Regulation of the leucine metabolism in *Mortierella alpina*

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Experimental procedures

Construction of plasmids for heterologous expression of *batA-L, batA-S, batC* and *leuA1*.

Coding sequences for the two versions of *batA (batA-L* and *batA-S,* with and without the codons for the mitochondrial import signal, respectively), *batC* and *leuA1* were amplified from cDNA of *M. alpina* ATCC32222 (grown on AMM for 5 days). Oligonucleotides oRS37/oRS039 (*batA-L*), oRS038/oRS039 (*batA-S*), oRS049/oRS046 (*batC*) and oRS40/oRS041 (*leuA1*) (Table S1), respectively, and Phusion DNA polymerase (Thermo Fisher) were used. The PCR protocol was: 98°C, 2 min; 98°C, 15 s, 60°C, 15 s; 72°C, 60 s; 35 cycles; terminal hold at 72°C, 5 min. The DNA fragments were ligated into pJET1.2 using the CloneJET PCR cloning kit (Thermo Fisher). The genes *batA-L* and *batA-S* were excised with *Nde*I/*Hind*III and ligated into the accordingly digested pET28a(+) vector to create plasmids pRS04 and pRS05, respectively. Similarly, *batC* and *leuA1* were excised with *Nhe*I/*Hind*III and ligated into the *Nhe*I/*Hind*III-linearized pJET28a(+) vector (final plasmids pRS08 and pRS06). These expression plasmids were used to transform *Escherichia coli* BL21, using 50 μg mL^{-1} kanamycin as selection marker.

Heterologous production of enyzmes in *Escherichia coli* and size exclusion chromatography (SEC).

Production of the N-terminally His$_6$-tagged proteins was performed in *Escherichia coli* BL21 in 400 mL 2xYT medium (20 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 10 g L^{-1} NaCl), supplemented with 50 mg mL^{-1} kanamycin, at 37°C and 180 rpm. At an OD$_{600}$ of 0.6–0.8, the temperature was shifted to 16°C, and gene expression was induced by addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After 16 h, cells were harvested by centrifugation and the pellet was resuspended in binding buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0). After cell lysis by sonication, the lysate was centrifuged, and the cell-free supernatant was transferred to an equilibrated gravity affinity column with 1 mL Protino Ni^{2+}-NTA resin (Macherey-Nagel). After 25 min incubation on ice, the resin was washed with binding buffer containing increasing concentrations of imidazole (10, 25 and 50 mM). Pure proteins were eluted with binding buffer with 250 mM imidazole. The enzymes were re-buffered in 50

mM Tris-HCl (pH 8.0) by gel filtration (PD10, Cytiva). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher) versus a serial dilution of the provided bovine serum albumine (BSA) standard solution. After supplementation with 10% glycerol, enzymes were stored at -80°C without loss of activity for up to 4 months. For determination of the oligomeric state by size exclusion chromatography (SEC), the native and denatured (95°C, 5 min) enzymes were re-buffered in SEC buffer (10 mM NaH_2PO_4, 140 mM NaCl, pH 7.5) and applied to a Superdex 200 increase 10/300GL column (Cytiva) on an FPLC Äkta Pure 25 instrument using an isocratic run. Commercial protein standards served as size reference (gel filtration HMW and LMW calibration kits, Cytiva).

Chromatographical quantification of α-amino acids and α-keto acids

To determine the intracellular amino acids, the fungus was grown in AMM with or without 10 mM of one of the BCAAs (Ile, Leu, Val) or 10 mM of each BCAA. Mycelium was harvested using Miracloth (Merck Millipore), extensively washed with 200 mL tap water and lyophilized. The dry fungal mass (50-400 mg) was ground to a fine powder with a micropistill and extracted twice with 100 μL methanol per 100 mg powder for 10 min each. After brief centrifugation (14,000 x g, 10 min), 1 μL thereof was applied to a SeQuant ZIC-HILIC column (Merck) using method A or method B (Table S3). The 20 standard L-amino acids and six α–keto acids (α-ketoisovalerate (KIV), α-ketomethylvalerate (KMV), α-ketocaproate (KIC), α-ketobutyrate (KB), α-ketoglutarate (KG), pyruvate) served as references. BAT activity was determined based on a calibration curve with L-glutamate monitored in a range of 0.01 – 10 mM using the extracted ion chromatogram (EIC) in positive mode.

Chromatographical quantification of malpinins

M. alpina was cultivated in 50 mL AMM without or with 10 mM of one of the BCAAs or 10 mM of each BCAA (Ile, Val, Leu). The fungal total fungal biomass was collected with Miracloth (Merck, Millipore), lyophilized and the dry biomass was weighted. The cell-free supernatant (50 mL) was adjusted to pH 6.5 by 4 M HCl and extracted twice with an equal volume of ethyl acetate. The solvent was removed by a rotary evaporator and the residue was dissolved in 2 mL methanol. After brief centrifugation (10,000

x g, 10 min), 10 μL of the crude extract was applied to a Zorbax Eclipse Plus C18 RRHD (Agilent) column using HPLC method C (Table S3). Metabolites were quantified from the extracted ion chromatograms (EIC) monitored in the positive detection mode. For quantification, a calibration curve of malpinin A (m/z 859 [M+H]$^+$) ranging from 0 to 500 μg/mL served as reference.

Phylogenetic analysis of BCAA aminotransferases and α-isopropylmalate synthases.

The evolutionary history was inferred by using the Maximum Likelihood method and Le_Gascuel_2008 model [1]. The bootstrap consensus tree inferred from 1000 replicates [2]. Evolutionary analyses were conducted in MEGA11 [3].

Statistical analysis

Statistical analyses were performed using the Dunnet-Test (One-Way and Two-Way-Anova) implemented in the GraphPad Prism 7.04 Software package. Significant values are denoted as: ns, not significant, * $p<0.05$, **$p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

cDNA sequences

Underlined sequences indicate codons encoding the mitochondrial import sequence.

>*batA* (1224 bp)

ATGTTTGTCCAAAGAACACTCCGCCAGGCTGTCAAGGCCCACTCCGCCTTCGCCTCCTCGCCC
GCCGCCTACAGCGCCAAGGTCCTCTCCTCGCGCTTCTACAGCACCTCCATCGCGCCCCTCGAT
GCCTCCAAGCTCGAGATCAACGCCTCCAAGAACAAGAAGCCACTGCAGGAGAACTCGCAGC
TGGTCTTTGGCAAGACCTTCTCCGACAACATGCTCACCATCGAGTGGGAGGCCGGCAAGGGCT
GGGCCAAGCCCGAGATCAAGGAGTATGGCAAGCTCCACCTCGACCCCTCCGCGGTCGTCTTC
CACTACTCGTTCGAGTGCTTCGAGGGCATGAAGGCCTACAAGGACAAGCACGGCAAGACCCG
TCTCTTCCGTCCCGACATGAACATGAAGCGTTTCAACAGGAGTGCTGCCCGCATTGCCCTTCC
CAACTTTGAGGACGAGGAGTTGATCAAGTTGATTGGCGAGTTCTTGAAGGTCGATGACCGCTG
GATCCCCAAGGGCCGTGGTTACTCGCTCTACCTCCGTCCCACCATGATCGGCACCCAAGAGTC
CCTGGGAGTGGGTGCATCGAACAAGGCCTTGATCTTTGTCATTGCCTCGCCTGTCGGTCCTTAC
TTCCCCTCGGGATTCAATGCCGTCAACCTGTTGGCGACCTCGGACAAGGTCCGTGCCTGGCCC
GGTGGAACGGGTGATGCCAAGGTCGGAGGCAACTATGCGCCTTGCATCAAGCCCCAGTTGGA
TGCCAACAAGGAGGGTTACCAGCAGAACCTGTGGCTGTTTGGCGCTGACCACCAGGTCACCG
AGGTCGGCACCATGAACTGCTTTGTCTTCTGGAAGAACGAGCAAGGAGAGAGAGAGCTGGTC
ACCCCTGACCTGGATGGCTCCATCCTGCCCGGAGTCACCCGTGACAGTATCCTGAGCCTTGCC
CGTCAATGGGGCGAGTTCAAGGTCTCGGAGCGCAAGTTCACCATGGCCGATCTCGTCAAGGC
TGATAAGGAAGGCCGTATCATTGAAATGTTTGGTGCTGGAACGGCTTGCATTGTCTGCCCTAT
CAAGAAGATCCACTTTGAGGGTAAGGACATCAACATCCCCCTGGATCCCACTGACAAGACCA
GCCAGGCTGGCAAGTTGACCAAGCGCATCAACGATGCCATCATGGACATCCAGTACGGTGAT
GTCGAGGGTCCCAAGGGCTGGTCCGTCGTTGTCTAA

>*batC* (1104 bp)

ATGACCTGCACACTGCCACCACCCCCAACCTCCGCCATCGACTGGGACAACCTCGGCTTCAA
GTGGGTCGACACCAACGGGCACGTCAAGTACATCCACAAGGACGGCCAGTGGGACCAGGGC
GAGTTTGTCCGCGACTCGTACATCAAGATGCACGTCTGTGCCCCAGCACTGAACTATGGCCAG
GAGTGCTTCGAGGGCATCAAGGCGTTCAGGTGCAAGGACGGCCAGGTCCGTATCTTCCGCCC
CGATGCCAACGCCAAGCGTATGCGCTACTCTGCAGACCTGGCCTGCATGGCCGCCGTGCCCG
AGGATCTTTTTGTGCAGGCATGCGAGCGCGCTGTGGCCAACAATCTCGAGTTTGTGCCCCCCT
ACGGCTCTGGCGGGGCCCTCTATCTGCGCCCTCTCCTCCTTGGCACTGGCCCCGAGATTGGCCT
TTATCCCGCTCCAGAGTTCACGTTCATTGTCATGGTCATTCCCGTTGGCAACTTTTACAAGTCC
GGAGTAAAGCCAGTGGACTGCCTTGTCGTTGACAACTTTGACCGAGCGGCGCCCCTGGGAAC
TGGAGCGGCTAAACTGGGTGGCAACTACGCGCCGGTTCTGAAGCACAGCAGCGCGGCCAAG
GAGCAGGGATACGCTATCACACTCCACCTCGACTCCAAGACACACTCGAACGTGGATGAATT
CAGCACTTCCAACTTTGTGGCCCTCACCTACCCCTCCGCCGAGCACGACAACAAGCCCGTCTT
TTGCGCCCCCGAAAGCCCCTCCATCCTCAACTCTGTCACCAAGCAATCACTGGTTCAGCTCGC
CAAAAGTTTTGGCTGGGCCGTTGAAAACAGCGTCGTGCCCTTCACTGACATCACAAGCAATCG
TTTCGAGGAAATTGCTGCCTGCGGGACTGCAGCGGTCATCACGCCTGTCAAATCCATCGAACG
TCAGGGAACAAAGACTCTTATCGGCACTTCCGCCACTCAGGAGGCCATTGGTGCCGGATTTCT
CCGCCTCTTTACAGCTATGAGGGGCATCCAGAATGGCGATCTTGAGGATCCATATGGTTGGAT
GCAGCCTGCCCAAGGCATTGCCCCTCGGCAGGAGGCGTAA

>*leuA1* (1689 bp)

ATGGCACAAGCAAATAAAGACAAGTTGATCATTTTCGACACTACTCTCAGAGATGGCGAGCA
GTCCCCAGGAGTAACCCTTACCGTGAACGAAAGATTGAAATCGCTAAGCAGCTCTCTCGCCT
CGGAGTTGATGTCCTCGAGGCTGGTTTCCCTGTGGCATCCCAGGGTGACTTTGATGCCGTTGAG
CGGATTGCGACTGAGGTTGGCCCCCTCATGGTCGGCCGTGAAGCCCTTGGCAAGCCCATTACA
ATTGCGGGTCTTGCTCGTGCCGTGCCAGCAGATATCCAGCGATGCTACGATGCCATCGCAAAG
GCCCCTCACAAACGCATTCACACCTTCCTCGCTACCTCGGACATCCACCTCGAGCACAAACTC
AAAATTTCGCGCGACGAATGCGTAAAGCGCGCAGTGGCCGCAGTATCCTTCGCCAAGACCTT
GGTCCAGGACGTCGAGTTTTCGCCTGAGGATGCAGGAAGGAGCGACCAGGACTTTTTGTGCA
AGGTCCTCTCTGCAGTAATCGAGGCCGGAGCAACAACTCTCAACATCCCTGACACAGTAGGC
TACAACATGCCGGAGGAGTATGGGTCAATGATCAAGTACTTGATCGCCAATACGAAGGGTTC
GGACACGGTCATCTGGTCTGCTCACTGCCACAACGACCTCGGACTTGCCACTGCCAACACCCT
CTCGGGCATCATGAACGGAATTCGCCAGGTTGAGGTCACCATCAATGGTATCGGCGAGCGTG
CGGGAAATACTGCCATGGAGGAGGTCGTCATGGCCATCCACACCCACCCCAACTTTTTCCCTG
TCTACCATACCATCAACACGCCCCTGTTCTTCAGGATCTCACAGCTCGTGTCATCGCTCTCGGG
AATGGCAGTACAATCTAACAAGGCCATTGTAGGGGCCAACGCATTCCTGCACGAGTCTGGTA
TTCATCAGGATGGTGTTCTTAAGAATAAGACCACATATGAGATCATCAGCCCCGAGACAGTA
GGTGCCAACACTGTCAACCTTGTCCTTGGCAAGCATTCTGGACGTGCTGCTTTCAAGTCTCGTC
TCCAGGAGCTTGGTTTTGGTGAGCTGACAGACGAAGAGTTCCAGGTGGCGTTCAACAGTTTCA
AGACCTTGGCCGATAGCAAGAAGCGTGTGACCGAGCAGGATTTGCTTGCCTTGCTGTCGGATC
AGGTCTCGGCCGCGTCCGGGGACAAGGCCACGTTCGTGATCAAGTCGCTCCAGGTGGTCTCG
GGCACATCGTTTGCAACCGCCACGGTCCAGTTGCAGAACAGCCAGACCGGCGATGTGGTGGT
GGATGCTGCCATTGGCAAGTCTGGACCAGTCGAGGCCGTCTTTTTGGCGATCCAGCGCTTGGT
GGGCAAGAGTATTCGCCTGCTCAAGTTTGATATCACGGCCGTAGGTGAGGGTGTCGATGCTTT
GGGCCAGGTTTCAGTCAAGATTGCAGAGGACACGTCGTCACCCGGTTCCTCGGACAGTGATG
AAAATGCAGAGGAGGCAAAGGACAATGAGAGCAAGGCCAATGGAATCAGCCAGGCCACGT
ACCACGGTCACGGTGCCGACTCGGATATTATCCAGGCTGCGACCAAAGCCTACCTCAATGCC
ATTAACCGCCTGGTAGCAAATGAGAACGAGCGTCTTCTCGGTGAACGCAAGGTCGATGTTTA
A

Table S1. Oligonucleotides.

oligonucleotide	5′-3′sequence	target gene	purpose	fragment size (gDNA) [bp]	fragment size (cDNA) [bp]	Primer efficiency or restriction site
oRS001	CGGTACCAGCATGTTTGTGG	*atmA*	expression analysis (qRT-pCR)	242	119	0.97
oRS002	CCTTCTTGAGCAACTCTTCG		expression analysis (qRT-pCR)			
oRS005	GGCCTACAAGGACAAGCACG	*batA*	expression analysis (qRT-pCR)	315	182	0.93
oRS006	CGAGTAACCACGGCCCTTG		expression analysis (qRT-pCR)			
oRS007	CAGAGTTCACGTTCATTGTCATGG	*batC*	expression analysis (qRT-pCR)	324	193	0.96
oRS008	GCGTATCCCTGCTCCTTGG		expression analysis (qRT-pCR)			
oRS009	GCAGCAGTCAAGGTCGTTGG	*ilvA*	expression analysis (qRT-pCR)	281	194	0.94
oRS010	GTTAGGTTGTGCTCCCGGAG		expression analysis (qRT-pCR)			
oRS011	GGTTTCTCCGATGGCTGATC	*ilvB*	expression analysis (qRT-pCR)	253	142	0.97
oRS012	GAACACGACCATGGGAGTTCC		expression analysis (qRT-pCR)			
oRS013	GAACCATCACCGAGACTCAGC	*ilvC*	expression analysis (qRT-pCR)	296	192	0.96
oRS014	CCTTTCCAGCATCAAGACACTCC		expression analysis (qRT-pCR)			
oRS015	TCGCTCCTTCTCGGTCATGG	*ilvE1*	expression analysis (qRT-pCR)	281	186	0.96
oRS016	GTGTCGTTCTTGAAGGTCTCC		expression analysis (qRT-pCR)			
oRS017	CTCGCTCTCCCTCTTGGCTC	*ilvE2*	expression analysis (qRT-pCR)	286	176	0.97
oRS018	CCTTGAAGGTTTCCTGCAGC		expression analysis (qRT-pCR)			
oRS019	CGAGGTGGCTGATCTGAGTC	*ilvF1*	expression analysis (qRT-pCR)	286	155	0.95
oRS020	GATCAACAGCAACTCGCGC		expression analysis			

			(qRT-pCR)			
oRS021	GGATGACGGTGGTGATGAAGG	*ilvF2*	expression analysis (qRT-pCR)	229	108	0,94
oRS022	CCTTGAACCCAGTGTAGTCCAAC		expression analysis (qRT-pCR)			
oRS023	TTGACCAAGAACGGCCAGC	*leuA2*	expression analysis (qRT-pCR)	141	141	0,94
oRS024	GAACTGTTCACTGCCATCGG		expression analysis (qRT-pCR)			
oRS025	CTTCAAGACTGAGCTCATCGG	*leuB1*	expression analysis (qRT-pCR)	389	139	0,92
oRS026	GTTTCCTGTCGCCCACTTGG		expression analysis (qRT-pCR)			
oRS027	GGTCGCCAAAGTCCGATCC	*leuB2*	expression analysis (qRT-pCR)	402	153	0.93
oRS028	CTCCTACTGCTCCCAAGAGG		expression analysis (qRT-pCR)			
oRS031	GCTGGTTTCCCTGTGGCATCC	*leuA1*	expression analysis (qRT-pCR)	376	135	0.94
oRS032	CGTAGCATCGCTGGATATCTGC		expression analysis (qRT-pCR)			
oRS033	AGATGAGACGCCCTATAGAAAGC	*leuE1*	expression analysis (qRT-pCR)	258	122	0.99
oRS034	GTTCGTTGTAAATTTTGCGGACC		expression analysis (qRT-pCR)			
oJW01	CATCGATCTGGCCTACATGG	*gpdA*	expression analysis (qRT-pCR)	229	80	1.04
oJW02	CCACCTTGCCCTTGTAGC		expression analysis (qRT-pCR)			
oJW03	GTATGTGCAAGGCCGGTTTCG	*actB*	expression analysis (qRT-pCR)	224	100	1.00
oJW04	CCCATACCGACCATCACACC		expression analysis (qRT-pCR)			
oJW175	TACGAGGAGCCAAGAGGTG	*malA*	expression analysis (qRT-pCR)	99	99	0.94
oJW176	GACAAAGAAGCCGTCGTTCC		expression analysis (qRT-pCR)			
oRS037	GTGCCGCGCGGCAGCCATATGATGTTTGTCCAAAGAACACTCCG	*batA-L*	Bat1L production in *E. coli*	1824	1266	*Nde*I
oRS038	GTGCCGCGCGGCAGCCATATGAGCACCTCCATCGCGCCC	*batA-S*	Bat1S production in *E. coli*	1763	1205	*Nde*I
oRS039	CTCGAGTGCGGCCGCAAGCTTTTAGACAACGACGGACCAGC	*batA*	Bat1L/S production in *E. coli*			*Hind*III

oRS049	CGCGGCAGCCATATGGCTAGCATGACCTGCACACTGCCACC	*batC*	BatC production in *E. coli*	1344	1104	*Nhe*I
oRS046	GCTCGAGTGCGGCCGCAAGCTTACGCCTCCTGCCGAGGG	*batC*	BatC production in *E. coli*			*Hind*III
oRS040	CGCGGCAGCCATATGGCTAGCATGGCACAAGCAAATAAAGACAAG	*leuA1*	LeuA1 production in *E. coli*	2126	1775	*Nhe*I
oRS041	CTCGAGTGCGGCCGCAAGCTTTTAAACATCGACCTTGCGTTCAC	*leuA1*	LeuA1 production *in E. coli*			*Hind*III

Table S2. Plasmids.

Plasmid name	vector backbone	expressed gene	position of His6-tag	reference
pRS02	pET28a(+)	*batA-L*	N-terminus	This study.
pRS04	pET28a(+)	*batA-S*	N-terminus	This study.
pRS06	pET28a(+)	*leuA1*	N-terminus	This study.
pRS08	pET28a(+)	*batC*	N-terminus	This study.

Table S3. UHPLC methods.

	method A	method B	method C
purpose	amino and keto acid quantification	amino and keto acid quantification	malpinin quantification
instrument	Agilent 1290 infinity II UHPLC	Agilent 1290 infinity II UHPLC	Agilent 1290 infinity II UHPLC
solvent A	20 mM NH_4CH_3COO, pH 6.8	20 mM NH_4CH_3COO, pH 6.8	water + 0.1% FA
solvent B	acetonitrile	acetonitrile	acetonitrile
gradient	0-13 min: 90-57% B, 13-15 min: 57-70% B, 15-16 min: 30%	0-12.5 min: 90-60% B, 12.5-15 min: 60-40% B, 15-16 min: 40% B	0-4 min: 5-72% B, 4-4.5 min: 72-95% B, 4.5-5 min: 95% B
temperature	45°C	45°C	30°C
flow	0.4 mL min^{-1}	0.4 mL min^{-1}	1 mL min^{-1}
column	SeQuant®ZIC®-HILIC 3.5 µm, 100 Å	SeQuant®ZIC®-HILIC 3.5 µm, 100 Å	Zorbax Eclipse Plus C18 RRHD (Agilent)
column dimension	250 x 2.1 mm, 3.5 µm	150 x 2.1 mm, 3.5 µm	50 mm × 2.1 mm, 1.8 µm
detection	Agilent 6130 single quadrupole MS, ESI ionization source	Agilent 6130 single quadrupole MS, ESI ionization source	Agilent 6130 single quadrupole MS, ESI ionization source

Table S4. Amino acid sequences used for phylogenetic analysis of LeuA1 homologs.

Organism	Protein	Accession number	reference
Anaeromyces robustus		NP_177544.1	
Arabidopsis thaliana	IPMS1	NP_197693.1	[4]
Arabidopsis thaliana	IPMS2	ORX84989.1	[4]
Aspergillus fumigatus	LeuC	EDP56737.1	[5]
Aspergillus nidulans	LeuA	XP_658444.1	[6]
Azotobacter vinelandii		ZP_00088628.1	
Bacillus cereus	LeuA	ZP_03238480.1	
Bacterial endosymbiont of Mortierella elongata FMR23-6		GAM51972.1	
Bacteroides fragilis	aIPMS	KXU51314.1	[7]
Basidiobolus meristosporus		ORX90720.1	
Blyttiomyces helicus		RKO85405.1	
Botrytis cinerea		XP_001553290.1	
Caulobacter vibrioides		WP_010919415.1	
Chytriomyces confervae		TPX76259.1	
Clostridium formicaceticum	aIPMS	AOY76370.1	[7]
Clostridium kluyveri	aIPMS	WP_012102499.1	[7]
Clostridium pasteurianum	aIPMS	KRU14690.1	[7]
Corynebacterium glutamicum		P42455.1	
Cryptococcus neoformans		XP_572334.1	
Cupriavidus necator	IPMS	WP_042884649.1	[8]
Deinococcus radiodurans		AAF11047.1	
Desulfovibrio carbinoliphilus		EHJ49596.1	
Escherichia coli		P09151.5	
Glycine max	IPMS	Glyma.03G005700.1	[9]
Haemophilus influenzae		P43861.1	
Klebsiella pneumoniae		WP_008806146.1	
Laccaria bicolor		XP_001874900.1	
Linderina pennispora		ORX68852.1	
Linnemannia elongata		OAQ34495.1	
Methanocaldococcus jannaschii		WP_010870707.1	
Micromonas pusilla		EEH52949.1	
Moorella thermoacetica	aIPMS	WP_011393738.1	[7]
Mortierella alpina	**LeuA1**	**ADAG01001098**	**This study.**
Mucor circinelloides		EPB85936.1	
Mycetohabitans rhizoxinica 1		WP_041754403.1	
Mycetohabitans rhizoxinica 2		WP_041753704.1	
Mycoavidus cysteinexigens		BBE09271.1	
Mycoavidus sp.		BBO59729.1	
Mycobacterium tuberculosis	MtIPMS	NP_218227.3	[10]
Neisseria meningitidis	aIPMS	WP_050184874.1	[11]
Neocallimastix californiae		ORY17376.1	
Neurospora crassa		XP_964875.1	
Phycomyces blakesleeanus	LeuA1	ADK54799.1	[12]
Physcomitrella patens	LeuA1	Pp3c3_21830V3.1	
Physcomitrella patens	LeuA2	Pp3c4_4420V3.1	
Piromyces finnis		ORX47300.1	
Podila verticillata		KFH70037.1	
Powellomyces hirtus		TPX55294.1	
Pseudomonas aeruginosa		WP_012075129.1	
Pyricularia oryzae	LEU4	XP_003716219.1	[13]
Pyricularia oryzae	LEU9	XP_003720428.1	[13]
Pyrococcus abyssi		WP_010868464.1	
Pyrococcus furiosus		AAL81061.1	
Ralstonia solanacearum		AOE89993.1	
Rhizoclosmatium globosum		ORY51790.1	

Rhizopus delemar		EIE92225.1	
Saccharomyces cerevisiae	LEU4	NP_014295.1	[14]
Saccharomyces cerevisiae	LEU9	NP_014751.1	[15]
Salmonella enterica ser. Typhimurium		P15875.4	
Schizosaccharomyces pombe	Leu3	O59736.2	[12]
Sinorhizobium meliloti		Q9X7L2.3	
Solanum pennellii 1		AAB61599.1	
Solanum pennellii 2		AAB61598.1	
Spinacia oleracea		XP_021839739.1	
Spizellomyces punctatus		XP_016611093.1	
Staphylococcus epidermidis		BBK81946.1	
Streptomyces coelicolor		O31046.2	
Synchytrium endobioticum		TPX34422.1	
Synchytrium microbalum		XP_031024433.1	
Synechococcus sp.		Q7U892.1	
Synechocystis spec.		WP_010873307.1	
Thalassiosira pseudonana		XP_002286323.1	
Thamnocephalis sphaerospora		RKP05169.1	
Thermosynechococcus elongatus		WP_011057237.1	
Trichodesmium erythraeum		Q112U2.1	
Ustilago maydis		KIS67652.1	
Yersinia pestis		NP_404175.1	

Table S5. Statistical analysis on the malpinin quantification in dependence on BCAA supply. Significance values are indicated as: ns, not significant; * p<0.05, ** p<0.01; *** p<0.001 and **** p<0.0001. Groups without significant changes are indicated with the same latter (a-d) in the compact letter display (Figure 3).

Compared groups	Mean Diff.	95.00% CI of diff.	Summary	P Value
AMM 5d vs. AMM 7d	4.382	1.906 to 6.859	***	0.0006
AMM 5d vs. AMM+ile 5d	-1.714	-4.19 to 0.7624	ns	0.2543
AMM 5d vs. AMM+ile 7d	-3.941	-6.417 to -1.464	**	0.0017
AMM 5d vs. AMM+leu 5d	-5.777	-8.254 to -3.301	****	<0.0001
AMM 5d vs. AMM+leu 7d	-2.892	-5.368 to -0.4155	*	0.0192
AMM 5d vs. AMM+val 5d	0.4465	-2.03 to 2.923	ns	0.9947
AMM 5d vs. AMM+val 7d	1.798	-0.4174 to 4.013	ns	0.1398
AMM 5d vs. AMM+ile/leu/val 5d	5.655	3.44 to 7.87	****	<0.0001
AMM 5d vs. AMM+ile/leu/val 7d	7.947	5.732 to 10.16	****	<0.0001
AMM 7d vs. AMM 5d	-4.382	-6.859 to -1.906	***	0.0006
AMM 7d vs. AMM+ile 5d	-6.096	-8.809 to -3.383	****	<0.0001
AMM 7d vs. AMM+ile 7d	-8.323	-11.04 to -5.61	****	<0.0001
AMM 7d vs. AMM+leu 5d	-10.16	-12.87 to -7.447	****	<0.0001
AMM 7d vs. AMM+leu 7d	-7.274	-9.987 to -4.561	****	<0.0001
AMM 7d vs. AMM+val 5d	-3.936	-6.648 to -1.223	**	0.0038
AMM 7d vs. AMM+val 7d	-2.585	-5.061 to -0.1082	*	0.039
AMM 7d vs. AMM+ile/leu/val 5d	1.273	-1.204 to 3.749	ns	0.5425
AMM 7d vs. AMM+ile/leu/val 7d	3.565	1.088 to 6.041	**	0.004
AMM+ile 7d vs. AMM 5d	3.941	1.464 to 6.417	**	0.0017
AMM+ile 7d vs. AMM 7d	8.323	5.61 to 11.04	****	<0.0001
AMM+ile 7d vs. AMM+ile 5d	2.227	-0.486 to 4.94	ns	0.1331
AMM+ile 7d vs. AMM+leu 5d	-1.837	-4.549 to 0.8761	ns	0.2732
AMM+ile 7d vs. AMM+leu 7d	1.049	-1.664 to 3.762	ns	0.7964
AMM+ile 7d vs. AMM+val 5d	4.387	1.675 to 7.1	**	0.0015
AMM+ile 7d vs. AMM+val 7d	5.738	3.262 to 8.215	****	<0.0001
AMM+ile 7d vs. AMM+ile/leu/val 5d	9.596	7.119 to 12.07	****	<0.0001
AMM+ile 7d vs. AMM+ile/leu/val 7d	11.89	9.411 to 14.36	****	<0.0001

Table S6. Gene expression data of *malA* and BCAA biosynthetic genes in *M. alpina*. C_t values of each set of 6 individual replicates are displayed.

gene	AMM 5d						AMM 7d					
gpdA	14.57	13.86	14.16	14.18	14.94	14.26	16.32	14.95	15.19	16.57	14.28	15.52
actB	17.23	16.12	16.77	17.26	17.39	16.86	18.08	17.60	17.60	18.20	17.20	17.41
malA	18.04	18.35	17.10	17.95	18.44	17.09	18.60	18.58	18.27	18.97	18.42	18.44
ilvA	22.02	20.91	22.00	21.64	21.82	22.05	23.12	21.79	22.08	23.27	21.57	22.56
ilvB	24.52	23.11	23.55	25.02	23.63	24.05	25.83	23.82	23.81	25.96	24.00	24.16
ilvC	18.71	18.47	18.21	18.91	18.31	19.01	19.36	20.03	19.73	19.67	19.59	19.41
ilvE1	17.72	18.05	17.33	17.57	18.00	17.43	20.29	18.17	18.76	20.29	18.45	18.59
ilvE2	17.30	17.70	17.37	17.35	16.88	17.34	19.02	18.49	18.14	19.21	18.15	18.18
ilvF1	20.23	19.80	20.41	20.24	19.46	20.29	22.33	20.58	19.82	22.00	20.40	19.75
ilvF6	20.08	21.14	20.24	20.15	20.07	20.06	21.41	21.08	20.38	21.36	20.23	19.48
leuA1	19.05	18.38	18.83	18.83	18.54	18.75	19.84	19.53	19.56	18.93	19.26	19.24
leuA2	16.89	15.84	17.31	17.06	16.46	17.09	18.38	17.02	17.42	18.12	17.99	17.17
leuB1	17.63	18.01	17.87	18.06	16.97	17.47	18.75	18.30	18.44	19.25	18.32	18.07
leuB2	18.12	17.39	17.60	18.24	17.54	17.86	19.25	18.43	18.45	19.14	18.66	18.37
batA	18.69	18.47	18.46	18.67	18.08	18.53	19.65	18.95	19.06	19.40	18.85	19.03
batC	18.34	18.75	18.47	18.26	18.32	18.42	20.14	19.12	19.39	20.15	19.35	19.19

gene	AMM + Ile 5d						AMM + Ile 7d					
gpdA	14.40	15.03	17.69	17.60	14.66	14.66	15.60	15.78	17.24	16.97	16.03	16.07
actB	17.07	17.14	20.04	19.67	17.33	17.68	17.78	18.02	18.63	18.64	17.58	17.64
malA	16.13	15.99	20.01	20.01	16.21	16.29	17.11	17.08	19.19	19.24	17.57	17.57
ilvA	19.76	19.56	22.15	22.05	20.53	20.87	20.23	20.42	21.01	20.88	20.41	20.36
ilvB	23.37	23.65	23.04	23.24	23.02	23.34	23.31	23.52	23.44	23.43	23.13	23.19
ilvC	17.43	16.10	20.40	20.54	18.55	18.38	18.23	18.49	19.91	19.48	18.85	18.76
ilvE1	15.81	15.51	18.18	17.81	16.38	15.66	16.20	16.11	16.99	16.95	16.01	16.05
ilvE2	17.05	17.08	19.05	19.29	17.17	17.04	17.53	17.37	18.44	18.80	17.58	17.49
ilvF1	18.12	18.27	19.71	19.55	18.81	18.60	19.00	18.97	19.71	19.68	19.33	19.18
ilvF6	18.88	19.02	19.07	18.95	19.52	18.81	19.37	19.29	19.78	18.58	19.63	19.50
leuA1	18.07	23.40	20.31	20.04	18.35	18.29	19.14	18.88	19.96	19.64	18.40	18.29
leuA2	15.50	15.39	17.70	17.80	16.31	16.48	16.45	16.33	17.41	17.31	17.07	16.83
leuB1	17.10	17.35	17.73	17.69	17.71	17.52	17.44	17.70	18.05	17.78	17.44	17.55
leuB2	17.19	17.17	17.68	17.80	17.70	17.46	17.67	17.59	18.10	18.08	17.41	17.59
batA	16.71	16.67	19.75	19.73	17.90	17.75	17.66	17.36	18.53	18.46	17.48	17.63
batC	17.03	17.10	20.03	19.84	17.80	18.03	18.25	18.29	18.88	19.14	18.34	18.25

gene	AMM + Leu 5d						AMM + Leu 7d					
gpdA	14.04	15.19	15.96	13.89	15.34	15.76	15.35	14.61	15.53	15.14	14.69	15.22
actB	17.07	18.96	20.07	17.01	18.92	19.65	18.02	17.75	18.37	18.06	17.76	18.26
malA	19.42	18.40	21.54	19.54	18.33	21.59	17.60	17.18	17.17	17.45	17.25	17.27
ilvA	21.07	22.47	23.28	20.85	22.95	23.06	23.07	21.64	22.30	23.12	21.59	22.26
ilvB	25.01	25.71	25.77	24.89	26.04	25.58	26.80	25.39	25.07	27.00	25.25	25.42
ilvC	19.09	19.78	21.22	18.41	20.07	21.17	20.33	19.31	19.96	19.92	19.20	19.98
ilvE1	16.40	18.82	19.14	16.51	17.73	19.26	19.25	17.18	18.00	19.52	17.11	18.12

gene												
ilvE2	17.20	19.09	19.26	16.87	18.93	19.46	18.87	17.84	18.38	19.16	17.67	18.58
ilvF1	19.69	21.26	21.29	19.71	20.88	21.27	21.49	20.87	21.16	21.39	20.51	21.13
ilvF6	20.06	21.07	22.15	20.07	21.44	22.06	21.65	21.32	21.51	20.80	21.19	21.85
leuA1	18.66	20.86	21.95	18.60	20.87	21.75	20.37	19.71	20.76	20.53	19.90	20.58
leuA2	17.24	18.12	18.33	17.22	18.03	18.34	17.97	17.77	18.08	18.29	17.76	18.06
leuB1	17.03	18.51	18.99	17.11	18.54	18.97	19.28	17.97	18.55	19.52	18.08	18.53
leuB2	17.62	19.27	19.49	17.88	19.12	19.57	19.56	18.14	19.09	19.35	18.22	19.04
batA	17.56	18.84	19.39	17.54	18.81	19.31	18.97	17.98	18.62	18.73	18.04	18.56
batC	18.21	18.98	19.52	18.08	18.99	19.33	19.60	18.45	19.00	19.71	18.20	18.72

gene	AMM + Val 5d						AMM + Val 7d					
gpdA	14.48	14.25	15.29	14.11	14.66	15.03	16.20	14.29	14.45	15.80	14.22	14.08
actB	17.32	17.07	17.71	17.38	17.17	17.33	18.29	17.84	17.30	18.35	17.76	17.47
malA	21.64	19.19	19.84	21.27	19.35	19.80	19.62	21.13	19.53	19.95	21.13	19.63
ilvA	20.02	20.02	20.33	19.99	20.20	20.26	21.32	20.82	20.33	21.32	20.91	20.33
ilvB	24.01	23.92	24.30	23.08	23.78	24.30	25.67	24.37	24.13	25.41	24.28	24.17
ilvC	18.89	17.39	18.26	18.83	17.92	18.25	19.35	19.12	18.14	19.53	18.94	18.47
ilvE1	16.41	16.31	16.13	16.62	16.04	16.22	18.36	16.61	16.57	17.98	16.34	16.44
ilvE2	17.55	16.90	17.36	18.32	16.89	17.62	18.94	16.98	17.12	19.21	16.87	17.10
ilvF1	19.14	19.05	19.20	19.15	18.98	19.05	19.98	19.52	19.29	20.13	19.41	19.31
ilvF6	19.17	20.47	20.47	19.29	20.64	20.62	21.01	21.00	20.49	21.07	20.97	20.48
leuA1	17.64	18.57	18.94	17.94	18.70	18.93	19.59	19.37	19.32	19.46	19.26	19.39
leuA2	16.05	16.55	16.82	16.00	16.60	16.81	17.35	17.38	16.51	17.30	17.35	16.56
leuB1	16.07	17.23	17.41	16.06	17.36	17.36	18.37	17.53	17.11	18.36	17.53	17.18
leuB2	17.14	17.39	17.42	17.17	17.35	17.65	18.63	17.37	17.50	18.89	17.53	17.39
batA	18.26	17.60	17.96	18.24	17.56	17.94	18.86	17.86	17.80	19.24	17.93	17.65
batC	17.50	17.34	17.87	17.50	17.56	17.72	18.39	18.27	17.93	18.37	18.21	18.00

gene	AMM + Ile/Leu/Val						AMM + Ile/Leu/Val					
gpdA	14.44	13.77	14.27	14.15	13.19	14.00	14.24	14.63	15.04	14.01	14.68	14.30
actB	17.24	16.25	16.48	17.03	16.33	16.35	17.11	17.09	16.94	16.92	17.18	16.74
malA	21.11	20.96	20.49	21.13	20.70	20.54	22.08	20.19	21.50	21.23	20.26	21.44
ilvA	21.60	21.35	21.48	21.53	21.26	21.37	22.09	22.97	21.65	22.19	22.62	21.90
ilvB	24.12	23.43	24.89	24.28	24.00	23.60	26.84	23.02	23.45	26.48	23.63	23.76
ilvC	19.22	18.73	19.14	19.17	18.91	18.89	19.55	20.09	20.06	19.40	20.21	20.14
ilvE1	17.69	17.17	17.18	17.52	17.08	17.04	18.06	19.42	18.04	18.08	19.42	18.04
ilvE2	17.05	16.40	17.00	17.28	16.64	17.24	16.78	17.96	17.06	17.08	17.95	17.40
ilvF1	21.30	21.08	20.91	21.22	21.19	21.22	16.12	18.13	19.71	13.90	17.90	19.77
ilvF6	21.00	21.03	20.34	21.14	21.10	20.44	21.36	20.71	21.54	22.70	20.70	21.94
leuA1	19.77	19.36	19.28	19.71	19.45	19.20	20.66	20.44	20.52	20.42	20.46	20.51
leuA2	18.46	17.99	18.08	18.53	17.93	18.02	18.70	18.85	17.86	18.97	18.50	17.57
leuB1	18.16	17.58	17.75	18.23	17.69	17.86	18.25	19.04	17.43	18.20	19.12	17.86
leuB2	18.19	17.96	17.97	18.32	18.01	17.67	18.27	18.91	18.66	18.29	18.87	18.23
batA	17.38	16.85	17.07	17.47	17.06	17.21	17.36	17.95	16.92	17.35	17.86	17.80
batC	18.83	18.17	18.62	18.78	18.25	18.35	18.87	19.95	18.29	18.79	19.71	18.89

Table S7. Statistical analysis of gene expression data according to One-Way-ANOVA tests.
Significance values are indicated as: ns, not significant; * p<0.05, ** p<0.01; *** p<0.001 and ****
p<0.0001.

	Mean Diff.	95.00% CI of diff.	Summary	P Value
malA				
5d vs. 7d	-0.258	-1.301 to 0.7846	ns	0.9916
5d vs. 5d	-1.316	-2.359 to -0.2739	**	0.0057
5d vs. 7d	-0.8179	-1.861 to 0.2246	ns	0.1998
5d vs. 5d	1.899	0.8568 to 2.942	****	<0.0001
5d vs. 7d	1.532	0.4897 to 2.575	***	0.0007
5d vs. 5d	0.7001	-0.3425 to 1.743	ns	0.3557
5d vs. 7d	-1.417	-2.46 to -0.3744	**	0.0022
5d vs. 5d	3.204	2.162 to 4.247	****	<0.0001
5d vs. 7d	3.034	1.991 to 4.076	****	<0.0001
ilvA				
5d vs. 7d	-0.3107	-1.353 to 0.7319	ns	0.9731
5d vs. 5d	-1.828	-2.871 to -0.7859	****	<0.0001
5d vs. 7d	-2.087	-3.13 to -1.045	****	<0.0001
5d vs. 5d	-0.6658	-1.708 to 0.3767	ns	0.4124
5d vs. 7d	-0.3605	-1.403 to 0.6821	ns	0.9368
5d vs. 5d	-1.893	-2.936 to -0.8505	****	<0.0001
5d vs. 7d	-1.571	-2.614 to -0.5288	***	0.0005
5d vs. 5d	0.04481	-0.9978 to 1.087	ns	0.9999
5d vs. 7d	0.3719	-0.6707 to 1.414	ns	0.9254
ilvB				
5d vs. 7d	-0.3166	-1.359 to 0.726	ns	0.9698
5d vs. 5d	-1.664	-2.707 to -0.6215	***	0.0002
5d vs. 7d	-1.605	-2.648 to -0.5627	***	0.0003
5d vs. 5d	0.3644	-0.6782 to 1.407	ns	0.933
5d vs. 7d	0.9477	-0.09492 to 1.99	ns	0.0938
5d vs. 5d	-0.4361	-1.479 to 0.6065	ns	0.841
5d vs. 7d	0.001112	-1.041 to 1.044	ns	>0.9999
5d vs. 5d	0.416	-0.6266 to 1.459	ns	0.8709
5d vs. 7d	0.4522	-0.5904 to 1.495	ns	0.8149
ilvC				
5d vs. 7d	0.0578	-0.9848 to 1.1	ns	0.9998
5d vs. 5d	-0.98	-2.023 to 0.0626	ns	0.0763
5d vs. 7d	-0.6049	-1.647 to 0.4377	ns	0.5237
5d vs. 5d	0.1273	-0.9153 to 1.17	ns	0.9995
5d vs. 7d	0.2165	-0.8261 to 1.259	ns	0.9966
5d vs. 5d	-0.6856	-1.728 to 0.3569	ns	0.3792
5d vs. 7d	-0.3907	-1.433 to 0.6519	ns	0.9042
5d vs. 5d	0.7381	-0.3044 to 1.781	ns	0.2986
5d vs. 7d	1.161	0.1189 to 2.204	*	0.0207

ilvE1				
5d vs. 7d	0.4549	-0.5876 to 1.498	ns	0.8101
5d vs. 5d	-2.06	-3.103 to -1.017	****	<0.0001
5d vs. 7d	-2.231	-3.274 to -1.189	****	<0.0001
5d vs. 5d	-0.8955	-1.938 to 0.1471	ns	0.1291
5d vs. 7d	-0.4194	-1.462 to 0.6231	ns	0.8659
5d vs. 5d	-1.734	-2.777 to -0.6919	****	<0.0001
5d vs. 7d	-1.329	-2.372 to -0.2864	**	0.0051
5d vs. 5d	-0.05648	-1.099 to 0.9861	ns	0.9998
5d vs. 7d	0.716	-0.3266 to 1.759	ns	0.3311
ilvE2				
5d vs. 7d	0.2383	-0.8043 to 1.281	ns	0.9935
5d vs. 5d	-0.497	-1.54 to 0.5455	ns	0.7341
5d vs. 7d	-0.4108	-1.453 to 0.6318	ns	0.8782
5d vs. 5d	-0.06724	-1.11 to 0.9753	ns	0.9997
5d vs. 7d	0.1393	-0.9033 to 1.182	ns	0.9995
5d vs. 5d	-0.2342	-1.277 to 0.8084	ns	0.994
5d vs. 7d	-0.3299	-1.373 to 0.7126	ns	0.9615
5d vs. 5d	-0.03858	-1.081 to 1.004	ns	0.9999
5d vs. 7d	-0.05913	-1.102 to 0.9835	ns	0.9998
ilvF1				
5d vs. 7d	-0.2281	-1.271 to 0.8145	ns	0.9963
5d vs. 5d	-2.126	-3.168 to -1.083	****	<0.0001
5d vs. 7d	-1.674	-2.717 to -0.6315	***	0.0002
5d vs. 5d	-0.5957	-1.638 to 0.4469	ns	0.5413
5d vs. 7d	0.05393	-0.9886 to 1.097	ns	0.9998
5d vs. 5d	-1.29	-2.332 to -0.2471	**	0.0072
5d vs. 7d	-1.149	-2.192 to -0.1067	*	0.0228
5d vs. 5d	1.384	0.341 to 2.426	**	0.0031
5d vs. 7d	-1.256	-2.422 to -0.09061	*	0.0277
ilvF2				
5d vs. 7d	-0.5958	-1.638 to 0.4468	ns	0.541
5d vs. 5d	-2.129	-3.171 to -1.086	****	<0.0001
5d vs. 7d	-1.829	-2.871 to -0.7861	****	<0.0001
5d vs. 5d	-0.379	-1.422 to 0.6636	ns	0.9178
5d vs. 7d	0.1122	-0.9304 to 1.155	ns	0.9996
5d vs. 5d	-0.5194	-1.562 to 0.5232	ns	0.6911
5d vs. 7d	-0.1846	-1.227 to 0.8579	ns	0.9979
5d vs. 5d	0.8662	-0.1764 to 1.909	ns	0.153
5d vs. 7d	1.032	-0.0109 to 2.074	ns	0.0539
leuA1				
5d vs. 7d	-0.3108	-1.353 to 0.7318	ns	0.973
5d vs. 5d	0.02203	-1.021 to 1.065	ns	>0.9999

5d vs. 7d	-0.6366	-1.679 to 0.406	ns	0.4644
5d vs. 5d	0.4566	-0.586 to 1.499	ns	0.8073
5d vs. 7d	0.5774	-0.4652 to 1.62	ns	0.5769
5d vs. 5d	-0.6159	-1.658 to 0.4267	ns	0.5027
5d vs. 7d	-0.06528	-1.108 to 0.9773	ns	0.9998
5d vs. 5d	1.04	-0.002379 to 2.083	ns	0.0509
5d vs. 7d	1.586	0.5438 to 2.629	***	0.0004
leuA2				
5d vs. 7d	-0.07575	-1.118 to 0.9668	ns	0.9997
5d vs. 5d	-1.18	-2.223 to -0.1376	*	0.0179
5d vs. 7d	-0.8249	-1.867 to 0.2177	ns	0.1924
5d vs. 5d	-0.1315	-1.174 to 0.9111	ns	0.9995
5d vs. 7d	0.2293	-0.8133 to 1.272	ns	0.9963
5d vs. 5d	-0.6408	-1.683 to 0.4018	ns	0.4566
5d vs. 7d	-0.4156	-1.458 to 0.6269	ns	0.8714
5d vs. 5d	1.674	0.6315 to 2.717	***	0.0002
5d vs. 7d	1.455	0.4125 to 2.498	**	0.0015
leuB1				
5d vs. 7d	-0.141	-1.184 to 0.9016	ns	0.9994
5d vs. 5d	-1.088	-2.13 to -0.0451	*	0.0362
5d vs. 7d	-0.953	-1.996 to 0.08963	ns	0.0908
5d vs. 5d	-0.694	-1.737 to 0.3486	ns	0.3655
5d vs. 7d	-0.0009555	-1.044 to 1.042	ns	>0.9999
5d vs. 5d	-1.058	-2.101 to -0.01564	*	0.0448
5d vs. 7d	-0.6899	-1.732 to 0.3527	ns	0.3721
5d vs. 5d	0.5375	-0.5051 to 1.58	ns	0.6557
5d vs. 7d	0.5035	-0.5391 to 1.546	ns	0.7219
leuB2				
5d vs. 7d	-0.0686	-1.111 to 0.974	ns	0.9997
5d vs. 5d	-1.222	-2.265 to -0.1796	*	0.0127
5d vs. 7d	-0.9943	-2.037 to 0.04833	ns	0.0695
5d vs. 5d	-0.2109	-1.254 to 0.8317	ns	0.9968
5d vs. 7d	0.1194	-0.9231 to 1.162	ns	0.9996
5d vs. 5d	-0.7678	-1.81 to 0.2748	ns	0.2586
5d vs. 7d	-0.6168	-1.659 to 0.4258	ns	0.501
5d vs. 5d	0.5572	-0.4854 to 1.6	ns	0.6169
5d vs. 7d	0.5996	-0.443 to 1.642	ns	0.5337
batA				
5d vs. 7d	-0.3043	-1.347 to 0.7383	ns	0.9764
5d vs. 5d	-1.32	-2.362 to -0.2773	**	0.0055
5d vs. 7d	-1.541	-2.584 to -0.4988	***	0.0007
5d vs. 5d	-0.8744	-1.917 to 0.1682	ns	0.1461
5d vs. 7d	-0.9486	-1.991 to 0.09397	ns	0.0933
5d vs. 5d	-1.096	-2.139 to -0.05382	*	0.034

5d vs. 7d	-0.9271	-1.97 to 0.1155	ns	0.1066
5d vs. 5d	-0.8974	-1.94 to 0.1452	ns	0.1276
5d vs. 7d	-1	-2.043 to 0.04263	ns	0.0669
batC				
5d vs. 7d	0.1448	-0.8977 to 1.187	ns	0.9994
5d vs. 5d	-1.06	-2.103 to -0.01738	*	0.0442
5d vs. 7d	-0.8489	-1.892 to 0.1936	ns	0.1686
5d vs. 5d	-1.165	-2.208 to -0.1229	*	0.0201
5d vs. 7d	-0.9252	-1.968 to 0.1174	ns	0.108
5d vs. 5d	-0.7768	-1.819 to 0.2658	ns	0.2472
5d vs. 7d	-0.429	-1.472 to 0.6136	ns	0.8519
5d vs. 5d	0.4131	-0.6295 to 1.456	ns	0.875
5d vs. 7d	0.5249	-0.5177 to 1.567	ns	0.6804

Table S8. Amino acid sequences used for phylogenetic analysis of BCAA aminotransferase homologs.

Organism	Protein	Accession number	reference
Aspergillus nidulans	BatA	CBF77764.1	[16]
Arabidopsis thaliana	BCAT1	BAH19488.1	[17]
Arabidopsis thaliana	BCAT2L	AEE28538.1	[17]
Arabidopsis thaliana	BCAT2S	AEE28539.1	[17]
Arabidopsis thaliana	BCAT3	Q9M401.1	[17]
Arabidopsis thaliana	BCAT4	Q9LE06.1	[17]
Arabidopsis thaliana	BCAT5	Q9FYA6.1	[17]
Bacillus subtilis	IlvE	sp|O31461	[18]
Bacillus subtilis	IlvE2/IlvK	sp|P39576	
Cryptococcus neoformans	BatA	XP_771917.1	[19]
Cryptococcus neoformans var. grubii	BatA	OXB33852.1	[19]
Chlamydomonas reinhardtii	BCA1	Cre02.g081400.t1.2	[20]
Chlamydomonas reinhardtii	BCA2	Cre05.g245900.t1.2	[20]
Chlamydomonas reinhardtii	BCA3	Cre10.g458050.t1.2	[20]
Chlamydomonas reinhardtii	BCA4	Cre13.g576400.t1.2	[20]
Escherichia coli	IlvE	sp|P0AB80	[21]
Fusarium graminearum	BatA	XP_011328206.1	
Glycine max	BCAT1	Glyma.06G050100.1	[22]
Glycine max	BCAT2	Glyma.04G049200.1	
Glycine max	BCAT3	Glyma.08G063000.1	
Haplosporangium gracile	BatA	KAF8942323.1	
human	mBCAT1	sp|P54687	[23]
Linnemannia elongata	BatA	KAF9288437.1	
Methanococcus aeolicus	IlvE	WP_011973904.1	
Mortierella alpina	BatA	**ADAG01001070**	**This study.**
Mortierella alpina	BatC	**ADAG01001070**	**This study**.
Mycobacterium tuberculosis	Bat1	PLV46681.1	
Mycobacterium tuberculosis	Bat2	CNG44365.1	
Pseudomonas aeruginosa	IlvE	sp|O86428|	
Phycomyces blakesleeanus	BatA	XP_018292219.1	
Pyricularia (syn. *Magnaporthe*) *oryzae*	BAT1	XP_003709286.1	[13]
Pyricularia (syn. *Magnaporthe*) *oryzae*	BAT3	XP_003708897.1	[13]
Physcomitrella patens	IlvE2	Pp3c3_15010V3.1	
Physcomitrella patens	IlvE3	Pp3c10_12890V3.1	
Populus trichocarpa	BCAT2	Potri.002G113600.1	
Populus trichocarpa	BCAT4	Potri.009G082600.1	
Podila verticillata	BatA	KAF9384042.1	
Streptomyces albus	Bat1	WP_032780549.1	
Saccharomyces cerevisiae	BAT1	NP_012078.3	[24]
Saccharomyces cerevisiae	BAT2	NP_012682.1	[24]
Solanum lycopersicum	BCAT1	XP_010314598.1	[25]
Solanum lycopersicum	BCAT2	XP_010323427.1	[25]
Solanum lycopersicum	BCAT3	XP_004231952.1	[25]
Solanum lycopersicum	BCAT4	XP_004234645.1	[25]
Solanum lycopersicum	BCAT5	XP_004253486.3	[25]
Solanum lycopersicum	BCAT6	XP_019069001.1	[25]
Trifolium pratense	BCAT1	Tp57577_TGAC_v2_mRNA23101	
Trifolium pratense	BCAT2	Tp57577_TGAC_v2_mRNA2817	
Trifolium pratense	BCAT3	Tp57577_TGAC_v2_mRNA31240	
Trifolium pratense	BCAT4	Tp57577_TGAC_v2_mRNA3185	
Volvox carteri	BCAT	Vocar.0026s0064.1	
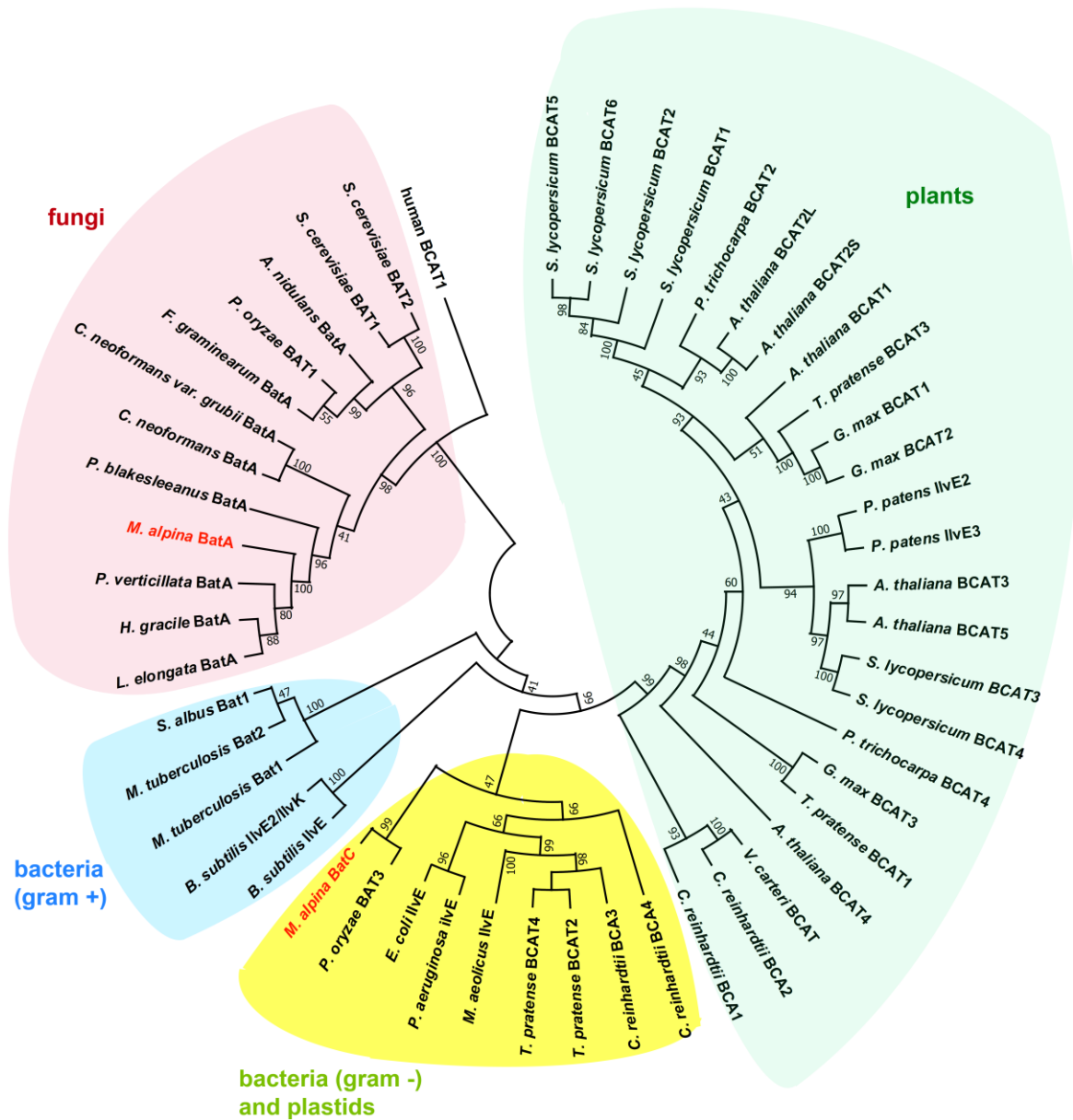
Figure S1. Phylogenetic analysis of BCAA aminotransferases (BATs). The evolutionary history was inferred by using the Maximum Likelihood method and Le_Gascuel_2008 model [1]. The bootstrap consensus tree inferred from 1000 replicates [2]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.7340)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.91% sites). Evolutionary analyses were conducted in MEGA11 [3]. This analysis involved 51 amino acid sequences (Table S8). The human BAT served as outgroup.
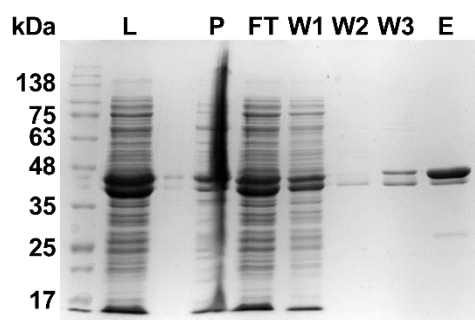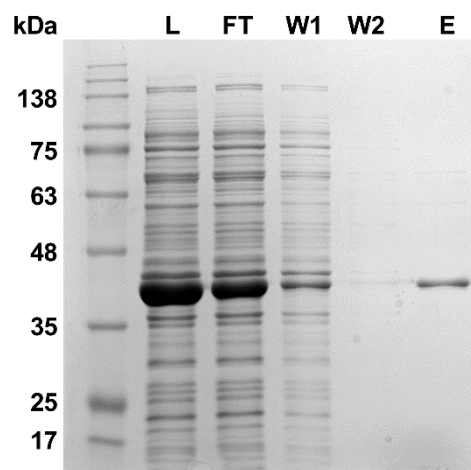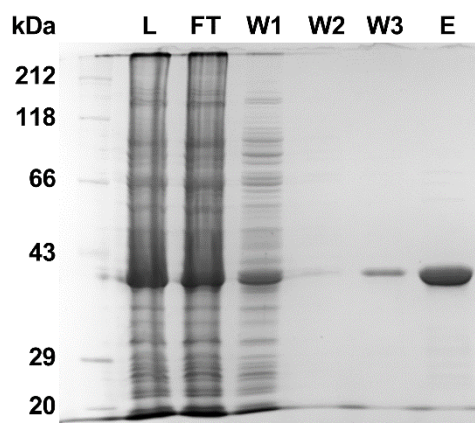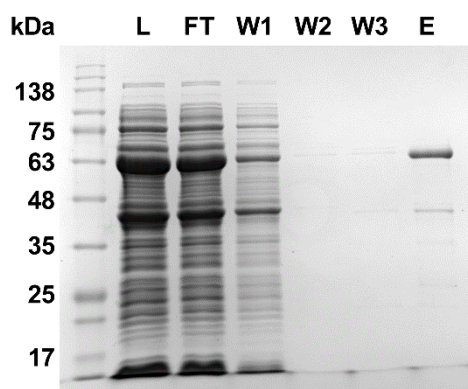
Figure S2. SDS polyacrylamide gel electrophoresis (SDS-PAGE) of His$_6$-tagged *Mortierella alpina* enzymes. (**A**) BatA-L (calculated protein mass 45.2 kDa), (**B**) BatA-S (41.6 kDa), (**C**) BatC (39.9 kDa) and (**D**) LeuA1 (60.1 kDa) are shown. The N-terminal His$_6$-tag adds approximately 2.0 kDa. Abbreviations: L, *E. coli* cell lysate; P, *E. coli* cell pellet, FT, flowthrough after Ni^{2+}-NTA binding; W1-3, wash steps with 10, 25, and 50 mM imidazole; E, elution with 250 mM imidazole. Note, that BatA-L was instable, resulting in main protein band at 45 kDa and two minor degraded proteins at ~40 kDa and ~28 kDa.
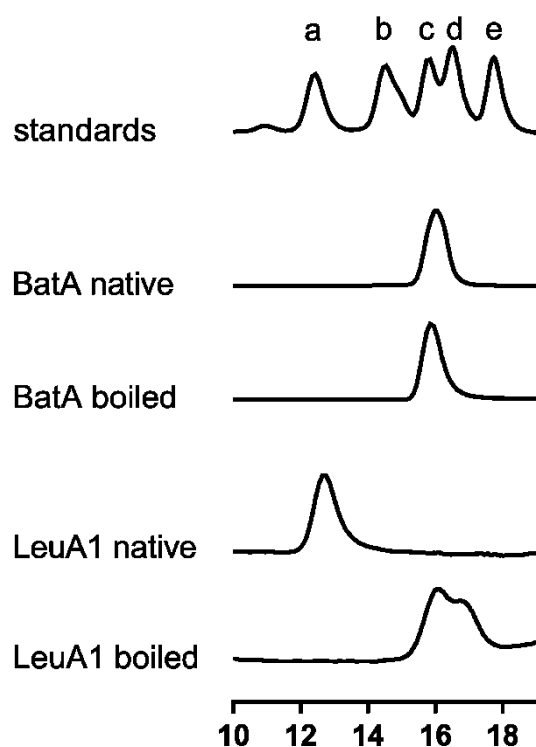
Figure S3. Size exclusion chromatography (SEC) of His₆-tagged proteins. BatA-S and LeuA1 were chromatographed on a Superdex 200 increase 10/300 GL column using an isocratic gradient. (SEC was performed with native and heat-denatured (95°C, 5 min) proteins. Native and denatured His₆-tagged BatA elutes at the same retention volume of 16.3 mL corresponding to a size of 50.1 kDa (calculated mass: 43.6 kDa), suggesting a monomeric enzyme. In contrast, native His₆-tagged LeuA1 forms a homohexamer in solution and elutes at 12.5 mL corresponding to 399.8 kDa (calc. 375 kDa), while its denatured monomer elutes at a retention volume of 16 mL with a corresponding mass of 62.9 kDa (calc. 62.5 kDa). Proteins of the GE Healthcare low and high molecular weight standard (upper lane) served as reference (a: ferritin, 440 kDa; b: aldolase, 158 kDa; c: conalbumin 75 kDa; d: ovalbumin, 43 kDa, e: carbonic anhydrase, 13.7 kDa).
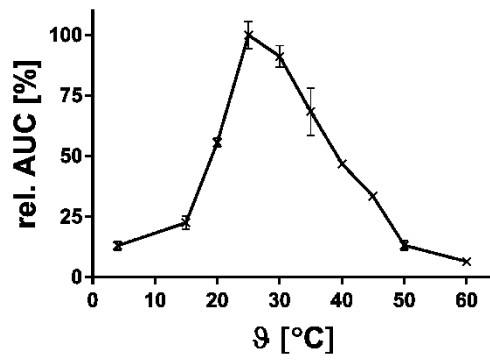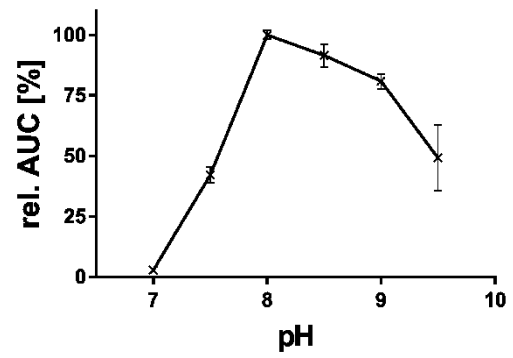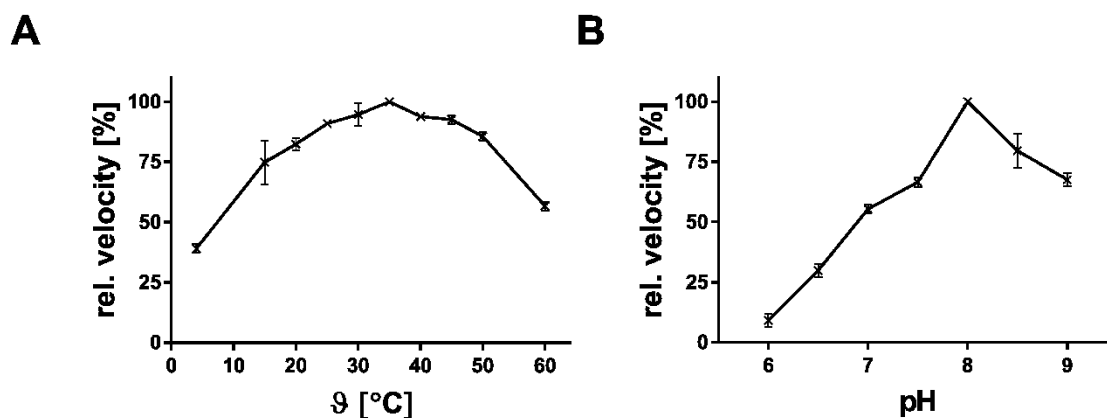
Figure S4. Impact of temperature and pH on BatA activity. Temperature (**A**) and pH dependence (**B**) of BatA. Error bars indicate the standard errors of the mean of three independent measurements.
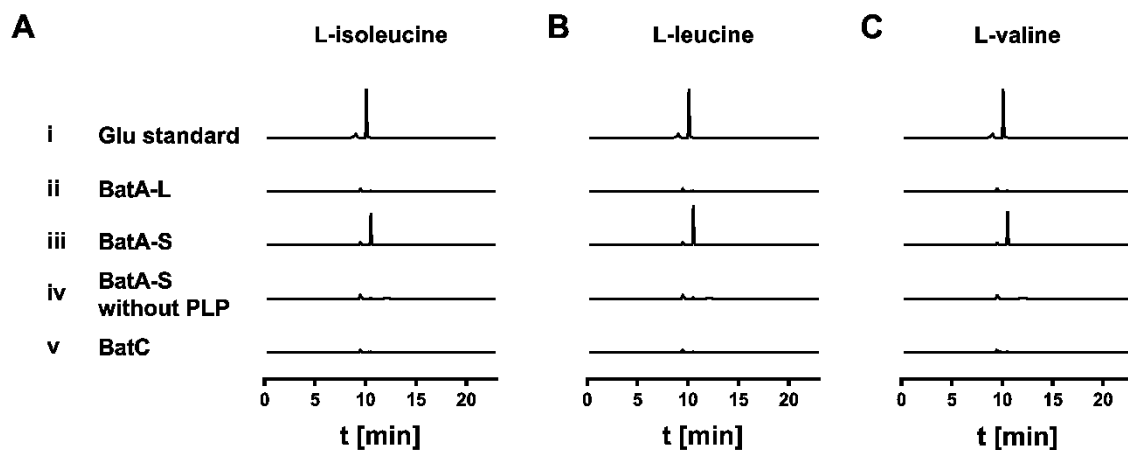
Figure S5. Activity of Bat-L, BatA-S, and BatC. Three different BCAAs (Ile (**A**), Leu (**B**), Val (**C**)) were used as substrates at a final concentration of 5 mM and the glutamate formation was monitored by UHPLC-MS. The extracted ion chromatogram (EIC) for the product glutamate (m/z 148.13 [M+H]$^+$) is shown in each trace. A commercial glutamate standard (trace i) served as reference. The activity was determined for BatA-L (trace ii), BatA-S (iii), Bat-S without additional pyridoxalphosphate (PLP) (iv) and BatC (v). Note that solely BatA-S converted all three BCAAs to glutamate in a PLP-dependent manner.

Figure S6. Impact of temperature and pH on LeuA1 activity. Temperature (**A**) and pH dependence (**B**) of LeuA1. Error bars indicate the standard errors of the mean of three independent measurements.
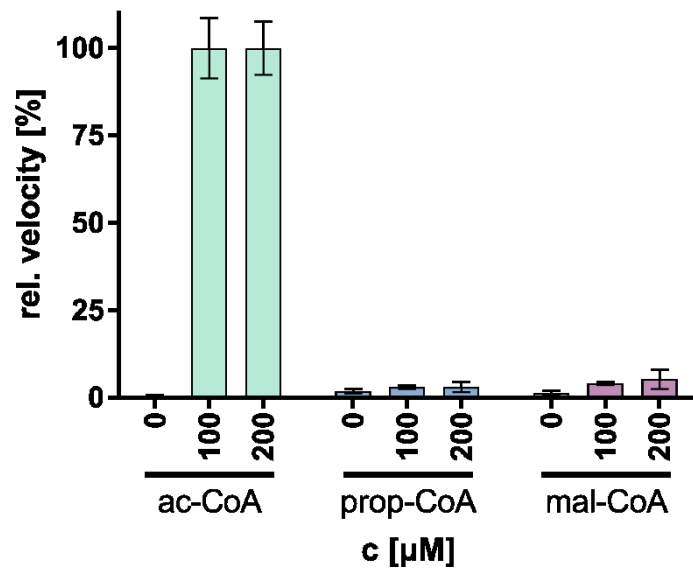
Figure S7. Acyl-CoA dependence of LeuA1 activity. LeuA1 was incubated with its native substrate KIV and the potential co-substrates Ac-CoA, Prop-CoA and Mal-CoA at concentrations of 0, 100, and 200 μM. Error bars indicate the standard errors of the mean of three independent measurements.
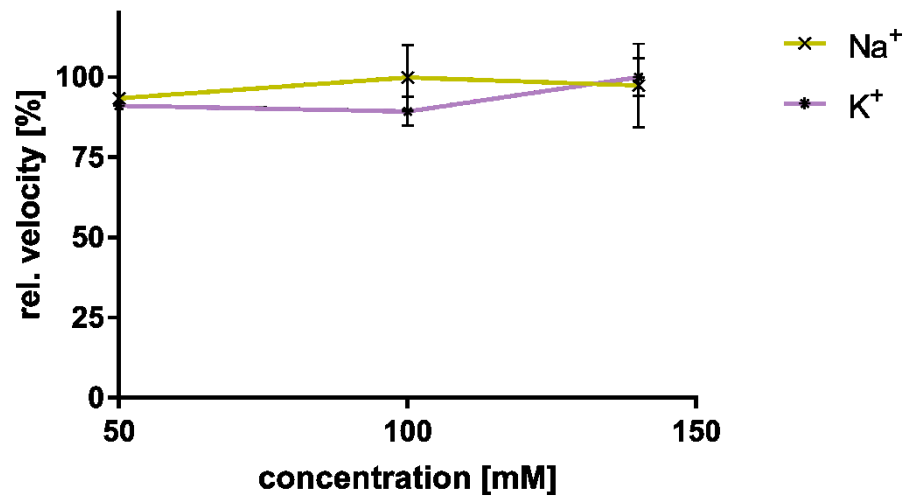
Figure S8. Sodium and potassium dependence of LeuA1 activity. LeuA1 was incubated with its native substrate KIV and Ac-CoA in presence of 50, 100, or 140 mM Na$^+$ or K$^+$. Enzyme activity is not affected. Error bars indicate the standard errors of the mean of three independent measurements.
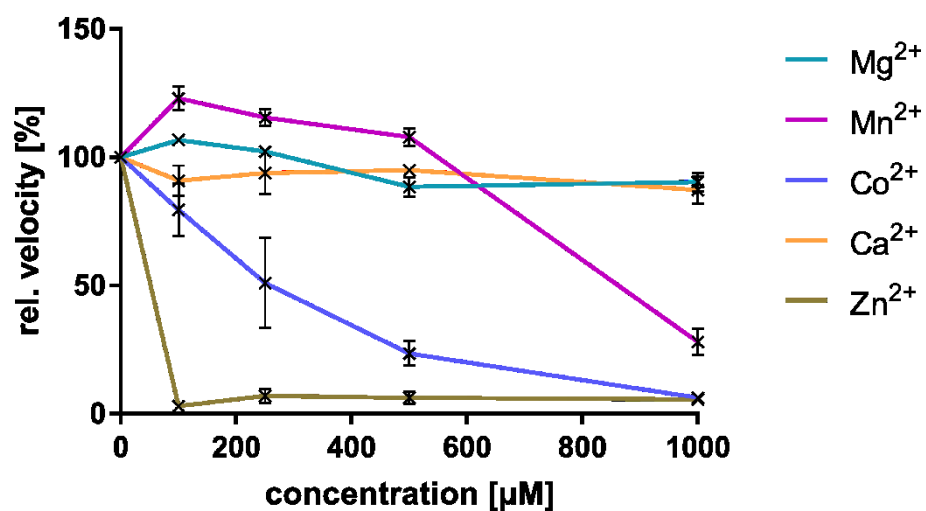
Figure S9. Impact of divalent cations on LeuA1 activity. LeuA1 was incubated in presence of 0, 100, 250, 500 and 1000 µM divalent ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}). Error bars indicate the standard errors of the mean of three independent measurements.
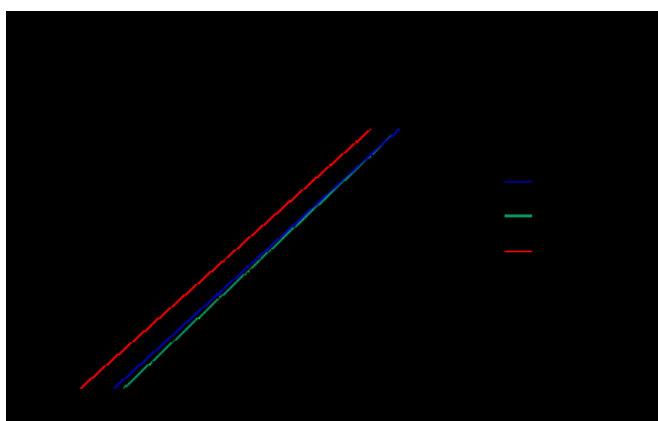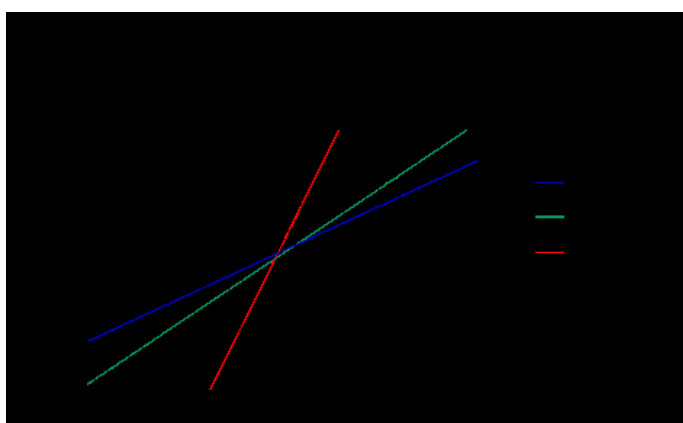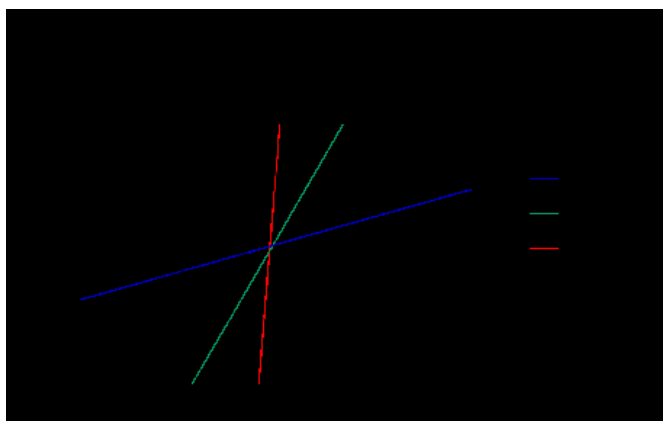
Figure S10. Regulation of LeuA1 by primary metabolites. Reactions were carried out using KIV and Ac-CoA as substrates. The Dixon plots are shown for reactions in the presence of (**A**) Leu (at 100 and 1000 μM), (**B**) KIC (at 100 μM and 1000 μM) and (**C**) Prop-CoA (at 10 and 100 μM). An untreated control was carried out in parallel (0 μM). Error bars indicate the standard errors of the mean of three independent measurements. Note, that Leu, KIC and prop-CoA are non-competitive (v_{max} decreased, K_M constant), competitive (v_{max} constant, K_M increased) and mixed-type inhibitors (v_{max} decreased, K_M decreased), respectively.

References

1. Le, S.Q.; Gascuel, O. An improved general amino acid replacement matrix. *Mol. Biol. Evol.* **2008**, *25*, 1307-1320.

2. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783-791.

3. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022-3027.

4. de Kraker, J.W.; Luck, K.; Textor, S.; Tokuhisa, J.G.; Gershenzon, J. Two *Arabidopsis* genes (IPMS1 and IMPS2) encode isopropylmalate synthase, the branchpoint step in the biosynthesis of leucine. *Plant Physiol.* **2007**, *143*, 970-986.

5. Orasch, T.; Dietl, A.M.; Shadkchan, Y.; Binder, U.; Bauer, I.; Lass-Flörl, C.; Osherov, N.; Haas, H. The leucine biosynthetic pathway is crucial for adaptation to iron starvation and virulence in *Aspergillus fumigatus*. *Virulence* **2019**, *10*, 925-934.

6. Bartnik, E.; Bonnet-Bidaud, A.; Kowalska, I.; Pieniazek, N.J. New leucine auxotrophs of *Aspergillus nidulans*. *Acta Microbiol. Pol.* **1980**, *29*, 29-33.

7. Wiegel, J. Alpha-isopropylmalate synthase as a marker for the leucine biosynthetic pathway in several Clostridia and in *Bacteroides fragilis*. *Arch. Microbiol.* **1981**, *130*, 385-390.

8. Wiegel, J.; Schlegel, H.G. Alpha-isopropylmalate synthase from *Alcaligenes eutrophus* H 16 I. Purification and general properties. *Arch. Microbiol.* **1977**, *112*, 239-246.

9. Nouwen, N.; Arrighi, J.F.; Cartieaux, F.; Chaintreuil, C.; Gully, D.; Klopp, C.; Giraud, E. The role of rhizobial (NifV) and plant (Fen1) homocitrate synthases in *Aeschynomene*/photosynthetic *Bradyrhizobium* symbiosis. *Sci. Rep.* **2017**, *7*, e448.

10. Koon, N.; Squire, C.J.; Baker, E.N. Crystal structure of leua from *Mycobacterium tuberculosis*, a key enzyme in leucine biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 8295-8300.

11. Huisman, F.H.; Hunter, M.F.; Devenish, S.R.; Gerrard, J.A.; Parker, E.J. The C-terminal regulatory domain is required for catalysis by *Neisseria meningitidis* alpha-isopropylmalate synthase. *Biochem. Biophys. Res. Commun.* **2010**, *393*, 168-173.

12. Larson, E.M.; Idnurm, A. Two origins for the gene encoding alpha-isopropylmalate synthase in fungi. *PLoS One* **2010**, *5*, e11605.

13. Que, Y.; Yue, X.; Yang, N.; Xu, Z.; Tang, S.; Wang, C.; Lv, W.; Xu, L.; Talbot, N.J.; Wang, Z. Leucine biosynthesis is required for infection-related morphogenesis and pathogenicity in the rice blast fungus *Magnaporthe oryzae*. *Curr. Genet.* **2020**, *66*, 155-171.

14. Beltzer, J.P.; Morris, S.R.; Kohlhaw, G.B. Yeast Leu4 encodes mitochondrial and nonmitochondrial forms of alpha-isopropylmalate synthase. *J. Biol. Chem.* **1988**, *263*, 368-374.

15. Casalone, E.; Barberio, C.; Cavalieri, D.; Polsinelli, M. Identification by functional analysis of the gene encoding alpha-isopropylmalate synthase II (Leu9) in *Saccharomyces cerevisiae*. *Yeast* **2000**, *16*, 539-545.

16. Steyer, J.T.; Downes, D.J.; Hunter, C.C.; Migeon, P.A.; Todd, R.B. Duplication and functional divergence of branched-chain amino acid biosynthesis genes in *Aspergillus nidulans*. *mBio* **2021**, *12*, e0076821.

17. Diebold, R.; Schuster, J.; Daschner, K.; Binder, S. The branched-chain amino acid transaminase gene family in *Arabidopsis* encodes plastid and mitochondrial proteins. *Plant Physiol.* **2002**, *129*, 540-550.

18. Berger, B.J.; English, S.; Chan, G.; Knodel, M.H. Methionine regeneration and aminotransferases in *Bacillus subtilis, Bacillus cereus*, and *Bacillus anthracis*. *J. Bacteriol.* **2003**, *185*, 2418-2431.

19. Chun, C.D.; Brown, J.C.S.; Madhani, H.D. A major role for capsule-independent phagocytosis-inhibitory mechanisms in mammalian infection by *Cryptococcus neoformans*. *Cell Host Microbe* **2011**, *9*, 243-251.

20. Merchant, S.S.; Prochnik, S.E.; Vallon, O.; Harris, E.H.; Karpowicz, S.J.; Witman, G.B.; Terry, A.; Salamov, A.; Fritz-Laylin, L.K.; Marechal-Drouard, L., *et al.* The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science* **2007**, *318*, 245-250.

21. Somers, J.M.; Amzallag, A.; Middleton, R.B. Genetic fine structure of the leucine operon of *Escherichia coli* k-12. *J. Bacteriol.* **1973**, *113*, 1268-1272.

22. Khoei, M.A.; Karimi, M.; Karamian, R.; Amini, S.; Soorni, A. Identification of the complex interplay between nematode-related lncRNAs and their target genes in *Glycine max* l. *Front Plant Sci* **2021**, *12*, e779597.

23. Yennawar, N.; Dunbar, J.; Conway, M.; Hutson, S.; Farber, G. The structure of human mitochondrial branched-chain aminotransferase. *Acta Crystallogr. D Biol. Crystallogr.* **2001**, *57*, 506-515.

24. Colon, M.; Hernandez, F.; Lopez, K.; Quezada, H.; Gonzalez, J.; Lopez, G.; Aranda, C.; Gonzalez, A. *Saccharomyces cerevisiae* Bat1 and Bat2 aminotransferases have functionally diverged from the ancestral-like *Kluyveromyces lactis* orthologous enzyme. *PLoS One* **2011**, *6*, e16099.

25. Maloney, G.S.; Kochevenko, A.; Tieman, D.M.; Tohge, T.; Krieger, U.; Zamir, D.; Taylor, M.G.; Fernie, A.R.; Klee, H.J. Characterization of the branched-chain amino acid aminotransferase enzyme family in tomato. *Plant Physiol.* **2010**, *153*, 925-936.