ChemBioChem

Supporting Information

Evaluation of an E. coli Cell Extract Prepared by Lysozyme-Assisted Sonication via Gene Expression, Phage Assembly and Proteomics

Elisabeth Falgenhauer, Sophie von Schönberg, Chen Meng, Andrea Mückl, Kilian Vogele, Quirin Emslander, Christina Ludwig, and Friedrich C. Simmel*
Experimental Section

**Chemicals.** Unless otherwise noted all chemicals were ordered from Sigma Aldrich (exceptions are listed in Table S1). The composition of 2x YTP medium, S30A and S30B buffer was adopted from Sun et al.[1] The TXTL buffer containing amino acids, nucleotides, tRNAs and other ingredients was also prepared according to Sun et al. and screening experiments for Mg-glutamate, K-glutamate, PEG and DTT concentrations were performed (see Figure S2).

**Cloning of plasmids.** All plasmids were cloned using a standard restriction/ligation protocol. Linear DNA fragments were ordered from IDT and cloned into the target vector (pSB1A3). The final plasmid sequences are listed in Tables S6-S9. Primers were ordered from Eurofins Genomics. Plasmids were sequenced by GATC Services/Eurofins Genomics.

**Bacterial strains and culture conditions.** Figure 1 gives a quick and Figure S1 a detailed overview over the cell extract preparation workflow. Bacterial cell extracts were prepared from *E.coli* Rosetta 2 (DE3) cells. Cells from glycerol stocks were grown overnight in an incubator shaker (Innova44, New Brunswick) in 2x YT+P medium containing selective antibiotic (Chloramphenicol, Cm) at 37 °C and 250 rpm. 2x YT medium is very common for this purpose and is often supplemented with phosphate and also with glucose,[1-2] which avoids phosphatase induction and results in reduced ATP hydrolysis activity.[2a] On the following day, cells were diluted 1:100 in 2x YTP+Cm medium and cultivated either in eight shaking flasks (666 mL each, 37 °C, and 250 rpm) or in a 2 L bioreactor.

**Cultivation of bacteria in a bioreactor.** Cells were cultured in a 2 L lab scale bioreactor (Minifors 2, Infors) with pO2 monitoring and pH control. Initially, the culture was agitated at 500 rpm and aerated with pressurized air at a rate of 2 L/min. To keep the oxygen saturation over 14%, we regulated the aeration rate stepwise up to 4 L/min and increased the stirrer speed up to 1000 rpm. We used a constant feeding with a glucose solution at a rate of 0.85 g/(L h) during the entire cultivation time. As we grow the bacteria in 2x YT medium supplemented with potassium phosphate (monobasic and dibasic), the medium is buffered and the external pH control during the cultivation is not obligatory. Due to the favorable growth conditions in the bioreactor, the exponential growth phase was prolonged and we were thus able to harvest the cells in an OD range from 5-6. A linear fit in a semi-logarithmic plot revealed that the bacteria were still in the exponential growth phase at this point (see Figure S3). Growth in a bioreactor allowed us to increase the biomass yield (wet pellet) to 10 g per liter culture volume compared to shaking flask cultivation, which typically yielded only 2-2.5 g/L.
**Cell harvest and washing.** Cells were harvested at OD 1.8-2.0 when cultivated in shaking flasks, or at OD 5-6 when grown in a bioreactor. After harvesting, we distributed the cell suspension to four bottles (750 mL each) for centrifugation (15 min, 4 °C, max. speed 4600 rcf, Rotanta 460R, Hettich). In the case of shaking flask cultivation, a second round of cell harvesting was necessary. The supernatant was decanted and cells were resuspended in S30A buffer (300 mL per centrifuge bottle). Centrifugation and washing (2 bottles were pooled, washing with 2x 350 ml) was repeated. Afterwards, we resuspended the cell pellets in 2x 40 mL S30A buffer and transferred the suspension into two 50 mL falcon tubes. Cells were centrifuged at 3000 rcf at 4 °C for 10 min. The supernatant was decanted and the pellet was again centrifuged at 3000 rcf at 4 °C for 3 min. The supernatant was then removed using a pipette. After determination of the wet pellet mass (typical 20 g from bioreactor cultivation), the pellets were flash frozen in liquid nitrogen and stored at -80 °C.

**Preparation of cell extract.** Cell pellets obtained in the previous step were thawed on ice and resuspended in S30A buffer (1 mL buffer per gram pellet mass) by vortexing. The cell suspension was then split into 4 mL aliquots and up to 1 mg/mL lysozyme (Lysozyme from chicken egg, >40,000 units/mg, Sigma Aldrich) was added. After mixing by pipetting up and down the cell suspension was incubated on ice for 15 min to allow the lysozyme to degrade the peptidoglycan layer. The incubation on ice should prevent a loss in activity due to a proteolytic degradation of released proteins. Cells were sonicated on ice using a SONOPULS mini20 (Bandelin) with a working frequency of 30 kHz at 10% amplitude. We applied 0-20 pulse cycles with durations of 10 sec each. Tubes were sonicated in series, so the cooling time between different rounds is 10 sec times the number of tubes. Thus, our typical pausing time between sonication pulses was 80-100 sec (for 8-10 samples) per tube. Since throughput with sonication is limited, we recommend to not to prepare larger volumes than 40 mL cell suspension at a time (at a constant sample volume of 4 mL per tube). After every second cycle, samples were mixed by pipetting up and down using a 5 mL pipette with the tip cut off. After lysis, samples were transferred into 2 mL tubes and centrifuged at 20,000 rcf for 30 min - 60 min at 4 °C until a sufficiently stable pellet had formed. Pellet-free supernatant was transferred into 2 mL screw cap tubes (1-1.5 mL volume per tube), leaving the caps unscrewed. The open tube containing the cell extract was inserted in a 15 mL Falcon tube as described by Sun et al. \(^1\) and incubated at 37 °C and 250 rpm for 80 min in an Innvova44 shaker for a run-off reaction. Afterwards, samples were transferred into individual reaction tubes and centrifuged at 12,000 rcf for 10 min at 4 °C. Pellet-free supernatant was transferred into 10 kDa MWCO dialysis tubing and dialyzed against S30B buffer at 4 °C for 3 h. Cell extract was then extracted from the tubing, distributed into 1.5 mL centrifugation tubes and centrifuged at 12,000 rcf for 10 min at 4 °C. Cell extract was finally...
aliquoted into the desired aliquot size (usually 30 µl), flash frozen in liquid nitrogen and stored at -80 °C.

**Bicinchoninic acid (BCA) assay.** Each cell extract batch was subjected to a Bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit, Reducing Agent Compatible Thermo Fisher Scientific) according to the manufacturer’s protocol to determine the total protein content of the prepared cell extracts. The results displayed in Figure 2A and C show the mean protein contents of three biological replicates, Figure S4 shows the protein content for each single extract.

**Transcription-translation of fluorescent proteins (TXTL test).** High copy number plasmids (iGEM part pSB1A3) containing a constitutive promoter (iGEM part J23106), an RBS (iGEM part B0034), the coding sequence for mScarlet-I (RFP), mVenus (YFP), GFPmut3 (iGEM part E0040) or mTurquoise-2 (CFP) and a Terminator (iGEM part B0015) were purified using a Qiagen Plasmid Midi Kit and afterwards phenol chloroform precipitated. Whereas Takahashi et al recommended to use a protein content of 10 mg/mL in the final cell-free protein expression reaction,[3] we used the same dilution factor for all tests independently of the protein content. Our samples contained 33.3% cell extract, 41.7% buffer solution and 25% plasmid mix or water for blank samples. The final plasmid concentration in each sample was 3 nM and the TXTL buffer composition resulted in sample concentrations of 4 mM Mg-glutamate, 60 mM K-glutamate, 1.5 mM each amino acid except leucine, 1.25 mM leucine, 50 mM HEPES, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA and 2.5% (w/v) PEG-8000.

**Fluorescence acquisition.** Transcription-translation of fluorescent proteins was monitored with a plate reader (FLUOstar Omega, BMG Lab Tech) at a temperature of 29 °C. Fluorescence measurements were performed every 3-6 min using the corresponding filter sets for RFP, YFP, GFP or CFP. Time traces were background corrected with blank values and molar concentrations were calculated using calibration curves for each of the four fluorescent proteins. To compare the fluorescence time traces, their maximum slopes (maximum protein expression rate) and end levels were determined. The mean end levels (mean of 3 biological replicates) are shown for the YFP reporter in Figure 2 as bar graphs for the single lysis settings. The data for RFP, GFP and CFP and the mean maximum protein expression rates (mean of 3 biological replicates) are shown in Figure S6.

**Phage assembly.** Phage assembly was performed according to the protocol of Rustad et al. [4] with the following adjustments: Phage DNA was mixed with cell extract, an energy solution and an amino acid solution as described in Sun et al. [1] using the same TXTL buffer composition as described in the TXTL test section.[5] For 6 reactions (of 13 µL volume
each), 2.5 µL PEG 8000 (36% w/v), 4 µL dNTPs (25 mM), 0.8 µL ATP (500 mM), 37.5 µL TXTL buffer, 2 µL GamS (150 µM), 28.5 µL cell extract and 1.6 µL DNA (10 nM) were mixed with nuclease-free water to a final volume of 80 µL. All constituents were mixed (except DNA) on ice and incubated for 5 min, followed by the addition of DNA. This 13-µL assembly mix was incubated for 4 h at 29 °C to express the bacteriophages.

**Plaque assay.** The plaque-assay was performed with the top-agar method, with 0.5% agarose in NZCYM (Carl Roth), a standard medium for *E. coli* cultures and bacteriophages.[6] The agar was melted and stored before use in a water bath at 48 °C. Separately, phage dilutions of $10^2$-$10^8$-fold in phage buffer (1x PBS, 1mM MgCl$_2$, 1mM MgSO$_4$) were prepared. 100 µL of each dilution was mixed with an equal volume of an overnight culture of the corresponding host bacterium. This mixture was added to the 0.5% agarose NZCYM medium aliquots and poured on a 1% NZCYM agar plate. After solidified at room temperature, the plates were incubated at 37 °C until plaques became visible.

**Sample preparation for mass spectrometry.** All cell extracts were dried to completeness using a centrifugal evaporator (Centrivap Cold Trap -50, Labconco, US). The resulting pellets were dissolved in lysis buffer (8 M Urea, 5 mM EDTA, 100 mM NH$_4$HCO$_3$) to a final concentration of 1 mg/mL. Next, 45 µg of each sample was reduced with 10 mM dithiothreitol (DTT) (30 min at 30 °C) and alkylated with 55 mM 2-chloroacetamide (CAA) (30 min in the dark at 25 °C). The samples were diluted 1:4 in 50 mM NH$_4$HCO$_3$ and double-digested with trypsin (1 h and 13 h at 30 °C, Trypsin gold Mass Spectrometry Grade, Promega), which was added twice at a ratio of trypsin:protein = 1:100 (by mass). The reaction was stopped with 1% formic acid (FA) and the resulting peptides were purified. For that in-house built C18 tips (5 disks of Sep-Pak Vac C18 material, Waters, US) were equilibrated with 250 µl 100% acetonitrile (ACN), 250 µl elution solution (40% ACN, 0.1% FA) and 250 µl washing solution (2% ACN, 0.1% FA) at 1500 g. The samples were loaded into the tips (centrifugation for 2 min at 500 g) and washed three times with washing solution for 2 min at 1500 rcf. Finally, the peptides were eluted with 100 µl elution solution for 2 min at 500g. The samples were dried to completeness and resuspended in washing solution 45 µl right before the MS measurement.

**Proteomics data acquisition.** Generated peptides were analyzed on an Dionex Ultimate 3000 RSLCnano system coupled to an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, Bremen, GER). For each analysis an injection amount of = 0.1 µg of peptides was delivered to a trap column (ReproSil-pur C18-AQ, 5 µm, Dr. Maisch, 20 mm x 75 µm, self-packed) at a flow rate of 5 µL/min in 100% solvent A (0.1% formic acid in HPLC grade water). After 10 min of loading, peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 µm, Dr. Maisch, 400 mm x 75 µm, self-packed)
and separated using a 50 min gradient from 4% to 32% of solvent B (0.1% formic acid in acetonitrile and 5% (v/v) DMSO) at 300 nL/min flow rate. Both nanoLC solvents contained 5% (v/v) DMSO. The Fusion Lumos Tribrid Mass Spectrometer was operated in data dependent acquisition and positive ionization mode. MS1 spectra (360–1300 m/z) were recorded at a resolution of 60,000 using an automatic gain control (AGC) target value of $4 \times 10^5$ and maximum injection time (maxIT) of 50 ms. After peptide fragmentation using higher energy collision induced dissociation (HCD), MS2 spectra of up to 20 precursor peptides were acquired at a resolution of 15,000 with an automatic gain control (AGC) target value of $5 \times 10^4$ and maximum injection time (maxIT) of 22 ms. The precursor isolation window width was set to 1.3 m/z and normalized collision energy to 30%. Dynamic exclusion was enabled with 20 s exclusion time (mass tolerance +/-10 ppm). MS/MS spectra of species that were singly-charged, unassigned or with charge states > 6+ were excluded.

**Proteomics data analysis.** Peptide identification and quantification was performed using the software MaxQuant (version 1.6.3.4) with its built-in search engine Andromeda.[7] MS2 spectra were searched against the *E. coli* (strain B / BL21-DE3) reference proteome from Uniprot (UP000002032, 4156 protein entries), supplemented with common contaminants (built-in option in MaxQuant). Trypsin/P was specified as proteolytic enzyme. Precursor tolerance was set to 4.5 ppm, and fragment ion tolerance to 20 ppm. Results were adjusted to 1% false discovery rate (FDR) on peptide spectrum match (PSM) level and protein level employing a target-decoy approach using reversed protein sequences. The minimal peptide length was defined as 7 amino acids, the "match-between-run" function was disabled. For full proteome analyses carbamidomethylated cysteine was set as fixed modification and oxidation of methionine and N-terminal protein acetylation as variable modifications.

Proteins were quantified using Label Free Quantification (maxLFQ).[8] The maxLFQ intensity was log transformed before downstream analysis. T-tests were used in the differential analysis (using R version 3.6.3). The false discovery rates (FDRs) were calculated from the p-value using the Benjamini-Hochberg method.[9] Proteins with FDR < 0.05 and unique proteins (proteins which were present in all three replicates of one sample, but not present in one of the three replicates of the other sample) were selected as significantly differentially expressed proteins and passed to the DAVID functional annotation[10] for enrichment analysis. To simplify the presentation, proteins in GO terms with FDR below 0.05 were roughly classified according to keywords using the UniProt database[11] related to protein expression and energy regeneration and their influence on gene expression was interpreted accordingly.
Figure S1: Detailed flow chart of the cell extract preparation protocol. See methods part in main text for more information.
Figure S2: Cell extract buffer screening experiments. Mg-glutamate, K-glutamate, PEG and DTT concentration was screened for 3 different cell extract batches prepared using the S16/L0.8 lysis setting. The fluorescence end levels for 2 different reporter plasmids (CFP and YFP) are shown. 4 mM Mg-glutamate, 60 mm K-glutamate, 2.5% PEG and 0 mM DTT were chosen.
Figure S3: Growth curves for cultivation in shaking flasks and in a bioreactor. (A) pO2-saturation plot. We fed glucose with a constant rate of 0.85 g/(l*h) and kept the oxygen level above 14% during the whole batch time by regulating the stirrer speed (500-1000 rpm) and the aeration rate (2-4 l/min) up. (B) OD was measured about every 30 min. Batches BR2 and BR3 show an elongated lag phase at the beginning. The desired OD was reached after about 200 min for batch BR1 and after about 280-300 min for batches BR2 and BR3. (C) Semi-logarithmic plot. The bacteria have doubling times between 32 and 38 min. (D) Growth curve for two different shaking flasks, cells are usually harvested between OD 1.8 and 2. (E) Semi-logarithmic plot. Bacteria were harvested in the late-log growth phase.
Figure S4: (A) For the shaking flask replicates lysozyme incubation had a higher impact on cell lysis than sonication cycles. The protein content was generally higher in samples without sonication cycles (S0/L0.5 and S0/L1) than in samples not incubated with lysozyme (S5/L0 and S15/L0). Samples which were treated with a combination of lysozyme incubation and sonication cycles showed a high protein content. (B) In contrast to the shaking flask replicates, the bioreactor replicates showed a higher deviation in protein content among the biological replicates than among the different lysis settings.
Figure S5: Expressed protein end level against maximum protein expression rates. The maximum expression rates correlates well with the expressed protein end levels.
Figure S6: End levels of expressed proteins and maximum protein expression rates. (A) Shaking flask samples, which were not treated with lysozyme but with 5 or 15 sonication cycles, show the lowest fluorescence end levels in a cell-free test for all tested reporter proteins. Samples which were lysed without sonication cycles show higher signals, so lysozyme has no negative effect on the protein synthesis. The fluorescence intensities of samples with nonzero lysis conditions are the highest, a signal decrease can be observed for 15 sonication cycles. (B) The maximum protein expression rates show the same trends. (C) For both lysozyme concentrations an increase of sonication cycles results in an increased fluorescence signal in the bioreactor samples. Independent of the lysozyme concentration, 12-16 sonication cycles appear optimal, while 20 cycles result in reduced signals. (D) Mean of bioreactor replicates BR1, BR2 and BR3. The maximum expression rate correlates with the expressed protein end levels.
**Figure S7:** Comparison of the best shaking flask replicates with the best bioreactor replicates, a bead beating batch and a commercial cell extract. (A) Normalized fluorescence data for all tested reporters. The commercial extract shows low levels for all reporter plasmids except the p70 control plasmid. (B) Not normalized fluorescence data for the YFP plasmid and the GFP control plasmid. The levels are very different, but these levels depend not just on protein concentration but also on quantum yield, brightness and other parameters. (C) p70-GFP control plasmid in commercial cell extract. We purified the p70-GFP control plasmid using our standard technique and performed a TXTL test. The commercial plasmid has a high signal whereas our self-purified one has almost no signal. The commercial cell extracts seems to be sensitive to residual chemicals in our plasmid.

**Figure S8:** Plaque assays for the shaking flask and bioreactor replicates. For all self-made cell extracts except for the S12/L0.8 samples comparable phage titers were measured. For the commercial kit one order of magnitude less phages could be assembled and the bead beating batch performed much worse, just 500 phages could be counted in one replicate, no plaques could be counted for the second replicate.
Figure S9: Comparing proteomes of extracts from shaking flask and bioreactor. (A) Volcano plot of shaking flask samples S5/L0.5 against bioreactor samples S5/L0.5. No protein had a FDR smaller than 0.05, but some unique proteins were found and used for enrichment analysis. (B) Summary of protein numbers. Compared to the bioreactor samples (S5/L0.5) the shaking flask extracts (S5/L0.5) contain 11 unique proteins, which were subjected to an enrichment analysis. (C) Result of enrichment analysis. For the shaking flask samples just 3 proteins were found in GO terms with GO FDR < 0.05. These proteins can be assigned to anaerobic growth conditions. In summary shaking flask extracts and bioreactor extracts, which were treated with the same lysis conditions show no differences in their proteome except for abundance of proteins related to anaerobic growth. This is an expected result as we in contrast to bioreactor cultivation did not provide additional oxygen in shaking flask cultivation.
Figure S10: Comparison of the proteomes of extracts prepared from bioreactor and shaking flask cultures and a commercial kit. (A) Volcano plot of shaking flask samples S0/L1 against S15/L1. The t-test of S0/L1 against S15/L1 extracts gives 1 and 6 proteins with FDR < 0.05 (i.e., –log10(FDR) > 1.3). In addition, 3 (S0/L1) and 47 (S15/L1) unique proteins could be found and were also subjected to an enrichment analysis. (B) Enrichment analysis derived from the comparison of S0/L1 against S5/L1 and from the comparison shown in (A). No GO terms with FDR<0.05 could be found for the S0/L1 extracts in both comparisons but in total 7 (S0/L1 against S5/L1) and 21 (S0/L1 against S15/L1) proteins were found in GO terms with FDR < 0.05 for the S5/L1 and S15/L1 samples respectively. For both cases these could be assigned to keywords related to DNA replication, relaxation, repair or recombination or were transcriptional regulators. (C) Volcano plot of bioreactor samples S5/L0.5 against the commercial kit. The commercial and bioreactor extracts differ in abundance of just 8 and 5 proteins with FDR
< 0.05 (i.e., \(-\log_{10}(FDR) > 1.3\)), but had 30 and 121 unique proteins, which were subjected to an enrichment analysis. (D) Enrichment analysis derived from the comparison shown in (C). GO terms with FDR < 0.05 included in total 11 proteins in the bioreactor samples, which were related in amino-acid biosynthesis, a nuclease or not relevant for cell-free gene expression. (E) Volcano plot of shaking flask samples S5/L0.5 against the commercial kit. The commercial and shaking flask extracts differ in abundance of 309 versus 356 proteins with FDR < 0.05 (i.e., \(-\log_{10}(FDR) > 1.3\)). Proteins with an FDR below 0.05 and proteins exclusively found only in shaking flask preparations or in commercial extract were subjected to an enrichment analysis. Proteins which were assigned to GO terms with an FDR below 0.05 are highlighted in the plot. (F) Fraction of proteins with FDR < 0.05 against fold change and fraction of proteins found in GO terms with FDR < 0.05 against fold change (unique proteins are not considered, as no fold change can be calculated). 27% of the proteins are less than 1.5 fold enriched, 67% less than 2 fold and 90% less than 3 fold.
Proteomic differences between our shaking flask replicates SF1, SF2 and SF3 with lysis setting S5/L0.5 and the commercial extract

The proteomics analysis revealed the greatest differences between our shaking flask batches S5/L0.5 and the commercial kit. The differences can be a result of differences in the culture conditions, the lysis procedure and other cell extract processing steps. Even though we had no detailed information about the preparation procedure of the commercial kit, we compared the two extracts, as the GFP expression yield was comparable. We found in total 172 (commercial extract, thereof were 15 unique proteins) and 31 (shaking flask batches, thereof were no unique proteins) proteins in GO terms with FDR<0.05, thereof were 90% of the proteins less than 3 fold enriched (67% less than 2 fold and 27% less than 1.5 fold enriched, see Figure S10F). The potential role of the single proteins is discussed in the following:

**Energy metabolism.** Compared to commercial extract, in our home-made batches 9 out of 10 enzymes from the glycolysis pathway were enriched, including glucokinase, which converts cytosolic glucose into glucose-6-phosphate (triosephosphate isomerase was the only enzyme that was not present at a higher abundance). Further, we found enzymes such as adenylate kinase or guanylate kinase enriched in our self-made batches, which potentially play a role in ATP and GTP regeneration in vitro. On the other hand, we detected higher abundance of the subunits IIB and IIC of *E. coli’s* major transmembrane carbohydrate transport system (the Pst system) in the commercial extract. This indicates that the lysis conditions used for the commercial extract might cause a higher fragmentation of the membrane and thus a more efficient release of trans-membrane proteins. In contrast to glycolysis, enzymes of the TCA cycle were enriched in the commercial extract. In shaking flask batches, we also detected an increased amount of dehydrogenases encoded by the *glpABC* operon, which belongs to the glycerol kinase pathway and is responsible for anaerobic energy generation. No differences were detected in the pentose phosphate pathway.

**Transcription/translation.** Together with core RNA polymerase, sigma factor RpoD (σ^70) dominates the transcription in exponentially growing cells. We found RpoD more highly abundant in the commercial extract. Furthermore, during envelope stress several sigma factors are upregulated in *E. coli*, including sigma factor RpoE (σ^24), which was also found more abundant in the commercial extract. Other stress factors such as translational regulator CsrA (envelope/periplasmic stress) and nitrogen-limitation factor RpoN (σ^54) were enriched in the commercial cell-free system, as well as the ribosomal subunit S22, which is associated with stationary bacterial growth. Apart from these stress indicators, a variety of other transcription regulators such as LacI, MarR, GntR, DeoR or LysR were enriched compared to our home-made shaking flask batches.

In addition, also translational capacity appeared to be enriched in the commercial system. In particular, we found 20 out of the 22 ribosomal proteins of the 30S subunit at higher abundance in the commercial extract, including S22 (see above) and the essential ribosomal protein S12, which takes part in both tRNA and ribosomal subunit interactions. In case of the 50S subunit, we found 24 out of 33 ribosomal proteins more abundant in the commercial extract, including the small ribosomal protein L34 (5.3 kDa). Also other translation-related proteins showed higher abundance in the commercial
extract such as initiation (IF-2, IF-3) and elongation (EF-4, EF-Tu, SelB) factors, but also the ribosomal silencing factor (RsfS) which inhibits ribosome association and prevents translation.

**Degradation of nucleic acids and proteins.** We also found notable differences between the cell extracts in degradation pathways. In the commercial extract ribonuclease 2 and E were enriched (p-value 0.05), which are mainly involved in mRNA degradation. Other ribonucleases participating in RNA maturation and processing (RNAse 3, G, PH and R) were also more abundant in the commercial extract. On the other hand, endoribonuclease L-PSP, also acting on mRNA, was found more highly concentrated in self-made batches. We also investigated the presence of proteases in the cell extracts. We found both subunits HslV and HslU (annotation at the transcript level) of the proteasome-like degradation complex HslVU (ClpQY) enriched in the commercial extract, which unfolds proteins under ATP consumption.

**Biosynthesis.** Interestingly, many proteins involved in amino acid biosynthesis were more abundant in our self-made CF system compared to the commercial extract, suggesting their potential use for amino acid production inside the extract starting from inexpensive precursors. Finally, the chaperone cofactor GroES was more highly expressed in the commercial batch, but not its chaperone complex GroEL, even though it is encoded by the same operon.
Table S1: List of chemicals which were not ordered from Sigma Aldrich.

| Chemical                          | Supplier          |
|----------------------------------|-------------------|
| 2-Chloroacetamide (CAA)          | Merck (GER)       |
| Acetonitrile (ACN)               | Merck (GER)       |
| CTP                              | Carl Roth (GER)   |
| GamS                             | Arbor Bioscience (US) |
| Glycerol                         | Carl Roth (GER)   |
| GTP                              | Carl Roth (GER)   |
| IPTG                             | Carl Roth (GER)   |
| Nuclease-free water              | Carl Roth (GER)   |
| Phosphate buffered saline (PBS)  | VWR Life Science (GER) |
| RTS Amino Acid Sampler           | Biozym Scientific (GER) |
| Tris                             | Carl Roth (GER)   |
| Trypsin                          | Roche (CH)        |
| UTP                              | Carl Roth (GER)   |

Table S2: All buffer and media compositions were adapted from Sun et al. [1]. Anyway the composition of the growth medium and the buffers needed for cell washing and cell extract dialysis are listed below. A detailed protocol for the TXTL buffer preparation is shown in the protocol of Sun et al.[1].

a) 2xYTP medium

| Component     | Concentration |
|---------------|---------------|
| 2xYT          | 31 g/l        |
| K2HPO4        | 40 mM         |
| KH2PO4        | 22 mM         |

b) S30A (cell washing, 2l are needed)

| Component              | Concentration |
|------------------------|---------------|
| Potassium glutamate    | 60 mM         |
| Magnesium glutamate    | 14 mM         |
| Tris                   | 50 mM         |

To reach pH 7.7, titrate with acetic acid. Add DTT to 2mM final concentration just before use. Store at 4 °C.

c) S30B (cell extract dialysis, 2l are needed)

| Component              | Concentration |
|------------------------|---------------|
| Potassium glutamate    | 60 mM         |
| Magnesium glutamate    | 14 mM         |
| Tris                   | 5 mM          |

To reach pH 8.2, titrate with 2 M Tris. Add DTT to 1 mM final concentration just before use. Store at 4 °C.
Table S3: Characteristics of reporter proteins. Codon adaption indices for mScarlet, mVenus, GFP and mTurquoise calculated by CAIcal.[12] The codon usage table of Escherichia coli B and of Shigella flex. 2a were used for the calculation. The second one is more related to MRE600, which is the origin strain of the purified tRNA used for the TXTL buffer. The translation rate was predicted using an RBS calculator.[13]

| Protein            | mScarlet | mVenus | GFP mut3 | mTurquoise 2 | p70-GFP (deGFP3) |
|--------------------|----------|--------|----------|---------------|------------------|
| Ex. Max (nm)       | 569      | 515    | 500      | 434           | 508              |
| Em. Max (nm)       | 593      | 527    | 513      | 474           | 518              |
| QY                 | 0.54     | 0.64   | 0.39     | 0.93          | 0.19             |
| Brightness         | 56.16    | 66.56  | 34.87    | 27.9          | 5.07             |
| maturation time (min) | 36     | 17.6   | 4.1      | 33.5          |                  |
| pKa                | 5.4      | 5.5    |          |               | 6.9              |

additional mutations

- CAI E. coli B: 0.636
- CAI Shigella flex. 2a: 0.765
- GC content full mRNA: 49.31
- GC content CDS: 49.5

Predicted translation rate

- 3052
- 905
- 746
- 357
- 9373

ΔG total

- -2.01
- 0.69
- 1.12
- 2.76
- -4.51

ΔG mRNA-rRNA

- -7.53
- -7.53
- -9.42
- -10.08
- -10.1

ΔG spacing

- 0.67
- 0.67
- 0.67
- 0.67
- 0.29

ΔG stacking

- 0
- 0
- 0
- 0
- 0

ΔG standby

- 4.9
- 4.9
- 4.9
- 4.9
- 0.01

ΔG start

- -2.76
- -2.76
- -2.76
- -2.76
- -2.76

ΔG mRNA

- -2.79
- -5.49
- -7.81
- -10.11
- -8.33
Table S4: T-test for TXTL data. We aimed to proof our hypothesis, that our data show an optimum in the number of sonication cycles. The data shown in Figure 2 B and D were split in two sets with fixed lysozyme concentration and different sonication cycles: For shaking flask cell extracts these were 0, 5, and 15 sonication cycles in combination with a lysozyme concentration of 0.5 or 1 mg/ml respectively (data set 1 and 2; samples S5/L0 and S15/L0 were excluded from the t-test). For the bioreactor extracts these were 4, 8, 12, 16, and 20 sonication cycles in combination with a lysozyme concentration of 0.5 or 0.8 mg/ml respectively (data set 4 and 5). As we observe the same trend in the data independent on the lysozyme concentration, we also introduced combined data sets (data set 3 and 6). A parabola \( y = a \cdot x^2 + b \cdot x + c \) with \( x \) being the number of sonication cycles and \( y \) being the TXTL end level of the YFP reporter was fitted to the data sets. The fit parameter \( a \) was tested against the hypothesis ‘Fit parameter \( a \) is zero’ and the according p-value was calculated. We could reject the null hypothesis for all data sets (p-value < 0.05) except for data set 4, which has a p-value of 0.06. So the observed optima in the TXTL data are statistically significant.

| Culture method   | Lysozyme (mg/ml) | Data set | p-value          |
|------------------|------------------|----------|------------------|
| Shaking flask    | 0.5              | 1        | 1.32·10^{-5}     |
|                  | 1                | 2        | 1.20·10^{-3}     |
|                  | 0.5 and 1 combined | 3   | 1.60·10^{-4}     |
| Bioreactor       | 0.5              | 4        | 6.43·10^{-2}     |
|                  | 0.8              | 5        | 4.46·10^{-2}     |
|                  | 0.5 and 0.8 combined | 6 | 8.30·10^{-3}     |
Table S5: Comparison of different cell extract preparation protocols.

|                          | This study         | Sun et al. [1]   | Kwon and Jewett [2b] | Fujiwara and Doi [19] |
|--------------------------|--------------------|------------------|-----------------------|------------------------|
| **Strain**               | Rosetta 2 (DE3)    | Rosetta 2 (DE3)  | BL21 star (DE3)       | BL21(DE3) codon plus (RIL) |
| **Culture method**       | Shaking flask or bioreactor | Shaking flask | Shaking flask or bioreactor | Shaking flask |
| **Culture medium**       | 2xYTP shaking flask; 2xYTGP bioreactor | 2xYTP         | 2xYTGP                | LB         |
| **IPTG induction**       | No                 | No               | Tested, but usually not used | Yes |
| **Lysis method**         | Lysozyme incubation + sonication | Bead beating   | Sonication            | Lysozyme incubation + osmotic shock + freeze thaw cycles |
| **Cell extract processing steps** (after lysis) | In total 3 centrifugation steps; run off reaction; dialysis | In total 3 centrifugation steps; run off reaction; dialysis | 1 centrifugation step; (run-off reaction and second centrifugation step was also tested) | 1 centrifugation step (buffer exchange was also tested) |
| **Total protein content (mg/ml)** | 15                | 30               | 40                    | 20-30                |
| **Expressed protein (mg/ml)** | 0.6               | 0.75             | 1                     | 0.25-0.5             |
| **TXTL test conditions** | J23106 promoter; 9 nM corresponds to 17 µg/ml plasmid concentration | Lamda promoter or T7 promoter | Addition of T7 polymerase; T7 promoter (13.3 µg/ml plasmid concentration) | T7 promoter (1,5 nM template concentration) or OR2OR1 (10nM plasmid concentration) |

Figure S11: Plasmid maps for TXTL tests. A mScarlet, mVenus, E0040 GFP or mTurquoise reporter was cloned in a pSB1A3 backbone containing a J23106 promoter, a B0034 ribosome binding site (RBS) and a B0015 terminator.
Table S6: pSB1A3-J23106-2003-mScarlet-B0015

ccccctggaagctcctcgtgcctctctcgtccacccgcgtccacctgatactctccgcttttcgcttctactcacttgacagcttggtggccttttcgtcatcacttactgtttttaaatagggagctgcggtttttaaatggtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
cgAggatcatgtcgacaggtgggtgtcggtttagtgtatggtcctactcttcgcgcgctacgcgtgaagcggataaacttcgacacagtcttttaagctgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcgtggttggttcgtcgcggagttgctcgcgtggagcgtggtgatgttgcagtaaacttcg
| Accession | Description |
|-----------|-------------|
| A0A14ONZ10_ECOBD | SelB translation factor |
| A0A14ON500_ECOBD | Multidrug efflux transporter ErmAB... |
| A0A14ON5K0_ECOBD | 2-octaprenyl-6-methoxyphenyl hydrox...
| A0A14ON763_ECOBD | RNA binding S1 domain protein |
| A0A14ON7F9_ECOBD | Biotin carboxylase |
| A0A14ON7L1_ECOBD | Amidohydrolase |
| A0A14ON843_ECOBD | FAD-dependent 2-octaprenylphosphyl hydrol... |
| A0A14ON8E5_ECOBD | [ProGluGln synthetase I], SpoTRelA |
| A0A14ON9K8_ECOBD | SuA5(Y/e)/YrdC/YwcC family protein |
| A0A14ONBW1_ECOBD | Signal peptidease I |
| A0A14ONBY0_ECOBD | Fumarate hydratase class I enzyme |
| A0A14ON919_ECOBD | Ancillary SecYEG translocase subunit... |
| A0A14ON9K0_ECOBD | Amidohydrolase |
| A0A14ON9T6_ECOBD | Ubiquinone biosynthesis O-methyltransferase... |
| A0A14ON9Z7_ECOBD | NADH-quinone oxidoreductase subunitIII |
| A0A14ONA7O_ECOBD | Cytochrome d ubiquinol oxidase subunitIII |
| A0A14ONB8_ECOBD | Misassigned aminoacyl-tRNA deacylase |
| A0A14ONBb6_ECOBD | Protease 4 |
| A0A14ONC78_ECOBD | PTS N-acetyl glucosamine transporter...
| A0A14ONCR0_ECOBD | Ribonuclease R |
| A0A14OND9_ECOBD | 3-methyl-2-oxobutanoate hydroxymethyltransferase... |
| A0A14ONEb0_ECOBD | 2-octaprenyl-3-methyl-6-hydroxy-2-
| A0A14ONEV1_ECOBD | Probable cytosol aminopeptidase |
| A0A14ONF24_ECOBD | DNA-binding protein HU-alpha |
| A0A14ONF90_ECOBD | DNA-binding protein HU-beta |
| A0A14ONFX6_ECOBD | Membrane protein insertion YidC |
| A0A14ONHC7_ECOBD | GTP-binding protein TypeA |
| A0A14ONH8_ECOBD | ATP-dependent protease subunit HslV |
| A0A14OS221_ECOBD | 2355 RNA (guanosine-2'-O-)-
methyltransferase... |
| A0A14OSA6_ECOBD | Xaa-Pro dipeptidase |
| A0A14OSF40_ECOBD | RNA/tnRNA (uracil-C(5))-methyltransferase... |
| TRMA_ECOBD | 10 kDa chaperonin |
| A0A14OEN65_ECOBD | ATP-dependent protease ATPase subunit... |
| A0A14ONF6E_ECOBD | ATP-dependent protease ATPase subunit...
| A0A14ONZS3_ECOBD | 505 ribosomal protein L22 |
| A0A14ONZT1_ECOBD | 505 ribosomal protein L6 |
| A0A14ONZ92_ECOBD | 305 ribosomal protein S9 |
| A0A14ON340_ECOBD | 305 ribosomal protein L27 |
| A0A14ON3G7_ECOBD | 505 ribosomal protein L3 |
| A0A14ON4H4_ECOBD | 505 ribosomal protein L14 |
| A0A14ON3L9_ECOBD | 505 ribosomal protein L8 |
| A0A14ON4K1_ECOBD | 305 ribosomal protein S3 |
| A0A14ON4M3_ECOBD | 505 ribosomal protein L17 |
| A0A14ON5s28_ECOBD | 305 ribosomal protein S19 |
Ribosomal RNA small subunit methyltransferase...
Phenylalanine-tRNA ligase alpha subunit...
Pseudouridylate synthase
Ribosome maturation factor RimM
Ribonuclease G
Pseudouridylate synthase
Phenylalanine-tRNA ligase beta subunit...
Pseudouridylase
Translational regulator CsrA
RNA polymerase beta subunit...
DNA-directed RNA polymerase subunit...
Exoribonuclease 2
Ribonuclease E
RNA chaperone ProQ
Serine-tRNA ligase
Ribonuclease S
Pseudouridylase
Queueine tRNA-ribosyltransferase
Poly(A) polymerase I
ATP-dependent RNA helicase RhlB
ATP-dependent RNA helicase DeaD
Catalase-peroxidase
LexA repressor
DNA-directed RNA polymerase subunit...
Catabolite activator protein
RNA polymerase sigma factor RpoD
ATP-binding transcriptional regulator
Transcription elongation factor Gre...
DNA-binding protein
RNA polymerase sigma factor
HTH-type transcriptional repressor...
Transcriptional regulator PhoB
Transcription termination/anti-termination...
RNA polymerase sigma factor-54 factor
DNA-binding transcriptional regulator...
Transcriptional regulator, TetR family...
DNA-binding protein
Transcription-repair-coupling factor
Transcription termination factor Rh...
Transcriptional regulator MraZ
HTH-type transcriptional regulator...
DNA-directed RNA polymerase subunit...
Transcription termination/anti-termination...
Table S11: Proteins enriched in the shaking flask extracts S5/L0.5 (derived from the comparison of the commercial extract and the shaking flask batches S5/L0.5)

| Uniprot ID | Fold change | -log10(FDR) | Protein name | Gene name | Keyword |
|-----------|-------------|-------------|--------------|-----------|---------|
| A0A14ON4Y5_ECOBD | 1.629766172 | 1.629766172 | Aspartate-semialdehyde dehydrogenase... | asd ECBD_0309 | amino-acid related |
| A0A14NS27_ECOBD | 1.975462238 | 1.975462238 | S-adenosylmethionine synthase | matK ECBD_0798, HO396_14190 | amino-acid related |
| A0A14N770_ECOBD | 2.503832349 | 2.503832349 | 4-hydroxy-... | dapA ECBD_1211, HO396_12065 | amino-acid related |
| A0A14N7T1_ECOBD | 1.651582735 | 1.651582735 | Succinyl-diaminopimelate desuccinyl... | dapE ECBD_1218, HO396_12030 | amino-acid related |
| A0A14N9W6_ECOBD | 2.165393138 | 2.165393138 | 2,3,4,5-tetrahydrodipicolinate re... | dapD ECBD_3453, HO396_00830 | amino-acid related |
| A0A14N8EB2_ECOBD | 1.671048798 | 1.671048798 | 4-hydroxy-... | dapB ECBD_3585, HO396_00160 | amino-acid related |
| A0A14N7B3_ECOBD | 2.505331739 | 2.505331739 | Glyceroldehyde-3-phosphate dehydro... | gapA ECBD_1855, HO396_08975 | carbohydrate metabolism and respiration |
| A0A14N8B59_ECOBD | 2.004735299 | 2.004735299 | Phosphofructokinase | pfkB ECBD_1922, HO396_08695 | glycolytic process |
| A0A14N8A0_ECOBD | 2.233174935 | 2.233174935 | 2,3-bisphosphoglycerate-independent... | pgml pgmM, pgml ECBD_0113, HO396_17735 | glycolytic process |
| A0A14N5G0_ECOBD | 1.995921991 | 1.995921991 | Enolase | eno ECBD_0950, HO396_13405 | glycolytic process |
| A0A14N8B11_ECOBD | 2.123646427 | 2.123646427 | Fructose-bisphosphate aldolase | frbA ECBD_0813, HO396_14115 | glycolytic process |
| A0A14N8E11_ECOBD | 2.672314268 | 2.672314268 | Phosphoglycerate kinase | pgk ECBD_0812, HO396_14120 | glycolytic process |
| A0A14N9C3_ECOBD | 2.488257014 | 2.488257014 | Glucokinase | gkh ECBD_1284, HO396_11975 | glycolytic process |
| A0A14N9D9_ECOBD | 1.935051911 | 1.935051911 | 2,3-bisphosphoglycerate-dependent p... | pgmA ECBD_2912, HO396_03615 | glycolytic process |
| A0A14N9V8_ECOBD | 2.631339246 | 2.631339246 | Pyruvate kinase | pkyK ECBD_1969, HO396_08460 | glycolytic process |
| A0A14NCD7_ECOBD | 2.715709578 | 2.715709578 | Glucose-6-phosphate isomerase | pgI ECBD_4012, HO396_25000 | glycolytic process |
| A0A14NDL0_ECOBD | 1.660343405 | 1.660343405 | Pyruvate dehydrogenase E1 component | ECBD_3505 | glycolytic process |
| Protein name | Gene name | GO (biological process) | Keyword |
|--------------|-----------|-------------------------|---------|
| Acetyltransferase component of pyruvate kinase | aceF | ECBD_3504, HO396_00565 | glycolytic process |
| Probable phosphoglycerate mutase Gp | ispG | gpcE, ECBD_1171, HO396_12260 | glycolytic process |
| 4-hydroxy-3-methylbut-2-en-1-yl diphytanyl glyceryl ether synthase | ispH | spg, ECBD_3587, HO396_00150 | other |
| Glycerol-3-Phosphate dehydrogenase | gpmB | ECBD_3625, HO396_21960 | other |
| Probable phosphoglycerate mutase | gpmB | ECBD_3625, HO396_21960 | other |
| 4-hydroxy-3-methylbut-2-enyl diphytanyl glyceryl ether synthase | ispH | spg, ECBD_3587, HO396_00150 | other |
| Dihydropteroate synthase | folP | ECBD_0565, HO396_15515 | other |
| tdtP-4-dehydrorhamnose reductase | tdtP | ECBD_1615, HO396_10030 | other |
| Adenylate kinase | adk | ECBD_3182, HO396_02190 | other |
| Thymidine kinase | tmk | ECBD_2503, HO396_05690 | other |
| Methionine synthase | metH | ECBD_4018, HO396_19970 | other |
| 4-hydroxy-3-methylbut-2-enyl diphytanyl glyceryl ether synthase | ispH | spg, ECBD_3587, HO396_00150 | other |

Table S12: Enriched proteins derived from the comparison of S0/L1 vs. S5/L1, S0/L1 vs. S15/L1 and of bioreactor S5/L1 against the commercial extract

S0/L1 vs. S5/L1

| Entry name | Protein name | Gene name | GO (biological process) | Keyword |
|------------|--------------|-----------|-------------------------|---------|
| A0A140NAS5_ECOBD | Transcriptional regulator, AsnC family | ECBD_2, 708 | Transcriptional regulator |
| A0A140N57_ECOBD | Nucleoid-associated protein YjK | ECBD_1, 471 | DNA relaxation |
| A0A140N231_ECOBD | Transcriptional regulator, IclR family | ECBD_0, 160 | Transcriptional regulator |

S0/L1 proteins in GO terms with GO FDR < 0.05

| Entry name | Protein name | Gene name | GO (biological process) | Keyword |
|------------|--------------|-----------|-------------------------|---------|
| none |

S0/L1 vs. S15/L1

| Entry name | Protein name | Gene name | GO (biological process) | Keyword |
|------------|--------------|-----------|-------------------------|---------|
| A0A140N231_ECOBD | Transcriptional regulator, IclR family | ECBD_0, 160 | regulation of transcription, DNA-templated |
| A0A140N3Y5_ECOBD | Transcriptional regulator, LysR family | ECBD_0, 633 | regulation of transcription, DNA-TEMPLATED |

S15/L1 proteins in GO terms with GO FDR < 0.05
### Bioreactor vs. Commercial extract

**Bioreactor proteins with GO FDR < 0.05**

| Entry name | Protein name | Gene name | GO (biological process) | Keyword |
|------------|--------------|-----------|-------------------------|---------|
| A0A140N479_ECOBD | 2-amino-3-ketobutyrate coenzyme A L-3-ketothiolase | kbl | biosynthetic process; L-threonine catabolic process to glyoxylate | Amino acid related |
| A0A140N487_ECOBD | Endoribonuclease L-PSL | ECBD_0 | 108 | Nuclease |
| A0A140N655_ECOBD | Selenide, water dikinase | selD | selenocysteine biosynthetic process | Other |
| A0A140N651_ECOBD | Amino-acid acetyltransferase | argA | arginine biosynthetic process | Amino acid related |
| A0A140N725_ECOBD | Phosphoanodenedine phosphosulfate reductase | cySH | hydrogen sulfide biosynthetic process; sulfate assimilation, phosphoanodenedine-sulfate reduction by phosphoanodenedine-sulfate reductase (thioredoxin) | Other |
| A0A140N770_ECOBD | 4-hydroxy-3,5-dihydroxypropionate | dapA | diaminopimelate biosynthetic process; lysine biosynthetic process via diaminopimelate | Amino acid related |
### Table S13: Summary of protein numbers for each comparison: total number of proteins found in the extracts, number of unique proteins, number of proteins with FDR<0.05 and numbers of proteins in GO terms with GO FDR<0.05

#### Shaking flask vs. bioreactor
same lysis setting (S5/L1), different culture conditions

|                      | SF S5/L0.5 | BR S5/L0.5 |
|----------------------|------------|------------|
| total number of proteins | 1480       | 1536       |
| unique proteins       | 65         | 0          |
| thereof n=3 n=0       | 11         | 0          |
| Proteins with FDR <0.05 | 0           | 8          |
| Proteins in GO with FDR <0.05 | 3           | 0          |
| thereof n=3 n=0       | 3           | 0          |

#### S0/L1 vs S5/L1
same lysozyme concentration, but 0 vs 5 sonication cycles

|                      | S0/L1      | S5/L1      |
|----------------------|------------|------------|
| total number of proteins | 1426      | 1469       |
| unique proteins       | 42         | 85         |
| thereof n=3 n=0       | 2          | 27         |
| Proteins with FDR <0.05 | 0           | 0          |
| Proteins in GO with FDR <0.05 | 0           | 7          |
| thereof n=3 n=0       | 0          | 7          |

#### S0/L1 vs S15/L1
same lysozyme concentration, but 0 vs. 15 sonication cycles

|                      | S0/L1      | S15/L1     |
|----------------------|------------|------------|
| total number of proteins | 1426      | 1469       |
|                           | unique proteins | thereof n=3 n=0 | Proteins with FDR <0.05 | thereof n=3 n=0 |
|---------------------------|-----------------|----------------|-------------------------|----------------|
| commercial extract vs. Bioreactor S5/L0.5 | 57              | 3              | 1                       | 0              |
|                           | 120             | 47             | 6                       | 19             |
| commercial versus shaking flask S5/L0.5 | 215             | 121            | 5                       | 0              |
|                           | 185             | 30             | 8                       | 5              |
|                           | 309             | 116            | 356                     | 172            |
|                           | 356             | 53             | 310                     | 31             |

|                           | thereof n=3 n=0 | thereof n=3 n=0 |
|---------------------------|----------------|----------------|
| commercial extract vs. Bioreactor S5/L0.5 | 121            | 30            |
| commercial versus shaking flask S5/L0.5 | 116            | 53            |
Supporting References

[1] Z. Z. Sun, C. A. Hayes, J. Shin, F. Caschera, R. M. Murray, V. Noireaux, J. Vis. Exp. 2013, e50762.
[2] a) T. W. Kim, J. W. Keum, I. S. Oh, C. Y. Choi, C. G. Park, D. M. Kim, J. Biotechnol. 2006, 126, 554-561; b) Y. C. Kwon, M. C. Jewett, Sci. Rep. 2015, 5, 8663.
[3] M. K. Takahashi, J. Chappell, C. A. Hayes, Z. Z. Sun, J. Kim, V. Singhal, S. Al-Khabouri, C. P. Fall, V. Noireaux, R. M. Murray, J. B. Lucke, ACS Synth. Biol. 2015, 4, 503-515.
[4] M. Rustad, A. Eastund, R. Marshall, P. Jardine, V. Noireaux, J. Vis. Exp. 2017, 1 - 9.
[5] J. Garamella, R. Marshall, M. Rustad, V. Noireaux, ACS Synth. Biol. 2016, 5, 344-355.
[6] F. R. Blatter, B. G. Williams, A. E. Blechli, K. Denniston-Thompson, H. E. Faber, L. Furlong, D. J. Grunwald, D. O. Kieber, D. M. Schumm, E. L. Sheldon, O. Smithies, Science 1977, 196, 161 - 169.
[7] a) J. r. Cox, N. Neuhauser, A. Michalski, R. A. Scheltema, J. V. Olsen, M. Mann, J. Proteome Res. 2011, 10, 1794-1805; b) Y. C. Kwon, M. C. Jewett, Sci. Rep. 2015, 5, 8663.
[8] [107x761]a) T. W. Kim, J. W. Keum, I. S. Oh, C. Y. Choi, C. G. Park, D. M. Kim, J. Biotechnol. 2006, 126, 554-561; b) Y. C. Kwon, M. C. Jewett, Sci. Rep. 2015, 5, 8663.
[9] Y. Benjamini, Y. Hochberg, J. Roy. Stat. Soc. B 1995, 57, 289-300.
[10] D. W. Huang, B. T. Sherman, R. A. Lempicki, Nat. Protocols 2008, 4, 44-57.
[11] A. Bateman, M. J. Martin, S. Orchard, M. Magrane, R. Agivetova, S. Ahmad, E. Alpi, E. H. Bowler-Barnett, R. Britto, B. Burton, G. H. C. de Castro, K. C. Echioukh, E. Coudert, B. Cuche, M. Dornier, A. Estreicher, M. L. Famiglietti, M. Feuermann, E. Gasteiger, S. Gehant, V. Gerritsen, A. Gos, N. Gruaz-Gumowski, U. Hinz, C. Hulo, N. Hyka-Nouspikel, F. Jungo, G. Keller, A. Kerhornou, V. Lara, P. Le Mercier, D. Lieberherr, T. Lombardot, X. Martin, P. Masson, Nucleic Acids Res. 2021, 49, D480-D489.
[12] P. Puigbo, I. G. Bravo, S. Garcia-Valle, Biol. Direct 2008, 3, 38.
[13] A. E. Borujeni, A. S. Channarasappa, H. M. Salis, Nucleic Acids Res. 2014, 42, 2646-2659.
[14] D. S. Bindels, L. Haarbosch, L. van Weeren, M. Postma, K. E. Wiese, M. Mastop, S. Amonier, G. Gotthard, A. Royant, M. A. Hink, T. W. Gadella, Jr., Nat. Methods 2017, 14, 53-56.
[15] G. J. Kremers, J. Goedhart, E. B. van Munster, T. W. Gadella, Jr., Biochemistry 2006, 45, 6570-6580.
[16] B. P. Cormack, R. H. Valdivia, S. Falkow, Gene 1996, 173, 33-38.
[17] J. Goedhart, D. von Stetten, M. Noirelcer-Savoye, M. Lelimalous, L. Joosen, M. A. Hink, L. van Weeren, T. W. Gadella, Jr., A. Royant, Nat. Commun. 2012, 3, 751.
[18] G. T. Hanson, T. B. McAnaney, E. S. Park, M. E. Rendell, D. K. Yarbrough, S. Chu, L. Xi, S. G. Boxer, M. H. Montrose, S. J. Remington, Biochemistry 2002, 41, 15477-15488.
[19] K. Fujiwara, N. Doi, Plos One 2016, 11, e0154614.