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Detection of endogenous MazF enzymatic activity in *Staphylococcus aureus*

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ABSTRACT

The *mazEF*$_{Sa}$ toxin–antitoxin (TA) system is ubiquitous in clinical isolates of *Staphylococcus aureus*, yet its physiological role is unclear. $MazF_{Sa}$ is a sequence-specific endoribonuclease that inhibits the growth of *S. aureus* and *Escherichia coli* on ectopic overexpression. $MazF_{Sa}$ preferentially cleaves RNA at UACAU sites, which are overrepresented in genes encoding pathogenicity factors. The exploitation of the inherent toxicity of $MazF_{Sa}$ by artificial toxin activation has been proposed as an antibacterial strategy; however, enzymatic activity of endogenous $MazF_{Sa}$ has never been detected, and tools for such analyses are lacking. Here we detail methods for detection of the ribonuclease activity of $MazF_{Sa}$, including a continuous fluorometric assay and a gel-based cleavage assay. Importantly, these methods allowed for the first detection of endogenous $MazF_{Sa}$ enzymatic activity in *S. aureus* lysate. These robust and sensitive assays provide a toolkit for the identification, analysis, and validation of stressors that induce MazF enzymatic activity and should assist in the discovery of artificial activators of the *mazEF*$_{Sa}$ TA system.

Toxin–antitoxin (TA)[1] systems were first discovered on plasmids, where they serve as plasmid maintenance systems via a post-segregational killing (PSK) mechanism [1–3]. In the canonical type II TA system, plasmid maintenance ensures continued production of the co-expressed inactive complex of antitoxin and toxin; however, if the plasmid is not inherited by a daughter cell, the labile antitoxin succumbs to degradation by the Lon or Clp proteases, releasing the toxin to kill the cell [4–7]. TA systems are also encoded on the chromosomes of nearly all free-living bacteria [8], although here their precise role remains a topic of much debate [9,10]. As observed for multiple different TA systems, rapid bacterial cell death is induced by the free toxin; these results have led to speculation that artificial activation of toxin proteins from TA systems could be a powerful antibacterial strategy [11–16].

In one study, the *mazEF*$_{Sa}$ TA genes were detected in 100% (78 of 78) clinical isolates of MRSA (methicillin–resistant *Staphylococcus aureus*), and the *mazEF*$_{Sa}$ transcript was also detected in these isolates [17]. Ectopic overexpression of $MazF_{Sa}$ in *S. aureus* decreased cell viability by 2-log colony-forming units (CFU)/ml after 60 min of induction [18]; however, there was only approximately 27% difference in cell death at the 60-min time point, suggesting that $MazF_{Sa}$ induces stasis and not cell death [18]. $MazF_{Sa}$ is a sequence-specific endoribonuclease that cleaves at U↓ACAU [19]. This sequence is highly abundant in certain transcripts, including those that code for virulence factors such as the serine-rich pathogen adhesion factor SraP [19]. Overexpression of $MazF_{Sa}$ in *S. aureus* results in time-dependent cleavage of other virulence transcripts, including *hla* and *spa*, whereas the essential housekeeping transcripts *recA* and *gyrB* were not cleaved [20]. Thus, the activation of $MazF_{Sa}$ could serve as an anti-virulence strategy by enhancing its ability to cleave virulence factor encoding transcripts. However, the cellular role of $MazF_{Sa}$ remains unclear, and the larger question about the cellular role of chromosomally encoded TA systems has not been answered. In fact, although a number of studies have examined the *mazEF*$_{Sa}$ gene cluster (via polymerase chain reaction [PCR]) [17], the inducibility of the *mazEF*$_{Sa}$ transcript with various stressors (via Northern blots and reverse transcription [RT]–PCR) [18,21], and the presence of the proteins (via Western blot) [22], there have been no studies that assess the enzymatic activity of $MazF_{Sa}$ in its cellular context, and tools for such evaluations are lacking. Ultimately, the ribonuclease activity of $MazF_{Sa}$ will dictate its ability to restrict growth

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[1] *Abbreviations used:* TA, toxin–antitoxin; PSK, post-segregational killing; MRSA, methicillin–resistant *Staphylococcus aureus*; PCR, polymerase chain reaction; mRNA, messenger RNA; NTA, nitrilotriacetic acid; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS, sodium dodecyl sulfate; TEAA, triethylammonium acetate; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; 6-FAM, 6-carboxyfluorescein; BHQ, black hole quencher; RFU, relative fluorescence units; DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid.

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or kill the cell, and non-enzymatic assessments (e.g., DNA, RNA, or protein levels) are only surrogates that do not directly report on actual enzyme activity. Further complicating matters is the fact that the transcripts for $MazE_{Sa}$ and $MazF_{Sa}$ are typically co-produced [18,21]; thus, elevation at the messenger RNA (mRNA) or protein level does not necessitate heightened $MazF_{Sa}$ enzymatic activity because levels of the $MazE_{Sa}$ antitoxin are also raised.

As described here, using a fluorogenic substrate, we have determined the kinetic parameters for $MazF_{Sa}$. In addition, an mRNA transcript was engineered to contain one optimal $MazF_{Sa}$ cleavage sequence, and this substrate provides a means for clear and rapid assessment of $MazF_{Sa}$ activity in vitro. A radiolabeled version of this substrate was used to detect the activity of endogenous $MazF_{Sa}$ in *S. aureus* lysate. This is the first time enzymatic activity of endogenous $MazF_{Sa}$ has been evaluated in the cellular milieu, and the tools described here should facilitate the evaluation of various stressors and assist in the discovery of artificial activators of $MazF_{Sa}$.

Materials and methods

Materials

Primers and oligonucleotides were synthesized by IDT. Ni^{2+}–NTA (nitrilotriacetic acid) resin was obtained from Qiagen. Pepstatin A, leupeptin, aprotinin, phenylmethanesulfonyl fluoride (PMSF), and lysozyme were purchased from Sigma. Restriction enzymes, bovine serum albumin (BSA), a low-range single-stranded RNA (ssRNA) ladder, RNase inhibitor–human placenta, and *Escherichia coli* strains DH5α and NiCo21(DE3) [23] were purchased from New England Biolabs. Subcloning efficiency DH5α chemically competent *E. coli* was purchased from Invitrogen. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and kanamycin were purchased from GoldBio. Lysing Matrix B was obtained from MP Biomedicals. [α-^{32}P]UTP was purchased from PerkinElmer. Sypro Red protein stain was purchased from VWR.

Cloning

E. coli DH5α and NiCo21(DE3) were used for cloning and protein expression, respectively. The $mazEF_{Sa}$ gene cassette was amplified by PCR using primers $mazEF_{Sa}$–*Nco*I-F (5′-ACACCCATGGATATGTTATCTTTAGTCAAAATAG-3′) and $mazEF_{Sa}$–*Xho*I-R (5′-CACACTCGAGATTTTTCTGGTGAGCTAC-3′) from the total DNA of the MRSA strain C2 [17]. The amplicon was inserted into the corresponding restriction sites of pET-28a to create pET-28a–$mazEF_{Sa}$, which codes for $MazEF_{Sa}$ containing a C-terminal histidine-6 tag on $MazF_{Sa}$. The $mazE_{Sa}$ antitoxin gene was amplified from the same strain using primers $mazE_{Sa}$–*Nde*I-F (5′-ACACCATATGTTATCTTTTATCAAATAGAAG-3′) and $mazE_{Sa}$–*Xho*I-stop-R (5′-ACACCTCGAGTCATTCATTCGTTGAATTAGAA-3′) and cloned into pET-28a, resulting in pET-28a–$mazE_{Sa}$, which encodes an N-terminal histidine-6-tagged $MazE_{Sa}$. The sequence of all clones was determined by DNA sequencing. *E. coli* carrying the recombinant plasmids was cultured in LB containing 50 μg/ml kanamycin.

Protein expression and purification

To express $MazEF_{Sa}(His)_6$, an overnight culture inoculated with a single colony of *E. coli* NiCo21(DE3) freshly transformed with pET-28a–$mazEF_{Sa}$ was diluted 100-fold into 2 L of LB + kanamycin. The culture was grown at 37 °C until the OD_{600} reached 0.6 to 0.8, at which point expression was induced with 1 mM IPTG (final concentration) for 4 h at 37 °C. Expression of $(His)_6MazE_{Sa}$ was performed the same except that IPTG was added when the OD_{600} reached 0.4 to 0.6. The 2-L cultures were harvested by centrifugation at 4 °C (8000*g* for 500-ml bottles and 10,000*g* for 50-ml conical tubes) and stored at −20 °C.

$MazF_{Sa}(His)_6$ was purified from the $MazEF_{Sa}(His)_6$ complex under denaturing conditions. A pellet corresponding to 2 L of culture was thawed in a room temperature water bath for 10 min. The pellet was resuspended in 20 ml of binding buffer (10 mM Tris, 500 mM NaCl, and 10 mM imidazole, pH 7.9) containing 8 M urea and lysed by 30 min of incubation at room temperature with inversion. Cell debris was pelleted by centrifugation at 40,000*g* at room temperature for 15 min. The clarified lysate was mixed with 1 ml of 1:1 Ni^{2+}–NTA resin slurry and batch loaded for 30 min at room temperature with inversion. The slurry was applied to a gravimetric flow column, and the resin was washed with 50 ml of binding buffer containing 8 M urea to fully disrupt the $MazE_{Sa}$–$MazF_{Sa}(His)_6$ complex. On-column refolding of $MazF_{Sa}(His)_6$ was achieved with seven washes of 10 ml urea/binding buffer, decreasing the concentration of urea by 1 M with each wash. Wash steps containing greater than 4 M urea were performed at room temperature; all subsequent wash steps were performed at 4 °C. Refolding was followed with 10-ml washes of binding buffer (containing no urea) and binding buffer containing 60 mM imidazole. $MazF_{Sa}(His)_6$ was eluted with 5 ml of binding buffer containing 250 mM imidazole. To remove proteins with high intrinsic affinity for Ni^{2+}–NTA resin [23], the eluate was diluted with 5 ml of binding buffer and applied to a 5-ml bed volume of chitin resin (New England Biolabs) followed by washing with 8 ml of binding buffer. The flow-through and wash fractions were combined and concentrated to approximately 1 ml using an Amicon Ultra-15 3-kDa molecular weight cutoff spin concentrator (Millipore) at 4 °C. After overnight dialysis in PBS (pH 6.5), the purity and concentration of $MazF_{Sa}(His)_6$ were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 4% to 20% TGX Mini-Protean gels (Bio-Rad). Concentration was determined by densitometry of protein bands in the gel and by BCA (bisinchoninic acid) assay (Pierce) using lysozyme (molecular mass = 14.3 kDa) as the standard for both quantification assays.

$(His)_6MazE_{Sa}$ was purified under native conditions, and all steps were performed on ice or at 4 °C. A pellet corresponding to 2 L of culture was thawed for 5 to 10 min and resuspended in cold binding buffer containing protease inhibitors (2 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF). The cells were lysed by 5 min of sonication with a 1-s pulse at 50% amplitude. The lysate was cleared by 15 min of centrifugation at 40,000*g* at 4 °C, and the supernatant was batch loaded with 0.5 ml of 1:1 Ni^{2+}–NTA resin slurry for 30 min at 4 °C with inversion. Protease inhibitors were included in the wash and elution buffers. The resin was washed with 20 ml of binding buffer, followed by 25 ml of binding buffer containing 60 mM imidazole. $(His)_6MazE_{Sa}$ was eluted with 5 ml of binding buffer containing 250 mM imidazole, concentrated to approximately 0.5 ml, and dialyzed against 50 mM sodium phosphate (pH 7.0), 150 mM NaCl, and 1 mM dithiothreitol (DTT). Purity and concentration were assessed using the same method described for $MazF_{Sa}(His)_6$.

HPLC analysis of oligonucleotide cleavage products

A 10-μl solution of 16 μM $MazF_{Sa}(His)_6$ and 32 μM 5′-AAGTCrUrACATCAG-3′ (where "r" denotes RNA base) was incubated overnight at 37 °C in 10 mM Tris, 500 mM imidazole, and 20% glycerol (pH 7.9). The reaction was diluted with 20 μl of 0.1 M triethylammonium acetate (TEAA, pH 7.0), and 10 μl was analyzed by high-performance liquid chromatography (HPLC) using an Alliance HPLC System (e2965 Separations Module, Waters) with detection at 260 nm (2489 UV/Visible Detector, Waters). The full-length oligonucleotide was separated from the