Supplementary Figures for:

Tinkering with osmotically controlled transcription allows enhanced production and excretion of ectoine and hydroxyectoine from a microbial cell factory

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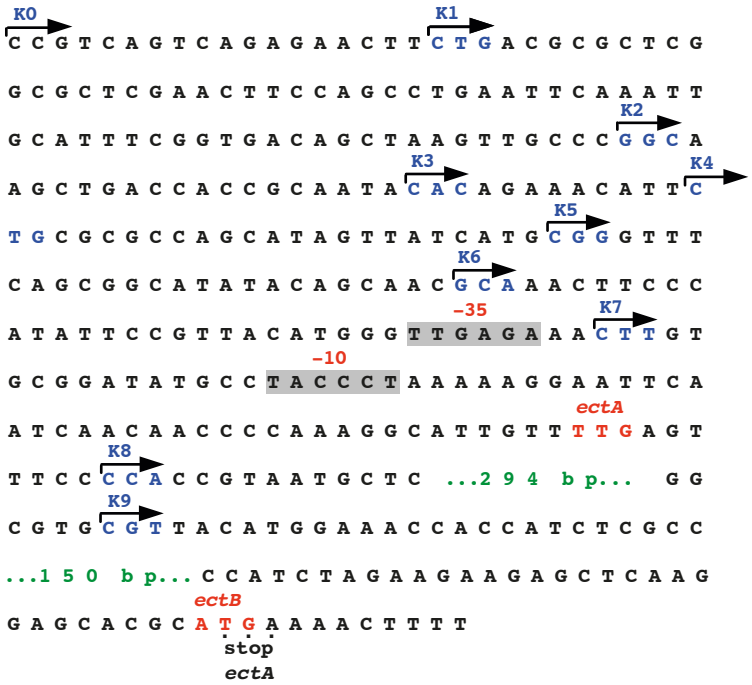
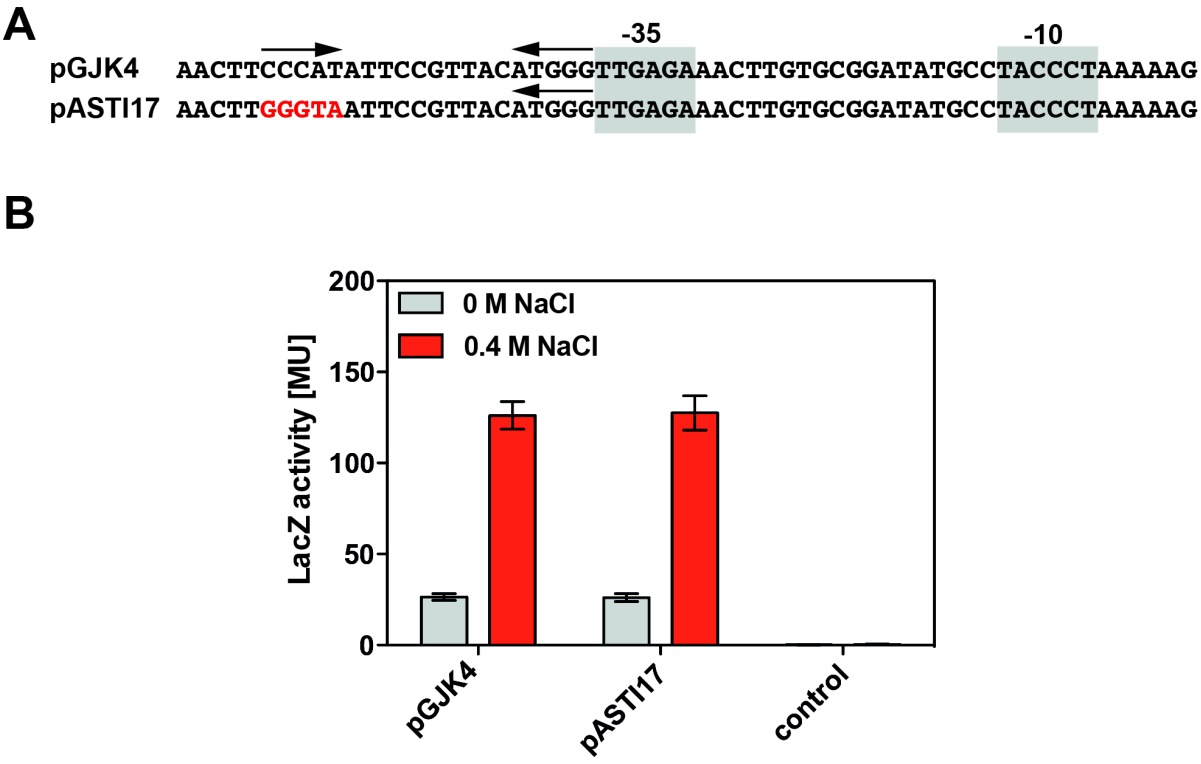
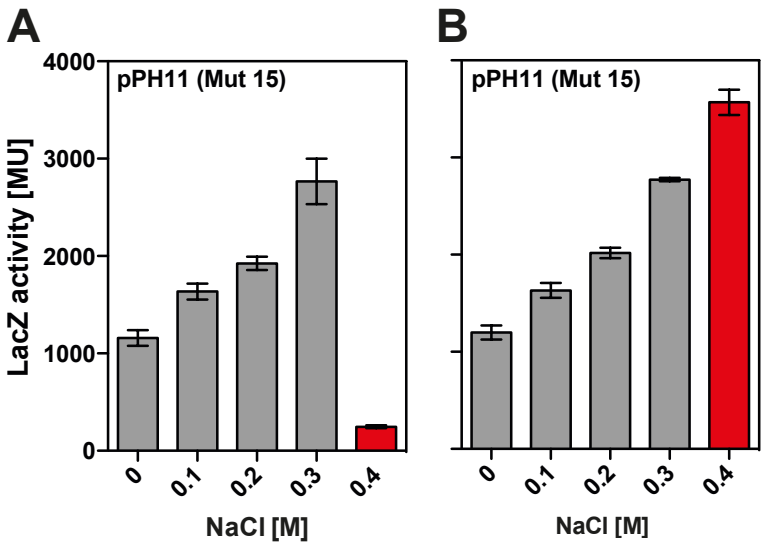


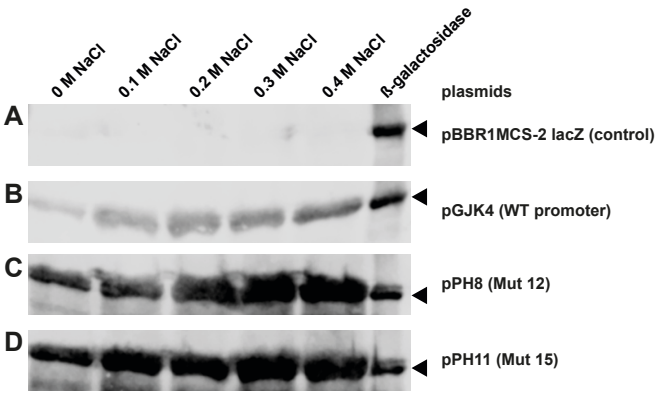
Supplementary Figure S1. Promoter region upstream of *ectA* present on the *ect-lacZ* fusion plasmid pGJK4. Plasmid pGJK4 carries an *ectA-ectB-lacZ* reporter fusion that is expressed from the *ect* promoter present upstream of the *ectA* gene (the -10 and -35 sequences are highlighted in grey). The *P. stutzeri* A1501 genomic DNA located in front of the *ectA* start codon has a length of 264 bp. This genomic segment was successively shortened as indicated by the K0 to K9 deletion endpoints (marked in blue). The coding regions of the *ectA* and *ectB* genes of *P. stutzeri* A1501 overlap; the stop codon (TGA) of *ectA* is indicated by three dots, and the start codon of *ectB* is marked in red.



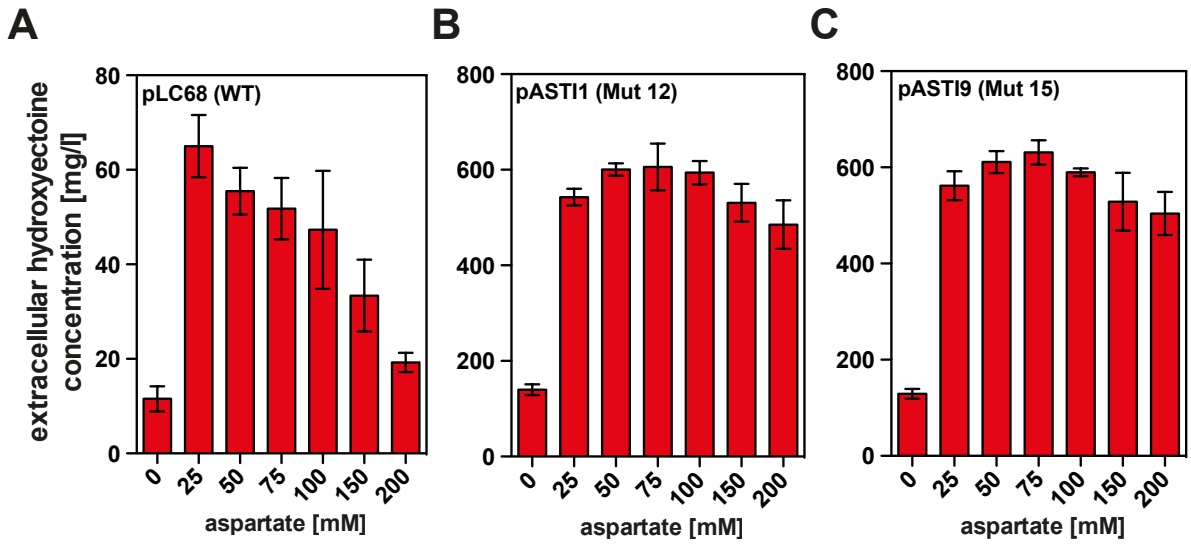
Supplementary Figure S2. Site-directed mutagenesis of an inverted repeat located upstream of the *ectA* promoter. (A) Sequence comparison of the inverted repeat upstream of the *ectA* promoter in the wildtype *ect-lacZ* reporter plasmid pGJK4 and its mutant derivative pASTI17. The mutated DNA sequence is indicated in red. (B) Cells of strain MC4100 carrying either the wildtype *ect-lacZ* reporter plasmid pGJK4, its mutant derivative pASTI17, or the cloning vector pBBR1MCS-2 *lacZ* (control) were grown in MMA without and with 0.4 M NaCl. When the cultures reached an optical density of OD_{578} 1.8-2, the cells were harvested by centrifugation and assayed for their ß-galactosidase activity. The data shown were derived from three independently grown cultures and each enzyme assay was performed at least twice. ß-galactosidase enzyme activity is given in Miller units (MU).



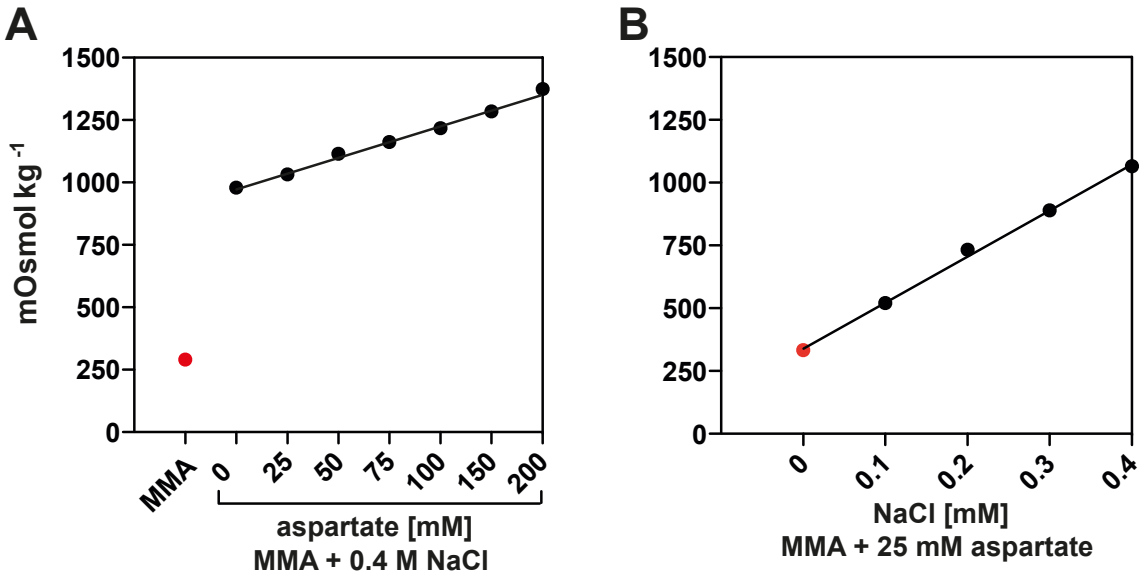
Supplementary Figure S3. Transcriptional activity of the *ect* promoter variant Mut 15 in response to increasing osmolarity. Cells of strain MC4100/(pPH11) carrying an *ect-lacZ* reporter fusion under the control of a mutant *ect* promoter changed in its spacer length, -10, and -35 regions to the consensus sequence of Sig^{70}-type promoters (Mut 15) were cultivated in MMA with increasing NaCl concentrations. When the cultures reached an optical density of OD_{578} 1.8-2, the cells were harvested by centrifugation and assayed for their ß-galactosidase activity. The data shown were derived from a set of in parallel grown cultures for each of the indicated NaCl concentration. Each enzyme assay was performed twice. ß-galactosidase enzyme activity is given in Miller units (MU). The bars shown in red highlight differences on LacZ reporter enzyme activity of individual cultures grown in parallel.



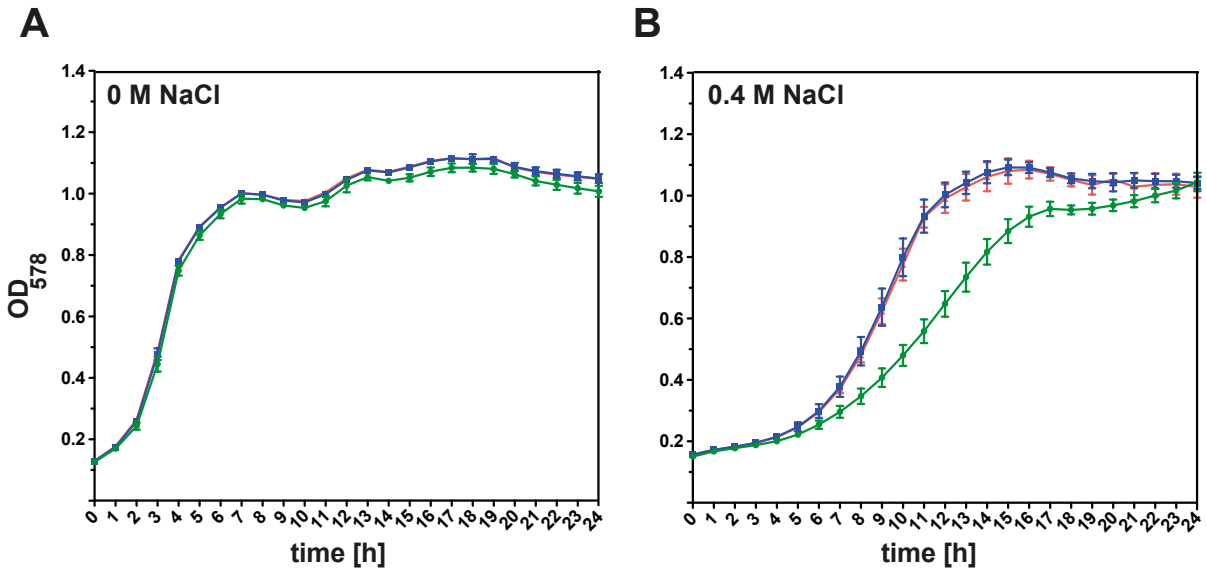
Supplementary Figure S4. Western blot analysis of *ect-lacZ* reporter fusions of the wild type *ect* promoter and two of its variants. MC4100 cells carrying the plasmids (A) pBBR1MCS-2 *lacZ*, (B) pGJK4 (wild-type *ect* promoter), (C) pPH8 (Mut 12 *ect* promoter variant) and (D) pPH11 (Mut 15 *ect* promoter variant) were cultivated in MMA with the addition of various NaCl concentrations. When the cultures reached an optical density of OD_{587} 1.8, the cells were harvested by centrifugation, and lysed by boiling for 5 min at 95° C in 2 x SDS/ bromophenol blue loading buffer. The proteins from the cell extracts were electrophoretically separated on a 7% SDS–polyacrylamide gel and were then analyzed by Western blotting using a primary rabbit antibody directed against a hybrid fusion protein (ProW-LacZ) derived from a component of the *E. coli* ProU ABC osmolyte transport system.



Supplementary Figure S5. Comparison of ectoine and hydroxyectoine production and extracellular accumulation in the presence of increasing L-aspartate concentrations. The *E. coli* mutant strain FF4169 (*otsA1*::Tn*10*) carrying (A) the ectoine/hydroxyectoine production plasmid with the wild type *ect* promoter (pLC68); (B) the ectoine/hydroxyectoine production plasmid with promoter variant Mut 12 (pASTI1); or (C) the ectoine/hydroxyectoine production plasmid with promoter mutant Mut 15 (pASTI9), were cultivated in MMA containing 0.4 M NaCl and increasing concentrations of L-aspartate. After 48 h of growth of the cultures at 37° C, the cells were harvested by centrifugation and the supernatant of the cultures were assayed for secreted ectoines by HPLC analysis. Only hydroxyectoine was present in the supernatant of the cultures..



Supplementary Figure S6. Osmolarity of the medium used for cultivation of the recombinant *E. coli* cell factory. (A) MMA was supplemented with 0.4 M NaCl and increasing concentrations of aspartate were then added. (B) MMA was supplemented with 25 mM aspartate and increasing concentrations of NaCl. The osmolarity values of the media were determined with a vapor pressure osmometer (Vapor Pressure 5500; Wescor, Inc., Utah).



Supplementary Figure S7. Effects of plasmid-based ectoine/hydroxyectoine production on the growth of the *E. coli* strain SK51. Cells of strain SK51 carrying either the empty cloning vector pHSG575 (green), or the ectoine/hydroxyectoine hyper-production plasmids pASTI1 (Mut 12; red), and pASTI9 (Mut 15; blue) were grown at 37 °C in (A) MMA or (B) MMA with 0.4 M NaCl in 48 well plates in a microplate reader. The optical density (OD_{578}) of the cultures was monitored every hour. The data shown were derived from four independently grown replicates of each strain.