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Characterization of MazF\textsubscript{Sa}, an Endoribonuclease from \textit{Staphylococcus aureus}\textsuperscript{v}

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The \textit{mazEF} homologs of \textit{Staphylococcus aureus}, designated \textit{mazEF}\textsubscript{\textit{sa}}, have been shown to cotranscribe with the \textit{sigB} operon under stress conditions. In this study, we showed that MazEF\textsubscript{\textit{sa}} as with their \textit{Escherichia coli} counterparts, compose a toxin-antitoxin module wherein MazF\textsubscript{\textit{sa}} leads to rapid cell growth arrest and loss in viable CFU upon overexpression. MazF\textsubscript{\textit{sa}} is a novel sequence-specific endoribonuclease which cleaves mRNA to inhibit protein synthesis. Using \textit{ctpA} mRNA as the model substrate both in vitro and in vivo, we demonstrated that MazF\textsubscript{\textit{sa}} cleaves single-strand RNA preferentially at the 5' side of the first U or 3' side of the second U residue within the consensus sequences VUUV (where V and V' are A, C, or G and may or may not be identical). Binding studies confirmed that the antitoxin MazE\textsubscript{\textit{sa}} binds MazF\textsubscript{\textit{sa}} to form a complex to inhibit the endoribonuclease activity of MazF\textsubscript{\textit{sa}}. Contrary to the system in \textit{E. coli}, exposure to selected antibiotics augmented \textit{mazEF}\textsubscript{\textit{sa}} transcription, akin to what one would anticipate from the environmental stress response of the \textit{sigB} system. These data indicate that the \textit{mazEF} system of \textit{S. aureus} differs from the gram-negative counterparts with respect to mRNA cleavage specificity and antibiotic stresses.

Many bacteria have chromosomally encoded toxin-antitoxin (TA) loci, in which the toxin and antitoxin genes exist in an operon and are coexpressed together to form a TA complex. The toxin is stable, whereas the antitoxin is a labile protein operon and are coexpressed together to form a TA complex. (TA) loci, in which the toxin and antitoxin genes exist in an extensively studied (7, 9, 12). Structural studies have disclosed that the MazE-MazF complex in \textit{E. coli} consists of two MazF dimers and one MazE dimer in a hexameric MazF\textsubscript{2}-MazE\textsubscript{2} configuration (17). In contrast, the RelBE complex from \textit{Pyrococcus horikochi} is a (RelE-RelB)\textsubscript{2} tetramer (32).

Inhibition of protein synthesis by MazF in \textit{E. coli} has been found to be attributable to cleavage of cellular mRNA. More specifically, MazF in \textit{E. coli} is a sequence-specific endoribonuclease, cleaving mRNA at ACA sites independently of ribosomes both in vitro and in vivo (6, 34). The cleavage occurs at the 5' end of ACA sequence to yield a 2',3'-cyclic phosphate as part of the end product. The 2'-OH group of the nucleotide preceding the ACA sequence is essential for MazF cleavage (37). In contrast, the RelE toxin of \textit{E. coli} was found to cleave mRNA positioned at the ribosomal A-site both in vitro and in vivo (26). Cleavage occurs between the second and third bases of the A-site codon (UAH, where H is usually G or A), with the cleavage efficiency depending on the specific codon on the ribosomal A-site. For instance, UAG and UAA are cleaved more efficiently than the UGA stop codon (26). The toxin systems from other prokaryotes also appeared to represent sequence-specific endoribonucleases. The PemK toxin from plasmid R100 in \textit{E. coli} cleaves mRNA at UAH (where H is A, C, or U) (36), and ChpBK cleaves at ACY (where Y is A, G, or U) in a single-stranded RNA (38), while the \textit{Bacillus subtilis} MazF homolog EndoA cleaves mRNA at a UAC sequence (27). Recently, two MazF homologs from \textit{Mycobacterium tuberculosis} were also found to be endoribonucleases. One of the MazF homologs from \textit{M. tuberculosis} cleaves mRNA at UAC triplets, while the other homolog cleaves U-rich regions within mRNA (39).

In examining the \textit{sigB} operon of \textit{Staphylococcus aureus}, Kullik et al. (18) noted that an open reading frame (ORF) immediately upstream of the \textit{sigB} operon may encode a \textit{mazF} homolog (designated the \textit{pemK} homolog). Senn et al. (30) subsequently demonstrated that the \textit{sigB} operon in \textit{S. aureus} strain COL comprises two additional ORFs (SA2059 and SA2058) in addition to \textit{rsbU}, \textit{rsbV}, and \textit{rsbW}, and \textit{sigB}. They also observed, as did Gertz et al. (13), that SA2058 and, to a much lesser extent, SA2059 share some degree of homology with MazF and MazE of \textit{E. coli}, respectively. SA2059 and SA2058 are cotranscribed with the \textit{sigB} operon under stress conditions, such as heat and high-salt conditions (30). For brevity and consistency, we propose to name SA2059 and SA2058 (designated as SA1873 and SAS067 in N315) in COL as MazE\textsubscript{\textit{sa}} and MazF\textsubscript{\textit{sa}}, respectively, in \textit{S. aureus}. Although it has been hinted that the \textit{S. aureus} MazF\textsubscript{\textit{sa}} may act as a TA module (30), there have been no experimental data supporting this hypothesis. This confusion has been generated in part as a consequence of a general lack of protein sequence similarity between MazE\textsubscript{\textit{sa}} and its \textit{E. coli} counterpart.

In this study, we provide definitive evidence that MazF\textsubscript{\textit{sa}} is a TA module in \textit{S. aureus}, with MazE\textsubscript{\textit{sa}} as the toxin. Our data demonstrate that MazF\textsubscript{\textit{sa}} is a sequence-specific endoribonuclease which cleaves \textit{ctpA} mRNA at a consensus U-rich sequence of VUUV (where V and V' are A, C, or G and may ...
or may not be identical) both in vivo and in vitro. MazF<sub>Sa</sub> showed high cellular toxicity in both E. coli and S. aureus upon induction and inhibited protein synthesis in a cell-free system. Collectively, our results suggest that the activated MazEF<sub>sa</sub> TA module cleaves mRNA cleavage at a specific site under stressful conditions to affect translation. This finding raises the possibility that inhibition of MazF<sub>sa</sub> may represent a novel approach to antibacterial therapy for S. aureus.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We used E. coli strains DH5α and BL21(DE3)pLysS and S. aureus strains Newman and 178RI (8) for these studies. S. aureus 178RI carries an isopropyl-β-D-thiogalactoside (IPTG)-inducible T7 polymerase gene integrated into the geh locus in the chromosome of RN4220. For transduction, phage geh and BamHI sites of cloning vectors pCDF1 and pET14b (Novagen) in and BglII-EcoRI-digested pG164 (8), respectively, to generate pBAD22-mazE<sub>sa</sub> make pCDF1-MazE(His)<sub>6</sub> and pET14b-MazF(His)<sub>6</sub> with the His<sub>6</sub> tag at the N

Construction of plasmids. The mazE<sub>sa</sub> and mazF<sub>sa</sub> (GenBank accession number Y16431) and mazE<sub>sa</sub> (GenBank accession number Y70645) genes were amplified by PCR using S. aureus Newman genomic DNA as a template and cloned into the NcoI and BamHI sites of cloning vectors pCDF1 and pET14b (Novagen) in E. coli to make pCDF1-MazE(His)<sub>6</sub>, and pET14b-MazF(His)<sub>6</sub> with the His<sub>6</sub> tag, at the N terminus, respectively. The mazE<sub>sa</sub> gene without the His tag was amplified by PCR and cloned into the NdeI/Xhol sites of pETDuet1 (Novagen). An NcoI-BamHII-digested DNA fragment from pET14b-MazF(His)<sub>6</sub> was then inserted to make pETDuet1-MazF(His)<sub>6</sub>, with the His tag only at the MazF<sub>sa</sub> N terminus. An NcoI-BamHII- and a BglII-EcoRI-digested DNA fragment from pET14b-MazF(His)<sub>6</sub> was further cloned into NcoI- and BamHI-digested pBAD22 (14) and BglII-EcoRI-digested pG164 (8), respectively, to generate pBAD22-MazF<sub>sa</sub> and pG164-MazF<sub>sa</sub>. The ctpA gene (encoding a carboxy-termini

Northern blot hybridization. Total RNA from S. aureus was prepared by using a TRIzol isolation kit (Invitrogen, CA) and a reciprocating shaker (4). For detection of specific transcripts, gel-purified DNA probes were radio labeled with [α-<sup>32</sup>P]dCTP by use of a random-primed DNA labeling kit (Roche Diagnostics Corporation) and hybridized under the above-described conditions at 4°C. The blots were subsequently washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) twice at room temperature and 1× SSC, 0.1% SDS twice at 65°C and autoradiographed as previously described (20).

Bacterial viability assay. Bacteria were stained with the membrane-permeable SYTO9 and the membrane-impermeable propidium iodide using the Live/Dead Blue Light bacterial viability kit (Molecular Probes, Eugene, OR) and quantitated with fluorescence microplate readers according to the manufacturer’s protocol. Bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red.

RESULTS

MazF<sub>sa</sub> is a TA module in S. aureus. A BLAST search of SA2058, encoding a 120-residue protein in the COL genome, identified this gene to have 20% identity and 40% similarity to the E. coli MazF protein. The upstream gene SA2059, which is cotranscribed with SA2058, encodes a 56-residue protein with only 12% identity and 21% similarity to the E. coli MazE protein. We have named SA2058 and SA2059 MazF<sub>sa</sub> and MazE<sub>sa</sub> for brevity and clarity, which were referred to by Mittenhuber (21) as Orf136-s.a and Orf6-s.a (Fig. 1A).

To examine if MazF<sub>sa</sub> and MazE<sub>sa</sub> function as a TA module,
we determined whether the MazF<sub>Sa</sub> protein, when expressed independently, is toxic to bacterial cells. For this purpose, the mazF<sub>Sa</sub> gene was cloned into the vector pBAD with an arabinose-inducible promoter and the shuttle vector pG164 with an IPTG-inducible promoter to generate pBAD-MazF(His)<sub>Sa</sub> and pG164-MazF(His)<sub>Sa</sub>, respectively, as described in Materials and Methods. The growth of <i>S. aureus</i> ACL6094 carrying the plasmid pBAD-MazF(His)<sub>Sa</sub> was mixed with MazF(His)<sub>Sa</sub>, and MazE(His)<sub>Sa</sub> proteins were purified as described in Materials and Methods. The proteins were analyzed by 10% SDS-PAGE. (i) lane 1, protein molecular mass markers; lane 2, MazE(His)<sub>Sa</sub>; lane 3, MazF(His)<sub>Sa</sub>; (ii) lane 4, purified MazE-MazF(His)<sub>Sa</sub> complex. The upper band is MazF(His)<sub>Sa</sub> and the lower band is the copurified MazE<sub>Sa</sub>, while the amount of free MazE(His)<sub>Sa</sub> increased with increasing concentrations of MazF(His)<sub>Sa</sub>. No obvious mobility was observed for MazF(His)<sub>Sa</sub> alone (Fig. 1E, lane 2), presumably due to its basic pI (9.5), which approaches the pH (9.4) of the running buffer used in native PAGE. Nevertheless, the MazEF<sub>Sa</sub> complex, appearing as a higher-molecular-weight species than that of MazE(His)<sub>Sa</sub> alone, was observed at the top of the gel (Fig. 1E, lanes 3 to 5). The quantity of the MazEF<sub>Sa</sub> complex rose with increasing concentrations of MazF(His)<sub>Sa</sub>, while the amount of free MazE(His)<sub>Sa</sub> at the bottom of the gel continued to diminish (Fig. 1E). These results indicated that MazEF<sub>Sa</sub> complex is toxic to both <i>E. coli</i> and <i>S. aureus</i> and that this toxicity can be reversed by coexpression of MazE<sub>Sa</sub> with MazF<sub>Sa</sub>.

To further characterize the MazEF<sub>Sa</sub> TA module, we expressed and purified MazF(His)<sub>Sa</sub> and MazE(His)<sub>Sa</sub> (both N-terminally tagged) (Fig. 1D) in <i>E. coli</i> as described in Materials and Methods. MazE(His)<sub>Sa</sub> and MazF(His)<sub>Sa</sub> were mixed together in a dose-dependent manner and subjected to native PAGE analysis. Despite the noticed migration of MazE(His)<sub>Sa</sub> in control, no obvious mobility was observed for MazF(His)<sub>Sa</sub> alone (Fig. 1E, lane 2), presumably due to its basic pl (9.5), which approaches the pH (9.4) of the running buffer used in native PAGE. Nevertheless, the MazEF<sub>Sa</sub> complex, appearing as a higher-molecular-weight species than that of MazE(His)<sub>Sa</sub> alone, was observed at the top of the gel (Fig. 1E, lanes 3 to 5). The quantity of the MazEF<sub>Sa</sub> complex rose with increasing concentrations of MazF(His)<sub>Sa</sub>, while the amount of free MazE(His)<sub>Sa</sub> at the bottom of the gel continued to diminish (Fig. 1E). These results indicated that MazEF<sub>Sa</sub> complex is toxic to both <i>E. coli</i> and <i>S. aureus</i> as a paired TA module, interact in vitro and possibly in vivo.

**MazF<sub>Sa</sub> inhibits protein synthesis in a cell-free system.** We then examined the effect of the purified MazF<sub>Sa</sub> on protein syn-

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**FIG. 1.** Inhibition of cell growth by <i>S. aureus</i> MazF<sub>Sa</sub>. (A) Schematic representation of genetic organization of the <i>S. aureus</i> mazEF<sub>Sa</sub> and sigB operons. (B) <i>S. aureus</i> ACL6094 cells were transformed with pG164-MazF(His)<sub>Sa</sub>. The cells were then streaked on a 03GL agar plate with or without induction with IPTG (1 mM) followed by incubation at 37°C. (C) Reduction of CFU with <i>S. aureus</i> ACL6094 harboring pG164-MazF(His)<sub>Sa</sub> under induction. The cultures were induced at an optical density at 650 nm of 0.4 with IPTG (1 mM). To determine the CFU, samples were withdrawn under induction with IPTG (1 mM) followed by incubation overnight at 37°C. (D) Characterization of MazF<sub>Sa</sub>. The MazE(His)<sub>Sa</sub> and MazF(His)<sub>Sa</sub> proteins were purified as described in Materials and Methods. The proteins were analyzed by 16% SDS-PAGE. (i) lane 1, protein molecular mass markers; lane 2, MazE(His)<sub>Sa</sub>; lane 3, MazF(His)<sub>Sa</sub>; (ii) lane 4, purified MazE-MazF(His)<sub>Sa</sub> complex. The upper band is MazF(His)<sub>Sa</sub> and the lower band is the copurified MazE<sub>Sa</sub>, while the amount of free MazE(His)<sub>Sa</sub> increased with increasing concentrations of MazF(His)<sub>Sa</sub>. No obvious mobility was observed for MazF(His)<sub>Sa</sub> alone (Fig. 1E, lane 2), presumably due to its basic pI (9.5), which approaches the pH (9.4) of the running buffer used in native PAGE. Nevertheless, the MazEF<sub>Sa</sub> complex, appearing as a higher-molecular-weight species than that of MazE(His)<sub>Sa</sub> alone, was observed at the top of the gel (Fig. 1E, lanes 3 to 5). The quantity of the MazEF<sub>Sa</sub> complex rose with increasing concentrations of MazF(His)<sub>Sa</sub>, while the amount of free MazE(His)<sub>Sa</sub> at the bottom of the gel continued to diminish (Fig. 1E). These results indicated that MazEF<sub>Sa</sub> complex is toxic to both <i>E. coli</i> and <i>S. aureus</i> as a paired TA module, interact in vitro and possibly in vivo.
In vitro cleavage of ctpA mRNA by MazFSo. To determine whether MazFSo has endoribonuclease activity, we prepared the ctpA mRNA using an in vitro transcription system as described in Materials and Methods. The ctpA mRNA was then incubated with MazFSo in a dose- and time-dependent manner. As shown in Fig. 3A, the ctpA mRNA was cleaved into small fragments with 15 pmol of MazFSo in a time-dependent manner, while the addition of MazEso inhibits the digestion of ctpA mRNA by MazFSo in a dose-dependent fashion (Fig. 3B). These results demonstrate that MazFSo is an endoribonuclease that cleaves mRNA to inhibit protein synthesis and that MazEso functions as an antitoxin to counteract the endoribonuclease activity of MazFSo.

The ctpA mRNA was noted to be cleaved into distinct, but not smearing, bands by MazFSo (Fig. 3A), indicating that MazFSo may be a sequence-specific endoribonuclease. To further map the cleavage site, we employed MazFSo to partially digest the ctpA mRNA and then subjected the digest to primer extension, using four different DNA primers, pEa to -d, covering the experimental ctpA mRNA as described in Materials and Methods. To determine the cleavage sites, each primer extension product was analyzed on a 6% sequencing gel with a DNA sequencing ladder prepared with the same primer (Fig. 4). The cleavage sites in the ctpA mRNA as determined by primer extension studies are shown in Table 1. Cleavages occurred preferentially in a U-rich region with a consensus sequence of VUUV in ctpA mRNA. The UU dinucleotides were found to be conserved among all cleavage sites. However, the
primary cleavages occur at either 5' side of the first U or 3' side of the second U residue in the VUUV' sequence, with most cleavages taking place 3' of the second U residue (Fig. 4; Table 1). However, not all of the VUUV' sequences in the ctpA mRNA were cleaved by MazFsa.

Cleavage specificity of MazFsa. To further define the specificity of cleavage sites, an 18-base synthetic RNA (5'-UUGGCAAUUCAUAUCAAU-3') with the AUUC sequence in the center was used for digestion with MazFsa. A clear cleavage was shown between the A and U of the sequence (Fig. 5).

**TABLE 1.** ctpA mRNA sequences around cleavage sites

<table>
<thead>
<tr>
<th>Primer</th>
<th>mRNA sequence around cleavage site(s)</th>
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<tbody>
<tr>
<td>pEa</td>
<td>103AAACGUUGGCAAUAUCAAUAUUA 130</td>
</tr>
<tr>
<td></td>
<td>+158CAUCACAGUUGCU_GCAAUAAUAAUAA 181</td>
</tr>
<tr>
<td>pEb</td>
<td>221CAAGCAAJACUJA AAUAAAUAUG 238</td>
</tr>
<tr>
<td></td>
<td>+250AAAUUUJJA AAUAUGUAUAUUAA JACAAA 277</td>
</tr>
<tr>
<td>pEd</td>
<td>477CCAGCAGAAGUCUGGCAJUUC GUCC 502</td>
</tr>
</tbody>
</table>

* The cleavage sites in the ctpA mRNA were determined by in vitro primer extension with primers pEa, pEb, and pEd (Fig. 4). The sequences around the MazFsa cleavage sites are indicated by bold characters, and the cleavages are indicated by arrows.

**FIG. 5.** Cleavage of synthetic RNA by MazFsa. All RNA substrates labeled at the 5' end with [γ-32P]ATP were digested with MazFsa and subjected to analysis in a 20% sequencing gel run with an RNA ladder by alkaline hydrolysis as described in Materials and Methods. (A) Cleavage of a synthetic 18-base RNA, AUUC. Lane 1, RNA ladder generated by alkaline hydrolysis; lane 2, RNA substrate without the addition of MazFsa; lane 3, RNA substrate digested by 15 pmol of MazFsa. The corresponding RNA sequence is shown to the right. The cleavage product and site are indicated by arrows. (B) Cleavage specificity of MazFsa with synthetic 18-base RNA substrates. Seven RNA substrates with the center AUUC sequence were changed to AGUC, AGUC, GUUG, UUUC, AUUG, GUUC, and AUUU sequences and named correspondingly. Lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17 contained no MazFsa; lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18 are with 15 pmol of MazFsa added. The background in the absence of MazFsa was attributed to impurities or incomplete synthesis of the full-length RNA substrates. Nevertheless, cleavage can be seen with MazFsa, as indicated by the arrows. (C) Predicted secondary structure formed by RB-1 (5'-UGCAAUUCAAUAUGUGAUG-3') using the RNA secondary prediction website (http://www.genebee.msu.su/services/rna2_reduced.html). (D) Cleavage of highly purified synthetic RNA substrates with different secondary structures. An 18-base sense RNA, AUUC, and AUUC antisense RNA RB-3, were digested separately by MazFsa. The 19-base RNAs RB-1 and RB-2 (an RB-1 variant) were also digested with MazFsa. Lanes 1, 3, 5, 7, and 9 are with 15 pmol of MazFsa added. The cleavage products are indicated by arrows. (E) Effects of RNA-RNA duplex formation on cleavage by MazFsa. Lane 1, RNA ladder generated by alkaline hydrolysis; lane 2, labeled sense RNA alone; lane 3, labeled sense RNA digested with 15 pmol of MazFsa; lanes 4 to 7, the labeled 18-base sense RNA, AUUC, was annealed with AUUC antisense RNA RB-3 in ratios of 1:0.2, 1:0.4, 1:0.8, and 1:1, respectively, as indicated and then digested with 15 pmol of MazFsa at 37°C for 30 min.
Seven additional 18-base synthetic RNA substrates were synthesized with the AUUC sequence in the center being replaced by AGUC, AUGC, AUUU, AUUG, GUUC, GUUG, or UUUU to examine consensus residues in VUUV. Our analyses showed that both U residues are essential for cleavage to occur; alterations in any of the two U residues in the center of the consensus sequence completely abolished the cleavage by MazF<sub>Sa</sub> (Fig. 5B, lanes 4 and 6). The cleavage efficiency was reduced significantly if the first and the fourth residues were changed to U (Fig. 5B, lanes 10 and 16). The fourth C residue could be changed to G without any significant loss of cleavage efficiency (Fig. 5B, lane 12). The first A could be changed to G with some degree of reduced cleavage efficiency (Fig. 5B, lanes 10 and 16). The second C could be changed to U (Fig. 5B, lane 10). The third C and the first A could be changed to G without any significant loss of cleavage efficiency (Fig. 5B, lanes 12 and 16). The third C could be changed to U (Fig. 5B, lane 14). The second A could be changed to G without any significant loss of cleavage efficiency (Fig. 5B, lane 14). The third A could be changed to G without any significant loss of cleavage efficiency (Fig. 5B, lane 14). The fourth A could be changed to G without any significant loss of cleavage efficiency (Fig. 5B, lane 14).

There are other VUUV sequences present in the <i>ctpA</i> mRNA, but cleavage did not occur with these sequences. We speculate that secondary structures of the substrate may affect the cleavage by MazF<sub>Sa</sub>. To test this hypothesis, a highly purified 19-base synthetic RNA, RB-1, which can form a hairpin structure with the AUUC sequence embedded within the stem region (Fig. 5C), was digested with MazF<sub>Sa</sub>. Cleavage was completely blocked with this hairpin structure, whereas clear cleavage occurred with the purified RB-2, the synthetic RNA without the hairpin structure that encompassed the AUUC sequence (Fig. 5D, lanes 5 and 7). We next examined the cleavage of MazF<sub>Sa</sub> on the AUUC antisense RNA, RB-3. Although there was an AUUG sequence in the single-stranded AUUC antisense RNA, cleavage by MazF<sub>Sa</sub> did not occur (Fig. 5D, lane 9), whereas the altered sense-strand RNA with the AUUG sequence (Fig. 5B, lane 12) was efficiently cleaved by MazF<sub>Sa</sub>. The reason for this discrepancy is unknown, but it is conceivable that the sequences adjacent to VUUV may play a role in promoting cleavage. The cleavage by MazF<sub>Sa</sub> was also blocked when the sense RNA with the AUUC sequence annealed with its antisense-strand RNA, RB-3, to form an RNA-RNA duplex in a dose-dependent manner (Fig. 5E). These results suggested that MazF<sub>Sa</sub> cannot cleave the VUUV sequences in the RNA-RNA duplex and hence is only specific for single-stranded RNA without any intramolecular base pairing involving VUUV.

**In vivo cleavage of <i>ctpA</i> RNA by MazF<sub>Sa</sub>**. To determine the MazF<sub>Sa</sub>-specific cleavage sites in mRNA in vivo, primer extension analysis of <i>ctpA</i> mRNA was performed with total RNA extracted from both <i>E. coli</i> and <i>S. aureus</i> carrying the corresponding plasmids at various time points after induction as described in Materials and Methods. A clear in vivo cleavage site was determined in <i>E. coli</i> with primer pEa, as shown in Fig. 6A. A primer extension product appeared at 30 min after induction of MazF<sub>Sa</sub>, with subsequent time points showing cleavage in a time-dependent manner (lanes 2 to 5). However, the effect of MazF<sub>Sa</sub> in <i>E. coli</i> likely occurs within seconds of initiation of the reaction, as this extension product can almost be detected at time zero (Fig. 6A, lane 1). In vivo cleavage was also detected in <i>S. aureus</i> 178RI carrying plasmid pG164-MazF(His)<sub>6</sub>/ctpA with primer pEa (Fig. 6B), but the cleaved <i>ctpA</i> mRNA with the extension product was faintly detected only after 30 min of induction (Fig. 6B). We speculate this may be due to a lower copy number of the cotranscribed <i>ctpA</i> mRNA. Nevertheless, the cleavage recognition site in vivo in both <i>E. coli</i> and <i>S. aureus</i> (Fig. 6A and B) was found to be identical to the one in vitro (Fig. 4A), but the cleavage site was shifted two bases upstream. Collectively, these results indicated that MazF<sub>Sa</sub> recognizes the same site on <i>ctpA</i> mRNA both in vivo and in vitro, but the exact cleavage site may differ by one to two bases between those in vivo and in vitro, which could be due to trimming of the RNA ends by cellular RNases.

Environmental stress triggers increasing expression of the <i>mazEF<sub>Sa</sub></i> transcript. The <i>mazEF<sub>Sa</sub></i> operon is located upstream of the <i>sigB</i> operon and is cotranscribed as a 3.6-kb transcript (Fig. 1A). This genetic arrangement suggests that <i>mazEF<sub>Sa</sub></i> may be related to environmental stresses. We thus examined the transcription of <i>mazEF<sub>Sa</sub></i> upon exposure to antibiotics. Increased expression of a 3.6-kb and a 0.5-kb transcript, as estimated from the migration pattern and corresponding to the
cotranscript with the sigB operon and the transcript of the mazEF<sub>Sa</sub> operon alone (Fig. 1A), respectively, was detected with the mazEF<sub>Sa</sub> probe upon exposure to doxycycline (DOX) for 45 min (Fig. 7). The increase of both transcripts was also found with exposure to sub-MIC levels of other antibiotics, e.g., erythromycin and penicillin (unpublished data). Interestingly, no reduction in CFU counts was observed with exposure to antibiotic at sub-MIC levels (data not shown). Similarly, we also found brief exposure of S. aureus cells to heat (48°C) activated transcription of the mazEF<sub>Sa</sub> promoter (unpublished data), thus confirming the finding of Senn et al., who also demonstrated increased transcription of mazEF<sub>Sa</sub> with sigB upon heat stress treatment (30). This is different from the mazEF system in E. coli, wherein brief exposure of antibiotic or heat disrupts mazEF transcription and translation, thus leading to proteolysis of the labile MazE and hence unlabeling the endoribonuclease activity of MazF (1, 15, 29). This mode of action of MazF in E. coli has been termed programmed cell death by one group of investigators (9), but this claim has been disputed in studies presented by Tsilibaris et al. (33).

**DISCUSSION**

Staphylococcus aureus is a major opportunistic pathogen that is a leading cause of nosocomial infections associated with surgical wounds and indwelling medical devices. Despite antimicrobial therapy, the morbidity and mortality associated with S. aureus infections remain high, due in part to the organism’s ability to develop resistance to antibiotics, including that to vancomycin (5, 20). In searching for antimicrobial targets within the stress-induced operon, we noticed, as did Kullik et al. (18), that the ORF (SA2058) upstream of rsbU, the first gene within the established sigB operon, shares sequence similarity with mazF of E. coli. Given that mazE and mazF in prokaryotes are often cotranscribed, we were puzzled with the functional identity of these two ORFs, since SA2059, directly upstream of SA2058, has little homology with mazE of E. coli.

In S. aureus, SA2059 and SA2058 have been shown to cotranscribe with the sigB operon, particularly under stressful conditions (30). Given that both mazEF and the sigB operons are modulated under stress and that SA2058 is homologous with mazF of E. coli, it is reasonable to speculate that SA2058 and SA2059 may represent a MazEF-like system in S. aureus, despite a lack of supporting experimental data. In this study, we demonstrated that MazEF<sub>Sa</sub> of S. aureus is a TA module wherein MazF<sub>Sa</sub> is the toxin and MazE<sub>Sa</sub> is the antitoxin that binds MazF<sub>Sa</sub> to inhibit its toxicity. Our data showed that MazF<sub>Sa</sub> is toxic to both E. coli and S. aureus after induction for its expression (Fig. 1). It inhibits protein synthesis in a cell-free system by cleaving mRNA substrates. MazE<sub>Sa</sub>, on the other hand, inhibits the toxicity of MazF<sub>Sa</sub> by preventing cleavage of the target mRNA and hence releases the inhibition in protein synthesis by MazF<sub>Sa</sub> (Fig. 2 and 3). This inhibition was due to the formation of the MazE<sub>Sa</sub>/MazF<sub>Sa</sub> complex (Fig. 1D), which prevents the free form of MazF<sub>Sa</sub> from cleaving the target RNA (Fig. 3B). However, the exact stoichiometry by which the C-terminal arm of MazE<sub>Sa</sub> mimics the similarly charged sugar-phosphate backbone of RNA to inhibit MazF<sub>Sa</sub> toxin activity by occupying the RNA binding site on the MazF<sub>Sa</sub> toxin as described for E. coli (19) will require further detailed crystal structure studies of the MazE/MazF<sub>Sa</sub> complex.

In S. aureus and some gram-positives bacteria (e.g., B. subtilis and Listeria monocytogenes), mazEF homologs are located immediately upstream of the sigB operon, which encodes σ<sup>B</sup>, the main alternative transcription factor involved in the stress response of many gram-positive bacteria, and a series of anti-sigma factors to control the concentration of free σ<sup>B</sup> (10, 13, 27, 30). In contrast, the mazEF genes in E. coli are located downstream of the relA gene. relA encodes a synthase for ppGpp and is upregulated in response to uncharged tRNA at the ribosomal A-site during amino acid starvation and other stressful conditions, including antibiotic exposure (1, 15, 29). It was shown that overproduction of ppGpp (by overproducing RelA<sup>+</sup>, a truncated version of ppGpp synthetase I of E. coli) in a strain derived from MC4100 represses expression from the mazEF promoter. Those authors then suggested that physiological conditions that confer increased levels of ppGpp would reduce synthesis of MazE antitoxin, hence enabling degradation of the more labile MazE antitoxin by the ClpPA protease system and unleashing the toxic effect of MazF to execute programmed cell death (PCD) (1). However, Christensen et al. (6) investigated whether the transcription pattern of mazEF during amino acid starvation induced by serine hydroxamate was stimulated strongly by amino acid starvation, and this stimulation depended on Lon. No TA locus-dependent cell killing was observed during this amino acid starvation. Penson et al. (25) also showed that the toxicity of MazF in E. coli can be rescued by the antitoxin MazE, expressed within 6 h after MazF induction. They further proposed that MazF does not mediate cell killing but rather induces a bacteriostatic condition. Both studies have shed doubt on the notion of PCD proposed by Aizenman et al. (1). Indeed, even with the overproduction of MazF, E. coli cells can retain transcriptional and translational competence for 4 days despite their growth arrest (31). Although Sat et al. (29), Amitai et al. (2), and Hazan et al. (15) suggested PCD is mediated by mazEF from E. coli upon exposure to some antibiotics, controversial results were presented for the same PCD experiments by Tsilibaris et al. (33); thus, the physiological roles of the toxin proteins remain under debate.

Our results demonstrated that the expression of the mazEF<sub>Sa</sub> transcripts was up-regulated (Fig. 7) when the culture was exposed to sub-MIC levels of some antibiotics, with no great loss of cell viability. Given the divergent structural ar-
rangement between E. coli and S. aureus with respect to MazEF and the stress operon, the regulation of the S. aureus MazEF_Sa TA module in response to stress warrants additional investigation (unpublished data). Pedersen et al. (25) reported that RelE-induced cell stasis exhibited increased sensitivity towards environmental stresses, e.g., heat shock, oxygen radicals, and osmotic stress. The above studies have led to the suggestion that TA complexes might constitute a novel approach toward the potential development of a new class of antimicrobial compounds which activate or mimic bacterial toxins. Compounds could function through several different mechanisms, such as preventing or reducing the association between a given TA pair or manipulating the signaling pathway that leads to toxin activation (9, 12, 22).

As with E. coli MazF, the MazF_Sa of S. aureus was also found to be a ribosome-independent endoribonuclease, but with very different sequence specificity compared with other MazF homologs. In particular, it cleaves the RNA substrate in a U-rich region with the consensus sequences VUUV as demonstrated both in vivo and in vitro (Fig. 4 and 5). Most commonly, the cleavage sites reside in the 5’ end of the first U residue and at the 3’ end of the second U (Fig. 4; Table 1). Importantly, the two U residues are essential for the MazF_Sa cleavage, since replacement of either U residue abolishes the cleavage while the V and V’ residues can be A, C, and G. When V or V’ residues were changed to U, the cleavage efficiency was significantly reduced (Fig. 5). Previously, the MazF of E. coli was demonstrated to cleave RNA substrates specifically at the 5’ end of ACA sequences (34). Similarly, two MazF homologs from Mycobacterium tuberculosis were also found to cleave at UAC triplets and (U/C)U (A/U)C(U/C) in the mRNA (39). Another PemK family toxin, EndoA from Bacillus subtilis, which shares homology with MazF of E. coli, was also shown to cleave at a UAC sequence (27). These results suggest that the cleavage sites of different MazF homologs in prokaryotes can differ. In particular, MazF_Sa is the first example of a toxin that cleaves most commonly at the 5’ or 3’ end an invariant UU residue with a consensus sequence of VUUV’. How various toxins contribute to bacterial cell physiology and metabolism in response to stress by cleaving mRNAs at specific sites is of general interest and merits further studies.

As there are other VUU’ sequences within the ctpA mRNA that are not amenable to cleavage, we investigated the role of the secondary structure of RNA, including the stem-loop structure and RNA duplex, in mRNA cleavage by MazF_Sa. Our data clearly showed that the VUU’ sequence can be cleaved as part of a loop, but not as part of the stem where the VUU’ sequence may form a partial RNA duplex (Fig. 5C and D). To confirm this, we incubated MazF_Sa with a perfect RNA-RNA duplex where the antisense RNA was complementary to the sense RNA strand, which is amenable to cleavage under in vitro conditions. As predicted from the stem-loop study and in concordance with the data for the MazF of E. coli (37), MazF_Sa can only cleave the single-stranded RNA at the predicted VUU’ site but not the perfect RNA-RNA duplex (Fig. 5E). Curiously, the MazF_Sa toxin cannot cleave the complementary antisense RNA strand with the 5’-GAAU UG-3’ sequence, where the first four bases are complementary to the AUUC consensus sequence in the sense strand and the last four nucleotides constitute the putative AUUG cleavage site (see the description of the AUUC antisense RB-3 in Materials and Methods). The reason for the difference in cleavage between the sense and the AUUC antisense strand is not entirely clear, but it may be due to the secondary structure, or the adjacent sequence may contribute to recognition of the putative site. Our data also demonstrated that MazF_Sa recognizes the same site on ctpA mRNA both in vivo and in vitro, but the exact cleavage site differed by one to two bases (Fig. 6). These differences may be due to changes in the buffering environment, which could affect the folding of the ctpA mRNA substrate. It is also quite possible that there may be another protein interacting with MazF_Sa besides MazE_Sa, which could change the conformation of this endoribonuclease, or that the RNA ends may be trimmed by RNases in vivo.

Recently, Moritz and Hergenrother showed that the mazEF TA system was found to be ubiquitous among plasmids obtained from vancomycin-resistant enterococci (22). Consistent with the early discovery of TA system in plasmids (11, 24), they proposed that the MazEF system functions to stabilize the plasmid in Enterococcus species. Since the vanA gene, the critical component of vancomycin resistance in enterococci (22), resides on the same plasmid as that of the mazEF genes in over 90% of the strains, this raises the possibility that TA systems may also serve to maintain the vancomycin-resistant gene in Enterococcus species. Given that gene transfer has been shown to occur between staphylococci and enterococci, it remains to be seen if the MazEFSa system plays an important role in maintenance of antibiotic resistance genes in S. aureus.

Although the MazF_Sa toxin shares sequence similarity to its counterparts in E. coli and B. subtilis, the antitoxin MazE_Sa was found to be homologous to MazE-like molecules only in Staphylococcus epidermidis, Staphylococcus hemolyticus and Staphylococcus saprophyticus, but not to other paralogs in gram-positive species (e.g., YdcD in B. subtilis). Studies in another TA module called yefMyoeB in Streptococcus pneumoniae showed that the toxicity of YoeB could be reverted by its cognate antitoxin YeFM, but not by the YeFM homolog from E. coli (23). The above findings clearly indicate that antitoxins are different between species within the same TA systems, while the toxins are more homologous.

Collectively, our findings indicate that the MazF_Sa of S. aureus differs in cleavage specificity from its E. coli counterpart. Based on the arrangement of mazEF_Sa together with the sigB operon as a single transcription unit and that the sigB operon is a known stress-induced transcription unit, we speculate that the toxic effect of MazF_Sa for S. aureus in response to stress likely diverges from that of E. coli. Finally, genomic mining reveals that MazF_Sa may be unique in staphylococcal species. Accordingly, we predict that a successful anti-MazF_Sa strategy will be active against other staphylococcal species as well.

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REFERENCES
