Characterization of a mazEF Toxin-Antitoxin Homologue from Staphylococcus equorum

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Toxin-antitoxin (TA) systems encoded in prokaryotic genomes fall into five types, typically composed of two distinct small molecules, an endotoxin protein and a cis-encoded antitoxin of ribonucleic or proteinaceous nature. In silico analysis revealed seven putative type I and three putative type II TA systems in the genome of the nonpathogenic species strain Staphylococcus equorum SE3. Among these, a MazEF system orthologue termed MazEFseq was further characterized. 5’ rapid amplification of cDNA ends (RACE) revealed the expression and the transcriptional start site of mazEseq, indicating an immediately upstream promoter. Heterologous expression of the putative toxin-encoding mazFseq gene imposed growth cessation but not cell death on Escherichia coli. In vivo and in vitro, MazFseq was shown to cleave at UACAU motifs, which are remarkably abundant in a number of putative metabolic and regulatory S. equorum gene transcripts. Specific interaction between MazFseq and the putative cognate antitoxin MazEseq was demonstrated by bacterial two-hybrid analyses. These data strongly suggest that MazEFseq represents the first characterized TA system in a nonpathogenic Staphylococcus species and indicate that MazEF modules in staphylococci may also control processes beyond pathogenicity.

Toxin-antitoxin (TA) systems are small episomally or chromosomally encoded genetic modules found in bacteria or archaea (1). TA systems typically consist of a small and stable toxic protein that can interfere with vital cellular functions and an unstable antitoxin, capable of inhibiting toxin activity (2). Depending on the biochemical nature and mode of action of the antitoxin, five classes can be distinguished. Type I TA systems possess an RNA antitoxin that posttranscriptionally inhibits toxin activity via antisense regulation. TA systems of types II, IV, and V contain a small proteinaceous antitoxin that can render the toxin inactive either by protein-protein interaction, by cleavage of toxin mRNA, or by binding to the toxin’s target structure. Type III TA systems (3) are characterized by an RNA antitoxin modulating toxicity in a posttranslational fashion (4). TA systems of types III, IV, and V have so far been characterized in only one instance each (5–7), but orthologues of type I, type II, and supposedly also type III representatives are widespread among plasmids and chromosomes of prokaryotes (3, 8). According to toxin sequence homology, at least 10 families of type II TA systems can be distinguished (4, 9–11). The genome of the notorious pathogen Staphylococcus aureus possibly bears a number of type I TA systems, from which one candidate has recently been characterized (12). Three type II TA systems, MazEFsa, YefM-YoeB-sa1, and YefM-YoeB-sa2, have been characterized (12). Three type II TA systems possibly bears a number of type I TA systems, from which one candidate has recently been characterized (12). Three type II TA systems, MazEFsa, YefM-YoeB-sa1, and YefM-YoeB-sa2, have been characterized (12). Three type II TA systems possibly bears a number of type I TA systems, from which one candidate has recently been characterized (12). 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and type II TA systems. Among these, a gene pair dubbed mazEF<sub>seq</sub> was genetically and biochemically characterized and yielded strong evidence for a first characterized functional TA system encoded in the S. equorum genome. We determined the transcriptional starting point of the mazEF<sub>seq</sub> locus and provide evidence for a toxic effect of mazF<sub>seq</sub> expression and for in vivo interaction of MazF<sub>seq</sub> and MazE<sub>seq</sub> in E. coli. It is shown that the purified putative toxin endolytically cleaves RNA in a sequence-specific manner, as it also does upon heterologous expression in vivo. Finally, we identified coding sequences that might posttranscriptionally be controlled by MazE<sub>seq</sub>-dependent cleavage due to unusually high relative abundances of respective target sites.

MATERIALS AND METHODS

Bioinformatical analysis. Access to the raw, partially assembled genome sequence of Staphylococcus equorum SE3 was kindly provided by F. Götz. Initially, a local BLAST protein database consisting of putative type I TA system toxins described by Fozo et al. (26) was created. Manual BLASTX searches were done against the S. equorum raw genome to identify homologues of type I TA toxin components. Furthermore, the antiRNA toxins of putative type I TA systems were analyzed to assess their structure and RNA-RNA interaction potential. The program RNAz (27, 28) was used to predict conserved secondary structures between the homologous RNAs. The input alignment for RNAz was calculated using ClustalW (29). For the prediction of RNA-RNA interactions, the program IntRNA (30, 31) was applied. In addition, a prediction of promoter regions, which is based on the detection of destabilized regions (32), was performed using nocoRNAc (33).

In silico screenings for type II TA systems were conducted using the web-based search tools RASTA Bacteria (34) and TADB (35). Obtained sequences were aligned using ClustalW (29) and viewed in Jalview (36) or BioEdit (37). For all programs, standard parameters were used.

Fine chemicals, nucleic acids, and enzymes. Chemicals were from Carl Roth (Karlruhe, Germany), Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ), and J. T. Baker (Philipsburg, NJ) at the highest purities available. Enzymes for DNA restriction and modification were purchased from NEB (Ipswich, MA), Fermentas (St. Leon-Rot, Germany), and TaKaRa Bio Inc. (Shiga, Japan). DNA polymerases were obtained from Genaxxon (Ulm, Germany) and Agilent Technologies (La Jolla, CA). Avian myeloblastosis virus reverse transcriptase (AMV RT), MS2 phage RNA, and RNase inhibitor for primer extension were purchased from Roche (Indianapolis, IN). For 5’ RACE (rapid amplification of cDNA ends) analysis, Ambion SUPERase-In, Ambion diethyl pyrocarbonate (DEPC)-treated double-distilled water (ddH<sub>2</sub>O), Ambion THE RNA storage solution, and the Topo TA cloning kit for sequencing with One Shot chemically competent T10 E. coli cells were purchased from Life Technologies (Darmstadt, Germany); Roti-Aqua-P/C1 (phenol-chloroform-isoamyl alcohol, 25:24:1; pH 4.5 to 5) was purchased from Carl Roth GmbH + Co. KG (Karlruhe, Germany); T4 RNA ligase was purchased from NEB (Ipswich, MA); tobacco acid phosphatase (TAP) was purchased from Epicentre (Madison, WI); and the first-strand cDNA synthesis kit for reverse transcription-PCR (RT-PCR) was purchased from Roche (Mannheim, Germany). Protein and DNA molecular weight markers and loading dyes were bought from NEB (Ipswich, MA), Invitrogen/Life Technologies (Carlsbad, CA), Promega (Madison, WI), and Fermentas (St. Leon-Rot, Germany). The Thermo Sequenase fluorescently labeled primer cycle sequencing kit with 7-deaza-dGTP was acquired from GE Healthcare (Freiburg, Germany). Isopropyl-β-D-thiogalactopyranoside (IPTG) was ordered from Merck (Darmstadt, Germany), and anhydrotriacyclon (ATc) was ordered from Acros Organics (Geel, Belgium). Lysostaphin was acquired from Dr. Petry Genmedics (Reutlingen, Germany). Nickel-nitritolriacetic acid (Ni-NTA) resin was purchased from Qiagen (Hilden, Germany). Synthetic DNA and RNA oligonucleotides were ordered from Biomers (Ulm, Germany) and Integrated DNA Technologies (Corvalis, IA).

Bacterial strains, plasmids, and growth conditions. The E. coli strains BL21(DE3) (38), DH5α (39), BW25113 (40), and BTH101 (41) (Euromedex, Soufflewysehrheim, France), as well as S. equorum SE3 (kindly provided by F. Götz), were used in this study (Table 1). E. coli cells were grown either in LB or, whenever sugar-sensitive inducible plasmids were used, in M9 minimal medium (44) supplemented with 0.2% (wt/vol) thiamine, 0.2% (wt/vol) Casamino Acids (CAA), and either 0.4% (wt/vol) glucose or 0.2% (vol/vol) glycerol as a carbon source. During the BACTH bacterial two-hybrid experiments (see below), cells were grown on LB agar plates supplemented with X-Gal (3-bromo-4-chloro-3-indo)lyl-β-D-galactopyranoside) and IPTG (both from Carl Roth, Karlsruhe, Germany) at final concentrations of 40 µg/ml or 0.5 mM, respectively. S. equorum was grown in basic medium (BM) (45). All cultures were grown with shaking (150 rpm) in baffled flasks at 37°C with aeration. Antibiotics were used where appropriate at the following concentrations: 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, and 50 µg/ml kanamycin.

Molecular cloning and isolation of nucleic acids. In general, cloning was carried out using standard protocols. E. coli cells were made chemically competent by BCI treatment (46). Plasmids and DNA fragments were isolated or purified using standard kits from Sigma-Aldrich (Munich, Germany), Peqlab (Erlangen, Germany), and Qiagen (Hilden, Germany) according to the manufacturers’ protocols. Genomic DNA of staphylococci was obtained either by breaking cells with lysostaphin (15 µg/ml for 90 min) and NaCl<sub>4</sub> treatment and chloroform-isooamyl alcohol extraction as described previously (47, 48) or by using the InstaGene kit (Bio-Rad, Munich, Germany). The nucleotide sequence of the S. equorum mazEF<sub>seq</sub> locus can be found under the GenBank accession number KC020193. Sequencing of cloned products was done at GATC (Constance, Germany) and Macrogen Corp. (New York, NY), and results were analyzed using Lasergene SeqMan (DNAStar, Madison, WI).

Construction and growth analysis of E. coli DH5α(pASK-IBA3 mazF<sub>seq</sub>). mazF<sub>seq</sub>, including its native ribosome-binding site (RBS), was PCR amplified from S. equorum SE3 genomic DNA (GenBank accession number KC020193) using the primers mazF<sub>f</sub> and mazF<sub>r</sub> (all oligonucleotides are listed in Table S1 in the supplemental material). The PCR product was digested with BglII and Ncol and ligated with the ATc-inducible vector pASK-IBA3 cut with Ncol and BamHI, yielding pASK-IBA3-mazF<sub>seq</sub> which was used to transform E. coli DH5α. Resulting strains carrying pASK-IBA3 with or without mazF<sub>seq</sub> were grown overnight in LB, and cultures from both strains were used to inoculate two flasks containing 17 ml of LB each to an optical density at 578 nm (OD<sub>578</sub>) of 0.07. Cells were cultivated with shaking at 37°C, and 60 min later, ATc (0.4 µM final concentration) was added to one flask of each strain. Growth was monitored on a hourly basis by determining both OD<sub>578</sub> values (measured as 10-fold or 20-fold dilutions, where necessary) and CFU counts. To this end, samples were 10-fold serially diluted from 10<sup>6</sup> to 10<sup>7</sup> in sterile saline solution (0.85% [wt/vol] NaCl) and plated on LB plates containing ampicillin. After 14 to 16 h of incubation at 37°C, the numbers of colonies were counted.

BACTH two-hybrid analysis of MazE<sub>bact</sub>-MazF<sub>seq</sub> interaction in vivo. The bacterial adenylate cyclase-based two-hybrid system (BACTH; Euromedex, Soufflewysehrheim, France) functions by fusing two putative binding partners to the Bordetella pertussis adenylate cyclase (CyaA) fragments T18 and T25. These form an active enzyme only when in close proximity, providing evidence for interaction of the fused proteins. The cyclic AMP (cAMP)-dependent activation of lactose-utilizing genes is visualized by blue colonies on X-Gal-containing medium. We cloned mazE<sub>seq</sub> in two ways to the T18-encoding fragment, to obtain N- and C-terminal translational fusions using the high-copy-number BACTH vectors pUT18 (mazE<sub>seq</sub> N terminally fused) and pUT18C (C-terminal fusion). Products amplified by PCR using the primers mazE<sub>bact_f</sub> and mazE<sub>_bact_r</sub> were cloned with the restriction enzymes BamHI and Xmal. Similarly, mazF<sub>seq</sub> was cloned in frame with the T25 open reading frame

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(ORF) into the low-copy-number BACTH vector pKT25 (C-terminal fusion) using the primers mazFseq-bach_f and mazFseq-bach_r and restriction enzymes as described above. Constructs were confirmed by sequencing, and the pUT18-, pUT18C-, and pKT25-derived vectors were used to consecutively transform strain E. coli BTH101. Single colonies of cells containing both plasmids (one each of the pUT and of the pKT series) were streaked onto LB–X–Gal–IPTG plates to provide evidence for protein–protein interaction. As negative controls, strains that harbor a plasmid containing the T18 fragment alone with either a plasmid containing only a MCS, multiple-cloning site.

**Table 1** Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description or information</th>
<th>Reference and/or source</th>
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<tbody>
<tr>
<td><strong>Bl21(DE3)</strong></td>
<td>F&lt;sup&gt;−&lt;/sup&gt; ompT gal dcm lon hsdS&lt;sup&gt;(r&lt;sup&gt;B&lt;/sup&gt;−, m&lt;sup&gt;B&lt;/sup&gt;−)&lt;/sup&gt; λ (DE3 [lacI lacUV5-7 gene 1 ind1 sam7 nin5])</td>
<td>38</td>
</tr>
<tr>
<td><strong>DH5α</strong></td>
<td>F&lt;sup&gt;−&lt;/sup&gt; endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG660dGalZΔM15 Δ(lacYIΔargF+U169 hsdR17(rK&lt;sup&gt;−&lt;/sup&gt; m&lt;sup&gt;K&lt;/sup&gt;−) λ)</td>
<td>39</td>
</tr>
<tr>
<td><strong>Top10</strong></td>
<td>F&lt;sup&gt;−&lt;/sup&gt; mcRA Δ(mer–hdamRMS-mccBC) 880lacZΔM15 ΔlacX74 recA1 araD139 araL-7697 galU galK rpsL (Str&lt;sup&gt;−&lt;/sup&gt;) endA1 nupGΔ</td>
<td>Invitrogen/Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td><strong>BTH101</strong></td>
<td>F&lt;sup&gt;−&lt;/sup&gt; cya-99 araD139 galE15 galK16 rpsL1 (Str&lt;sup&gt;−&lt;/sup&gt;) hsdR2 mcrA1 mcrB1</td>
<td>41; Euromedex, Soufflwegersheim, France</td>
</tr>
<tr>
<td><strong>BW25113</strong></td>
<td>rpsB3 ΔlacZ4787 hsdR514 Δ(arabAD)567 Δ(rhaBAD)568 ρ1-</td>
<td>40</td>
</tr>
<tr>
<td><strong>S. equorum SE3</strong></td>
<td>Isolated from red-rind cheese</td>
<td>German Federal Dairy Research Centre, Max Rubner Institute, Kiel, Germany</td>
</tr>
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**Plasmids**

| pASK-IBA3 | bla, ATc-inducible promoter | IBA-GmbH, Göttingen, Germany |
| pASK-IBA3-mazFseq | mazFseq cloned downstream of the ATc-inducible promoter | This study |
| pBAD33 | cat, arabinose-inducible promoter | 42 |
| pBAD33 SD | mazFseq, cloned downstream of an arabinose-inducible promoter with a synthetic E. coli Shine-Dalgarno sequence | This study |
| pColdIII | bla, IPTG- and cold shock-inducible promoter | 43; TaKaRa Bio Inc., Shiga, Japan |
| pColdIII-mazFseq | mazFseq cloned downstream of the IPTG- and cold shock-inducible promoter | This study |
| pUT18 | bla, MCS<sup>a</sup> in N terminus of T18 fragment | 41; Euromedex, Soufflwegersheim, France |
| pUT18C | bla, MCS in C terminus of T18 fragment | 41; Euromedex, Soufflwegersheim, France |
| pUT18C-zip | Derivative, bla, leucine zipper C terminally fused to T18 fragment | 41; Euromedex, Soufflwegersheim, France |
| pKT25 | aphaIII, MCS in C terminus of T25 fragment | 41; Euromedex, Soufflwegersheim, France |
| pKNT25 | aphaIII, MCS in N terminus of T25 fragment | 41; Euromedex, Soufflwegersheim, France |
| pKT25-zip | Derivative, aphaIII, leucine zipper C terminally fused to T25 fragment | 41; Euromedex, Soufflwegersheim, France |
| pUT18-mazE | Derivative, bla, mazEseq<sup>a</sup> N terminally fused to T18 fragment | This study |
| pUT18C-mazE | Derivative, bla, mazEseq<sup>a</sup> C terminally fused to T18 fragment | This study |
| pUT18C-yeIF1 | Derivative, bla, yeIF1<sup>a</sup> N terminally fused to T18 fragment | This study |
| pUT18C-yeIF1 | Derivative, bla, yeIF1<sup>a</sup> C terminally fused to T18 fragment | This study |
| pKT25-mazF | Derivative, aphaIII, mazFseq<sup>a</sup> C terminally fused to T25 fragment | This study |
| pKT25-yeIF1 | Derivative, aphaIII, yeIF1<sup>a</sup> C terminally fused to T25 fragment | This study |

*<sup>a</sup>MCS, multiple-cloning site.

**Overexpression and purification of (His)<sub>6</sub>-tagged MazFseq**

MazFseq-(His)<sub>6</sub> was purified as previously described (49). Prior to use, primers were labeled using a T4 polynucleotide kinase and [γ<sup>−32P</sup>]ATP. Primer extension reactions were carried out with added MS2 RNA fragments, AMV RT, and labeled primers for 1 h at 47°C. The extension reaction was terminated by the addition of 6 μl of stop solution 1 (95% formamide, 20 mM EDTA, 0.05% bromophenol...
blue, and 0.05% xylene cyanol EF), incubation for 2 min at 95°C, and storage on ice for 5 min. After being heated to 90°C for 5 min, samples were electrophoresed on a 6% polyacrylamide gel with 8 M urea, which was subsequently dried for 2 h in a gel vacuum dryer before an X-ray film (Kodak, Rochester, NY) was exposed for 20 to 48 h.

In vivo primer extension assays were performed according to the literature (20, 50), but instead of using 32P labeling, primers were ordered with a DY-681 (Dyomics, Jena, Germany) infrared dye modification at the 5’ end. Plasmid pBAD33 SD mazFseq was constructed by cloning PCR-amplified mazFseq (primers mazF_BAD_f and mazF_BAD_r) into pBAD33 via SacI and XbaI. E. coli BW21513 cells containing pBAD33 SD mazFseq or the empty pBAD33 vector were grown in 250-ml flasks containing 100 ml BM to an OD660 of 0.6. The cells were then split, and to one flask of each construct, arabinose was added to a final concentration of 0.2% (wt/vol). After 40 min, 12 ml of culture from each flask was harvested and the cells were broken by treatment with glass beads (Roth, Karlsruhe, Germany) and a tissue lyser (Thermo Savant; FastPrep FP120 Bio101) twice for 20 s. Total RNA was extracted from the lysate using TRIzol (Ambion, Life Technologies, Darmstadt, Germany) according to the manufacturer’s protocol, the RNA pellets were dissolved in TREA RNA storage solution (Ambion, Life Technologies, Darmstadt, Germany), and the concentration was determined by NanoDrop measurement. Reverse transcription was conducted in the presence of 16 µg total RNA, 1 pmol DY-681-labeled primer (OmpF_ec or TufA_ec), 10 mmol deoxynucleoside triphosphates, 160 ng SUPERase-In RNase inhibitor in a total volume of 10 ml, 0.05% bromophenol blue. Sequencing ladders were generated either to digestion by purified MazFseq-(His)6 or to chemical ligation of cellular RNA molecules (20,50), but instead of using 32P labeling, primers were ordered with DY-681-labeled primer (10 pmol/l) were mixed and incubated for 30 min at 37°C and stopped by the addition of 6 µl stop solution 2 (95% formamide, 10 mM EDTA, 0.05% bromophenol blue). Sequencing ladders were generated from genomic DNA prepared from E. coli BW21513 pBAD33 cells using the Thermo Sequenase fluorescently labeled primer cycle sequencing kit with 7-deaza-dGTP (GE Healthcare, Freiburg, Germany) and the primers OmpF_ec and TufA_ec. All samples were heated for 2 min at 95°C and then loaded onto a 25 cm 8M urea, 10% polyacrylamide gel and run on a Li-Cor Long Read 4200 system (Lincoln, NE) using the IRD700 channel according to the manufacturer’s recommendations.

For RNA oligonucleotide digestion tests, γ-32P-labeled fragments were subjected either to digestion by purified MazFseq-(His)6, or to chemical hydrolysis, to provide a molecular weight standard ladder for electrophoresis. In a total volume of 10 µl, 0.5 µl RNase inhibitor, 0.25 µg MazFseq-(His)6, 1 µl Tris-HCl (pH 7.8, 100 mM), and 0.5 µl radioactively labeled primer (10 pmol/µl) were mixed and incubated for 30 min at 37°C for enzymatic cleavage by MazFseq-(His)6. The reaction was stopped as before. A nucleotide ladder was created by mixing 2 µl of labeled RNA with 1 µl NaOH (1 M) and 7 µl DEPC-treated H2O and incubating the mixture for 1 min at 75°C, followed by terminating the reaction with 40 µl stop buffer. The samples were loaded onto a 20% SDS gel with 8 M urea and further processed as described above.

**Transcription start site determination by 5’ RACE.** An S. equorum culture was grown in BM to an OD660 of 1.4, and the RNA was isolated as described above (in vivo primer extension). 5’ RACE was essentially done as described before (51). Specifically, 7.5 µg of total RNA was incubated with 12.5 U of tobacco acid pyrophosphatase (TAP), 20 U of SUPERase-In (Ambion, Life Technologies, Darmstadt, Germany), and 1× TAP buffer for 1 h at 37°C. This modified RNA was extracted with acidic P/C/I solution, ethanol precipitated, dissolved in 55 µl DEPC-treated dH2O, and incubated with 500 pmol of an RNA adapter fragment (see Table S1 in the supplemental material) at 95°C for 5 min. Ligation of cellular RNA molecules with the RNA adapter was done for 90 min at 16°C with 50 U of T4 RNA ligase I, 20 U of SUPERase-In, 1× T4 RNA ligase buffer, 1 mM ATP, and 1 ng/ml bovine serum albumin (BSA) in a total volume of 80 µl. The ligated RNA was phenol-chloroform extracted and ethanol precipitated as before and resuspended in 10 µl DEPC-treated dH2O. Five microliters was used for reverse transcription by using the Roche first-strand cDNA synthesis kit for RT-PCR (containing AMV RT) with a primer specific for mazFseq (mazE-RACE-outer) in a total volume of 20 µl. One microliter of 10-fold-diluted cDNA was PCR amplified using 1 µM (each) primers specific for the adapter (RACE-PCR) and for mazFseq (mazE-RACE-inner), 1 mM dNTPs, 1× Taq buffer E, and 5 U Taq polymerase (Genaxxon, Ulm, Germany) in a 50-µl reaction mixture. Amplification of cDNA was confirmed on a 2% agarose gel, and 2 µl of the PCR product was used for ligase- and restriction-independent TOPO cloning followed by transformation of One Shot chemically competent E. coli Top10 cells (Invitrogen/Life Technologies). Sequencing of the resulting plasmids allowed determination of the transcriptional start site of mazEseq.

**Analysis of TACAT sequence abundance in the S. equorum SE3 genome.** A screen for TACAT motifs in coding sequences (CDSs) of the S. equorum SE3 genome, including an assessment of stochastic or cumulative occurrence, was performed as described previously (19, 52). Briefly, the probability P of a CDS containing K (actual number) or fewer TACAT sites is dependent on the base composition and length (L) and was calculated as follows:

\[ P = \sum_{i=0}^{K} \binom{L-4}{i} (1 - p)^{L-4-i} \frac{(L - 4)!}{i!(L - 4 - i)!} \]

The expected number of TACAT sites is \( \bar{P}(L - 4) \) where \( p \), the probability of the pentad sequence occurring in a CDS stochastically, is \( p = (\%A^2)(\%T^2)(\%C^2) \). The complete set of 2,581 possible CDSs of S. equorum SE3 was exported from an automatically annotated data set using GenDB (53) and passed on to a custom Perl script (the source code is available upon request) calculating P for each CDS and sorting the results in a descending order.

**RESULTS AND DISCUSSION**

*In silico* analysis indicates several putative type I and type II TA systems in the genome of *Staphylococcus equorum*. In this study, we were interested in mining the genome of the nonpathogenic, food industry-relevant bacterium *S. equorum* SE3 for putative TA systems, for two main reasons. First, it has been proposed that the abundance of TA systems may be higher in free-living organisms than in host-associated species (8), which would suggest a greater number of such systems in *S. equorum* than in *S. aureus*. Second, one of the TA systems of *S. aureus* was suggested to regulate pathogenicity factors (19), which raises the question whether nonpathogenic staphylococci also possess TA systems and, if so, what their functions might be. In order to identify putative type I TA systems in the raw genome of *S. equorum* SE3, we first conducted manual BLAST searches. Using the putative *S. saprophyticus* type I toxin Fst described by Fozo et al. (26) as a query revealed five possible homologues in *S. equorum* (Fig. 1). Respective systems have previously been suggested to reside on a number of other staphylococcal plasmids and chromosomes (54, 55) but have, with one recent exception (12), not been characterized yet. To assess if the putative antitoxin RNAs of these systems may be transcribed, we performed an *in silico* prediction of promoters by the detection of destabilized regions (33). For three of the five systems, a region with low stability was detected, which complements the presence of −10 and −35 box motifs (Fig. 1). The putative antitoxin RNAs do not overlap the coding sequence of the assumed toxin. Therefore, we performed an *in silico* RNA-RNA interaction prediction between the antitoxin RNAs and the toxin mRNAs. For all five pairs of RNAs, a stable interaction was predicted with free energy values ranging from −16.6 to −33.5 kcal/mol (see Table S2 in the supplemental material). In all cases, the predicted interaction site partly covers the putative ribosome-binding site of the toxin mRNA, providing evidence for a possible regulatory role of the candidate antitoxin RNA. In addition, two possible TpsA/RatA type I TA systems were identified, sharing distinct features with
putative staphylococcal systems (26). To collect further evidence that the candidate antitoxin RNAs of these systems are functional, we created a ClustalW sequence alignment of the two antitoxin RNAs with the RatA homologue in *S. aureus* (56). According to a prediction by the program RNAz (27), the aligned RNAs contain a stable conserved secondary structure (classifier P value, 0.99). This suggests that the two putative antitoxin RNAs identified in *S. equorum* carry out a similar function. Manual BLAST searches for the two representatives of type IV and type V TA systems YeeU-CbtA (6) and GhoST (7) yielded no apparent homologues in *S. equorum*.

For the revelation of putative type II TA systems in the *S. equorum* SE3 genome, the publicly accessible online tools RASTA Bacteria (34) and TADB (35) were used. This approach yielded three candidate loci bearing two distinct putative *yefM-yoeB* systems (Fig. 2) and an apparent *mazEF* orthologue (see below), which reflects the complete set of validated type II TA systems in *S. aureus* (16, 19, 20). Thus, we identified a total of 10 putative TA systems in the *S. equorum* SE3 genome (summarized in Table 2), seven of which belonged to type I and three to type II TA systems. The two putative *yefM-yoeB* paralogues are clearly homologous to the *yefM-yoeB*-sa1 and -sa2 systems from *S. aureus* (20), and the

![FIG 1](http://jb.asm.org/) Five different putative type I Fst TA systems from *S. equorum* discovered in silico, aligned with ClustalW using manual adjustments similar to those in the work of Jensen et al. (57). Promoter elements are indicated by black shading, the RBS is indicated by a black frame, direct repeats “b” (DR b) are indicated by dashed lines and arrows, direct repeats “a” (DR a) are indicated by solid lines and arrows, the toxin coding region is indicated by gray shading, and bidirectional terminators are indicated by thick black underlining.

![FIG 2](http://jb.asm.org/) Alignment of YefM-YoeB-sa1 homologues (SA2195 and SA2196) (A) and YefM-YoeB-sa2 homologues (SA2245 and SA2246) (B) from *S. aureus* (sa) N315 with their *S. equorum* (seq) SE3 homologues. Sequences were aligned using MAFFT (63), and residues were shaded according to their BLOSUM62 score in BioEdit (37).
degree of similarity to \textit{S. aureus} counterparts was even more pronounced in the case of the putative \textit{mazEF} system (16), as detailed in Fig. 3. An examination of the adjacent genomic context surrounding the candidate \textit{mazEF} genes also revealed an identical gene synteny in \textit{S. equorum} and \textit{S. aureus}. The respective gene pair was hence termed \textit{mazEF}_{seq} and was further experimentally characterized.

\textbf{P}_{mazE seq} lies adjacent to the \textit{mazEF}_{seq} locus. To provide evidence about the expression of the \textit{mazEF}_{seq} genes and their transcriptional start point(s), 5' RACE was conducted using total RNA isolated from exponential-phase \textit{S. equorum} SE3 cells. Sequencing of three independent plasmids containing cDNA from reverse transcription revealed an identical fragment, unambiguously indicating a single transcriptional starting point (TSP) 50 bp upstream of the \textit{mazE seq} start codon (Fig. 3). Starting at the seventh base preceding the TSP, the sequence TAGTCA(N)_{17}TATTAT was found, likely representing the $\mathbf{35}$ and $\mathbf{10}$ sites of a $\mathbf{A}$-dependent promoter separated by a stretch of optimal distance (58). The presumable $\mathbf{35}$ hexamer was flanked by almost-perfect inverted repeats of the sequence AAAAT(A)GTA, harboring a putative binding site for the \textit{S. aureus} global regulator SarA (59).

Starting at the 34th base downstream of the TSP and located eight bases upstream of the start codon, a TGGAGGT stretch represents the probable ribosome-binding site for \textit{mazE seq}. These features reflect almost identical \textit{cis} elements and transcriptional start sites of the orthologous \textit{S. aureus} system (14,18).

Heterologous overexpression of \textit{MazF seq} in \textit{E. coli} impairs growth. Tools for the genetic manipulation of \textit{S. equorum} have not been established to date. Therefore, the putative toxin-encoding gene \textit{mazF seq} was cloned into the tetracycline-inducible \textit{E. coli} cloning vector pASK-IBA3. Induction of \textit{mazF seq} expression in \textit{E. coli} resulted in an up to 10-fold reduction of OD$_{578}$ values in comparison to those of noninduced strains and those carrying the empty plasmid during the course of the experiment (Fig. 4A). An

| TABLE 2 Identities and similarities of putative \textit{Staphylococcus equorum} SE3 TA systems$^a$
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Putative TA system</td>
<td>% identity</td>
<td>% similarity</td>
<td>pI value</td>
<td>Size (amino acids)</td>
</tr>
<tr>
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<td>82$^b$</td>
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<td>85</td>
<td>4.31</td>
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</table>

$^a$ Fst from pSK1 (57).
$^b$ \textit{S. aureus} N315 TxpA SAS059.
$^c$ \textit{mazEF}, SACOL2058 and SACOL2059.
$^d$ yefM-yoeB-sa1, SA2195 and SA2196.
$^e$ yefM-yoeB-sa2, SA2245 and SA2246.

$^f$ Identities and similarities to confirmed (type II) and putative (type I) \textit{S. aureus} homologues (16,20, 54–57). T, putative toxin; A, putative antitoxin.

\textbf{FIG 3} (Top) Alignment of MazF and MazE from \textit{S. equorum} (seq) SE3 with their \textit{S. aureus} (sa) COL homologues (SACOL2058 and SACOL2059). (Middle) Genetic organization of the \textit{mazEF}-sigB locus in \textit{S. equorum} SE3. Gene synteny is identical in \textit{S. aureus} COL (not shown). (Bottom) Transcriptional start point of \textit{mazE} in \textit{S. equorum} SE3 (seq) compared to \textit{S. aureus} COL (sa) (14,18). Letters “a” and “b” and the corresponding arrows indicate inverted repeats surrounding the $\mathbf{-35}$ region, putatively representing SarA binding sites. Repeat “a” contains a one-base insertion (gray “A”) in comparison to repeat “b”. “…$^+1$” indicates the transcriptional starting point, which is 50 bp upstream of the \textit{mazE seq} start codon.
observation of concomitant CFU values revealed that the expression of MazFseq exerted a bacteriostatic but not a bactericidal effect on *E. coli*, as reflected by rather constant colony counts up to 8 h after induction (Fig. 4B). This observation is in agreement with results from the work of Syed et al. (60), when expression of *Streptococcus mutans* mazF induced growth arrest in *E. coli*. Of note, Fu and colleagues (16) reported bacteriostatic effects upon mazFsa overexpression in *S. aureus*. In our experiments, heterologous overexpression of mazFseq in *S. aureus* from inducible plasmids did not lead to a growth defect (unpublished results). This was possibly due to cross-interaction of the *S. aureus* antitoxin orthologue with the *S. equorum* toxin or due to weaker expression than that in the previous study, in which a different induction system for mazFsa had been used (16).

**MazFseq interacts with MazEseq in vivo.** To check for protein-protein interaction between MazEseq and MazFseq a bacterial two-hybrid analysis using the BACTH system was conducted (41). It makes use of two fragments of adenylate cyclase (T18 and T25) that form an active enzyme only when in close proximity, thus providing evidence for interaction of the fused proteins. Catabolite gene activator protein (CAP)/cAMP-dependent activation of lactose utilization in *E. coli* is visualized by blue colonies on X-Gal-containing medium. Pronounced blue coloring was evident with strains producing MazFseq C terminally fused to the T25 fragment with either N- or C-terminally fused MazEseq-T18, suggesting that MazFseq is bound by MazEseq in vivo (Fig. 5). This adds to data from the work of Fu et al. (16) demonstrating complex formation of purified MazEsa and MazFsa components in vitro. Indeed, the interaction of cognate toxin and antitoxin pairs under nonstress conditions is a characteristic trait of type II TA systems (61), as exemplified by hexameric (MazF2-MazE2-MazF2) complexes in *E. coli* (62). Extensive negative controls yielded colorless colonies, ruling out unspecific β-galactosidase activation observed with the mazE-mazF plasmid couples.

**MazFseq is a sequence-specific endoribonuclease that preferentially cleaves within the UACAU motif.** To determine the catalytic activity and sequence specificity of MazFseq an affinity-purified C-terminally hexahistidine-tagged fusion protein (Fig. 6A) was incubated with 3.5-kb MS2 phage RNA in the presence of CspA in vitro, followed by primer extension reactions. In total, seven restriction sites could be determined (Fig. 6B). In five of these cases, UACAU sites were cleaved, mainly after and occasionally before the first uracil. Of note, one out of six UACAU motifs in MS2 RNA appeared to be unaffected (Fig. 6B, C, and D; not all data are shown). In addition, cleavage was observed at the one-base aberrant sequence U\H11625CAU, as well as

**FIG 4** Growth behavior of *E. coli* DH5α(pASK-IBA3) or DH5α(pASK-IBA3 mazFseq). Induction with 0.4 μM ATc at t = 1 h is indicated by arrows. Diamonds connected by solid lines represent growth of *E. coli* DH5α(pASK-IBA3), and circles and dashed lines represent growth of DH5α(pASK-IBA3 mazFseq). Filled symbols indicate noninduced strains, whereas open symbols denote cultures grown in the presence of ATc. (A) Optical density measurement. (B) CFU analysis.

**FIG 5** Bacterial two-hybrid study to confirm the specific in vivo interaction of MazFseq with MazEseq. Negative controls (sectors 1 to 6), also including components of the putative YeM-YoeB-seq1 TA system, did not show blue coloration, whereas the leucine zipper positive control (sector 9) yielded colored colonies. Strains BTH101(pUT18-mazE pKT25-mazF) (sector 7) and BTH101(pUT18C-mazE pKT25-mazF) (sector 8) showed considerable blue coloration, indicating protein-protein interaction.
as at CG/C/ACU (Fig. 6B). To rule out sequence specificity extending the five-base motif, synthetic RNA oligonucleotides containing UACAU sites flanked by different combinations of bases were treated with MazFseq. Restrictions were observed at UACAU sequences preceded by either U or C at the 5' end and trailed by either C, A, or G at the 3' end (Fig. 6B). Specific MazFseq-dependent restriction in all of these cases suggested that the main MazFseq recognition site is the pentad sequence UACAU, in agreement with the S. aureus orthologue (19). Regarding the high degree of similarity between MazFseq and MazFsa, this finding is not unexpected; instead, it speaks in favor of UACAU as the major restriction site of both staphylococcal MazF RNA interferases, after an initial report on MazFsa cleavage specificity had reported on a slightly different motif (16). In agreement with studies by Zhu et al. (19,52), cleavage of some UACAU sites by MazFseq in vitro was enhanced in the presence of CspA, evidently due to the removal of higher RNA structures that might impede access of MazFseq to the target site.

We note that the affinity-purified fraction of the MazFseq protein had not been purified to homogeneity (Fig. 6A). To confirm that the RNase activity observed in the in vitro primer extension assays was solely conferred by MazFseq, in vivo primer extensions were conducted. For this, total RNA was extracted from E. coli BW25113 cells in which MazFseq had been induced before. Primer extensions were done using the mRNAs of ompF and tufA, which are confirmed targets for MazF from Firmicutes expressed in E. coli (50). Judging from respective RNA preparations, defined bands were visible at the UACAU sites directly after the first U in the in vivo primer extensions (Fig. 7A and B). These signals were missing in the absence of MazFseq, backing up the results from the in vitro primer extension assays and suggesting that the specific cleavage of the mRNA is due to the activity of the MazFseq protein.

### FIG 6
Sequence-specific RNA cleavage of purified MazFseq-(His)_6. (A) SDS-PAGE of MazFseq-(His)_6 (15.0 kDa) purified by Ni-NTA affinity chromatography. MazFseq-(His)_6 protein is indicated by the arrow. (B) Confirmed MazFseq-(His)_6 target sites in MS2 RNA. (C and D) In vitro primer extensions of MS2 phage RNA subjected to MazFseq-(His)_6 treatment with different radioisotope-labeled primers. In both cases, RNA restriction (arrowheads) was more pronounced in the presence of CspA. Restriction occurred before or after the 5' uracil of the recognition sequence. Labeling of the sequencing ladder is complementary to the chain terminator deoxyxynucleotide triphosphate (ddNTP) used (e.g., ddATP for U lane, etc.). (E) Synthetic RNA oligonucleotides containing the UACAU sequence (preceded and trailed by different bases) were incubated with purified MazFseq-(His)_6 to verify that the recognition site is confined in length to the pentad sequence UACAU. All four test oligonucleotide RNAs were cut by MazFseq-(His)_6, with the resulting RNA fragments indicated by arrowheads.

### FIG 7
In vivo primer extension experiments for MazFseq carried out with different primers (OmpF_ecn and TufA_ecn) and RNA templates prepared from E. coli BW25113 carrying pBAD33 (empty) or carrying pBAD33 SD mazFseq (mazFseq). Lanes 1, empty Ara; lanes 2, empty Ara; lanes 3, mazFseq Ara; lanes 4, mazFseq + Ara. Reverse transcription from the RNA of BW25113 cells with either the empty pBAD33 vector or the uninduced MazFseq construct revealed no cleavage at the UACAU sites, whereas mRNA cleavage at UACAU could be observed in the presence of MazFseq (arrowheads). The result for only one of the two UACAU sites in tufA RNA is shown here. The sequencing ladder was done from E. coli BW25113 genomic DNA, and the figure inscription represents the bases from the template strand, not the ddNTPs used (e.g., the T lane was created with ddATP etc.).
Elevated local concentrations of MazF<sub>seq</sub> target sites in coding sequences. To screen for mRNAs that might be particularly susceptible to MazF<sub>seq</sub> cleavage in <i>S. equorum</i>, a statistical analysis of the occurrences of the UACAU motif in its 2,581 possible CDSs was conducted as previously described (19, 52). According to the equation detailed in Materials and Methods, Table 3 summarizes the top 10 candidate sensitive MazF<sub>seq</sub> target genes and their closest <i>S. aureus</i> orthologues. A number of the putative target CDSs code for hypothetical proteins, whereas the others are likely involved in catabolism of sugars, amino acids, and lipids. Intriguingly, the CDS of rsbV, putatively coding for an anti-anti-sigma<sup>B</sup> factor, might also have a higher susceptibility to MazF<sub>seq</sub> due to the unusually high abundance of UACAU sites (three copies within 327 bases). Assuming negative regulation of mazEF<sub>seq</sub> expression by sigma<sup>B</sup> as in <i>S. aureus</i> (14), the conceivable cleavage of rsbV by MazF<sub>seq</sub> would add a coherent second mode of mazEF<sub>seq</sub> control. As in <i>S. aureus</i> (18), a lack of RsbV would arguably result in unbound anti-sigma<sup>B</sup> factor RsbW, which might thus complex with sigma<sup>B</sup>. This would abolish the repressive effect of sigma<sup>B</sup> on mazEF<sub>seq</sub> expression to impose a positive feedback loop. These assumptions warrant further studies, which may also confirm target genes of MazF<sub>seq</sub> and unravel the regulation of mazEF<sub>seq</sub> activity.

Conclusions. To our knowledge, this study describes the first characterization of a putative TA system in a nonpathogenic <i>Staphylococcus</i> species. We show that in <i>S. equorum</i> SE3, the mazEF<sub>seq</sub> genes are expressed and are highly similar in sequence, length, position, synteny, and TSP to their <i>S. aureus</i> counterparts. Although both the trigger(s) and the function(s) of the MazEF system in <i>S. equorum</i> remain enigmatic to date, regulation of staphylococcal pathogenicity, as proposed by Zhu et al. (19), is probably not the sole function of this TA system, which has orthologues in at least seven further <i>Staphylococcus</i> species (our unpublished results). In the future, it will be interesting to reveal the instances of activity control, and indeed, besides sigB <i>S. equorum</i> also possesses sarA, clpP, and clpC orthologues (unpublished results) possibly involved in regulating MazF<sub>seq</sub> abundance.

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