

Gene Name/fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Thiolase *(thl)*	thl_pMTL_ Px_F	*C.acetobutylicum* ATCC 824 gDNA	ctgtatccatatgaaagaagttgtaatagctag	1199
	thl_pMTL_(B)_R		tctcacaaagctagcacttttctagcaatattg	
Acetoacetyl-CoA reductase *(phbB)*	phbB_pMTL_(B)_F	*Cupriavidus necator* gDNA	aaagtgctagctttgtgagagacaagcag	784
	phbB_pMTL_(B)_R		agtaacaaagttattgcatgtgctgccc	
Thioesterase II *(tesB)*	tesB_pMTL_(B)_F	*Escherichia coli* gDNA	catgcaataactttgttactggagagttatatg	901
	tesB_pMTL_(B)_R		ttatttttatttaattgtgattacgcatcac	
Vector Backbone	Vec_pMTL_(B)_F	pMTL82151	aatcacaattaaataaaaataagaagcctgcatttg	5010
	Vec_pMTL_ Px_R		cttctttcatatggatacagcggccgcg	

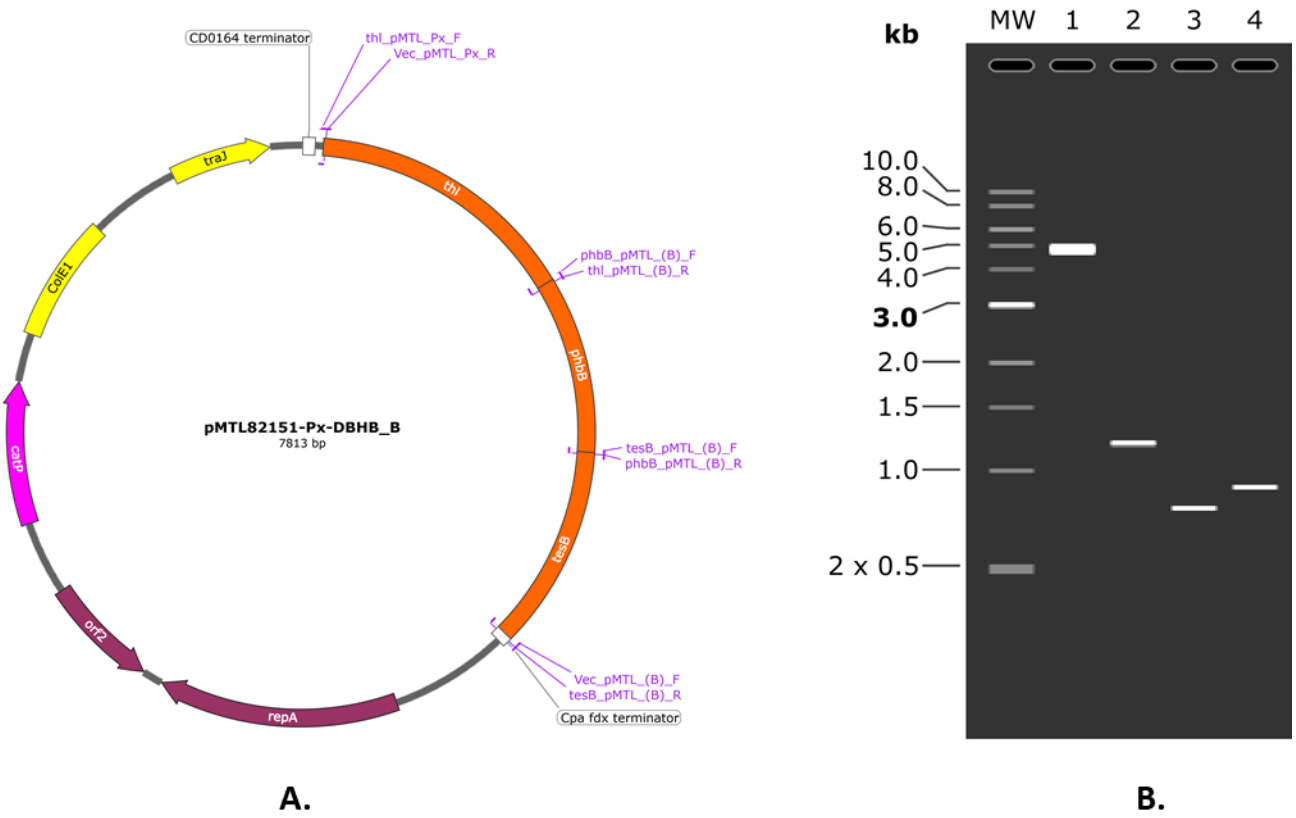

Figure 5 - pMTL82151-Px-DBHB$_B$ Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-thl; 3-phbB; 4-tesB.

pMTL82151-Pntnh-DBHB$_B$

Table 8- Primer table to amplify fragments needed to assemble pMTL82151-pntnh-DBHB$_B$ by HiFi assembly.

Gene Name/ Fragment	Primer name	Template	Primer 5′ to 3′	Amplicon size (bp)
Pntnh promoter	Pntnh_pMTL_F	*C. botulinum* ATCC 3502 gDNA	atgagtcgacccctaaaatttaaatatatcaaatttttattagtatgtttac	873
	Pntnh_pMTL_R		cttctttcatatctaaccaccctcctaaatttatttc	
Thiolase *(thl)*	thl_pMTL_ Pntnh_F	*C.acetobutylicum* ATCC 824 gDNA	gtggttagatatgaaagaagttgtaatagctag	1199
	thl_pMTL_(B)_R		tctcacaaagctagcacttttctagcaatattg	
Acetoacetyl-CoA reductase *(phbB)*	phbB_pMTL_(B)_F	*Cupriavidus necator* gDNA	aaagtgctagctttgtgagagacaagcag	784
	phbB_pMTL_(B)_R		agtaacaaagttattgcatgtgctgccc	
Thioesterase II *(tesB)*	tesB_pMTL_ (B)_F	*Escherichia coli* gDNA	catgcaataactttgttactggagagttatatg	901
	tesB_pMTL_ (B)_R		ttatttttatttaattgtgattacgcatcac	
Vector Backbone	Vec_pMTL_(B)_F	pMTL82151	aatcacaattaaataaaaataagaagcctgcatttg	4997
	Vec_pMTL_ Pntnh_R		aaattttagggtcgactcatagctgtttc	

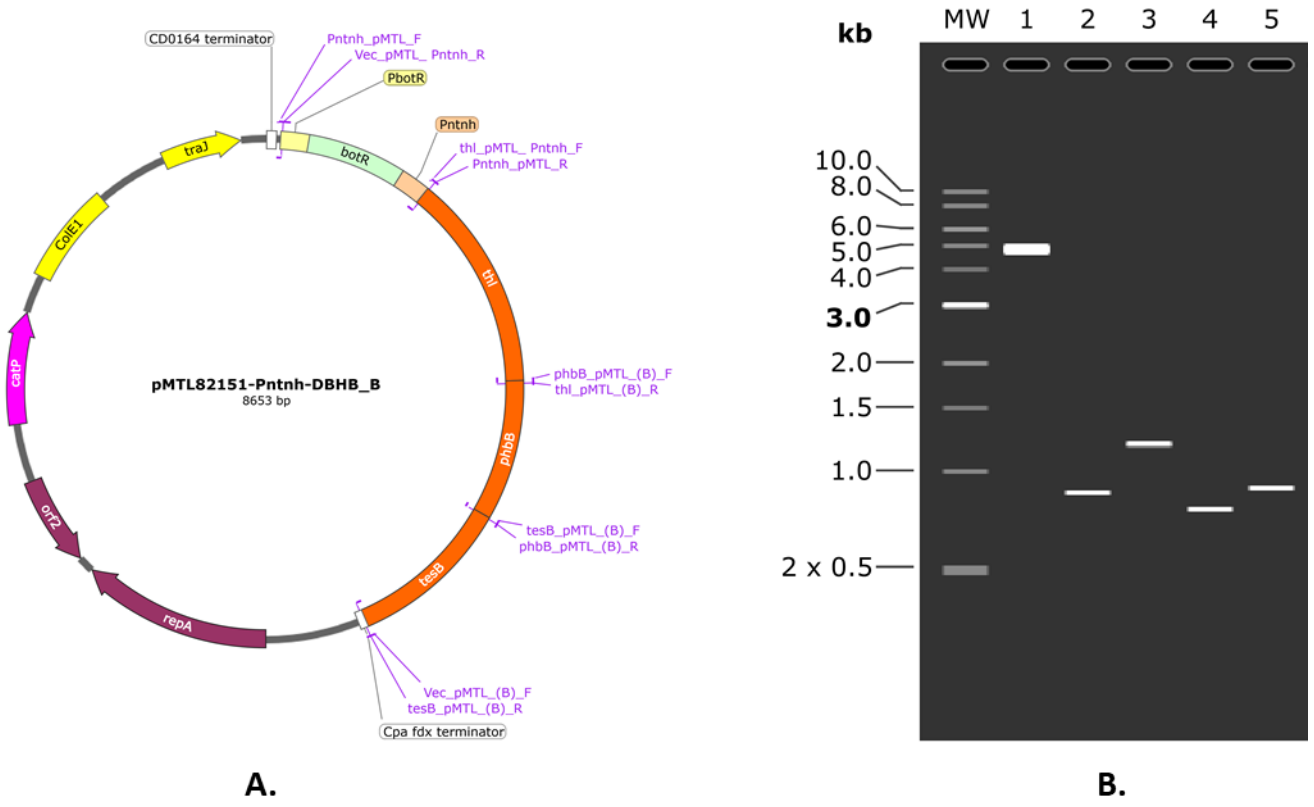

Figure 6 - pMTL82151-Pntnh-DBHB$_B$ Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Pntnh; 3-thl; 4-phbB; 5-tesB.

pMTL82151-Plac-DBHB$_B$

Table 9 - Primer table to amplify fragments needed to assemble pMTL82151-plac-DBHB$_B$ by HiFi assembly.

Gene Name/ Fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Plac promoter	Plac_pMTL_F	*C. perfringens* gDNA	atgagtcgactaatttagatattaattctaaattaagtgaaattaatatag	1421
	Plac_pMTL_R		cttctttcattttaccctcccaatacatttaaaataattatg	
Thiolase *(thl)*	thl_pMTL_ Plac_F	*C.acetobutylicum* ATCC 824 gDNA	ggagggtaaaatgaaagaagttgtaatagctag	1199
	thl_pMTL_(B)_R		tctcacaaagctagcacttttctagcaatattg	
Acetoacetyl-CoA reductase *(phbB)*	phbB_pMTL_(B)_F	*Cupriavidus necator* gDNA	aaagtgctagctttgtgagagacaagcag	784
	phbB_pMTL_(B)_R		agtaacaaagttattgcatgtgctgccc	
Thioesterase II *(tesB)*	tesB_pMTL_ (B)_F	*Escherichia coli* gDNA	catgcaataactttgttactggagagttatatg	901
	tesB_pMTL_(B)_R		ttatttttatttaattgtgattacgcatcac	
Vector Backbone	Vec_pMTL_(B)_F	pMTL82151	aatcacaattaaataaaaataagaagcctgcatttg	4997
	Vec_pMTL_ Plac_R		atctaaattagtcgactcatagctgtttc	

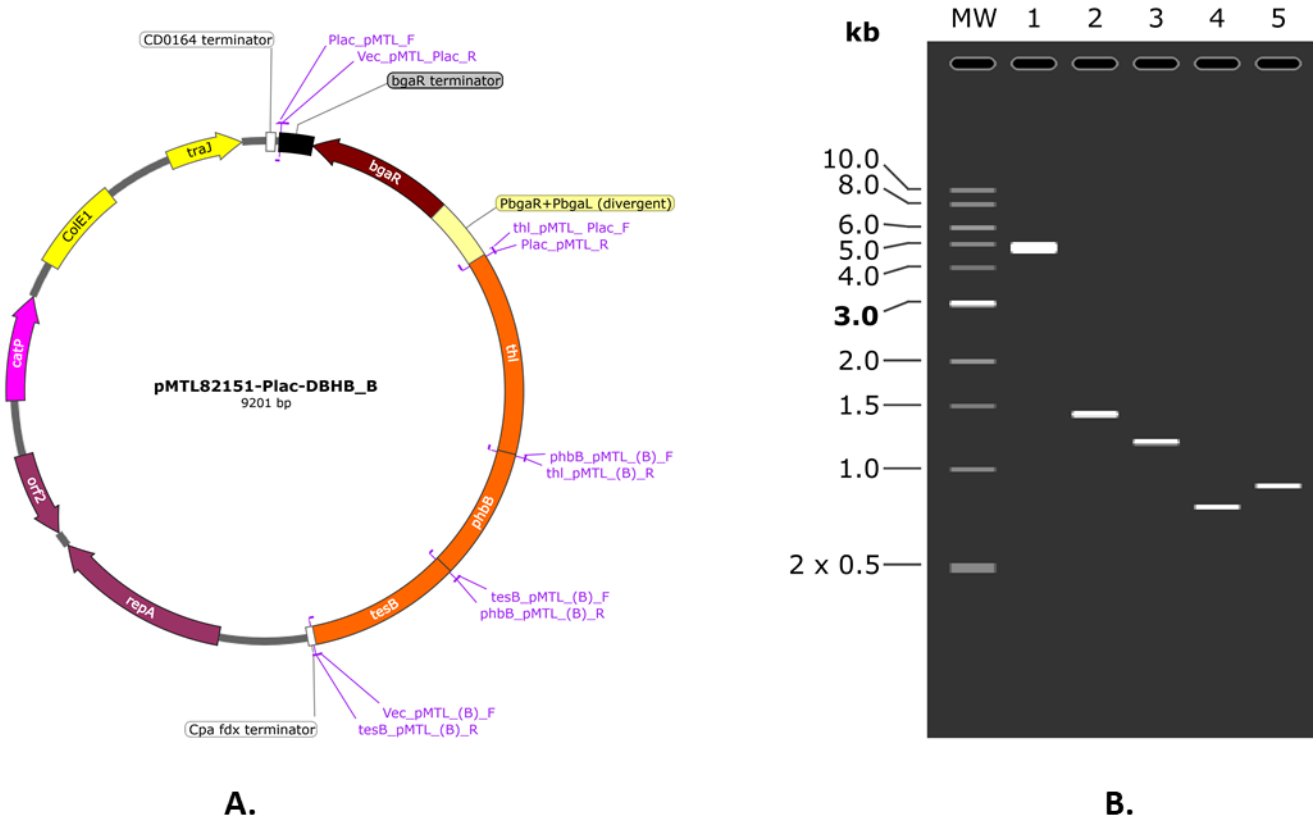

Figure 7 - pMTL82151-Plac-DBHBB Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Plac; 3-thl; 4-phbB; 5-tesB.

pMTL82151-Pfdx-DBHB$_B$

Table 10 - Primer table to amplify fragments needed to assemble pMTL82151-pfdx-DBHB$_B$ by HiFi assembly.

Gene Name/fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Pfdx promoter	Pfdx_pMTL_F	*C. sporogenes* 10696 gDNA	atgagtcgacgtgtagtagcctgcgaaataag	223
	Pfdx_pMTL_R		cttctttcatatgtaacacacctccttaaaaattac	
Thiolase *(thl)*	thl_pMTL_ Pfdx_F	*C.acetobutylicum* ATCC 824 gDNA	tgtgttacatatgaaagaagttgtaatagctag	1199
	thl_pMTL_ Pfdx(B)_R		tctcacaaagctagcacttttctagcaatattg	
Acetoacetyl-CoA reductase *(phbB)*	phbB_pMTL_(B)_F	*Cupriavidus necator* gDNA	aaagtgctagctttgtgagagacaagcag	784
	phbB_pMTL_(B)_R		agtaacaaagttattgcatgtgctgccc	
Thioesterase II *(tesB)*	tesB_pMTL_(B)_F	*Escherichia coli* gDNA	catgcaataactttgttactggagagttatatg	901
	tesB_pMTL_(B)_R		ttatttttatttaattgtgattacgcatcac	
Vector Backbone	Vec_pMTL_(B)_F	pMTL82151	aatcacaattaaataaaaataagaagcctgcatttg	4997
	Vec_pMTL_ Pfdx_R		gctactacacgtcgactcatagctgtttc	

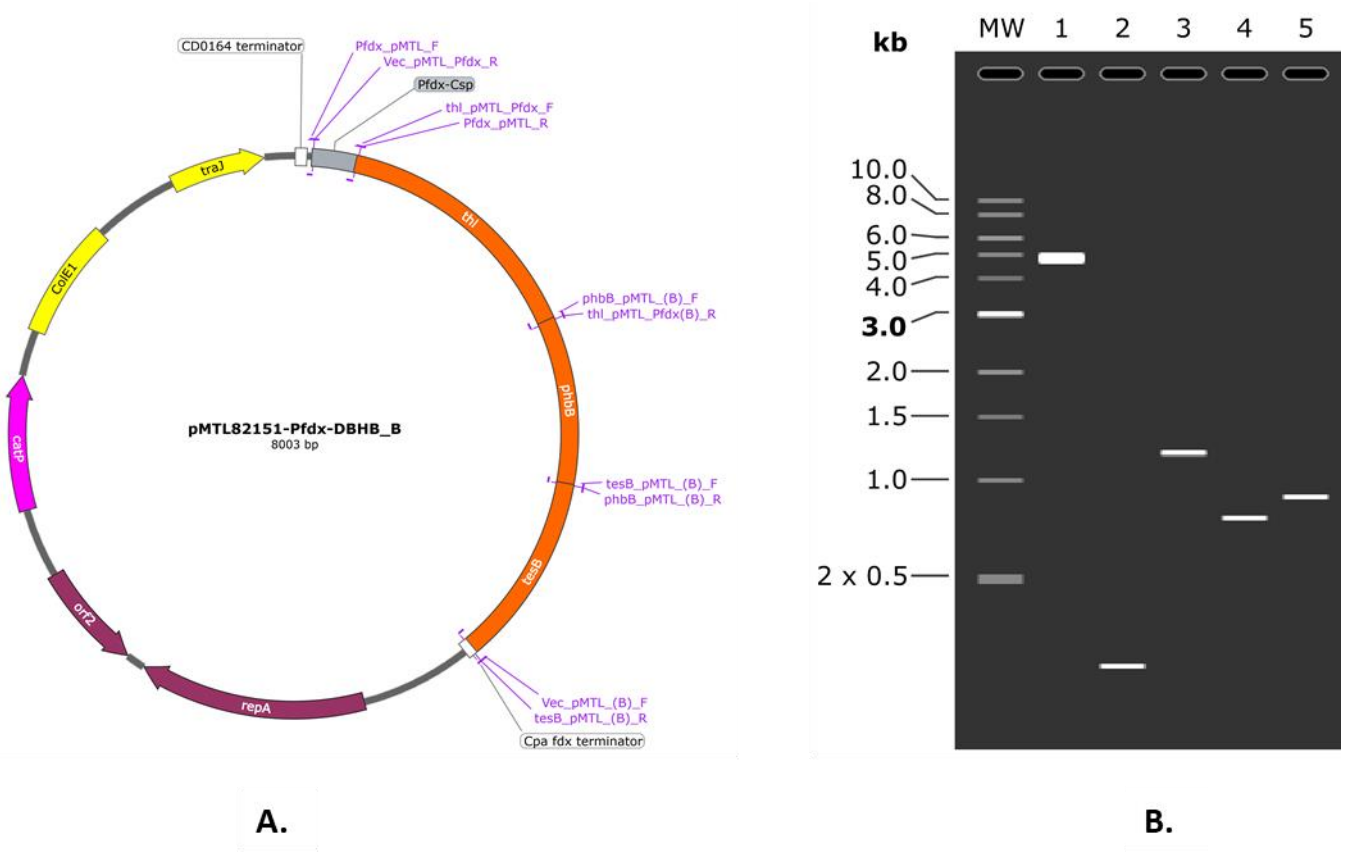

Figure 8 - pMTL82151-Pfdx-DBHB$_B$ Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Pfdx; 3-thl; 4-phbB; 5-tesB.

Shuttle vector screening primers

To confirm the successful construction of the shuttle vectors by HiFi assembly in E.coli, primers needed to be designed that bind in the vector backbone to allow amplification across the entire inserted KPO operons. The screening primer sequences and expected band sizes are found in table 11 below. These primers can also be used to confirm the presence of the shuttle vectors in *C. sporogenes* after conjugation. Figure 9 demonstrates what this would look like when run on an agarose gel by electrophoresis.

Table 11 - Screening primers sequences and band sizes for shuttle vectors to confirm assembly and/or presence in C. sporogenes

Screening primer name	Primer 5' to 3'	Amplicon size (bp)							
		pX_A	pX_B	$Pntnh_A$	$Pntnh_B$	$Pfdx_A$	$Pfdx_B$	$Plac_A$	$Plac_B$
scr_pri_pMTL_F	ttttttttatcaggaaacagc	3088	2901	3285	3049	3278	3091	4476	4289
scr_pri_pMTL_R	agaagcctgcaaatgcaggc								

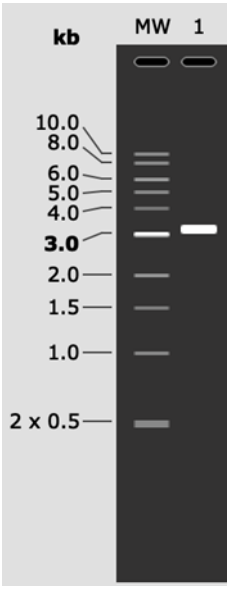

Figure 9 - A 1% agarose gel stimulation demonstrating the band size expected if the pMTL82151-Pfdx-DBHBA is correctly assembled vector (or present in C. sporogenes genome) – MW- Ladder; 1- pMTL82151-Pfdx-DBHB$_A$

Stage 2 – Genomic Integration Vector Design

Once tested using shuttle vectors the most suitable pathway ($DBHB_A$ in our 'dry lab' project) would be integrated into the C. sporogeneses genome to allow stable expression of the KPO without the need to maintain the plasmid vector by antibiotic selection. To integrate the $DBHB_A$ KPO with the three promotor variants for alternative expression profiles we would use the RiboCas system. Below in table 12 outlines the three vectors that would be created.

Table 12 - List of RiboCas vectors designed to integrate the DBHBA KPO into the C. sporogenes genome

Vector	Vector Map	Primer table (Primers to amplify fragments with overhangs suitable for HiFi assembly)	Gel of expected fragments needed to assemble vector (run on a 1% agarose gel)
pRECas1-Pfdx-DBHB$_A$	Figure 10-A.	Table 13	Figure 10-B.
pRECas1-Pntnh-DBHB$_A$	Figure 11-A.	Table 14	Figure 11-B.
pRECas1-Plac-DBHB$_A$	Figure 12-A.	Table 15	Figure 12-B.

RiboCas DBHB$_A$ KPO vector maps, primer tables and agarose gel simulations

pRECas1-Pfdx-DBHB$_A$

Table 13 - Primer table to amplify fragments needed to assemble pRECas1-Pfdx-DBHB$_A$ by HiFi assembly.

Gene Name/ Fragment	Primer Name	Template	Primer 5' to 3'	Amplicon size (bp)
Pfdx promotor	Pfdx_pRECas1_F	*C. sporogenes* 10696 gDNA	attgtggcagactttattttatctattgatttatttcatttttttaatgtgtagtagcctgcgaaat	260
	Pfdx_pRECas1_R		cttctttcatatgtaacacacctccttaaaaattac	
Thiolase *(thl)*	thl_pRECas1_Pfdx_F	*C.acetobutylicum* ATCC 824 gDNA	tgtgttacatatgaaagaagttgtaatagctag	1199
	thl_pRECas1_R		ccttttaaatctagcacttttctagcaatattg	
Acetoacetate CoA-transferase subunit A/B *(ctfA/ & ctfB)*	ctfA/B_pRECas1_F	*C.acetobutylicum* ATCC 824 gDNA	aaagtgctagatttaaaaggagggattaaaatgaac	1363
	ctfA/B_pRECas1_R		tcgtctttctctaaacagccatgggtctaag	
3-hydroxubutanoate dehydrogenase *(bdhA)*	bdhA_pRECas1_F	*Streptococcus dysgalactiae subsp. equisimilis* AC2713 gDNA	ggctgtttagagaaagacgaggatagtatc	529
	bdhA_pRECas1_R		aagcctgcaaatgcaggcttcttatttttatctacaaataagctgattttcc	
Right Homology Arm (RHA)	RHA_pRECas1_F	*C. sporogenes* 10696 gDNA	agcctgcatttgcaggcttcttatttttatatttaaaaataaggagtgtc	840
	RHA_pRECas1_R		gcggcgcgccaactccccataaataatcttg	
Vector Backbone	Vec_pRECas1_F	pRECas1-ΔpyrE::PbotR-botR (from Nottingham IGEM 2019 project)	atggggagttggcgcgccgccattattt	8806
	Vec_pRECas1_R		aaggtcaatcgacgtcataaaaataagaagcctgcaaatg	
Left Homology Amr (LHA)	LHA_ pRECas1_F	*C. sporogenes* 10696 gDNA	ttatgacgtcgattgaccttaaatcctaaagaag	856
	LHA_pRECas1_R		taaagtctgccacaattgtggcagactttattttatctattggcagttatatcttctccttatact	

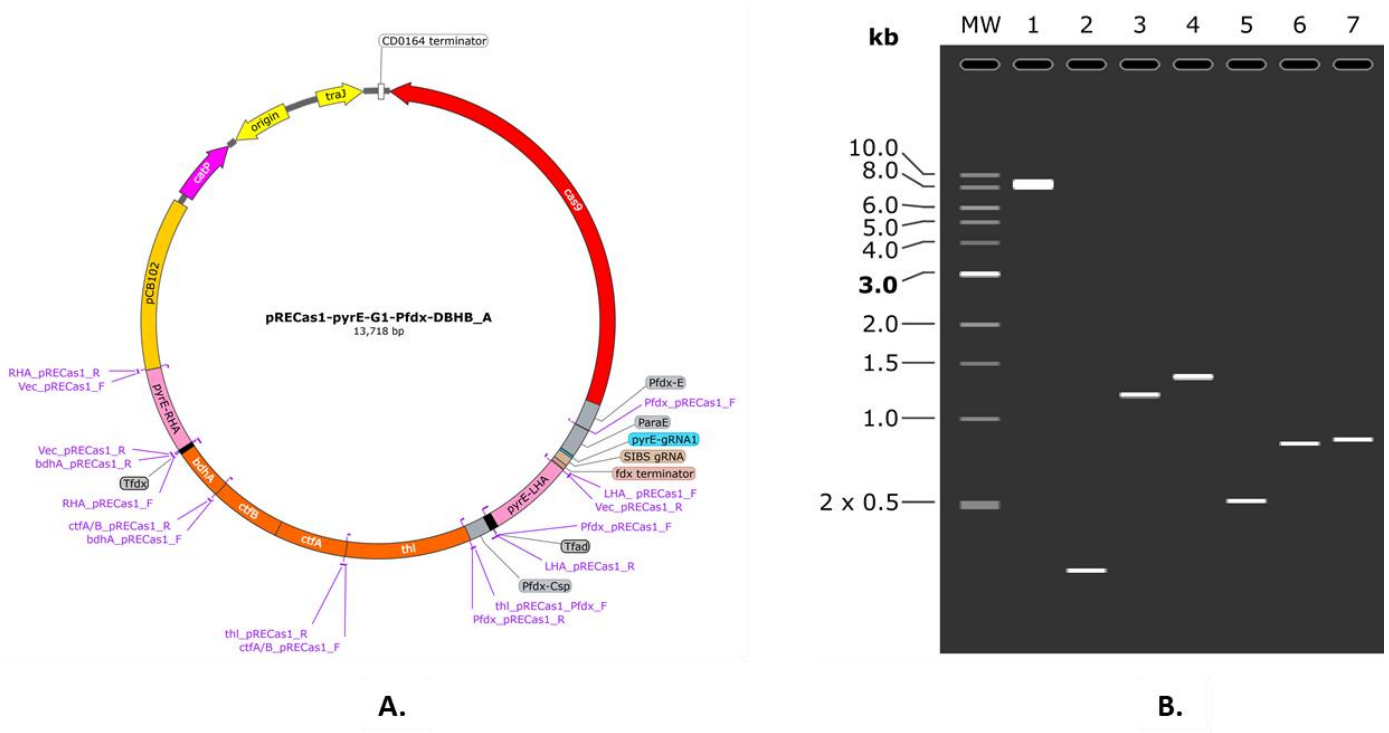

Figure 10 -pRECas1-Pfdx-DBHB$_A$ Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Pfdx; 3-thl; 4-ctfA/B; 5-bdhA; 6-RHA; 7-LHA.

pRECas1-Pntnh-DBHB$_A$

Table 14 - Primer table to amplify fragments needed to assemble pRECas1-Pntnh-DBHB$_A$ by HiFi assembly.

Gene Name/ Fragment	Primer Name	Template	Primer 5' to 3'	Amplicon size (bp)
Pntnh promotor	Pntnh_pRECas1_F	*C. botulinum* ATCC 3502 gDNA	attgtggcagactttattttatctattgatttatttcatttttta atcctaaaatttaaatatatc	910
	Pntnh_pRECas1_R		cttctttcatatctaaccaccctcctaaatttatttc	
Thiolase *(thl)*	thl_pRECas1_Pntnh_F	*C.acetobutylicum* ATCC 824 gDNA	gtggttagatatgaaagaagttgtaatagctag	1199
	thl_pRECas1_R		ccttttaaatctagcacttttctagcaatattg	
Acetoacetate CoA-transferase subunit A/B *(ctfA/ & ctfB)*	ctfA/B_pRECas1_F	*C.acetobutylicum* ATCC 824 gDNA	aaagtgctagatttaaaaggagggattaaaatgaac	1363
	ctfA/B_pRECas1_R		tcgtctttctctaaacagccatgggtctaag	
3-hydroxubutanoate dehydrogenase *(bdhA)*	bdhA_pRECas1_F	*Streptococcus dysgalactiae subsp. equisimilis* AC2713 gDNA	ggctgtttagagaaagacgaggatagtatc	529
	bdhA_pRECas1_R		aagcctgcaaatgcaggcttcttatttttatctacaaataag ctgattttcc	
Right Homology Arm (RHA)	RHA_pRECas1_F	*C. sporogenes* 10696 gDNA	agcctgcatttgcaggcttcttatttttatatttaaaaataag gagtgtc	840
	RHA_pRECas1_R		gcggcgcgccaactccccataaataatcttg	
Vector Backbone	Vec_pRECas1_F	pRECas1-ΔpyrE::PbotR-botR (from Nottingham IGEM 2019 project)	atggggagttggcgcgccgccattattt	8806
	Vec_pRECas1_R		aaggtcaatcgacgtcataaaaataagaagcctgcaaatg	
Left Homology Amr (LHA)	LHA_ pRECas1_F	*C. sporogenes* 10696 gDNA	ttatgacgtcgattgaccttaaatcctaaagaag	856
	LHA_pRECas1_R		taaagtctgccacaattgtggcagactttattttatctattgg cagttatatcttctccttatact	

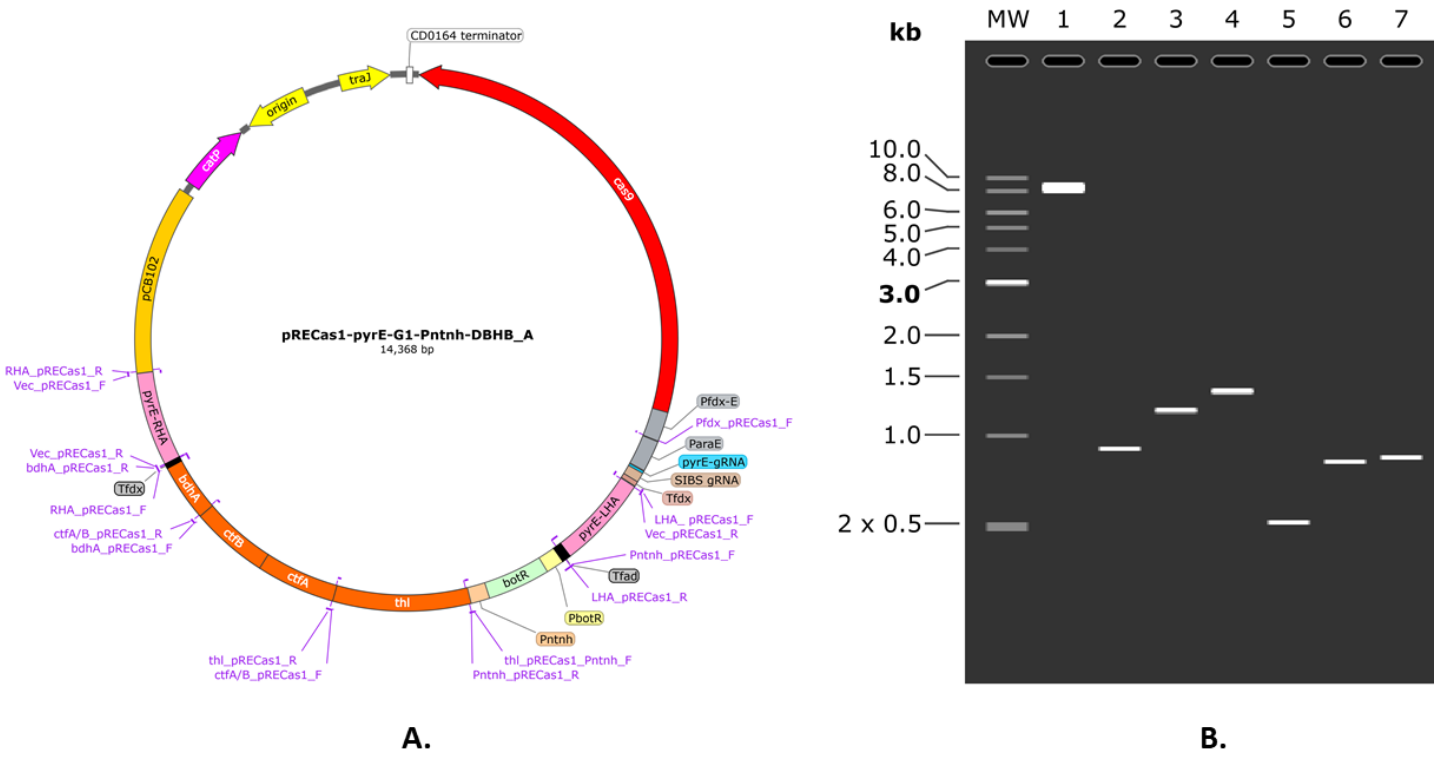

Figure 11 - pRECas1-Pntnh-DBHB$_A$ Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Pntnh; 3-thl; 4-ctfA/B; 5-bdhA; 6-RHA; 7-LHA.

pRECas1-Plac-DBHB$_A$

Table 15 - Primer table to amplify fragments needed to assemble pRECas1-Plac-DBHB$_A$ by HiFi assembly.

Gene Name/ Fragment	Primer Name	Template	Primer 5' to 3'	Amplicon size (bp)
Plac promotor	Plac_pRECas1_F	*C. perfringens* gDNA	attgtggcagactttattttatctattgatttatttcatttttta attaatttagatattaattct	1458
	Plac_pRECas1_R		cttctttcattttaccctcccaatacatttaaaataattatg	
Thiolase *(thl)*	thl_pRECas1_Plac_F	*C.acetobutylicum* ATCC 824 gDNA	ggagggtaaaatgaaagaagttgtaatagctag	1199
	thl_pRECas1_R		ccttttaaatctagcacttttctagcaatattg	
Acetoacetate CoA-transferase subunit A/B *(ctfA/ & ctfB)*	ctfA/B_pRECas1_F	*C.acetobutylicum* ATCC 824 gDNA	aaagtgctagatttaaaaggagggattaaaatgaac	1363
	ctfA/B_pRECas1_R		tcgtctttctctaaacagccatgggtctaag	
3-hydroxubutanoate dehydrogenase *(bdhA)*	bdhA_pRECas1_F	*Streptococcus dysgalactiae subsp. equisimilis* AC2713 gDNA	ggctgtttagagaaagacgaggatagtatc	529
	bdhA_pRECas1_R		aagcctgcaaatgcaggcttcttatttttatctacaaataag ctgattttcc	
Right Homology Arm (RHA)	RHA_pRECas1_F	*C. sporogenes* 10696 gDNA	agcctgcatttgcaggcttcttatttttatatttaaaaataag gagtgtc	840
	RHA_pRECas1_R		gcggcgcgccaactccccataaataatcttg	
Vector Backbone	Vec_pRECas1_F	pRECas1-ΔpyrE::PbotR-botR (from Nottingham IGEM 2019 project)	atggggagttggcgcgccgccattattt	8806
	Vec_pRECas1_R		aaggtcaatcgacgtcataaaaataagaagcctgcaaatg	
Left Homology Amr (LHA)	LHA_ pRECas1_F	*C. sporogenes* 10696 gDNA	ttatgacgtcgattgaccttaaatcctaaagaag	856
	LHA_pRECas1_R		taaagtctgccacaattgtggcagactttattttatctattgg cagttatatcttctccttatact	

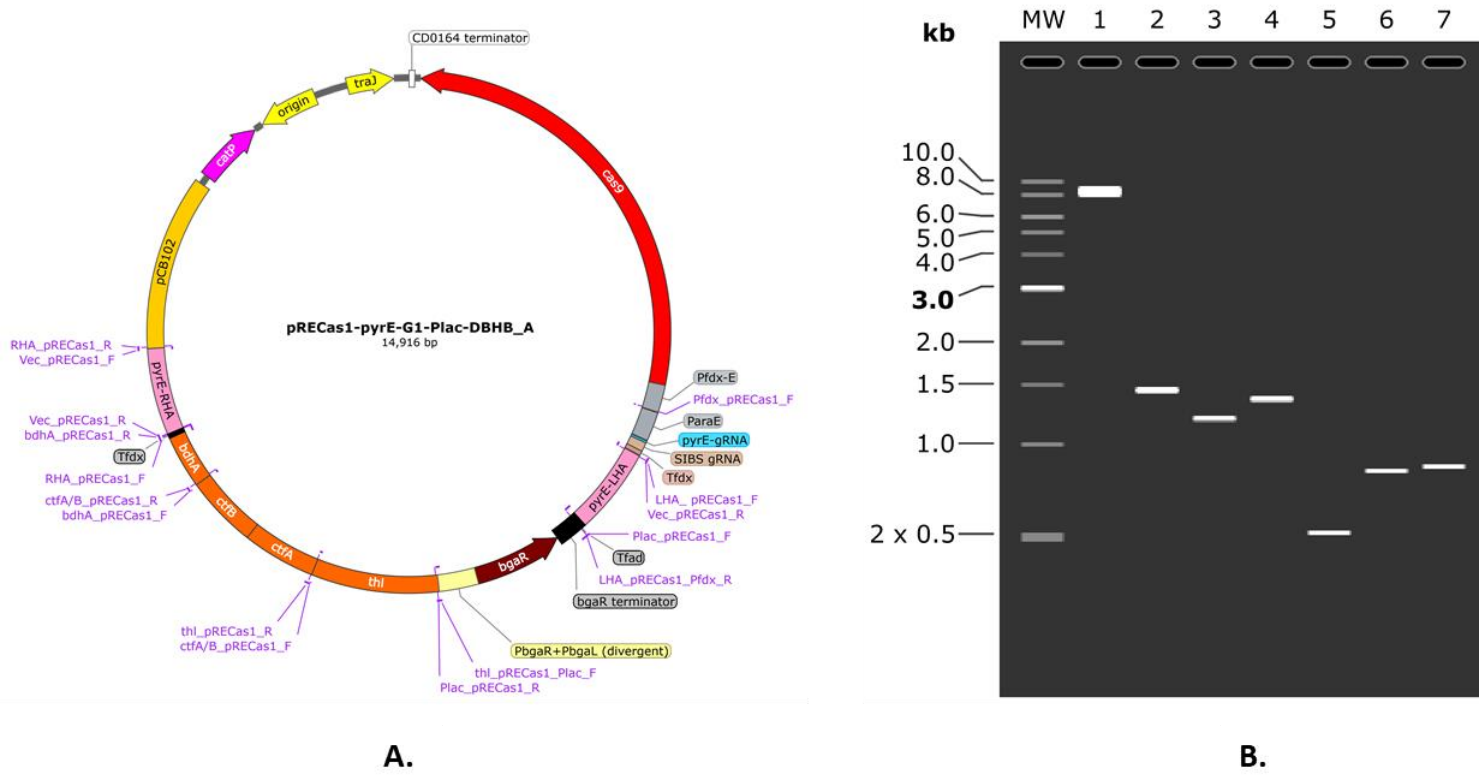

Figure 12- pRECas1-Plac-DBHBA Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1-Vector backbone2-Plac; 3-thl; 4-ctfA/B; 5-bdhA; 6-RHA; 7-LHA.

Genomic integration screening primers

To confirm the successful construction of the integration vectors by HiFi assembly in E.coli, primers needed to be designed that bind in the vector backbone to allow amplification across the region of the vector including the $DBHB_A$ operon, homology arms and sgRNA region. These primers and the expected band sizes for the three vectors are shown in table 16 below.

Table 16 - Screening primers to confirm successful assembly of the RiboCas integration vectors in E.coli.

Vector screening primer name	Primer 5' to 3'	Amplicon size (bp)		
		$Pntnh_A$	$Pfdx_A$	$Plac_A$
sgRNA-F	cctttcatttacaattcatacg	5277	5319	6517
pCB102-R	ctgttatgccttttgactatc			

A second set of screening primers needed to be designed to allow screening of *C. sporogenes* colonies following conjugation with the RiboCas integration vectors. These primers bind in the *C. sporogenes* genome either side of the *pyrE* locus in which the $DBHB_A$ KPO is to be integrated into. The primer sequences can be found in table 17 below and the binding locations of these primers is demonstrated in figure 13.

Table 17 - Screening primers to confirm successful integration of the DBHBA variants into the C. sporogenes genome.

Genomic screening primer name	Primer 5' to 3'	Amplicon size (bp)		
		$Pntnh_A$	$Pfdx_A$	$Plac_A$
scr_pri_pRECas1_F	actgcagtacaaataggaacagc	3623	3741	4639
scr_pri_pRECas1_ R	tctttgctaaaaatacttggttttgc			

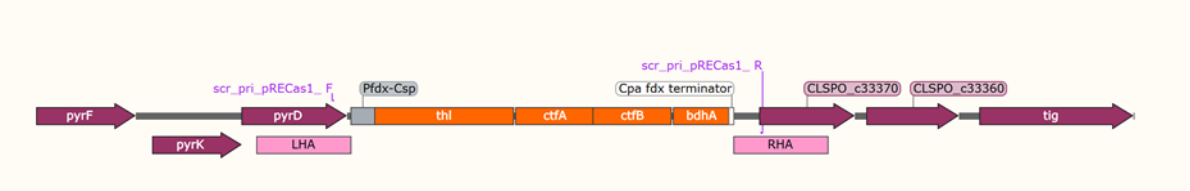

Figure 13- C. sporogenes pyrE locus with $DBHB_A$ KPO driven be the native fdx promotor replacing the pyre gene with the genomic screening primers binding sites shown.

How this would be implemented in the wet lab

If the wet lab was available to use these are the steps we would have followed to create the vectors we have designed, which we have outlined above. The process of making both the shuttle and genomic integration vectors is the same. The difference in the process comes when conjugating into *C. sporogenes* – which is described below.

Step 1

Extraction of gDNA (from organisms stated in primers tables) using genomic DNA extraction kit (See Protocol 1 in Experiments Page).

Step 2

Amplification of DNA fragments needed to construct vectors using KOD Hot Start Master Mix and primers from table(s) 2-5, 7-10 for shuttle vectors and tables 13-15 for integration vectors. See protocol 2 for example reaction setup and thermocycler conditions.

Step 3

Load PCR reactions on 1% agarose gel and septate by electrophoresis (Protocol 3).

Step 4

Extract bands (expected as shown in figures 1-B. to 8-B. and 10-B.to 12-B.) using a DNA gel extraction kit (as outlined in protocol 4) and quantify DNA concentrations obtained using Thermo Scientific Nanodrop (see protocol 5).

Step 5

Setup the NEBuilder HiFi DNA assembly reaction with the amplified fragments to be assembled following the ratios suggested in manufactures protocol (protocol 6).

Step 6

After HiFi incubation transform reaction mixture into the NEB 5-alpha competent 2 "cloning" *E. coli* cells provided in the HiFi cloning kit following the manufactures transformation protocol (protocol 7) and plating onto Luria-Bertani (LB) agar supplemented with chloramphenicol (at 25 µg/mL) – to select for successful transformants (plates made following protocol 8).

Step 7

Screen any colonies that appear on the transformation plates by colony PCR using primers in table 11 for shuttle vectors and table 16 for RiboCas integration vectors - following protocol 9.

Analyse PCR products by gel electrophoresis (protocol 3) looking for band sizes outlined in table 11 and 16 for the respective vectors.

Step 8

Inoculate 5 mL of LB broth containing chloramphenicol (at a final concentration of 12.5 µg/mL) with a colony identified from the gel as being correctly assembled (with the expected band size). From this liquid culture extract the plasmid from the bacteria suing a miniprep kit- following protocol 10. The plasmid DNA can then be sent to be sanger sequenced, preparing the samples as per the sequencing company's requirements (using the same primers used in the colony PCR), to ensure the correct sequence is has been assembled.

Step 9

The extracted plasmid is then transformation into a conjugation donor *E.coli* strain CA434 by electroporation following protocol 11 and again plate on LB+ Chloramphenicol (25 µg/mL) selection plates.

Step 10

Once the plasmid vectors (both the shuttle and genomic integration vectors) have been assembled and transformed into the conjugation donor *E.coli* strain as outlined above they can be conjugated into *C. sporogenes* 10696. The steps carried out will differ depending on if conjugating the preliminary shuttle vector or the vectors for genomic integration of the KPO operon.

<u>For the shuttle vectors</u>

Follow the conjugation protocol outlined in protocol 12. Once trans-conjugants appear on the selection plates pick single colonies and screen by colony PCR and gel electrophoresis (as in protocol 9 and 3 respectively) using primers in table 16 for presence of the shuttle vector plasmid. Restreak confirmed trans-conjugant colonies onto TYG selective plates with thiamphenicol (15µg/mL) to ensure the shuttle plasmid is maintained.

<u>For the genomic integration vectors</u>

Follow the conjugation protocol outlined in protocol 12. Once trans-conjugants appear on the selection plates pick single colonies and restreak onto reduced TYG plates supplemented with Thiamphenicol , D-Cylcoserine and Theophylline (final concentrations of 15µg/mL, 250µg/mL and 15µg/mL respectively). After incubation in the anaerobic cabinet overnight any colonies that appear to the theophylline plates should be successful mutants. Screen these colonies to grow by colony PCR and gel electrophoresis (as in protocol 9 and 3 respectively) using primers in table 17 for successful integration of the KPO operon in the *C.*

sporogenes genome. Choose a mutant colony confirmed by the colony PCR lose the plasmid as described in protocol 13.

The mutant with the plasmid lost can then be stored at -80^{o}C in a TYG-glycerol (10% v/v) stock. The genomic DNA can be extracted as in protocol 1 and sent externally for sanger sequencing to confirm the operon has been correctly inserted with no errors.

Further steps

Growth and spore assays to characterise the $DBHB_A$ KPO integrated strains and gas chromatography analysis of strain supernatants to determine whether our *C. sporogenes* strains produce the desired concentrations of DBHB and which promotor variant may be most suitable.