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Detection of Endogenous MazF Enzymatic Activity in *Staphylococcus aureus*

Julia J. Williams¹ and Paul J. Hergenrother^{2,3}

¹Department of Microbiology, University of Illinois at Urbana-Champaign, Roger Adams Laboratory, 600 S. Mathews Ave., Urbana, IL 61801

²Department of Biochemistry, University of Illinois at Urbana-Champaign, Roger Adams Laboratory, 600 S. Mathews Ave., Urbana, IL 61801

³Department of Chemistry, University of Illinois at Urbana-Champaign, Roger Adams Laboratory, 600 S. Mathews Ave., Urbana, IL 61801

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* upon 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. Herein 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.

Keywords

MazF_{Sa}; toxin-antitoxin; ribonuclease; enzyme assay

Toxin-antitoxin (TA) 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 coexpressed 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

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*Corresponding author: Telephone: (217) 333-0363; Fax: (217) 244-8024, hergenro@uiuc.edu.

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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, 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 CFU/mL after 60 min of induction [18]; however, there was only ~27% difference in cell death at the 60 minute 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 PCR) [17], the inducibility of the *mazEF_{Sa}* transcript with various stressors (via Northern blots and 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 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 mRNA or protein level does not necessitate heightened MazF_{Sa} enzymatic activity, as levels of the MazE_{Sa} antitoxin are also raised.

As described herein, using a fluorogenic substrate we have determined the kinetic parameters for MazF_{Sa}. Additionally, 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 herein should facilitate the evaluation of various stressors and assist in the discovery of artificial activators of MazF_{Sa}.

Experimental Procedures

Materials

Primers and oligonucleotides were synthesized by IDT. Ni²⁺-NTA resin was obtained from Qiagen. Pepstatin A, leupeptin, aprotinin, phenylmethanesulfonyl fluoride (PMSF) and lysozyme were purchased from Sigma. Restriction enzymes, BSA, Low-Range ssRNA Ladder, RNase Inhibitor—Human Placenta, and *E. coli* strains DH5⁺ and NiCo21(DE3) [23] were purchased from New England Biolabs (NEB). Subcloning Efficiency DH5 Chemically Competent *E. coli* was purchased from Invitrogen. IPTG and kanamycin were purchased from GoldBio. Lysing Matrix B was obtained from MP Biomedicals. [γ-³²P]UTP was purchased from Perkin-Elmer. Sypro RED protein stain was purchased from VWR.

Cloning

E. coli DH5⁺ and NiCo21(DE3) were used for cloning and protein expression, respectively. The *mazEF_{Sa}* gene cassette was amplified by PCR using primers *mazEF_{Sa}*-NcoI-F

(5 ACACCCATGGATATGTTATCTTTAGTCAAAATAG-3') and *mazEF_{Sa}-XhoI*-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}-NdeI*-F (5 ACACCATATGTTATCTTTATCAAATAGAAG-3') and *mazE_{Sa}-XhoI*-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 were 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+Kan. The culture was grown at 37°C until the OD₆₀₀ reached 0.6–0.8, at which point expression was induced with 1 mM IPTG (final concentration) for 4 hours at 37°C. Expression of (His)₆MazE_{Sa} was performed the same, except IPTG was added when the OD₆₀₀ reached 0.4–0.6. The 2 L cultures were harvested by centrifugation at 4°C (8,000×*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 culture was thawed in a room temperature water bath for 10 minutes. The pellet was resuspended in 20 mL Binding Buffer (10 mM Tris, 500 mM NaCl, 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 minutes. The clarified lysate was mixed with 1 mL 1:1 Ni²⁺-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 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 binding buffer containing 250 mM imidazole. To remove proteins with high intrinsic affinity for Ni²⁺-NTA resin [23], the eluate was diluted with 5 mL binding buffer and applied to a 5 mL bed volume of chitin resin (NEB) followed by washing with 8 mL binding buffer. The flow-through and wash fractions were combined and concentrated to ~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 MazF_{Sa}(His)₆ were assessed by SDS-PAGE using 4–20% TGX Mini-Protean gels (Bio-Rad). Concentration was determined by densitometry of protein bands in the gel, and by BCA 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 culture was thawed for 5–10 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 sonication with 1 s pulse at 50% amplitude. The lysate was cleared by 15 min centrifugation at 40,000×*g* at 4°C and the supernatant was batch loaded with 0.5 mL 1:1 Ni²⁺-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 binding buffer, followed with 25 mL binding buffer containing 60

mM imidazole. (His)₆MazE_{Sa} was eluted with 5 mL binding buffer containing 250 mM imidazole, concentrated to ~0.5 mL and dialyzed against 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, 1 mM DTT. Purity and concentration were assessed using the same method described for MazF_{Sa}(His)₆.

HPLC analysis of oligonucleotide cleavage products

A 10 μ L solution of 16 μ M MazF_{Sa}(His)₆ and 32 μ M 5-AAGTCrUrACATCAG-3 (“r” denotes RNA base) was incubated overnight at 37°C in 10 mM Tris, 500 mM imidazole, 20% glycerol (pH 7.9). The reaction was diluted with 20 μ L of 0.1 M triethylammonium acetate (TEAA) (pH 7.0) and 10 μ L was analyzed by high performance liquid chromatography 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 cleavage products on a YMCbasic S5 column (4.6 $\times$ 150 mm, 5 μ m, Waters) with a linear gradient of 0.1 M TEAA to 0.05 M TEAA/50% acetonitrile over 25 min. Fractions were analyzed by MALDI mass spectrometry.

Fluorometric oligonucleotide cleavage assay

The fluorogenic substrate 5'-6FAM-AGTCrUrACATCAG-BHQ-3 (6-FAM, 6-carboxyfluorescein, BHQ, black hole quencher; “r” denotes RNA base) was diluted in McIlvaine's phosphate-citrate (PC) buffer (71 mM phosphate, 14.5 mM citrate, pH 6.5). Wells of a 384-well sterile black tissue-culture plate (ThermoFisher) were filled with 15 μ L of each dilution (0.025–4 μ M, final concentration). After the substrate equilibrated for 30 min at room temperature, 15 μ L of MazF_{Sa}(His)₆ was added to the wells containing intact substrate. The fluorescence of the wells was measured every 30 s for 45 min using a Criterion Analyst AD (Molecular Devices) with 485 $\pm$ 15 nm excitation and 530 $\pm$ 15 nm emission filters and a 505 nm cutoff dichroic mirror. The fluorophore was excited with a 1000 W continuous lamp with 10 reads per well. The Z-height was set to 1 nm.

A calibration curve of the independently-synthesized substrate halves corresponding to the cleavage products, 5'-6-FAM-AGTCG and ACATCAG-BHQ-3 [24], was constructed to quantify the amount of cleavage product formed based on the RFU value. Wells containing 0.0625–2 μ M of the cleavage fragments were prepared alongside the intact oligonucleotide following the same protocol, except PC buffer was added instead of MazF_{Sa}(His)₆. The calibration curve was constructed by plotting the average RFU measured over the 45 min experiment against amount of oligonucleotide in the well.

In vitro transcription of an RNA substrate

Quickchange site-directed mutagenesis was performed to modify the MazF_{Sa} cleavage sites in the pET-200-*mazEF* recombinant plasmid, referred to as pKm6EF [24]. MazF_{Sa} optimal cleavage site 1 was removed by changing TACAT to GGCAT using the following primers Site1-QC-F (5'-GTTTAACTTTAAGAAGGAGATAGGCATATGCGGGTTCTCATCATC and Site1-QC-R GATGATGAGAACCCCGCATATGCCTATCTCCTTCTTAAAGTTAAAC-3), suboptimal cleavage site 2 was removed by changing TACGT to CACAT using Site2-QC-F (5'-GGATCCCGGCCACGTTAATGCAGGCGCTCAATC-3) and Site2-QC-R (5'-GATTGAGCGCCTGCATTAACGTGCGGGGATCC-3), and the suboptimal cleavage site 3 was optimized by changing from TACGT to TACAT using Site3-QC-F (5'-GGTAATGGTAAGCCGATACATACCCGATATGG and Site3-QC-R (5'-CCATATCGGGTATGATCGGCTTACCATTACC-3). The quickchange PCR was performed following the guidelines in the QuikChange manual (Stratagene) with annealing temperatures of 58°C for the Site 1 and Site 2 PCRs and 61°C for the Site 3 PCR. The PCR product was purified and digested with *DpnI* and transformed into Sub-cloning Efficiency

CaCl₂-treated *E. coli* following the manufacturer's instructions except the cells were heat shocked for 30 s. The transformation was plated on LB containing 50 µg/mL kanamycin. Each mutation was confirmed by sequencing of both strands. The plasmid containing the optimized substrate is referred to as pET-200-1 2 3opt.

To prepare the RNA substrate, the pET-200-1 2 3opt plasmid was digested with *Hind*III for 3 hours at 37°C. The digested plasmid was purified using the QiaPrep Spin Column (Qiagen) and eluted with 30 µL DEPC-treated water. The digested plasmid (0.5–1 µg) served as the template for *in vitro* transcription using the T7 High Yield RNA Synthesis Kit (NEB) according to the manufacturer's instructions. Each transcription reaction was divided into two equal portions and purified according to the RNeasy Mini handbook (Qiagen) RNA Cleanup protocol using reagents from the Total RNA Kit I (Omega Bio-Tek). RNA was eluted twice with 30 µL 0.1 mM EDTA prepared in DEPC-treated water, and all elution fractions from both columns were pooled. The RNA was quantified by A₂₆₀ and checked for integrity by gel electrophoresis on a 5% acrylamide 8 M urea 1X TBE gel (denaturing gel) run in 1X TBE (pH 8.3) and post-stained for 15 min with 0.05 µg/mL ethidium bromide.

Synthesis of radiolabeled RNA transcript

Synthesis and purification of the radiolabeled RNA substrate followed the same protocol as described for the unlabeled transcript, except that 3.87 µCi of [γ-³²P]UTP was added in addition to the standard amounts of unlabeled NTPs.

Gel-based RNA cleavage assay

To assess the cleavage of the 3-site and 1-site substrates, 50 nM MazF_{Sa}(His)₆ was incubated with 50 nM RNA in Buffer A (71 mM sodium phosphate, 14.5 mM citrate, 137 mM NaCl, 2 mM KCl, pH 6.5) at room temperature. To stop the reaction, a 10 µL aliquot was added to 10 µL SDS-formamide loading dye (87% formamide, 2.75% SDS, 16 mM EDTA, 0.025% bromophenol blue). Samples were heated to 95°C for at least 5 min prior to analysis on a 3.5% acrylamide denaturing gel and visualized with ethidium bromide.

For the time course assay, the same conditions were followed, except 1 nM MazF_{Sa}(His)₆ and 25 nM RNA were prepared in assay buffer containing 0.01% BSA and the products were separated on 5% acrylamide denaturing gels

Activity against the radiolabeled substrate was assessed following the same conditions, except 1, 5 or 10 nM MazF_{Sa}(His)₆ was mixed with 100 nM radiolabeled RNA substrate in Buffer A containing 0.05% BSA. The products were separated on 5% acrylamide denaturing gels, which were exposed to an Imaging Screen-K (BioRad) for 10 min prior to visualization.

Detection of MazF_{Sa} activity in *S. aureus* cell lysate

A single colony of MRSA strain NRS26 [17] grown on tryptic-soy agar was inoculated into 10 mL tryptic-soy broth (TSB) and grown overnight at 37°C with aeration. A fresh 22 mL culture was seeded with a 100-fold dilution of the overnight culture and grown to an OD₆₀₀ of ~1.3, at which point 10 mL portions were harvested by centrifugation at 4°C, flash frozen in liquid nitrogen and stored at –80°C. The pellet was resuspended in 0.3 mL assay buffer and lysed by vortexing for 4 min with 0.15 g Lysing Matrix B silica beads (MP Biomedical). The lysate was cleared by 30 s full speed centrifugation at room temperature and mixed with 1X RNase inhibitor. After 7, 15, 35, and 65 min post-lysis, the radiolabeled RNA substrate was added to 100 nM (final concentration) and the samples were heated at 95°C for 1 min. After 10 min reaction at room temperature with RNA, 10 µL aliquots were quenched with SDS-formamide loading dye, electrophoresed, and imaged as described above. To inhibit

endogenous MazF_{Sa} activity in the most active lysate, 200 nM purified (His)₆MazE_{Sa} was added to lysate 65 min post-lysis and incubated for 10 min prior to the addition of RNA for a 10 min reaction. For negative and positive controls, 100 nM radiolabeled RNA was incubated without or with 10 nM MazF_{Sa}(His)₆ in assay buffer with 0.01% BSA at 95°C for 1 min followed by room temperature for 1 min.

Results

Design of a fluorogenic RNA-DNA chimeric substrate for kinetic assessment of MazF_{Sa}

MazF_{Sa} optimally cleaves at the sequence U ACAU in single-stranded RNA and was also shown to cleave the synthetic RNA substrate 5'-AAGUCUACAUCAG-3' [19]. Based on this substrate, a chimeric DNA-RNA substrate consisting of DNA bases at all positions except for the U and the A surrounding the cleavage site was designed (Fig. 1A). The DNA bases afford enhanced stability and protection from general ribonucleases. The synthetic chimeric substrate 5'-AAGTCrUrACATCAG-3' was subjected to cleavage by MazF_{Sa}(His)₆ and analyzed by HPLC. The HPLC trace in Fig. 1B shows the cleavage of approximately 83% of the input chimeric substrate by MazF_{Sa}. MALDI mass spectrometry of fractions confirmed the expected mass of the intact substrate and cleavage products (Fig. 1B).

A continuous fluorometric assay developed for MazF_{Ec} [24] was modified for kinetic evaluation of MazF_{Sa}. The chimeric substrate from Fig. 1A was synthesized with a 6-FAM fluorescein on the 5' end and a quencher (Black Hole Quencher, BHQ) on the 3' end. Cleavage of this fluorogenic substrate by MazF_{Sa} resulted in increased fluorescence over time, allowing progress curves to be generated at various substrate concentrations (Fig. 1C). Over the course of 30 min in the kinetic assays, MazF_{Sa} cleaved approximately 2–14% of the fluorogenic substrate. The continuous nature of the fluorometric assay enabled the characterization of MazF_{Sa}(His)₆ enzyme kinetics ($V_{\max} = 0.12$ pmol/min, $K_M = 0.56$ μ M, $k_{\text{cat}}/K_M = 0.0079$ M⁻¹s⁻¹) (Fig. 1D). As has been noted previously, the absolute rate of processing of oligonucleotide substrates by recombinantly expressed and purified toxins is low and thus requires higher concentrations of enzyme than normally used in standard kinetic assays [24, 25]. Therefore, although the Michaelis-Menten equation was fit to the data to determine an apparent K_M and $V_{\max}$, the enzymatic parameters observed for MazF_{Sa}(His)₆ do not follow standard Michaelis-Menten kinetics.

Construction of an RNA substrate for gel-based endoribonuclease activity

Although the fluorogenic substrate is useful for kinetic assessment, MazF_{Sa} cleaves mRNA transcripts as its endogenous substrate. Thus, a method for the analysis of MazF_{Sa}(His)₆ endoribonuclease activity against mRNA was developed. An RNA substrate was generated by *in vitro* transcription as described in the Materials and Methods. The resultant transcript from this template contained three MazF_{Sa} cleavage sites, including one preferentially cleaved site (site 1, UACAU), a sub-optimal site at which cleavage was not detected (site 2, UACGU) and a second sub-optimal site that was cleaved (site 3, UACAU) (Fig. 2, Left). The primary cleavage site was approximately 66 bases from the 5' end of the full length transcript, complicating analysis of the cleavage products by gel electrophoresis (Fig. 2, Left). To improve the resolution of the cleavage products on gels, the original template was engineered by site-directed mutagenesis to remove sites 1 and 2 and optimize site 3, such that the new substrate contained only one cleavage site of the optimal sequence UACAU (Fig. 2, Right). Cleavage of this substrate by MazF_{Sa} resulted in easily distinguishable products (Fig. 2, Right).

The sensitivity of this substrate for MazF_{Sa}(His)₆ activity was tested with lower and perhaps more physiologically relevant concentrations of MazF_{Sa}. A time course experiment with 1

nM MazF_{Sa}(His)₆ and 25 nM RNA resulted in rapid processing, with approximately 50% cleavage of the substrate in 5 minutes (Fig. 3). To our knowledge, this is the most sensitive assay for *in vitro* assessment of the ribonuclease activity of purified MazF_{Sa}. Furthermore, MazF_{Sa}(His)₆ activity was inhibited by (His)₆MazE_{Sa}, (+E lane, Fig. 3) demonstrating the specificity of the cleavage of this substrate.

Radiometric assay for MazF_{Sa} activity

As a prelude to experiments with endogenous MazF_{Sa} from *S. aureus* lysate, the activity of MazF_{Sa}(His)₆ was assessed with a ³²P-labeled version of the single site substrate (from Fig. 2, Right) in a time course experiment. As shown in Fig. 4, the sensitivity of the assay allows for clear differentiation in the activity of increasing concentrations of MazF_{Sa}. In this experiment, minimal cleavage was detected by 1 nM MazF_{Sa}(His)₆ after 11 min incubation, moderate processing was observed by 5 nM MazF_{Sa}(His)₆, and there was significant processing by 10 nM MazF_{Sa}(His)₆ (Fig. 4).

Detection of endogenous MazF_{Sa} activity in *S. aureus* cell lysate

The highly sensitive and specific cleavage of the radiolabeled substrate allowed for detection of endogenous MazF_{Sa} activity in *S. aureus* cell lysate. Lysate was prepared from the *S. aureus* strain NRS26, in which the *mazEF_{Sa}* genes and transcript were previously detected [17]. The radiolabeled substrate was added to the lysate 7, 15, 35 and 65 min post-lysis. As shown in Fig. 5, cleavage of the RNA substrate increased with longer post-lysis incubation times, consistent with MazE_{Sa} being susceptible to proteolysis by endogenous proteases in the lysate, resulting in the release of active MazF_{Sa}. Addition of recombinant (His)₆MazE_{Sa} to lysate 65 min post-lysis inhibited MazF_{Sa} activity, demonstrating the specificity of the RNA cleavage by endogenous MazF_{Sa} (far right lane in Fig. 5). This is the first detection of the enzymatic activity of endogenous MazF_{Sa}.

Discussion

Toxin-antitoxin systems are an ingenious method to maintain a plasmid in a bacterial host. Although their role on the bacterial chromosome is unclear, at the biochemical level Type II TA systems operate via the same mechanism whether plasmid or chromosomal; that is, a stable toxin protein is inhibited by a proteolytically labile (and co-transcribed/translated) antitoxin protein. Important work has shown that the genes for TA systems are present in a variety of pathogenic bacteria [17, 26–30]. Fewer publications have reported on the detection of TA transcripts in such bacteria [17, 26, 27] and fewer still on the detection of the actual TA proteins [18, 21]. While the presence of the DNA, mRNA, and proteins is clearly informative, it does not tell the whole story, especially for TA systems. Specifically, transcription and translation does not mean that the protein toxin is enzymatically active, given the co-transcription and co-translation of the proteic antitoxin.

Here we describe an assay that allows, for the first time, for the detection of MazF_{Sa} enzymatic activity from its endogenous source. The principles applied for the design of this substrate and this assay should be readily transferable to the study of other toxins from TA pairs, especially those that are ribonucleases. There are many questions about the roles of TA systems in basic bacteriology [9, 10, 31–34], their function in response to stress [35, 36] and their potential as novel antibacterial targets [11–14, 16]. The tools described herein for the direct detection of endogenous toxin activity should significantly facilitate the answering of those questions.

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Abbreviations

TA	Toxin-Antitoxin
PSK	Post-segregational killing

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Fig. 1.

Cleavage of an oligomeric substrate by MazF_{Sa}(His)₆. (A) Sequence of the non-fluorogenic substrate, “r” denotes RNA; MazF_{Sa} recognition sequence is underlined. MazF_{Sa} cleaves this substrate to the products shown. (B) Products from a 16 hour incubation of the oligonucleotide substrate from Fig. 1A (32 μM) in the absence (black) or presence (blue) of MazF_{Sa}(His)₆ (16 μM) were separated by HPLC and analyzed by MALDI mass spectrometry. Approximately 83% of the substrate was cleaved by MazF_{Sa} over the course of the incubation. The peak at the longest retention time corresponds to intact oligonucleotide (expected molecular weight [MW] = 3960.6). The peaks at shorter retention times correspond to 5 and 3 fragments generated from MazF_{Sa}(His)₆ cleavage (expected MWs = 1854.3 and 2105.4), respectively). (C) Representative set of progress curves showing cleavage of the fluorogenic substrate derivative (5'-6-FAM-AAGTCrUrACATCAG-BHQ-3) by MazF_{Sa}(His)₆. Enzyme (0.8 μM) was added to the indicated concentration of substrate and the fluorescence was monitored over time in 384-well plates. Approximately 2–14% of the substrate was cleaved over 30 min time course. (D) Slopes from the progress curves were plotted against oligonucleotide substrate concentration. The Michaelis-Menten equation was fit to the data and apparent K_M and V_{max} values were determined using GraphPad. Error bars represent standard deviation, n=3.

Fig. 2.

Cleavage of an mRNA transcript substrate by MazF_{Sa}(His)₆. A transcript was produced as described in Materials and Methods. Incubation of the full length transcript (1070 nucleotides [nts]) with MazF_{Sa}(His)₆ resulted in the cleavage products shown in the gel and depicted in the schematic below the gel. Left: Cleavage of the 3-site substrate (50 nM) produced 4 cleavage products within 240 s incubation with MazF_{Sa}(His)₆ (50 nM). The products from MazF_{Sa}(His)₆ cleavage at site 1 (UACAU) and site 3 (UACGU) are depicted below the gel. MazF_{Sa}(His)₆ did not cleave at site 2 (UACGU) within 240 s. Right: Cleavage of the 1-site substrate under the same conditions results in two well-separated products. As shown below the gel, in the 1-site substrate the original cleavage sites 1 and 2 were removed and site 3 was changed to the optimal MazF_{Sa} recognition sequence, UACAU. Products were separated by denaturing gel electrophoresis and visualized by ethidium bromide staining.

Fig. 3.

Time course of MazF_{Sa} endoribonuclease activity on an mRNA transcript. MazF_{Sa}(His)₆ (1 nM) was incubated at room temperature with the single site substrate (from Fig. 2, Right; 25 nM). Activity is detected within 10 s and >50% processing is achieved by 5 min. To inhibit MazF_{Sa} activity, (His)₆MazE_{Sa} was incubated with MazF_{Sa}(His)₆ for 15 min prior to reaction with the single site mRNA substrate for 60 seconds (+E lane). Products were separated by denaturing gel electrophoresis and visualized by ethidium bromide staining.

Fig. 4.

Radiometric assay for MazF_{Sa} activity. MazF_{Sa}(His)₆ (1, 5, or 10 nM) was incubated at room temperature with ³²P-labeled single-site substrate (100 nM). The resultant products were separated by denaturing gel electrophoresis and visualized by phosphorimaging.

Fig. 5.

Detection of endogenous MazF_{Sa} activity in *S. aureus* cell lysate. Radiolabeled RNA transcript (100 nM) was added to *S. aureus* lysate 7, 15, 35 or 65 min post-lysis and incubated at room temperature for 10 min. CONTROLS: Far Left -- RNA only (R) and RNA processed *in vitro* by 10 nM MazF_{Sa}(His)₆ (+F); Far Right -- *S. aureus* lysate 65 min post-lysis was incubated with 200 nM (His)₆MazE_{Sa} prior to reaction with radiolabeled RNA for 10 min (65+E). Products were separated by denaturing gel electrophoresis and visualized by phosphorimaging.