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RESEARCH ARTICLE

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Characterization of the all-*E. coli* transcription-translation system myTXTL by mass spectrometry

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Rationale: Cell-free transcription-translation (TXTL) is becoming a popular technology to prototype and engineer biological systems outside living organisms. TXTL relies commonly on a cytoplasmic extract that provides the molecular components necessary to recapitulate gene expression *in vitro*, where most of the available systems are derived from *E. coli*. The proteinic and enzymatic composition of lysates, however, is typically unknown. In this work, we analyzed by mass spectrometry the molecular constituents of the all-*E. coli* TXTL platform myTXTL prepared from the *E. coli* strain BL21 Rosetta2.

Methods: Standard TXTL reactions were assembled and executed for 10–12 hours at 29°C. In addition to a no-DNA control, four DNA programs were executed in separate reactions to synthesize the reporter protein deGFP as well as the phages MS2, phix174 and T7. The reactions were treated according to standard procedures (trypsin treatment, cleaning) before performing liquid chromatography/mass spectrometry (LC/MS). Data analysis was performed using Sequest and protein identification using Scaffold.

Results: A total of 500–800 proteins were identified by LC/MS in the blank reactions. We organized the most abundant protein sets into several categories pertaining, in particular, to transcription, translation and ATP regeneration. The synthesis of deGFP was easily measured. The major structural proteins that compose the three phages MS2, phix174 and T7 were also identified.

Conclusions: Mass spectrometry is a practical tool to characterize biochemical solutions as complex as a cell-free TXTL reaction and to determine the presence of synthesized proteins. The data presented demonstrate that the composition of TXTL based on lysates can be used to validate some underlying molecular mechanisms implicated in cell-free protein synthesis. The composition of the lysate shows significant differences with respect to similar studies on other *E. coli* strains.

1 | INTRODUCTION

Cell-free transcription-translation (TXTL) has become a multi-purpose technology to engineer biological systems *in vitro*.[1-3] TXTL offers experimental settings ideal to accelerate the design-build-test cycle of DNA programs executed for utilizations far beyond the traditional synthesis of proteins. Cell-free expression is now used to perform bioengineering from the molecular to the cellular scale, in reaction volumes spanning at least seventeen orders of magnitude (fL to 100 L), and for applications as various as synthetic and quantitative biology, biological physics, and biomanufacturing.[2] Although rapidly expanding as a highly versatile technology to execute gene circuits and produce proteins *in vitro*, TXTL is also often viewed as a "black box" because the molecular composition of most of the available

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TXTL systems is unknown. Except for the PURE system[4] made of purified proteins and ribosomes, the other platforms rely on cell lysates that provide the molecular components necessary to recapitulate gene expression *in vitro*, among hundreds of other unidentified proteins. While this has not prevented the translation of TXTL into many different applications, knowing the protein composition of extract-based TXTL would help extend the scope and capabilities of this technology by, for example, facilitating model-driven engineering of TXTL systems or customizing TXTL platforms for specific applications based on the parts provided by the lysates. As importantly, identifying the protein composition of TXTL lysates is necessary to comprehend or validate mechanisms underlying the process of gene expression *in vitro*, especially at the level of the metabolism supporting ATP regeneration. Liquid chromatography/mass spectrometry (LC/MS) was recently employed to report the protein composition of TXTL systems using *E. coli* lysates. The composition of a lysate prepared from the *E. coli* strain BL21 Star was compared with the PURE system considered in the field as a minimal *in vitro* protein synthesis kit.[5] The composition of a lysate prepared from the *E. coli* strain A19 was also thoroughly analyzed and discussed.[6] Some LC/MS results were also reported to characterize the composition of a lysate prepared from non-growing stressed *E. coli* cells.[7] While these studies pioneered the utilization of LC/MS for cell-free expression systems, the associated TXTL systems represent conventional hybrid systems, based on the T7 bacteriophage for transcription and on the translation machinery of an organism such as *E. coli*. It is also well established that the strength and capabilities of TXTL systems depend on the strain used to prepare the lysate. Consequently, performing LC/MS for lysates based on other widely used *E. coli* strains is necessary to document their protein composition and to understand molecular mechanisms supporting cell-free expression that have only been hypothesized so far. Taking advantage of a resource like LC/MS to analyze the products of large DNA programs executed in TXTL is also an area that has not been investigated.

In this work, we performed LC/MS to determine the protein composition of myTXTL, an all-*E. coli* TXTL platform based on the endogenous TX and TL machineries provided by a lysate from BL21 Rosetta2 cells. This system was developed to be highly versatile and adaptable to a broad range of applications.[8-14] As opposed to bacteriophage transcription, the *E. coli* core RNA polymerase and sigma factor 70 drive transcription from hundreds of promoters. We determined the protein composition of this system by LC/MS and we organized the most relevant protein sets into several categories pertaining principally to transcription, translation, and metabolism related to ATP regeneration (Figure 1). In addition to discussing several other categories related to membrane proteins, chaperones, nucleases, and proteases, we also highlighted several major proteins that are not present in the lysate or are at negligible levels. Furthermore, we executed four DNA programs to synthesize the reporter protein deGFP as well as the phages MS2 (RNA, 4 genes), phix174 (linear dsDNA, 11 genes), and T7 (linear dsDNA, 60 genes) (Figure 1). We show that the structural proteins of the three phages are all identified by LC/MS. Although not quantitative, the LC/MS data are referenced in each category with respect to the protein EF-Tu, the most abundant protein in *E. coli* and in the lysate, so as

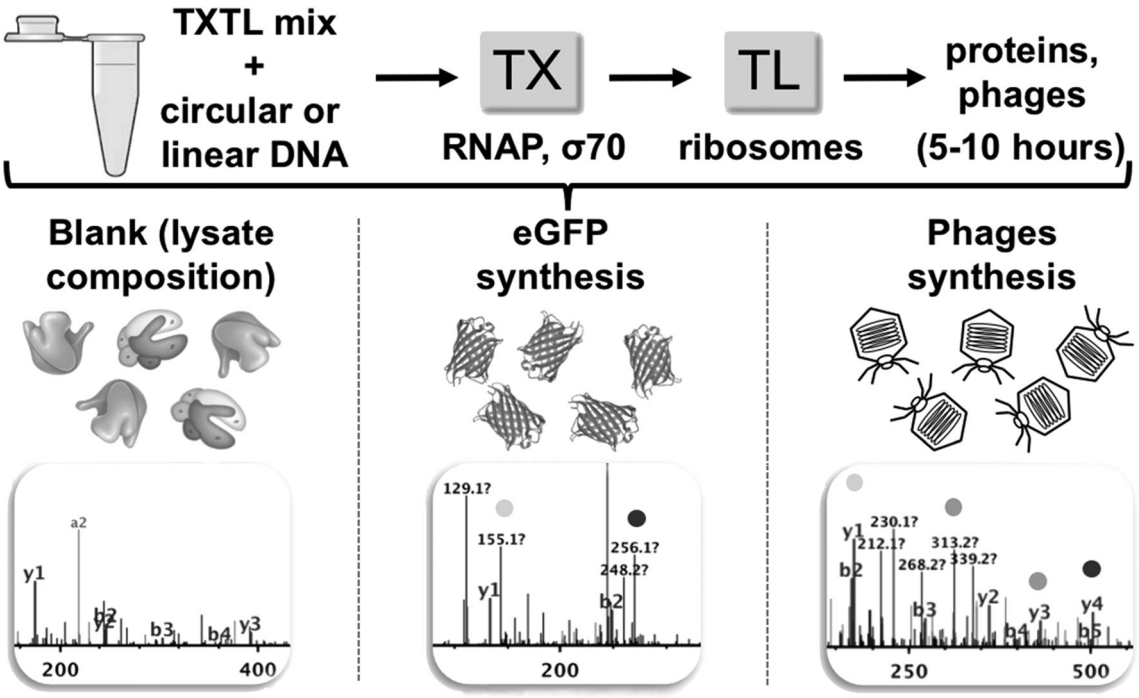

FIGURE 1 The experimental approach. Blank reactions (no genetic template added) were used to determine the lysate composition. Several DNA programs were executed to synthesize the reporter protein deGFP and three phages: MS2, phix174, and T7

to know whether each detected protein is in high or low abundance in the lysate. We expect that the data reported in this work will serve as a general and extensive resource for the users of the myTXTL extract-based all-*E. coli* TXTL system.

2 | EXPERIMENTAL

2.1 | Cell-free transcription-translation system and reactions

The preparation and description of the all-*E. coli* TXTL system (commercialized by Arbor Biosciences under the name myTXTL) were previously reported in several articles.[8,15-19] Briefly, the lysate was prepared following standard steps: growth in a rich medium (2xYT supplemented with 22 mM K_2HPO_4 and 40 mM KH_2PO_4), cell pelleted by centrifugation in mid-log phase (OD600 24), lysis using a French press, clarification at 30,000 *g*, pre-incubation for 80 min at 37°C, dialysis for several hours (typically 3 h), and storage at −80°C. Transcription and translation are performed by the endogenous molecular components provided by an *E. coli* cytoplasmic extract, without the addition of exogenous purified TXTL proteins or enzymes. Transcription is booted up by the *E. coli* core RNA polymerase and sigma factor 70 (RpoD). The energy buffer for ATP regeneration is composed of the following: 50 mM Hepes pH 8, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM coenzyme A, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 20–40 mM maltodextrin. A typical cell-free reaction is composed of 33% (v/v) of *E. coli* lysate, the other 66% of the reaction volume includes the energy mix, the amino acid solution[20] (3 mM of each of the 20 amino acids) and plasmids. Mg-glutamate, K-glutamate, and PEG8000 concentrations were adjusted to 5 mM, 60 mM and 2%, respectively.[8] Cell-free reactions were carried out in a volume of 5 to 20 µL at 29–30°C by simply adding the DNA to the TXTL mixture. A blank reaction (also named no-DNA reaction) contains all the ingredients except for the genetic template (plasmid, linear dsDNA, mRNA).

2.2 | Fluorescence measurements

deGFP (25.4 kDa, 1 mg/mL = 39.37 µM) is a variant of the reporter eGFP that is more translatable in cell-free systems. The excitation and emission spectra, as well as fluorescence properties of deGFP and eGFP, are identical, as reported previously.[16] The fluorescence of deGFP produced in the batch mode cell-free reaction was measured on an H1m plate reader (Biotek Instruments, 384-well plate). End-point measurements were carried out after 10–12 h of incubation. Pure recombinant eGFP with His tags (from two sources: Cell Biolabs Inc. and Biovision) was used for quantification (linear calibration on plate reader). Error bars are the standard deviations from three repeats.

2.3 | Phage synthesis

Cell-free expression and synthesis of bacteriophages was reported previously.[8,11,17] The TXTL reaction settings were similar to standard reactions. The genomes were purchased from Sigma (MS2, 100 nM in TXTL reaction), New England Biolabs (phix174, 5 nM in TXTL reaction), and Boca Scientific (T7, 0.25 nM in TXTL reaction).

2.4 | Sample preparation for LC/MS

All samples were prepared as follows: 5 µL of sample (50 µg, samples were in a 10 µg/µL concentration) was mixed with 20 µL of denaturing buffer (7 M urea, 2 M thiourea, 0.4 M triethylammonium bicarbonate (TEAB) pH 8.5, 20% acetonitrile and 4 mM tris(2-carboxyethyl)phosphine (TCEP)). The samples were vortexed briefly, and then each sample was transferred to a pressure cycling technology (PCT) tube and capped for the Barocycler NEP2320 (Pressure Biosciences, Inc., South Easton, MA, USA). Pressure cycled between 35 kpsi for 20 s and 0 kpsi for 10 s for 60 cycles at 37°C. The PCT tube was uncapped and 200 mM methyl methanethiosulfonate (MMTS) was added to a final concentration of 8 mM MMTS to alkylate cysteine residues, recapped, inverted several times and incubated for 15 min at room temperature. The samples were transferred to a new 1.5 mL microfuge Eppendorf Protein LoBind tube. All samples were diluted four-fold with ultra-pure water to dilute the urea concentration and trypsin (Promega, Madison, WI, USA) was added in a 1:40 ratio of trypsin to total protein. Samples were incubated overnight for 16 h at 37°C. After incubation they were frozen at −80°C for 0.5 h and dried in a vacuum centrifuge. A 10 µg aliquot of each sample was cleaned with an MCX STAGE tip[21] and the eluate was dried *in vacuo*.

2.5 | LC/MS

0.5 µg of each peptide mixture was analyzed by capillary LC/MS on an Eksigent nanoLC 1D plus system with an Orbitrap Velos mass spectrometer (Thermo Scientific) as previously described.[22] Briefly: spray voltage was set to 2 kV, the heated capillary was maintained at 260°C, the orbital trap was set to acquire survey mass spectra (300–1800 *m/z*) with a resolution of 30,000 at 400 *m/z* with automatic gain control (AGC) 1 × 10E6, 500 ms minimum injection time and lock mass at 445.1200 *m/z* (polysiloxane). The six most intense ions (2+ charged and higher) from the full scan were selected for fragmentation by higher-energy collisional dissociation with normalized collision energy 40%, activation time 0.1, and detector settings of 7500 resolution, AGC 1 × 10E5 ions, 500 ms maximum injection time and FT first mass mode fixed at 111 m/z. Lock mass was not employed. The dynamic exclusion settings were: repeat count = 1, exclusion list size = 200, exclusion duration = 30 s, exclusion mass width (high and low) was 15 ppm and early expiration was disabled.

2.6 | Data analysis

MS data were analyzed using Sequest (XCorr Only) (Thermo Fisher Scientific, San Jose, CA, USA; version IseNode in Proteome Discoverer 2.2.0.388). Sequest (XCorr Only) with the protein sequences from NCBI Reference Sequence *Escherichia coli* BL21(DE3) (taxonomy ID 469008). The common contaminant proteins (https://www.thegpm.org/crap/) and custom protein sequences (deGFP, MS2, phix174, T7) were added as templates. Sequest (XCorr Only) was searched with a fragment ion mass tolerance of 0.100 Da and a precursor ion tolerance of 50 parts per million (ppm). Methylthiolation of cysteine was specified in Sequest (XCorr Only) as a fixed modification. We specified the following variable amino acid modifications in Sequest (XCorr Only): pyro-glutamate formation from N-terminal glutamine, deamidation of asparagine and glutamine, oxidation of methionine, dioxidation of methionine and N-terminal protein acetylation.

2.7 | Criteria for protein identification

We used Scaffold (version 4.8.4, Proteome Software Inc., Portland, OR, USA) to validate tandem mass spectrometry (MS/MS)-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 97.0% probability to achieve a false discovery rate (FDR) less than 2.0% and contained at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm.[23] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

3 | RESULTS AND DISCUSSION

3.1 | Broad considerations

LC/MS was performed to determine the protein and enzymatic composition of TXTL and synthesized proteins. We did not perform LC/MS to search for the metabolites in the lysate. The last step in the lysate preparation consists of a dialysis step of 3 h against a simple buffer (S30B: 14 mM magnesium glutamate, 150 mM potassium glutamate, 10 mM Tris) through a dialysis membrane of molecular mass cutoff 10 kDa. Metabolites below a few kDa are removed, therefore their amounts are expected to be negligible.

We performed two LC/MS repeats (labeled as rep1. and rep2. in the tables) for three different *E. coli* lysates prepared in 2015, 2017 and 2018. The samples were treated identically for each repeat and the same amounts were used. The comments and tables focus on our most recent batch prepared in 2018. The two other lysates are added only as a resource that can be used, for example, to make batch-to-batch comparisons. The synthesis of deGFP and the three phages MS2, phix174 and T7 was quantitatively comparable in the three batches, as discussed below and described previously.[8] The LC/MS data counts are rescaled with respect to the total number of counts, which explains why the number of counts of our reference protein EF-Tu (WP_000031784.1) changes when one or more proteins are synthesized in TXTL, in contrast to the no-DNA reaction. We used the no-DNA reaction to discuss the components related to the DNA-dependent synthesis of proteins *in vitro*.

3.2 | Transcription

As expected based on the work already performed with this TXTL system,[8,15,24,25] all subunits of the *E. coli* core RNA polymerase (beta, beta', and alpha) were present in the lysate including the subunit omega whose physiological functions are not completely understood in bacteria (Table 1). The transcription termination proteins Rho and NusA seem to be abundant in the lysate compared to NusG. As anticipated too, the primary sigma factor 70 (RpoD) is also present. It is this transcription machinery, core RNAP and RpoD, that is used to boot up all circuitries in the all-*E. coli* TXTL system. As predicted previously,[8,24] none of the six other sigma factors (19, 24, 28, 32, 38 and 54) were detected in the lysate in line with the *E. coli* cells being grown in a rich medium and collected during the exponential phase when no other sigma factor than RpoD is required. Among the major global transcriptional regulators, small amounts of CRP and FIS were detected, while other global regulators such as LrP, H-NS, IhF, FnR, ArcA, CrA, and SoX were not. Interestingly, the RNA polymerase releasing factors RapA and GreA were present, which could be important for transcription. Several known transcription factors were present, such as trace amounts of LacI and DksA. Finally, detectable amounts of the response regulators for three different two-component systems were measured: OmpR, PhoP and BasR.

3.3 | Translation

The concentration of ribosomes in TXTL is estimated to be around 1–2.5 μM.[26] On DNA gels (data not shown), the two parts of the ribosomes are the only clearly apparent nucleotide species in the lysate. Using mass spectrometry, we identified all the proteins of the S30 ribosomal subunit except S22 (Table 2), which makes sense because S22 is a stationary phase induced ribosome-associated protein. For the S50 subunit, 30 out of 33 proteins were detected (Table 3). L34, 35, 36 were not measured possibly due to a very low abundance and also to the rather small size of these three proteins (5.3, 7.2, 4.3 kDa, respectively). L34 appeared at very low levels when the experiment was repeated for the three different lysates (Table S1, supporting information). The three initiation factors IF1, 2 and 3 are present well above background (Table 2). Only two of the peptide chain release factors are detected (Table 2). In addition to the elongation factor EF-Tu, the most abundant protein in the lysate, five other translation elongation factors were detected: G, T, P, 4 and YeiP (Table 3). The ribosome recycling factor RFF (RF4), which is

TABLE 1 Major TX components measured by LC/MS in the all-*E. coli* TXTL lysate

#	Identified proteins	Access. No.	MW	Rep. 1	Rep. 2
TX					
REF	EF-Tu	WP_000031784.1	43 kDa	90	103
1	DNA-directed RNA pol. Subunit beta	WP_000263098.1	151 kDa	66	83
2	DNA-directed RNA pol. Subunit beta'	WP_000653944.1	155 kDa	59	81
3	DNA-directed RNA pol. Subunit alpha	WP_001162094.1	37 kDa	31	34
4	DNA-directed RNA pol. Subunit omega	WP_000135058.1	10 kDa	5	8
5	RNA polymerase sigma factor RpoD	WP_000437375.1	70 kDa	7	10
TX termination					
1	Transcription term. Factor rho	WP_012767773.1	47 kDa	40	40
2	Transcription term. Protein NusA	WP_001031057.1	55 kDa	24	22
3	Transcription term./antiterm. Protein NusG	WP_001287516.1	21 kDa	6	9
TX factors					
1	RNA polymerase-binding TX factor DksA	WP_001155227.1	18 kDa	6	7
2	Transcription elongation factor GreA	WP_001148001.1	18 kDa	6	8
3	YebC/PmpR family transcriptional regulator	WP_000907234.1	26 kDa	5	4
4	Transcriptional repressor	WP_000131702.1	17 kDa	3	3
5	cAMP-activated global TX regulator CRP	WP_000242755.1	24 kDa	3	4
6	LacI family transcriptional regulator	WP_000857361.1	34 kDa	4	3
7	PurR family transcriptional regulator	WP_000190982.1	38 kDa	2	3
8	Trifunctional transcriptional regulator	WP_001703864.1	144 kDa	0	5
9	RNA-binding TX accessory protein	WP_000980709.1	85 kDa	2	2
10	YebC/PmpR family transcriptional regulator	WP_000532923.1	26 kDa	3	1
11	Fis family transcriptional regulator	WP_000462905.1	11 kDa	1	2
12	RNA polymerase-associated protein RapA	WP_001117011.1	110 kDa	1	2
13	Transcription-repair coupling factor	WP_012767721.1	130 kDa	0	3
14	Transcriptional regulator	WP_000378442.1	21 kDa	1	1
15	TCS response regulator OmpR	WP_001157756.1	27 kDa	1	4
16	TCS response regulator PhoP	WP_001265481.1	26 kDa	2	3
17	TCS response regulator BasR	WP_000697915.1	25 kDa	1	3

abundant in living cells,[27] appeared as the other notable ribosomal-associated protein. The presence of the ribosome-associated inhibitor A (YfiA) was somewhat unexpected because it is expressed during the stationary phase.[28] Finally, the 20 tRNA ligases were detected (Table S1, supporting information) as expected because the synthesis of milligrams of proteins in TXTL requires the addition of the twenty standard free amino acids. Note that for the lysine tRNA ligase, two ligases were detected because the BL21 strains carry the gene encoding for WP_001295090.1, which has 88.5% analogy (amino acids) with respect to the K12 *E. coli* strain protein (WP_000003071.1).

3.4 | Metabolism

Glycolysis is active in TXTL reactions.[19,29] We focused on this pathway because it is used for ATP regeneration in TXTL. We also made tables for two other pathways related to ATP regeneration and metabolites: the TCA cycle (Krebs cycle) and the pentose phosphate pathway. These metabolic pathways are the three central energy-related series of biochemical reactions in *E. coli*. Starting with glycolysis, we observed that the glucose-specific PTS enzyme II, which is a membrane enzyme complex that transports exogenous glucose into the cytoplasm,[30] was not detected because membrane proteins are removed during the preparation of lysates. This enzyme converts glucose into glucose-6-phosphate during the uptake of glucose. The absence of this enzyme explains why, in our TXTL system, glucose as a carbon source is not efficient for protein expression (data not shown). Except for this transporter, all the glycolysis enzymes in *E. coli* were observed in all six samples (Table 4). Glycogen phosphorylase (WP_000081903.1), which processes short-chain maltodextrins into glucose-1-phosphate, was detected. Glucose-1-phosphate is in turn transformed into glucose-6-phosphate by a phosphoglucomutase (WP_001320171.1), which is also detected in the samples. Therefore, maltodextrins are processed to form intermediate of glycolysis (Figure 2), which

TABLE 2 Composition of the S30 ribosome subunit and other TL factors measured by LC/MS in the all-*E. coli* TXTL lysate

#	Identified proteins	Access. No.	MW	Rep. 1	Rep. 2
Subunit S30					
REF	EF-Tu	WP_000031784.1	43 kDa	90	103
1	30S ribosomal protein S1	WP_000140327.1	61 kDa	91	81
2	30S ribosomal protein S2	WP_000246882.1	27 kDa	35	43
3	30S ribosomal protein S3	WP_000529945.1	26 kDa	33	39
4	30S ribosomal protein S5	WP_000940120.1	18 kDa	37	40
5	30S ribosomal protein S4	WP_000135224.1	23 kDa	31	31
6	30S ribosomal protein S7	WP_001138043.1	18 kDa	25	35
7	30S ribosomal protein S11	WP_001029684.1	14 kDa	20	28
8	30S ribosomal protein S12	WP_000246815.1	14 kDa	18	25
9	30S ribosomal protein S13	WP_000090775.1	13 kDa	21	21
10	30S ribosomal protein S19	WP_001138117.1	10 kDa	21	22
11	30S ribosomal protein S10	WP_001181004.1	12 kDa	16	15
12	30S ribosomal protein S6	WP_001216675.1	15 kDa	13	11
13	30S ribosomal protein S9	WP_000829818.1	15 kDa	12	16
14	30S ribosomal protein S21	WP_001144069.1	9 kDa	10	11
15	30S ribosomal protein S15	WP_000059466.1	10 kDa	8	11
16	30S ribosomal protein S16	WP_000256450.1	9 kDa	5	10
17	30S ribosomal protein S20	WP_001274021.1	10 kDa	7	8
18	30S ribosomal protein S8	WP_000062611.1	14 kDa	7	7
19	30S ribosomal protein S18	WP_000135199.1	9 kDa	7	8
20	30S ribosomal protein S14	WP_001118930.1	12 kDa	7	8
21	30S ribosomal protein S17	WP_000130100.1	10 kDa	7	9
22	30S ribosomal protein S12 RimO	WP_000049367.1	50 kDa	2	3
23	30S ribosomal protein S12 YcaO	WP_001295344.1	66 kDa	0	1
TL initiation					
1	Translation initiation factor IF-2	WP_000133044.1	97 kDa	38	54
2	Translation initiation factor IF-3	WP_001700733.1	21 kDa	11	13
3	Translation initiation factor IF-1	WP_001040187.1	8 kDa	5	6
TL elongation					
1	Elongation factor G	WP_000124700.1	78 kDa	86	96
2	Elongation factor Ts	WP_000818114.1	30 kDa	36	50
3	Elongation factor P	WP_000257278.1	21 kDa	7	4
4	Elongation factor 4	WP_000790168.1	67 kDa	3	6
5	Elongation factor P-like protein YeiP	WP_001136827.1	22 kDa	3	2
TL termination					
1	Peptide chain release factor 3 prfC	WP_000175940.1	60 kDa	2	3
2	Peptide chain release factor 1 prfA	WP_000804726.1	41 kDa	1	1

explains why the use of maltodextrins or maltose as carbon sources in this TXTL effectively increases protein synthesis.[19] It is interesting to note, however, that none of the enzymes related to the maltodextrin system (e.g. malZ, malQ, malP) were detected. This observation supports the idea that maltodextrins are transformed into glucose-1-phosphate only by the glycogen phosphorylase (WP_000081903.1) (Figure 2). All enzymes of the Krebs cycle (TCA cycle) were observed and for the vast majority with a high number of counts, which is expected because it is a central and essential pathway for aerobic organisms (Table 4). All enzymes of the pentose phosphate pathway were detected except for the 6-phosphogluconolactonase, which was not detected in any of the samples (Table 4).

The kinases of the common ATP regeneration systems (phosphoenolpyruvate, phosphoglycerate) are present in the lysates. The relatively large counts measured for the phosphoglycerate kinase explains why this phosphate donor has been the most efficient for the myTXTL system. The presence of an adenylate kinase and a nucleoside-diphosphate kinase also provide the necessary mechanisms for ATP and GTP regeneration in the system.

TABLE 3 Composition of the S50 ribosome subunit and other TL components measured by LC/MS in the all-*E. coli* TXTL lysate

#	Identified proteins	Access. No.	MW	Rep. 1	Rep. 2
Subunit S50					
REF	EF-Tu	WP_000031784.1	43 kDa	90	103
1	50S ribosomal protein L2	WP_000301864.1	30 kDa	38	40
2	50S ribosomal protein L17	WP_001216368.1	14 kDa	31	30
3	50S ribosomal protein L9	WP_001196062.1	16 kDa	31	31
4	50S ribosomal protein L5	WP_001096200.1	20 kDa	24	37
5	50S ribosomal protein L6	WP_000091945.1	19 kDa	31	33
6	50S ribosomal protein L1	WP_001096684.1	25 kDa	29	31
7	50S ribosomal protein L24	WP_000729185.1	11 kDa	30	26
8	50S ribosomal protein L3	WP_000579833.1	22 kDa	22	25
9	50S ribosomal protein L7/L12	WP_000028878.1	12 kDa	22	19
10	50S ribosomal protein L28	WP_000091955.1	9 kDa	20	22
11	50S ribosomal protein L19	WP_000065253.1	13 kDa	19	23
12	50S ribosomal protein L25	WP_000494183.1	11 kDa	20	19
13	50S ribosomal protein L10	WP_001207201.1	18 kDa	20	19
14	50S ribosomal protein L13	WP_000847559.1	16 kDa	19	18
15	50S ribosomal protein L23	WP_000617544.1	11 kDa	20	19
16	50S ribosomal protein L22	WP_000447529.1	12 kDa	19	19
17	50S ribosomal protein L4	WP_000424395.1	22 kDa	15	15
18	50S ribosomal protein L15	WP_001238917.1	15 kDa	16	17
19	50S ribosomal protein L14	WP_000613955.1	14 kDa	13	16
20	50S ribosomal protein L11	WP_001085926.1	15 kDa	12	15
21	50S ribosomal protein L18	WP_000358960.1	13 kDa	5	11
22	50S ribosomal protein L21	WP_000271401.1	12 kDa	13	8
23	50S ribosomal protein L30	WP_001140433.1	7 kDa	11	11
24	50S ribosomal protein L29	WP_000644741.1	7 kDa	10	10
25	50S ribosomal protein L20	WP_000124850.1	13 kDa	11	13
26	50S ribosomal protein L32	WP_000290727.1	6 kDa	8	9
27	50S ribosomal protein L16	WP_000941212.1	15 kDa	6	7
28	50S ribosomal protein L33	WP_001051798.1	6 kDa	8	8
29	50S ribosomal protein L27	WP_000940595.1	9 kDa	7	8
30	50S ribosomal protein L31	WP_000710769.1	8 kDa	5	6
31	50S ribosomal protein L3 N(5)-GM	WP_001295704.1	35 kDa	1	1
Other TL proteins					
1	Ribosome-recycling factor (RFF, RF4)	WP_000622418.1	21 kDa	12	12
2	Ribosome-associated inhibitor A (YfiA)	WP_000178456.1	13 kDa	8	7
3	Ribosome-binding factor A	WP_001040205.1	15 kDa	3	3
4	Ribosome hibernation promoting factor	WP_001176599.1	11 kDa	2	2
5	Ribosome maturation factor	WP_001300397.1	17 kDa	2	2
6	Ribosome silencing factor RsfS	WP_001161664.1	12 kDa	1	3
7	Ribosome assembly protein YhbY	WP_001054420.1	11 kDa	1	1
8	Ribosome maturation factor RimM	WP_000043335.1	21 kDa	1	1

Interestingly, the five cytoplasmic components of the ATP synthase making the F1 part are found in the lysates: atpA, atpC, atpD, atpG, atpH. The presence of a large fraction of the ATP synthase could be exploited to regenerate ATP in synthetic cell systems for instance. It would be interesting to express the membrane components making the F0 part (subunits a, b and c) and a proton pump like the bacteriorhodopsin to determine whether ATP regeneration can be achieved using proteins already present in the lysate.

In addition to metabolic pathways related to ATP regeneration, we sorted out the proteins involved in the synthesis of fatty acids (Table S1, supporting information). A majority of these proteins are found soluble in the cytoplasm. We found fifteen proteins involved in fatty acid biosynthesis. The MS counts suggest that most of these enzymes are present in relatively large amounts. This finding agrees with recent results demonstrating that *E. coli* cell extracts can convert acetyl-CoA into fatty acids of medium size.[31]

TABLE 4 Composition of the central metabolic pathways measured by LC/MS in the all-*E. coli* TXTL lysate

#	Identified proteins	Access. No.	MW	Rep. 1	Rep. 2
Glycolysis					
REF	EF-Tu	WP_000031784.1	43 kDa	90	103
1	Glucose-6-phosphate isomerase	WP_000789986.1	62 kDa	1	4
2	ATP-dependent 6-phosphofructokinase	WP_000591795.1	35 kDa	2	4
3	Fructose 1,6-bisphosphatase	WP_000853753.1	37 kDa	6	7
4	Fructose-bisphosphate aldolase	WP_000034372.1	39 kDa	17	23
5	Triose-phosphate isomerase	WP_001216325.1	27 kDa	8	8
6	Glyceraldehyde-3-phosphate dehydrogenase	WP_000048667.1	36 kDa	52	56
7	Phosphoglycerate kinase	WP_000111269.1	41 kDa	51	40
8	Phosphoglycerate mutase	WP_000942344.1	24 kDa	1	1
9	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	WP_001295305.1	29 kDa	23	24
10	Enolase	WP_000036723.1	46 kDa	35	40
11	Phosphoenolpyruvate synthase	WP_000069410.1	87 kDa	27	33
12	Pyruvate kinase I	WP_001295403.1	51 kDa	17	19
13	Pyruvate kinase II	WP_000091148.1	51 kDa	18	20
TCA cycle					
1	Pyruvate dehydrogenase E1 component	WP_000003829.1	100 kDa	49	53
2	Type II citrate synthase	WP_000785834.1	48 kDa	22	37
3	Aconitate hydratase B	WP_001307570.1	94 kDa	81	97
4	Aconitate hydratase A	WP_000099534.1	98 kDa	1	2
5	NADP-dependent isocitrate dehydrogenase	WP_000444487.1	46 kDa	43	50
6	2-oxoglutarate dehydrogenase subunit E1	WP_001181473.1	105 kDa	70	87
7	Succinyl-CoA ligase subunit alpha	WP_000025458.1	30 kDa	26	30
8	Succinyl-CoA ligase subunit beta	WP_001048602.1	41 kDa	44	56
9	Succinate dehydrogenase flavoprotein subunit	WP_000775540.1	64 kDa	17	23
10	Succinate dehydrogenase iron–sulfur subunit	WP_001235264.1	27 kDa	10	8
11	Fumarate hydratase class I	WP_000066639.1	60 kDa	25	26
12	Malate dehydrogenase	WP_001295272.1	32 kDa	32	37
Pentose phosphate					
1	Glucose-6-phosphate dehydrogenase	WP_000301720.1	56 kDa	13	12
2	Phosphogluconate dehydrogenase	WP_000043460.1	51 kDa	26	24
3	Ribulose-phosphate 3-epimerase	WP_000816280.1	25 kDa	1	4
4	Ribose-5-phosphate isomerase	WP_000189743.1	23 kDa	5	6
5	Transketolase	WP_000098614.1	72 kDa	37	45
6	Transaldolase	WP_000130189.1	35 kDa	28	37
Other proteins					
1	Glycogen phosphorylase	WP_000081903.1	91 kDa	5	5
2	Phosphoglucomutase	WP_001320171.1	58 kDa	10	10

3.5 | Other categories

Beyond the components for TX, TL, and metabolism, we focused our analysis on four other categories of proteins that we considered worth listing for TXTL: membrane proteins, chaperones, nucleases, and proteases. Several high-speed centrifugations are carried out during the preparation of the lysate. The buffers used for the extract preparation do not contain any reagent that would facilitate the resuspension of hydrophobic molecular components. Consequently, it is not surprising that traces of only two membrane proteins were detected (Table S1, supporting information): the outer membrane protein A and the outer membrane protein assembly factor BamC. This observation also suggests that the amount in the lysate of phospholipids such as liposomes or other forms is negligible. This conclusion is supported by the fact that membrane proteins, such as MscL-eGFP, are poorly expressed in the absence of added membranes.[32] The lysate contained substantial amounts of chaperones – notably DnaK, GroEL, GroES and ClpB (Table S1, supporting information). This is expected because chaperones like GroEL and DnaK are also among the most abundant proteins in

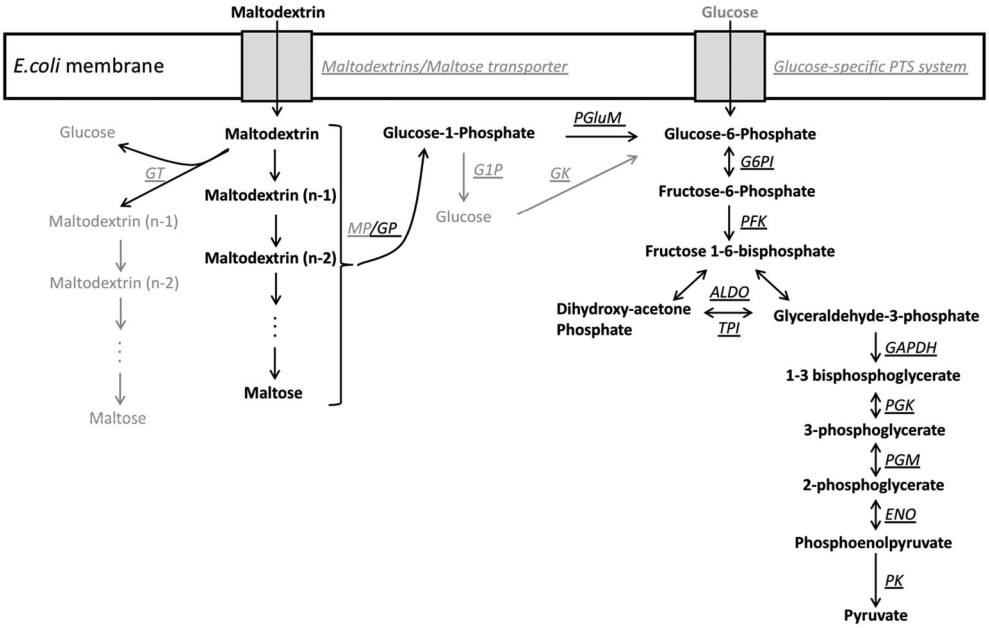

FIGURE 2 LC/MS analysis of maltodextrin and glucose metabolic pathways in TXTL. The enzymes detected by mass spectrometry are underlined, in italics and dark. The enzymes that are not detected are underlined in italics and grey. The intermediates processed in the TXTL metabolism are bold and dark while the intermediates not processed are bold and grey. For each step of maltodextrin degradation by the glycogen phosphorylase, one unit of glucose-1-phosphate is generated (n units). G6PI: glucose-6-phosphate isomerase, PFK: 6-phosphofructokinase, ALDO: fructose bisphosphate aldolase, TPI: triose-phosphate isomerase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglycerate mutase, ENO: enolase, PK: pyruvate kinase, MP: maltodextrin phosphorylase, GP: glycogen phosphorylase, GP: 4-alpha-glucanotransferase, G1P: glucose-1-phosphatase, GK: glucokinase, PGluM: phosphoglucomutase

E. coli.[33] The two pathways for periplasmic chaperone activity are represented by SurA and Skp. Interestingly, redox-specific chaperones are also present (YbbN and Hsp33). Some other chaperones are detected but at low abundance. Two other interesting categories to consider for TXTL are nucleases (DNases and RNases) and proteases. It is well established that circular closed DNA templates, such as plasmids, are stable in TXTL, as opposed to linear dsDNA, which are rapidly degraded by the RecBCD complex.[34,35] RecBCD is not detected in the lysate presumably because of its low concentration of ~10 nM (10 copies per cell).[36] Taking into account a dilution of 10 times with respect to the *E. coli* cytoplasm, the concentration of RecBCD in the lysate is estimated to be around 1 nM and on the order of 0.3 nM in a TXTL reaction. The low abundance of ribonucleases (Table S1, supporting information) explains the rather long mean lifetime previously found for mRNA,[8] around 18 min for the deGFP mRNA. The only notable ribonuclease is RNase II. The major ribonuclease, RNase E, in not detected in the lysate, as expected because it is a membrane-binding protein.[37] For proteases, the presence of ClpP and ClpX was expected and detected, as evidenced previously by the ability of this TXTL to degrade proteins tagged with ClpXP degrons.[38] The other major protease is DegP found in the periplasm.

Except traces of FtsZ (not detected in all the lysates), cytoskeletal proteins from the Fts and MreB families were not detected. Only the two cytoplasmic proteins of the secretion system, SecA and SecB, are present. Nine periplasmic ABC transporter binding proteins were detected.

3.6 | BL21 Rosetta2 versus A19 lysates

We compared the composition of the BL21 Rosetta2 lysate to the composition of the A19 lysate recently published.[6] While the major trends in composition are conserved, one can also observe notable differences. The glycolysis pathway is entirely present in the BL21 Rosetta2 lysate, as opposed to the A19 system. Glycolysis can be exploited in the BL21 Rosetta2 system by just adding a carbon source such as maltose or maltodextrin.[19] The other major difference between the two extracts is the abundance of membrane proteins detected in the A19 system compared to BL21. For instance, none of the proteins from the Omp family nor from the ABC transporters are present in BL21. The BL21 Rosetta2 strain is deficient in OmpT and Lon, which explains the absence of these two proteins in myTXTL lysates. Finally, only the primary sigma factor rpoD is found in the BL21 Rosetta2 lysates, whereas both rpoD and rpoN (sigma 54) are found in the A19 extract.

3.7 | Synthesis of deGFP

The reporter protein deGFP[15,38] was synthesized in a standard TXTL reaction (2018 batch) using the plasmid P70a-deGFP (5 nM in TXTL reaction) described previously.[8] As anticipated, deGFP was measured as the most abundant protein after an incubation time of more than 8 h (Table 5). deGFP is typically synthesized with this TXTL system at a final concentration of 1.5–2 mg/mL (60–80 μM). Synthesis of

TABLE 5 LC/MS data for the TXTL synthesis of deGFP in the all-*E. coli* TXTL system

#	Identified proteins	Access. Number	MW	Rep. 1	Rep. 2
REF	EF-Tu	WP_000031784.1	43 kDa	160	134
1	deGFP		25 kDa	172	162

TABLE 6 LC/MS data for the TXTL synthesis of the bacteriophages MS2, phix174, and T7

#	Identified proteins	Access. Number	MW	Rep. 1	Rep. 2
MS2					
REF	EF-Tu	WP_000031784.1	43 kDa	113	
1	MS2 assembly protein	NP_040647.1	44 kDa	12	
2	MS2 rna replicase beta chain	NP_040650.1	61 kDa	5	
3	MS2 coat protein	NP_040648.1	14 kDa	4	
phix174					
REF	EF-Tu	WP_000031784.1	43 kDa	72	66
1	phix174 protein F	AAA32578.1	48 kDa	111	85
2	phix174 protein B	AAA32572.1	14 kDa	52	36
3	phix174 protein H	AAA32580.1	34 kDa	33	32
4	phix174 protein A	AAA32570.1	59 kDa	32	19
5	phix174 protein G	AAA32579.1	19 kDa	23	23
6	phix174 protein D	AAA32575.1	17 kDa	12	11
7	phix174 protein K	AAA32573.1	6 kDa	8	9
8	phix174 protein C	AAA32574.1	10 kDa	8	8
T7					
REF	EF-Tu	WP_000031784.1	43 kDa	82	75
1	T7 DNA ligase	NP_041963.1	41 kDa	59	55
2	T7 major capsid protein	NP_041998.1	37 kDa	36	29
3	T7 internal virion protein D	NP_042004.1	144 kDa	38	8
4	T7 ss-DNA-binding protein	NP_041970.1	26 kDa	17	24
5	T7 tail fiber protein	NP_042005.1	62 kDa	26	11
6	T7 capsid assembly protein	NP_041996.1	34 kDa	19	16
7	T7 lysozyme	NP_041973.1	17 kDa	14	20
8	T7 tail tubular protein B	NP_042000.1	89 kDa	13	8
9	T7 hypothetical protein T7p37	NP_041990.1	9 kDa	11	9
10	T7 hypothetical protein T7p31	NP_041984.1	19 kDa	8	8
11	T7 DNA polymerase	NP_041982.1	80 kDa	9	6
12	T7 hypothetical protein T7p27	NP_041980.1	10 kDa	6	7
13	T7 internal virion protein C	NP_042003.1	84 kDa	8	2
14	T7 DNA primase/helicase	NP_041975.1	63 kDa	2	8
15	T7 head-tail connector protein	NP_041995.1	59 kDa	7	2
16	T7 protein kinase	NP_041959.1	41 kDa	6	1
17	T7 hypothetical protein T7p38	NP_041991.1	9 kDa	4	2
18	T7 host range protein	NP_041993.1	10 kDa	1	5
19	T7 hypothetical protein T7p39	NP_041992.1	15 kDa	3	3
20	T7 hypothetical protein T7p14	NP_041967.1	22 kDa	2	3
21	T7 hypothetical protein T7p13	NP_041966.1	10 kDa	2	2
22	T7 exonuclease	NP_041988.1	35 kDa	2	2
23	T7 tail tubular protein	NP_041999.1	22 kDa	1	2
24	T7 host recBCD nuclease inhibitor	NP_041987.1	6 kDa	2	1
25	T7 hypothetical protein T7p18	NP_041971.1	16 kDa	0	2

deGFP in the three tested lysates was comparable (Table S1, supporting information). The three lysates did not show any major differences in composition (Table S1, supporting information).

3.8 | Synthesis of bacteriophages

Our last experiment consisted of executing the genetic programs encoded within three phage genomes to fully synthesize viral particles.[8,11,17] Reactions for each phage were analyzed by LC/MS (Table 6). As reported previously,[8] in TXTL the number of plaque-forming units is around 10^{12}–10^{13} for MS2, 10^{12} for phix174, and 10^{11} for T7. The MS2 genome is composed of 3569 nucleotides of single-stranded RNA encoding four proteins: the maturation protein (A-protein), the lysis protein, the coat protein, and the replicase protein.[39] Except for the lysis protein, the three other proteins were detected. The absence of the lysis protein is explained by the fact that it is a membrane protein that precipitates in TXTL. TXTL reactions are centrifuged at low speed (15,000 *g*) after incubation, which eliminates most of the precipitated proteins when no membranes are added to the reaction. The phage phix174 has a circular single-stranded DNA genome of 5386 nucleotides encoding eleven proteins.[40] We used the phix174 dsDNA genome as template for the TXTL reaction. Protein A* can hardly be distinguished from A because it is an in-frame truncated version of A. Out of the other ten proteins, eight were detected by LC/MS. The phix174 lysis membrane protein E was not present, likely for the same reasons as the MS2 lysis protein. Protein J was the only structural protein not detected, possibly due to its small size (4.2 kDa). The three other structural proteins in the final virion, H, F and G, were abundant based on the LC/MS counts compared to EF-Tu. The third phage, T7, has a linear dsDNA of 40 kbp encoding about 60 proteins.[41-43] The final T7 virion is composed of eleven major structural proteins[44]: gp 6.7, 7.3, 8, 10A, 10B, 11, 12, 14, 15, 16, 17. Proteins 10A and 10B are hardly distinguishable because 10A is a slightly truncated version of 10B. Only the protein gp14 was not detected. We hypothesize that this protein was not detected because it is found in the outer membrane of host cells during infection,[45] which indicates that this protein is hydrophobic and may precipitate during treatment and cleaning of the samples before LC/MS. About fifteen other T7 proteins were detected including the most relevant: RNA and DNA polymerases, ligase, and RecBCD inhibitor.

4 | CONCLUSIONS

Cell-free transcription-translation is growing as a versatile tool to execute DNA programs and synthesize, in a few hours, single or tens of proteins in one-pot reactions. New approaches are necessary to broaden the advantages of the TXTL technology. In particular, new methods are necessary to analyze the products of TXTL reactions and to deepen our understanding of TXTL biochemical mechanisms governed predominantly by the molecular components present in the lysates. In this work, we applied LC/MS to a unique TXTL system that preserves the transcriptional and translational machinery of exponentially growing *E. coli* cells and showed that proteomics analysis could validate predicted molecular mechanisms and understand others, especially the metabolism behind ATP regeneration. Surprisingly, major differences in protein composition are revealed between lysates prepared from different *E. coli* strains. We assume that these differences are due to the strain used and to the preparation of the lysates. TXTL reactions do not need any special treatment before being analyzed by LC/MS, which makes the whole approach straightforward. LC/MS has become a standard technical resource for determining the protein composition of lysates specific to each strain, for characterizing the synthesized peptides and proteins in TXTL reactions. As suggested recently,[46] one can envision that LC/MS could be also useful to determine the composition of individual TXTL-based synthetic cells.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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