

The MazF-regulon: a toolbox for the post-transcriptional stress response in *Escherichia coli*

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ABSTRACT

Flexible adaptation to environmental stress is vital for bacteria. An energy-efficient post-transcriptional stress response mechanism in *Escherichia coli* is governed by the toxin MazF. After stress-induced activation the endoribonuclease MazF processes a distinct subset of transcripts as well as the 16S ribosomal RNA in the context of mature ribosomes. As these ‘stress-ribosomes’ are specific for the MazF-processed mRNAs, the translational program is changed. To identify this ‘MazF-regulon’ we employed Poly-seq (polysome fractionation coupled with RNA-seq analysis) and analyzed alterations introduced into the transcriptome and translome after *mazF* overexpression. Unexpectedly, our results reveal that the corresponding protein products are involved in all cellular processes and do not particularly contribute to the general stress response. Moreover, our findings suggest that translational reprogramming serves as a fast-track reaction to harsh stress and highlight the so far underestimated significance of selective translation as a global regulatory mechanism in gene expression. Considering the reported implication of toxin-antitoxin (TA) systems in

persistence, our results indicate that MazF acts as a prime effector during harsh stress that potentially introduces translational heterogeneity within a bacterial population thereby stimulating persister cell formation.

INTRODUCTION

During their lifetime, free-living bacteria have to deal with sudden environmental changes, e.g. in temperature, pH and nutrient availability, or to cope with the immune response and antibiotic treatment when invading a host. A general means to overcome adverse stress conditions is the stringent response, a bacterial survival mechanism by which the metabolism is reduced to a minimum. During the stringent response the alarmone guanosine tetra- or pentaphosphate (p)ppGpp is synthesized to trigger substantial alterations of the transcriptional program (1) by favoring alternative sigma factors that guide the RNA polymerase to the respective promoters (2). In addition, a variety of specific transcription factors can change the transcriptional landscape to ensure the physiological adaptation to the given conditions (3). Besides the transcriptional regulation, an increasing number of studies suggest that regulation at the post-transcriptional and translational level is likewise crucial for the modulation of protein synthesis, underlined by the rather imperfect correlation between tran-

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scriptomes and translomes (4). Hitherto, known mechanisms for translational regulation involve e.g. regulatory small RNAs (sRNAs), riboswitches and regulatory proteins that can mask or expose ribosome binding sites or affect the RNA stability. However, in contrast to the global regulatory effect governed by alternative transcription these post-transcriptional mechanisms are rather specific for individual targets.

In striking contrast, we recently identified a post-transcriptional regulatory mechanism in *Escherichia coli* that has the potential to globally affect protein synthesis in response to a variety of different stress conditions (5). When cells encounter stress the toxin-antitoxin (TA) module *mazEF* is activated by proteolysis of the antitoxin MazE. Consequently, the free toxin MazF cleaves RNAs specifically at single-stranded ACA-sites leading to the rapid degradation of bulk mRNA and overall reduction of protein synthesis (6). Besides, MazF generates a subset of leaderless mRNAs (lmRNAs) by cleaving specific transcripts at ACA-sites upstream of the AUG start codon. Surprisingly, the 16S rRNA incorporated in mature ribosomes is likewise targeted by MazF. The endoribonuclease removes 43 nucleotides (nts) from the 16S rRNA 3'-end comprising the anti-Shine-Dalgarno (aSD) sequence (5). Thereby, 70S^{Δ43} ribosomes are generated that are incapable to initiate translation on canonical mRNAs containing a long and structured 5'-untranslated region (UTR) due to the lack of the SD/aSD interaction. However, the modified 70S^{Δ43} ribosomes were shown to selectively translate lmRNAs (5) constituting the so called stress translation machineries (STMs) (7).

Several studies addressing the physiological significance of chromosomally encoded TA systems, which are abundant in free-living bacteria but lost from strictly host-associated bacteria (8), suggest their implication in the general stress response and biofilm formation (9). Furthermore, the role of TA systems in growth arrest, (programmed) cell death and cell survival is widely discussed (10,11) and their influence on bacterial persistence, in particular during antibiotic treatment, has been shown (12–14). Persisters are supposed to be a metabolically inactive, dormant fraction of a bacterial population that is—despite being genetically identical to their non-persistent kin—tolerant to lethal concentrations of antibiotics (15). Thus, despite this transient nature of the tolerance phenotype, bacterial persistence poses a severe health problem during antibiotic treatment of pathogenic bacteria, which possess an usual high number TA loci (8,16). However, at present the underlying mechanisms are still poorly understood. Considering that MazF activity results in the processing of specific mRNAs as well as modification of the translational machinery, we hypothesized that this post-transcriptional stress response mechanism might contribute to the differentiation of some cells of a population into persister cells. Hitherto, only a few highly abundant proteins have been identified, that remain to be synthesized after *mazF* activation employing 2D gel electrophoresis and mass spectrometry (17). As Vesper *et al.* have shown that about 50% of the ribosomes are cleaved by MazF after serine hydroxamate (SHX) treatment mimicking amino acid starvation (5), it is conceivable that this mechanism targets many more transcripts. To determine the

so-called ‘MazF-regulon’, i.e. the entity of processed and selectively translated mRNAs after *mazF* overexpression, we employed a Poly-seq analysis, combining polysome fractionation and next generation RNA sequencing. In contrast to the ribosome profiling analysis developed by Ingolia *et al.* (18), our approach is suitable to isolate intact, full length mRNAs from polysomes and thereby enables the concomitant analysis of the translome and the processing state of the polysome-associated mRNA. Hence, our results provide insights into the linkage between transcription and translation levels and represent a snapshot of the altered transcriptional and translational landscape in dependence of MazF activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions used in this study

Escherichia coli strain MC4100 F' (19) was used for the analysis in the absence of *mazF* overexpression. For the analysis upon *mazF* overexpression the same strain was transformed with plasmid pSA1 harboring the *lacI^q* gene as well as *mazF* under the control of the T5 promoter and the lac operator (17). Bacterial strains were grown at 37°C in Luria-Bertani (LB) broth, supplemented with 100 µg/ml ampicillin when required for plasmid maintenance. Growth was monitored by photometric measurement of the optical density at 600 nm.

Purification of total and polysome-associated RNA upon *mazF* overexpression

E. coli strains MC4100 F' and MC4100 F' pSA1 were grown at 37°C in LB. At OD₆₀₀ of 0.5, strain MC4100 F' pSA1 was treated with 100 µM IPTG for 15 min and then harvested by centrifugation. MC4100 F' was harvested without treatment at an OD₆₀₀ of 0.6. For total RNA preparation, 50 ml of cell cultures were harvested by centrifugation for 10 min at 4000 rpm and 4°C in an Eppendorf 5810 R centrifuge (Rotor FA 45–6–30) and cell pellets were frozen in liquid nitrogen. Total RNA was isolated using TRIzol®-reagent (Invitrogen) following the manufacturer's protocols.

For preparation of polysome-associated RNA 1.2 l of cell culture per sample were quickly chilled by pouring into 3x 500 ml centrifuge bottles (Nalgene) containing 100 g of fresh ice, while kept in an ice-water-bath (1:1 v/v) containing 10 g/l NaCl and immediately harvested by centrifugation at 4000 rpm for 10 min at 4°C in a Sorvall RC5-C (FiberLite F10S-6x500y rotor, Piramon Technologies). Cell pellets were kept on ice and gently resuspended in ice-cold TICO-lysis-buffer (TICO-buffer: 20 mM HEPES, 6 mM MgOAc, 6 mM NH₄OAc, 4 mM β-Mercapto-EtOH plus 4 mg/ml Lysozyme) to a final concentration of 200 OD₆₀₀-units per ml, transferred to a 50 ml conical centrifuge tube (Starlab), and slowly frozen at –20°C to avoid shearing of RNA. For gentle cell disruption the suspension was slowly thawed on ice and slowly refrozen at –20°C for three times. DNase I (RNase-free, Roche) was added in a concentration of 0.05 units per OD₆₀₀-unit and incubated for 10 min on ice after each thawing step. The S30 extracts were cleared in aliquots of 1 ml by centrifugation in 1.5 ml reac-

tions tubes (Sarstedt) at 30,000 g for 1 h at 4°C in a Sigma 3K30 centrifuge (rotor 12154) and stored at -80°C.

A total of 50–100 A₂₆₀-units of S30 extracts (in a maximum of 1 ml) were loaded onto a 10–30% sucrose gradient in TICO-buffer in SW28 tubes (SETON) to separate ribosomal subunits, monosomes and polysomes by centrifugation at 28,000 rpm for 3 h at 4°C in a Beckmann L-70 ultracentrifuge (Beckmann SW28 rotor). Upon fractionation, polysome fractions (Figure 1B, fractions 20–32, ~13 ml) were pooled and concentrated to 300 µl in H₂O-DEPC by precipitation with 10% sodium acetate (pH 5.2) and 50% 2-propanol over night at -20°C followed by centrifugation at 13,000 rpm for 1 h at 4°C in a Eppendorf 5810 R centrifuge (Rotor FA 45–6–30). RNA was isolated using TRIzol®-reagent (Invitrogen) following the manufacturer's protocols.

To remove accidentally co-purified genomic DNA from total or polysome derived RNA, the samples were treated with DNase I (RNase-free, Roche), extracted again with phenol/chloroform and ethanol-precipitation. Complete removal of DNA was verified by PCR (Primers for chromosomal *grcA*: 13/G1, data not shown). Ribosomal RNA was depleted using Ribo-Zero™ Magnetic Kit (Gram-Negative Bacteria, Epicentre) following the manufacturer's protocol. For further analysis, the depleted rRNA, bound to the magnetic beads, was recovered by phenol/chloroform extraction and ethanol-precipitation. For an overview of the purification process and efficiencies see the Supplementary Table S1.

Library preparation and next-generation sequencing

For the comparative RNA-seq analysis the following samples were used: Total RNA from untreated MC4100 F' cells ('T-') and from MC4100 F' pSA1 cells 15 min after induction of *mazF* overexpression by IPTG ('T+'), and polysome-associated mRNA from untreated MC4100 F' cells ('P-') and from MC4100 F' pSA1 cells 15 min after induction of *mazF* overexpression by IPTG ('P+'). Libraries from two biological replicates (R1 and R2) were prepared using 50–100 ng of the rRNA-depleted RNA using NEBNext® Ultra Directional RNA Library Prep Kit for Illumina (New England BioLabs), following the manufacturer's protocol. The quality of the resulting adaptor ligated cDNA was checked with the Agilent DNA Kit on an Agilent 2100 Bioanalyzer. Library preparation resulted in samples with average fragment sizes of 200–240 bp (data not shown). Samples were pooled (one set of four ('T-', 'T+', 'P-', 'P+') per replicate for one multiplex) and sequenced on Illumina HiSeq2000 with a single read length of 100 bp (VBCF NGS Unit; www.vbcf.ac.at). Sequence reads were mapped to the *E. coli* BW2952 MC4100 reference sequence (accession NC_012759).

Computational analysis

The sequencing resulted in a total of ~220 million raw reads per multiplex/replicate. Sequencing adapters were removed from the de-multiplexed samples with *cutadapt* (20). Quality control before and after adapter removal was performed with *FastQC* (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). The BW2952 MC4100 reference genome

and annotations (accession NC_012759) were obtained from the NCBI FTP server and reads were mapped against the reference genome with *segemehl* (v0.1.7) (21,22). Uniquely mapped reads were extracted for the downstream analysis and processed for UCSC visualization. Read count numbers for each sample were determined with the *htseq-count* utility from the *HTSeq* package (23) and differential gene expression analysis was performed with *DESeq* (24). Cutoff values for considering changes as significant are $\text{padj} < 0.05$ and $\log_2\text{fold change} < -0.6$ for down-regulation and > 0.6 for up-regulation. Visualization of aligned reads and coverage profiles were done with the UCSC genome browser (25). Coverage profiles of individual samples were normalized (26).

To cluster candidates according to their functions we used the function assignments provided by *EcoGene 3.0* (27). We downloaded a table of gene names, protein products and functions for all 4506 annotated genes (status of December 2014) and used the provided information to cluster the genes into the following functional classes: Metabolism and energy supply (ME), Cell cycle (CC), Protein synthesis (PS), Response regulation (RR), Cell structure (CS), Not classified (NC). See Supplementary Table S3 for a detailed list of the defined functional classes and subclasses. The matching of lists of candidates with the classification annotation list was performed with the R statistics software (28).

RESULTS

Purification of total and polysome-associated mRNA

In light of the hypothesized role of the *mazEF* module in cell survival and persist cell formation, our observation that MazF activity leads to reprogramming of protein synthesis prompted us to simultaneously analyze alterations introduced by MazF in the *E. coli* transcriptome and translome. As an initial approach we ectopically overexpressed *mazF* in *E. coli* strain MC4100 F' harboring plasmid pSA1 (17). The cells were grown in LB medium until mid-exponential phase, and 15 min after induction of *mazF* overexpression by addition of IPTG, total RNA ('T+') was isolated from two biological replicates for transcriptome analysis. Likewise, total RNA was prepared from untreated MC4100 F' cells ('T-'). Concomitantly, we prepared S30 extracts, which were separated on sucrose density gradients to subsequently isolate mRNAs from the polysome fractions ('P-' and 'P+') as schematically depicted in Figure 1A. In contrast to sequencing analysis of total RNA after *mazF* overexpression, which reveals the processing state of all RNAs in general, this additional step allows the determination of the entity of mRNAs that are selectively translated by the 70S^{Δ43} ribosomes and therefore associated to polysomes.

Polysomes are assemblies of 70S ribosomes translating simultaneously the same mRNA molecule (29), thus mRNAs associated to polysomes represent the translome. In contrast to the state of the art method for polysome-based translome analysis described by Ingolia *et al.* (18), we isolated full length mRNAs from polysomes without the use of translational inhibitors, to avoid a bias on the

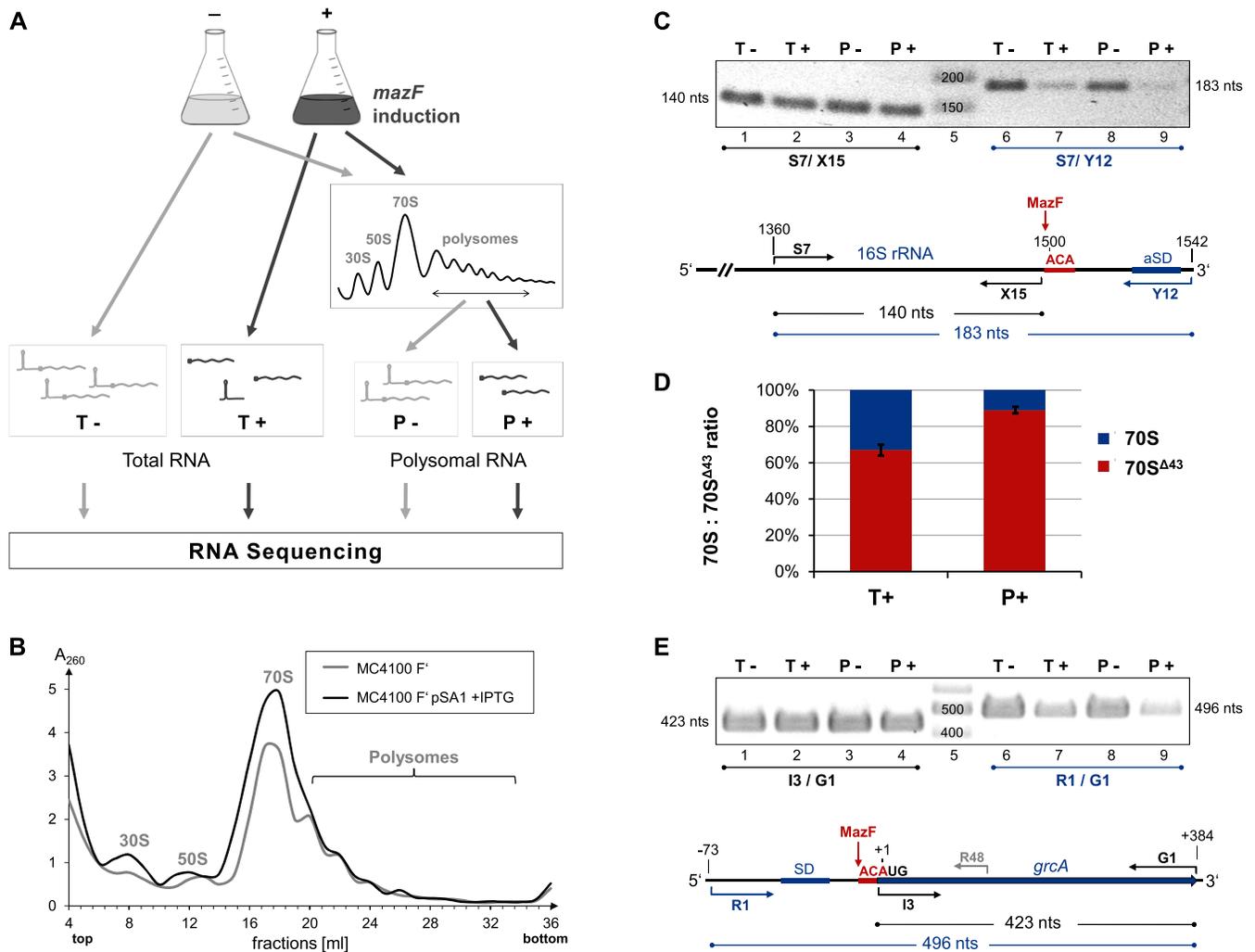


Figure 1. The RNA purification and validation of the method. (A) Schematic depiction of the workflow. *E. coli* MC4100 F⁻ (light gray, ‘-’) and MC4100 F⁻ pSA1 (dark gray, ‘+’) were cultured and *mazF* overexpression was induced in MC4100 F⁻ pSA1 by addition of IPTG at OD₆₀₀ of 0.5. Fifteen minutes thereafter total RNA was extracted (‘T’) and S30 extracts were subjected to sucrose density gradient centrifugation to obtain ribosome profiles shown in (B). RNA was isolated from the pooled polysome fractions (‘P’). All samples of two independent experiments were subjected to RNA-seq. (C) Processing of the 16S rRNA was determined by RT-PCR analysis performed on rRNA from total and polysomal RNA using forward primer S7 and reverse primers X15 or Y12 that bind upstream or downstream of the MazF-cleavage site, as indicated below. Lanes 1–4: RT-PCR with S7 and X15 served as internal control. Lanes 6–9: RT-PCR with S7 and Y12. (D) Signals obtained in the RT-PCR analysis shown in (C) were quantified and normalized. The 70S:70S^{Δ43} ratios calculated for the total (‘T+’) and polysomal (‘P+’) RNA purified after *mazF* overexpression are given. (E) RT-PCR on *grcA* mRNA in total and polysomal RNA using forward primers R1 or I3 that bind upstream or downstream of the MazF-cleavage site, respectively, as indicated below, and reverse primer G1. Lanes 1–4: RT-PCRs with I3 and G1 served as internal control. Lanes 6–9: RT-PCR with R1 and G1.

stress response. Furthermore, we disrupted the cells gently using lysozyme and three freeze-and-thaw cycles to avoid shearing of the RNA and degradation of the non-immobilized polysomes. The ribosomal subunits, monosomes and polysomes were separated by sucrose density gradient centrifugation of cell lysates. As shown in Figure 1B, the overall inhibition of translation after *mazF* induction is indicated by less pronounced polysome peaks (black line) when compared to ribosome profiles obtained from exponentially growing cells (gray line). The polysome fractions (Figure 1B, fractions 20–34) were pooled omitting the monosome peak in order to select for actively translated mRNAs. The respective RNA was isolated and upon depletion of rRNA *via* magnetic beads (Ribozero®, Epicen-

ter; see Supplementary Table S1, rows ‘P-’ and ‘P+’, column ‘rRNA depletion’) subjected to RNA-seq (see Materials and Methods).

Validation of rRNA and mRNA processing by MazF

First, we confirmed the formation of the 70S^{Δ43} ribosomes upon *mazF* overexpression. To this end, the rRNA recovered from magnetic beads used for depletion of the above mentioned RNA samples was subjected to reverse transcription PCR (RT-PCR). To distinguish between full length 16S rRNA (nts 1–1542) and MazF-processed 16S^{Δ43} rRNA (nts 1–1499), two different reverse primers specific for the 16S rRNA sequence upstream (X15) or downstream (Y12) of the MazF cleavage site were used in combination

with the forward primer S7, which anneals to a central region of the 16S rRNA (Figure 1C). Employing primer pair S7/X15, which anneals to both, intact and truncated 16S rRNA, we obtained comparable amounts of the expected product in all samples tested, without treatment (lanes 1 and 3) and upon overexpression of *mazF* (lanes 2 and 4), revealing that the same amount of rRNA was used in all RT-PCR analyses. Using primer pair S7/Y12, which is specific for the full length 16S rRNA, we obtained significantly weaker signals when using rRNA purified from cells upon *mazF* overexpression (lane 7 and 9) when compared to the sample taken from untreated cells (lane 6 and 8). Remarkably, quantification and normalization of the data indicated that 15 min after *mazF* induction more than 65% of the ribosomes are processed. Intriguingly, about 90% of the ribosomes present in the polysome fractions are 70S^{Δ43} ribosomes (Figure 1D). Together, these results not only prove the formation of 70S^{Δ43} ribosomes by MazF in general, they further underline that translationally active ribosomes after *mazF* overexpression are predominantly 70S^{Δ43} ribosomes, which lack the 3'-terminal 43 nts of the 16S rRNA due to MazF cleavage.

Next, the quality of isolated total and polysome-associated mRNA was assessed *via* RT-PCR using the *grcA* mRNA that has been previously identified as MazF target (formerly *yfiD*) (5). The encoded protein GrcA represents the glycine radical co-factor A that reactivates pyruvate formate lyase after oxidative stress (30). Active MazF cleaves at an ACA-site at position -2 relative to the A of the AUG start codon resulting in the selective translation of the leaderless *grcA* mRNA by the 70S^{Δ43} ribosomes. We confirmed the MazF-processing by primer extension (Supplementary Figure S2A) and RT-PCR analysis using polysomal RNA (Figure 1E). To discriminate between full length *grcA* mRNA comprising the 5'-UTR and the leaderless *grcA* mRNA variant we performed RT-PCR with reverse primer G1, hybridizing within the *grcA* coding region, in combination with either I3, annealing at the 5'-end of the *grcA* coding region downstream of the MazF cleavage site, or R1, binding to the 5'-UTR upstream of the MazF cleavage site (Figure 1E). RT-PCR performed with primers I3/G1 specific for both full length and leaderless *grcA* yielded the same amounts of the 423 nts long PCR product in all four samples tested (Figure 1E, lanes 1–4). In contrast the amount of the PCR products using primers R1/G1 specific for the full length *grcA* mRNA was significantly reduced in RNA extracted from cells after *mazF* overexpression (lanes 7). Using mRNA purified from polysomes the amount of this product is even further reduced (Figure 1E, lane 9) indicating that the actively translated *grcA* mRNA upon MazF activation is predominantly leaderless.

Taken together, these data reveal that the employed polysome purification procedure is appropriate to extract sufficient amounts of intact mRNA for downstream applications like RNA-sequencing. Thus, the polysome-associated mRNAs as well as the total RNAs were used to generate cDNA libraries that were subjected to deep sequencing as described in Material and Methods to identify transcripts that are selectively translated upon *mazF* overexpression thereby constituting the 'MazF-regulon'.

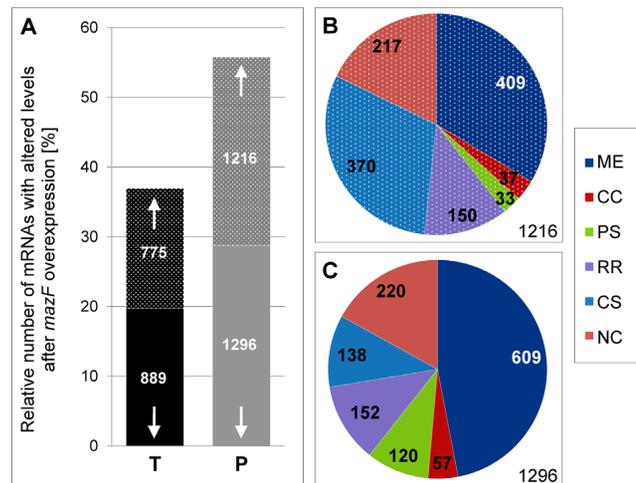


Figure 2. Alteration of mRNA levels after *mazF* overexpression in total and polysome-associated mRNAs. (A) The ratio between mRNAs with significantly increased (dotted) and decreased (plain) levels in total RNA (black) and polysome-associated mRNA (gray) after *mazF* overexpression is shown relative to the total number of *E. coli* genes, according to *EcoGene3.0* (27). Absolute numbers are indicated in each bar. (B) Distribution of polysome-associated mRNAs with significantly increased levels into the different functional clusters. (C) Distribution of polysome-associated mRNAs with significantly decreased levels into the different functional clusters. The absolute numbers of RNAs assigned to each functional cluster are indicated and represent the numbers given in Supplementary Table S2, columns 'P up A' and 'P down A'. (ME = metabolism and energy supply, CC = cell cycle, PS = protein synthesis, RR = response regulation, CS = cell structure, NC = not classified).

Selective translation plays a crucial regulatory role after *mazF* overexpression

First, we characterized MazF-mediated changes introduced in the transcriptome and translome employing a differential gene expression (DGE) analysis with *DESeq* (24) on the read count data obtained from total and polysome-associated RNA-seq data mapped with the short read aligner *segemehl* (21,22). We only considered transcripts with an adjusted *P*-value (*padj*) < 0.05 and a log2fold change > 0.65 or < -0.65 (3-fold change) significantly differentially abundant between the two conditions (\pm *mazF* overexpression). We found that upon *mazF* overexpression the levels of 1664 transcripts are significantly changed in total RNA, amongst those are 889 down-regulated and 775 up-regulated (Figure 2A). These numbers indicate that MazF induces a plethora of changes within only 15 min, as this number corresponds to 37% of the genome. This effect is even more pronounced in the polysome-associated mRNA fraction, where the levels of 2511 transcripts, representing 56% of the genome, are significantly altered (Figure 2A). Upon *mazF* overexpression 1296 mRNAs are less abundant in polysomes, whereas 1216 transcripts are more abundant. Additionally, we observed, that the transcript level alterations in total RNA and polysome-associated RNA do not entirely overlap (Supplementary Figure S1A and B).

Given these substantial alterations in total and polysome-associated mRNA levels, we next determined the physiological functions of the proteins encoded by the affected mR-

NAs applying a functional cluster analysis based on information provided by *EcoGene 3.0* (27) as specified in detail in Materials and Methods (also see Supplementary Table S3). We observed that almost half of the mRNAs, whose translation is reduced after *mazF* overexpression are functionally involved in the general cell ‘metabolism and energy supply’ (Figure 2C, dark blue). This result goes in line with the observations that activation of the toxin MazF leads to down-regulation of cellular metabolism (14). Our analysis further revealed that the levels of a rather large fraction of mRNAs that classify into ‘protein synthesis’ are decreased in polysomes after *mazF* overexpression and that correspondingly only the levels of 33 transcripts of this functional cluster are increased (Figure 2B and C, respectively, light green). Taken together, these results suggest that the ‘protein synthesis’ cluster is an example for negative regulation on the basis of selective protein synthesis during stress (also shown in Supplementary Figure S1C). By contrast, a large fraction of mRNAs that show augmented levels in polysomes after *mazF* overexpression, is involved in ‘cell structure’ (Figure 2B, light blue and Supplementary Figure S1C) indicating their selective translation after the stress.

Notably, the cluster specific MazF-induced transcript level alterations are only apparent when analyzing polysome-associated mRNA (Supplementary Table S2 and Supplementary Figure S1C). Likewise, the difference in mRNA abundance between total and polysome-associated mRNA is more pronounced after *mazF* overexpression (see Supplementary Figure S1D). Taken together, these observations strongly support the notion that the translational adaptation by the means of specialized ribosomes plays a significant role in the MazF-triggered stress response and suggest that MazF induces a first-level, fast-track stress response by generating the 70S^{Δ43} ribosomes.

The ‘MazF-regulon’

Finally, we analyzed the processing state of selectively translated mRNAs present in the polysomes after *mazF* overexpression. To this end, we screened the read count density profiles visualized in the UCSC genome browser (25) for variations in the transcript coverage (Table 1). In contrast to the expected generation of lmrRNAs, this analysis revealed that MazF processing not only occurs directly upstream of the AUG start codon as shown for the *grcA* mRNA (Figure 1E and Supplementary Figure S2A), but also can take place up to 100 nts upstream of the start codon yielding a processed but still leadered mRNA harboring a SD sequence. Nonetheless, these MazF-processed but leadered mRNAs are still predominantly associated to polysomes, i.e. they are actively translated. To validate the MazF-mediated processing at the observed ACA-sites in the 5′-UTR of 15 selected mRNAs with cleavage sites between one to 25 nts upstream of the start codon we performed primer extension analysis on total RNA (Figure 3 and Supplementary Figure S2). Further, we confirmed that in correspondence to the sequencing data the *erfK* and *infA* mRNAs despite comprising ACA-sites in their 5′-UTR are not cleaved by MazF at these positions (data not shown).

Further analysis of the MazF-regulon, comprising the 330 processed and significantly polysome-associated

mRNAs (listed in Table 1) revealed no particular functional clustering of the corresponding protein products (Figure 4A). We observed that transcripts with functions in ‘metabolism and energy supply’ and ‘protein synthesis’ are slightly overrepresented compared to the distribution of functional clusters among all *E. coli* genes (Figure 4B), whereas ‘not classified’ RNAs and RNAs with function in ‘cell structure’ are slightly underrepresented. This shows that the MazF-mediated stress response has a more wide-ranging impact than expected. Interestingly, 52 of the 330 (16%) processed mRNA, constituting the MazF-regulon, are essential. As only 7% of the *E. coli* genes are essential, this high number supports our hypothesis that the MazF-regulon represents a subset of mRNAs, essential or important for the bacterial population to survive during and to recover after stress.

Selective translation of MazF-processed mRNAs

The unexpected observation that the MazF-regulon not only comprises lmrRNAs but also processed transcripts with 5′-UTRs that still harbor a SD sequence is difficult to reconcile with the selective translation by 70S^{Δ43} ribosomes that lack the aSD sequence. Thus, we tested for translation initiation complex formation by 70S^{Δ43} ribosomes employing the full length and the MazF processed variants of the *rpsU* and the *groL* mRNAs as examples for a lmrRNA generated by MazF cleavage directly upstream of the AUG start codon (5) and a MazF-processed mRNA that still harbors a 5′-UTR comprising the SD-sequence generated by cleavage 25 nts upstream of the start codon, respectively (Figure 5). As shown in Figure 5A, toeprinting analysis employing the canonical *rpsU* mRNA comprising the 47 nts long 5′-UTR revealed that in contrast to 30S subunits (lane 2) isolated 70S^{Δ43} ribosomes do not form translation initiation complexes (lane 3). However, on the leaderless *rpsU* transcript the 70S^{Δ43} ribosomes are proficient to selectively form initiation complexes at the 5′-terminal AUG start codon (lane 4) whereas only a very weak toeprinting signal was detectable when canonical 30S subunits were used (lane 5). These results are in line with the selective translation of lmrRNAs by 70S^{Δ43} ribosomes described by Vesper *et al.* (5). Using the two *groL* mRNA variants comprising either the canonical 5′-UTR of 152 nts or only 25 nts after MazF-processing, respectively (Figure 5B and C), the analysis revealed that 70S^{Δ43} ribosomes are able to form a translation initiation complex on the MazF-processed transcript despite the presence of a 25 nts long 5′-UTR (Figure 5C, lane 8). As expected, we did not observe a toeprinting signal of the 70S^{Δ43} ribosomes when using the full length *groL* mRNA (lane 3). This result exemplifies that 70S^{Δ43} ribosomes are proficient to selectively translate MazF-processed transcripts even if they harbor a truncated 5′-UTR comprising the SD sequence.

DISCUSSION

The MazF-mediated stress response poses a novel prime example for a fast and energy-efficient post-transcriptional regulation mechanism in bacteria. Solely by triggering the degradation of one protein, namely the antitoxin MazE,

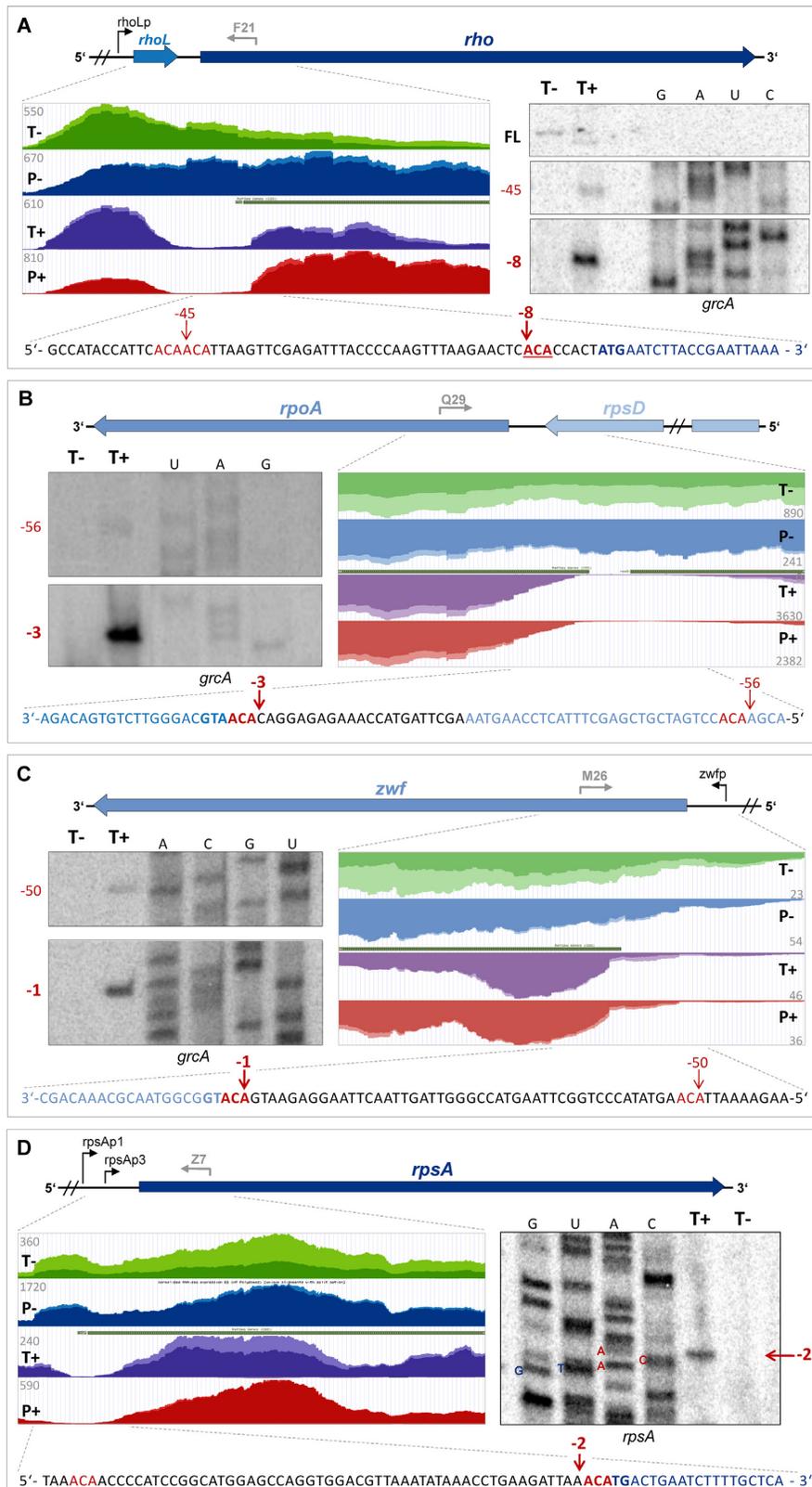


Figure 3. Validation of the MazF target mRNAs (A) *rho*, (B) *rpoA*, (C) *zwf* and (D) *rpsA*, respectively by primer extension analysis. Gene loci of the respective transcripts are schematically depicted by blue arrows. Positions of primers used for the analysis are indicated by gray arrows. The coverage profiles of sequencing reads performed on total RNA ('T', green and blue) and RNA extracted from polysomes ('P', blue and red) from *E. coli* cells during exponential growth ('-', green and blue) or 15 min after *mazF* overexpression ('+', purple and red) aligned to the respective genes and the corresponding primer extension analyses are shown. Sequencing reactions were performed using *in vitro* transcribed *grcA* (A, B and C) or *rpsA* mRNA (D), respectively. Below the nucleotide sequences of the respective regions are given. The coding region is highlighted in blue, the AUG start codon is shown in bold and the MazF cleavage sites are highlighted in red.

Table 1. The MazF-regulon. All MazF-processed and significantly polysome-associated mRNAs identified by the Poly-seq analysis are listed

Gene	cleaved ACA [Distance to start in nts]	Protein product	Classification
<i>mutH</i>	2	methyl-directed mismatch repair protein	CC
<i>mscL</i>	2	mechanosensitive channel protein, high conductance	CS
<i>tatC</i>	2	TatABCE protein translocation system subunit	CS
<i>aroG</i>	2	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, phenylalanine repressible	ME
<i>cycA</i>	2	D-alanine/D-serine/glycine transporter	ME
<i>ptrB</i>	2	protease II	ME
<i>sppA</i>	2	protease IV (signal peptide peptidase)	ME
<i>yggG</i>	2	Phe-Phe periplasmic metalloprotease, OM lipoprotein; low salt-inducible; Era-binding heat shock protein	ME
<i>srlB</i>	2	glucitol/sorbitol-specific enzyme IIA component of PTS	ME
<i>pdxY</i>	2	pyridoxamine kinase	ME
<i>nadC</i>	2	quinolinate phosphoribosyltransferase	ME
<i>grcA</i>	2	autonomous glycyl radical cofactor	ME
<i>zwf</i>	2	glucose-6-phosphate 1-dehydrogenase	ME
<i>gatZ</i>	2	D-tagatose 1,6-bisphosphate aldolase 2, subunit	ME
<i>glpK</i>	2	glycerol kinase	ME
<i>mltD</i>	2	predicted membrane-bound lytic murein transglycosylase D	ME
<i>fabD</i>	2	malonyl-CoA-[acyl-carrier-protein] transacylase	ME
<i>ispD</i>	2	4-diphosphocytidyl-2C-methyl-D-erythritol synthase	ME
<i>amn</i>	2	AMP nucleosidase	ME
<i>nrdA</i>	2	ribonucleoside-diphosphate reductase 1, alpha subunit	ME
<i>nupG</i>	2	nucleoside transporter	ME
<i>proS</i>	2	prolyl-tRNA synthetase	ME
<i>yajG</i>	2	putative lipoprotein	NC
<i>ybgL</i>	2	UPF0271 family protein	NC
<i>yjeI</i>	2	DUF4156 family lipoprotein	NC
<i>rpmB</i>	2	50S ribosomal subunit protein L28	PS
<i>rpsA</i>	2	30S ribosomal subunit protein S1	PS
<i>rpsU</i>	2	30S ribosomal subunit protein S21	PS
<i>rsuA</i>	2	16S rRNA pseudouridine(516) synthase	PS
<i>rpoN</i>	2	RNA polymerase, sigma 54 (sigma N) factor	PS
<i>srmB</i>	2	ATP-dependent RNA helicase	PS
<i>engA</i>	2	GTPase; multicopy suppressor of ftsJ	RR
<i>ygiW</i>	2	hydrogen peroxide and cadmium resistance periplasmic protein; stress-induced OB-fold protein	RR
<i>uspD</i>	2	stress-induced protein	RR
<i>ftsA</i>	3	ATP-binding cell division protein involved in recruitment of FtsK to Z ring	CC
<i>ftsE</i>	3	cell division ATP-binding protein	CC
<i>mltA</i>	3	membrane-bound lytic murein transglycosylase A	CS
<i>btuB</i>	3	vitamin B12/cobalamin outer membrane transporter	CS
<i>yadS</i>	3	UPF0126 family inner membrane protein	CS
<i>exbB</i>	3	membrane spanning protein in TonB-ExbB-ExbD complex	CS
<i>ffh</i>	3	Signal Recognition Particle (SRP) component with 4.5S RNA (ffs)	CS
<i>ynaI</i>	3	mechanosensitive channel protein, very small conductance	CS
<i>ptsH</i>	3	phosphohistidinoprotein-hexose phosphotransferase component of PTS system (Hpr)	ME
<i>srlA</i>	3	glucitol/sorbitol-specific enzyme IIC component of PTS	ME
<i>visC</i>	3	2-octaprenylphenol hydroxylase, FAD-dependent	ME
<i>yqjH</i>	3	putative siderophore interacting protein	ME
<i>kdsC</i>	3	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase	ME
<i>artI</i>	3	arginine transporter subunit	ME
<i>grxD</i>	3	glutaredoxin-4	NC
<i>yeaQ</i>	3	UPF0410 family protein	NC
<i>yoaH</i>	3	UPF0181 family protein	NC
<i>ytfK</i>	3	DUF1107 family protein	NC
<i>rplB</i>	3	50S ribosomal subunit protein L2	PS
<i>rpoA</i>	3	RNA polymerase, alpha subunit	PS
<i>trmJ</i>	3	tRNA mC32,mU32 2'-O-methyltransferase, SAM-dependent	PS
<i>cpxA</i>	3	response regulator in two-component regulatory system with CpxA	RR
<i>mdoG</i>	4	OPG biosynthetic periplasmic beta-1,6 branching glycosyltransferase	CS
<i>ydeE</i>	4	putative transporter	CS
<i>lgt</i>	4	phosphatidylglycerol-prolipoprotein diacylglycerol transferase	ME
<i>glnP</i>	4	glutamine transporter subunit	ME
<i>wecH</i>	4	O-acetyltransferase for enterobacterial common antigen (ECA)	ME
<i>yqaE</i>	4	cyaR sRNA-regulated protein	NC
<i>greA</i>	4	transcript cleavage factor	PS
<i>tig</i>	5	peptidyl-prolyl cis/trans isomerase (trigger factor)	CC
<i>yhhQ</i>	5	DUF165 family inner membrane protein	CS
<i>ynaJ</i>	5	DUF2534 family putative inner membrane protein	CS

Table 1. Continued

Gene	cleaved ACA [Distance to start in nts]	Protein product	Classification
<i>ilvL</i>	5	ilvG operon leader peptide	ME
<i>radA</i>	5	DNA repair protein	ME
<i>uxuR</i>	5	fructuronate-inducible hexuronate regulon transcriptional repressor; autorepressor	ME
<i>yafV</i>	5	putative NAD(P)-binding C-N hydrolase family amidase	ME
<i>yfcF</i>	5	glutathione S-transferase	ME
<i>ppiD</i>	5	periplasmic folding chaperone, has an inactive PPIase domain	NC
<i>ycdJ</i>	5	putative metalloenzyme	NC
<i>yacL</i>	5	UPF0231 family protein	NC
<i>ytfJ</i>	5	putative transcriptional regulator	NC
<i>yceA</i>	5	putative rhodanese-related sulfurtransferase	NC
<i>yeaO</i>	5	DUF488 family protein	NC
<i>yhdV</i>	5	putative outer membrane protein	NC
<i>rplR</i>	5	50S ribosomal subunit protein L18	PS
<i>rtcB</i>	5	RNA-splicing ligase	PS
<i>rnd</i>	5	ribonuclease D	PS
<i>emrA</i>	5	multidrug efflux system	RR
<i>treR</i>	5	trehalose 6-phosphate-inducible trehalose regulon transcriptional repressor	RR
<i>yceN</i>	6	putative lipid II flippase	CS
<i>ynjC</i>	6	putative ABC transporter permease	CS
<i>thrL</i>	6	thr operon leader peptide	ME
<i>glgB</i>	6	1,4-alpha-glucan branching enzyme	ME
<i>caiC</i>	6	putative crotonobetaine/carnitine-CoA ligase	ME
<i>vacJ</i>	6	ABC transporter maintaining OM lipid asymmetry, OM lipoprotein component	ME
<i>apt</i>	6	adenine phosphoribosyltransferase	ME
<i>gsk</i>	6	inosine/guanosine kinase	ME
<i>nrdB</i>	6	ribonucleoside-diphosphate reductase 1, beta subunit, ferritin-like protein	ME
<i>ydfZ</i>	6	selenoprotein, function unknown	NC
<i>ygiB</i>	6	DUF1190 family protein	NC
<i>rlmB</i>	6	23S rRNA mG2251 2'-O-ribose methyltransferase, SAM-dependent	PS
<i>yddM</i>	6	putative DNA-binding transcriptional regulator	RR
<i>yjhU</i>	6	putative DNA-binding transcriptional regulator; KpLE2 phage-like element	RR
<i>katG</i>	6	catalase-peroxidase HPI, heme b-containing	RR
<i>ysaZ</i>	7	putative L-valine exporter, norvaline resistance protein	ME
<i>mukE</i>	7	chromosome condensin MukBEF, MukE localization factor	NC
<i>pgm</i>	8	phosphoglucomutase	ME
<i>uhpA</i>	8	response regulator in two-component regulatory system with UhpB	ME
<i>coaA</i>	8	pantothenate kinase	ME
<i>pflA</i>	8	pyruvate formate-lyase 1-activating enzyme; [formate-C-acetyltransferase 1]-activating enzyme; PFL activase	ME
<i>fadH</i>	8	2,4-dienoyl-CoA reductase, NADH and FMN-linked	ME
<i>htrG</i>	8	SH3 domain protein	NC
<i>rho</i>	8	transcription termination factor	PS
<i>ampH</i>	9	D-alanyl-D-alanine-carboxypeptidase/endopeptidase; penicillin-binding protein; weak beta-lactamase	CS
<i>yidC</i>	9	membrane protein insertase	CS
<i>hisQ</i>	9	histidine ABC transporter permease	ME
<i>glmM</i>	9	phosphoglucomutase	ME
<i>ugpQ</i>	9	glycerophosphodiester phosphodiesterase, cytosolic	ME
<i>lrp</i>	9	leucine-responsive global transcriptional regulator	ME
<i>ycbZ</i>	9	putative peptidase	NC
<i>yeaT</i>	9	transcriptional activator of dmlA	RR
<i>murA</i>	10	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	CS
<i>rplL</i>	10	50S ribosomal subunit protein L7/L12	PS
<i>sohA</i>	10	antitoxin of the SohA(PrIF)-YhaV toxin-antitoxin system	RR
<i>yhcM</i>	11	divisome ATPase	CC
<i>shiA</i>	11	shikimate transporter	CS
<i>rbsK</i>	11	ribokinase	ME
<i>yjcE</i>	11	putative cation/proton antiporter	NC
<i>zipA</i>	12	FtsZ stabilizer	CC
<i>ivbL</i>	12	ilvB operon leader peptide	ME
<i>ndk</i>	12	multifunctional nucleoside diphosphate kinase and apyrimidinic endonuclease and 3'-phosphodiesterase	ME
<i>yhiR</i>	12	23S rRNA m(6)A2030 methyltransferase, SAM-dependent	PS
<i>phoB</i>	12	response regulator in two-component regulatory system with PhoR	RR
<i>yjgJ</i>	12	transcriptional repressor for divergent <i>bdcA</i>	RR
<i>iadA</i>	13	isoaspartyl dipeptidase	ME
<i>metL</i>	13	Bifunctional aspartokinase/homoserine dehydrogenase 2	ME
<i>yhhK</i>	13	PanD autocleavage accelerator, pantothenate synthesis	ME
<i>fpr</i>	13	ferredoxin-NADP reductase; flavodoxin reductase	ME

Table 1. Continued

Gene	cleaved ACA [Distance to start in nts]	Protein product	Classification
<i>gnd</i>	13	6-phosphogluconate dehydrogenase, decarboxylating	ME
<i>yjyG</i>	13	dUMP phosphatase	ME
rpsP	13	30S ribosomal subunit protein S16	PS
<i>ydeP</i>	13	putative oxidoreductase	RR
<i>hcaR</i>	14	hca operon transcriptional regulator	ME
<i>dipZ</i>	14	thiol:disulfide interchange protein and activator of DsbC	ME
<i>fdoG</i>	14	formate dehydrogenase-O, large subunit	ME
<i>rhcC</i>	14	Rhc protein with putative toxin domain; putative neighboring cell growth inhibitor	RR
<i>dnaQ</i>	15	DNA polymerase III epsilon subunit	CC
<i>fimE</i>	15	tyrosine recombinase/inversion of on/off regulator of fimA	CS
<i>ftiY</i>	15	cystine transporter subunit	CS
<i>dcuA</i>	15	C4-dicarboxylate antiporter	ME
<i>murP</i>	15	N-acetylmuramic acid permease, EIIBC component, PTS system	ME
<i>yciO</i>	15	putative RNA binding protein	NC
<i>yadH</i>	15	putative ABC transporter permease	NC
<i>bdm</i>	15	biofilm-dependent modulation protein	RR
<i>yfiB</i>	16	OM lipoprotein putative positive effector of YfiN activity	CS
<i>ycdS</i>	16	putative ABC transporter periplasmic binding protein	CS
<i>btuE</i>	16	glutathione peroxidase	ME
<i>sixA</i>	16	phosphohistidine phosphatase	ME
<i>yjfJ</i>	16	PspA/IM30 family protein	NC
<i>yeiS</i>	16	DUF2542 family protein	NC
<i>yhcB</i>	16	DUF1043 family inner membrane-anchored protein	NC
<i>yjbR</i>	16	DUF419 family protein	NC
<i>rpsG</i>	16	30S ribosomal subunit protein S7	PS
<i>lepA</i>	16	back-translocating elongation factor EF4, GTPase	PS
<i>lexA</i>	16	transcriptional repressor of SOS regulon	RR
<i>yggE</i>	16	oxidative stress defense protein	RR
<i>yjyD</i>	17	UPF0053 family inner membrane protein	CS
<i>yjiY</i>	17	putative transporter	CS
<i>ptsI</i>	17	PEP-protein phosphotransferase of PTS system (enzyme I)	ME
atpE	17	F0 sector of membrane-bound ATP synthase, subunit c	ME
<i>ymiA</i>	17	fructosamine kinase family protein	NC
<i>imp</i>	17	LPS assembly OM complex LptDE, beta-barrel component	RR
<i>seqA</i>	18	negative modulator of initiation of replication	CC
<i>frdA</i>	18	anaerobic fumarate reductase catalytic and NAD/ flavoprotein subunit	ME
<i>fabI</i>	18	enoyl-[acyl-carrier-protein] reductase, NADH-dependent	ME
<i>nlpC</i>	18	putative C40 clan peptidase lipoprotein	ME
<i>rpsT</i>	18	30S ribosomal subunit protein S20	PS
<i>feaR</i>	18	transcriptional activator for <i>tynA</i> and <i>feaB</i>	RR
<i>dnaN</i>	19	DNA polymerase III, beta subunit	CC
<i>sstT</i>	19	sodium:serine/threonine symporter	CS
<i>nepI</i>	19	putative transporter	CS
<i>fbp</i>	19	fructose-1,6-bisphosphatase I	ME
<i>galU</i>	19	glucose-1-phosphate uridylyltransferase	ME
<i>gatB</i>	19	galactitol-specific enzyme IIB component of PTS	ME
<i>cysQ</i>	19	3'(2'),5'-bisphosphate nucleotidase	ME
<i>nuoM</i>	19	NADH:ubiquinone oxidoreductase, membrane subunit M	ME
<i>wbbK</i>	19	lipopolysaccharide biosynthesis protein	ME
<i>thyA</i>	19	thymidylate synthetase	ME
<i>pepB</i>	19	aminopeptidase B	ME
<i>infC</i>	19	translation initiation factor IF-3	PS
<i>efp</i>	19	polyproline-specific translation elongation factor EF-P	PS
<i>prfA</i>	19	peptide chain release factor RF-1	PS
<i>marA</i>	19	multiple antibiotic resistance transcriptional regulator	RR
<i>nagZ</i>	19	beta N-acetyl-glucosaminidase	RR
<i>yobA</i>	19	CopC family protein	RR
<i>mdtK</i>	20	multidrug efflux system transporter	CS
<i>ilvD</i>	20	dihydroxyacid dehydratase	ME
<i>mtlA</i>	20	mannitol-specific PTS enzyme: IIA, IIB and IIC components	ME
<i>gapA</i>	20	glyceraldehyde-3-phosphate dehydrogenase A	ME
<i>rsd</i>	20	stationary phase protein, binds sigma 70 RNA polymerase subunit	RR
<i>rfaB</i>	21	lipopolysaccharide 1,6-galactosyltransferase; UDP-D-galactose:(glucosyl)lipopolysaccharide-1,6-D-galactosyltransferase	CS
<i>aroH</i>	21	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, tryptophan repressible	ME
<i>kefG</i>	22	potassium-efflux system ancillary protein for KefB, glutathione-regulated	CS
<i>aroP</i>	22	aromatic amino acid transporter	ME
<i>clpX</i>	22	ATPase and specificity subunit of ClpX-ClpP ATP-dependent serine protease	ME
<i>araF</i>	22	L-arabinose ABC transporter periplasmic binding protein	ME

Table 1. Continued

Gene	cleaved ACA [Distance to start in nts]	Protein product	Classification
<i>accB</i>	22	acetyl CoA carboxylase, BCCP subunit	ME
<i>folE</i>	22	GTP cyclohydrolase I	ME
<i>fadD</i>	22	acyl-CoA synthetase (long-chain-fatty-acid-CoA ligase)	ME
<i>lepB</i>	23	leader peptidase (signal peptidase I)	CS
<i>glpF</i>	23	glycerol facilitator	ME
<i>garR</i>	23	tartronate semialdehyde reductase	ME
<i>metF</i>	23	5,10-methylenetetrahydrofolate reductase	ME
<i>yjcZ</i>	23	YjcZ family protein; yjhH motility defect suppressor	NC
<i>yebZ</i>	23	inner membrane protein	RR
<i>clcB</i>	24	H(+)/Cl(-) exchange transporter	CS
<i>pepP</i>	24	proline aminopeptidase P II	ME
<i>panC</i>	24	pantothenate synthetase	ME
<i>pdxJ</i>	24	pyridoxine 5'-phosphate synthase	ME
acnB	24	aconitate hydratase 2; aconitase B; 2-methyl-cis-aconitate hydratase	ME
<i>ynjH</i>	24	DUF1496 family protein	NC
<i>yfiH</i>	24	UPF0124 family protein	NC
<i>frr</i>	24	ribosome recycling factor	PS
<i>ygdD</i>	25	UPF0382 family inner membrane protein	CS
<i>pabC</i>	25	4-amino-4-deoxychorismate lyase component of para-aminobenzoate synthase multienzyme complex	ME
<i>napB</i>	25	nitrate reductase, small, cytochrome C550 subunit, periplasmic	ME
<i>aphA</i>	25	acid phosphatase/phosphotransferase, class B, non-specific	ME
<i>ytfB</i>	25	OapA family protein	NC
rpmI	25	50S ribosomal subunit protein L35	PS
groL	25	Cpn60 chaperonin GroL, large subunit of GroSL	RR
<i>sbcB</i>	26	exodeoxyribonuclease I; exonuclease I	CC
<i>bacA</i>	26	undecaprenyl pyrophosphate phosphatase	RR
<i>ygcJ</i>	26	CRISP RNA (crRNA) containing Cascade antiviral complex protein	RR
<i>ubiE</i>	27	bifunctional 2-octaprenyl-6-methoxy-1,4-benzoquinone methylase/S-adenosylmethionine:2-DMK methyltransferase	ME
<i>yniC</i>	27	2-deoxyglucose-6-P phosphatase	ME
<i>yodD</i>	27	uncharacterized protein	RR
<i>cysU</i>	28	sulfate/thiosulfate ABC transporter permease	CS
<i>ydqQ</i>	28	DUF1375 family outer membrane protein	NC
<i>yigZ</i>	28	UPF0029 family protein	NC
<i>yhaH</i>	29	DUF805 family inner membrane protein,	CS
<i>metQ</i>	29	DL-methionine transporter subunit	ME
<i>speA</i>	29	biosynthetic arginine decarboxylase, PLP-binding	ME
<i>nrfA</i>	29	nitrite reductase, formate-dependent, cytochrome	ME
<i>udk</i>	29	uridine-cytidine kinase	ME
<i>hfq</i>	29	global sRNA chaperone; HF-I, host factor for RNA phage Q beta replication	RR
<i>aer</i>	29	fused signal transducer for aerotaxis sensory component/methyl accepting chemotaxis component	RR
<i>yjbB</i>	30	putative Na ⁺ /Pi-cotransporter	CS
<i>dsbA</i>	30	periplasmic protein disulfide isomerase I	PS
<i>tnaB</i>	31	tryptophan transporter of low affinity	ME
<i>otsB</i>	31	trehalose-6-phosphate phosphatase, biosynthetic	RR
<i>pstS</i>	32	phosphate ABC transporter periplasmic binding protein	CS
<i>rimM</i>	32	ribosome maturation factor	PS
<i>otsA</i>	32	trehalose-6-phosphate synthase	RR
<i>argT</i>	34	lysine/arginine/ornithine transporter subunit	ME
<i>livJ</i>	34	branched-chain amino acid ABC transporter periplasmic binding protein	ME
<i>pepN</i>	34	aminopeptidase N	ME
<i>pagB</i>	34	lipid A phosphoethanolamine transferase	ME
<i>hokD</i>	34	Qin prophage; small toxic polypeptide	RR
<i>hflB</i>	35	protease, ATP-dependent zinc-metallo	ME
<i>narP</i>	35	response regulator in two-component regulatory system with NarQ	ME
<i>yejG</i>	35	uncharacterized protein	NC
<i>rimN</i>	35	tRNA(ANN) t(6)A37 threonylcarbamoyladenosine modification protein, threonine-dependent ADP-forming ATPase	PS
<i>ygaW</i>	36	alanine exporter, alanine-inducible, stress-responsive	ME
<i>hybC</i>	36	hydrogenase 2, large subunit	ME
<i>yciK</i>	36	putative EmrKY-TolC system oxoacyl-(acyl carrier protein) reductase	ME
<i>rpoD</i>	36	RNA polymerase, sigma 70 (sigma D) factor	PS
<i>cmoA</i>	36	carboxy-SAM synthase	PS
<i>hslO</i>	36	heat shock protein Hsp33	RR
<i>gatC</i>	37	pseudogene, galactitol-specific enzyme IIC component of PTS	ME
<i>plsC</i>	37	1-acyl-sn-glycerol-3-phosphate acyltransferase	ME
<i>gloA</i>	38	glyoxalase I, Ni-dependent	ME

Table 1. Continued

Gene	cleaved ACA [Distance to start in nts]	Protein product	Classification
<i>ygcK</i>	38	CRISP RNA (crRNA) containing Cascade antiviral complex protein	RR
<i>iscX</i>	39	Fe(2+) donor and activity modulator for cysteine desulfurase	NC
<i>cheZ</i>	39	chemotaxis regulator, protein phosphatase for CheY	RR
<i>yfbV</i>	40	UPF0208 family inner membrane protein	CS
<i>yaaJ</i>	40	putative transporter	ME
<i>dgkA</i>	40	diacylglycerol kinase	ME
<i>sbmA</i>	41	peptide antibiotic transporter	RR
<i>hslU</i>	41	molecular chaperone and ATPase component of HslUV protease	RR
<i>aroB</i>	42	3-dehydroquinate synthase	ME
<i>yqeF</i>	42	short chain acyltransferase	ME
<i>cysW</i>	43	sulfate/thiosulfate ABC transporter permease	CS
<i>dmsD</i>	43	twin-arginine leader-binding protein for DmsA and TorA	ME
<i>rlmL</i>	43	23S rRNA m(2)G2445 and m(7)G2069 methyltransferases, SAM-dependent	PS
<i>lptB</i>	44	lipopolysaccharide export ABC transporter ATPase	CS
<i>hcaT</i>	45	putative 3-phenylpropionic transporter	ME
<i>fabG</i>	45	3-oxoacyl-[acyl-carrier-protein] reductase	ME
<i>csiR</i>	45	transcriptional repressor of <i>csiD</i>	RR
<i>yjgP</i>	46	lipopolysaccharide export ABC permease	CS
<i>dkgA</i>	47	2,5-diketo-D-gluconate reductase A	ME
<i>acpP</i>	49	acyl carrier protein (ACP)	ME
<i>rluB</i>	49	23S rRNA pseudouridine(2605) synthase	PS
<i>yheV</i>	50	DUF2387 family putative metal-binding protein	NC
<i>gldA</i>	51	glycerol dehydrogenase, NAD ⁺ dependent; 1,2-propanediol:NAD ⁺ oxidoreductase	ME
<i>rfaQ</i>	51	lipopolysaccharide core biosynthesis protein	ME
<i>crr</i>	52	glucose-specific enzyme IIA component of PTS	ME
<i>fimA</i>	53	major type 1 subunit fimbriae (pilin)	CS
<i>rffA</i>	53	TDP-4-oxo-6-deoxy-D-glucose transaminase	ME
<i>tap</i>	54	methyl-accepting protein IV	RR
<i>lamB</i>	55	maltose outer membrane porin (malto porin)	ME
<i>dusA</i>	56	tRNA-dihydrouridine synthase A	PS
<i>dnaA</i>	58	chromosomal replication initiator protein DnaA, DNA-binding transcriptional dual regulator	CC
<i>yjjP</i>	58	DUF1212 family inner membrane protein	CS
<i>rpoB</i>	58	RNA polymerase, beta subunit	PS
<i>thrS</i>	59	threonyl-tRNA synthetase	ME
<i>yjgR</i>	60	DUF853 family protein with NTPase fold	NC
<i>yheN</i>	60	sulfurtransferase for 2-thiolation step of mnm(5)-s(2)U34-tRNA synthesis	PS
<i>leuE</i>	61	leucine efflux protein	ME
<i>mtgA</i>	62	biosynthetic peptidoglycan transglycosylase	CS
<i>yfeX</i>	63	porphyrinogen oxidase, cytoplasmic	ME
<i>nemA</i>	63	chromate reductase, quinone reductase, FMN-linked; N-Ethylmaleimide reductase; old yellow enzyme	ME
<i>csdE</i>	64	CsdA-binding activator; Fe-S protein	ME
<i>fre</i>	65	NAD(P)H-flavin reductase	ME
<i>yrfG</i>	65	GMP/IMP nucleotidase	ME
<i>ygiC</i>	66	ATP-Grasp family ATPase	ME
<i>sspA</i>	66	stringent starvation protein A, phage P1 late gene activator, RNAP-associated acid-resistance protein, inactive glutathione S-transferase homolog	RR
<i>wbbJ</i>	67	putative lipopolysaccharide biosynthesis O-acetyl transferase	ME
<i>rdgB</i>	68	dITP/XTP pyrophosphatase	CS
<i>yceH</i>	71	UPF0502 family protein	NC
<i>lldR</i>	75	dual role activator/repressor for <i>lldPRD</i> operon	ME
<i>ysaA</i>	75	putative hydrogenase, 4Fe-4S ferredoxin-type component	ME
<i>rpsJ</i>	75	30S ribosomal subunit protein S10	PS
<i>aaeB</i>	76	p-hydroxybenzoic acid efflux system component	CS
<i>ytjA</i>	76	uncharacterized protein	NC
<i>greB</i>	76	transcript cleavage factor	PS
<i>clpP</i>	78	proteolytic subunit of ClpA-ClpP and ClpX-ClpP ATP-dependent serine proteases	ME
<i>glpP</i>	78	glutamate/aspartate:proton symporter	ME
<i>glnE</i>	83	fused deadenyltransferase/adenylyltransferase for glutamine synthetase	PS
<i>rne</i>	83	endoribonuclease; RNA-binding protein; RNA degradosome binding protein	PS
<i>deaD</i>	83	ATP-dependent RNA helicase	PS
<i>luxS</i>	85	S-ribosylhomocysteine lyase	RR
<i>pstB</i>	91	phosphate ABC transporter ATPase	CS
<i>pepA</i>	95	multifunctional aminopeptidase A: a cyteinyglycinase, transcription regulator and site-specific recombination factor	ME
<i>dut</i>	95	deoxyuridinetriphosphatase	ME

Table 1. Continued

Gene	cleaved ACA [Distance to start in nts]	Protein product	Classification
<i>rpsB</i>	97	30S ribosomal subunit protein S2	PS
<i>rpoS</i>	108	RNA polymerase, sigma S (sigma 38) factor	PS

The distance of the MazF cleavage sites to the AUG start codon, the encoded protein products as well as their respective functional clusters are given. mRNAs whose cleavage was experimentally verified by primer extension analysis are indicated in bold. (ME = metabolism and energy supply, CC = cell cycle, PS = protein synthesis, RR = response regulation, CS = cell structure, NC = not classified).

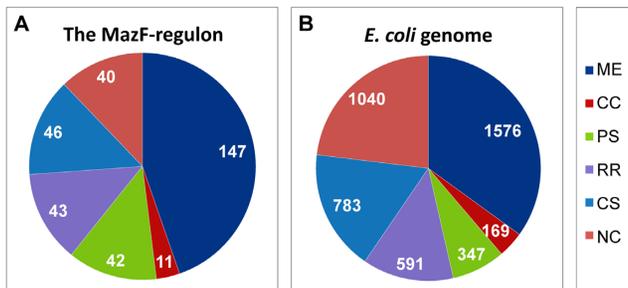


Figure 4. Functional cluster analysis of the MazF-regulon. (A) Functional cluster analysis was performed with all 330 MazF-processed and selectively translated mRNAs, comprising the MazF-regulon, according to function assignments provided by *EcoGene 3.0* (27) (B) shows the distribution of the functional clusters within the entity of all 4506 *E. coli* genes. (ME = metabolism and energy supply, CC = cell cycle, PS = protein synthesis, RR = response regulation, CS = cell structure, NC = not classified).

protein synthesis is modulated due to selective translation of a subset of processed mRNAs by the concomitantly generated 70S^{A43} ribosomes (5). Recently, several lines of evidence indicate that the activation of TA modules affects persister cell formation. Thus, we aimed to decipher the entity of MazF-processed and selectively translated mRNAs, the ‘MazF-regulon’, in order to shed light on physiological alterations, which are potentially required for the reprogramming of distinct cells toward persistence. With this end in view, we established a method to isolate intact, full length mRNAs from polysomes, avoiding physiological interference by translation blocking agents and subsequent RNA-sequencing analysis (Figure 1A and Supplementary Table S1). Given the high potential for reciprocal activation of the different TA systems present in *E. coli* as well as their induction by antibiotic treatment, we chose artificial ectopic *mazF* pulse-expression to enrich for immediate MazF targets and to primarily study direct effects of the toxin. Moreover, this approach facilitates the determination of the isolated MazF-mediated effects without induction of additional stress response mechanisms like, e.g. alternative transcription, which would likewise be triggered by physiological stress conditions. As shown in Figure 1, our validation experiments revealed that our approach allows the identification of processed mRNAs entailed by the overexpression of *mazF*. Collectively, this study resulted in the identification of 330 transcripts that are cleaved by MazF within their 5′-UTR and consequently efficiently associated to polysomes upon *mazF* overexpression.

Interestingly, these MazF-processed transcripts are not particularly involved in the stress response, but encode pro-

teins with a broad variety of functions (Figure 4 and Table 1) indicating the widespread effects of MazF activity and consequently translational selectivity in response to stress. In light of the fact that TA systems and in particular the *mazEF* system, are required for persistence (14), we envisage the following model to explain the potential impact of the MazF-mediated stress response on persistence. Our study addresses the entire bacterial population, thus we cannot conclude how transcription and translation are altered in every individual cell. However, given the functional diversity of the proteins encoded by MazF-processed transcripts it is conceivable that MazF induces heterogeneous effects within single cells of the population. Moreover, given the fast reaction to stress conditions triggered solely by degradation of the antitoxin MazE, we hypothesize that the variations introduced by the TA system might differ from cell to cell with regard to their current status, e.g. during different phases of the division cycle or their intrinsic age (31). Thus, MazF could act as a prime effector in response to stress, which might have the potential to amplify the cell variations within a population in an undirected manner and hence would consequently give rise to a variety of heterogeneous cells with distinct physiologies. Moreover, considering the proposed reciprocal activation of different TA systems present in *E. coli* we hypothesize that the interconnected activity of different TA systems further increases the phenotypic variability and in turn stimulates persister cell formation either by induction of stochastic variations in gene expression or by amplification of the molecular noise. This stochastic increase in variability could ensure that a few cells within a population are equipped with a unique toolbox, i.e. a combination of proteins and/or RNAs required to sustain challenging conditions.

The underestimated significance of translational regulation and ribosome specificity

Considering the general stress response, which is mediated primarily at the transcriptional level, one would expect a direct correlation between the transcriptional regulation of a particular mRNA and its translational efficiency as exemplified by its presence in the polysome fraction. However, this assumption is not supported by our first comparative analysis of polysome-associated *versus* total RNA. Interestingly, we observed that the changes in mRNA levels in response to *mazF* overexpression are more pronounced in the polysome-associated mRNA when compared to total RNA (Figure 2A and Supplementary Figure S1C and D). Further, almost 50% of the mRNAs that are differentially associated to polysomes upon *mazF* overexpression are not

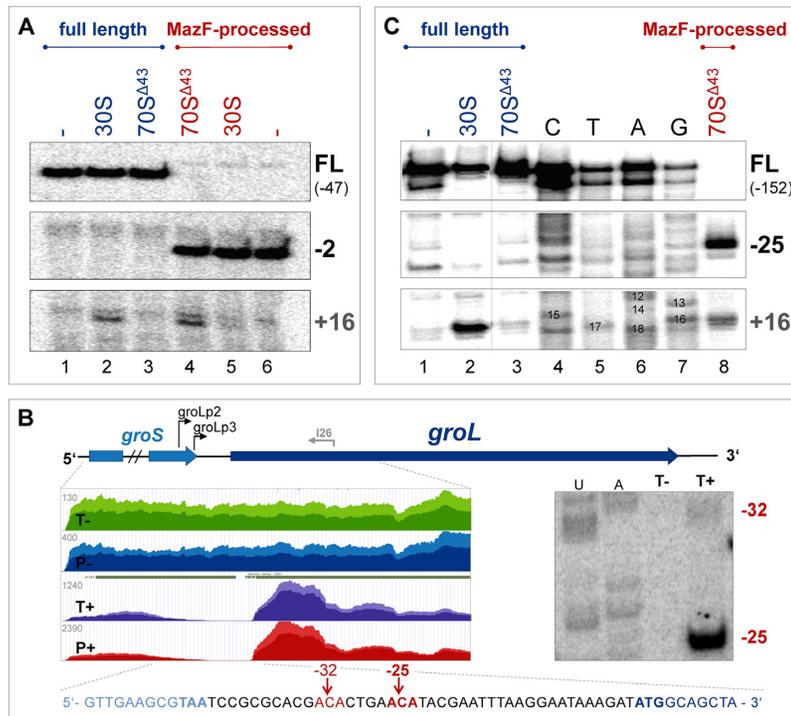


Figure 5. Selective translation initiation of MazF-processed mRNAs by 70S Δ 43 ribosomes. (A) Toeprinting analysis on full length (blue) and leaderless (red) *rpsU* mRNA using 30S (lanes 2 and 5) and 70S Δ 43 ribosomes (lanes 3 and 4). Lanes 1 and 6 (‘-’): no ribosomes added. (B) Validation of MazF induced cleavage of *groL* by primer extension analysis as described in Figure 3. (C) Toeprinting analysis on full length (blue) and leaderless (red) *groL* mRNA using 70S Δ 43 ribosomes (lanes 3 and 8). Primer extension analysis on full length *groL* mRNA in the absence of ribosomes (lane 1) and in the presence of 30S ribosomal subunits (lane 2) served as control. The sequencing reaction was performed on full length *groL* mRNA.

significantly regulated at the total RNA level (Supplementary Figure S1A and B). Taken together, our data indicate that in contrast to relaxed conditions, regulation at the level of translation plays a major role in response to stress. This notion was recently strongly supported by Picard *et al.* who analyzed the translational response of the lactic acid bacterium *Lactococcus lactis* during isoleucine starvation by ribosome profiling coupled with microarray analysis (32). The authors present evidence that translational regulation significantly contributes to the stress response. Correspondingly, Taylor *et al.* investigated the extent of translational regulation in protein synthesis of *Shewanella oneidensis MR-1* during oxygen limitation by comparing RNA sequencing and proteome data (32). They report that the alteration of translational efficiency contributes to about 75% of the changes in protein levels.

In our analysis, the entire set of transcripts encoding ribosomal proteins (RPs) intriguingly exemplifies the stress-responsive regulation by selective translation. Here, 46 out of 54 RP-encoding mRNAs are significantly reduced in polysomes after *mazF* overexpression. This is also reflected by the large fraction of ‘protein synthesis’ transcripts, which are reduced in polysomes after *mazF* overexpression (Supplementary Figure S1C). However, only 14 out of these are also reduced in the total RNA pool. In addition, eleven RP-coding mRNAs (encoding proteins bS1, uS2, uS7, uS10, bS16, bS20, uL2, bL7, uL18, bL28 and bL35 (33)) are processed by MazF and found to be associated with polysomes (Table 1). In contrary, over 50% of all ‘cell structure’ tran-

scripts are particularly augmented in polysomes after *mazF* overexpression (Supplementary Figure S1C). Together, our observations highlight the significance of translational selectivity, at the level of ribosome heterogeneity and put forward the notion that the immediate response to harsh stress conditions does not rely on the generation of additional regulatory protein or RNA factors.

Selected MazF targets in the spotlight

With respect to their physiological functions associated with the stress response, important candidates for MazF-cleavage are the *rho*, *rpoA*, *zwf* and *rpsA* mRNAs encoding transcription termination factor Rho, the α -subunit of RNAP, the glucose-6-phosphate 1-dehydrogenase and RP bS1, respectively.

The transcription termination factor Rho (Figure 3A) promotes dissociation of the RNA polymerase (RNAP) and the nascent mRNA from the template DNA by ATP-dependent helicase activity upon binding to the so-called *rut* (*rho utilization*) sites in the nascent transcript (34,35). It has been shown that transcription and translation are coupled by indirect interaction of the ribosome and RNAP under favorable conditions (36,37). Thereby, frequent *rut* sites within coding regions of mRNAs, that would recruit Rho and hence lead to premature transcription termination, are obscured by the ribosome. When translation is shut down due to stress-induced MazF activity, Rho can access these *rut* sites and promote transcription termination (38). It is conceivable that sustained production of Rho *via* selective

translation of its MazF-processed mRNA might link decreased protein synthesis to early transcription termination in order to save resources for the stressed cells. Furthermore, Rho has been linked to additional regulatory functions in gene expression (38) which might likewise be important during the stress response.

The α -subunit of RNAP (*rpoA*, Figure 3B) is essential for assembly of the core RNAP and involved in the regulation of transcription initiation *via* the α -subunit. Recently, RNAP α was shown to interact with RP uL2 that acts as a transcriptional regulator (39). As we likewise identified the *rplB* transcript coding for uL2 as a MazF target, one could surmise that the transcriptional regulation *via* uL2-RNAP α might be of importance during stress response or stress recovery.

The *zwf* gene (Zwischenferment, Figure 3C) encodes the glucose-6-phosphate 1-dehydrogenase. Interestingly, the penta-peptide NNWDN (Asn-Asn-Trp-Glu-Asn; residues 199–203 of Zwf) is excised from the protein by the ClpPX protease (40) and is likely to be converted to NNWNN (Asn-Asn-Trp-Asn-Asn) by the asparagine synthase A (AsnA) (41). NNWNN represents the quorum sensing molecule ‘extracellular death factor’ (EDF), which is secreted into the extracellular environment and thus relays cell density information to the MazEF complex, thereby triggering MazF toxicity (42). Deletion of the genes *zwf* or *asnA* both individually prevented production of active EDF (41). Thus, the removal of the *zwf* 5'-UTR by MazF might ensure the continuous synthesis of the corresponding protein in order to preserve EDF production (43).

Protein bS1 (*rpsA*, Figure 3D) is crucial for efficient translation initiation in Gram-negative bacteria (44–46), but is dispensable for the translation of l-mRNAs (47,48). As the MazF-mediated stress response mechanism is based on translation of l-mRNAs, bS1 would not be required during stress. However, continuous synthesis of bS1 under these conditions from the leaderless transcript might be crucial to ensure its required presence during recovery from stress when translation of canonical mRNAs becomes prevalent again.

The ‘MazF-regulon’

Surprisingly and in contrast to our expectations, the determination of the ‘MazF-regulon’ revealed that MazF processing of mRNAs does not only result in the formation of l-mRNAs. In addition, we identified processing events that leave truncated 5'-UTRs with various lengths. Despite the presence of these 5'-UTRs that comprise the SD sequence these processed transcripts are selectively translated after *mazF* overexpression. Further toeprinting analyses using the leaderless *rpsU* mRNA and the MazF-processed *groL* mRNA that comprises a 25 nts long 5'-UTR verified that 70S Δ^{43} ribosomes are able to form translation initiation complexes at both MazF-processed transcripts, the leaderless and the leadered mRNA (Figure 5). Taken together, our results indicate that the translational selectivity not only relies on the presence of a 5'-terminal AUG start codon. We hypothesize that MazF-processing by itself primes mRNAs to selective translation by 70S Δ^{43} ribosomes, rather than being rendered leaderless. Noteworthy, the cleavage by MazF

leaves the mRNAs with a 5'-hydroxyl. Consequently, the processed transcripts are not targeted by RNase E and are thus stabilized (49). However, conceptually related to the selective recognition of the 5' monophosphate by RNase E, we hypothesize that the 5'-hydroxyl might represent a primary feature stimulating the selective interaction with the 70S Δ^{43} ribosome in the absence of the SD-aSD interaction. Thus, our results raise the possibility that translation of MazF-processed transcripts initiates with the recognition of the 5'-hydroxyl group by the 70S Δ^{43} ribosomes that are equipped with the initiator tRNA. Subsequently, the 70S Δ^{43} ribosomes would scan the mRNA downstream to the AUG start codon. As structures within the 5'-UTR would interfere with the scanning process, the removal of structured regions by MazF processing might also stimulate the translational efficiency of the 70S Δ^{43} ribosomes. However, the underlying mechanism still remains to be elucidated and is currently under study in our laboratory. Nevertheless, our data suggest that the previously described STM (7) has to be redefined. The STM rather comprises 70S Δ^{43} stress-ribosomes that translate MazF-processed transcripts, independent of the length of the 5'-UTR.

Taken together, our work provides insights into a fast and energy-saving regulatory mechanism that allows bacteria to reprogram protein synthesis in response to harsh changes in environmental conditions. As mentioned above, for this initial approach we ectopically expressed *mazF* in *E. coli* strain MC4100 that harbors the *relA1* mutation (19), to enrich for direct MazF targets. Considering that activation of TA-systems mainly require the stringent response mediated by RelA, it is important to note that our results represent a comprehensive but artificial overview of the MazF-regulon. However, this knowledge will allow and facilitate the determination of distinct MazF-regulons under various physiological stress conditions using the ‘wild type’ *E. coli* strain MG1655, which is currently ongoing in our group.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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