

Supplementary Information

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Improvement of dicarboxylic acid production with *Methylobacterium extorquens* by reducing the product reuptake

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Electronic supplementary material as Online Resource

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Table S1 Details of genomic sequencing and mapping procedure

Strain	Genetic entity	Total number of mapped Illumina reads	Base coverage of reference sequence	Average base coverage [mean \pm standard deviation]
<i>M. extorquens</i> AM1 wild type	NC_012807	317 955	100 %	514.6 \pm 186.0
	NC_012808	17 352 981	99.998 %	225.9 \pm 212.4
	NC_012809	235 559	100 %	445.3 \pm 141.6
	NC_012810	180 717	100 %	519.8 \pm 180.6
	NC_012811	3 828 584	99.99 %	216.9 \pm 70.7
<i>M. extorquens</i> AM1 DFS mutant 1	NC_012807	353 562	100 %	1061.5 \pm 258.9
	NC_012808	18 846 841	100 %	452.3 \pm 86.2
	NC_012809	216 117	100 %	764.7 \pm 110.5
	NC_012810	139 576	100 %	750.8 \pm 101.3
	NC_012811	4 279 978	99.9998 %	447.9 \pm 76.9
<i>M. extorquens</i> AM1 DFS mutant 2	NC_012807	295 048	100 %	869.9 \pm 195.3
	NC_012808	15 563 801	100 %	367.0 \pm 83.7
	NC_012809	166 635	100 %	580.1 \pm 73.9
	NC_012810	104 196	100 %	550.5 \pm 76.2
	NC_012811	3 425 166	99.997 %	352.7 \pm 58.6

Table S2 Genetic differences between the sequenced strains and the used reference genome. The listed mutations were found in all sequenced strains including *M. extorquens* AM1 wild type. N/A indicates that there is no annotated gene function (annotation date 04/11/2021)

Genetic entity	Locus tag	Gene	Polymorphism type	Change	Effect on open reading frame
NC_012808	MEXAM1_RS02205	N/A	Insertion	(C)3 \rightarrow (C)4	Frame Shift
NC_012808	MEXAM1_RS10680	N/A	Deletion	(G)3 \rightarrow (G)2	Frame Shift
NC_012808	MEXAM1_RS34015	N/A	SNP (transversion)	T \rightarrow G	None
NC_012808	MEXAM1_RS12610	N/A	SNP (transition)	C \rightarrow T	None
NC_012808	MEXAM1_RS12610	N/A	SNP (transition)	T \rightarrow C	None
NC_012808	MEXAM1_RS13695	treS	Deletion	(C)4 \rightarrow (C)3	Frame Shift
NC_012808	MEXAM1_RS14240	N/A	Deletion	(G)3 \rightarrow (G)2	Frame Shift
NC_012808	MEXAM1_RS32900	N/A	Deletion	-TGCCG	Frame Shift
NC_012808	Intergenic region	N/A	SNP (transition)	A \rightarrow G	None
NC_012811	MEXAM1_RS28080	N/A	Insertion	+C	Frame Shift
NC_012811	MEXAM1_RS29025	N/A	Deletion	(G)3 \rightarrow (G)2	Frame Shift

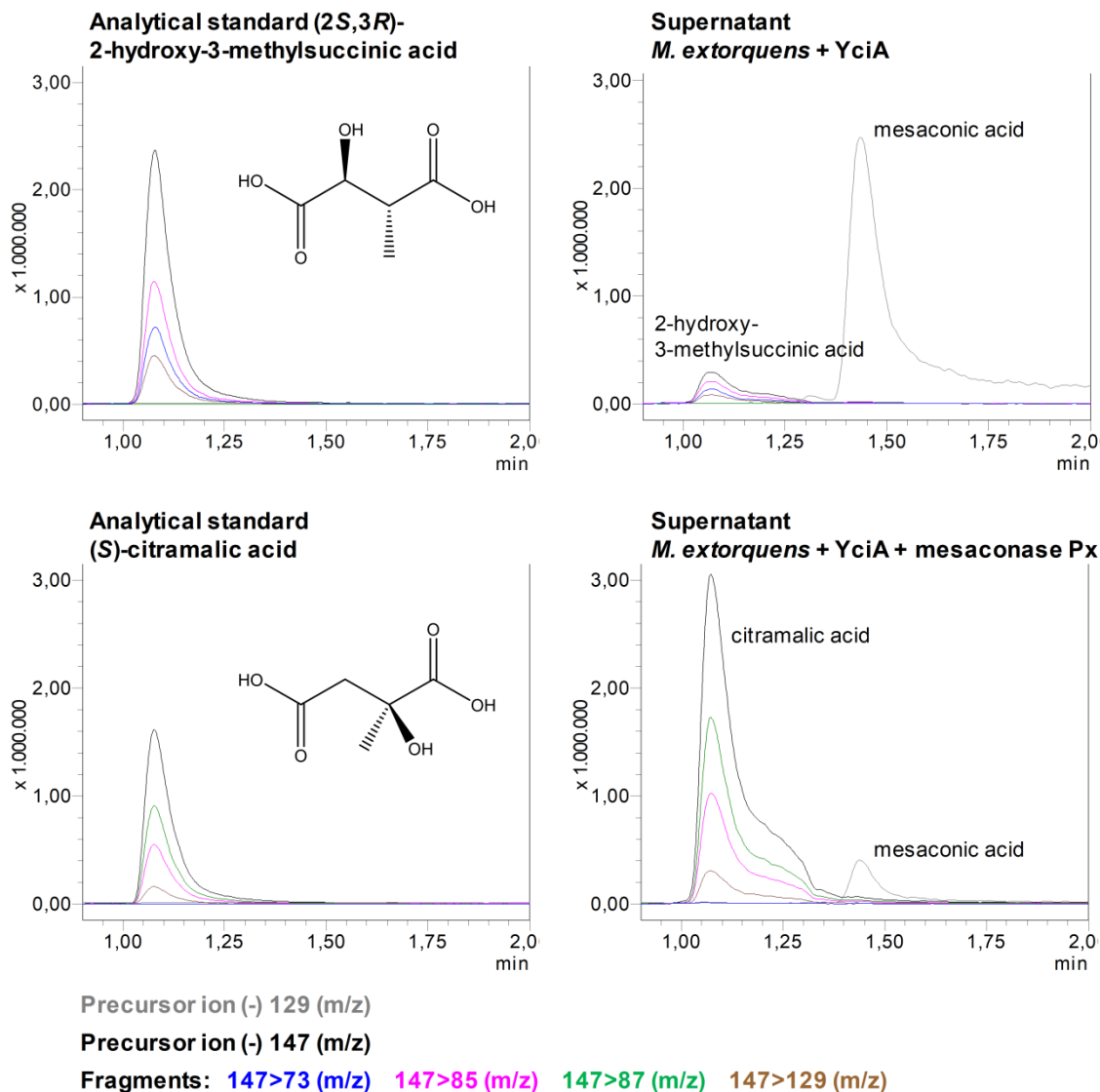


Fig. S1 Analytical evidence for production of citramalic acid by an *M. extorquens* AM1 strain expressing a mesaconase from *Paraburkholderia xenovorans* (mesaconase_Px) in addition to YciA from *Haemophilus influenzae*. Shown are chromatograms of precursor 129 m/z (mesaconic acid) and precursor 147 m/z (2-hydroxy-3-methylsuccinic acid or citramalic acid). To distinguish between 2-hydroxy-3-methylsuccinic acid (present at low concentrations in *M. extorquens* AM1 cultures) and citramalic acid, the analytes were fragmented using the identical LC-MS/MS MRM method detecting fragments 73, 85, 129 and 87. Here, the fragment of 87 (m/z) is characteristic for citramalic acid and can therefore be used for unambiguous identification. In supernatant of cultures with *M. extorquens* AM1 expressing thioesterase gene *yciA*, mesaconic acid as well as 2-hydroxy-3-methylsuccinic acid can be detected. With the additional introduction of a mesaconase, the mesaconase peak becomes smaller and conversion to citramalic acid can be observed.

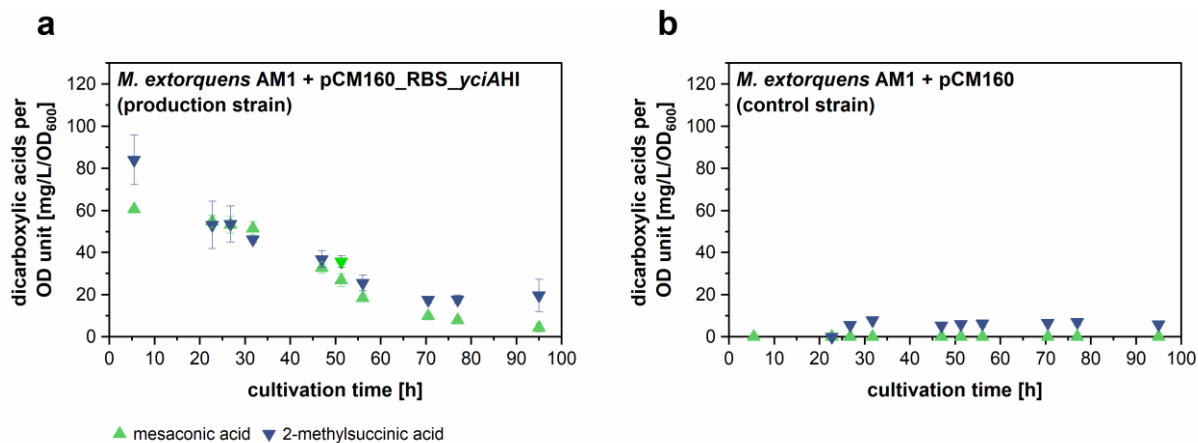


Fig. S2 Dicarboxylic acid production per OD₆₀₀ (transformed data from figure 2). Meseaconic acid and 2-methylsuccinic acid concentrations per OD₆₀₀ in supernatant of *M. extorquens* AM1 + pCM160_RBS_yciAHI (a) or *M. extorquens* AM1 + pCM160 (b) growing in methanol minimal medium. Error bars represent standard deviations from three independent replicates

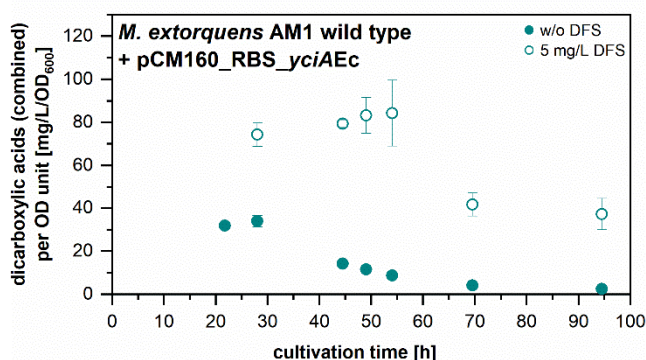


Fig. S3 Dicarboxylic acid production per OD₆₀₀ (transformed data from figure 5). Meseaconic acid and 2-methylsuccinic acid concentrations (combined) per OD₆₀₀ in supernatant of *M. extorquens* AM1 harboring pCM160_RBS_yciAEc in methanol minimal medium (filled symbols) and in methanol minimal medium with addition of 5 mg/L 2,2-difluorosuccinic acid (DFS) after 5 h of cultivation (empty symbols). Error bars represent standard deviations from two independent replicates

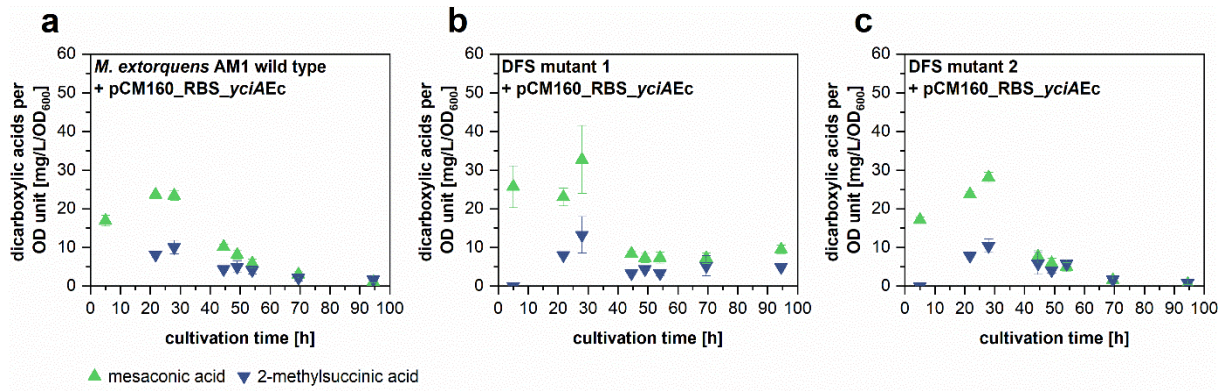


Fig. S4 Dicarboxylic acid production per OD₆₀₀ (transformed data from figure 6). Mesaconic acid and 2-methylsuccinic acid concentrations per OD₆₀₀ in supernatant of *M. extorquens* AM1 wild type + pCM160_RBS_yciAEc (**a**), DFS mutant 1 + pCM160_RBS_yciAEc (**b**) and DFS mutant 2 + pCM160_RBS_yciAEc (**c**), growing in methanol minimal medium. Error bars represent standard deviations from three independent replicates

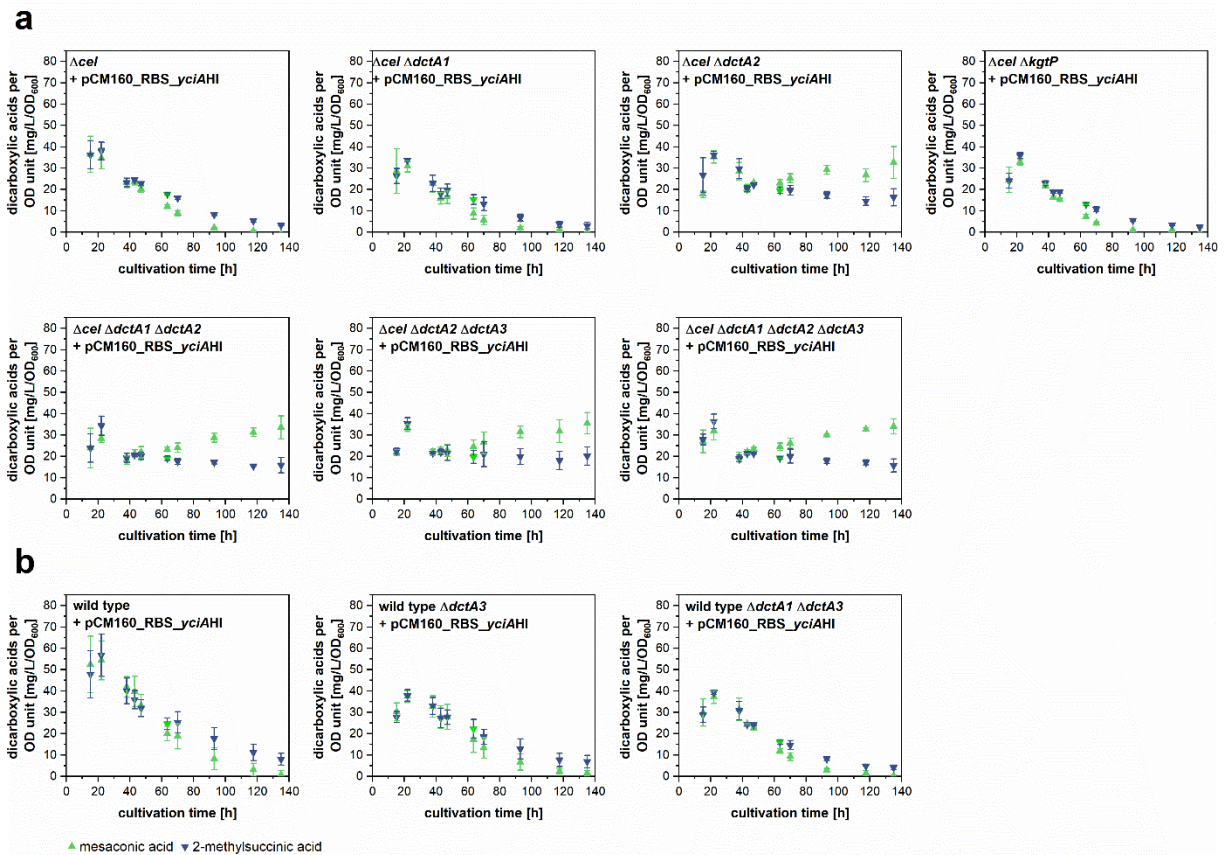


Fig. S5 Dicarboxylic acid production per OD₆₀₀ (transformed data from figure 7). Mesaconic acid and 2-methylsuccinic acid concentrations per OD₆₀₀ in supernatant of *M. extorquens* AM1 cells without and with single, double or triple transporter deletions. Strains heterologously express the thioesterase encoding gene *yciAHI* in methanol minimal medium. Error bars represent standard deviations from three independent replicates. Strains were constructed based on either **a** *M. extorquens* AM1 wild type or **b** *M. extorquens* AM1 *Δcel*/strain

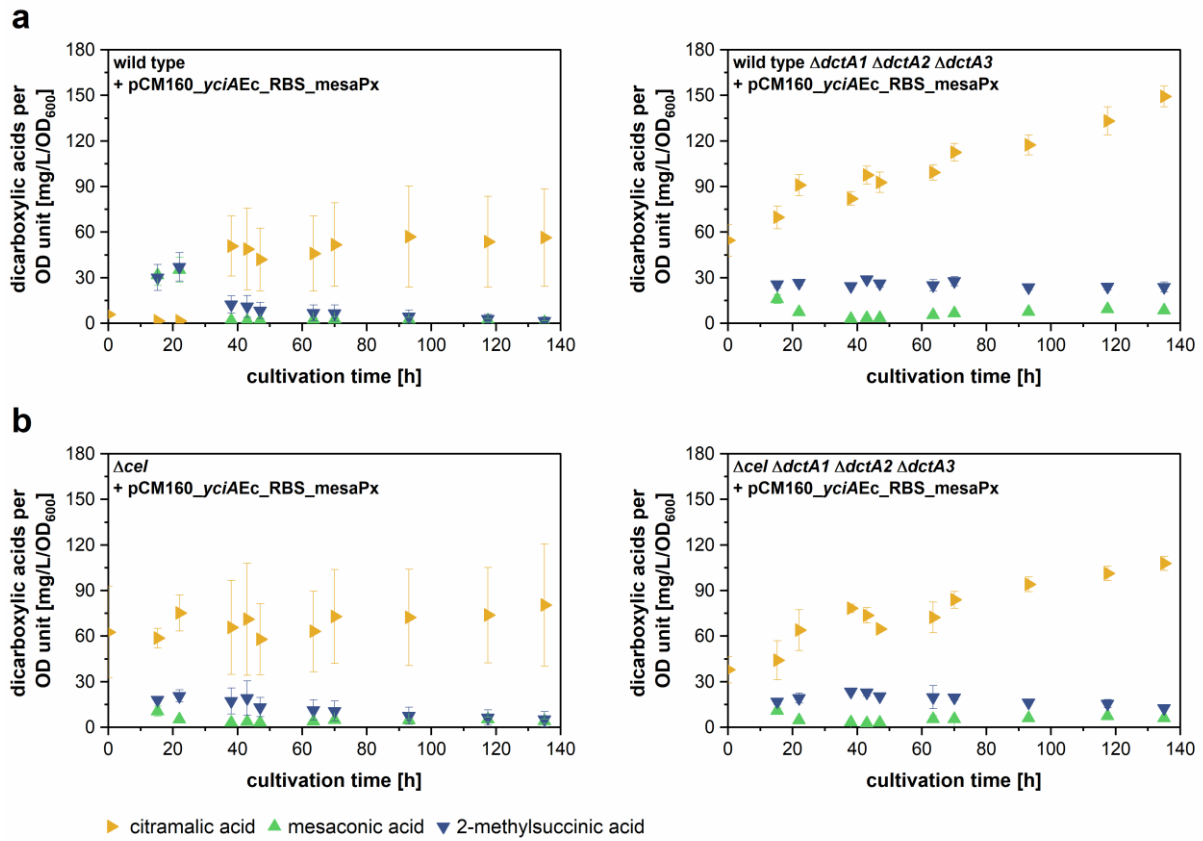


Fig. S6 Dicarboxylic acid production per OD₆₀₀ (transformed data from figure 8). Mesaconic acid, 2-methylsuccinic acid and citramalic acid concentrations per OD₆₀₀ in supernatant of *M. extorquens* AM1 wild type and triple *dctA* transporter deletion strain expressing thioesterase encoding gene *yciA*H1 and mesaconase in methanol minimal medium