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 translatome 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-
scriptomes and translatomes (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 endonuclease removes 43 nucleotides (nts) from the 16S rRNA 3′-end comprising the anti-Shine-Dalgarno (aSD) sequence (5). Thereby, 70SΔ43 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 70SΔ43 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 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 translatome 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 lacF 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 OD_600_ 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 OD_600_ 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 NaN₃ and immediately harvested by centrifugation at 4000 rpm for 10 min at 4°C in a Sorvall RC5-C (FiberLite FL08-6×500y rotor, Piramon Technologies). Cell pellets were kept on ice and gently resuspended in ice-cold TICO-lys-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 A_{260} 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 an Eppendorf 5810 R centrifuge (Rator 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: I3/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 (‘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 adapter 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 (VBVF NGS Unit; www.vbvf.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 p.adj < 0.05 and log2fold 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 persister 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 translatome. 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 A13 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 translatome. In contrast to the state of the art method for polysome-based translatome 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
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®, Epicenter; see Supplementary Table S1, rows ‘P-’ and ‘P+’, column ‘rRNA depletion’) subjected to RNA-seq (see Materials and Methods).

Validation of RNA and mRNA processing by MazF

First, we confirmed the formation of the 70S\(^{443}\) 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\(^{443}\) 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/Y15, 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 polysomes are 70S ribosomes (Figure 1D). Together, these results not only prove the formation of 70S ribosomes by MazF in general, they further underline that translationally active ribosomes after mazF overexpression are predominantly 70S 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 formylase 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 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 mRNAs 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 Eco-Genes3.0 (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 translatome 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 (±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-
NALAs 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 adaption 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\(^{43}\) 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 lmRNAs, 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’ mRNAs and RNAs with function in ‘cell structure’ are slightly underrepresented. This shows that the MazF-mediated stress response has a more wide-ranging impact then 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 lmRNAs but also processed transcripts with 5’-UTRs that still harbor a SD sequence is difficult to reconcile with the selective translation by 70S\(^{43}\) ribosomes that lack the aSD sequence. Thus, we tested for translation initiation complex formation by 70S\(^{43}\) ribosomes employing the full length and the MazF processed variants of the rpsU and the groL mRNAs as examples for a lmRNA 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\(^{43}\) ribosomes do not form translation initiation complexes (lane 3). However, on the leaderless rpsU transcript the 70S\(^{43}\) 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 lmRNAs by 70S\(^{43}\) 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\(^{43}\) 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\(^{43}\) ribosomes when using the full length groL mRNA (lane 3). This result exemplifies that 70S\(^{43}\) 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 purple) 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 groA (A, B and C) or rpsA mRNAs (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 |
|------|----------------------------------------|-----------------|----------------|
| mutH | 2                                      | methyl-directed mismatch repair protein | CC             |
| mscL | 2                                      | mechanosensitive channel protein, high conductance | CS             |
| tatC | 2                                      | TatABCE protein transllocation system subunit | CS             |
| aroG | 2                                      | 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, phenylalanine repressible | ME             |
| cycA | 2                                      | D-alanine/D-serine/glycine transporter | ME             |
| ptrB | 2                                      | protease II | ME             |
| sppA | 2                                      | protease IV (signal peptide peptidase) | ME             |
| yggG | 2                                      | Phe-Phe periplasmic metalloprotease, OM lipoprotein; low salt-inducible; Era-binding heat shock protein | ME             |
| srlB | 2                                      | glucitol/sorbitol-specific enzyme IIA component of PTS | ME             |
| pdsY | 2                                      | pyridoxamine kinase | ME             |
| nadC | 2                                      | quinolinate phosphoribosyltransferase | ME             |
| gecA | 2                                      | autonomous glycol radical cofactor | ME             |
| zwf  | 2                                      | glucose-6-phosphate 1-dehydrogenase | ME             |
| gatZ | 2                                      | D-tagatose 1,6-bisphosphate aldolase 2, subunit | ME             |
| gplK | 2                                      | glycerol kinase | ME             |
| mhdD | 2                                      | predicted membrane-bound lytic murein transglycosylase D | ME             |
| lfdD | 2                                      | malonyl-CoA-[acyl-carrier-protein] transacylase | ME             |
| ispD | 2                                      | 4-diphosphocytidyl-2C-methyl-D-erythritol synthase | ME             |
| ann  | 2                                      | AMP nucleosidase | ME             |
| nadA | 2                                      | ribonucleoside-diphosphate reductase 1, alpha subunit | ME             |
| nupG | 2                                      | nucleoside transporter | ME             |
| proS | 2                                      | prolyl-tRNA synthetase | ME             |
| yafG | 2                                      | putative lipoprotein | NC             |
| ybgL | 2                                      | UPF0271 family protein | NC             |
| yjeI | 2                                      | DUF4156 family lipoprotein | NC             |
| rpmB | 2                                      | 505 ribosomal subunit protein L28 | PS             |
| rpsA | 2                                      | 308 ribosomal subunit protein S1 | PS             |
| rpsU | 2                                      | 308 ribosomal subunit protein S21 | PS             |
| rsaA | 2                                      | 16S rRNA pseudouridin[516] synthase | PS             |
| rpoN | 2                                      | RNA polymerase, sigma 54 (sigma N) factor | PS             |
| srnB | 2                                      | ATP-dependent RNA helicase | PS             |
| engA | 2                                      | GTPase; multicopy suppressor of ftsJ | RR             |
| ygiW | 2                                      | hydrogen peroxide and cadmium resistance periplasmic protein; stress-induced OB-fold protein | RR             |
| uspD | 2                                      | stress-induced protein | RR             |
| ftsA | 3                                      | ATP-binding cell division protein involved in recruitment of FtsK to Z ring | CC             |
| ftsE | 3                                      | cell division ATP-binding protein | CC             |
| mbA  | 3                                      | membrane-bound lytic murein transglycosylase A | CS             |
| btuB | 3                                      | vitamin B12/coenzyme inner membrane transporter | CS             |
| ydIS | 3                                      | UPF0126 family inner membrane protein | CS             |
| exbB | 3                                      | membrane spanning protein in TonB-ExbB-ExbD complex | CS             |
| ffh  | 3                                      | Signal Recognition Particle (SRP) component with 4.5S RNA (fals) | CS             |
| ynaI | 3                                      | mechanosensitive channel protein, very small conductance | CS             |
| ptsH | 3                                      | phosphohistidinoprotein-hexose phosphotransferase component of PTS system (Hpr) | ME             |
| srlA | 3                                      | glucitol/sorbitol-specific enzyme IC component of PTS | ME             |
| vscC | 3                                      | 2-octaprenylphenol dehydrogenase, FAD-dependent | ME             |
| yqiH | 3                                      | putative siderophore interacting protein | ME             |
| kdsC | 3                                      | 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase | ME             |
| arlI | 3                                      | arginine transporter subunit | ME             |
| grxD | 3                                      | glutaredoxin-4 | NC             |
| yeaQ | 3                                      | UPF0410 family protein | NC             |
| yoaH | 3                                      | UPF0181 family protein | NC             |
| ytfK | 3                                      | DUF1107 family protein | NC             |
| rplB | 3                                      | 505 ribosomal subunit protein L2 | PS             |
| rpoA | 3                                      | RNA polymerase, alpha subunit | PS             |
| trnJ  | 3                                    | tRNA mC32,mU32 2'-O-methyltransferase, SAM-dependent | PS             |
| cpxR | 3                                      | response regulator in two-component regulatory system with CpxA | RR             |
| mdsE | 4                                      | OPG biosynthetic periplasmic beta-1,6 branching glycosyltransferase | CS             |
| ydeE | 4                                      | putative transporter | CS             |
| lgt  | 4                                      | phosphatidylglycerol-prolipoprotein diacylglyceroltransferase | ME             |
| glpP | 4                                      | glutamine transporter subunit | ME             |
| wecH | 4                                      | O-acetyltransferase for enterobacterial common antigen (ECA) | ME             |
| yqaE | 4                                      | cyaR sRNA-regulated protein | NC             |
| greA | 4                                      | transcript cleavage factor | PS             |
| lig  | 5                                      | peptidyl-prolyl cis/trans isomerase (trigger factor) | CC             |
| ybbQ | 5                                      | DUF165 family inner membrane protein | CS             |
| ynaJ | 5                                      | DUF2534 family putative inner membrane protein | CS             |
| Gene  | Cleaved ACA [Distance to start in nts] | Protein Product                                      | Classification |
|-------|----------------------------------------|------------------------------------------------------|----------------|
| ilvL  | 5                                      | ilvG operon leader peptide                            | ME             |
| radA  | 5                                      | DNA repair protein                                    | ME             |
| uxaR  | 5                                      | fructose-1,6-bisphosphate aldolase                    | ME             |
| yufV  | 5                                      | putative NAD(P)-binding C-N hydrolase family amidase  | ME             |
| yfcF  | 5                                      | glutathione S-transferase                             | ME             |
| pplD  | 5                                      | periplasmic folding chaperone, has an inactive PPlase domain | NC             |
| ydcJ  | 5                                      | putative metalloenzyme                                 | NC             |
| yacL  | 5                                      | UPF0231 family protein                                | NC             |
| ypfJ  | 5                                      | putative transcriptional regulator                     | NC             |
| yceA  | 5                                      | putative rhodanese-related sulfuryltransferase        | NC             |
| yeoO  | 5                                      | DUF488 family protein                                 | NC             |
| yhvD  | 5                                      | putative outer membrane protein                       | NC             |
| rplR  | 5                                      | 50S ribosomal subunit protein L18                      | PS             |
| rtcB  | 5                                      | RNA-splicing ligase                                   | PS             |
| rmd  | 5                                      | ribonuclease D                                        | PS             |
| emrA  | 5                                      | multidrug efflux system                               | RR             |
| mcrA  | 5                                      | trehalose &-phosphate-inducible trehalose regulon transcription repressor | RR             |
| yceN  | 6                                      | putative lipid II flippase                            | CS             |
| ynjC  | 6                                      | putative ABC transporter permease                     | CS             |
| thrL  | 6                                      | thr operon leader peptide                              | ME             |
| gglB  | 6                                      | 1,4-alpha-glucan branching enzyme                     | ME             |
| caIC  | 6                                      | putative coenzymetagin-CoA ligase                     | ME             |
| yutJ  | 6                                      | ABC transporter maintaining OM lipid asymmetry, OM lipoprotein component | ME             |
| apt   | 6                                      | adenine phosphoribosyltransferase                     | ME             |
| gsk   | 6                                      | inosine/guanosine kinase                              | ME             |
| mrdB  | 6                                      | ribonucleoside-diphosphate reductase 1, beta subunit, ferritin-like protein | ME             |
| ydvJ  | 6                                      | selemoprotein, function unknown                       | NC             |
| ygbB  | 6                                      | DUF1190 family protein                                | NC             |
| rmbB  | 6                                      | 23S rRNA mG2251 2′-O-ribose methyltransferase, SAM-dependent | PS             |
| yddM  | 6                                      | putative DNA-binding transcriptional regulator        | RR             |
| yhiU  | 6                                      | putative DNA-binding transcriptional regulator; KpLE2 phage-like element | RR             |
| kattG | 6                                      | catalase-peroxidase HPI, heme b-containing             | RR             |
| ygaZ  | 7                                      | putative L-valine exporter, norvaline resistance protein | ME             |
| mukE  | 7                                      | chromosome condensin MueBEF, MueE localization factor  | NC             |
| pgm   | 8                                      | phosphoglucomutase                                    | ME             |
| uhpA  | 8                                      | response regulator in two-component regulatory system with UhpB | ME             |
| coaA  | 8                                      | pantothenate kinase                                   | ME             |
| pfIA  | 8                                      | pyruvate formate-lyase 1-activating enzyme; [formate-C-acetyltransferase 1]-activating enzyme; PFL activase | ME             |
| fadH  | 8                                      | 4,4-dienediol-CoA reductase, NADH and FMN-linked      | ME             |
| hioG  | 8                                      | SH3 domain protein                                    | ME             |
| rho   | 8                                      | transcription termination factor                       | PS             |
| ampH  | 9                                      | D-alanyl-D-alanine-carboxypeptidase/endopeptidase; penicillin-binding protein; weak beta-lactamase | CS             |
| yicC  | 9                                      | membrane protein insertase                            | CS             |
| hisQ  | 9                                      | histidine ABC transporter permease                    | ME             |
| glmM  | 9                                      | phosphoglucomutase                                    | ME             |
| apoQ  | 9                                      | glycerophosphodiester phosphodiesterase, cytosolic   | ME             |
| lrp   | 9                                      | leucine-responsive global transcriptional regulator   | ME             |
| ycbZ  | 9                                      | putative peptidase                                    | NC             |
| yeaT  | 9                                      | transcriptional activator of dmlA                     | RR             |
| nuA   | 10                                     | UDP-N-acetylglucosamine 1-carboxyvinyltransferase     | CS             |
| rplL  | 10                                     | 50S ribosomal subunit protein L7/L12                   | PS             |
| suA   | 10                                     | antitoxin of the SohA(PrlF)-YhaV toxin-antitoxin system | RR             |
| yhcM  | 11                                     | divisome ATPase                                       | CC             |
| shiA  | 11                                     | shikimate transporter                                 | CS             |
| rbsK  | 11                                     | ribokinase                                           | ME             |
| yjeE  | 11                                     | putative cation/proton antiporter                     | NC             |
| zipA  | 12                                     | FisZ stabilizer                                      | CC             |
| ivL   | 12                                     | ilvB operon leader peptide                            | ME             |
| ndk   | 12                                     | multifunctional nucleoside diphosphate kinase and apyrimidin wide nucleoside and 3′-phosphodiesterase | ME             |
| yiiR  | 12                                     | 23S rRNA m(6)A2030 methyltransferase, SAM-dependent    | PS             |
| phoB  | 12                                     | response regulator in two-component regulatory system with PhoR | RR             |
| yjGA  | 12                                     | transcriptional repressor for divergent bdcA         | RR             |
| iadA  | 13                                     | isoaspartyl dipeptidase                               | ME             |
| melL  | 13                                     | Bifunctional aspartokinase/homoserine dehydrogenase 2 | ME             |
| yhhK  | 13                                     | PanD autocleavage accelerator, panothenate synthesis  | ME             |
| fpr   | 13                                     | ferredoxin-NADP reductase; flavodoxin reductase       | ME             |
Table 1. Continued

| Gene | cleaved ACA [Distance to start in nts] | Protein product | Classification |
|------|---------------------------------------|-----------------|----------------|
| gnd  | 13                                    | 6-phosphogluconate dehydrogenase, decarboxylating | ME |
| yjjG | 13                                    | dUMP phosphatase  | ME |
| rpsP | 13                                    | 30S ribosomal subunit protein S16 | PS |
| ydeP | 13                                    | putative oxidoreductase | RR |
| hcaR | 14                                    | hca operon transcriptional regulator | ME |
| dipZ | 14                                    | thiol/disulfide interchange protein and activator of DsbC | ME |
| fdoG | 14                                    | formate dehydrogenase-O, large subunit | ME |
| rhsC | 14                                    | Rhs protein with putative toxin domain; putative neighboring cell growth inhibitor | RR |
| dnaQ | 15                                    | DNA polymerase III epsilon subunit | CC |
| ftuE | 15                                    | tyrosine recombination/inversion of on/off regulator of fimA | CS |
| fiY  | 15                                    | cystine transporter subunit | CS |
| dcaA | 15                                    | C4-dicarboxylate antipporter | ME |
|mutP | 15                                    | N-acetylmuramic acid permease, EIIBC component, PTS system | ME |
| yciO | 15                                    | putative RNA binding protein | NC |
| yadH | 15                                    | putative ABC transporter permease | NC |
| hdn | 15                                    | biofilm-dependent modulation protein | RR |
| yfB | 16                                    | OM lipoprotein putative positive effector of YfN activity | CS |
| ydeS | 16                                    | putative ABC transporter periplasmic binding protein | CS |
| btlE | 16                                    | glutathione peroxidase | ME |
| sixA | 16                                    | phosphohistidine phosphatase | ME |
| yjJ | 16                                    | PspA/IM30 family protein | ME |
| yelS | 16                                    | DUF2542 family protein | NC |
| yfB | 16                                    | putative oxidoreductase | RR |
| ydeS | 16                                    | putative ABC transporter periplasmic binding protein | CS |
| btlE | 16                                    | glutathione peroxidase | ME |
| atpE | 16                                    | back-translocating elongation factor EF4, GTPase | PS |
| lexA | 16                                    | transcriptional repressor of SOS regulon | RR |
| yggE | 16                                    | oxidative stress defense protein | RR |
| yfD | 17                                    | UPF0853 family inner membrane protein | CS |
| yfyF | 17                                    | putative transporter | CS |
| ptsI | 17                                    | PEP-protein phosphotransferase of PTS system (enzyme I) | ME |
| atpE | 17                                    | F0 sector of membrane-bound ATP synthase, subunit c | ME |
| yniA | 17                                    | fructose kinase family protein | NC |
| impA | 17                                    | LPS assembly OM complex LptDE, beta-barrel component | RR |
| seqA | 18                                    | negative modulator of initiation of replication | CC |
| frdA | 18                                    | anaerobic fumarate reductase catalytic and NAD/oxidoreductase subunit | ME |
| fabI | 18                                    | enoyl-[acyl-carrier-protein] reductase, NADH-dependent | ME |
| nlpC | 18                                    | putative C40 clan peptidase lipoprotein | ME |
| rpsT | 18                                    | 30S ribosomal subunit protein S20 | PS |
| feaR | 18                                    | transcriptional activator for ynaA and feaB | RR |
| dnaQ | 19                                    | DNA polymerase III, beta subunit | CC |
| sstT | 19                                    | sodium:serine/threonine symporter | CS |
| tepB | 19                                    | putative transporter | CS |
| fbp | 19                                    | fructose-1,6-bisphosphatase 1 | ME |
| galU | 19                                    | glucose-1-phosphate uridylyltransferase | ME |
| catB | 19                                    | galactolact-specific enzyme IIB component of PTS | ME |
| cysQ | 19                                    | 3′(2′),5′-bisphosphate nucleotidase | ME |
| nuoM | 19                                    | NADH:ubiquinone oxidoreductase, membrane subunit M | ME |
| wkbK | 19                                   | lipopolysaccharide biosynthesis protein | ME |
| thyA | 19                                    | thymidylate synthetase | ME |
| pepB | 19                                    | aminopeptidase B | ME |
| infC | 19                                    | translation initiation factor IF-3 | PS |
| efp | 19                                    | polyproline-specific translation elongation factor EF-P | PS |
| prfA | 19                                    | peptide chain release factor | PS |
| marA | 19                                    | multiple antibiotic resistance transcriptional regulator | RR |
| nagZ | 19                                    | beta N-acetyl-glucosaminidase | RR |
| yobA | 19                                    | CopC family protein | RR |
| mdr2 | 19                                    | multidrug efflux system transporter | RR |
| ilvD | 20                                    | dihydroxyacid dehydratase | ME |
| mttA | 20                                    | mannitol-specific PTS enzyme: IIA, IIB and IIC components | ME |
| gapA | 20                                    | glyceraldehyde-3-phosphate dehydrogenase A | ME |
| rrsD | 20                                    | stationary phase protein, binds sigma 70 RNA polymerase subunit | RR |
| rfaB | 21                                    | lipopolysaccharide 1,6-galactosyltransferase; UDP-D-galactose:glucosyllipopolysaccharide-1,6-D-galactosyltransferase | CS |
| aroH | 21                                    | 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, tryptophan repressible | ME |
| kefG | 22                                    | potassium-efflux system ancillary protein for KefB, glutathione-regulated | ME |
| arop | 22                                    | aromatic amino acid transporter | ME |
| clpX | 22                                    | ATPase and specificity subunit of ClpX-ClpP ATP-dependent serine protease | ME |
| arAF | 22                                    | L-arabinose ABC transporter periplasmic binding protein | ME |
Table 1. Continued

| Gene  | cleaved ACA [Distance to start in nts] | Protein product                                      | Classification |
|-------|----------------------------------------|------------------------------------------------------|----------------|
| accB  | 22                                     | acetyl-CoA carboxylase, BCCP subunit                  | ME             |
| folE  | 22                                     | GTP cyclohydrolase I                                  | ME             |
| fudD  | 22                                     | acyl-CoA synthase (long-chain-fatty–CoA ligase)       | ME             |
| lepB  | 22                                     | leader peptidase (signal peptidase I)                 | CS             |
| glpF  | 23                                     | glycerol facilitator                                  | ME             |
| garR  | 23                                     | tartronate semialdehyde reductase                    | ME             |
| metF  | 23                                     | 5,10-methyltenetetrahydrofolate reductase            | ME             |
| yjcZ  | 23                                     | YjcZ family protein; yhjH motility defect suppressor  | NC             |
| yehZ  | 23                                     | inner membrane protein                                | RR             |
| cleB  | 24                                     | H(+)/Cl(−) exchange transporter                      | CS             |
| pcpP  | 24                                     | proline aminopeptidase P II                           | ME             |
| pncC  | 24                                     | pantothenate synthetase                               | ME             |
| pdxJ  | 24                                     | pyridoxine 5′-phosphate synthase                     | ME             |
| acnB  | 24                                     | aconitate hydratase 2; aconitase B; 2-methyl-cis-aconitate hydratase | ME |
| ynjH  | 24                                     | DUF1496 family protein                                | NC             |
| yfH   | 24                                     | UPF0124 family protein                                | NC             |
| frr   | 24                                     | ribosome recycling factor                             | PS             |
| ygdD  | 25                                     | UPF0382 family inner membrane protein                 | CS             |
| pabC  | 25                                     | 4-amino-4-deoxychorismate lyase component of para-aminobenzoate synthase | multi-enzyme complex |
| napB  | 25                                     | nitrate reductase, small, cytochrome C550 subunit, periplasmic | ME |
| apha  | 25                                     | acid phosphatase/phosphotransferase, class B, non-specific | ME |
| yitB  | 25                                     | OupA family protein                                   | NC             |
| groL  | 25                                     | Cpn60 chaperonin GroL, large subunit of GroSL         | RR             |
| sbeB  | 26                                     | exodeoxyribonuclease 1; exonuclease I                 | CC             |
| bacA  | 26                                     | undecaprenyl pyrophosphate phosphatase                | RR             |
| ygcJ  | 26                                     | CRISP RNA (crRNA) containing Cascade antiviral complex protein | RR |
| ublE  | 26                                     | bifunctional 2-octaprenyl-6-methoxy-1,4-benzoquinone methylase/ S-adenosylmethionine:2-DMK methyltransferase | ME |
| yniC  | 27                                     | 2-deoxyglucose-6-P phosphatase                        | ME             |
| yodD  | 27                                     | uncharacterized protein                               | RR             |
| cySU  | 28                                     | sulfate/thiosulfate ABC transporter permease          | CS             |
| yidQ  | 28                                     | DUF1375 family outer membrane protein                 | NC             |
| yigZ  | 28                                     | UPF0029 family protein                                | NC             |
| yhaH  | 29                                     | DUF805 family inner membrane protein, DL-methionine transporter subunit | CS |
| metQ  | 29                                     | DUF805 family inner membrane protein, DL-methionine transporter subunit | CS |
| speA  | 29                                     | biosynthetic arginine decarboxylase, PLP-binding      | ME             |
| nraF  | 29                                     | nitrite reductase, formate-dependent, cytochrome      | ME             |
| udk   | 29                                     | uridine-cytidine kinase                               | ME             |
| hfg   | 29                                     | global sRNA chaperone; HF-I, host factor for RNA phase Q beta replication | RR |
| aer   | 29                                     | fused signal transducer for aerotaxis sensory component/methyl accepting | RR |
| yhbB  | 30                                     | putative Na+/Pi-cotransporter                        | CS             |
| dshA  | 30                                     | periplasmic protein disulfide isomerase I             | ME             |
| tnaB  | 31                                     | tryptophan transporter of low affinity                | ME             |
| otsB  | 31                                     | trehalose-6-phosphate phosphatase, biosynthetic       | RR             |
| ptaS  | 32                                     | phosphate ABC transporter periplasmic binding protein | CS             |
| rimM  | 32                                     | ribosome maturation factor                            | PS             |
| otsA  | 32                                     | trehalose-6-phosphate synthase                        | RR             |
| argT  | 34                                     | lysine/arginine/ornithine transporter subunit         | ME             |
| hvd   | 34                                     | branched-chain amino acid ABC transporter periplasmic binding protein | ME |
| pepN  | 34                                     | aminopeptidase N                                      | ME             |
| pagB  | 34                                     | lipid A phosphoethanolamine transferase              | ME             |
| hokD  | 34                                     | Qin prophage; small toxic polypeptide                 | RR             |
| hftB  | 35                                     | protease, ATP-dependent zinc-metallo                  | ME             |
| narP  | 35                                     | response regulator in two-component regulatory system with NarQ | ME |
| yefG  | 35                                     | uncharacterized protein                               | NC             |
| vimN  | 35                                     | tRNA(ANN)(t(6)A)37 threonylcarbamoyladenosine modification protein, threonine-dependent ADP-forming ATPase | PS |
| ygaW  | 36                                     | alanine exporter, alanine-inducible, stress-responsive | ME             |
| hybC  | 36                                     | hydrogenase 2, large subunit                           | ME             |
| yciK  | 36                                     | putative EmrKY-ToIC system oxaacyl-(acyl carrier protein) reductase | ME |
| rpoD  | 36                                     | RNA polymerase, sigma 70 (sigma D) factor             | PS             |
| emoA  | 36                                     | carboxy-SAM synthase                                  | PS             |
| lsdO  | 36                                     | heat shock protein Hsp33                               | RR             |
| gutC  | 37                                     | pseudogene, galactitol-specific enzyme IIC component of PTS | ME |
| ptsC  | 37                                     | 1-acetyl-sn-glycerol-3-phosphate acyltransferase      | ME             |
| gloA  | 38                                     | glyoxalase I, Ni-dependent                            | ME             |
Table 1. Continued

| Gene   | cleaved ACA [Distance to start in nts] | Protein product                                                                 | Classification |
|--------|---------------------------------------|-------------------------------------------------------------------------------|---------------|
| ygcK   | 38                                    | CRISP RNA (crRNA) containing Cascade antiviral complex protein                | RR            |
| iscX   | 39                                    | Fe(2+) donor and activity modulator for cysteine desulphurase                | NC            |
| cheZ   | 39                                    | chemotaxis regulator, protein phosphatase for CheY                           | RR            |
| yfbV   | 40                                    | UPF0208 family inner membrane protein                                        | CS            |
| yaaJ   | 40                                    | putative transporter                                                          | ME            |
| dkgA   | 40                                    | diacylglycerol kinase                                                         | ME            |
| sbmA   | 41                                    | peptide antibiotic transporter                                               | RR            |
| hslU   | 41                                    | molecular chaperone and ATPase component of HslUV protease                   | RR            |
| aroB   | 42                                    | 3-dehydroquinase synthase                                                   | ME            |
| yqJF   | 42                                    | short chain acyltransferase                                                  | ME            |
| cyoW   | 43                                    | sulfate/thiosulfate ABC transporter permease                                 | CS            |
| dhnD   | 43                                    | twin-arginine leader-binding protein for DmsA and TorA                       | ME            |
| rblL   | 43                                    | 23S rRNA m(2)G2445 and m(7)G2069 methyltransferases, SAM-dependent           | PS            |
| lptB   | 44                                    | lipopolysaccharide export ABC transporter ATPase                             | CS            |
| hcaT   | 45                                    | putative 3-phenylpropionic transporter                                        | ME            |
| fabG   | 45                                    | 3-oxoacyl-[acyl-carrier-protein] reductase                                    | ME            |
| csaR   | 45                                    | transcriptional repressor of csD                                              | RR            |
| ygiP   | 46                                    | lipopolysaccharide export ABC permease                                        | CS            |
| dkgA   | 47                                    | 2.5-diketo-D-gluconate reductase A                                            | ME            |
| acpP   | 49                                    | acyl carrier protein (ACP)                                                   | ME            |
| rluB   | 49                                    | 23S rRNA pseudouridine(m2) synthase                                          | PS            |
| yheV   | 50                                    | DUF2387 family putative metal-binding protein                                | NC            |
| gldA   | 51                                    | glycerol dehydrogenase, NAD+ dependent; 1,2-propanediol:NAD+ oxidoreductase  | ME            |
| rfaQ   | 51                                    | ATPase                                                                       | ME            |
| hcaT   | 52                                    | glucose-specific enzyme IIA component of PTS                                 | ME            |
| fimA   | 53                                    | major type 1 subunit fimbrin (pilin)                                         | CS            |
| rfaA   | 53                                    | TDP-4-oxo-6-deoxy-D-glucose transaminase                                     | ME            |
| tap    | 54                                    | methyl-accepting protein IV                                                 | RR            |
| lamB   | 55                                    | maltose outer membrane porin (maltoporin)                                   | ME            |
| dasA   | 55                                    | tRNA-dihydouridine synthase A                                               | CS            |
| dnaA   | 58                                    | chromosomal replication initiation protein DnaA, DNA-binding transcriptional | CC            |
| yjgP   | 58                                    | DUF1212 family inner membrane protein                                        | CS            |
| rpoB   | 58                                    | RNA polymerase, beta subunit                                                 | PS            |
| thrS   | 59                                    | threonyl-tRNA synthetase                                                     | ME            |
| ygiR   | 60                                    | DUF6535 family protein with NTPase fold                                       | NC            |
| yheN   | 60                                    | sulfurtransferase for 2-thiolation step of mmm(5)-s(2)U34-tRNA synthesis      | PS            |
| leuE   | 61                                    | leucine efflux protein                                                       | ME            |
| mtgA   | 62                                    | biosynthetic peptidoglycan transglycosylase                                  | CS            |
| yfeX   | 63                                    | porphyrinogen oxidase, cytoplasmian                                          | ME            |
| nemA   | 63                                    | chromate reductase, quinone reductase, FMN-linked; N-Ethylmaleimide reductase; | ME            |
| csdE   | 64                                    | CsdA-binding activator; Fe-S protein                                         | ME            |
| fre    | 65                                    | NAD(P)H-flavin reductase                                                     | ME            |
| yrfG   | 65                                    | GMP/IMP nucleotidase                                                         | ME            |
| ygiC   | 66                                    | ATP-Grasp family ATase                                                       | ME            |
| sspA   | 66                                    | stringent starvation protein A, phage PI late gene activator, RNAP-associated | RR            |
| wbbJ   | 67                                    | putative lipopolysaccharide biosynthesis O-acetyl transferase                | ME            |
| rdgB   | 68                                    | dTTP/XTP pyrophosphatase                                                     | CS            |
| yceH   | 71                                    | UPF0502 family protein                                                       | NC            |
| lldR   | 75                                    | dual role activator/repressor for lldPRD operon                             | ME            |
| ysaA   | 75                                    | putative hydrogenase, 4Fe-4S ferredoxin-type component                       | ME            |
| rpsJ   | 75                                    | 30S ribosomal subunit protein S10                                            | PS            |
| aaeB   | 76                                    | p-hydroxybenzoic acid efflux system component                               | CS            |
| ytlA   | 76                                    | uncharacterized protein                                                     | NC            |
| gveB   | 76                                    | transcript cleavage factor                                                  | PS            |
| clpP   | 78                                    | proteolytic subunit of ClpA-ClpP and ClpX-ClpP ATP-dependent serine proteases | ME            |
| glpP   | 78                                    | glutamate/aspartate/proton symporter                                        | ME            |
| glnE   | 83                                    | fused deadenyltransferase/adenylyltransferase for glutamine synthetase       | PS            |
| rne    | 83                                    | endoribonuclease; RNA-binding protein;RNA degradosome binding protein       | PS            |
| deaD   | 83                                    | ATP-dependent RNA helicase                                                   | PS            |
| luxS   | 85                                    | S-ribosylhomocysteine lyase                                                  | RR            |
| pstB   | 91                                    | phosphate ABC transporter ATPase                                            | CS            |
| pepA   | 95                                    | multifunctional aminopeptidase A; a cytoenylglycinase, transcription regulator and site-specific recombination factor | ME            |
| dat    | 95                                    | deoxyuridinetriphosphatase                                                  | ME            |
protein synthesis is modulated due to selective translation of a subset of processed mRNAs by the concomitantly generated 70S \textsuperscript{43} 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, 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 \textit{E. coli} genes. (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 by the concomitantly generated 70S \textsuperscript{43} 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, 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 \textit{E. coli} genes. (ME = metabolism and energy supply, CC = cell cycle, PS = protein synthesis, RR = response regulation, CS = cell structure, NC = not classified).

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 \textit{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 \textit{mazF} overexpression are not
significant regulatory 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) intriguing 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-encoding mRNAs (encoding proteins bS1, uS2, uS7, uS10, hS16, bS20, uL2, bL7, uL18, bL28 and bL35 (33)) are processed by MazF and found to associate with polysomes (Table 1). In contrary, over 50% of all ‘cell structure’ transcripts 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 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, RNAPα 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-RNAPα 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 pentapeptide 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 pentapeptide NNWDN (Asn-Asn-Trp-Glu-Asn; residues 199–203 of Zwf) that 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 ImRNAs (47,48). The MazF-mediated stress response mechanism is based on translation of ImRNAs. 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 ImRNAs. 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-rpsU 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-rpsU 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 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 ribosomes that are equipped with the initiator tRNA. Subsequently, the 70S 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 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 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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REFERENCES

1. Potrykus, K. and Cashel, M. (2008) (p)ppGpp: still magical? Annu. Rev. Microbiol., 62, 35–51.
2. Sharma, U.K. and Chatterji, D. (2010) Transcriptional switching in Escherichia coli during stress and starvation by modulation of sigma activity. FEMS Microbiol. Rev., 34, 646–657.
3. Balleza, E., Lopez-Bojorquez, L.N., Martinez-Antonio, A., Resendis-Antonio, O., Lozada-Chavez, L., Balderran-Martinez, Y.L., Encarnacion, S. and Collado-Vides, J. (2009) Regulation by transcription factors in bacteria: beyond description. FEMS Microbiol. Rev., 33, 133–151.
18. Ingolia, N.T., Ghaemmaghami, S., Newman, J.R.S. and Weissman, J.S. (2011) Regulation of growth and death
11. Yamaguchi, Y. and Inouye, M. (2011) Regulation of growth and death
16. Ramage, H.R., Connolly, L.E. and Cox, J.S. (2009) Comprehensive
14. Tripathi, A., Dewan, P.C., Siddique, S.A. and Varadarajan, R. (2014)
12. Keren, I., Kaldalu, N., Spoering, A., Wang, Y. and Lewis, K. (2004)
19. Casadaban, M.J. (1976) Transposition and fusion of the lac genes to
22. Hoffmann, S., Otto, C., Kurutz, S., Sharma, C.M., Khaitovich, P,
23. Anders, S., Pyl, P.T. and Huber, W. (2014) HTSeq—a Python
26. Wolfinger, M.T., Fallmann, J., Stadler, P.F. and Hackermüller, J. (2009) Fast mapping of
28. R Development Core Team (2008) R: a language and environment
24. Hoffmann, S., Otto, C., Doose, G., Tanzer, A., Langenberger, D.,
25. Kent, W.J., Sagnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H.,
27. Zhou, J. and Rudd, K.E. (2013) EcoGene 3.0. Nucleic Acids Res., 41,
29. Miller, O.L. Jr, Hamkalo, B.A. and Thomas, C.A. Jr (1970)
30. Wagner, A.F., Schultz, S., Bomek, J., Pils, T., Lehmann, W.D. and
31. Nystöm, T. (2007) A bacterial kind of aging. PLoS Genet., 3, e224.
32. Picard, F., Loubière, P., Girbal, L. and Cocaign-Bousquet, M. (2013) The significance of translation regulation in the stress response. BMC Genomics, 14, 588.
33. Ban, N., Beckmann, R., Cate, J.H., Dinman, J.D., Dragon, F., Ellis, S.R.,
34. Peters, J.M., Vangello, A.D. and Landick, R. (2011) Bacterial transcription terminators: the RNA 3’-ends. J. Mol. Biol., 412, 793–813.
35. Boudvillain, M., Nollmann, M. and Margeat, E. (2010) Keeping up to speed with the transcription termination factor Rho motor. Transcription, 1, 70–75.
36. Burmann, B.M., Schweimer, K., Luo, X., Wahl, M.C., Stitt, B.L.,
37. Proshkin, S., Rahmouni, A.R., Mironov, A. and Nudler, E. (2010) Cooperation between translating ribosomes and RNA polymerase in transcription elongation. Science, 328, 504–508.
38. Boudvillain, M., Figueroa-Bossi, N. and Bossi, L. (2013) Terminator still moving forward: expanding roles for Rho factor. Curr. Opin. Microbiol., 16, 118–124.
39. Rippa, V., Cirilli, C., Di Palo, B., Doti, N., Amoresano, A. and
dutta, A. (2010) The ribosomal protein L2 interacts with the RNA polymerase alpha subunit and acts as a transcription modulator in Escherichia coli. J. Bacteriol., 192, 1882–1889.
40. Kolodkin-Gal, I. and Engelberg-Kulka, H. (2008) The extracellular death factor: physiological and genetic factors influencing its production and response in Escherichia coli. J. Bacteriol., 190, 3169–3175.
41. Kolodkin-Gal, I., Hazan, R., Gaathon, A., Carmeli, S. and
42. Kolodkin-Gal, I., Kolodkin-Gal, I., Hazan, R., Gaathon, A., Carmeli, S. and
43. Kumar, S., Kolodkin-Gal, I., Vesper, O., Alam, N.,
44. Qu, X., Lancaster, L., Noller, H.F., Bustamante, C. and Tinoco, I. (2012) Ribosomal protein S1 unwinds double-stranded RNA in multiple steps. Proc. Natl. Acad. Sci. U.S.A., 109, 14458–14463.
45. Moll, I. and Engelberg-Kulka, H. (2007) The extracellular death factor EDF induces the endonucleolytic activities of the toxins MazF and CbpBK. Mol. Cell, 41, 625–635.
46. Boni, I.V., Iszak, M., Myschenko, M.L. and Tareva, N.V. (1991) Ribosome-messenger recognition: mRNA target sites for ribosomal protein S1. Nucleic Acids Res., 19, 155–162.
47. de Smit, M.H. and van Duin, J. (1994) Translational initiation on structured messengers. Another role for the Shine-Dalgarno interaction. J. Mol. Biol., 235, 173–184.
48. Qu, X., Lancaster, L., Noller, H.F., Bustamante, C. and Tinoco, I. (2012) Ribosomal protein S1 unwinds double-stranded RNA in multiple steps. Proc. Natl. Acad. Sci. U.S.A., 109, 14458–14463.
49. Moll, I., Grill, S., Gründling, A. and Bläsi, U. (2002) Effects of ribosomal proteins S1, S2 and the DeaD/CsdA DEAD-box helicase on translation of leaderless and canonical mRNAs in Escherichia coli. Mol. Microbiol., 44, 1387–1396.
50. Tedin, K., Resch, A. and Bläsi, U. (1997) Requirements for ribosomal protein S1 for translation initiation of mRNAs with and without a 5’ leader sequence. Mol. Microbiol., 25, 189–199.
51. Celesnik, H., Deana, A. and Belasco, J.G. (2007) Initiation of RNA decay in Escherichia coli by 5’ pyrophosphate removal. Mol. Cell, 27, 79–90.