RNA BIOLOGY
2017, VOL. 14, NO. 1, 15–19
http://dx.doi.org/10.1080/15476286.2016.1255396

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POINT OF VIEW

# Cloaked dagger: tRNA slicing by an unlikely culprit

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**ABSTRACT**

The unusually high number of toxin-antitoxin (TA) systems in *Mycobacterium tuberculosis*, the etiological agent of tuberculosis, is thought to contribute to the unique ability of this pathogen to evade killing by the immune system and persist as a latent infection. One TA family, designated *mazEF* (for the MazE antitoxin and MazF toxin), comprises 10 of the >80 TA systems in the *M. tuberculosis* genome. Here we discuss the significance of our recent *Nucleic Acids Res*. paper that reports a surprising enzymatic activity for the MazF-mt9 toxin—sequence- and structure-specific cleavage of tRNA to generate tRNA halves—that underlies the growth-regulating properties of this toxin. This activity is distinct from all characterized MazF family members in *M. tuberculosis* and other bacteria; instead it is strikingly similar to that documented for members of another toxin family, VapC, despite the absence of sequence or structural similarity.

**Abbreviations:** TA, toxin-antitoxin

**ARTICLE HISTORY**
Received 6 September 2016
Revised 12 October 2016
Accepted 25 October 2016

**KEYWORDS**
Antitoxin; endoribonuclease; tuberculosis; toxin; tRNA

## MazF toxins and latent tuberculosis infection

The *M. tuberculosis* genome harbors an unusual abundance of TA systems (>80) relative to other free-living bacteria.[1] Therefore, TA systems have been implicated in *M. tuberculosis* stress survival and/or the switch to a non-replicating persistent state characteristic of latent tuberculosis infection. Among the >80 *M. tuberculosis* TA systems, the *vapBC* and *mazEF* families (assigned by amino acid sequence similarity) are the largest, with 50 and 10 members, respectively.[2] By comparison, most free-living bacteria have just one *mazEF* and when a *vapBC* module is present, there is only one.[1]

*M. tuberculosis* has the highest number of *mazEF* TA systems of any pathogen. Fig. 1 summarizes the general features of *mazEF* TA systems and illustrates the multiple mechanisms enlisted by MazF toxins to modulate bacterial cell growth. In addition to this mechanistic view, the association between *M. tuberculosis* MazF toxin activity and virulence, stress survival and nonreplicating persistence is based on a collection of published work. *M. tuberculosis* MazF toxin transcripts or protein levels are influenced by stresses associated with infection: nutrient starvation,[3-6] hypoxia,[6-8] macrophage infection[7,9-11] or antibiotic treatment.[6,12-14] MazF toxin expression increases the number of persisters[15] and collection, simultaneous deletion of 3 *mazF* genes reduces persisters.[6] Finally, a triple *mazF* deletion hinders mycobacterial growth and lessens the pathology in the lungs and liver in the guinea pig model, suggesting that some MazF toxins may act cooperatively as virulence factors.[6]

## An evolving view of the mode of action of MazF toxins

All MazF toxins are unified by their hallmark single-strand, sequence-specific endoribonuclease activity. For example, all characterized MazF orthologs from bacteria and archaea arrest growth by cleaving single-stranded RNA at specific 3-, 5-, or 7-base recognition sequences.[16-22] Our studies and others to date portend that each of the 10 *M. tuberculosis* MazF toxins are likely to recognize distinct RNA sequences, some of different lengths.[22-26] While initially thought to exclusively target mRNAs—and thus surgically alter the transcriptome as a means to a desired end—we and others have demonstrated that MazF toxins can also target 23S and 16S rRNA to modify or inactivate the ribosome.[24,25,27] The focus of this Point of View article is on the unexpected function of one MazF toxin in *M. tuberculosis*, MazF-mt9, which regulates cell growth through very selective, precise cleavage of tRNA.

## tRNA as a new MazF target

The breadth of RNA targets for MazF toxins further widened when we recently discovered that one of the *M. tuberculosis* MazF toxins, MazF-mt9, was a tRNase that specifically targeted cleavage of $tRNA^{Lys}$.[23] From the start, we found that this toxin behaves very differently. We initially tried to identify the cleavage consensus sequence of MazF-mt9 using conventional approaches, i.e. *in vivo* and/or *in vitro* primer extension experiments after expression of, or incubation with, the toxin. We only identified a UU↓U sequence (where ↓ represents the location of

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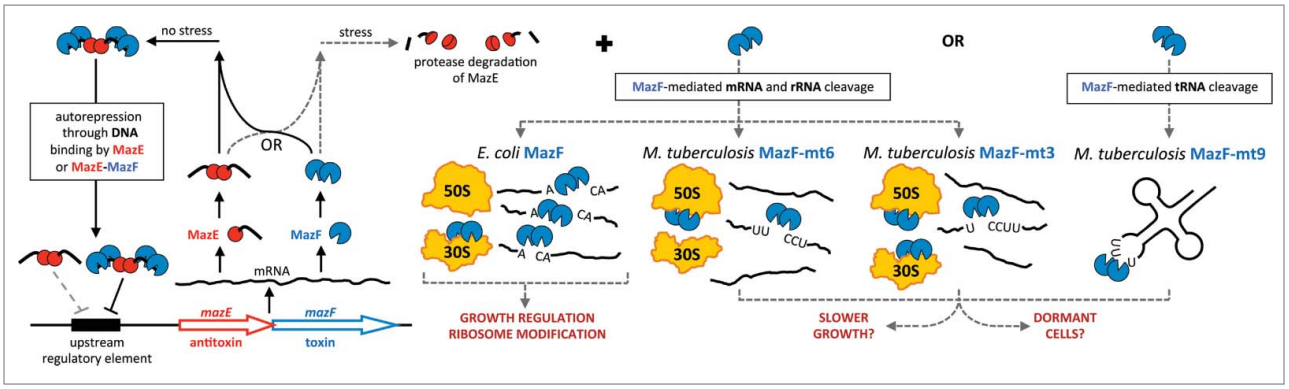


**Figure 1.** MazF toxins can specifically target mRNA, rRNA, or tRNA. All *mazEF* operons encode the intracellular toxin MazF downstream of the gene for the antitoxin MazE. In the absence of stress, the MazE antitoxin neutralizes the toxicity of MazF by forming a stable complex with the toxin. The MazEF complex and to a lesser extent, the MazE dimer, autoregulate the operon. Thus, the amount of free, active MazF toxin is dynamic and dependent on the concentration of the unstable MazE antitoxin that is susceptible to the action of cellular proteases. Many MazF family members appear to regulate growth by cleaving single-stranded mRNA at unique and specific 3-, 5- or 7-base sequences. However, *E. coli* MazF also cleaves 16S rRNA in the 30S ribosomal subunit. *M. tuberculosis* toxins MazF-mt6 and MazF-mt3 also cleave 23S rRNA in the 50S subunit or both 16S and 23S rRNA, respectively. By contrast, *M. tuberculosis* MazF-mt9 cleaves tRNA.

RNA cleavage) in common in the 11 RNAs tested. However, only 2% of the UUU sequences in these 11 RNAs were cleaved. In contrast, other MazF toxins typically cleave at all consensus sequences, as we demonstrated for MazF-mt6.[24] These results indicated that while that presence of a UUU sequence is necessary, it was not sufficient for MazF-mt9 cleavage.

Then the question remains: what is the context required for MazF-mt9 cleavage at selected UUUs? This was answered only after exploiting a powerful modified RNA-seq technology.[24] In contrast to conventional RNA-seq routinely used to survey the entire transcriptome, our RNA-seq method enabled differential detection of subpopulations of RNA depending on the chemical moiety present at the 5′ end of the transcripts (i.e., 5′-OH for MazF family members). These RNA-seq experiments revealed that the proper context for the UU↓U cleavage site deduced from primer extension analysis was within a tRNA, more specifically $tRNA^{Lys}$ (Fig. 2), representing the first example of a MazF toxin that targets tRNA.

This finding contrasts with the literature that relates to MazF cleavage specificity, representing >45 publications that reach the conclusion that MazF toxins only cleave mRNA, not tRNA. In fact, due to the preponderance of double-stranded regions and conserved tertiary fold, it was assumed that tRNA is specifically immune from MazF cleavage. A model invoking mRNA as the exclusive target for MazF family members was so well accepted in the field that all MazF toxins had been almost universally referred to as "mRNA interferases." Given that there are now examples of MazF toxins with specificity for tRNA, 23S rRNA, and 16S rRNA in addition to mRNA, use of the term mRNA interferase for MazF family members in general is obsolete as it does not accurately reflect the true spectrum of MazF targets.

## Context matters

MazF-mt9 requires a precise structural context for target recognition and cleavage.[23] The determinants for toxin cleavage were analyzed by mutagenesis of the anticodon stem loop of $tRNA^{Lys}$ to either 1) alter the cleavage consensus sequence, 2) abolish the stem or 3) reconstitute the stem using a different complementary sequence (Fig. 3).

An intact UU↓U consensus is essential for MazF-mt9 cleavage of $tRNA^{Lys}$ (Fig. 3A). No cleavage occurs when the UUU is mutated to AAA (Fig. 3B). In addition, the UUU recognition sequence must also be in the proper context spatially and structurally. First, the UUU must be in the anticodon position of an intact anticodon stem loop. No cleavage occurs when the UUU is moved further up the anticodon loop (i.e. 3′), also resulting in a mutated anticodon (UUU to AAU, Fig. 3C). Therefore the UUU must be in the anticodon position within the anticodon loop, and in concordance with all other MazF toxins, the actual site of cleavage must be single-stranded. Second, the UUU must be in the anticodon position of the loop and adjacent to an intact stem. Disruption of stem formation prevents toxin cleavage (Fig. 3D). However, restoration of the stem using a different, but complementary sequence restored MazF-mt9 cleavage (Fig. 3E).

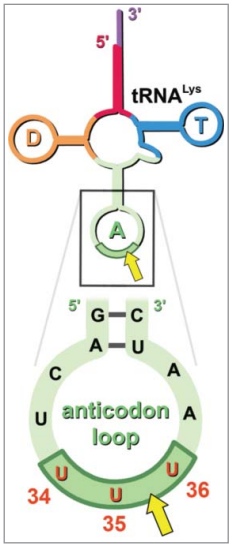


**Figure 2.** MazF-mt9 cleaves $tRNA^{Lys}$ in its anticodon stem loop. The site of cleavage is within the red UUU anticodon ($^{5'}$UU↓U$^{3'}$, yellow arrow). The sequence shown is identical between *E. coli* and Mtb. In the full length tRNA image above the boxed sequence, the D-arm is highlighted *orange*, the anticodon arm in *light green*, the anticodon in *dark green*, the T-arm in *blue*. Numbering in the anticodon indicates mature tRNA nucleotide position.

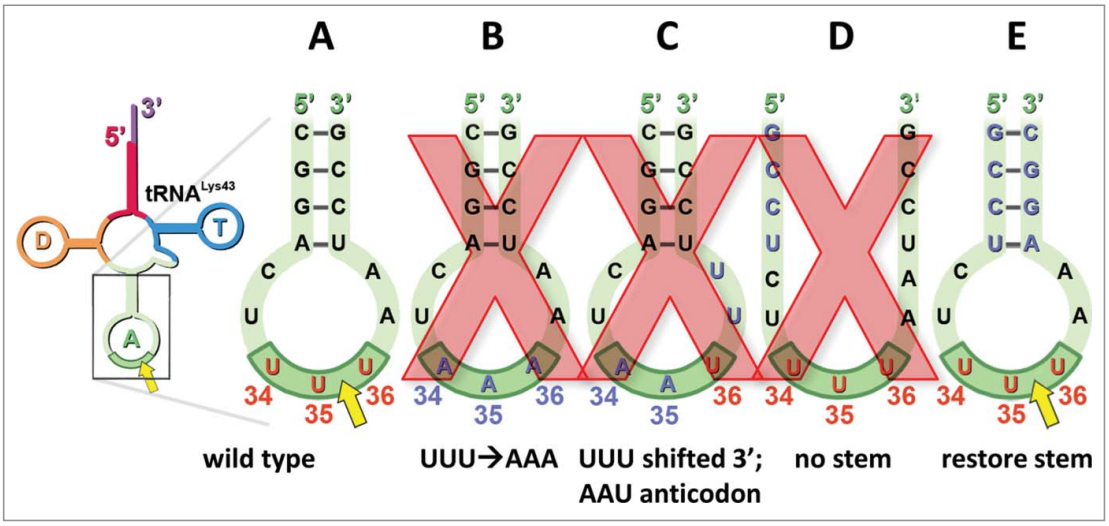


**Figure 3.** MazF-mt9 cleavage of tRNA is sequence- and structure-dependent. A yellow arrow indicates that cleavage occurs at the position shown. Wild type sequence at the anticodon, *red*; mutated sequences, *blue*. Mutants with a *red X* over the structure were not cleaved by MazF-mt9. Full-length tRNA colored as in Fig. 2. Numbering in the anticodon indicates mature tRNA nucleotide position.

No other MazF toxin is known to exhibit properties that are consistent with structure-dependent cleavage.

## This MazF acts like a VapC

Although MazF-mt9 is the first example of a MazF toxin that targets tRNA, it is not the first toxin known to target tRNA for cleavage at the anticodon stem loop. The Gerdes laboratory[28,29] and our laboratory[30] have previously shown that some VapC toxins a have similar specificity. Yet VapC toxins have an entirely different amino acid sequence with no similarity to MazF toxins, use a completely different mechanism for catalysis that involves a PIN domain comprising 4 highly conserved acidic residues coordinated with a metal ion, and the structures of VapC toxins do not share any obvious features with the structures of MazF.[31-38] This conundrum will only be solved once a high resolution crystal structure of MazF-mt9 bound its tRNA substrate is obtained and compared to other MazF toxins as well as tRNA-cleaving VapC toxins.

## A cautionary tale: One is not always like the others

Once again, as we had earlier demonstrated for MazF-mt3 and MazF-mt6, we have debunked the notion that the function of

**Table 1.** Compilation of MazF RNA cleavage recognition sequences and targets.

| Toxin name | Alias | Organism | RNA target | Cleavage specificity | # of sites (mut) | Reference(s) | Other reported consensus | # of sites (mut) | Alternate reference(s) |
|---|---|---|---|---|---|---|---|---|---|
| MazF | ChpAK | *Escherichia coli* | 16S rRNA, mRNA | ↓ACA or A↓CA | 47 (16) | Zhang et al. 2003 & 2005[19,20]; Vesper et al. 2011[27]; Miyamoto et al. 2016a[39] | A↓AC or U↓AC | 12 (0) | Munoz-Gomez et al., 2004[40] |
| MazF-mt1 | MazF9 | *Mycobacterium tuberculosis* | mRNA | U↓AC | 2 (5) | Zhu et al. 2006[26] | None | N/A | N/A |
| MazF-mt3 | MazF6 | *M. tuberculosis* | 23S/16S rRNA, mRNA | U↓CCUU | 273 (0) | Schifano et al. 2013[24] | UU↓CCU or CU↓CCU | 12 (0) | Zhu et al. 2008[22] |
| MazF-mt6 | MazF3 | *M. tuberculosis* | 23S rRNA, mRNA | UU↓CCU | 21 (0) | Schifano et al. 2014[25] | U-rich or YU↓WCY | 3 (0) | Zhu et al. 2006[26] |
| MazF-mt7 | MazF4 | *M. tuberculosis* | mRNA | U↓CGCU | 23 (0) | Zhu et al. 2008[22] | None | N/A | N/A |
| MazF-mt9 | MazF7 | *M. tuberculosis* | tRNA, (mRNA) | UU↓U + stem | 220 (0) | Schifano et al. 2016[23] | None | N/A | N/A |
| MazF-hw | HQ2202A | *Haloquadra walsbyi* | mRNA | UU↓ACUCA | 5 (8) | Yamaguchi et al. 2012[17] | None | N/A | N/A |
| MazF-bs | EndoA, YdcE | *Bacillus subtilis* | mRNA | U↓ACAU | 12 (4) | Park et al. 2011[41] | ↓UAC or U↓AC | 1 (5) | Pellegrini et al. 2005[42] |
| MazF-cd | CD3461 | *Clostridium difficile* | mRNA | U↓ACAU | 11 (0) | Rothenbacher et al. 2012[16] | None | N/A | N/A |
| MazF-sa | SA2058 | *Staphylococcus aureus* | mRNA | U↓ACAU | 9 (3) | Zhu et al. 2009[21] | V↓UUV′ | 7 (12) | Fu et al. 2007[43] |
| MazF-seq | None | *Staphylococcus equorum* | mRNA | U↓ACAU | 9 (4) | Schuster et al. 2013[44] | None | N/A | N/A |
| MazF-ne | NE1181 | *Nitrosomonas europaea* | mRNA | A↓AU | 25 (7) | Miyamoto et al. 2016b[45] | None | N/A | N/A |
| MazF-pp | PP0771 | *Pseudomonas putida* | mRNA | U↓AC | 25 (7) | Miyamoto et al. 2016a[39] | None | N/A | N/A |

The number of MazF-mediated cleavage sites used in each reference to determine a consensus cleavage recognition sequence is indicated, along with the number of mutants ("mut," in parentheses) that were tested in cleavage assays. The total cleavage sites and the tested mutants listed for *E. coli* MazF were combined from the _indicated 4 references. mRNA in parentheses for MazF-mt9 indicates that this class of RNA is not the primary target of this toxin Y = C, U; W = A, U; V = A, C, G.

one MazF toxin mirrors that of the family as a whole. MazF-mt9 not only targets a different class of RNA distinct from other MazFs (Table 1), its mode of recognition is also different. No other MazF toxin is known to select its RNA target based on both sequence and structure. Each RNA species – tRNA, mRNA, and rRNA – folds in a unique way and interacts with specific enzymes, structural proteins, or cofactors to bestow a distinct function in protein synthesis and possibly other cellular processes. As a result, cleavage of one or more of these subtypes of RNA by a particular MazF toxin should yield distinct outcomes. Since many of the 10 MazF toxins in *M. tuberculosis* have yet to be characterized in detail, perhaps even more surprises lie ahead.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

## Funding

This work was supported in part by National Institutes of Health (NIH) grants R21AI072399, R56AI119055 to Woychik, NA and Husson, RN as well as New Jersey Health Foundation Signature Initiatives Research Grant #PC38-15 and a Busch Biomedical Research Grant to Woychik, NA; Schifano, JM was supported in part by NIH training grant T32AI007403, Virus-Host Interactions in Eukaryotic Cells to awarded to Brewer, G.

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