Supplementary Material

Interrogating the Role of the Two Distinct Fructose-Bisphosphate Aldolases of *Bacillus methanolicus* by Site-Directed Mutagenesis of Key Amino Acids and Gene Repression by CRISPR Interference

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Supplementary tables

Table S1: Bacterial strains and plasmids used in this study.

Strain	Relevant characteristics	Reference
Escherichia coli DH5α	General cloning host, F- *thi*-1 *endA*1 *hsdR*17(r^-, m^-) *supE*44 *_lacU*169 (80*lacZ*_M15) *recA*1 *gyrA*96 *relA*1	(Hanahan, 1983)
Escherichia coli BL21 (DE3)	Protein production host, F- *ompT hsdSB* (r_B- m_B-) *gal dcm* (DE3)	(Studier and Moffatt, 1986)
Bacillus methanolicus MGA3	Wild type strain (ATCC 53907)	(Schendel et al., 1990)
Plasmid		
pET16b	Ap^R; T7*lac*; pBR322 origin, vector for N-terminal His-tagged protein overproduction	Novagen
pET16b-*fba*^{C}	Ap^R; T7*lac*; pBR322 origin, *fba*^{C} sequence was inserted in *Nde*I CA^TATG cloning site	(Stolzenberger et al., 2013a)
pET16b-*fba*^{P}	Ap^R; T7*lac*; pBR322 origin, *fba*^{P} sequence was inserted in *Nde*I CA^TATG cloning site	(Stolzenberger et al., 2013a)
pET16b-*glpX*^{C}	Ap^R; T7*lac*; pBR322 origin, g*lpX*^{C} sequence from *B. methanolicus* was inserted in *Nde*I CA^TATG cloning site	(Stolzenberger et al., 2013a)
pET16b-*fba*^{C}-*sdm1*	pET16b derivative for the production of *B. methanolicus* His_{10}-tagged Fba^C E51V mutant from *E. coli* BL21 (DE3), Ap^R; T7*lac*; pBR322 origin SDM of *fba*^{C} E51V	This study
pET16b-*fba*^{C}-*sdm2*	pET16b derivative for the production of *B. methanolicus* His_{10}-tagged Fba^C T140R mutant from *E. coli* BL21 (DE3), Ap^R; T7*lac*; pBR322 origin, SDM of *fba*^{C} T140R	This study
pET16b-*fba*^{C}-*sdm5*	pET16b derivative for the production of *B. methanolicus* His_{10}-tagged Fba^C E51V/T140R mutant from *E. coli* BL21 (DE3), Ap^R; T7*lac*; pBR322 origin, SDM of *fba*^{C} E51V/T140R	This study
pET16b-*fba*^{P}-*sdm3*	pET16b derivative for the production of *B. methanolicus* His_{10}-tagged Fba^P V51E mutant from *E. coli* BL21 (DE3), Ap^R; T7*lac*; pBR322 origin, SDM of *fba*^{P} V51E	This study
pET16b-*fba*^{P}-*sdm4*	pET16b derivative for the production of *B. methanolicus* His_{10}-tagged Fba^P R140T mutant from *E. coli* BL21 (DE3), Ap^R; T7*lac*; pBR322 origin, SDM of *fba*^{P} R140T	This study
pET16b-*fba*^{P}-*sdm6*	pET16b derivative for the production of *B. methanolicus* His_{10}-tagged Fba^P V51E/R140T mutant from *E. coli* BL21 (DE3), Ap^R; T7*lac*; pBR322 origin, SDM of *fba*^{P} V51E/R140T	This study
pET28a-*glpX*	pET28a derivative for the production of *C. glutamicum* His-tagged GlpX from *E. coli* BL21 (DE3), Knt^R; T7*lac*; pBR322 origin, *glpX* sequence from *C. glutamicum* was inserted in *Nde*I CA^TATG cloning site	(Rittmann et al., 2003)

pNW33Nkan	Cm^R, Km^R; pNW33N derivative in which the kanamycin-resistance gene was inserted	(Irla et al., 2016)
piCas	Cm^R, Km^R; pNW33N derivative, m2p controlled expression of *dcas9*, followed by terminator sequence of *S. pyogenes*. Contains another m2p promoter, which lacks 5'UTR, followed by *Ava*I/*Xba*I cloning site and dCas9 handle and terminator sequence of *S. pyogenes*	(Schultenkämper et al., 2019)
piCas-*tfba*^{C}	Cm^R, Km^R; piCas derivative, 20 bp complementary to *fba*^{C} gene was inserted in *Ava*I/*Xba*I cloning site.	This study
piCas-*tfba*^{P}	Cm^R, Km^R; piCas derivative, 20 bp complementary to *fba*^{P} gene was inserted in *Ava*I/*Xba*I cloning site.	This study
piCas-*ttkt*^{C}	Cm^R, Km^R; piCas derivative, 20 bp complementary to *tkt*^{C} gene was inserted in *Ava*I/*Xba*I cloning site.	This study
piCas-*ttkt*^{P}	Cm^R, Km^R; piCas derivative, 20 bp complementary to *tkt*^{P} gene was inserted in *Ava*I/*Xba*I cloning site.	This study

Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance; Ap^R, ampicillin resistance;

Table S2: Oligonucleotide sequences used in the present study.

Oligonucleotide ID	Characteristic	Sequence [5'-3']
tfbaC_fwd	Fwd primer for annealing oligonucleotides and synthesizing 20 bp region complementary to *fba*^{C} as target for CRISPRi with overlaps to piCas plasmid	*aaacgttttatgataaatat*aata tctgcccccagctctt
tfbaC_rev	Rev primer for annealing oligonucleotides and synthesizing 20 bp region complementary to *fba*^{C} as target for CRISPRi with overlaps to piCas plasmid	*tttctagctctaaaactcga*aag agctgggggcagatatt
tfbaP_fwd	Fwd primer for annealing oligonucleotides and synthesizing 20 bp region complementary to *fba*^{P} as target for CRISPRi with overlaps to piCas plasmid	*aaacgttttatgataaatat*ccat ggacagaacctaacgc
tfbaP_rev	Rev primer for annealing oligonucleotides and synthesizing 20 bp region complementary to *fba*^{P} as target for CRISPRi with overlaps to piCas plasmid	*tttctagctctaaaactcga*gcgt taggttctgtccatgg
ttktC_fwd	Fwd primer for annealing oligonucleotides and synthesizing 20 bp region complementary to *tkt*^{C} as target for CRISPRi with overlaps to piCas plasmid	*aaacgttttatgataaatat*gatg gaaagtgtacgtatcg
ttktC_rev	Rev primer for annealing oligonucleotides and synthesizing 20 bp region complementary to *tkt*^{C} as target for CRISPRi with overlaps to piCas plasmid	*tttctagctctaaaactcga*cgat acgtacactttccatc
ttktP_fwd	Fwd primer for annealing oligonucleotides and synthesizing 20 bp region complementary to *tkt*^{P} as target for CRISPRi with overlaps to piCas plasmid	*aaacgttttatgataaatat*gatc aatatctatttttgt
ttktP_rev	Rev primer for annealing oligonucleotides and synthesizing 20 bp region complementary to *tkt*^{P} as target for CRISPRi with overlaps to piCas plasmid	*aaacgttttatgataaatat*gatc aatatctatttttgt
proI_fwd	Fwd primer for amplifying *proI of Bacillus methanolicus*, quality check for contaminating genomic DNA in RNA samples	caaggccgcttgaaaaggggga aatgacaaatgaagaagcttactt ttgtc
proI_rvs	Rev primer for amplifying *proI of Bacillus methanolicus*, quality check for contaminating genomic DNA in RNA samples	gcggccgcggggtacccgggg atccttactgctttactgttactg
parA_fwd	qRT-PCR fwd primer for analysis for *parA* expression	tccagcctgaaggatatagc
parA_rev	qRT-PCR rev primer for analysis for *parA* expression	tcttcggcactgttgaagga
fbaC_fwd	qRT-PCR fwd primer for analysis for *fba*^{C} expression	agccgttcagtctgttttc

fbaC_rev	qRT-PCR rev primer for analysis for *fba*^{C} expression	catcccccatttatcacattcc
fbaP_fwd	qRT-PCR fwd primer for analysis for *fba*^{P} expression	cggtggacaagaagatgatgtag
fbaP_rev	qRT-PCR rev primer for analysis for *fba*^{P} expression	acgcaggtgcaaagcag
dCas9_fwd	qRT-PCR fwd primer for analysis for *dcas9* expression	cgtcgccgttatactggttg
dCas9_rev	qRT-PCR rev primer for analysis for *dcas9* expression	ctatcgccttgtccagacac
tktC_fwd	qRT-PCR fwd primer for analysis for *tkt*^{C} expression	gagaagacggaccaacacac
tktC_rev	qRT-PCR rev primer for analysis for *tkt*^{C} expression	taggagcaacgcatcaggag
tktP_fwd	qRT-PCR fwd primer for analysis for *tkt*^{P} expression	aaagaagcagcagagaagaag
tktP_rev	qRT-PCR rev primer for analysis for *tkt*^{P} expression	cgacacggtaaacaggaac
SDM1_fwd	Backbone amplification of pET16b-*fba*^{C} with a mutation in position 51 from E to V (SDM1), fwd primer	tcattttaggagtttctgtgggcgccggccgctatatgg
SDM1_rev	Backbone amplification of pET16b-*fba*^{C} with a mutation in position 51 from E to V (SDM1), rev primer	catatagcggccggcgcccacagaaactcctaaaatgac
SDM2_fwd	Backbone amplification of pET16b-*fba*^{C} with a mutation in position 140 from T to R (SDM2), fwd primer	aagcagagcttggacgcgttggcggccaagag
SDM2_rev	Backbone amplification of pET16b-*fba*^{C} with a mutation in position 140 from T to R (SDM2), rev primer	tcttggccgccaacgcgtccaagctctgcttc
SDM3_fwd	Backbone amplification of pET16b-*fba*^{P} with a mutation in position 51 from V to E (SDM 3), fwd primer	gttattatcggggtatctgaaggtgctgctaattacatg
SDM3_rev	Backbone amplification of pET16b-*fba*^{P} with a mutation in position 51 from V to E (SDM 3), rev primer	catgtaattagcagcaccttcagataccccgataataac
SDM4_fwd	Backbone amplification of pET16b-*fba*^{P} with a mutation in position 140 from R to T (SDM4), fwd primer	ggcagagctaggtaccatcggtggacaag
SDM4 rev	Backbone amplification of pET16b-*fba*^{P} with a mutation in position 140 from R to T (SDM4), rev primer	cttgtccaccgatggtacctagctctgcc
P212	Sequencing primer of pET16a plasmids (fwd)	gctaacgcagtcaggcaccgtgta
P213	Sequencing primer of pET16a plasmids (rev)	gactcactataggggaattgtgagcg

Overlapping regions are shown in italics; fwd: forward; rev: reverse; SDM: site directed mutagenesis.

Table S3: SDMs characteristics.

Name	Performed mutation(s)	Primer pair	Template	Relevant characteristics
FBA$^{C;\ E51V}$	FBA^{C} E51V	SDM1_fwd/ SDM2_rev	pET16b-*fba*^{C}	Mutation in FBP binding site
FBA$^{C;\ T140R}$	FBA^{C} T140R	SDM2_fwd/ SDM2_rev	pET16b-*fba*^{C}	Mutation in Zinc binding site
FBA$^{P;\ V51E}$	FBA^{P} V51E	SDM3_fwd/ SDM3_rev	pET16b-*fba*^{P}	Mutation in FBP binding site

FBA$^{P;\ R140T}$	FBA^{P} R140T	SDM4_fwd/ SDM4_rev	pET16b-*fba*^{P}	Mutation in Zinc binding site
FBA$^{C;\ E51V,T40R}$	FBA^{C} E51V/T140R	SDM2_fwd/ SDM2_rev	pET16b-*fba*$^{C-}$ *sdm1*	Mutation in FBP and Zinc binding site (Double mutant)
FBA$^{P;\ V51E,\ R140T}$	FBA^{P} V51E/R140T	SDM3_fwd/ SDM3_rev	pET16b-*fba*$^{P-}$ *sdm4*	Mutation in FBP and Zinc binding site (Double mutant)

Table S4: Amino acid sequences of FBA$^{C/P}$ and SDMs.

Name	Sequence
FBA^{C}	MPLVSMTEMLNKAKAEGYAVGQFNLNNLEFTQAILLAAEEEKSPVILGVS**E**GAGRY MGGFKTVVNMVKGLMEDYKITVPVAIHLDHGSSFEKCKEVIDAGFTSVMIDASHHPF EENVEVTKKVVEYAHARGVSVEAELG**T**VGGQEDDVIADGVIYADPKECEELVKRTGI DCLAPALGSVHGPYKGEPNLGFKEMEEIGRITGVPLVLHGGTGIPTKDIQRAISLGTAK INVNTENQIASAKKVREVLAENPNMYDPRKYLGPARDAIKETVIGKMREFGSSGKA
FBA$^{C;\ E51V}$	MPLVSMTEMLNKAKAEGYAVGQFNLNNLEFTQAILLAAEEEKSPVILGVS**V**GAGRY MGGFKTVVNMVKGLMEDYKITVPVAIHLDHGSSFEKCKEVIDAGFTSVMIDASHHPF EENVEVTKKVVEYAHARGVSVEAELG**T**VGGQEDDVIADGVIYADPKECEELVKRTGI DCLAPALGSVHGPYKGEPNLGFKEMEEIGRITGVPLVLHGGTGIPTKDIQRAISLGTAK INVNTENQIASAKKVREVLAENPNMYDPRKYLGPARDAIKETVIGKMREFGSSGKA
FBA$^{C;\ T140R}$	MPLVSMTEMLNKAKAEGYAVGQFNLNNLEFTQAILLAAEEEKSPVILGVS**E**GAGRY MGGFKTVVNMVKGLMEDYKITVPVAIHLDHGSSFEKCKEVIDAGFTSVMIDASHHPF EENVEVTKKVVEYAHARGVSVEAELG**R**VGGQEDDVIADGVIYADPKECEELVKRTG IDCLAPALGSVHGPYKGEPNLGFKEMEEIGRITGVPLVLHGGTGIPTKDIQRAISLGTA KINVNTENQIASAKKVREVLAENPNMYDPRKYLGPARDAIKETVIGKMREFGSSGKA
FBA$^{C;}$ E51V,T140R	MPLVSMTEMLNKAKAEGYAVGQFNLNNLEFTQAILLAAEEEKSPVILGVS**V**GAGRY MGGFKTVVNMVKGLMEDYKITVPVAIHLDHGSSFEKCKEVIDAGFTSVMIDASHHPF EENVEVTKKVVEYAHARGVSVEAELG**R**VGGQEDDVIADGVIYADPKECEELVKRTG IDCLAPALGSVHGPYKGEPNLGFKEMEEIGRITGVPLVLHGGTGIPTKDIQRAISLGTA KINVNTENQIASAKKVREVLAENPNMYDPRKYLGPARDAIKETVIGKMREFGSSGKA
FBA^{P}	MPLVSMKDMLNHGKENGYAVGQFNINNLEFGQAILQAAEEEKSPVIIGVS**V**GAANY MGGFKLIVDMVKSSMDSYNVTVPVAIHLDHGPSLEKCVQAIHAGFTSVMIDGSHLPL EENIELTKRVVEIAHSVGVSVEAELG**R**IGGQEDDVVAESFYAIPSECEQLVRETGVDCF APALGSVHGPYKGEPKLGFDRMEEIMKLTGVPLVLHGGTGIPTKDIQKAISLGTAKIN VNTESQIAATKAVREVLNNDAKLFDPRKFLAPAREAIKETIKGKMREFGSSGKA
FBA$^{P;\ V51E}$	MPLVSMKDMLNHGKENGYAVGQFNINNLEFGQAILQAAEEEKSPVIIGVS**E**GAANY MGGFKLIVDMVKSSMDSYNVTVPVAIHLDHGPSLEKCVQAIHAGFTSVMIDGSHLPL EENIELTKRVVEIAHSVGVSVEAELG**R**IGGQEDDVVAESFYAIPSECEQLVRETGVDCF APALGSVHGPYKGEPKLGFDRMEEIMKLTGVPLVLHGGTGIPTKDIQKAISLGTAKIN VNTESQIAATKAVREVLNNDAKLFDPRKFLAPAREAIKETIKGKMREFGSSGKA
FBA$^{P;\ R140T}$	MPLVSMKDMLNHGKENGYAVGQFNINNLEFGQAILQAAEEEKSPVIIGVS**V**GAANY MGGFKLIVDMVKSSMDSYNVTVPVAIHLDHGPSLEKCVQAIHAGFTSVMIDGSHLPL EENIELTKRVVEIAHSVGVSVEAELG**T**IGGQEDDVVAESFYAIPSECEQLVRETGVDCF APALGSVHGPYKGEPKLGFDRMEEIMKLTGVPLVLHGGTGIPTKDIQKAISLGTAKIN VNTESQIAATKAVREVLNNDAKLFDPRKFLAPAREAIKETIKGKMREFGSSGKA
FBA$^{P;\ V51E,}$ R140T	MPLVSMKDMLNHGKENGYAVGQFNINNLEFGQAILQAAEEEKSPVIIGVS**E**GAANY MGGFKLIVDMVKSSMDSYNVTVPVAIHLDHGPSLEKCVQAIHAGFTSVMIDGSHLPL EENIELTKRVVEIAHSVGVSVEAELG**T**IGGQEDDVVAESFYAIPSECEQLVRETGVDCF APALGSVHGPYKGEPKLGFDRMEEIMKLTGVPLVLHGGTGIPTKDIQKAISLGTAKIN VNTESQIAATKAVREVLNNDAKLFDPRKFLAPAREAIKETIKGKMREFGSSGKA

Amino acid positions 51 and 140 are highlighted in red.

Table S5: X-ray data collection and refinement statistics.

Data sets		**FBA^C**	**FBA^P**
PDB accession code		7NC7	7NCC
Space group		C 2 2 2_1	C 2 2 2_1
Cell constants	a, b, c [Å] α, β, γ [°]	71.97, 98.26, 139.37 90, 90, 90	91.58, 122.12, 160.96 90, 90, 90
Wavelength [Å]		1.54	1
Resolution limits [Å]		27.41 – 2.20 (2.28 – 2.20)	45.79 – 2.00 (2.05 – 2.00)
Completeness (%)		99.8 (98.0)	99.3 (99.4)
Unique reflections		24421 (2158)	60641 (4427)
Multiplicity (%)		4.1 (4.2)	13.4 (13.7)
R_{merge}[a]		0.395 (1.006)	0.156 (0.925)
$R_{p.i.m.}$		0.229 (0.551)	0.044 (0.256)
Mean I/σ (I)		6.7 (1.7)	11.7 (3.0)
$CC_{1/2}$		0.996 (0.521)	0.999 (0.661)
Refinement statistics			
R_{work}[b] / R_{free}		0.192 / 0.248	0.194 / 0.258
No. atoms		4324	4614
	Protein	4155	4288
	Ligand/ion	20	44
	Water	149	282
B-factor [Å^2]		39.13	38.72
	Protein	39.12	38.26
	Ligand/ion	65.05	47.25
	Water	36.07	44.39
R.m.s. deviations			
	bond lengths [Å]	0.014	0.018
	bond angels [°]	1.66	2.13
Ramachandran plot			
	Favored (%)	96.81	96.63
	Allowed (%)	3.19	3.37
	Outliers (%)	0	0

[a] $R_{merge} = \Sigma_{hkl} [(\Sigma_i |I_i - \langle I \rangle|) / \Sigma_i I_i]$

[b] $R_{work} = \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$

R_{free} is the cross-validation R value for a test set of 5 % of unique reflections

Supplementary figures

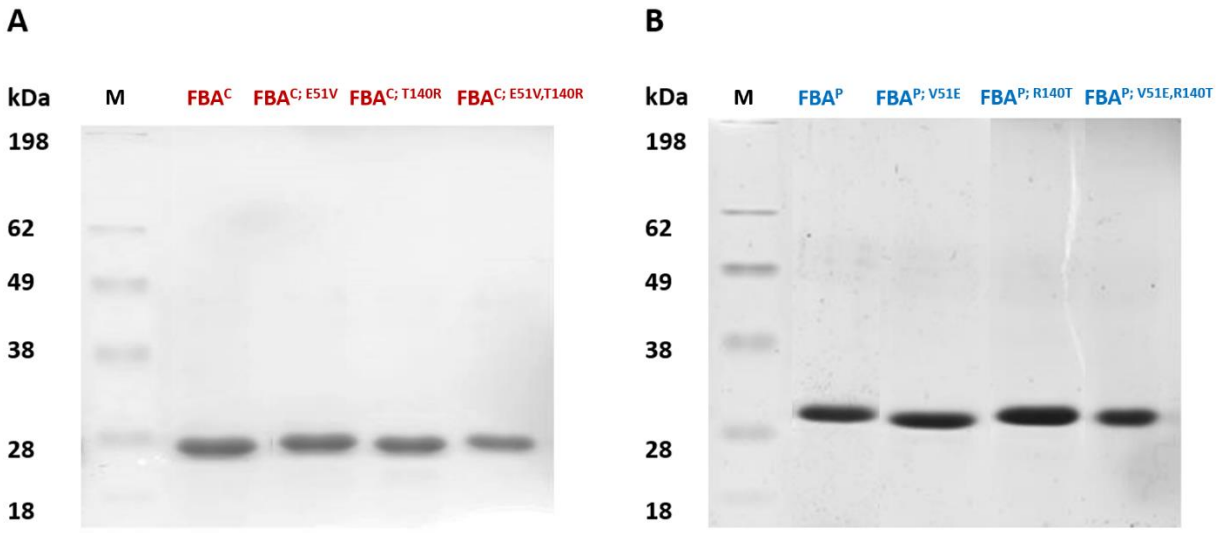

Fig. S1: Purification of distinct FBAs and mutants. SDS gels showing the purified FBA^{C} with mutants $FBA^{C; E51V}$, $FBA^{C; T140R}$ and $FBA^{C; E51V,T140R}$ (**A**), FBA^{P} with $FBA^{P; V51E}$, $FBA^{P; R140T}$ and $FBA^{P; V51E, R140T}$ (**B**) after His-Tag cleavage. Additionally, SeeBlue™ pre-stained protein standard (Thermo Fisher Scientific), was applied as marker (M).

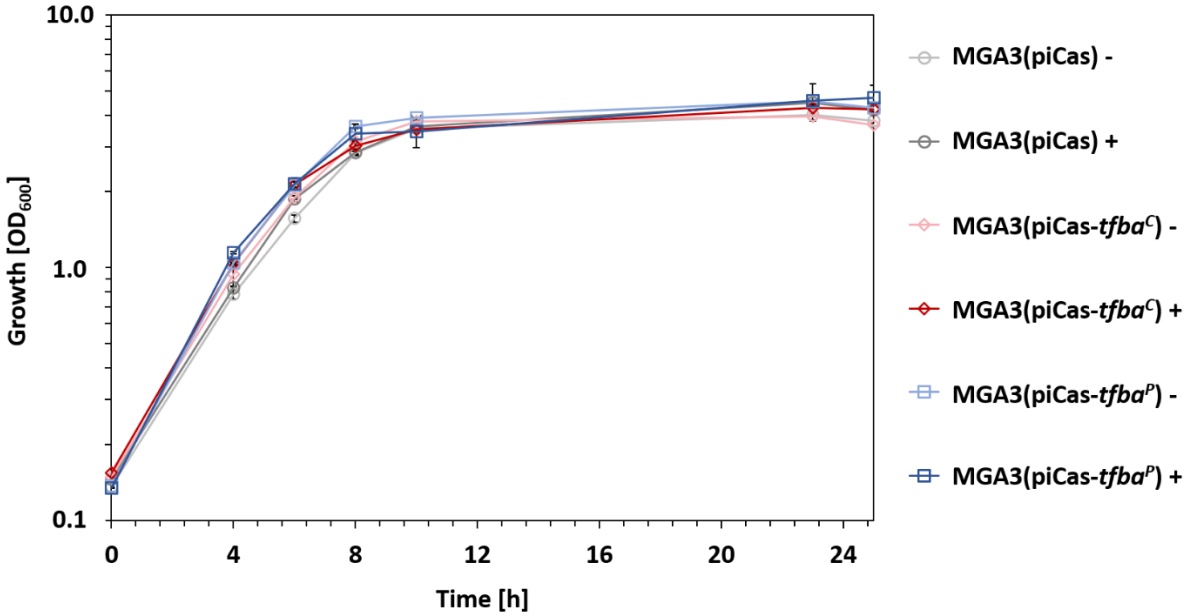

Fig. S2: Growth of *B. methanolicus* MGA3 (piCas-$tfba^{C/P}$) strains. Cultivation was performed with methanol as sole carbon source (-) and additionally with mannitol for *dCas9* induction (+). Error bars indicating standard deviations (n=3)

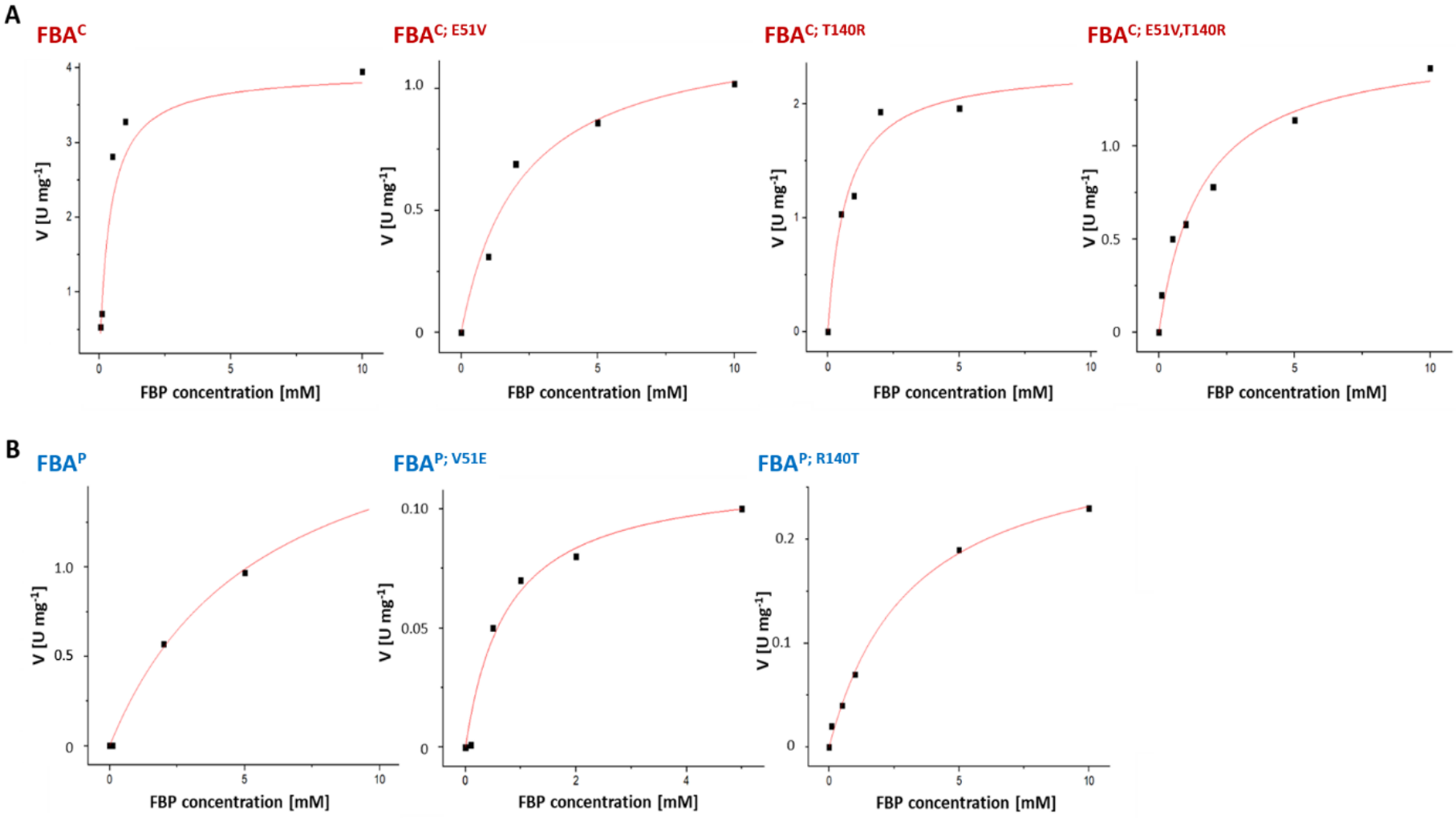

Fig. S3: Michaelis Menten Plots to determine K_M for distinct FBAs and mutants in glycolysis (FBP cleavage). The specific activity (axis of ordinate) for FBA^C and SDMs (**A**) and FBA^P and SDMs (**B**) were applied against the FBP substrate concentration (axis of abscissa). The kinetic parameters were calculated according to Michaelis Menten kinetics (Michaelis et al., 2011).

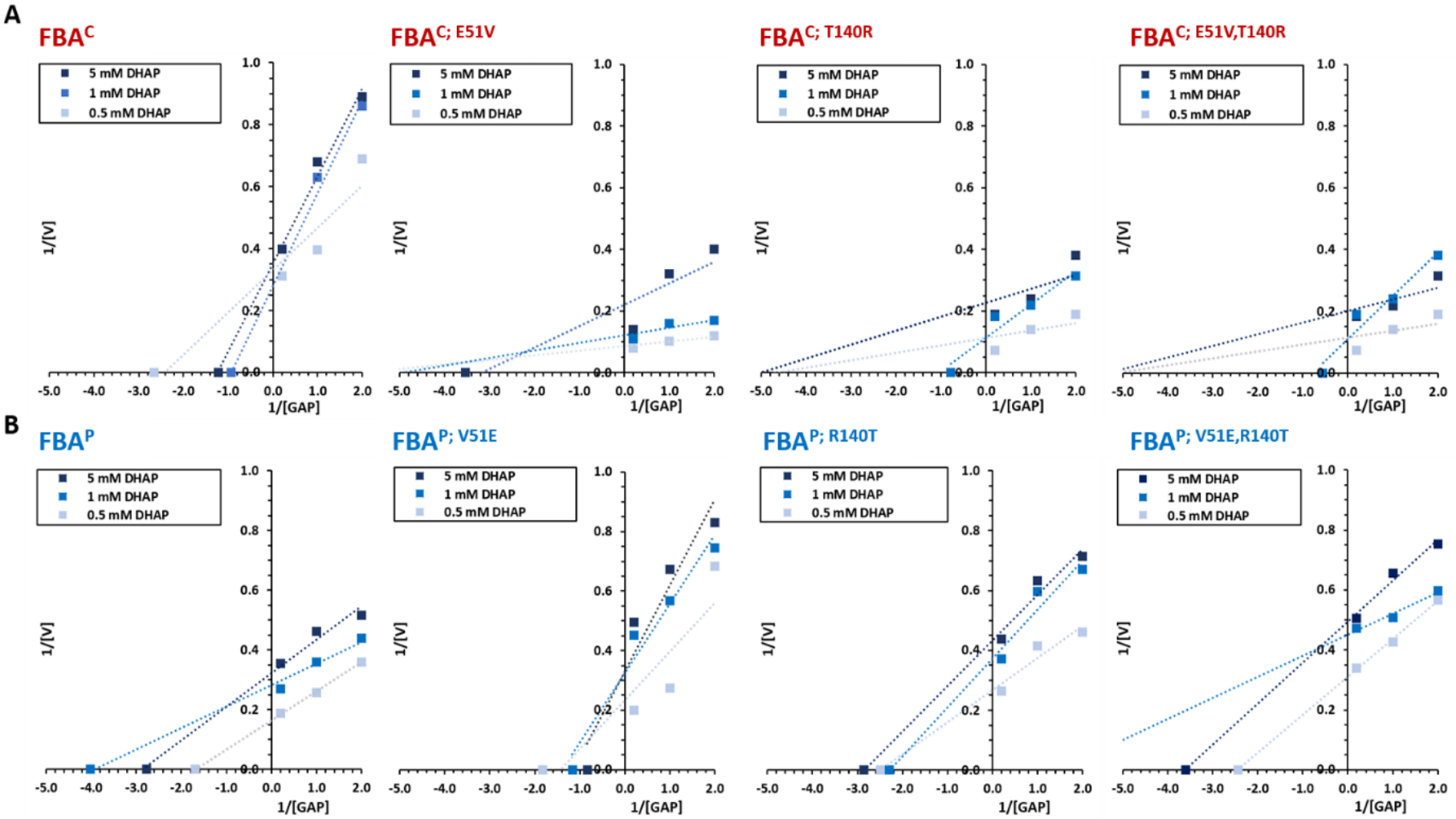

Fig. S4: Lineweaver Burk Plots to determine K_M for distinct FBAs and mutants in gluconeogenesis (FBP synthesis) with GAP as substrate. Asymmetric intersecting initial velocity patterns for an equilibrium-ordered mechanism. The reciprocals of the specific activity (axis of ordinate) for FBA^C and SDMs (**A**) and FBA^P and SDMs (**B**) were applied against the reciprocals of the GAP substrate concentration (axis of abscissa). The kinetic parameters were calculated according to Lineweaver and Burk kinetics (Lineweaver and Burk, 1934).

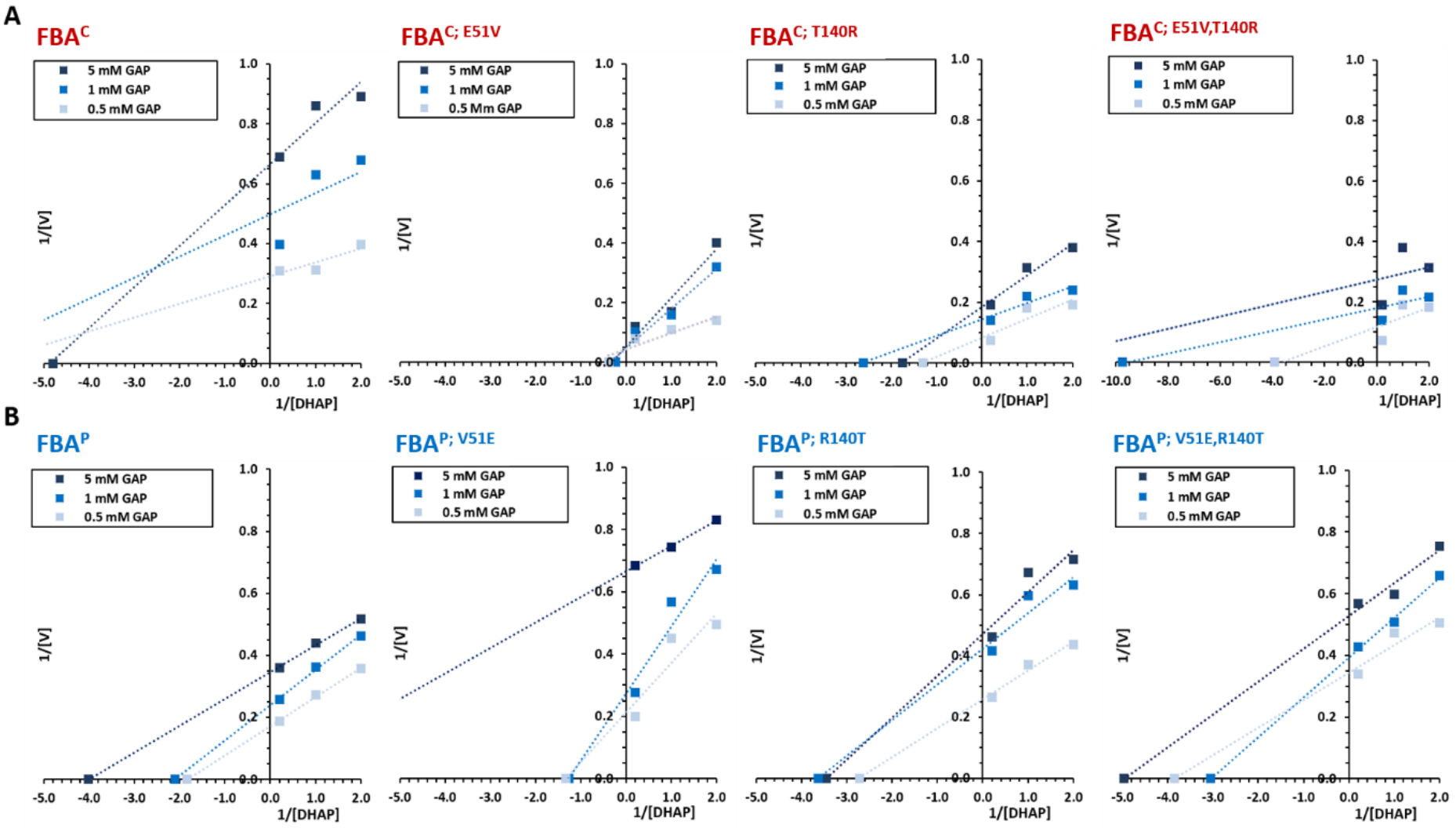

Fig. S5: Lineweaver Burk Plots to determine K_M for distinct FBAs and mutants in gluconeogenesis (FBP synthesis) with DHAP as substrate. Asymmetric intersecting initial velocity patterns for an equilibrium-ordered mechanism. The reciprocals of the specific activity (axis of ordinate) for FBA^C and SDMs (**A**) and FBA^P and SDMs (**B**) were applied against the reciprocals of the DHAP substrate concentration (axis of abscissa). The kinetic parameters were calculated according to Lineweaver and Burk kinetics (Lineweaver and Burk, 1934).