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RESEARCH ARTICLE

A Bias in the Reading of the Genetic Code of *Escherichia coli* is a Characteristic for Genes that Specify Stress-induced MazF-mediated Proteins

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Abstract: ***Background*:** *Escherichia coli* (*E. coli*) *mazEF*, a stress-induced toxin-antitoxin (TA) system, has been studied extensively. The MazF toxin is an endoribonuclease that cleaves RNAs at ACA sites. Thereby, under stress, the induced MazF generates a Stress-induced Translation Machinery (STM), composed of MazF processed mRNAs and selective ribosomes that specifically translate the processed mRNAs.

***Materials and Methods*:** Based on the data from the EcoCyc website of the National Center for Biotechnology Information (NCBI), the sequence of all *E. coli* MG1655 genes were scanned for ACA sites upstream from the initiation codons. Among these sequences, the fuzznuc program of the "European Molecular Biology Open Software Suite" (EMBOSS) was used to find the "ACA" pattern. The distribution of the ACA threonine codon, both in-frame and out-of-frame, was determined by using the HTML Script Program (Supplementary Material).

***Results*:** Here it is reported that for most of the *E. coli* proteins mediated by stress-induced MazF, the ACA threonine codon in their mRNAs is not in-frame but rather out-of-frame; in these same RNAs, the three synonymous threonine codons, ACG, ACU, and ACC, are in-frame. In contrast, for proteins translated by the canonical translation system, in the majority of mRNAs, the ACA codon is located in-frame.

***Conclusion*:** The described bias in the genetic code is a characteristic of *E. coli* genes specifying for stress-induced MazF-mediated proteins.

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1. INTRODUCTION

E. coli mazEF, abundant in the chromosomes of most bacteria [1-4], is the most studied chromosomal toxin-antitoxin *(*TA) module [1, 2]. *E. coli mazF* specifies for the stable toxin MazF, and *mazE* specifies for the labile antitoxin, MazE, which is degraded by the ATP-dependent ClpAP serine protease [1]. Various stressful conditions can prevent the production of the antitoxin MazE: the absence of the anti-toxin permits the uninterrupted activity of the toxin MazF [5-7]. MazF is a sequence-specific endoribonuclease that preferentially cleaves single-stranded mRNAs either at the 3′ or at the 5′ side of the first A in ACA sequences [8, 9]. Moreover, under stress, by cleaving ACA sites immediately adjacent to or within the span of 100 nucleotides upstream from the AUG start codons of any given mRNA, the induced MazF generates leaderless mRNAs [10]. Concomitantly, MazF targets 16S rRNA within 30S ribosomal subunits at the decoding center, consequently removing 43 nucleotides from the 3′ terminus [10]. Since this region comprises the anti-Shine-Dalgarno (aSD) region, the generated deficient ribosomes are selectively able to translate the generated, processed mRNAs [10-12]. This Stress-induced Translation Machinery (STM) is responsible for the selective synthesis of specific proteins resulting from MazF induction [13].

Previously, when the STM system was characterized [14], it was found that MazF cleaves ACA sites located in frame 0 of the coding region of the processed mRNAs, while out-of-frame ACAs are resistant to such cleavage. Moreover, under stressful conditions, when MazF cleaves in-frame ACAs, a bias in the reading of the genetic code is caused. The result of this bias is that the amino acid threonine is no longer encoded by ACA, but rather by its synonym codons ACC, ACU, or ACG [14]. Recently, a proteomic study was carried out [13] in which the stress-induced MazF-mediated proteins of *E. coli* were identified. It was found that the mRNAs of nearly all the identified proteins are characterized by the presence of an ACA site within a span of 1 to 100 nucleotides upstream from the AUG initiator [13]. Thus, under stressful conditions, the induced MazF processes the mRNAs that are translated by the STM. Here it is shown that in half of these proteomic identified proteins, the threonine

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codon ACA is not located in-frame, while its synonymous codons, ACG, ACU and ACC, are mostly in-frame. In contrast, in the majority of mRNAs of the proteins translated by the canonical translation system, an ACA is mostly located in-frame. Thus, it is suggested that *E. coli* genes specifying for stress-induced MazF-mediated proteins are mostly characterized by the described bias in the reading of the genetic code.

Recently, Aoi *et al.* reported that, in *Nitrospira* strain ND1, the MazF toxin specifically recognizes the motifs AACU, AACG, and AAUU [15]. However, since, in this case, MazF target is not a coding triplet, a bias in the reading of the genetic code should not be characteristic for genes that specify stress-induced MazF mediated proteins of *Nitrospira* strain ND.

2. METHODS

2.1. Computational Analysis

The distribution of the in-frame and out-of-frame threonine codon (ACA) was determined using the HTML Script Program (Supplementary Materials).

The sequences of all *E. coli* MG1655 genes were scanned for ACA sites upstream from the initiation codons using the *E. coli* K-12 sub-strain MG1655 genomic sequence and the EcoCyc database. For this aim, gene annotations were downloaded from the NCBI Genome database (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/005/845/GCF_000005845.2_ASM584v2/). Gene annotations were downloaded from the Genome database of the National Center for Biotechnology Information (NCBI) (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/005/845/GCF_000005845.2_ASM584v2/).

All protein coding gene coordinates and strand information were scanned to find genes that had a coding-free region of at least 100 non-coding nucleotides upstream from their translation start site. Among these sequences, The EMBOSS-*fuzznuc* function of the *The European Molecular Biology Open Software Suite* (EMBOSS) was used to search for the pattern "ACA" [16].

3. RESULTS

3.1. The Distribution of the Threonine Synonymous Codons ACA, ACG, ACU, and ACC in Relation to the Open Reading Frames in mRNAs of Stress-induced MazF-mediated *E. coli* Proteins

The amino acid threonine is encoded by four synonymous codons: ACA, ACG, ACU, and ACC. The locations of all four of these codons, in the mRNAs of stress-induced MazF-mediated *E. coli* proteins (Table **1**), were identified. Note that because all these mRNAs carry the MazF cleavage site ACA within the region of 1 to 100 nucleotides upstream from the AUG initiator codon, under stressful conditions, they can be translated by the STM system [10, 11]. As shown in Table **1**, about 50% of these mRNAs (18 out of 36) include no in-frame ACA site. However, the three synonymous codons ACG, ACU, and ACC, which are not cleaved by MazF, are located in-frame. Moreover, while they carry no in-frame ACA codons, these mRNAs, do carry out-of-frame ACA codon(s).

The positions of out-of-frame ACA sites in the genes of some specific MazF-mediated proteins (highlighted yellow in Fig. **1**) were identified. These genes, including *ftsH*, *osmE*, *groS* and *ibpA*, did not carry any in-frame ACA sites (Table **1**).

3.2. The Distribution of the Four Synonymous Threonine Codons, ACA, ACG, ACU, and ACC, in Relation to the Open Reading Frames in the mRNAs of *E. coli* Proteins that are not Mediated by MazF

The locations of the four synonymous threonine codons, ACA, ACG, ACU, and ACC, in mRNAs of *E. coli* proteins that are not mediated by stress-induced MazF (Table **2**), were identified. Because none of these mRNAs carry an ACA MazF cleavage site within a span of 1 to 100 nucleotides upstream from the AUG initiator codon, they were not considered to be translated by the Stress-induced Translation Machinery (STM), but rather by the "regular" canonical translation system. As shown, 88% (44 out of 50) of the described mRNAs do carry at least one in-frame ACA site. This value is significantly higher than the corresponding value of 50% that was obtained in the mRNAs of genes, which are stress-induced MazF-mediated (Table **1**). Fig. (**2**) shows the positions of in-frame (red) and out-of-frame (yellow) ACA sites in some specific proteins (randomly selected from Table **2**) that are not mediated by MazF.

4. DISCUSSION

Here the distribution of the four synonymous threonine codons ACA, ACG, ACU, and ACC, in relation to the open reading frames in mRNAs of the stress-induced MazF-mediated *E. coli* proteins, was studied. The results clearly show that in the mRNAs of 50% of these proteins, the threonine codon ACA is not located in-frame, while its synonymous codons, ACG, ACU and ACC are in-frame (Table **1**). However, all (100%) of these mRNA molecules carry out-of-frame ACAs. In addition, the mRNAs of each of these proteins carry an ACA codon within a span of 100 nucleotides upstream from the AUG initiator (Table **1**), thus permitting translation by the Stress-induced Translation Machinery (STM). In contrast, in the majority of mRNAs of the proteins translated by the canonical translation system, ACAs are located in-frame (Table **2**). Furthermore, none of them carry an ACA site within a span of 100 nucleotides upstream from the AUG initiator (Table **2**).

The frequencies that were found for the four synonymous threonine codons in *E. coli* are ACA: 17%, ACU: 19%, ACC: 40%, and ACG: 25% (Table **3**, column B). In the mRNAs of *E. coli* proteins not mediated by MazF, similar frequencies were found: ACA: 12%, ACU: 19. 5%, ACC: 44%, and ACG: 24.5% (Table **3** column C). In contrast, in the mRNAs of MazF-mediated *E. coli* proteins, it was observed that the frequency of ACA was reduced from 12% to 5% (Table **3**, column D). It is suggested that this significant reduction of more than 50% is a confirmation of the bias against ACA in the genetic code of *E. coli* genes specifying for stress-induced MazF-mediated proteins.

It is emphasized that although most of the mRNAs of the stress-induced MazF-mediated proteins carried out-of-frame ACA codons were found, 50% of these mRNAs also carried

Table 1. The locations of ACA sites in mRNAs of stress-induced MazF-mediated *E. coli* proteins.

#	Gene Names	Protein Product	# ACA In-frame	# ACA Out of Frame	# ACG In-frame	# ACC In-frame	# ACU In-frame	Distance of ACA from AUG Initiator
1	*bola*	DNA-binding transcriptional regulator	0	4	1	2	4	29
2	*dnaK*	Chaperone protein	0	22	4	23	17	33
3	*ftsH*	ATP dependent zinc metallo-protease ftsH	0	18	8	10	9	1
4	*galF*		0	9	3	3	5	15
5	*groL*	60 kDa chaperonin	0	13	0	25	8	25
6	*groS*	10 kDa chaperonin	0	3	0	2	1	32
7	*grpE*	Protein GrpE	0	2	4	1	3	90
8	*hslU*	ATP-dependent protease ATPase subunit	0	14	0	16	5	19
9	*IbpA*	Small heat shock protein	0	3	0	2	0	30
10	*ihfB*	Integration host factor	0	1	1	3	2	40
11	*metK*	S-adenosyl methionine synthase	0	16	1	15	8	40
12	*osmE*	Osmotically- inducible lipoprotein E	0	7	1	6	2	14
13	*pflB*	Formate acetyltransferase 1	0	34	1	27	18	1
14	*RpoD*	RNA polymerase sigma factor	0	22	7	29	2	35
15	*sra*	Stationary phase induced ribosome associated protein	0	1	0	2	0	18
16	*ybeZ*	phoH- like protein	0	16	3	10	4	20
17	*ydfG*	NADP dependent3-hydroxy acid dehydrogenase	0	5	9	7	3	7
18	*yeeX*	Uncharacterized protein	0	5	0	1	1	19
19	*def*	Peptide deformylase	1	4	1	1	0	15
20	*dps*	DNA protection during starvation protein	1	9	0	8	3	20
21	*galU*	UTP-glucose-1-phosphate uridylyltransferase	1	5	4	6	2	18
22	*lon*	Lon protease	1	22	6	20	5	50
23	*osmY*	Osmotically-inducible protein Y	1	7	0	14	4	13
24	*pckA*	Phosphoenolpyruvate carboxykinase	1	20	2	27	14	23
25	*rho*	Transcription termination factor	1	12	3	13	6	7
26	*rpmA*	50 S ribosomal protein	1	2	0	1	1	89

(Table 1) contd….

#	Gene Names	Protein Product	# ACA In-frame	# ACA Out of Frame	# ACG In-frame	# ACC In-frame	# ACU In-frame	Distance of ACA from AUG Initiator
27	*rraB*	Regulator of ribonuclease activity	1	6	1	3	1	18
28	*ybeD*	Uncharacterized protein	1	3	1	2	4	1
29	*clpX*	ATP-dependent Clp protease ATP-binding subunit ClpX	2	14	6	10	1	20
30	*infC*	Translation initiation factor IF-3	2	0	3	0	0	18
31	*osmC*	peroxiredoxin	2	3	4	5	0	2
32	*ygaU*	Uncharacterized protein	2	4	2	2	3	37
33	*clpB*	Chaperone protein ClpB	3	25	9	11	9	86
34	*dksA*	RNA polymerase binding transcription factor	3	2	2	1	0	1
35	*tyrB*	Aromatic-amino-acid ami-notransferase	3	7	4	4	4	74
36	*ycaC*	Uncharacterized protein	3	5	3	8	2	93

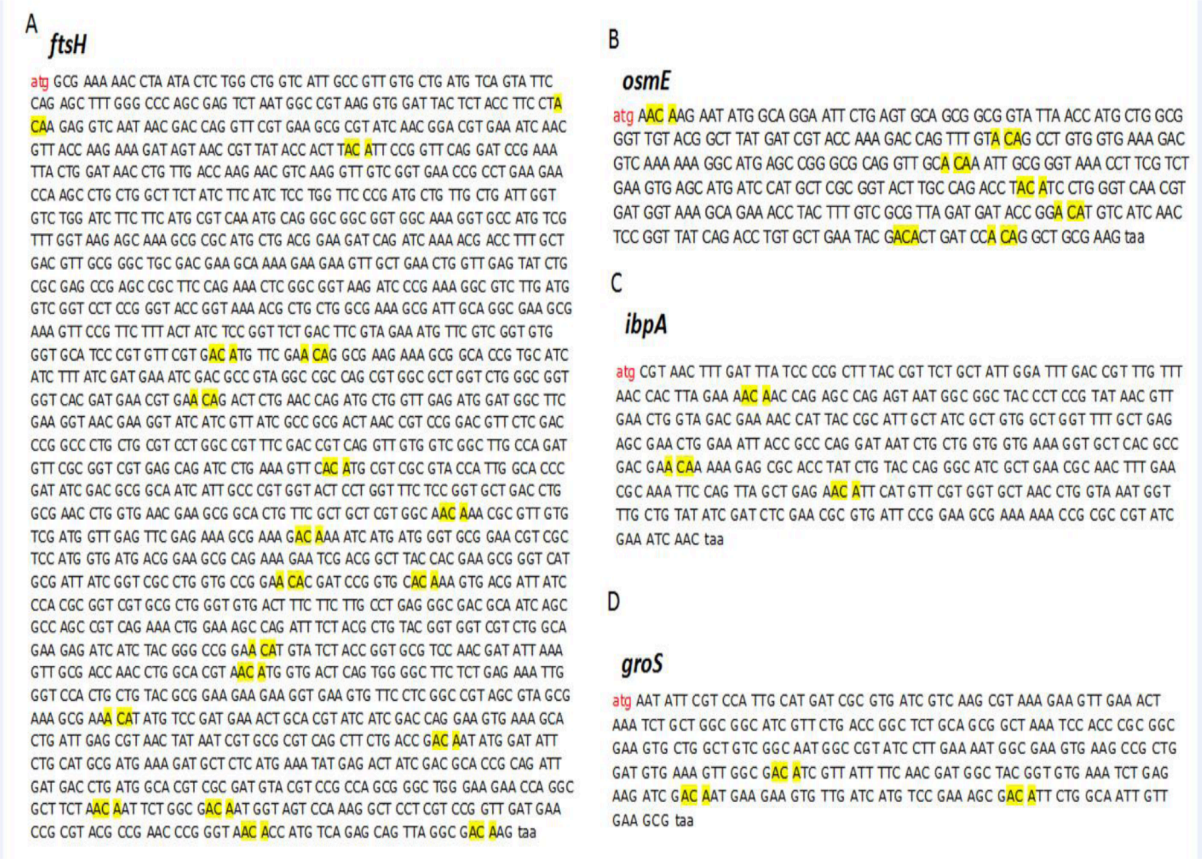

Fig. (1). The locations of ACA sites in the mRNAs that encode stress-induced MazF-mediated proteins in *E. coli*. DNA sequences: (**A**) *ftsH,* (**B**) *osmE,* (**C**) *ibpA* and (**D**) *groS.* Out-of-frame ACA codons are highlighted in yellow. Sequences were taken from ecocyc.org (database *for E. coli*, Strain K-12). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Absence of ACA sites upstream to the AUG initiator and the locations of the threonine synonymous codons (ACA, ACG, ACU, and ACC) in mRNAs of encoding proteins that are not stress-induced MazF-mediated *E. coli* proteins.

#	Gene Name	Protein Product	# ACA In-frame	# ACA Out-of-Frame	# ACG In-frame	# ACC In-frame	# ACU In-frame	# ACA Up-stream AUG Initiator*
1	*mrdA*	Peptidoglycan DD-transpeptidase MrdA	7	20	10	19	2	0
2	*speF*	ornithine decarboxylase, degradative	5	23	6	8	5	0
3	*mrcA*	peptidoglycan glycosyltransferase/ peptidoglycan DD-transpeptidase MrcA	5	27	12	22	6	0
4	*pgrR*	DNA-binding transcriptional repressor	5	5	5	3	4	0
5	*bamA*	outer membrane protein assembly factor BamA	5	27	9	36	7	0
6	*cynR*	DNA-binding transcriptional dual regulator CynR	4	12	5	4	2	0
7	*dnaG*	DNA primase	4	19	12	13	3	0
8	*mocA*	molybdenum cofactor cytidylyltransferase	4	4	0	6	3	0
9	*nemA*	*N*-ethylmaleimide reductase	4	6	5	5	3	0
10	*yneK*	protein YneK	4	17	5	5	4	0
11	*anmK*	anhydro-*N*-acetylmuramic acid kinase	4	13	4	11	3	0
12	*dmlA*	D-malate/3-isopropylmalate dehydrogenase (decarboxylating)	3	7	3	6	3	0
13	*rlmF*	23S rRNA m^6A1618 methyltransferase	3	4	3	5	0	0
14	*csdA*	cysteine sulfinate desulfinase	3	9	5	6	6	0
15	*hdeD*	acid-resistance membrane protein	3	2	2	0	0	0
16	*melA*	α-galactosidase	3	11	11	11	4	0
17	*envY*	DNA-binding transcriptional activator EnvY	3	9	6	12	5	0
18	*gfcB*	lipoprotein GfcB	3	7	5	4	4	0
19	*nadC*	quinolinate phosphoribosyltransferase (decarboxylating)	3	7	6	7	4	0
20	*speC*	ornithine decarboxylase, biosynthetic	3	17	9	10	5	0
21	*ybfK*	uncharacterized protein YbfK	2	2	1	0	0	0
22	*ycdU*	uncharacterized protein YcdU	2	5	5	2	4	0
23	*hemA*	glutamyl-tRNA reductase	2	10	6	5	5	0
24	*trmL*	tRNA (cytidine/uridine-2'-*O*)-ribose methyltransferase	2	5	2	4	1	0
25	*aslB*	putative anaerobic sulfatase maturation enzyme	2	12	7	3	3	0
26	*gstB*	glutathione *S*-transferase GstB	2	2	3	2	2	0
27	*cirA*	ferric dihyroxybenzoylserine outer membrane transporter	2	23	17	26	5	0
28	*yaiC*	diguanylate cyclase DgcC	1	9	7	11	3	0

(Table 2) contd….

#	Gene Name	Protein Product	# ACA In-frame	# ACA Out-of-Frame	# ACG In-frame	# ACC In-frame	# ACU In-frame	# ACA Up-stream AUG Initiator*
29	*Mog*	molybdopterin adenylyltransferase	1	3	7	3	3	0
30	*allR*	DNA-binding transcriptional repressor AllR	1	9	6	5	1	0
31	*msyB*	acidic protein that suppresses heat sensitivity of a *secY* mutant	1	4	1	2	0	0
32	*yeaK*	mischarged aminoacyl-tRNA deacylase	1	3	1	3	2	0
33	*rcsA*	DNA-binding transcriptional activator RcsA	1	7	6	7	1	0
34	*sdaB*	L-serine deaminase II	1	13	1	13	6	0
35	*hslV*	HslV hexamer	1	6	2	5	4	0
36	*zapA*	cell division protein ZapA	1	5	0	2	4	0
37	*dapF*	diaminopimelate epimerase	1	2	2	7	1	0
38	*sdiA*	DNA-binding transcriptional dual regulator SdiA	1	4	4	3	3	0
39	*oxyR*	DNA-binding transcriptional dual regulator OxyR	1	7	2	6	3	0
40	*caiA*	crotonobetainyl-CoA reductase	1	13	3	13	2	0
41	*pflB*	pyruvate formate-lyase (inactive)	1	34	1	28	18	0
42	*osmB*	osmotically-inducible lipoprotein OsmB	1	1	2	3	1	0
43	*sdiA*	DNA-binding transcriptional dual regulator SdiA	1	4	4	3	3	0
44	*proQ*	RNA chaperone ProQ	1	9	3	3	0	0
45	*uxaC*	D-glucoronate/D-galacturonate isomerase	0	16	1	14	9	0
46	*ackA*	acetate kinase	0	19	1	14	6	0
47	*Pgk*	phosphoglycerate kinase	0	11	0	13	7	0
48	*sugE*	quaternary ammonium compound efflux pump	0	0	4	2	2	0
49	*fimE*	regulator for fimA	0	0	2	5	4	0
50	*roxA*	ribosomal protein-arginine oxygenase	0	9	1	3	2	0

*100 nucleotides upstream to AUG initiator.

in-frame ACA codons (Table **1**). Therefore, it is suggested that, in such cases, it may provide a stress-operating mechanism for processing mRNAs by MazF within the coding sequence, thereby reducing high levels of proteins that are probably produced because of elevated levels of transcription. Finally, might an ACA codon located out-of-frame have a regulatory function for the synthesis of a protein under stressful conditions? If stress also induces frame-shifting, the out-of-frame MazF ACA cleavage may serve as a mechanism to prevent STM translation through frame-shifting. Accordingly, the destructive effect of frame-shifting due to extensive stress locates the originally out-of-frame ACAs into the open reading frame, thereby exposing them to MazF cleavage. Thus, the out-of-frame ACAs serve as safeguards for accurate translation under stress.

Genomic screening is generally used to detect *cis* and *trans* elements as well as mutations. This is the first report showing the use of genomic screening for the detection of proteins involved in a metabolic state, specifically, stress-induced MazF-mediated proteins in *E. coli.* The presence of an ACA MazF cleavage site within the span of 100 nucleotides upstream from the AUG initiator, along with the described bias in the genetic code, will probably also be found in additional bacteria in which MazF cleaves ACA sites.

Fig. (2). The locations of ACA sites in mRNAs encoding proteins that are not mediated by MazF in *E. coli*. DNA sequences: (**A**) *rlmF*, (**B**) *nemA*, (**C**) *mocA,* (**D**) *envY* and (**E**) *gfcB*. In-frame ACAs are highlighted in red. Out-of-frame ACAs are highlighted in yellow. Sequences were taken from ecocyc.org (data base *for E. coli*, Strain K-12). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 3. Frequency of each synonymous codons for threonine: ACA, ACG, ACU, and ACC in MazF-mediated mRNAs compared with non MazF-mediated mRNAs.

A	B	C	D
Codon Triplet	**Published data***	**mRNAs of proteins not mediated by MazF (Table 2)**	**mRNAs of MazF-mediated proteins (Table 1)**
ACA	17%	12%	5.0%
ACU	19%	19.5%	25.4%
ACC	40%	44%	53.8%
ACG	25%	24.5%	15.8%

* Published data from www.genscript.com/tools/codon-frequency-table.

CONCLUSION

This study has revealed that in the mRNAs of most of the stress-induced MazF-mediated *E. coli* proteins translated by the Stress-induced Translation Machinery (STM), the threonine codon ACA is not located in-frame but rather out-of-frame, while its synonymous codons, ACG, ACU, and ACC are in-frame. In contrast, in most of the mRNAs of the proteins translated by the canonical translation system, an in-frame ACA was found. Thus, it is suggested that this bias in the reading of the genetic code is characteristic of *E. coli* genes specifying for stress-induced MazF-mediated proteins. This is the first report showing the use of genomic screening for the detection of proteins involved in a metabolic state, stress-induced MazF-mediated proteins in *E. coli.*

AUTHORS' CONTRIBUTIONS

Experiments were performed by A.N. and A.O.G. The paper was written jointly by A.N., A.O.G., and H.E.K. The project was directed by H.E.K.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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