

## In silico Work Performed

### 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<sub>A</sub>

The following plasmid vectors variants in table 1 below have been designed for testing of the DBHB<sub>A</sub> operon.

Table 1 - List of pMTL8000 modular shuttle vectors designed to test KPO DBHB<sub>A</sub>.

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 <sub>A</sub>	No promoter as a negative control - no expression of KPO genes.	Figure 1-A.	Table 2	Figure 1-B.
pMTL82151-Pntnh-DBHB <sub>A</sub>	Expression of KPO genes dependent upon sigma factor <i>botR</i> expression.	Figure 2-A.	Table 3	Figure 2-B.
pMTL82151-Plac-DBHB <sub>A</sub>	Expression of KPO genes dependent on presence of lactose (inducible system)	Figure 3-A.	Table 4	Figure 3-B.
pMTL82151-Pfdx-DBHB <sub>A</sub>	Constitutive expression of KPO genes using the native <i>fdx</i> promoter as a positive control.	Figure 4-A.	Table 5	Figure 4-B.

## DBHB<sub>A</sub> KPO vector maps, primer tables and agarose gel simulations

### pMTL82151-Px-DBHB<sub>A</sub>

Table 2 - Primer table to amplify fragments needed to assemble pMTL82151-pX-DBHB<sub>A</sub> by HiFi assembly

Gene Name/ Fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Thiolase ( <i>thl</i> )	thl_pMTL_Px_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	ctgtatccatgatgaaagaag ttgtaatagctag	1199
	thl_pMTL_(A)_R		ccttttaaatctagcacttttc tagcaatattg	
Acetoacetate CoA- transferase subunit A/B ( <i>ctfA/ &amp; ctfB</i> )	ctfA/B_pMTL_(A)_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	aaagtgctagattaaaagg agggattaaaatgaac	1363
	ctfA/B_pMTL_(A)_R		tcgtctttctctaaacagcca tgggtctaag	
3-hydroxybutanoate dehydrogenase ( <i>bdhA</i> )	bdhA_pMTL_(A)_F	<i>Streptococcus</i> <i>dysgalactiae</i> subsp. <i>equisimilis</i> AC2713 gDNA	ggctgttagagaaagacg aggatagtatc	509
	bdhA_pMTL_(A)_R		ttattttatgctacaataa gctgattttcc	
Vector Backbone	Vec_pMTL_(A)_F	pMT82151	ttattttagcataaaaata agaagcctgcatt	5010
	Vec_pMTL_Px_R		cttcttcatatggatacagc ggccgcg	

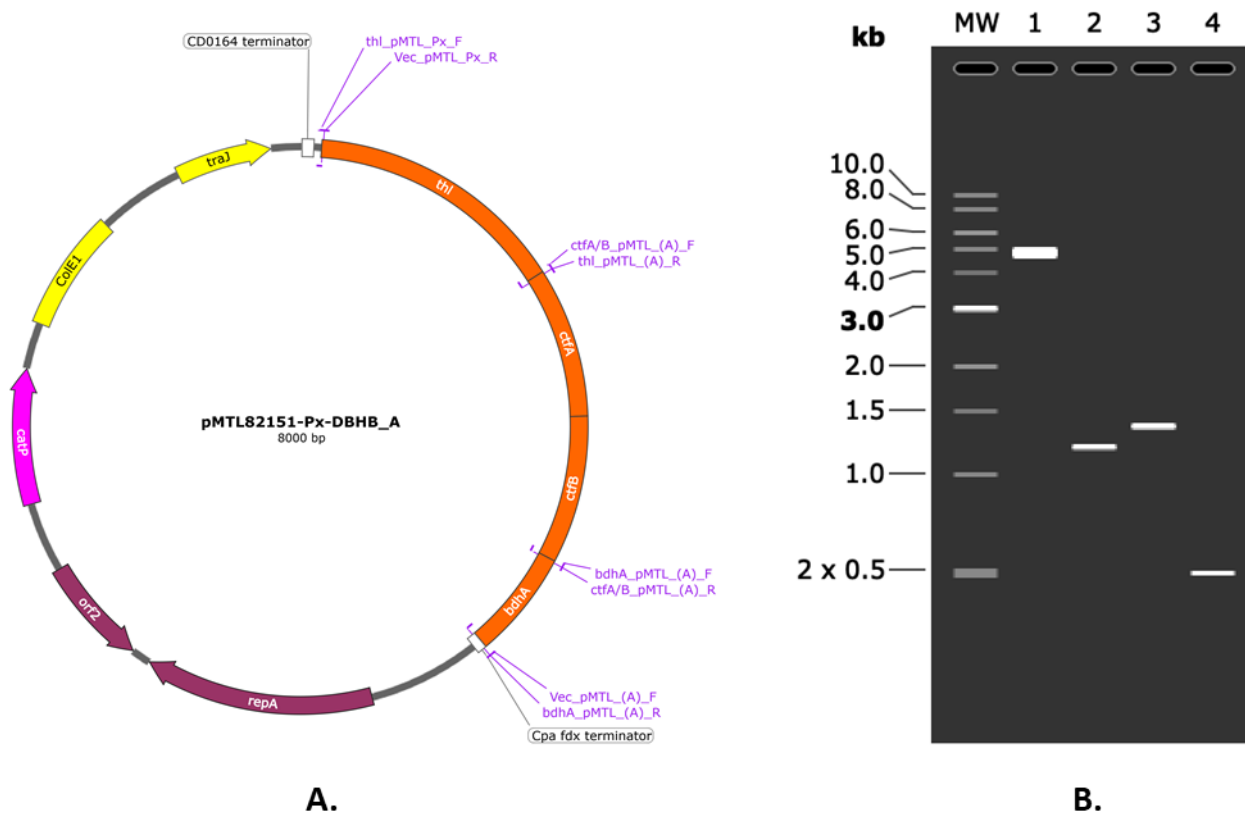


Figure 1 - pMTL82151-Px-DBHB<sub>A</sub> Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-*thl*; 3-*ctfA/B*; 4-*bdhA*.

## pMTL82151-Pntnh-DBHB<sub>A</sub>

Table 3 – Primer table to amplify fragments needed to assemble pMTL82151-pntnh-DBHB<sub>A</sub> by HiFi assembly.

Gene Name/ Fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Pntnh promoter	Pntnh_pMTL_F	<i>C. botulinum</i> ATCC 3502 gDNA	atgagtcgaccctaaaatttaaatatatacaaat tttattagtagtttac	873
	Pntnh_pMTL_R		ctctttcatatctaaccacctcctaaattattt c	
Thiolase ( <i>thl</i> )	thl_pMTL_ Pntnh_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	gtggttagatagaagaagtgtgaatagctag	1199
	thl_pMTL_(A)_R		ccttttaaatctagcacttttctagcaatattg	
Acetoacetate CoA- transferase subunit A/B ( <i>ctfA/</i> & <i>ctfB</i> )	ctfA/B_pMTL_ (A)_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	aaagtgctagattaaaaggaggattaaaatg aac	1363
	ctfA/B_pMTL_(A)_ R		tcgtctttctctaaacagccatgggtctaag	
3-hydroxybutanoate dehydrogenase ( <i>bdhA</i> )	bdhA_pMTL_ (A)_F	<i>Streptococcus</i> <i>dysgalactiae</i> <i>subsp. equisimilis</i> AC2713 gDNA	ggctgttagagaaaagacgaggatagatc	509
	bdhA_pMTL_(A)_ R		ttattttatgctacaataagctgattttcc	
Vector Backbone	Vec_pMTL_(A)_F	pMTL82151	ttattgtagcataaaaaataagaagcctgcatt	4997
	Vec_pMTL_ Pntnh_R		aaatttagggtcgactcatagctgtttc	

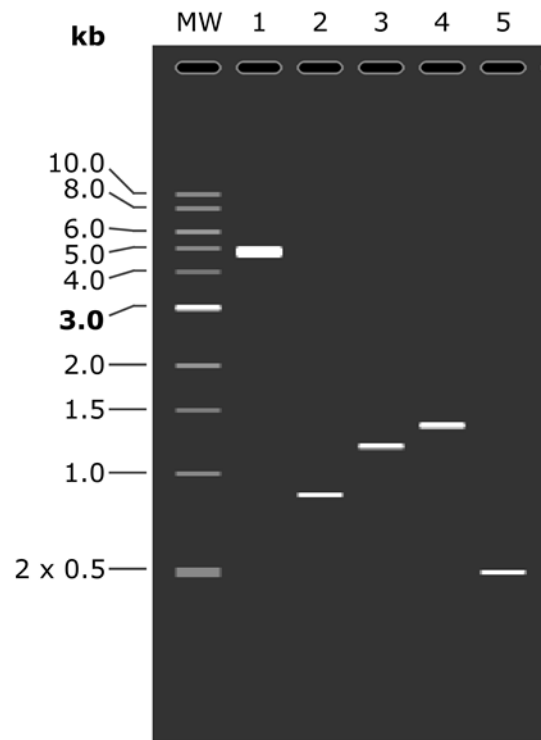


Figure 2 - pMTL82151-Pntnh-DBHB<sub>A</sub> Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Pntnh; 3--thl; 4-ctfA/B; 5-bdhA.

### pMTL82151-Plac-DBHB<sub>A</sub>

Table 4 - Primer table to amplify fragments needed to assemble pMTL82151-plac-DBHB<sub>A</sub> by HiFi assembly.

Gene Name/ Fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Plac promoter	Plac_pMTL_F	<i>C. perfringens</i> gDNA	atgagtgcgactaatatg attaattctaattaagtga attaatatag	1421
	Plac_pMTL_R		cttctttcattttaccctcca atacatttataaataattg	
Thiolase ( <i>thl</i> )	thl_pMTL_Plac_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	ggagggtaaaaatgaaagaa ggtgtaatagctag	1199
	thl_pMTL_(A)_R		ccttttaaatctagcacttttc tagcaatattg	
Acetoacetate CoA- transferase subunit A/B ( <i>ctfA/ &amp; ctfB</i> )	ctfA/B_pMTL_(A)_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	aaagtgcgactttaaaagg agggattaaaatgaac	1363
	ctfA/B_pMTL_(A)_R		tcgtctttctctaaacagcca tgggtctaag	
3-hydroxybutanoate dehydrogenase ( <i>bdhA</i> )	bdhA_pMTL_(A)_F	<i>Streptococcus</i> <i>dysgalactiae</i> subsp. <i>equisimilis</i> AC2713 gDNA	ggctgttagagaaagacga ggatagtatc	509
	bdhA_pMTL_(A)_R		ttatattatgctacaataa gctgattttcc	
Vector Backbone	Vec_pMTL_(A)_F	pMTL82151	ttattttagcataaaaaataa gaagcctgcatt	4997
	Vec_pMTL_Plac_R		atctaaattagtcgactcata gctgtttc	

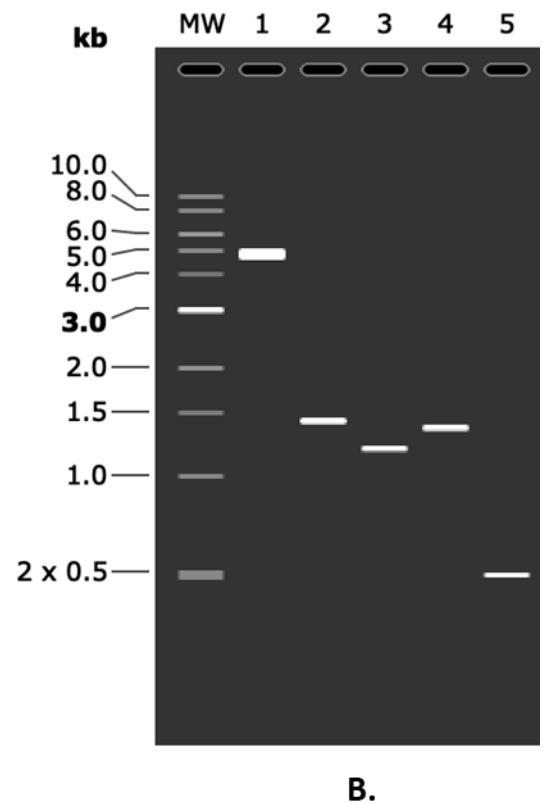
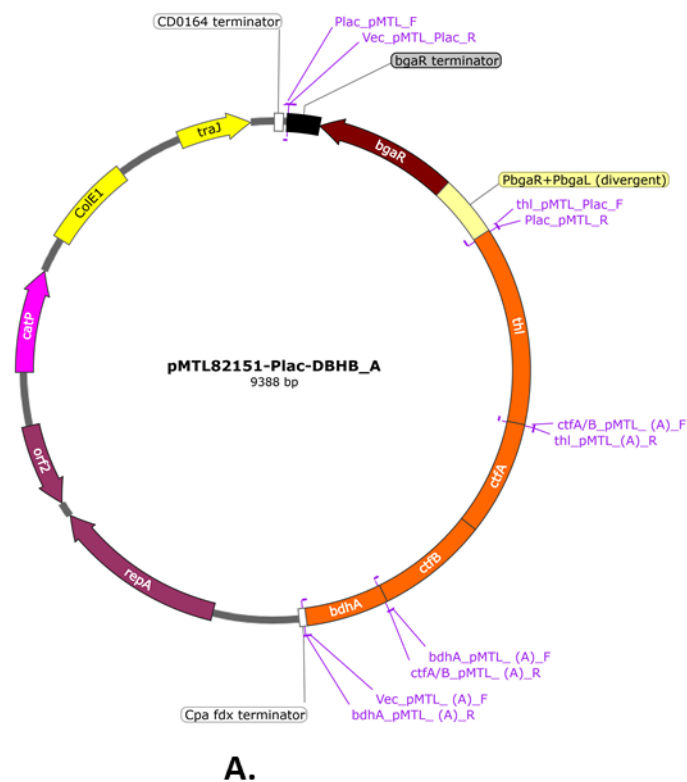
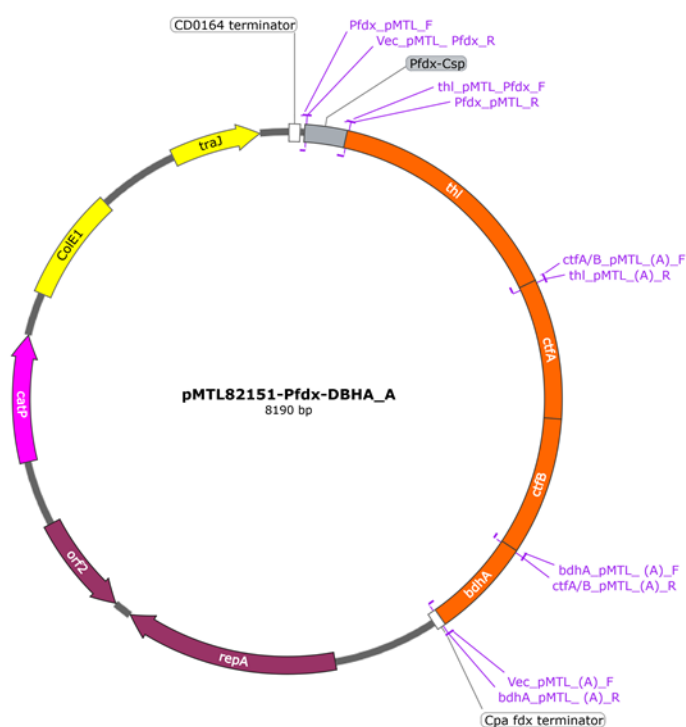


Figure 3 - pMTL82151-Plac-DBHB<sub>A</sub> Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Plac; 3--*thl*; 4-*ctfA/B*; 5-*bdhA*.

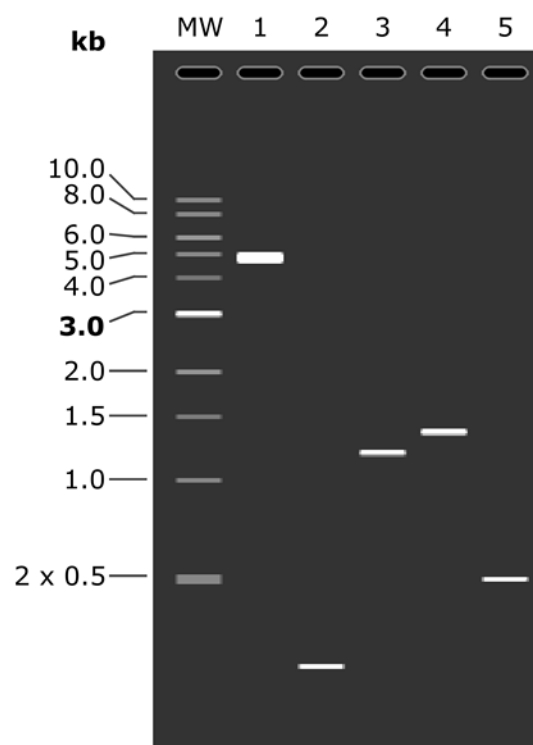
## pMTL82151-Pfdx-DBHB<sub>A</sub>

Table 5 - Primer table to amplify fragments needed to assemble pMTL82151-pfdx-DBHB<sub>A</sub> by HiFi assembly.

Gene Name/fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Pfdx promoter	Pfdx_pMTL_F	<i>C. sporogenes</i> 10696 gDNA	atgagtcgacgtgtagtagc ctgcgaaataag	223
	Pfdx_pMTL_R		cttctttcatatgtaacacac ctccttaaaaattac	
Thiolase ( <i>thl</i> )	thl_pMTL_Pfdx_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	tgtgttcatatgaaagaag ttgtaatagctag	1199
	thl_pMTL_(A)_R		ccttttaaatctagcacttttc tagcaatattg	
Acetoacetate CoA-transferase subunit A/B ( <i>ctfA/ &amp; ctfB</i> )	ctfA/B_pMTL_(A)_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	aaagtgcctagattaaaagg agggattaaaatgaac	1363
	ctfA/B_pMTL_(A)_R		tcgtctttctctaaacagcca tgggtctaag	
3-hydroxybutanoate dehydrogenase ( <i>bdhA</i> )	bdhA_pMTL_(A)_F	<i>Streptococcus dysgalactiae subsp. equisimilis</i> AC2713 gDNA	ggctgttagagaaagacga ggatagatc	509
	bdhA_pMTL_(A)_R		ttatattatgctacaataa gctgattttcc	
Vector Backbone	Vec_pMTL_(A)_F	pMTL82151	ttattgtagcataaaaaataa gaagcctgcatt	4997
	Vec_pMTL_Pfdx_R		gctactacagctgactcat agctgtttc	



A.



B.

Figure 4 - pMTL82151-pfdx-DBHB<sub>A</sub> 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<sub>B</sub>

The following plasmid vectors variants in table 6 below have been designed for testing of the DBHB<sub>B</sub> operon.

Table 6 - List of pMTL8000 modular shuttle vectors designed to test KPO DBHB<sub>B</sub>.

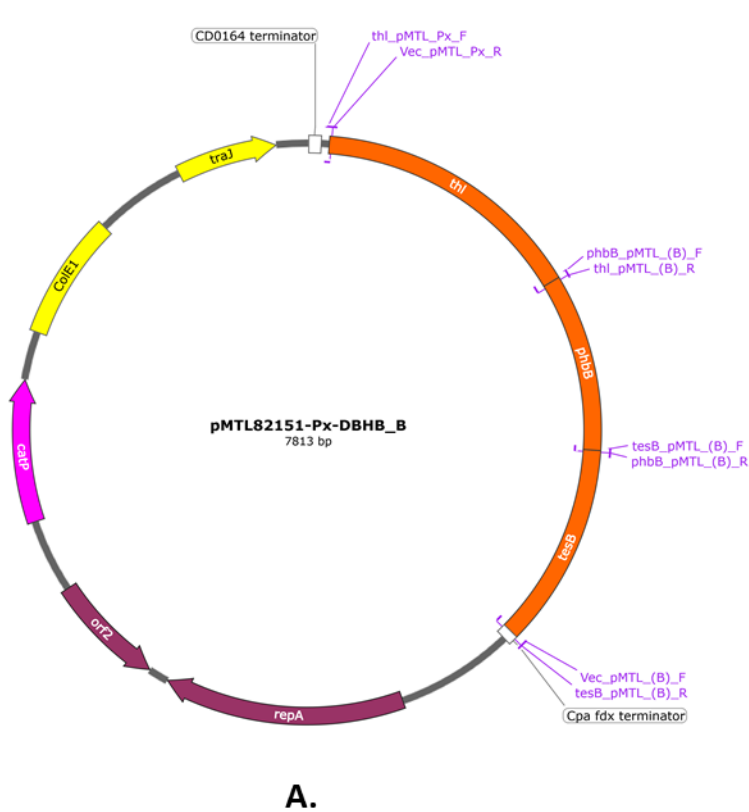
Vector	Description	Vector Map	Primer table (Primers to amplify fragments with overhangs suitable for HiFi assembly)	Gel of expected fragments needed to assemble vector (run on a 1% agarose gel)
pMTL82151-Px-DBHB <sub>B</sub>	No promoter as a negative control - no expression of KPO genes.	Figure 5-A.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

## DBHB<sub>B</sub> KPO vector maps, primer tables and agarose gel simulations

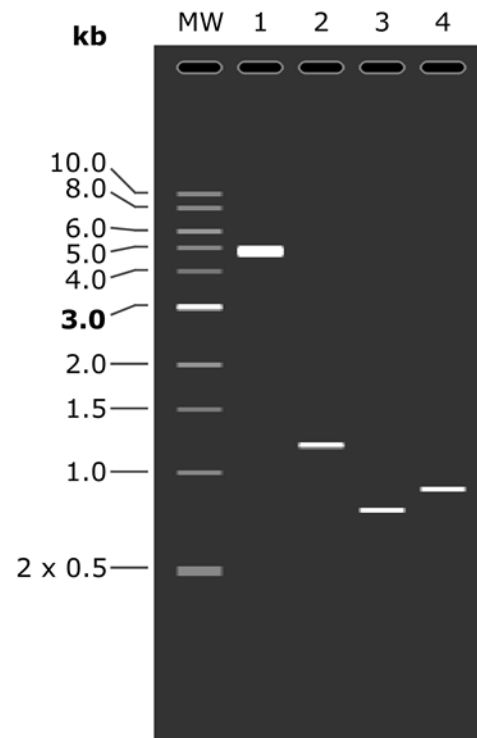
### pMTL82151-Px-DBHB<sub>B</sub>

Table 7 - Primer table to amplify fragments needed to assemble pMTL82151-pX-DBHB<sub>B</sub> by HiFi assembly.

Gene Name/fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Thiolase ( <i>thl</i> )	thl_pMTL_Px_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	ctgtatccatatgaaagaagttgtaatagctag	1199
	thl_pMTL_(B)_R		tctcacaagctagcacttttctagcaatattg	
Acetoacetyl-CoA reductase ( <i>phbB</i> )	phbB_pMTL_(B)_F	<i>Cupriavidus necator</i> gDNA	aaagtgctagcttgtgagagacaagcag	784
	phbB_pMTL_(B)_R		agtaacaaagttattgcatgtgctgccc	
Thioesterase II ( <i>tesB</i> )	tesB_pMTL_(B)_F	<i>Escherichia coli</i> gDNA	catgcaataactttgtactggagagtatatg	901
	tesB_pMTL_(B)_R		ttatttttattaattgtgattacgcatcac	
Vector Backbone	Vec_pMTL_(B)_F	pMTL82151	aatcacaattaataaaaaataagaagcctgcattg	5010
	Vec_pMTL_Px_R		cttcttcatatggatacagcggccg	



A.



B.

Figure 5 - pMTL82151-Px-DBHB<sub>B</sub> 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<sub>B</sub>**

Table 8- Primer table to amplify fragments needed to assemble pMTL82151-pntnh-DBHB<sub>B</sub> by HiFi assembly.

Gene Name/ Fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Pntnh promoter	Pntnh_pMTL_F	<i>C. botulinum</i> ATCC 3502 gDNA	atgagtcgacccctaaaatttaatatcaaatTTTTATTAGTATGT ttac	873
	Pntnh_pMTL_R		cttctttcatacttaaccaccctcctaaatttatttc	
Thiolase ( <i>thl</i> )	thl_pMTL_ Pntnh_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	gtggtagatatgaaagaagttgtaaatagctag	1199
	thl_pMTL_(B)_R		tctcacaagctagcacttttctagcaatattg	
Acetoacetyl- CoA reductase ( <i>phbB</i> )	phbB_pMTL_(B)_F	<i>Cupriavidus necator</i> gDNA	aaagtgctagcctttgtgagagacaagcag	784
	phbB_pMTL_(B)_R		agtaacaaagttattgcatgtgctgcc	
Thioesterase II ( <i>tesB</i> )	tesB_pMTL_(B)_F	<i>Escherichia coli</i> gDNA	catgcaataactttgttactggagagtatatg	901
	tesB_pMTL_(B)_R		ttatTTTTATTAAATTGtattacgcatcac	
Vector Backbone	Vec_pMTL_(B)_F	pMTL82151	aatcacaattaaataaaaaataagaagcctgcatttg	4997
	Vec_pMTL_ Pntnh_R		aaatTTTAGGTCgactcatagctgtttc	

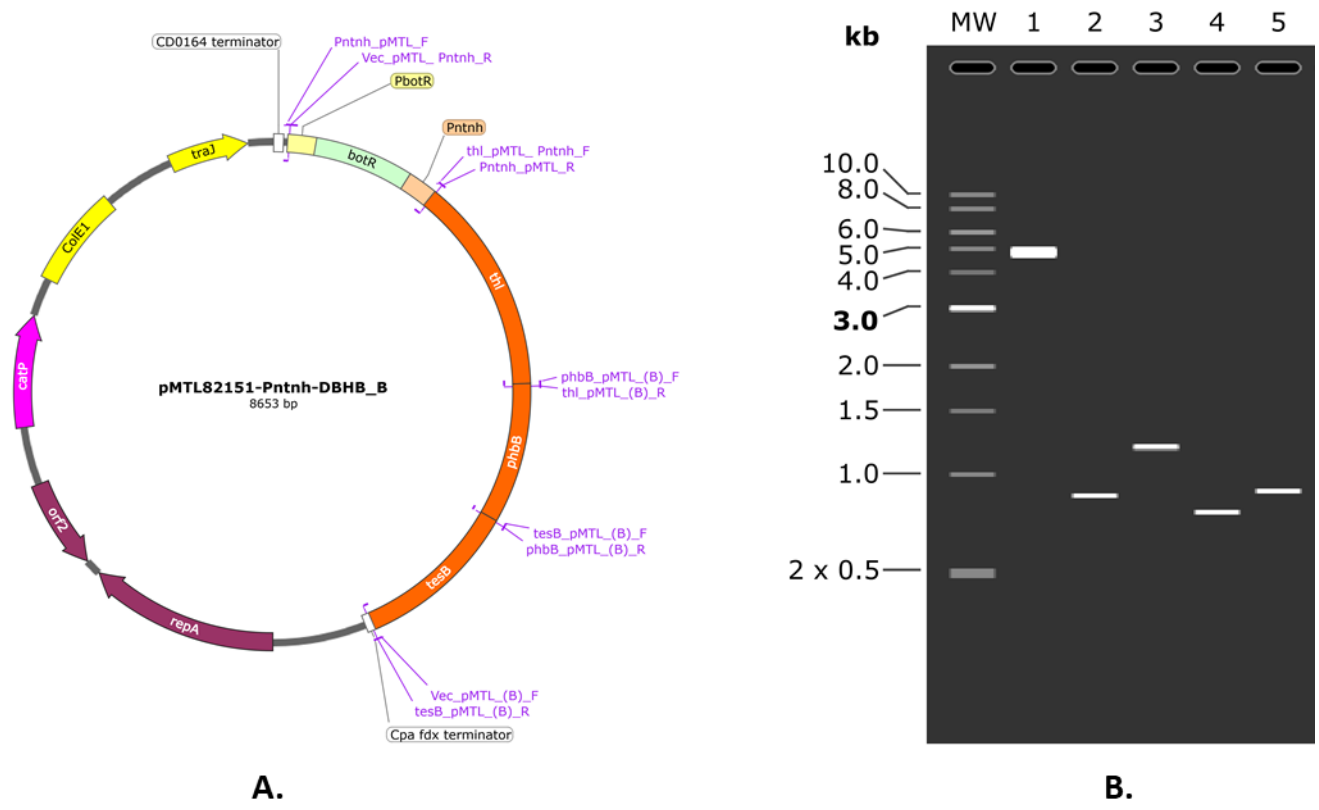


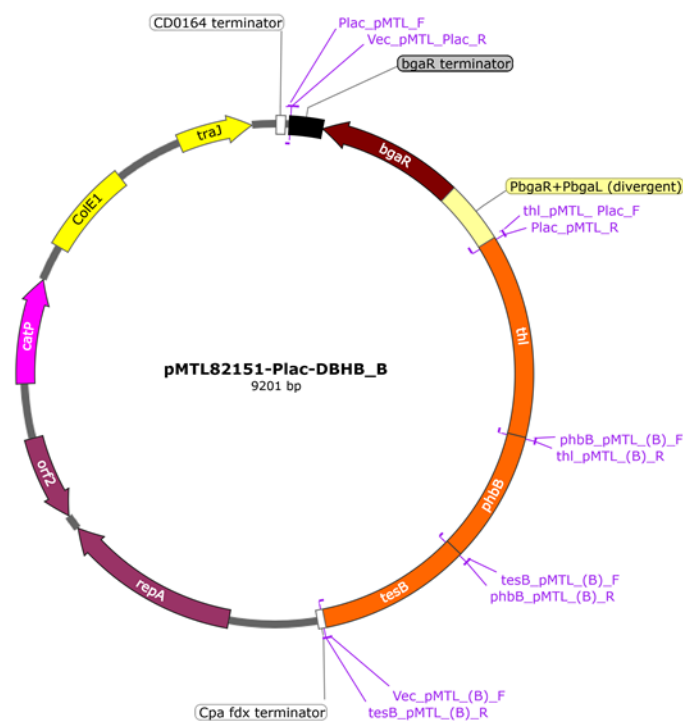
Figure 6 - pMTL82151-Pntnh-DBHB<sub>B</sub> Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Pntnh; 3-thl; 4-phbB; 5-tesB.



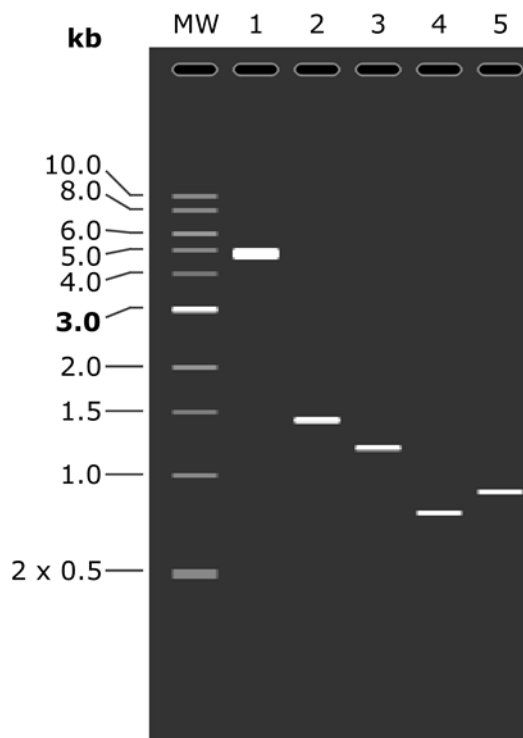
**pMTL82151-Plac-DBHB<sub>B</sub>**

Table 9 - Primer table to amplify fragments needed to assemble pMTL82151-plac-DBHB<sub>B</sub> by HiFi assembly.

Gene Name/ Fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Plac promoter	Plac_pMTL_F	<i>C. perfringens</i> gDNA	atgagtcgactaatttagatattaattctaattaagtgaattaatag	1421
	Plac_pMTL_R		cttctttcattttaccctccaatacatttaaataattatg	
Thiolase ( <i>thl</i> )	thl_pMTL_Plac_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	ggagggtaaaatgaagaagttgtaatagctag	1199
	thl_pMTL_(B)_R		tctcacaagctagcacttttctagcaatattg	
Acetoacetyl- CoA reductase ( <i>phbB</i> )	phbB_pMTL_(B)_F	<i>Cupriavidus</i> <i>necator</i> gDNA	aaagtctagctttgtgagagacaagcag	784
	phbB_pMTL_(B)_R		agtaacaaagtattgcatgtgctgccc	
Thioesterase II ( <i>tesB</i> )	tesB_pMTL_(B)_F	<i>Escherichia coli</i> gDNA	catgcaataactttgttactggagagttatag	901
	tesB_pMTL_(B)_R		ttatTTTTTtaattgtgattacgcatcac	
Vector Backbone	Vec_pMTL_(B)_F	pMTL82151	aatcacaataaataaaaataagaagcctgcatttg	4997
	Vec_pMTL_ Plac_R		atctaaattagtcgactcatagctgtttc	



**A.**



**B.**

Figure 7 - pMTL82151-Plac-DBHB<sub>B</sub> 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<sub>B</sub>**

Table 10 - Primer table to amplify fragments needed to assemble pMTL82151-pfdx-DBHB<sub>B</sub> by HiFi assembly.

Gene Name/fragment	Primer name	Template	Primer 5' to 3'	Amplicon size (bp)
Pfdx promoter	Pfdx_pMTL_F	<i>C. sporogenes</i> 10696 gDNA	atgagtcgacgtgtagtagcctgcgaaataag	223
	Pfdx_pMTL_R		cttctttcatatgtaacacacctcttaaaaattac	
Thiolase ( <i>thl</i> )	thl_pMTL_Pfdx_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	tgtgttacatgatgaagaagttgtaatagctag	1199
	thl_pMTL_Pfdx(B)_R		tctcacaagctagcacttttctagcaatattg	
Acetoacetyl-CoA reductase ( <i>phbB</i> )	phbB_pMTL_(B)_F	<i>Cupriavidus necator</i> gDNA	aaagtgcctagctttgtgagagacaagcag	784
	phbB_pMTL_(B)_R		agtaacaaagtattgcatgtgctgcc	
Thioesterase II ( <i>tesB</i> )	tesB_pMTL_(B)_F	<i>Escherichia coli</i> gDNA	catgcaataactttgttactggagagttatag	901
	tesB_pMTL_(B)_R		ttatntttatntaattgtgattacgcatcac	
Vector Backbone	Vec_pMTL_(B)_F	pMTL82151	aatcacaattaataaaaaataagaagcctgcat	4997
	Vec_pMTL_Pfdx_R		gctactacagctgactcatagctgtttc	

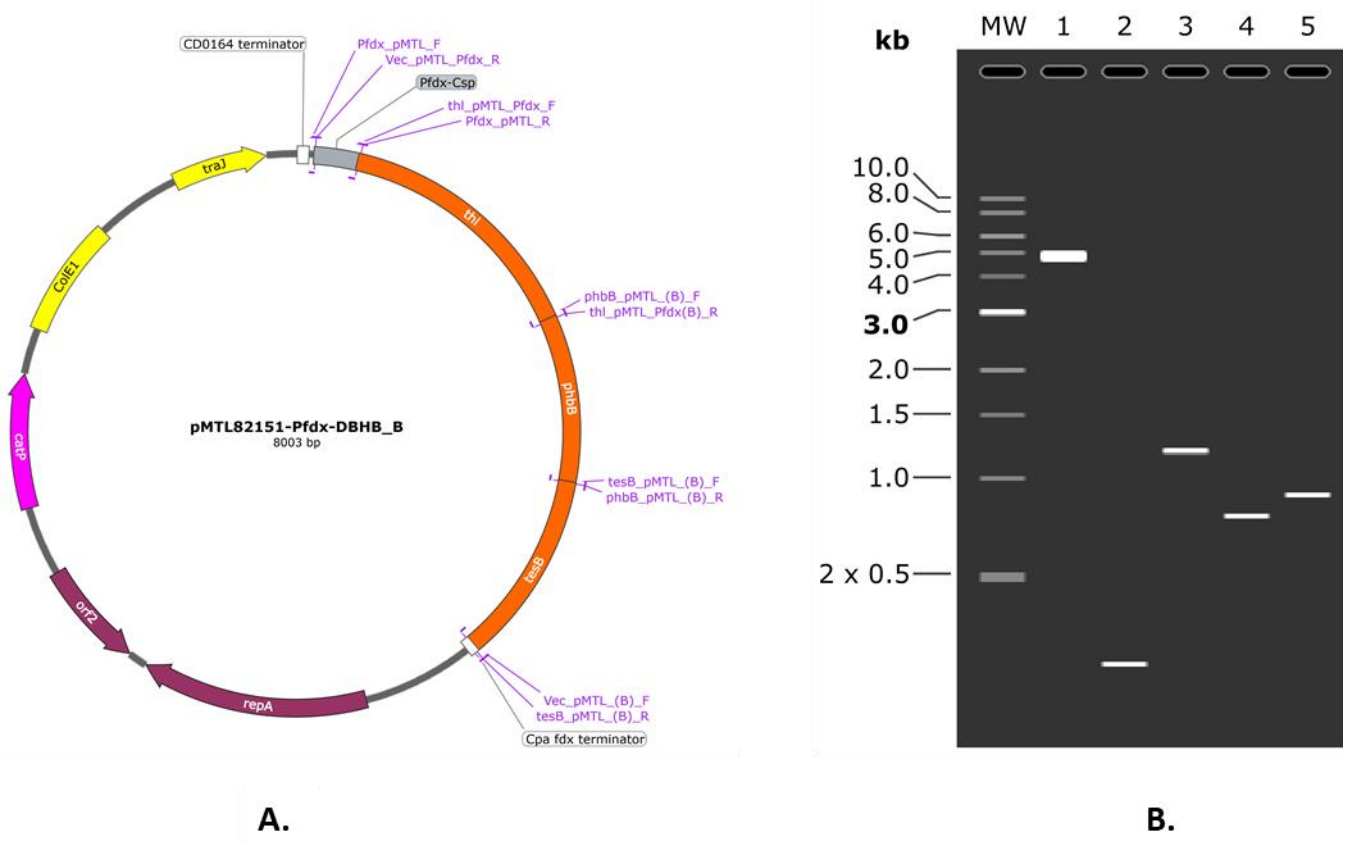


Figure 8 - pMTL82151-Pfdx-DBHB<sub>B</sub> Vector plasmid map annotated with primer binding sites (A.) and 1% agarose gel stimulation (B.) of expected fragment sizes needed to create vector – MW- Ladder; 1- Vector Backbone; 2-Pfdx; 3-thl; 4-phbB; 5-tesB.

### 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 <sub>A</sub>	pX <sub>B</sub>	Pntn <sub>H</sub> <sub>A</sub>	Pntn <sub>H</sub> <sub>B</sub>	Pfd <sub>X</sub> <sub>A</sub>	Pfd <sub>X</sub> <sub>B</sub>	Plac <sub>A</sub>	Plac <sub>B</sub>
scr_pri_pMTL_F	ttttttatcaggaaacagc	3088	2901	3285	3049	3278	3091	4476	4289
scr_pri_pMTL_R	agaagcctgcaaatgcaggc								

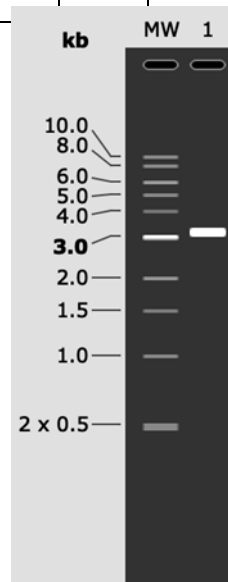


Figure 9 - A 1% agarose gel stimulation demonstrating the band size expected if the pMTL82151-Pfdx-DBH<sub>A</sub> is correctly assembled vector (or present in *C. sporogenes* genome) – MW- Ladder; 1- pMTL82151-Pfdx-DBH<sub>A</sub>

## Stage 2 – Genomic Integration Vector Design

Once tested using shuttle vectors the most suitable pathway (DBH<sub>A</sub> in our 'dry lab' project) would be integrated into the *C. sporogenes* genome to allow stable expression of the KPO without the need to maintain the plasmid vector by antibiotic selection. To integrate the DBH<sub>A</sub> KPO with the three promoter 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 DBHB<sub>A</sub> KPO into the *C. sporogenes* genome

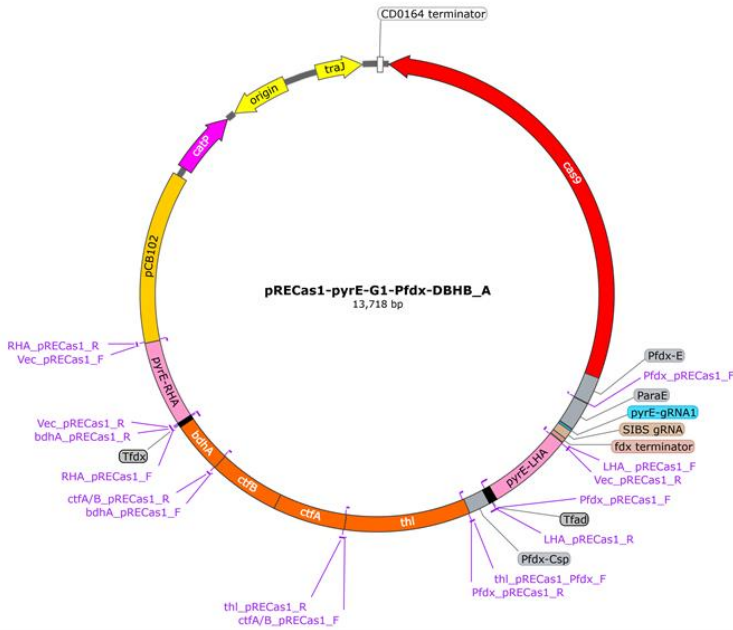
Vector	Vector Map	Primer table (Primers to amplify fragments with overhangs suitable for HiFi assembly)	Gel of expected fragments needed to assemble vector (run on a 1% agarose gel)
pRECas1-Pfdx-DBHB <sub>A</sub>	Figure 10-A.	Table 13	Figure 10-B.
pRECas1-Pntnh-DBHB <sub>A</sub>	Figure 11-A.	Table 14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

### **RiboCas DBHB<sub>A</sub> KPO vector maps, primer tables and agarose gel simulations**

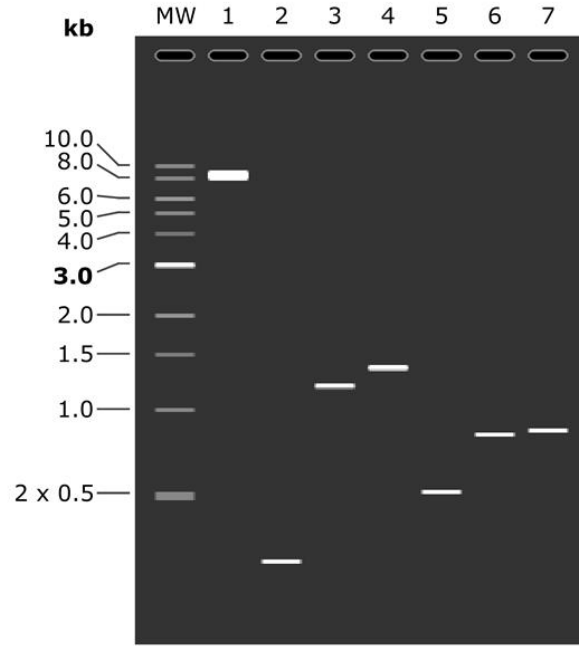
#### **pRECas1-Pfdx-DBHB<sub>A</sub>**

Table 13 - Primer table to amplify fragments needed to assemble pRECas1-Pfdx-DBHB<sub>A</sub> by HiFi assembly.

Gene Name/ Fragment	Primer Name	Template	Primer 5' to 3'	Amplicon size (bp)
Pfdx promoter	Pfdx_pRECas1_F	<i>C. sporogenes</i> 10696 gDNA	attgtggcagactttatttctattgattttttcatttttaat	260
	Pfdx_pRECas1_R		gtgtagtagcctcgcaaat	
Thiolase ( <i>thl</i> )	thl_pRECas1_Pfdx_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	cttctttcatatgtaacacacctcttaaaattac	1199
	thl_pRECas1_R		tgtgttacatagaaagaagtgtaatagctag	
Acetoacetate CoA-transferase subunit A/B ( <i>ctfA/ &amp; ctfB</i> )	ctfA/B_pRECas1_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	ccttttaaatctagcacttttctagcaatattg	1363
	ctfA/B_pRECas1_R		aaagtgcagatttaaaaggaggattaaatgaac	
3-hydroxybutanoate dehydrogenase ( <i>bdhA</i> )	bdhA_pRECas1_F	<i>Streptococcus dysgalactiae subsp. equisimilis</i> AC2713 gDNA	tcgtctttcttaaacagccatgggtctaag	529
	bdhA_pRECas1_R		ggctgttagagaaagacgaggatagatc	
Right Homology Arm (RHA)	RHA_pRECas1_F	<i>C. sporogenes</i> 10696 gDNA	aagcctgcaaatgcaaggcttctattttatctacaataagctgattttcc	840
	RHA_pRECas1_R		agcctgcattgcaggcttctattttatatttaaaataagg agtgtc	
Vector Backbone	Vec_pRECas1_F	pRECas1-ΔpyrE::PbotR-botR (from Nottingham IGEN 2019 project)	gcggcgcgccaactcccataaataatcttg	8806
	Vec_pRECas1_R		atggggagttggcgcgccgattatt	
Left Homology Arm (LHA)	LHA_pRECas1_F	<i>C. sporogenes</i> 10696 gDNA	aagtcfaatcgacgtcataaaaataagaagcctgcaaatg	856
	LHA_pRECas1_R		ttatgacgtcattgacctaaatcctaagaag	
			taaagtctgccacaattgtggcagactttattttctattggc agttatatcttcttact	



**A.**



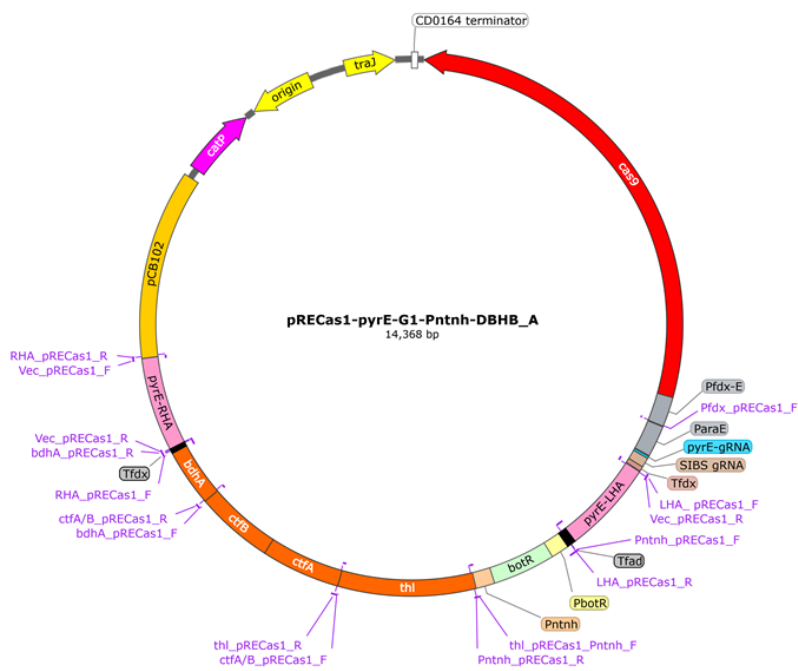
**B.**

Figure 10 -pRECas1-Pfdx-DBHB<sub>A</sub> 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.

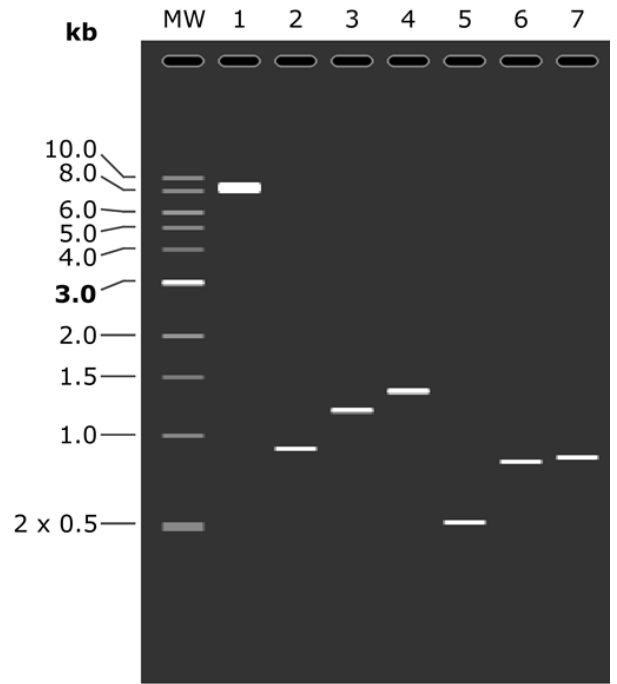
### pRECas1-Pntnh-DBHB<sub>A</sub>

Table 14 - Primer table to amplify fragments needed to assemble pRECas1-Pntnh-DBHB<sub>A</sub> by HiFi assembly.

Gene Name/ Fragment	Primer Name	Template	Primer 5' to 3'	Amplicon size (bp)
Pntnh promotor	Pntnh_pRECas1_F	<i>C. botulinum</i> ATCC 3502 gDNA	attgtggcagactttatctattgattatttcattttta atcctaaaatttaatatc	910
	Pntnh_pRECas1_R		cttctttcatatctaaccacccctcctaaattatttc	
Thiolase ( <i>thl</i> )	thl_pRECas1_Pntnh_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	gtggtagatagaagaagttgtaatagctag	1199
	thl_pRECas1_R		ccttttaaatctagcacttttctagcaatattg	
Acetoacetate CoA- transferase subunit A/B ( <i>ctfA</i> & <i>ctfB</i> )	ctfA/B_pRECas1_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	aaagtgcagattaaaaggaggattaaaatgaac	1363
	ctfA/B_pRECas1_R		tcgtctttctctaaacagccatgggtctaag	
3- hydroxybutan oate dehydrogenas e ( <i>bdhA</i> )	bdhA_pRECas1_F	<i>Streptococcus</i> <i>dysgalactiae</i> subsp. <i>equisimilis</i> AC2713 gDNA	ggctgttagagaagacgaggatagatc	529
	bdhA_pRECas1_R		aagcctgcaaatgcaggcttctattttatctacaataag ctgattttcc	
Right Homology Arm (RHA)	RHA_pRECas1_F	<i>C. sporogenes</i> 10696 gDNA	agcctgattgcaggcttctattttatatttaaaaaataag gagtgtc	840
	RHA_pRECas1_R		gcggcgcccaactcccataaataatcttg	
Vector Backbone	Vec_pRECas1_F	pRECas1- ΔpyrE::PbotR-botR (from Nottingham IGEM 2019 project)	atggggagttggcgcgccccattattt	8806
	Vec_pRECas1_R		aaggtcaatcgacgtcataaaaaataagaagcctgcaaatg	
Left Homology Arm (LHA)	LHA_pRECas1_F	<i>C. sporogenes</i> 10696 gDNA	ttatgacgtcgattgaccttaaatcctaaagaag	856
	LHA_pRECas1_R		taaagtctgccacaattgtggcagactttattttatctattgg cagtatatcttctctatact	



**A.**



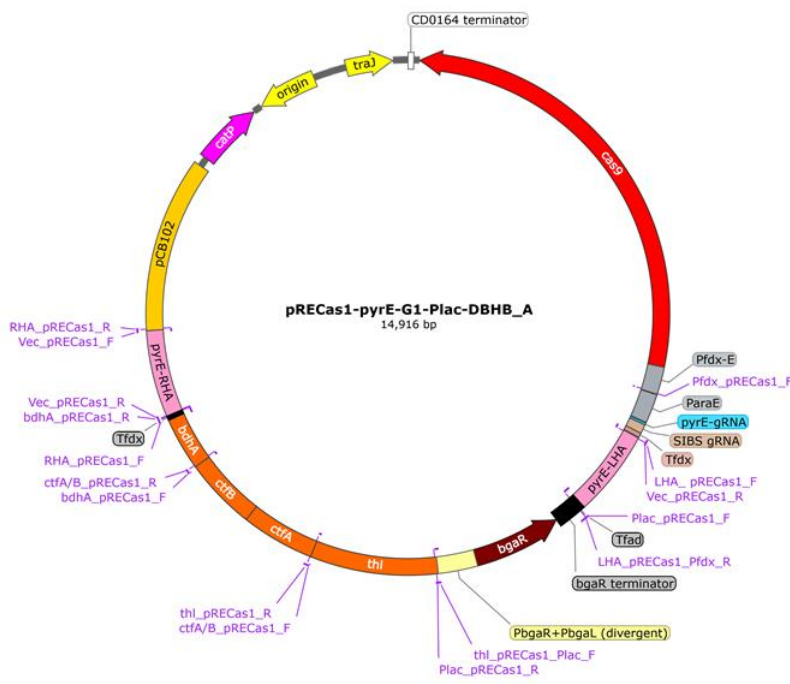
**B.**

Figure 11 - pRECas1-Pntnh-DBHB<sub>A</sub> 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.

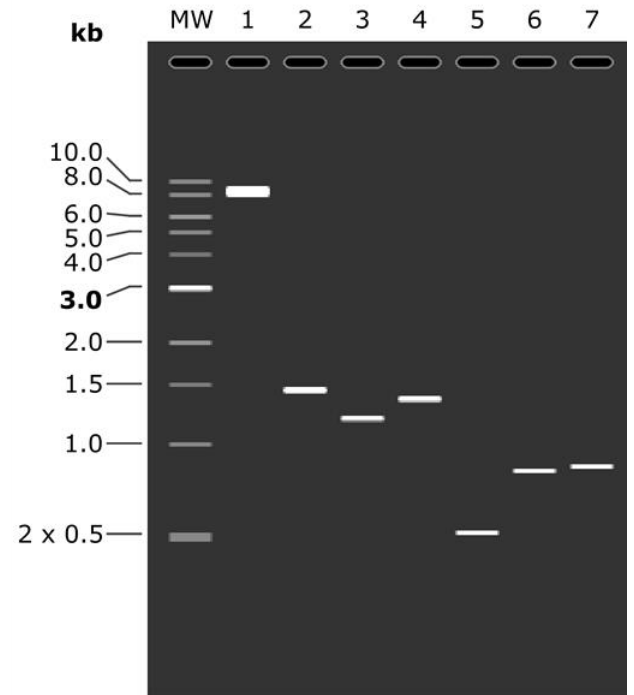
### pRECas1-Plac-DBHB<sub>A</sub>

Table 15 - Primer table to amplify fragments needed to assemble pRECas1-Plac-DBHB<sub>A</sub> by HiFi assembly.

Gene Name/ Fragment	Primer Name	Template	Primer 5' to 3'	Amplicon size (bp)
Plac promoter	Plac_pRECas1_F	<i>C. perfringens</i> gDNA	atttggcagactttatctattgattatttcattttta attaatttagatattaattct	1458
	Plac_pRECas1_R		cttctttcattttaccctccaatacatttaaataattatg	
Thiolase ( <i>thl</i> )	thl_pRECas1_Plac_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	ggagggtaaaatgaaagaagttgtaatagctag	1199
	thl_pRECas1_R		ccttttaaatctagcacttttctagcaatattg	
Acetoacetate CoA- transferase subunit A/B ( <i>ctfA</i> & <i>ctfB</i> )	ctfA/B_pRECas1_F	<i>C. acetobutylicum</i> ATCC 824 gDNA	aaagtgcagatttaaaggaggattaaaataaac	1363
	ctfA/B_pRECas1_R		tcgtctttcttaaacagccatgggtctaag	
3- hydroxybutano ate dehydrogenas e ( <i>bdhA</i> )	bdhA_pRECas1_F	<i>Streptococcus</i> <i>dysgalactiae</i> subsp. <i>equisimilis</i> AC2713 gDNA	ggctgttagagaaagacgaggatagatc	529
	bdhA_pRECas1_R		aagcctgcaaatgcaggcttctattttatctacaataag ctgattttcc	
Right Homology Arm (RHA)	RHA_pRECas1_F	<i>C. sporogenes</i> 10696 gDNA	agcctgcatttgaggcttctattttatattaaaataag gagtgct	840
	RHA_pRECas1_R		gcggcgcgccaactccccataaataacttg	
Vector Backbone	Vec_pRECas1_F	pRECas1- ΔpyrE::PbotR-botR (from Nottingham IGEM 2019 project)	atggggagttggcgcgccattattt	8806
	Vec_pRECas1_R		aaggtcaatcgactcataaaaataagaagcctgcaaatg	
Left Homology Arm (LHA)	LHA_pRECas1_F	<i>C. sporogenes</i> 10696 gDNA	ttatgacgtcattgaccttaaatcctaaagaag	856
	LHA_pRECas1_R		taaagtctgccacaattgtggcagactttatttattctattgg cagtatacttctcttatact	



A.



B.

Figure 12- pRECas1-Plac-DBHB<sub>A</sub> 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; 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<sub>A</sub> 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 <sub>A</sub>	Pfdx <sub>A</sub>	Plac <sub>A</sub>
sgRNA-F	cctttcattacaattcatcag	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<sub>A</sub> 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 DBHB<sub>A</sub> variants into the *C. sporogenes* genome.

Genomic screening primer name	Primer 5' to 3'	Amplicon size (bp)		
		Pntn <sub>H</sub> <sub>A</sub>	Pfdx <sub>A</sub>	Plac <sub>A</sub>
scr_pri_pRECas1_F	actgcagtacaaataggaacagc	3623	3741	4639
scr_pri_pRECas1_R	tctttgctaaaaataacttggttttgc			

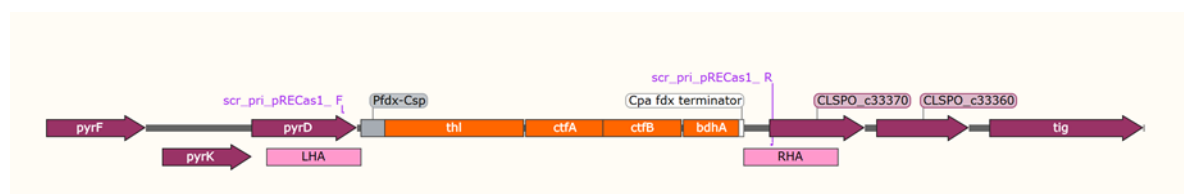


Figure 13- *C. sporogenes* pyrE locus with DBHB<sub>A</sub> KPO driven by the native fdx promoter replacing the pyre gene with the genomic screening primers binding sites shown.

## 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 using 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.

### For the shuttle vectors

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.

### For the genomic integration vectors

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°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<sub>A</sub> 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.