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Supplemental information

Activating a dormant metabolic pathway for high-temperature L-alanine production in *Bacillus licheniformis*

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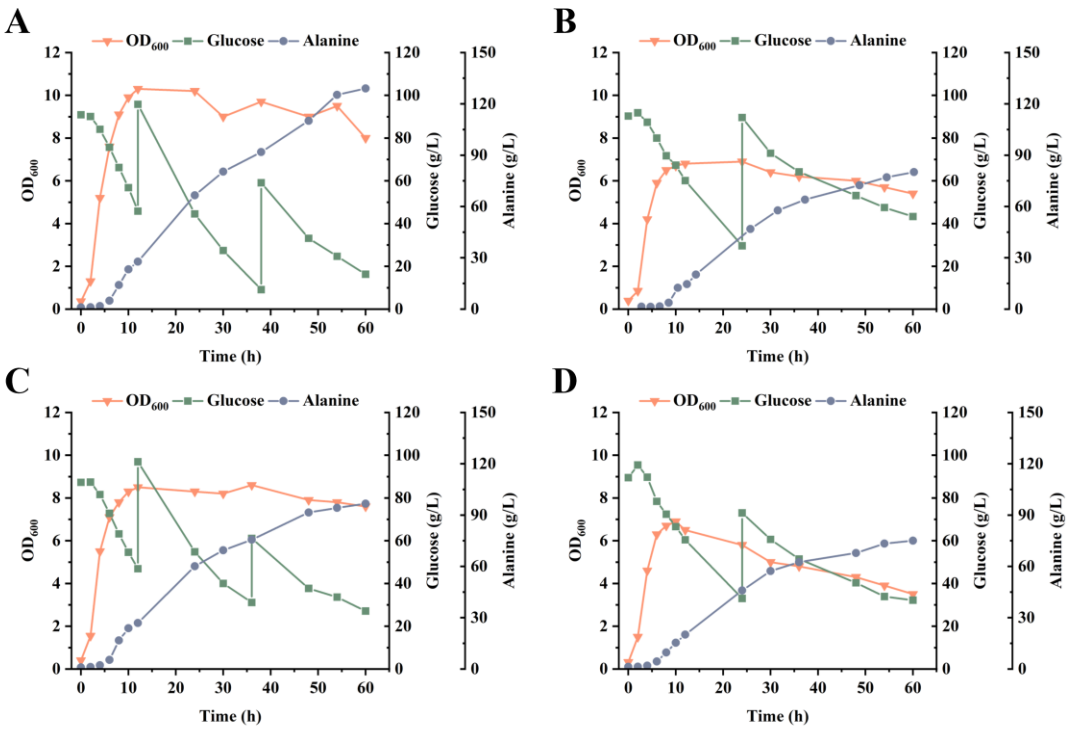
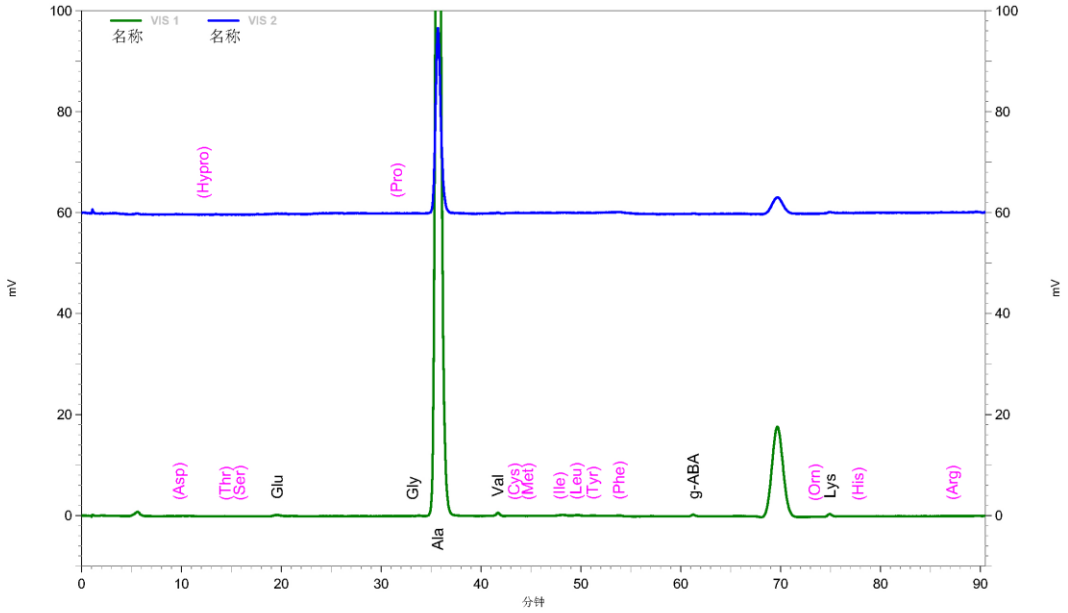


Figure S1. Fed-batch fermentations of *Bacillus licheniformis* overexpressing alanine dehydrogenases from different sources, related to Figure 1. (A–D) Fed-batch fermentation of *B. licheniformis* BLA1-*ald1*, BLA1-*ald2*, BLA1-*STald*, and BLA1-*BSald*.



VIS 1 结果

Peak #	Retention Time	Name	Area	Resolution	Mol concentration (nmol/mL)	CalcMol (ng/mL)
		Asp			0.00 BDL	0.00
		Thr			0.00 BDL	0.00
		Ser			0.00 BDL	0.00
3	19.580	Glu	36372	13.96	12.05	1771.93
4	33.140	Gly	1056	22.21	0.34	25.24
5	35.680	Ala	30681140	4.37	9611.76	856311.98
6	41.687	Val	59514	7.65	17.98	2105.07
		Cys			0.00 BDL	0.00
		Met			0.00 BDL	0.00
		Ile			0.00 BDL	0.00
		Leu			0.00 BDL	0.00
		Tyr			0.00 BDL	0.00
		Phe			0.00 BDL	0.00
7	61.233	g-ABA	31851	30.78	9.90	1020.42
		Orn			0.00 BDL	0.00
9	74.927	Lys	56914	3.74	14.67	2144.12
		His			0.00 BDL	0.00
		Arg			0.00 BDL	0.00
总数			30866847		9666.68	

VIS 2 结果

Peak #	Retention Time	Name	Area	Resolution	Mol concentration (nmol/mL)	CalcMol (ng/mL)
		Hypro			0.00 BDL	0.00
		Pro			0.00 BDL	0.00
总数						

C

Figure S2. Concentrations of different amino acid by-products in *B. licheniformis* BLA3, related to Figure 2. The concentrations of free amino acids were detected by L-8900 automatic amino acid analyzer. Fermentation end product samples were diluted 100-fold before being assayed.

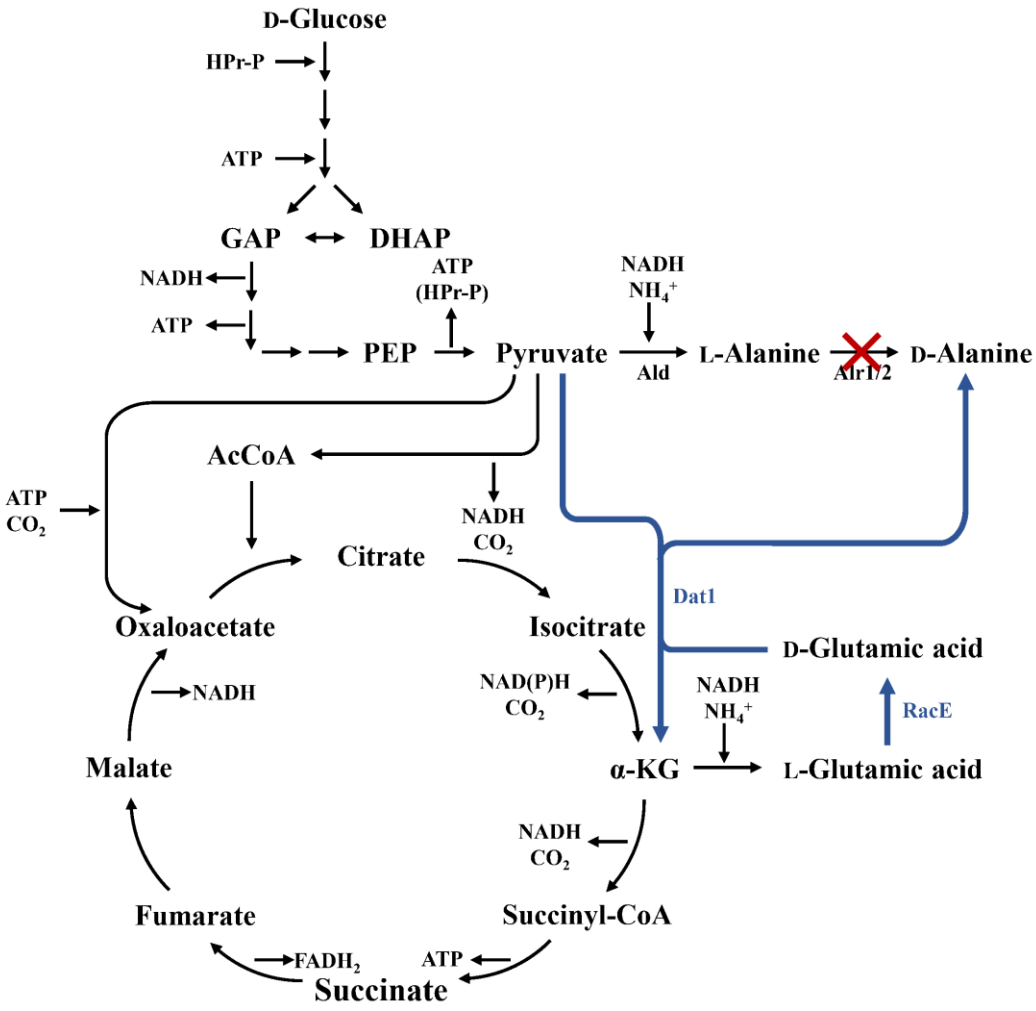


Figure S3. The alternate D-alanine synthesis pathway in *B. licheniformis*, related to Figure 4. The main reaction of the alternate D-alanine synthesis pathway was marked in blue in the picture. L-Glutamate was converted to D-glutamate catalyzed by the glutamate racemase RacE, and subsequently converted to D-alanine catalyzed by the D-amino acid aminotransferase Dat1.

Figure S4. Genome upstream and downstream genes of *alsT5* in *B. licheniformis*, related to Figure 5. The *alsT5* gene is located in the upstream region of the *ald1* gene encoding alanine dehydrogenase, suggesting that it may be involved in alanine transport.

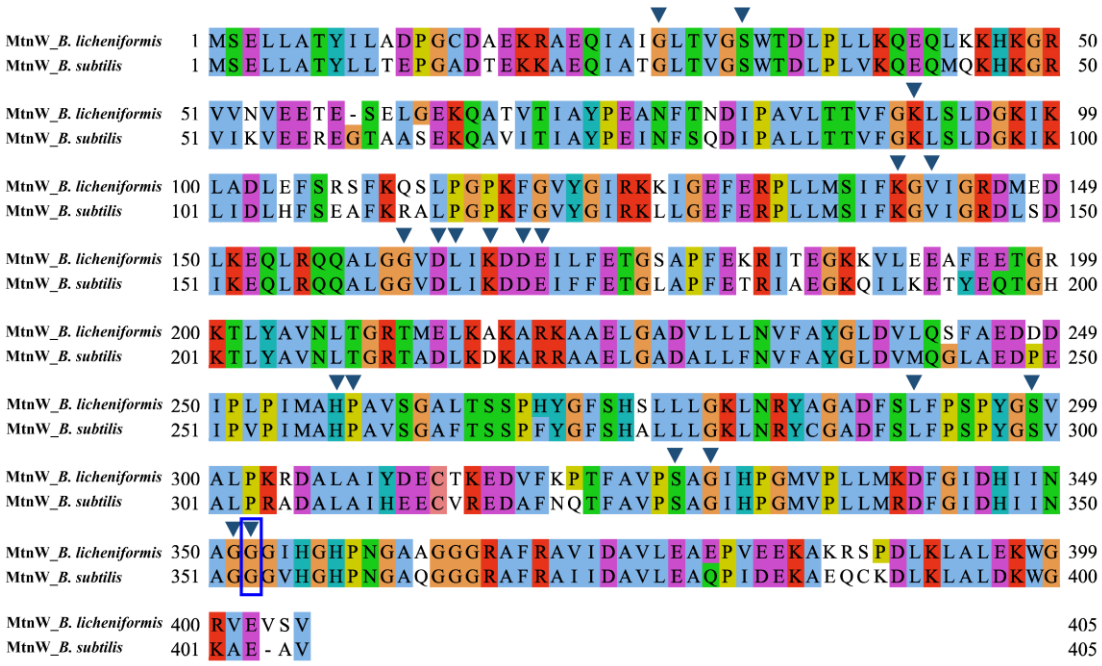


Figure S5. Amino acid sequences alignment between *B. licheniformis* MtnW and *B. subtilis* MtnW, related to Figure 5. The residues involved in the substrate binding and catalytic activity of MtnW were marked by dark blue triangles. Site where SNP occurred in MtnW in evolved strain *B. licheniformis* BLA3-E1 was highlighted by a blue box.

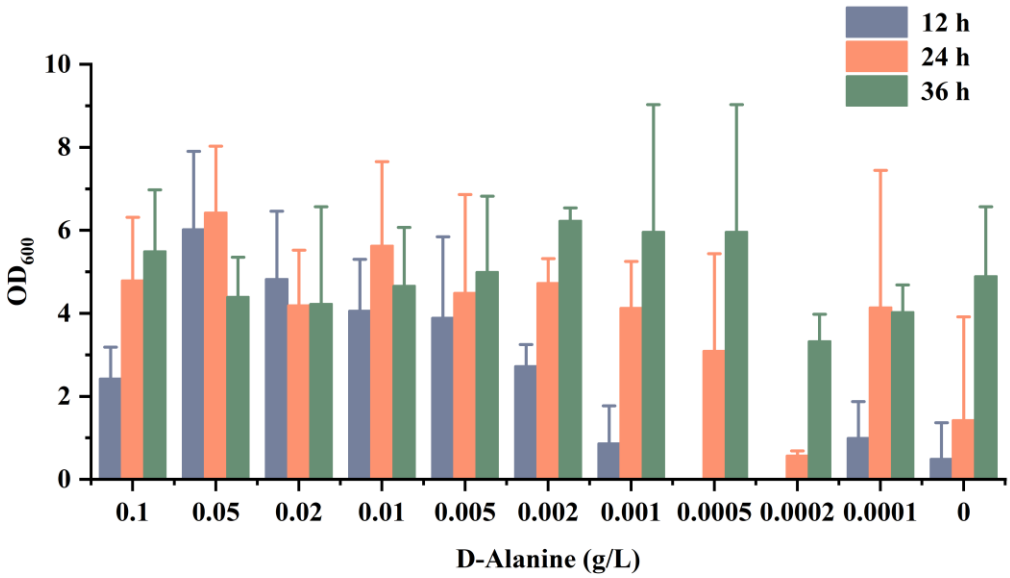


Figure S6. Identification of the effects of *mtnW* on the exogenous D-alanine dependence of the *B. licheniformis* BLA3Δ*dltB*, related to Figure 5.

Characterization of the D-alanine dependence on *B. licheniformis* BLA3Δ*dltB*Δ*mtnW* by testing the growth performance in media containing various concentrations of D-alanine. Triplicate experiments were carried out for physiological measurements, and error bars represent standard deviations.

Table S1. Concentrations of organic acid and alcohol by-products in different *B. licheniformis*, related to Figure 2.

Strain	Succinic acid	Lactic acid	Glycerol	Formic acid	Acetic acid
BLA3	0.9 g/L	0.4 g/L	6.6 g/L	1.6 g/L	2.7 g/L
BLA4	0.8 g/L	1.3 g/L	ND	1.8 g/L	1.7 g/L

ND, undetectable.

Table S2. Summary of SNPs and InDels occurring in BLA3-E1 compared to BLA3, related to Figure 5.

Type	Position and content	Description
SNP	338665, G to T	Hypothetical protein gene, missense mutation, L59F
SNP	609159, C to A	Intergenic region between sortase gene and inositol transport protein gene *iolT*
SNP	920866, A to G	Intergenic region between hypothetical protein gene and 16S ribosomal RNA gene
SNP	1491092, G to T	2,3-diketo-5-methylthiopentyl-1-phosphate enolase gene *mtnW*, missense mutation, G352V
SNP	2210832, G to T	Sporulenol synthase gene *sqhC*, missense mutation, A432S
SNP	2525390, C to T	Biotin carboxylase gene *accC2*, missense mutation, R149H
SNP	3139007, C to T	Sodium/glucose cotransporter gene *sglT*, missense mutation, P263L
SNP	3233706, C to T	Sodium:alanine symporter family protein gene *alsT5*, missense mutation, V165I
SNP	3612548, C to T	Two-component sensor histidine kinase gene *degS*, missense mutation, E65K
InDel	3234341, CAAAATTTTTTCA TGCAAA to CAA	Intergenic region between sodium:alanine symporter family protein gene *alsT5* and proline-responsive transcriptional activator gene *putR*
InDel	3905875, GC to G	Teichoic acid D-alanyltransferase *dltB*, frame shift mutation after the position 408

Table S3. Strains and plasmids used in this study, related to STAR Methods.

Strain or plasmid	Description
plasmids	
pKVM1	*E. coli*/*B. licheniformis* shuttle vector, Amp^r and Em^r
pKVM1-PFYAK	Vector for introducing the *pfkA* and *pyk* genes from *B. coagulans* into strain BN11 with the strong promoter P_{als}
pKVM1Δldh_{Ti}	Vector for deleting the ldh_{Ti} gene in strain BN11-PFYAK
pKVM1-*ald1*	Vector for introducing another copy of *ald1* gene into strain BLA1 with the strong promoter P_{als}
pKVM1-*ald2*	Vector for introducing another copy of *ald2* gene into strain BLA1 with the strong promoter P_{als}
pKVM1-*GSald*	Vector for introducing the codon-optimized *GSald* gene into strain BLA1 with the strong promoter P_{als}
pKVM1-*STald*	Vector for introducing the codon-optimized *STald* gene into strain BLA1 with the strong promoter P_{als}
pKVM1-*BSald*	Vector for introducing the codon-optimized *BSald* gene into strain BLA1 with the strong promoter P_{als}
pKVM1Δ*alr1*	Vector for deleting the *alr1* gene in strain BLA2
pKVM1Δ*alr2*	Vector for deleting the *alr2* gene in strain BLA2 or BLA2Δ*alr1*
pKVM1Δ*dgp*	Vector for deleting the *dgp* gene in strain BLA3
pKVMΔ*dltB*	Vector for deleting the *dltB* gene in strain BLA3
pKVMΔ*mtnW*	Vector for deleting the *mtnW* gene in strain BLA3 or BLA3Δ*dltB*
pKVMΔ*alsT5*	Vector for deleting the *alsT5* gene in strain BLA3 or BLA3-E1
pKVMΔ*dat1*	Vector for deleting the *dat1* gene in strain BLA3 or BLA3-E1
pKVM-*dat1*	Vector for introducing another copy of *dat1* gene into strain BLA3 with the strong promoter P_{als}

strains	
B. licheniformis	Efficient D-lactate producer, *B. licheniformis* ATCC 14580
BN11	Δ*hsdR1*Δ*hsdR2*Δ*ldh*Δ*alsS*Δ*alsD*::ldh_{Ti}
E. coli S17-1	Conjugative strain able to host λ-pir-dependent plasmids
BN11-PFYAK	BN11::P_{als}PFYAK
BLA1	BN11-PFYAKΔldh_{Ti}
BLA1-P*ald1*	BLA1::P_{als}*ald1*
BLA1-P*ald2*	BLA1::P_{als}*ald2*
BLA2	BLA1::P_{als}*GSald*
BLA1-P*STald*	BLA1::P_{als}*STald*
BLA1-P*BSald*	BLA1::P_{als}*BSald*
BLA2Δ*alr1*	BLA2Δ*alr1*
BLA2Δ*alr2*	BLA2Δ*alr2*
BLA3	BLA2Δ*alr1*Δ*alr2*
BLA4	BLA3Δ*dgp*
BLA3-E1	Evolved BLA3 which can grow without adding D-alanine
BLA3Δ*dltB*	BLA3Δ*dltB*
BLA3Δ*mtnW*	BLA3Δ*mtnW*
BLA3Δ*alsT5*	BLA3Δ*alsT5*
BLA3-E1Δ*alsT5*	BLA3-E1Δ*alsT5*
BLA3Δ*dltB*Δ*mtnW*	BLA3Δ*dltB*Δ*mtnW*
BLA3Δ*dat1*	BLA3Δ*dat1*
BLA3-E1Δ*dat1*	BLA3-E1Δ*dat1*
BLA3-P*dat1*	BLA3::P_{als}*dat1*