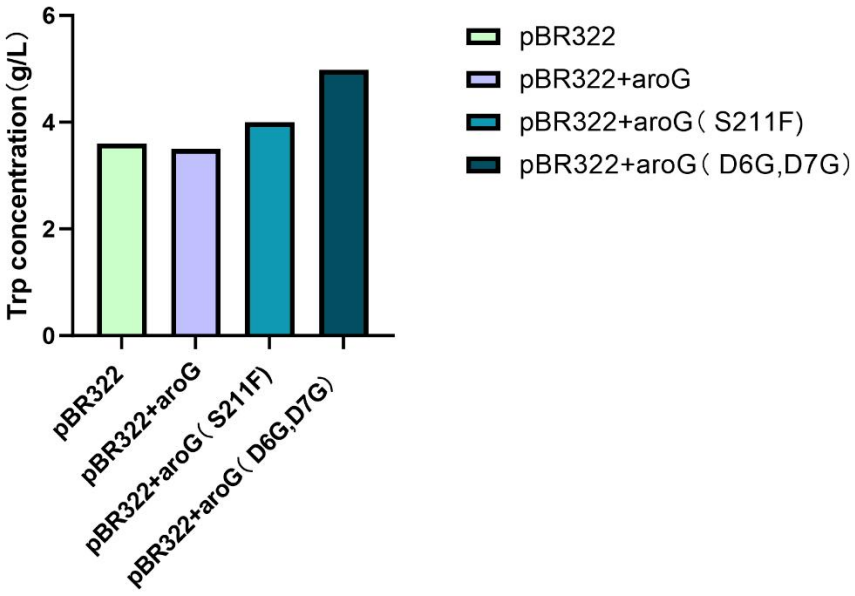
Supplementary Materials

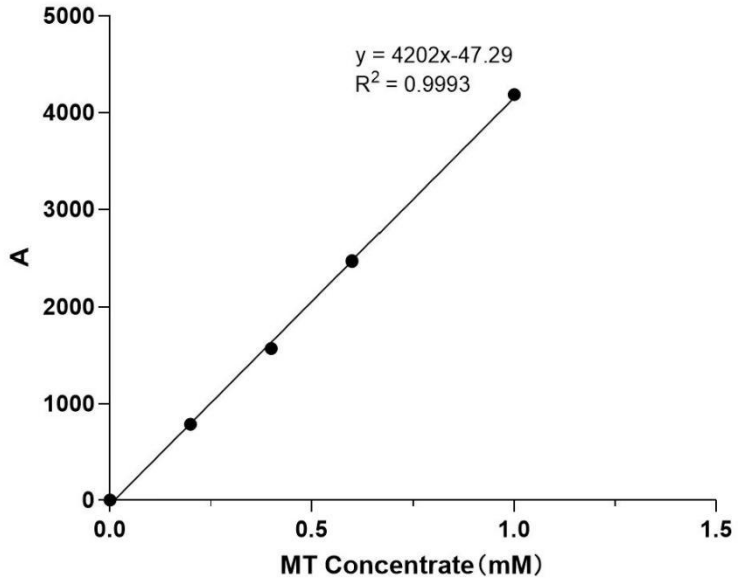
Strain	Functional Category	Gene	Potential Role in Tryptophan Overproduction
3-4	Global Regulation	*pnp*	Degrades competing pathway mRNAs
3-4	Global Regulation	*trmA*	Influences trp operon attenuation
3-4	Precursor Supply	*fbp*	Increases precursor (E4P) supply by blocking gluconeogenesis
3-4	Precursor Supply	*argF*	Increases precursor (PEP) supply by reducing arginine synthesis
3-4	Redox Balance (NADPH)	*ligA*	Increases NADPH availability via enhanced NAD^+ cycling
3-4	Redox Balance (NADPH)	*gudP*	Increases NADPH by altering glutamate transport
3-4	Competing Pathways	*dtpA*	Increases free amino acid pool by reducing peptide consumption
10-7	Enzyme Activity	*trxA*	Maintains the activity of tryptophan synthesis enzymes (TrpE/TrpA).
10-7	Competing Pathways	*pheT*	Alters precursor (chorismate) flux, favoring the tryptophan branch.
10-7	Precursor Supply	*ptsP*	Reduces PEP consumption for sugar transport, increasing PEP availability.
10-7	Precursor Supply	*gapA*	Alters glycolytic flux, impacting precursor (PEP) supply.

Supplementary Table 1. Additional candidate genes mutations and their potential roles in tryptophan overproduction.



Supplementary Figure 1. *aroG* mutant tryptophan yield.

By performing targeted mutations on the *aroG* gene, the L-tryptophan yield reached 5 g/L, which is 171 times higher than that of the wild-type strain.



Supplementary Figure 2. The standard curve of melatonin.

Supplementary Table 2. Strains and plasmids used in this study.

Strains and plasmids	Description	Source
E. coli DH5α	F⁻*ΔlacU169(Φ80 lacZ ΔM15) hsdR17 recA1 endA1 supE44*	Invitrogen
E. coli BW25113	*F-, λ-, E. coli K-12 strain BD792 (CGSC6159) lacZ*	Invitrogen

E. coli BWΔtrpR	BW25113 strain knocked out *trpR*	This study
E. coli BWΔtrpRΔtnaAB	BW25113 strain knocked out *trpR and tnaAB*	This study
E. coli BW*ΔtrpRΔtnaABΔpheA*	BW25113 strain knocked out *trpR, tnaAB, pheA*	This study
E. coli BW*ΔtrpRΔtnaABΔptsG*	BW25113 strain knocked out *trpR, tnaAB, ptsG*	This study
E. coli BW*ΔtrpRΔtnaABΔpykA*	BW25113 strain knocked out *trpR, tnaAB, pykA*	This study
E. coli BW*ΔtrpRΔtnaABΔpykF*	BW25113 strain knocked out *trpR, tnaAB, pykF*	This study
E. coli BWΔtrpRΔtnaABΔpheA–PBR322	BW25113 derivative with *trpR, tnaAB,* and *pheA deleted,* carrying plasmid *PBR322*	This study

E. coli BW*ΔtnaABΔtrpR*	BW25113 derivative with *tnaA and trpR* genes deleted	This study
E. coli BW*cys3.cys4* Δ*cysE*	BW25113 derivative with *cys3 and* cys4 genes expressed	This study
BW*ΔCysE-PLB1s-Cys3-Cys4*	BW25113 Δ*cysE* carrying pLB1s plasmid expressing *cys3* and *cys4* genes	This study
Bw Δ*trpR* Δ*tnaAB* Δ*pheA* pBR322-pYB1a-*BH4* pSB1c-*SgAANAT-CrTDC*	BW25113 Δ*trpR* Δ*tnaAB* Δ*pheA* carrying pBR322, pYB1a-*BH4,* and pSB1c-*SgAANAT-CrTDC* plasmids	This study
Bw*ΔCysE/*pYB1a-*AtCOMT/*pLB1s-*Cys3-Cys4*	BW25113 Δ*cysE* carrying pYB1a-*AtCOMT* and pLB1s-*Cys3-Cys4* plasmids	This study

Bw*ΔCysE/*pYB1a-*AtCOMT*/*pLB1s-*Cys3-Cys*	BW25113 Δ*cysE* carrying pYB1a-*AtCOMT* (mutant) and* pLB1s-*Cys3-Cys4* plasmids	This study

Plasmids

pLB1s-*VioABCDE* */*pSB1c-*VioABCDE*	*Spe/Chl, araC, VioA, VioB, VioC, VioD, VioE*	This study
pYB1a- *hucR-eGFP*	*Amp, araC, hucR,eGFP*	This study
pYB1a- *hucR-CmR*	*Amp, araC, hucR,CmR*	
Donor-*pheA U500D500*	*pheA/ptsG/pykA/pykF U500, pheA/ptsG/pykA/pykF D500, SmR*	This study
Donor-*ptsG U500D500*		
Donor-pykA		

U500D500		
Donor-*pykF* *U500D500*		
pTarget-*pheA* pTarget-*ptsG* pTarget-*pykA* pTarget-*pykF*	*J23119(SpeI)* promoter, *pheA/ptsG/pykA/pykF, gRNA scaffold, Spe*	This study
pLB1s-PBAD-*tnaC-mCherry-Cmr*	*araC, pir, I2* and *I1 region, His tag, tnaC, mCherry, CmR, TrrnB, oriR6k*	This study
pYB1a-P23119-*trpEDCBA*	*trpE, D, C B, A, TrrnB, P15A_ORI, AmpR*	This study
pYB1a-P23119-*trpE^{S40F}DCBA*	*trpE^{S40F}, D, C, B , A, TrrnB, P15A_ORI, AmpR*	This study
PBR322-*trp^{fbr}EDCBA-aroG^{fbr}-serA^{fbr}*	*TcR, Lac I, trpD, trpC, trpB, serA, aroG*	2

MP6-K	*araC, UGI, PmCDA1, KanR, CloDF13 ori, araBAD* promoter	This study
pYB1a-*BH4*	*Amp, araC,* expresses *human GTP cyclohydrolase I (BH4* biosynthesis*)*	This study
pYB1a-*ccdB*	*Amp，araC，ccdB* negative selection gene	This study
pYB1a-*AtCOMT**（*pYB1a-AtCOMTC296F/Q310L/V314T*）	*Amp，araC，AtCOMT**expresses *mutant Arabidopsis thaliana catechol-O-methyltransferase (C296F/Q310L/V314T)*	This study
pYB1a-*AtCOMT*	*Amp，araC，*expresses wild-type *Arabidopsis thaliana catechol-O-methyltransferase*	This study
pSB1c-*SgAANAT*-	*Chl,* expresses *Streptomyces*	This study

CrTDC	*griseus arylalkylamine N-acetyltransferase (SgAANAT)* and *Catharanthus roseus tryptophan decarboxylase (CrTDC)*	
pSB1c-*SgAANAT*	*Chl,* expresses *Streptomyces griseus arylalkylamine N-acetyltransferase (SgAANAT)*	This study
PLB1s-*Cys3-Cys4*	*Spe*，*Chl,* expresses *cys3 and cys4* genes	This study
PLB1s-P23119-*AtCOMT*-SgAANAT-CrTDC*	*Spe*，*Chl,* P23119 promoter drives expression of *mutant AtCOMT*, SgAANAT,* and *CrTDC*	This study
PLB1s-*AtCOMT*-SgAANAT-CrTDC*	*Spe*，*Chl,* expresses *mutant AtCOMT*, SgAANAT,* and	This study

	CrTDC	
PLB1s-P23119-*AtCOMT**-*CrTDC*	*Spe*，*Chl3*，P23119 promoter drives expression of *mutant AtCOMT** and *CrTDC*	This study

Supplementary Table 3. Primers used in this study.

pLB1s-VioABCDE /pSB1c-VioABCDE construction	
pLB1s-F	TTAGCGAATAAAGATCTGGTACTAGTGGTGAATTCG
pLB1s-R	GTTGGTCATTTCCTCCTGTTAGCCCAAAAAACG
pSB1c-F	TTAGCGAATAAAGATCTGGTACTAGTGGTGAATTCG
pSB1c-R	GTTGGTCATTTCCTCCTGTTAGCCCAAAAAACG
VioA-F	AACAGGAGGAAATGACCAACTACAGTGATATC
VioA-R	TG

VioB-F	ATGCTCATCTCCTTTATGCACGTTCGGTCAGAC
VioB-R	TGCATAAAGGAGATGAGCATCCTGGATTTTCC
VioC-F	GTGCATCTCCTTTATGCTTCACGGCTCATTTTAC
VioC-R	GAAGCATAAAGGAGATGCACAAGATCATCATC
VioD-F	GTTG
VioD-R	AGGATCTTCATCTCCTTTAATTCACGCGGCCCA
VioE-F	G
VioE-R	TTAAAGGAGATGAAGATCCTGGTTATTGGC
	TGAGGAGGCATCTCCTTTAACGACCCAGGGCAT
	AG
	TTAAAGGAGATGCCTCCTCATGCCAC
	TACCAGATCTTTATTCGCTAACAAACACGCTG

pYB1a- hucR-eGFP construction

pYB1a -F	GTATCATTATCCATGCGGGCACTC
pYB1a -R	ACAGGGTGTTTAAGGCTCACCTTCACGGGTGG
HucR-F	GTGAGCCTTAAACACCCTGTTCCAGACC
HucR-R	ATGAGTGCCCGCATGGATAATG

pYB1a- hucR-CmR construction

CmR -F	CGCTTTTTATCGCAACTCTC

CmR -R	GATCTACCCTCGAGTTACGCCCCGCCCTG
HucR-F	GCGTAACTCGAGGGTAGATCTGGTACTAGTGGT GAATTC
HucR-R	CTCCATCCTCGAGGCTGCCGCG
Donor -pheA U500D500 construction	
pheA-U500-F	TCCTCGAGGTAAACACATCTGATTAATCCACAT ATCATTC
pheA-U500-R	CACCTTTTCAAGTGTTGCCTTTTTGTTATCAATA AAAAAG
pheA-D500-F	AGGCAACACTTGAAAAGGTGCCGGATGATGTG
pheA-D500-R	TACTGCAGTACATACCAATGGTTTCTGGAGCAA ATTC
DO-pheA-F	CCATTGGTATGTACTGCAGTAGTTTTGCTGAAA TAC
DO-pheA-R	AGATGTGTTTACCTCGAGGAAAATGTCGTAAAC
pheA1000-F	AAACACATCTGATTAATCCACATATCATTC
pheA1000-R	CATACCAATGGTTTCTGGAGC
Donor -ptsG U500D500 construction	

ptsG-U500-F	TCCTCGAGGTATCGGTTACTGGTGGAAACTG
ptsG-U500-R	GTCTTACGGAAATTGAGAGTGCTCCTGAGTATG
ptsG-D500-F	ACTCTCAATTTCCGTAAGACGTTGGGGAGAC
ptsG-D500-R	TACTGCAGTAGTGGATGGGACAGTCAGTAAAG
DO-ptsG-F	GTCCCATCCACTACTGCAGTAGTTTTGCTGAAA TAC
DO-ptsG-R	AGTAACCGATACCTCGAGGAAAATGTCGTAAA C
ptsG1000-F	ATCGGTTACTGGTGGAAACTGAC
ptsG1000-R	GTGGATGGGACAGTCAGTAAAGG
Donor -pykA U500D500 construction	
pykA-U500-F	TCCTCGAGGTACGCATGAGTTGTATGAATTGTA G
pykA-U500-R	GGCAACGTACGTAATACTCCGTTGACTGAAACA ACC
pykA-D500-F	GGAGTATTACGTACGTTGCCGGATGCGGCGAA AAC
pykA-D500-R	TACTGCAGTAGTACTGGGGATATTATTTACCCG ATCAGG
DO-pykA-F	

DO-pykA-R	TCCCCAGTACTACTGCAGTAGTTTTGCTGAAATAC
pykA1000-F	ACTCATGCGTACCTCGAGGAAAATGTCGTAAAC
pykA1000-R	ACGCATGAGTTGTATGAATTGTAGC
	GTACTGGGGATATTATTTACCCGATCAG
Donor -pykF U500D500 construction	
pykF-U500-F	TCCTCGAGGTCAAAAATCAAACAAAATCAGACAAATAACGC
pykF-U500-R	AAAAGCAATAGACAGTCTTAGTCTTTAAGTTGAGAAGG
pykF-D500-F	TAAGACTGTCTATTGCTTTTGTGAATTAATTTGTATATCGAAGC
pykF-D500-R	TACTGCAGTAGAGCTGCGTCATCTTTAGCAG
DO-pykF-F	GACGCAGCTCTACTGCAGTAGTTTTGCTGAAATAC
DO-pykF-R	TTGATTTTTGACCTCGAGGAAAATGTCGTAAAC
pykF1000-F	CAAAAATCAAACAAAATCAGACAAATAACGC
pykF1000-R	GAGCTGCGTCATCTTTAGCAG
pTarget -pheA construction	
pTarget-pheA-F	CTAGTCATACCAGCTTGTCGATTGTGTTTTAGA

	GCTAGAAATAGC
pTarget-pheA-R	
	TAAAACACAATCGACAAGCTGGTATGACTAGT ATTATACCTAGGAC
pTarget -ptsG construction	
pTarget-ptsG-F	CTAGTCGGCGACATTCCGCGTTATAGTTTTAGA GCTAGAAATAGC
pTarget-ptsG-R	
	TAAAACTATAACGCGGAATGTCGCCGACTAGT ATTATACCTAGGAC
pTarget -pykA construction	
pTarget-pykA-F	CTAGTCATCATCCTCGCCTCTGACGGTTTTAGA GCTAGAAATAGC
pTarget-pykA-R	
	TAAAACCGTCAGAGGCGAGGATGATGACTAGT ATTATACCTAGGAC
pTarget -pykF construction	
pTarget-pykF-F	CTAGTCGAAGCCTCTGACGGCATCAGTTTTAGA GCTAGAAATAGC
pTarget-pykF-R	
	TAAAACTGATGCCGTCAGAGGCTTCGACTAGTA TTATACCTAGGAC
pLB1s-PBAD-tnaC-mCherry -Cmr construction	
cmr-F	AAGTAAAGGAGATGGAGAAAAAAATCACTGGA TATACC
cmr-R	
	CTCACCGAATTCACCACTAGTACCTTACGCCCC GCCCTGCC
D21T--F	
	CAAAATTGTCACTCACCGCCCTTG

D21T--R	
	CGGTGAGTGACAATTTTGTTGTCAATATTG
LS-tnaC-F	
	CGGCCTGGTGCCGCGCGGCAGCCTCGAGATGAATATCTTACATATATGTGTGACCTC
mcherry-R	
	TTTCTCCATCTCCTTTACTTGTACAGCTCGTCCATGCCG
pYB1a-P23119-trpEDCBA construction	
pyb1a-F	GCGCAGTTAACTCGAGGGTAGATCTGGTACTAGTGG
pyb1a-R	GTGTTTGCATGGTACCCATGGTTAATTCCTCCTG
trpE-F	
	CATGGGTACCATGCAAACACAAAAACCGACTCTC
JC2	
	CAAACCGTTCTTGAGGTACTGCG
trpD-R	
	GTTTGCATCATTTACCCTCGTGCC
trpA-R	
	TACCCTCGAGTTAACTGCGCGTCGCCGCTTTC
MP6-K construction	
kana-F	CCCATGGCGTTTATAAAAACTCATCGAGCATCAAATG
kana-R	GGAAGCTAAAATGAGCCATATTCAACGGGAAAC

ZT-F	TATGGCTCATTTTAGCTTCCTTAGCTCCTGAAA ATCTCGATAACTCAAAAAATACGCCC
ZT-R	GTTTTTATAAACGCCATGGGCATGTAGTCAAAA GCC

Sequencing Data Quality Assessment

Whole-genome sequencing (WGS) enables detailed analysis of gene function and genomic loci[1,2]. WGS of the wild-type strain BW-RT and the two evolved strains (3-4 and 10-7) generates robust data quality, with >94% bases achieving Q30, mean sequencing depths >175×, and unique mapping rates >92%.

Strain	Q30	Mean sequencing depths	Unique mapping rates
BW-RT	94.82%	187.92×	97.53%
3-4	94.78%	190.26×	92.36%
10-7	94.97%	175.8×	92.80%

Supplementary Table 4. Quality metrics for WGS data.

This table summarizes key indicators for the wild-type (BW-RT) and two evolved strains (3-4 and 10-7) derive via directed evolution for tryptophan overproduction. Metrics include: (i) the percentage of bases with Phred quality score ≥30 (Q30), indicating high base-calling accuracy; (ii) mean sequencing depth (×), reflecting coverage reliability; and (iii) unique mapping rate (%) to the reference genome (E. coli K-12 MG1655), ensuring precise variant detection. All values exceed standard thresholds (>94% Q30, >175× depth, >92% mapping), supporting robust downstream genomic variation analysis.

References

1.Priya, V. K., Sarkar, S., & Sinha, S. (2014). Evolution of tryptophan biosynthetic pathway in microbial genomes: a comparative genetic study. *Systems and synthetic biology*, *8*(1), 59-72.

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2. Hou, M., Gao, S., Wu, J., Chen, S., & Zhang, K. (2025). Metabolic engineering of Escherichia coli to enhance L-tryptophan biosynthesis. *Systems Microbiology and Biomanufacturing*. https://doi.org/10.1007/s43393-025-00338-3