Supplement to

Metabolic engineering of *Corynebacterium glutamicum* for high-level ectoine production – design, combinatorial assembly and implementation of a transcriptionally balanced biosynthetic pathway

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Strains and plasmids

The strains and plasmids are listed in **Supplementary Table S1**. *Corynebacterium glutamicum* ATCC 13032 *lysC*^{fbr} was obtained from previous work.[1] It contains a feedback-resistant variant of aspartokinase (Cgl0251). *Escherichia coli* DH5α, *E. coli* NM522 (Invitrogen, Carlsbad, CA, USA), and *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) were used for cloning.[2] All strains were stored as cryostocks in 60% glycerol at -80 °C. The integrative plasmid pClik int *sacB*[3, 4] and the episomal plasmids pClik 5a MCS,[5] pCGH36A,[38] and PCES208[38] have been previously described. The codon-optimized *ectABC* genes from *Pseudomonas stutzeri* under control of the *tuf* promoter were taken from previous work.[6]

Recombinant DNA work in *C. glutamicum*

DNA fragments were amplified by PCR (Phusion Flash PCR Master Mix, Thermo Fischer Scientific, St. Leon-Roth, Germany) using sequence-specific primers (**Supplementary Table S2**). For heterologous expression of the previously developed ectoine cluster,[6] pClik 5a MCS was linearized using the restriction enzyme *Nde*I (FastDigest, Thermo Fischer Scientific), followed by *in vitro* assembly with the codon-optimized construct P_{tuf} *ectABC*.[7] After amplification in *E. coli* DH5α, methylation in *E. coli* NM522, and isolation (QIAprep Spin MiniPrep Kit, Quiagen, Hilden, Germany), the functional plasmid was transformed into *C. glutamicum* using electroporation.[8] Genomic deletion of *lysE*, which encodes the L-lysine exporter (Cgl1262), was performed as described previously.[9] The genetic modifications were validated by PCR and sequencing (GATC Biotech AG, Konstanz, Germany).

Construction and expression of a combinatorial ectoine pathway

A combinatorial plasmid library was constructed and assembled in the shuttle vector pCES208 (*E. coli* – *C. glutamicum*). All primers used for this purpose are listed in **Supplementary Table S5**. Each library construct was transformed into *E. coli* XL1-

Blue (Stratagene, La Jolla, CA, USA) using electroporation. First, we constructed a combinatorial library of *ectA* using 19 synthetic promoters (**Supplementary Table S3)**,[10] three bicistronic designed elements (BCDs, **Supplementary Table S4**), namely, BCD2, BCD8 and BCD21,[11] and the transcriptional terminator *rrnBT1T2*.[12] The selected promoters differed in relative expression strength: H36 (100%), H5 (69%), H3 (68%), H34 (68%), H30 (67%), H28 (65%), H72 (62%), H4 (60%), H17 (58%), I29 (48%), I9 (45%), I12 (40%), I16 (36%), I15 (35%), I64 (33%), I51 (31%), L10 (18%), L80 (16%), and L26 (13%).[10] Similarly, the BCD elements included a strong (BCD2, 100%), a medium (BCD21, 23%), and a weak variant (BCD8, 8%).[12] For library construction, the codon-optimized *ectA* gene[6] was amplified by PCR with the primers BCD-F-BamHI, EctA-R and either BCD2-EctA-F, BCD8-EctA-F or BCD21-EctA-F (**Supplementary Table S5**). All PCR products were pooled, digested with *Bam*HI and *Not*I, and cloned into pCGH36A containing the synthetic promoter P_{H36} and *rrnBT1T2*.[11] For the promoter library, 19 synthetic promoters[10] were cloned into *Kpn*I and *Bam*HI sites of pCGH36A-EctA, yielding pCGH36A-*ectA*-Lib. In the same way, the combinatorial libraries for *ectB* and *ectC*, i.e., pCGH36A-*ectB*-Lib and pCGH36A-*ectC*-Lib were constructed. Next, each *ectA*, *ectB*, and *ectC* module was assembled in the pCES208 shuttle vector as follows. First, the *ectA* module containing promoters-BCDs-*ectA*-*rrnBT1T2* was amplified from pCGH36A-*ectA*-Lib by PCR using the primers XhoI-EctA-F and EctA-R-NotI. The PCR products were digested with *Xho*I and *Xba*I and cloned into the *Sal*I and *Spe*I sites of pCES208, yielding p*EctA*-Lib. Next, for the assembly of the *ectA* and *ectB* modules, the *ectB* module was amplified from pCGH36A-*ectB*-Lib by PCR with the primers XhoI-EctB-F and EctB-SalI-XbaI-R. The PCR products were digested with *Xho*I and *Xba*I and cloned into the *Sal*I and *Xba*I sites of the p*EctA* library, yielding *pEctAB*-Lib. Finally, the *ectC* module was amplified from pCGH36A-*ectC*-Lib by PCR with the primers SalI-EctC-F and EctC-XbaI-R, and the PCR products were cloned into the *Sal*I and *Xba*I sites of the p*EctAB* library, yielding p*EctABC*-Lib (**Supplementary Fig. S1**). The plasmid library

was transformed into *C. glutamicum* using electroporation.[8] The genetic modifications were validated by sequencing (GATC Biotech AG, Konstanz, Germany).

Media and cultivation

The first preculture was conducted in liquid BHI medium (37 g L^{-1} Becton Dickson, Heidelberg, Germany). The second preculture and the main culture were grown in minimal medium containing the following per liter: 10 g glucose, 15 g $(NH_4)_2SO_4$, 1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 55 mg $CaCl_2$, 20 mg $FeSO_4 \cdot 7H_2O$, 0.5 mg biotin, 1 mg thiamin·HCl, 1 mg calcium pantothenate, 100 mL buffer solution (2 M potassium phosphate, pH 7.8), 10 mL trace element solution (200 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 200 mg L^{-1} $MnSO_4 \cdot H_2O$, 50 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 20 mg L^{-1} $CuC \cdot 2H_2O$, 20 mg L^{-1} $Na_2B_4O_7 \cdot 10H_2O$, 10 mg L^{-1} $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, adjusted to pH 1 with HCl), and 1 mL chelating agent solution (30 mg of 3,4-dihydroxybenzoic acid with 50 µL of 6 M NaOH). All solutions were sterilized by autoclaving or sterile filtration. Antibiotics were added from filter-sterilized stocks to final concentrations of 50 mg mL^{-1} kanamycin or 12.5 mg mL^{-1} tetracycline when needed. Cultivations were generally carried out at 30 °C. After 24 h of incubation on BHI agar, a single colony was picked to inoculate the first preculture in liquid BHI medium (baffled shake flasks with 10% filling volume) and incubated for 10 h in an orbital shaker at 230 rpm (Multitron, Infors AG, Bottmingen, Switzerland). Cells from the first preculture were harvested by centrifugation (4 min, 8800 x*g*, 30 °C), washed twice with medium, and used to inoculate the second preculture, which was harvested during the exponential growth phase to inoculate the main culture as described above. All cultures were conducted in triplicate.

High-throughput screening of transformants

Cells were cultivated in a microbioreactor with online optical density measurement (BioLector 1, m2plabs, Baesweiler, Germany) using 48-well flower plates (m2plabs,

Baesweiler, Germany). Each well was filled with 500 µL of minimal glucose medium (see above). The incubations were conducted at 1,300 rpm, 30 °C and 85% humidity.

Fed-batch production in stirred tank bioreactors

The fed-batch production of ectoine was conducted in 1 L laboratory-scale bioreactors (SR0700ODLS, DASGIP AG, Jülich, Germany) controlled by process control software (DASGIP AG, Jülich). The initial batch medium (300 mL) contained the following per liter: 100 g glucose , 72.4 g sugar cane molasses (Hansa Melasse, Bremen, Germany), 35 g yeast extract (Difco, Becton Dickinson), 20 g $(NH_4)_2SO_4$, 100 mg $MgSO_4$, 11 mg $FeSO_4 \cdot 7H_2O$, 10 mg citrate, 250 µL H_3PO_4 (85%), 60 mg Ca-pantothenate, 18 mg nicotinamide, 15 mg thiamin HCl, 9 mg biotin, and 200 mg Antifoam 204 (Sigma-Aldrich, Taufkirchen, Germany). The molasses contained 0.2% NaCl, according to the supplier. After the initial glucose concentration had dropped below 15 g L^{-1}, feeding was initiated. The feed was added pulsewise to keep the glucose level above 10 g L^{-1}. The feed solution contained the following per liter: 670 g glucose, 162.5 g sugar cane molasses, 40 g $(NH_4)_2SO_4$ and 2 ml antifoam. The pH was monitored online (Mettler Toledo, Giessen, Germany) and the solution was maintained at pH 7.0 by the automatic addition of 25% NH_4OH (MP8 pump system, Eppendorf, Hamburg, Germany). The temperature was maintained at 30 °C. The pO_2 was monitored online (Hamilton, Höchst, Germany) and maintained above 30% saturation by adjusting the stirrer speed, aeration rate and oxygen level in the inlet gas.

Substrate and product quantification

The cell concentration was determined as the optical density (OD_{660}) using photometric measurements at a wavelength of 660 nm. The concentration of cell dry mass (CDM) was calculated from the optical density using a correlation factor of CDM [g L^{-1}] = 0.32 x OD_{660}.[7] Ectoine was quantified by HPLC (1290 Infinity, Agilent

Technologies, Waldbronn, Germany) using a reversed-phase column (Zorbax Eclipse Plus C18, 4.6 x 100 mm, 3.5 µm, Agilent) as the stationary phase, demineralized water (0.5 mL min^{-1}, 25°C) as the mobile phase and UV detection at 210 nm. Glucose and organic acids were quantified by HPLC (Agilent 1260 Infinity).[13] Amino acids were analyzed by HPLC after derivatization with *ortho*-phthalaldehyde.[14]

Proteome analysis

Quantitative shotgun proteomics was conducted as previously described, using ^{15}N-labeling for quantification.[15, 16] For preparation of the ^{15}N standard, strains P3.4 and P11.37 were grown in mineral salt medium as described above, where the naturally labeled $(NH_4)_2SO_4$ in the second preculture and in the main culture was replaced by 99% $(^{15}NH_4)_2SO_4$ (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). During the exponential growth phase, cells were harvested by centrifugation (5 min, 10000 x*g*, 4 °C), washed with 20 mL of HEPES-KOH buffer (50 mM, pH 7.4, 4 °C), centrifuged again (5 min, 10000 x*g*, 4 °C), and then resuspended in lysis buffer (50 mM NH_4HCO_3, 1 mM NaCl, 4 °C) to obtain a concentration of 4 (mg CDM) mL^{-1}. Aliquots of 1.5 mL were directly frozen in liquid nitrogen. Before mixing with samples, the ^{15}N standards generated for P3.4 and P11.37 were pooled (1:1). Sample preparation was carried out consistently. Unlabeled samples and the ^{15}N reference standard were mixed at a $^{15}N/^{14}N$ ratio of 0.8 based on the CDM. Subsequently, cells were transferred into 2 mL tubes (FastPrep-24 Lysing Matrix Tubes, 0.1 mm silica spheres; MP Biomedicals, Santa Ana, CA, USA) and disrupted 3x 30 s at 6500 m s^{-1} in a ribolyzer (Precellys®-24 Homogenisator, Peqlab Biotechnology GmbH, Erlangen, Germany) with 1 min breaks on ice between disruption cycles. Cell debris was removed by centrifugation (5 min, 17000 x*g*, 4 °C), and the supernatant was transferred to a new tube and frozen in liquid nitrogen. The protein concentration was determined via the bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, Thermo Fischer Scientific). Subsequent sample processing and LC-ESI-MS/MS

measurements were conducted as described previously.[15, 16] The libraries BioFSharp. Mz (https://github.com/CSBiology/BioFSharp.Mz) and MzLite (https://github.com/CSBiology/MzLite) were used for MS data analysis. Peptide identification was based on the computed cross correlation between theoretical and measured spectra with a mass tolerance of 25 ppm and up to three missed cleavages. The peptide sequences were generated by digesting the *Corynebacterium glutamicum* proteome *in silico* (UniProt Protein ID UP000000582 complemented with the engineered protein sequences of EctA, EctB, and EctC)[6], considering ^{14}N light and ^{15}N heavy stable isotopes. Light-to-heavy peptide ratios were calculated based on the quantification of the peak areas of ^{15}N peptide ions and ^{14}N peptide ions. The efficiency of ^{15}N incorporation into the labeled peptides was estimated according to previous work.[17]

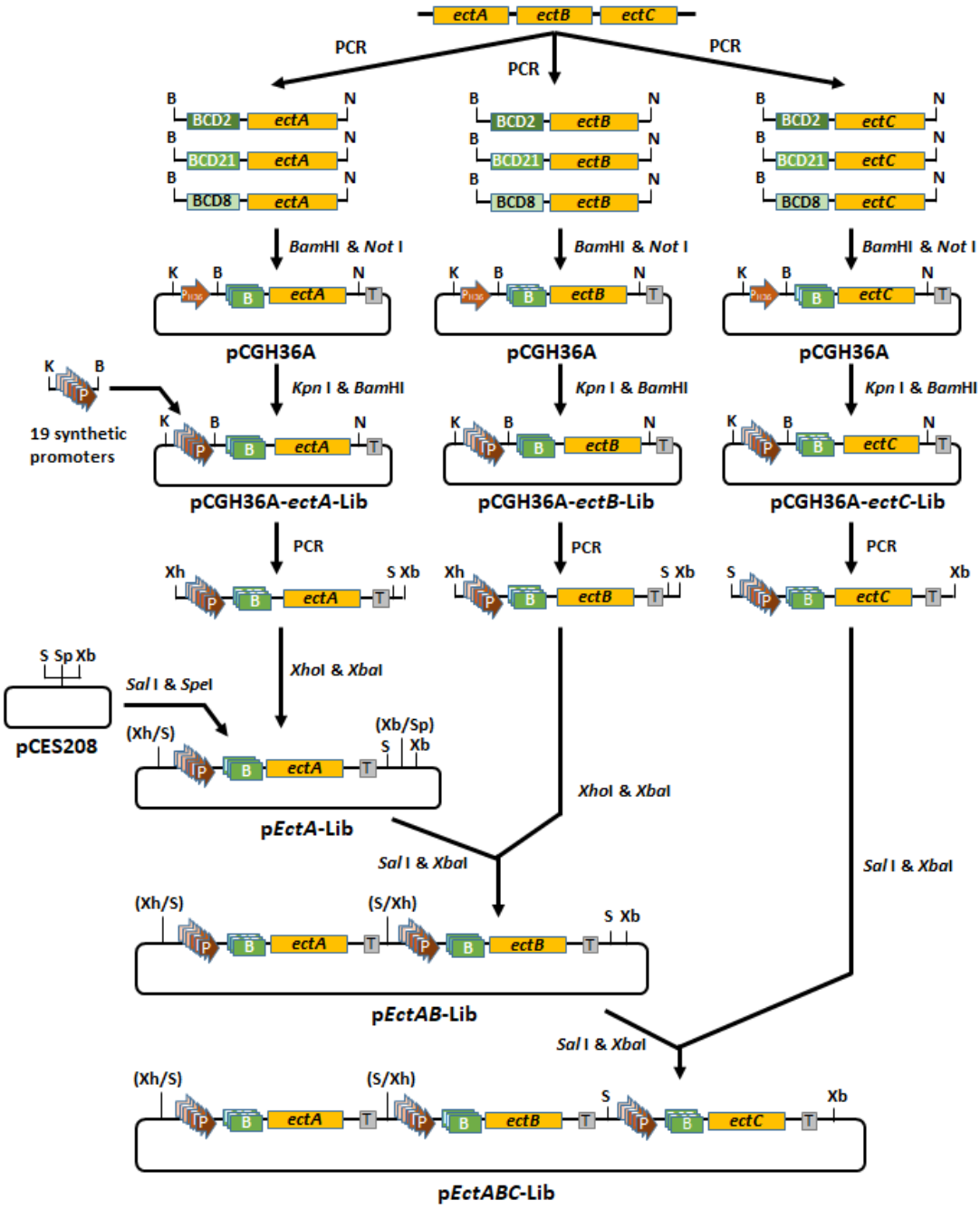


Figure S1. Construction of the EctABC library. Each gene expression module consists of a synthetic promoter (P), a bicistronic design element (BCD) (B), a corresponding *ect* gene and the rrnBT1T2 transcription terminator (T). Abbreviation of restriction enzymes: B, *Bam*HI; K, *Kpn*I; N, *Not*I; Xb, *Xba*I; Xh, *Xho*I, S, *Sal*I, Sp, *Spe*I. The sequences of *Xho*I and *Xba*I-digested cohesive ends are complimentary to those of *Sal* I and *Spe*I, respectively.

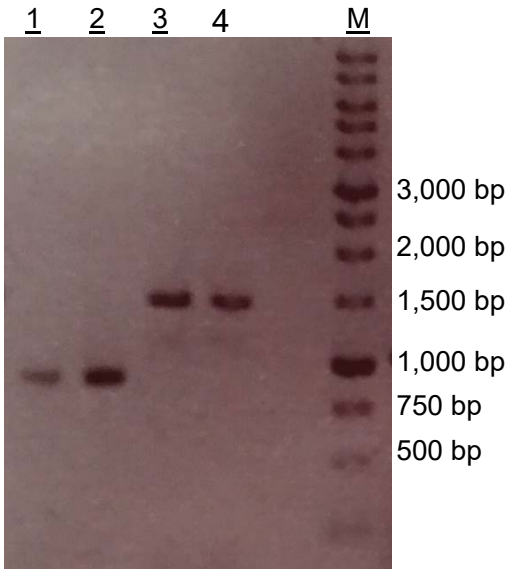
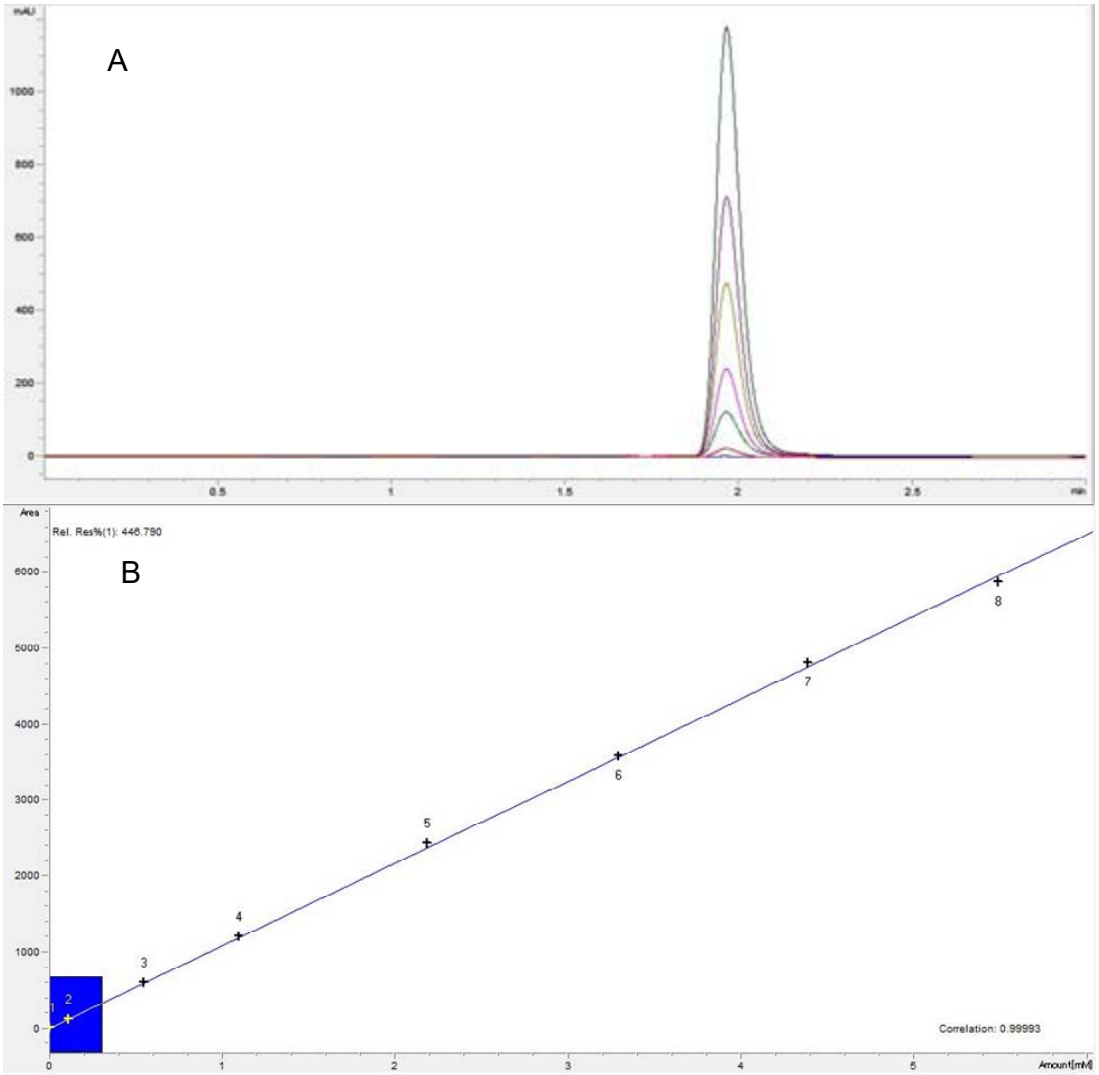


Figure S2: Gel electrophoresis of PCR fragments for validation of the deletion of the L-lysine exporter *lysE* in *C. glutamicum lysC*. The different lanes show mutants with the desired deletion (1, 2) and revertants (3,4). M = marker.



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215 **Figure S3:** HPLC analysis of ectoine, ranging from 0.01 mM to 5 mM (A) and the

216 corresponding calibration curve using UV detection (B).

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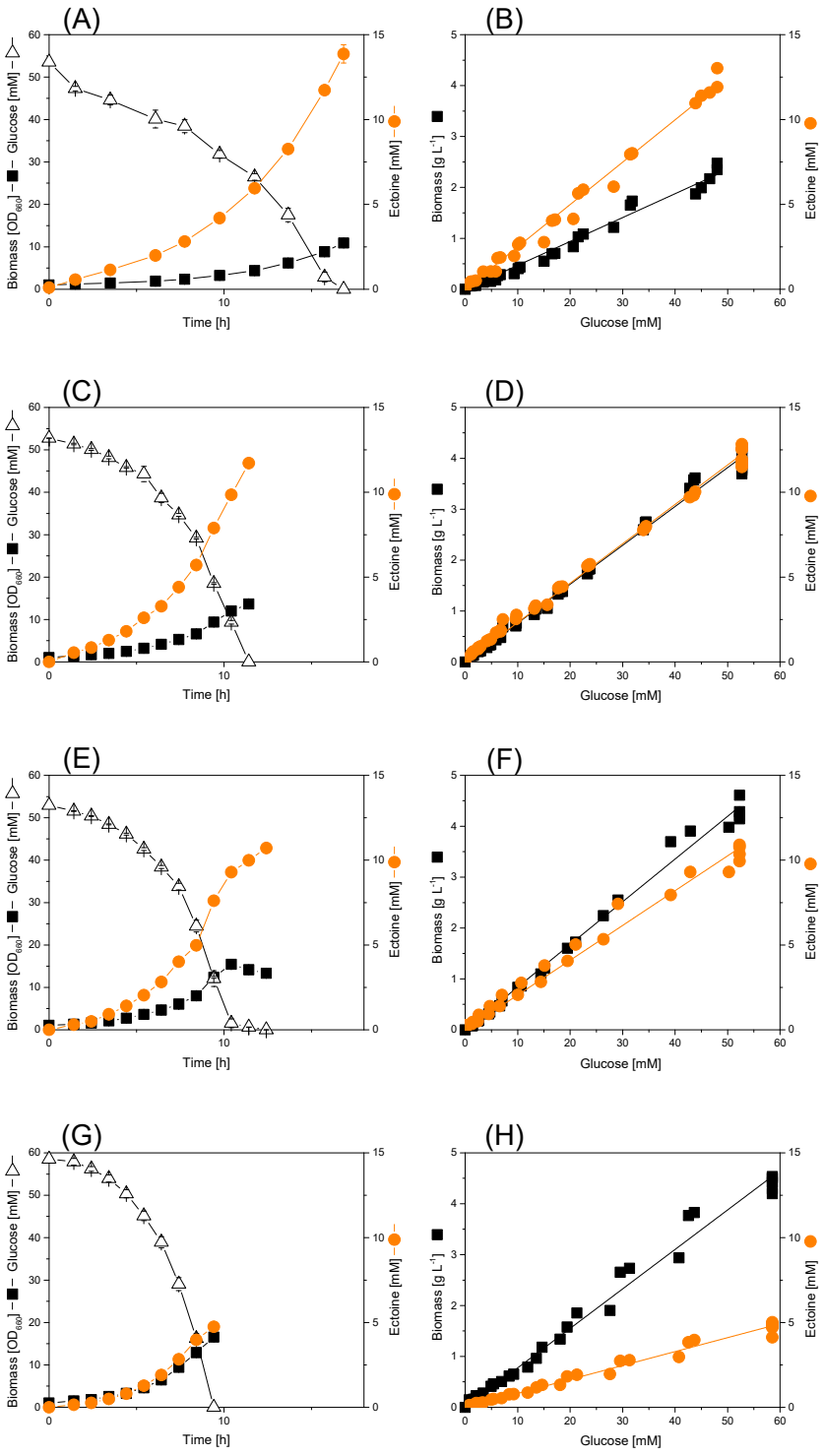

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222 **Figure S4: Growth and ectoine production in different synthetic mutants of**
223 ***Corynebacterium glutamicum.*** The strains shown are P3.4 (A,B), P3.32 (C,D),
224 P11.28 (E,F), and P11.37 (G,H). Errors represent standard deviations from three
225 biological replicates. Due to its superior performance, strain P3.4 is also designated
226 *C. glutamicum ectABC*^{opt} in this work.

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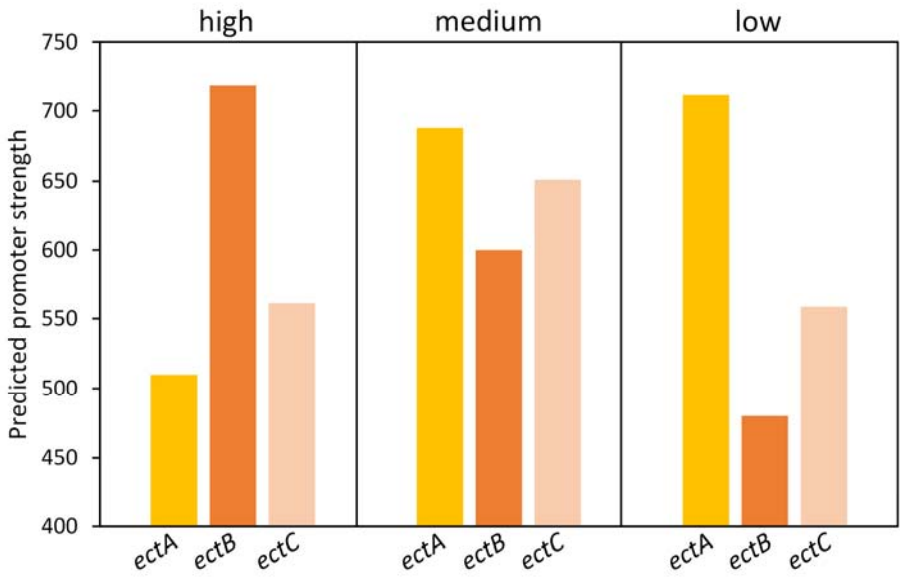

Figure S5: Impact of the transcriptional balancing of *ectA*, *ectB*, and *ectC* on ectoine production in *Corynebacterium glutamicum*. The data show the predicted promotor strength in high- (P3.4 – P3.12), medium- (P11.19-P11.37), and low-titer producers (P3.10-P11.23). Due to its superior performance, strain P3.4 is also designated *C. glutamicum ectABC*^{opt} in this work.

237 **Table S1. Strains and plasmids used in this study.**

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Name	Description	Reference
C. glutamicum lysC^{fbr}	Wild-type ATCC 13032 with the nucleotide replacement S301Y in the *lysC* gene, which encodes aspartokinase	[1, 18]
C. glutamicum lysC^{fbr} Δ*lysE*	*lysC*^{fbr} + deletion of the lysine exporter *LysE* (Ncgl1214)	This work
C. glutamicum lysC^{fbr} Δ*lysE ectABC*^{basic}	*lysC*^{fbr} Δ*lysE* + pClik 5a MCS P_{tuf}*ectABC*	This work
C. glutamicum PX.Y[a]	*C. glutamicum lysC*^{fbr} Δ*lysE* expressing a variant from the ectoine cluster library	This work
E. coli DH5α	Heat-shock-competent cells for the amplification of transformation vectors	Invitrogen
E. coli XL1-Blue	Electroporation competent cells for library construction	Stratagene
E. coli NM522	Heat-shock-competent cells for amplification and methylation of transformation vectors	Invitrogen
pTc15AcglM	Expression of the *C. glutamicum*-specific methyltransferase	[2]
pClik 5a MCS	Episomal expression vector	[19]
pClik 5a MCS tuf_p *ectABC*	Episomal vector for the expression of the codon-optimized ectoine gene cluster *ectABC* from *P. stutzeri*[6]	This work
pCES208	Episomal expression vector	[10, 20]
pCES208 PX.X	Ectoine gene cluster library plasmids based on pCES208	This work

pClik int *sacB* *ectABCD*		Codon-optimized biosynthetic ectoine/hydroxyectoine tuf_p *ectABCD* cluster of *P. stutzeri* for integration into the *ddh* gene (NCgl2528), which encodes diaminopimelate dehydrogenase	[6]
pClik int *sacB* Δ*lysE*		Integrative vector for deletion of the lysine exporter *LysE*	[9]

[a] The code PX.Y for the library mutants refers to an internal numbering of the strains, reflecting plate number and colony number from the isolation.

Table S2. Oligonucleotides used for construction and sequencing of the native ectoine cluster

Name	Sequence (5'- 3')
Sequencing primer	
Pr1_seq_*EctA*	CGAGCTGGTGCAGGTTGTAG
Pr2_seq_*EctB*	TCGATGTAGCGCTTGCCATCC
Pr3_seq_*EctC*	GAAGGAGAAGCCCACCTTATC
Construction primer	
Pr1_$_p$*tufEctABC*_FW	GGGCCCGGTACCACGCGTCATGGCCGTTACCCTGCGAA
Pr2_$_p$*tufEctABC*_RV	CCCTAGGTCCGAACTAGTCATATTACACGGTTTCTGCTTCCAGTG

Table S3. Synthetic promoters used for library construction. According to their strength, they are classified into high (H), intermediate (I), and low strength (L).

Name	Sequence (5'- 3')
H36	GGTACCTCTATCTGGTGCCCTAAACGGGGGAATATTAACGGGCCCAGGGTGGTCGCACCTTGGTTGGTAGGAGTAGCATGGGATCC
H4	GGTACCTGGATTTAGCAATTGGAGTGGCGTATCATGGACGTCCAATTGAGGTATAATAACAGGAGAAGAGGAGAAGCAGGGGATCC
H5	GGTACCGGTGGTCGTGCTGACTCTACGGGGGAGGAAGTTCAGCTGGTACTGCTCGCGTTGGCTGATAAAGGAGTAGAGTTGGATCC
H30	GGTACCAAAGTAACTTTTCGGTTAAGGTAGCGCATTCGTGGTGTTGCCCGTGGCCCGGTTGGTTGGGCAGGAGTATATTGGGATCC
H72	GGTACCGGAGACAATTTGTGCTTCGACGATTTTGTTGGTTAGCACGATCATTTACTGGCGCGCCTCCTAGGAGTATTCTTGGATCC
H17	GGTACCCCGAGTAGCCGGCCCGAGGGTTAAGGTTAGATTGTTGATCGTCGTGGCACGGTGGGACTTGTAGGAGTAAGTTGGGATCC
H3	GGTACCTTCGCTTGTAGTTTGGGGGTGTCGCTTATGGTTTAGATCTTCCGTTGCAGACGAGTGATTTGAGGATTAGAGTCGGATCC
H28	GGTACCGGGGTTTGGCCGATCGGTATTCTCCTTACATTCGGCTTTAAGTTAGCAATTACTTTATGCTTAGGAGTATCGTTGGATCC
H34	GGTACCCTGCAAGGCAATGTTCGATGTTGGGCTTCATTTTGAGGGTTTGGTTGAGTTTCAAGGGTCGTAGGATAATAATGGGATCC
I29	GGTACCCCTTTTTGAGTGATGAATTTGGTCTTGGTTCGGTTGGTGTTAGTGGGGGTGTATTGGGGTAATAGGAGTATGCTTGGATCC
I9	GGTACCGACATAGAGAAGGTCTTTTTCTGTTATAGTGTGGAAGCGTATGGACCGCGCTATGGGAGGGTAGGATTTGGATGGGATCC
I12	GGTACCAGTAGTACAGAGATATAGTTCCGGTGGGCGTGTTTGGGATGTGCTTCTGGTCGTTGCCCAATAGGAGTACGATTGGATCC
I15	GGTACCGTGGTAGTGCTTTGATCGGCTGTAGATAGTGACTTGGATTTTAGATTGTTGTCGGGTCTCTGAGGATATATTCTGGATCC
I64	GGTACCGGATTTCTTCGTGGTGTCGGGCTAGTAAGCTACGGTTGGTGGCCTTTTGTTACCCGTCGTTTAGGACTAGAGTCGGATCC
I51	GGTACCCTGTGTCGTAGGTCTCAAACGGCGTGGAGTTACGGGCTCCCGCATGGCGTGTCACTAGCGTAAGGAGCTAGAGTGGATCC
L10	GGTACCGCAGACGGTTATGGTCGCCGCTAGGTCTTGGGGAGTTTTGTTCGGTAGTTATTTATTGTTGAAGGAGATAGATTGGATCC
L80	GGTACCTTATTGTGGATGTGCTCGTATACCATTGGGGGCATGTCAGCGGCGGTTAGTAGTGTAGATGTAGGAGGGCATTGGGATCC
L26	GGTACCGTGAGTTTAGAGCAGGGGGGGGGGTTCTTTATGTATGTTCGACGTCGCTTTAGTATGCGTTAGGATTACTATCGGATCC

Table S4. Bicistronic design elements used for library construction.

	Name	Sequence (5'- 3')
High strength	BCD2	GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTTCGTACTGAAACATCTTAATCATGCTAAGGAGGTTTTCTAATG
Low strength	BCD21	GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTTCGTACTGAAACATCTTAATCATGCGAGGGATGGTTTCTAATG
Medium strength	BCD8	GGGCCCAAGTTCACTTAAAAAGGAGATCAACAATGAAAGCAATTTTCGTACTGAAACATCTTAATCATGCATCGGACCGTTTCTAATG

Table S5. Oligonucleotides used for library construction

Name	Sequence (5'- 3')
BCD-F-BamHI	ATTAATGGATCCGGGCCCAAGTTCACTTAAAAAGGAGATC AACAATGAAAGCAATTTTCGTACTGAAAC
BCD2-EctA-F1	GAAAGCAATTTTCGTACTGAAACATCTTAATCATGCTAAGG AGGTTTTCTAATGCCAACCCTGAAGCGCAACT
BCD21-EctA-F1	GAAAGCAATTTTCGTACTGAAACATCTTAATCATGCGAGGG ATGGTTTCTAATGCCAACCCTGAAGCGCAACT
BCD8-EctA-F1	GAAAGCAATTTTCGTACTGAAACATCTTAATCATGCATCGG ACCGTTTCTAATGCCAACCCTGAAGCGCAACT
BCD2-EctB-F1	GAAAGCAATTTTCGTACTGAAACATCTTAATCATGCTAAGG AGGTTTTCTAATGAAAACCTTCGAACTGAACGAATCC
BCD21-EctB-F1	GAAAGCAATTTTCGTACTGAAACATCTTAATCATGCGAGGG ATGGTTTCTAATGAAAACCTTCGAACTGAACGAATCC
BCD8-EctB-F1	GAAAGCAATTTTCGTACTGAAACATCTTAATCATGCATCG GACCGTTTCTAATGAAAACCTTCGAACTGAACGAATCC
BCD2-EctC-F1	GAAAGCAATTTTCGTACTGAAACATCTTAATCATGCTAAGG AGGTTTTCTAATGATCGTGCGCACCCTG
BCD21-EctC-F1	GAAAGCAATTTTCGTACTGAAACATCTTAATCATGCGAGGG ATGGTTTCTAATGATCGTGCGCACCCTG
BCD8-EctC-F1	GAAAGCAATTTTCGTACTGAAACATCTTAATCATGCATCGG ACCGTTTCTAATGATCGTGCGCACCCTG
EctA-R-NotI	ATTAATGCGGCCGCTCATTATGCGTGTTCTTTCAGTTCTTC TTCCAG
EctB-R-NotI	ATTAATGCGGCCGCTCATTAGGATGCCTGGTTTTCGGTCT
EctC-R-NotI	ATTAATGCGGCCGCTCATTACACGGTTTCTGCTTCCAGT
XhoI-EctA-F	GCTCCTCGAGAAAGGGAACAAAAGCTGGGTAC
EctA-SalI-XbaI-R	CGAGTCTAGAGTCGACTCACCGACAAAACAACAGATAAAA
XhoI-EctB-F	GCTCCTCGAGAAAGGGAACAAAAGCTGGGTAC
EctB-SalI-XbaI-R	CGAGTCTAGATTGTCGACTCACCGACAAACAACAGATAAA A
SalI-EctC-F	GCTCGTCGACAAAGGGAACAAAAGCTGGGTAC
EctC-XbaI-R	CGAGTCTAGATCACCGACAAACAACAGATAAAA

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