

## 23S rRNA as an a-Maz-ing new bacterial toxin target

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*Mycobacterium tuberculosis*, the causative agent of tuberculosis in humans, is a bacterium with the unique ability to persist for years or decades as a latent infection. This latent state, during which bacteria have a markedly altered physiology and are thought to be dormant, is crucial for the bacteria to survive the stressful environments it encounters in the human host. Importantly, *M. tuberculosis* cells in the dormant state are generally refractory to antibiotics, most of which target cellular processes occurring in actively replicating bacteria. The molecular switches that enable *M. tuberculosis* to slow or stop its replication and become dormant remain unknown. However, the slow growth and dormant state that are hallmarks of latent tuberculosis infection have striking parallels to the “quasi-dormant” state of *Escherichia coli* cells caused by the toxin components of chromosomal toxin-antitoxin (TA) modules. An unusually large number of TA modules in *M. tuberculosis*, including nine in the *mazEF* family, may contribute to initiating this latent state or to adapting to stress conditions in the host. Toward filling the gap in our understanding of the physiological role of TA modules in *M. tuberculosis*, we are interested in identifying their molecular mechanisms to better understand how toxins impart growth control. Our recent publication<sup>1</sup> uncovered a novel function of a MazF toxin in *M. tuberculosis* that had not been associated with any other MazF ortholog. This toxin, MazF-mt6, can disrupt protein synthesis by cleavage of 23S rRNA at a single location in an evolutionarily conserved five-base sequence in the ribosome active center.

### Background

MazEF toxin-antitoxin (TA) systems are present in the genomes of many free-living bacteria.<sup>2</sup> TA systems have been implicated in stress survival, persistence, and latent tuberculosis infection,<sup>3–5</sup> in part because their general function is to facilitate reversible growth inhibition in response to stress. TA modules are autoregulated operons comprising adjacent genes that encode two small (~10 kDa) proteins, an intracellular toxin and its cognate antitoxin (Fig. 1). The cytotoxic activity of MazF can be triggered by stresses such as nutrient limitation, DNA damage, high temperature, oxidative stress, or exposure to various antibiotics.<sup>6–9</sup> Although the MazF toxin and the MazE antitoxin are part of the same transcript, the amount of active, free MazF is variable because its activity can be inhibited upon binding to MazE. The dynamic interplay between the inactive MazEF complex and the active, free MazF toxin is due to the intrinsic instability of the antitoxin, which is readily degraded by cellular proteases (Fig. 1).<sup>6,7</sup> Therefore, the activity of the toxin is dependent on the concentration of antitoxin, the concentration of the antitoxin is influenced by the milieu of cellular proteases, and the level of toxin and antitoxin synthesized is dependent on the transcriptional regulation of the operon, which is responsive to cellular signals that are not yet defined for *M. tuberculosis* TA systems.

*E. coli* MazF mediates bacterial cell growth control through its dynamic association and dissociation with its cognate antitoxin MazE.<sup>10</sup> Earlier biochemical studies on *E. coli* MazF demonstrated

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**Abbreviations:** TA, toxin-antitoxin; RRF, ribosome recycling factor; H70, helix/loop 70 in 23S rRNA; PTC, peptidyl transferase center

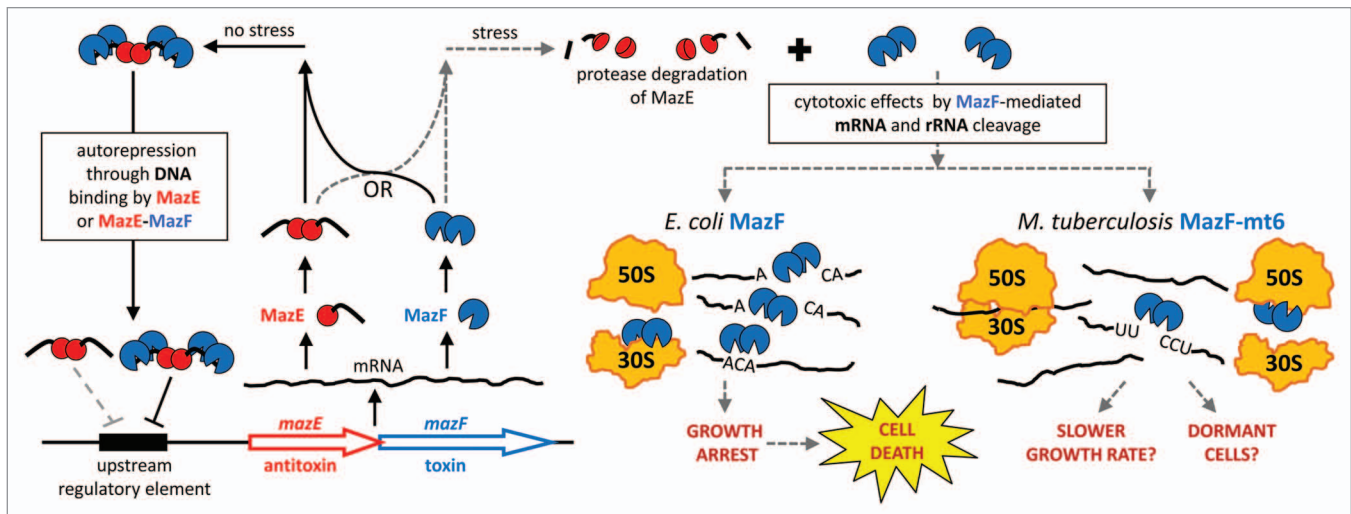
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**Figure 1.** MazF toxins inhibit translation and arrest growth by cleaving mRNA and rRNA. All *mazEF* operons consist of two adjacent genes, one that encodes the intracellular toxin MazF and another that produces its inhibitor, antitoxin MazE.<sup>37</sup> In the absence of stress, the MazE protein forms a stable complex with the MazF toxin to neutralize its toxicity.<sup>6,38</sup> The MazEF heterohexameric complex (2 MazE: 2 MazF)<sup>39</sup> and to a lesser extent, the MazE dimer, repress the operon by binding to an upstream regulatory element that consists of a palindromic DNA sequence.<sup>10,40</sup> The intrinsically unstable MazE antitoxin can be degraded by cellular proteases,<sup>6,7</sup> which frees and activates the MazF toxin. The toxic activity of MazF is triggered by stresses such as nutrient limitation, DNA damage, high temperature, oxidative stress, or exposure to various antibiotics.<sup>6-9</sup> MazF orthologs exert their cytotoxic effects by cleaving single-stranded RNA at unique and specific 3-, 5-, or 7-base sequences.<sup>11-23</sup> Although MazF toxins were initially proposed to solely cleave mRNA as “mRNA interferases,”<sup>11-20</sup> *E. coli* MazF also cleaves 16S rRNA in the 30S ribosomal subunit,<sup>24</sup> while *M. tuberculosis* toxin MazF-mt6 also cleaves 23S rRNA in the 50S subunit.<sup>1</sup> Cleavage of mRNAs or rRNAs disrupts protein synthesis,<sup>1,12,24</sup> which can induce a state of reversible dormancy.<sup>41</sup> Expression of MazF triggers this “quasi-dormant” state, during which cells stop dividing but are able to transcribe mRNA and synthesize proteins.<sup>42</sup> Due to its recognition of a relatively short RNA sequence (ACA), *E. coli* MazF cleaves a majority of cellular mRNAs. In addition, not only can MazF render certain mRNAs leaderless by cleaving an ACA sequence upstream of the start codon, but it also removes 43 nt from the 3' end of 16S rRNA to create “stress ribosomes” that selectively translate either naturally present or MazF-generated leaderless mRNAs.<sup>24</sup> *E. coli* MazF has also been shown to initiate cell death if its toxic effects are not neutralized by MazE in sufficient time.<sup>6,8,9,43</sup> In contrast, *M. tuberculosis* toxin MazF-mt6 is expected to cleave only a subpopulation of mRNAs due to its recognition of the five-base RNA sequence UUCCU. However, MazF-mt6-mediated cleavage of 23S rRNA in free 50S ribosomal subunits is sufficient to inhibit protein synthesis,<sup>1</sup> so combined cleavage of mRNA and rRNA may elicit rapid growth arrest. The effect of MazF-mt6 on *M. tuberculosis* cells in vivo has not been rigorously investigated, but two possible endpoints are shown.

that this toxin is a single-stranded, sequence-specific endoribonuclease that targets ACA sequences and that its cleavage at ACAs appeared to be specific for mRNA.<sup>11,12</sup> Consequently, Inouye and colleagues coined the term “mRNA interferase” for *E. coli* MazF, and this term was often extended to MazF orthologs from other bacteria.<sup>11-20</sup> Despite high sequence similarity between MazF toxins in various prokaryotes, nearly every MazF ortholog recognizes a unique 3- to 7-base RNA sequence.<sup>11-23</sup> Therefore, it was thought that if one could determine the precise cleavage recognition sequence of a given MazF ortholog, one could predict how the toxin specifically alters the transcriptome in vivo.

Implicit in this prediction, the length and base content of the recognition sequence required for MazF toxin-mediated RNA cleavage should dictate the degree of post-transcriptional editing.

Expression of MazF toxins with short RNA recognition sequences, such as the ACA-cleaving *E. coli* MazF, should result in wholesale destruction of mRNAs. In contrast, MazF toxins with larger recognition sequences are predicted to selectively degrade a subpopulation of transcripts that possess one or more copies of the recognition sequence and spare those lacking this sequence. Since it has been demonstrated that the rate of transcript degradation can be correlated with the number of cleavage sites,<sup>15,18,22</sup> statistical analyses can be performed to predict the degree of vulnerability of mRNA transcripts.<sup>18,19,22</sup> However, this kind of statistical analysis is only applicable when mRNAs are the sole targets of MazF toxins. In 2011, Moll, Engelberg-Kulka, and colleagues demonstrated that mRNA is not the only target of *E. coli* MazF.<sup>24</sup> This toxin also specifically cleaves 16S rRNA at a single ACA sequence, resulting in the production of a

population of truncated 16S rRNAs lacking the last 43 nucleotides at the 3' end. Furthermore, their studies also showed that these shortened 16S rRNAs assemble into specialized “stress ribosomes” that selectively translate leaderless transcripts. Therefore, mRNAs are clearly not the only MazF target in *E. coli*.

It was also thought that the function of the representative *E. coli* MazF toxin mirrors that of the family as a whole. Our paper<sup>1</sup> demonstrates that this is somewhat true, but the devil is in the details. We focused on one of the nine MazF family members in the *M. tuberculosis* genome. Although *M. tuberculosis* harbors an extremely high number (> 80) of predicted TA modules relative to most other prokaryotes,<sup>2,25</sup> the physiological role of this large repertoire of TA loci in *M. tuberculosis* is unclear because their fundamental properties, enzymatic activities, and intracellular targets are only

now beginning to be studied in molecular detail.

## 23S rRNA as a New Target for MazF Toxins

In Schifano et al.,<sup>1</sup> we first demonstrate that the consensus recognition sequence for MazF-mt6 cleavage of RNA is UU↓CCU, where “↓” indicates the cleavage site. Coincidentally, the 23S rRNA band diminishes in intensity when MazF-mt6 is ectopically expressed in *E. coli* or the mycobacterial model organism *Mycobacterium smegmatis*. The loss of 23S rRNA appears to result from cleavage at a single site, since the loss of full-length 23S rRNA coincides with the increase of two stable degradation products whose estimated sizes total that of the intact rRNA. Even though *E. coli* and *M. tuberculosis* 23S rRNAs each contain three UUCCU sequences, only the lone site located in a single-stranded region is targeted in each case. Therefore, the MazF-mt6 toxin not only cleaves mRNA, it also directly targets 23S rRNA for cleavage at a single UUCCU site evolutionarily conserved in *E. coli*, *M. smegmatis*, and *M. tuberculosis*. Because of this conservation, we employed an in vitro coupled transcription/translation system with purified *E. coli* components and a separate ribosome fraction to determine how addition of MazF-mt6 before or after ribosome assembly influences the translation step. This also enabled us to distinguish the net effect of mRNA plus 23S rRNA cleavage by MazF-mt6 vs. 23S rRNA cleavage alone on translation. We observed complete translation inhibition in both cases, indicating that MazF-mt6-mediated cleavage of 23S rRNA alone is capable of disabling protein synthesis. This result is in agreement with the critical function of the target region, helix/loop 70 of domain IV, which facilitates tRNA binding in the ribosomal A-site (Fig. 2).

The vital role of this highly conserved UUCCU sequence in 23S rRNA of *M. tuberculosis* is illuminated by a wealth of data for its counterpart in *E. coli* 23S rRNA, <sup>1939</sup>UUCCU<sup>1943</sup> (Fig. 2). First, a U1940A mutation results in 50S ribosomal subunits defective in assembly.<sup>26</sup> Second, the X-ray crystal structure of a 50S

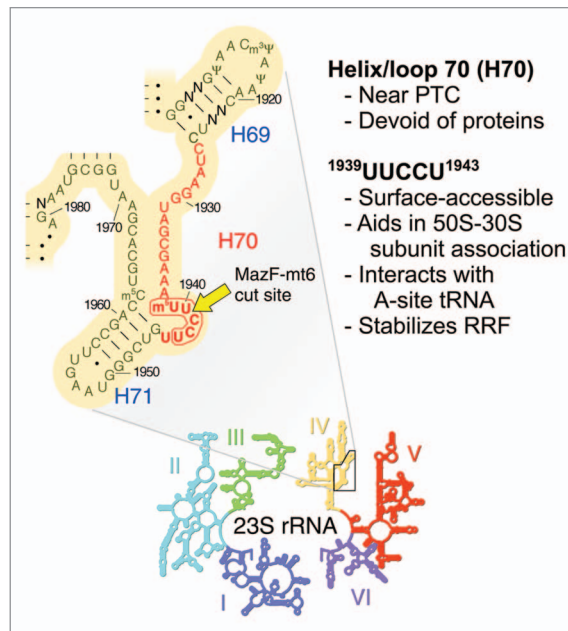
subunit revealed that helix/loop 70 forms part of the front rim of the peptidyl transferase active site cleft.<sup>27</sup> Third, crystallographic studies revealed that helix/loop 70 contacts and positions the CCA tail of the tRNA acceptor stem and that residues C1941, C1942, and U1943 within the MazF-mt6 cleavage sequence also contact the acceptor stem.<sup>28,29</sup> Fourth, this region also directly interacts with ribosome recycling factor (RRF), forming hydrogen bonds to distinct amino acids.<sup>30</sup> Higher resolution X-ray crystal structures of the 50S subunit bound to a domain of RRF also uncovered an interaction between U1941 and a highly conserved amino acid in RRF that is important for 50S binding and function.<sup>31</sup> Finally, a slowdown in cell growth occurs upon cleavage of 23S rRNA by an unknown *E. coli* RNase at helix/loop 70 within the same UUCCU sequence cleaved by MazF-mt6.<sup>32</sup>

In addition, there is ample precedent for potent translation inhibition through disruption of rRNA in the large ribosomal subunit. Interestingly, 23S rRNA

and its eukaryotic counterpart are targeted by several deadly plant or bacterial toxins—ricin, saporin, Shiga toxin, α-sarcin, and pokeweed antiviral toxin—that either remove a specific adenine from the rRNA backbone or cleave between the two adjacent nucleotides in the universally conserved helix 95, also known as the sarcin-ricin loop.<sup>33</sup> However, these toxins cleave intact 70S ribosomes, while MazF-mt6 only cleaves rRNA in free 50S subunits. There are also several antibiotics—sparsomycin, clindamycin, chloramphenicol, linezolid, the pleuromutilins, and the macrolides—that perturb translation through binding to 23S rRNA in *E. coli* 50S subunits.<sup>34</sup> Recently, an *M. tuberculosis* toxin in the VapC family has also been shown to cleave 23S rRNA, but unlike MazF-mt6, this toxin cleaves at the sarcin-ricin loop.<sup>35</sup>

## Rethinking the Role of MazF

The physiological activities of MazF-mt6 and other family members in



**Figure 2.** MazF-mt6 cleaves *M. tuberculosis* 23S rRNA at a single UUCCU in the ribosomal A site. To pinpoint this cleavage, the sequence and secondary structure of a conserved region of domain IV (above) in 23S rRNA (inset below) are shown. Helix/loop 70 (H70), whose nucleotides are bolded, is 100% identical between *E. coli*, *M. smegmatis*, and *M. tuberculosis*. Non-conserved residues are labeled N, *E. coli* position numbers are adjacent to tick marks, the MazF-mt6 recognition sequence is encircled by a bold line, and the lone MazF-mt6 cleavage site is indicated by a gray arrow. This structure is adapted from *E. coli* 23S rRNA gene *rrlB*, courtesy of Harry Noller and the University of California, Santa Cruz ([http://rna.ucsc.edu/rnacenter/ribosome\\_images.html](http://rna.ucsc.edu/rnacenter/ribosome_images.html)) and published in Yusupov et al.<sup>29</sup> PTC, peptidyl transferase center.

*M. tuberculosis* were originally thought to exclusively stem from their cleavage of mRNA.<sup>19,20</sup> Consequently, the characterized *M. tuberculosis* MazF toxins with five-base recognition sequences were proposed to selectively edit the transcriptome to alter protein expression.<sup>19</sup> Our data revealed that this is not the complete extent of MazF-mt6 activity, because cleavage at a functionally essential and evolutionarily conserved region of 23S rRNA also blocks translation.<sup>1</sup> Therefore, this toxin appears to have evolved to selectively target both UCCU-containing mRNAs and ribosomes. Lesson learned? Proceed with caution when inclined to designate MazF family members as mRNA interferases since we and others<sup>1,24</sup> have now demonstrated that MazF toxins do not possess inherent specificity for exclusive mRNA cleavage. Instead, there are at least three requirements dictating MazF cleavage of RNA: (1) the cleavage recognition sequence must be present, (2) the RNA must be single-stranded, and (3) the RNA must be accessible to the toxin.

Because cleavage of 23S rRNA alone can arrest translation, the significance of dual cleavage of mRNA and ribosomes and the consequences of their interplay are unclear. In *E. coli*, 99% of mRNAs (4192 of 4243) contain the ACA MazF motif, and are therefore susceptible to MazF cleavage,<sup>36</sup> so widespread mRNA degradation likely occurs in conjunction with the production of “stress ribosomes.” In contrast, only 38% of *M. tuberculosis* mRNAs (1530 of 4022) contain one or more UCCU MazF-mt6 cleavage sequences, suggesting mRNA cleavage is not as prevalent with this toxin. Since MazF-mt6 appears to preferentially target 23S rRNA in free 50S ribosomal subunits, the simultaneous cleavage of rRNA with a subset of mRNAs may facilitate the rapid release of ribosomal subunits that are then inactivated (Fig. 1). This “one-two punch” by the MazF-mt6 enzyme should enable a faster physiological response—by reducing the level of ribosomes below the threshold required for active growth—than by mRNA cleavage alone.

Stay tuned, as the scope of new RNA targets and associated molecular detail for MazF toxins expands, our understanding of their role in *M. tuberculosis*, other

pathogens, and yes—even *E. coli*—will surely surprise us again.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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