Supplementary Materials for **"The Ethylmalonyl-CoA Pathway for Methane-based Biorefineries: A Case Study of Using Methylosinus trichosporium OB3b, an Alpha-proteobacterial Methanotroph, for Producing 2-Hydroxyisobutyric Acid and 1,3-Butanediol from Methane"**

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SUPPLEMENTARY MATERIALS

Table S1. List of primers used in this study.

Number	Name	Sequence (5' - 3')
1	rcm _EcoRI_FWD	AAATGGGTCGCGGATCCGATGTTAGGTAAATTATTGAAAGAAGTTGA
2	rcm_EcoRI_REV	GCTTGTCGACGGAGCTCGTTACCCTATTTTTTTCCCTACATGCT
3	P89-RCM-FWD	TTCACACAGGAAACAGCTATGTTAGGTAAATTATTGAAAGAAGTTGA
4	P89-RCM-REV	GCATCTTCCCGACAACTAAGTTCCTCCTTTCAGCAAAAAACC
5	P89BB_FWD	TAGTTGTCGGGAAGATGCGT
6	P89BB_REV	AGCTGTTTCCTGTGTGAATACCT
7	pET28-Adhac_FWD	AAATGGGTCGCGGATCCGATGAAAGTTACAAATCAAAAAGAACT
8	pET28-Adhac_REV	GCTTGTCGACGGAGCTCGTAAAATGATTTTATATAGATATCCT
9	pET28-Adhe_FWD	AAATGGGTCGCGGATCCGATGGCTGTTACTAATGTCGCTGA
10	pET28-Adhe_REV	GCTTGTCGACGGAGCTCGTTAAGCGGATTTTTTCGCTT
11	pET28-Adhac-bld_FWD	CTCCGTCGACAAGCTTGCAGGAGATATAAATGATTAAAGACACGCTAGTTTCT
12	pET28-Adhac-bld_REV	GTGGTGGTGCTCGAGTGCACCGGCGAGTACACATCTTC
13	pET28-Adhe-bld_FWD	CTCCGTCGACAAGCTTGCAGGAGATATAAATGATTAAAGACACGCTAGTTTCT
14	pET28-Adhe-bld_REV	GTGGTGGTGCTCGAGTGCACCGGCGAGTACACATCTTC
15	P89-Adhac-bld_FWD	TTCACACAGGAAACAGCTATGAAAGTTACAAATCAAAAAGAACT
16	P89-Adhac-bld_REV	GCATCTTCCCGACAACTAGGATATAGTTCCTCCTTTCAGCA
17	P89-Adhe-bld_FWD	TTCACACAGGAAACAGCTATGGCTGTTACTAATGTCGCTGA
18	P89-Adhe-bld_REV	GCATCTTCCCGACAACTAATAGTTCCTCCTTTCAGCAA
19	16S-RT-F	TATTGGACAATGGGCGCAAG
20	16S-RT-R	CAGTGATTCCGAACAACGCT
21	phaA3-RT -F	TGACCGACGCCTTCAACAATTACC
22	phaA3 -RT -R	CGTCCTGGTCGACGATCACGT
23	phaB-RT -F	GAACTCGCTGTTCAACGTCA
24	phaB-RT -R	GGCGGAATAATTGGTTTGGC
25	Cro-RT -F	CACGGTGATCGGCATGTATC
26	Cro-RT -R	GATCTCGACCACCACATGGA
27	Ccr-RT -F	AACCGCAAGGATTTCAAGGG
28	Ccr-RT -R	CGATGTCGACGTCCTTCTTG
29	Epi-RT -F	ACGGTGGTGTTCGTCGAAC
30	Epi-RT -R	GGCTTGCCATGGGCGC
31	Ecm-RT -F	CTCTCGGACAAGGATTTCGC
32	Ecm-RT -R	TTCTCATCCGTGACGCCATA
33	Mcd-RT -F	GTCGTCGGCAATAAGACCTG

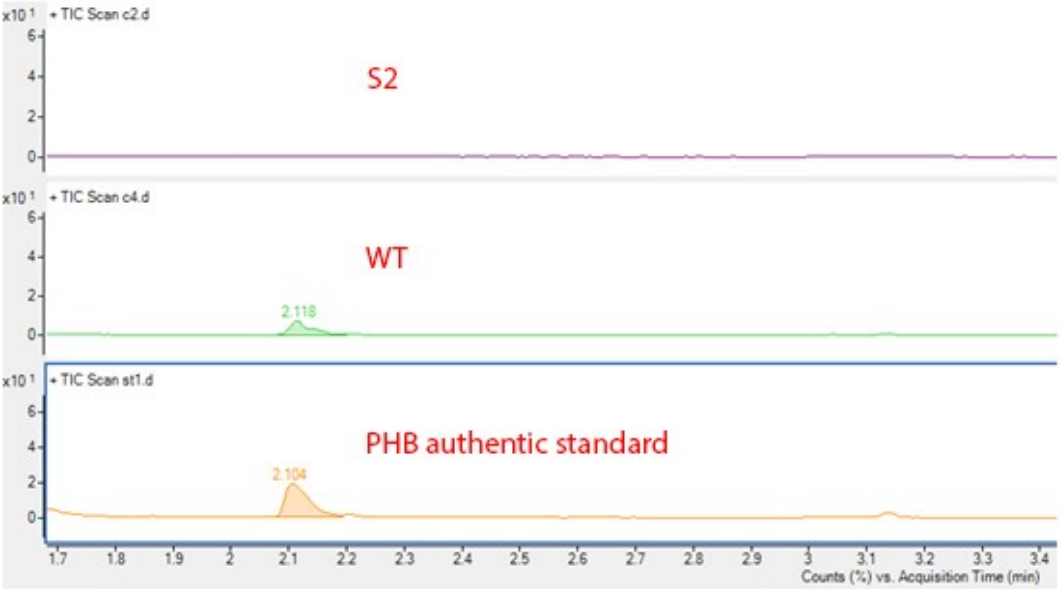
34	Mcd-RT -R	GCCCTTATAGCCCTTCTCGT
35	Mch-RT -F	ACTATGAGGTCGGCGAGAAG
36	Mch-RT -R	AGATGATGCGCTTTCCGAAC
37	phaC-RT -F	AACTTGCTCTCGTCGCTCTG
38	phaC-RT -R	CGACAATCCCTTCTTCGATTGC
37	F1-phaC- FWD	GGCGGGTCCTATTTCGCC
39	F1-phaC- REV	CAACAGCTCATTTCAGAGATGTGGCCGAGGAATGCTG
40	F2-phaC- FWD	GCCGAGGAGCAGGACTGAGCAGTCCCACAATGCCCT
41	F2-phaC- REV	ATGAGACGAATTTTCGCGCTTG
42	ZeoR-FWD	CTCTGAAATGAGCTGTTG
43	ZeoR-REV	TCAGTCCTGCTCCTCGGC

Table S2. List of RNA-seq datasets used in this study

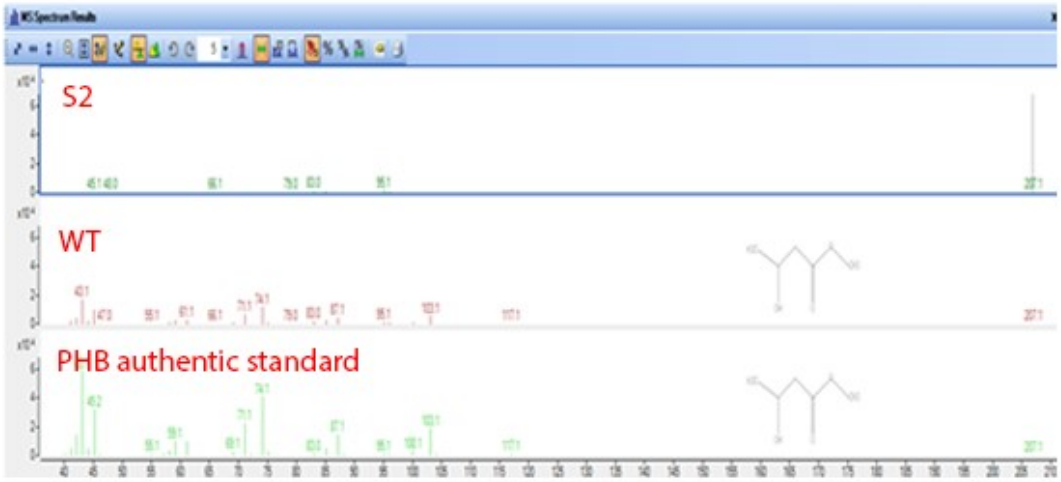
Sample name	Overall alignment rate	Number of reads	Culture condition	Source
NMS_Cu10_Rep1	96.92%	25595084	NMS 10uM $CuSO_4$	This study
NMS_Cu10_Rep2	96.14%	24578150	NMS 10uM $CuSO_4$	This study
REF_Cu_10_Rep1	88.85%	29349680	NMS 10uM $CuSO_4$	SRX2391218
REF_Cu_10_Rep2	89.72%	32054084	NMS 10uM $CuSO_4$	SRX2391219
REF_Cu_10_Rep3	81.33%	39629235	NMS 10uM $CuSO_4$	SRX2391220
REF_Cu_0_Rep1	88.85%	29349680	NMS no $CuSO_4$	SRX2391212
REF_Cu_0_Rep2	75.59%	28603376	NMS no $CuSO_4$	SRX2391213
REF_Cu_0_Rep3	77.83%	22867261	NMS no $CuSO_4$	SRX2391214

Fig. S1) Detection of PHB formation in wildtype and ΔC strain (strain S2) by GC-MS. (a). GC–MS elution profiles showing the detection of methyl-3-hydroxybutyrate from the methanolized 3-hydroxybutyrate standard (PHB standard). No PHB formation was detected in ΔC strain (b). Mass fragmentation patterns for the peaks detected in the GC–MS. (c). Comparison of PCR products from mutant strains and wildtype. WT= *M. trichosporium* OB3b wildtype, S1, S2, S3 = *M. trichosporium* OB3b recombinant strains obtained after gene knockout. +

a.)


x10 1 + TIC Scan c2.d
S2
x10 1 + TIC Scan c4.d
WT
2.118
x10 1 + TIC Scan st1.d
PHB authentic standard
2.104
Counts (%) vs. Acquisition Time (min)

b.)


S2
WT
PHB authentic standard

c.)

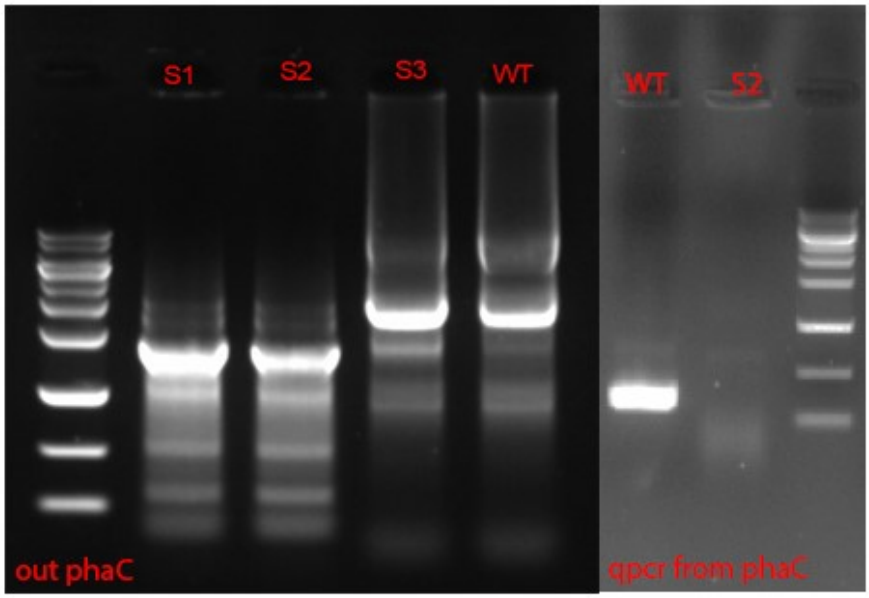

S1
S2
S3
WT
WT
S2
out phaC
qpcr from phaC

Fig. S2) Growth performance of phaC null mutant compared to wildtype. WT = *Methylosinus trichosporium* OB3b wildtype, ΔC strain = phaC-knockout mutant of *M. trichosporium* OB3b.

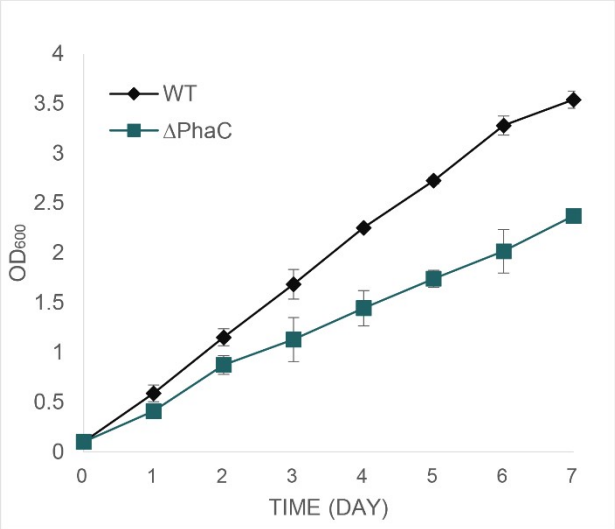
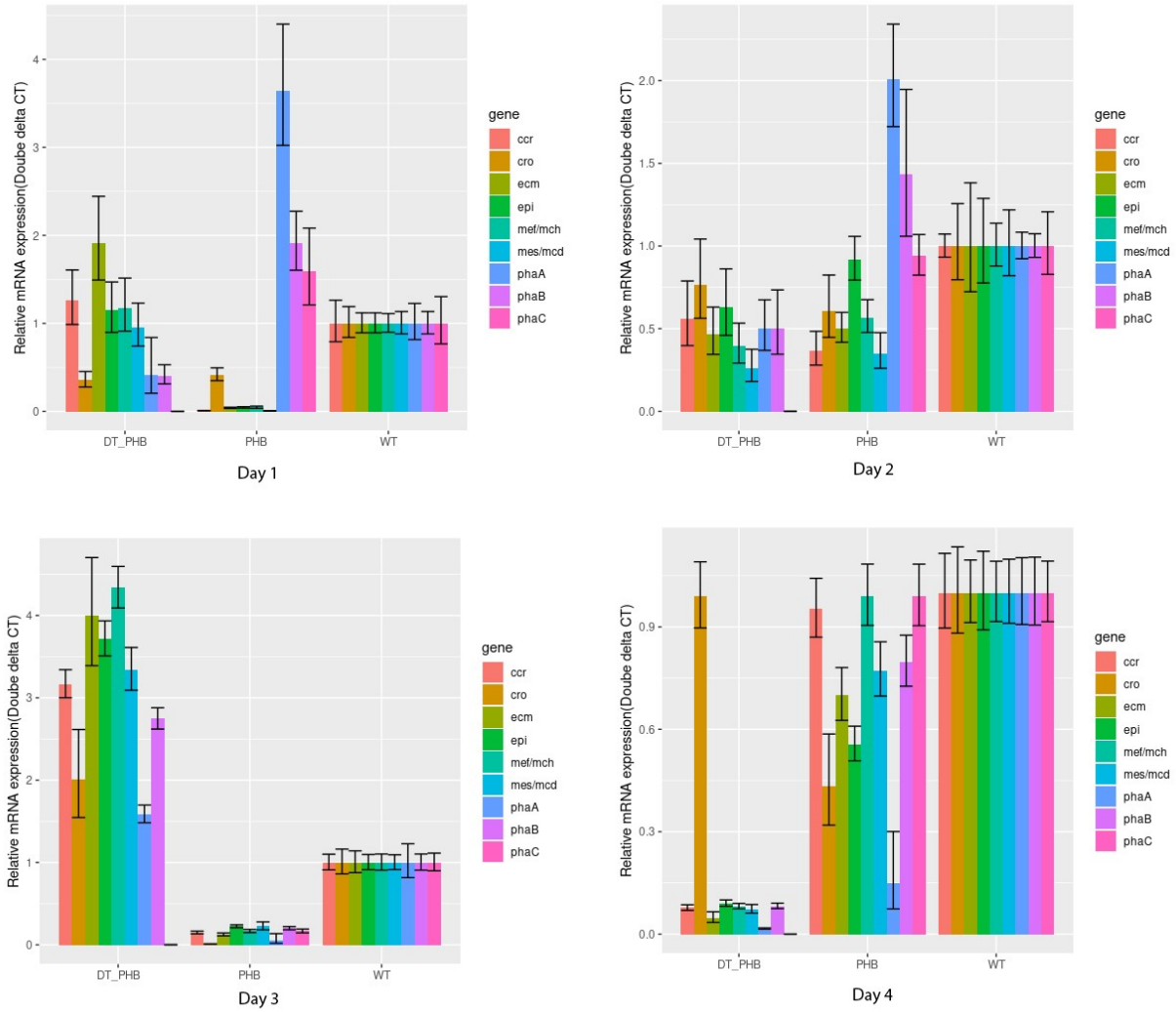


Fig. S3) Expression patterns of *M. trichosporium* OB3b phaC null mutant strain compared to *M. trichosporium* OB3b wildtype during PHB accumulation process. Data were analyzed by PCR package for R. mRNA expression of *M. trichosporium* OB3b phaC-knock out strain (DT_PHB) and *M. trichosporium* OB3b wildtype in nitrogen limiting condition are compared and normalized against *M. trichosporium* OB3b wildtype cultured in NMS media. (n =3)



Under nitrogen-limiting conditions, in *M. trichosporium* OB3b wildtype *phaA* and *phaB* were upregulated after day 1. From day 1 to day 2, the other genes in the EMC pathway were downregulated. After day 3 all genes were downregulated and recovered on day 4.

For the *phaC* null mutant of *M. trichosporium* OB3b the expression patterns of all genes involved in the EMC pathways were different from the wildtype. On day 3 all genes in the EMC pathways were upregulated significantly and then decrease dramatically on day 4 in a similar way to day 3 of wildtype strain excepting the *cro* gene. The upregulation of *cro* in both strains is likely responsible for mediating (R)-3-hydroxy-butanoyl-CoA and crotonyl-CoA abundance when ccr and ecm were down regulated. It appeared that the EMC can actively shut down and reactivate in response to external stimuli. This might be a mechanism for actively controlling the concentration of toxic intermediate metabolites, such as glyoxylate.

Fig. S4) Product formation of 2-hydroxyisobutyric acid and 1,3-butanediol in recombinant strains. 2HIBA standard = 200mg/L ; 1,3BDO standard = 400mg/L.