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Overexpression of $MazF_{Sa}$ in *Staphylococcus aureus* Induces Bacteriostasis by Selectively Targeting mRNAs for Cleavage▽

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The role of chromosomally encoded toxin-antitoxin (TA) loci in bacterial physiology has been under debate, with the toxin proposed as either an inducer of bacteriostasis or a mediator of programmed cell death (PCD). We report here that ectopic expression of $MazF_{Sa}$, a toxin of the TA module from *Staphylococcus aureus*, led to a rapid decrease in CFU counts but most cells remained viable as determined by differential Syto 9 and propidium iodide staining after $MazF_{Sa}$ induction. This finding suggested that the toxin $MazF_{Sa}$ induced cell stasis rather than cell death. We also showed that $MazF_{Sa}$ selectively cleaves cellular mRNAs in vivo, avoiding "important" transcripts such as *recA*, *gyrB*, and *sarA* mRNAs in $MazF_{Sa}$-induced cells, while these three mRNAs can be cleaved in vitro. The results of Northwestern blotting showed that both *sarA* and *recA* mRNAs bind strongly to a putative RNA-binding protein. These data suggest that *S. aureus* likely undergoes stasis by protecting selective mRNA with RNA-binding proteins upon the expression of $MazF_{Sa}$ in vivo.

Many bacteria have chromosomally encoded toxin-antitoxin (TA) loci in which the toxin and antitoxin genes exist in an operon and are coexpressed to form a TA complex. The toxin is stable, while the antitoxin is labile and can be degraded in vivo by host proteases (e.g., ClpP or Lon in *Escherichia coli*). Under conditions of stress whereby transcription of the TA operon is repressed and which hence preclude the continuous synthesis of the labile antitoxin, the more-stable toxin can unleash its toxic effect to inhibit cell growth. However, metabolic stresses, such as amino acid and carbon source starvation, have been shown to induce transcription of *E. coli mazEF* and other TA loci in *E. coli* (7, 9, 14). Studies with several toxin systems indicate that many toxins are probably sequence-specific endoribonucleases. For instance, MazF of *E. coli* cleaves mRNA at ACA sites both in vitro and in vivo (30), while the RelE toxin, also from *E. coli*, cleaves mRNA positioned at the ribosomal A site both in vitro and in vivo, with cleavage occurring between the second and third bases of the A site codon (UAR, where R is usually G or A) (22). The PemK toxin from plasmid R100 in *E. coli* also cleaves mRNA at the UAH site (where H is A, C, or U) (29). ChpBK from *E. coli* cleaves at ACD (where D is A, G, or U) in single-stranded mRNA (31), whereas EndoA, a *Bacillus subtilis* MazF homolog, cleaves mRNA at the UAC site (23). Recently, two MazF homologs from *Mycobacterium tuberculosis* were also found to be endoribonucleases, with one cleaving mRNA at UAC triplets and the other at U-rich regions (32).

Ectopic overexpression of MazF in *E. coli* has been shown to reduce cell viability as measured by CFUs (1, 2, 16). However, cell viability could be fully restored by the expression of the antitoxin MazE after induction of the MazF toxin (21). This cell viability phenomenon has been known to be reversible for up to ~6 h after toxin induction, beyond which there is a point of no return and a gradual loss of colony formation (2). Overexpression of MazF in *E. coli* blocks protein synthesis by degrading many cellular mRNAs, eventually leading to growth arrest (30). More recently, Suzuki et al. (28) developed a single-protein production system based on MazF-induced cells which exhibited growth arrest but retained the full spectrum of biosynthetic functions required for transcription and translation of a codon-modified mRNA for up to 4 days.

The physiological roles of toxin proteins remain ill defined. Although various propositions have been advanced, two theories remain most prominent, with one contending that TA modules induce programmed cell death (PCD) and the other supporting the role of MazF in causing bacterial stasis. The evidence for the first model comes from studies of the *mazEF* TA genes in *E. coli*, located downstream of *relA*. The *relA* gene encodes a ppGpp synthase 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, 14, 21). In *E. coli* strain MC4100, overproduction of ppGpp by hyperexpressing RelA′, a truncated version of ppGpp synthase, represses *mazEF* promoter expression. Based on the above-described evidence, these authors suggested that stress-induced upregulation of ppGpp in *E. coli* strain MC4100 reduced synthesis of the labile MazE antitoxin and promoted its degradation in the presence of an activated ClpPA system and, hence, unleashed the toxic effect of MazF to advance to PCD (1).

Another model, proposed by Gerdes et al. (14), supported the role of TA systems in bacterial stasis under conditions of stress. In support of their hypothesis, Christensen et al. (6) found transcription of *mazEF* to increase during amino acid starvation induced by serine hydroxamate, accompanied by no cell killing. Pedersen et al. (21) also showed that the toxicity of MazF in *E. coli* is reversible and can be rescued by the antitoxin MazE within 6 h after MazF induction. Another TA

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module, HipAB, from *E. coli* was recently shown to induce a bacteriostatic state by the expression of the toxin HipA, which can also be reversed by the expression of its cognate antitoxin, HipB (18). Finally, the toxin of an orphan family of TA loci, evolutionarily unrelated to previously described TA families and found in plasmids of the Inc18 group, induces loss of cell proliferation in both *Bacillus subtilis* and *E. coli*, resulting in viable but nonreplicative cells within a defined time window beyond which cell death might ensue (19). Based on these studies, these authors proposed that the MazF toxin may not induce cell killing but rather may promote bacterial stasis.

In a recent study, we showed that the two open reading frames immediately upstream of the *sigB* operon in *Staphylococcus aureus*, herein designated $mazE_{Sa}$ and $mazF_{Sa}$, represent a TA system (11). We demonstrated that the toxin $MazF_{Sa}$ is a sequence-specific endoribonuclease which cleaves model substrate *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. Overexpression of $MazF_{Sa}$ was also found to reduce the CFU counts (11), while coexpression of $MazEF_{Sa}$ had no effect on reducing CFUs. In this report, we demonstrate that ectopic overexpression of $MazF_{Sa}$ induces *S. aureus* to undergo bacteriostasis rather than cell death. Importantly, $MazF_{Sa}$ does not cleave all mRNAs containing VUUV′ sequences. The mRNAs of some essential genes and also a global regulator are found to be protected in vivo. We thus propose that this "protective effect" is mediated by RNA-binding proteins, since these mRNAs, while protected in vivo, can be cleaved by $MazF_{Sa}$ in vitro. Thus, our report here suggests the selectivity of $MazF_{Sa}$ for target mRNAs and helps explain why the expression of toxin is not immediately bactericidal and, hence, can be reversed by the expression of antitoxin within a defined time window. Additionally, our data also explain why cells undergoing MazF-induced growth arrest can still retain transcriptional and translational competence.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We used *E. coli* strains DH5α and BL21(DE3)/pLysS and *Staphylococcus aureus* ALC6094 for these studies. *S. aureus* ALC6094 is a derivative of strain Newman that contains the *lacUV5*-regulated T7 polymerase gene integrated into the *geh* locus in the chromosome (8, 11). For our studies with $MazF_{Sa}$, ALC6094 was transformed with the plasmid pG164, which carries an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 promoter driving the expression of $mazF_{Sa}$ (8, 11). In some studies, we also deployed a *sarA* deletion mutant of ALC6094. Cultures were routinely grown in LB for *E. coli* and in Trypticase soy broth or O3GL medium for *S. aureus*, with aeration at 37°C. For pulse-chase labeling experiments, *S. aureus* ALC6094 was grown in chemically defined medium (CDM) as described previously (15). The media were supplemented with either ampicillin (70 μg/ml) or chloramphenicol (10 μg/ml). Rifampin (rifampicin) at 200 μg/ml was also used to determine RNA decay in the culture at an optical density at 650 nm (OD_{650}) of 0.4.

Bacterial viability assay. Bacteria were stained with membrane-permeant Syto 9 and membrane-impermeant propidium iodide by using a Live/Dead *Bac*Light bacterial viability kit (Molecular Probes, Eugene, OR). The kit is based on a combination of two probes; Syto 9 is a membrane-permeant nucleic acid stain (green fluorescence at 530 nm upon excitation at 488 nm) used to label all cells, whereas propidium iodide (red fluorescence at 620 nm upon excitation at 488 nm) enters only cells with compromised membranes. Bacteria with intact cell membranes indicative of live cells were stained fluorescent green, whereas bacteria with damaged membranes were stained fluorescent red due to acquisition of propidium iodide. The green/red fluorescence ratios were quantitated in an FL600 fluorescence reader (BioTek Instruments, Winooski, VT). The percentage of live cells was calculated from the standard curve according to the manufacturer's protocol. In brief, appropriate dilutions from the samples were prepared, stained with Live/Dead probes, and measured for fluorescence in triplicate. Culture that was heat-killed at 60°C for 1 h was the positive control and was defined as dead cells, whereas a fresh culture without any $MazF_{Sa}$ induction was defined as 100% live cells. Suspensions containing various proportions of live and dead cells (0, 10, 50, 90, or 100%) were used to prepare the standard curves (relationship between the percentage of live bacteria and the green/red fluorescence ratio). The analysis of relative viability of the samples was derived from the standard curves.

Construction of plasmids. Using *S. aureus* Newman chromosomal DNA as the template, the following genes or gene fragments were amplified by PCR: *sarA*, *recA* (600 bp of the C terminus), *hla*, and *sigB* (GenBank accession number NC_002745). The list of primers used for these and other studies described below can be obtained from the authors. The PCR products were digested with NcoI and BamHI and cloned into the NcoI and BamHI sites of pET14b (Novagen) to produce pET14b-sarA, pET14b-recA, pET14b-hla, and pET14b-sigB. The construction of plasmids pG164-MazF(His_6) and pETDuet1-MazEF(His_6), encoding $MazF_{Sa}$ and $MazEF_{Sa}$, respectively, was described previously (11). We also constructed a derivative of pG164-MazF(His_6) in which a fragment containing a modified pEPSA5 promoter (10) driving the *sarA* coding region was cloned downstream of the transcription terminator of *mazF*. The expression from the modified pEPSA5 promoter lacking the XylR repressor was moderate but constitutive, while the expression from the T7 promoter in pG164 remained IPTG-inducible (8, 10, 11). The recombinant plasmid, designated pG164-MazF(His_6)-pEPSA5-sarA, was then introduced into *S. aureus* strain RN4220 by electroporation. The plasmid harvested from RN4220 was then transformed into *S. aureus* strain ALC6094 and its isogenic *sarA* mutant. All of the above DNA techniques were performed according to standard procedures (25).

Protein expression and purification. To express $MazF_{Sa}(His_6)$, $MazE_{Sa}$ and $MazF_{Sa}(His_6)$ were coexpressed in *E. coli* BL21(DE3)/pLysS harboring the plasmid pETDuet1-MazEF(His_6) after IPTG induction (1 mM) for 6 h. The cells were harvested and subjected to lysis by ultrasonification. The MazE-$MazF_{Sa}$-(His_6) complex was purified with a nickel-nitrilotriacetic acid resin affinity column (Novagen) according to the manufacturer's protocol. $MazF_{Sa}(His_6)$ was further purified from the denatured MazE-$MazF_{Sa}(His_6)$ complex (in 6 M guanidine-HCl) and refolded by stepwise dialysis as described previously (11).

Production of anti-$MazF_{Sa}$, anti-SarA, and anti-SigB antibodies and Western blotting. The purified $MazF_{Sa}(His_6)$ protein (100 μg) was used to immunize three BALB/c × SJL/J (F1 cross) mice to obtain the anti-$MazF_{Sa}$ antibodies as described previously (5). Anti-SarA and anti-SigB monoclonal antibodies were obtained as described previously (4, 5). Western blot analysis was carried out on cellular lysates as described previously (11, 12).

In vitro cleavage of mRNA by $MazF_{Sa}$. The *sarA* and *recA* mRNAs were transcribed from BamHI-linearized pET14b-sarA and pET14b-recA plasmids by using a T7 large-scale transcription kit (Promega) as described previously (11). Five micrograms of mRNAs was digested with 15 pmol of $MazF_{Sa}$ at 37°C for various time periods in 20-μl reaction mixtures containing 40 U RNase inhibitor, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 1 mM dithiothreitol (DTT). The reaction mixture was then extracted with phenol-chloroform, ethanol precipitated to remove proteins, and subjected to agarose gel electrophoresis, followed by detection of the cleaved fragments by Northern blotting with [α-^{32}P]dCTP-radiolabeled *sarA* and *recA* DNA probes (see below).

Northern blot hybridization. Total RNA from *S. aureus* cells was prepared by using Trizol (Invitrogen, CA) and a reciprocating shaker with 0.1-mm silica-zirconia beads as previously described (3). For detection of specific transcripts, gel-purified DNA probes were radiolabeled with [α-^{32}P]dCTP by use of a random-primed DNA-labeling kit (Roche Diagnostics GmbH) and hybridized under aqueous-phase conditions at 65°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).

Pulse-chase labeling of de novo protein synthesis with [^{35}S]methionine. *S. aureus* ALC6094 harboring pG164-MazF(His_6) was inoculated for overnight growth in O3GL medium at 37°C, followed by 1:100 dilution into the CDM without methionine the next morning. When the culture reached an OD_{650} of 0.4, IPTG (1 mM) was added to induce the expression of $MazF_{Sa}$, while another aliquot without IPTG was used as the noninduced culture control. At selected time intervals (0 to 90 min) after induction, 1 ml of the induced or noninduced culture was taken and added to a test tube containing 15 μCi [^{35}S]methionine. After isotopic labeling for 15 min at 37°C, 0.2 ml of regular methionine (40 mg/ml) was added to chase the culture for 5 min. The labeled cells were pelleted and washed with Tris-buffered saline (TBS), followed by resuspension in lysis buffer (TBS containing 2 mM phenylmethylsulfonyl fluoride proteinase inhibitor and 50 mM EDTA). The cells were then lysed with 0.1-mm silica-zirconia beads

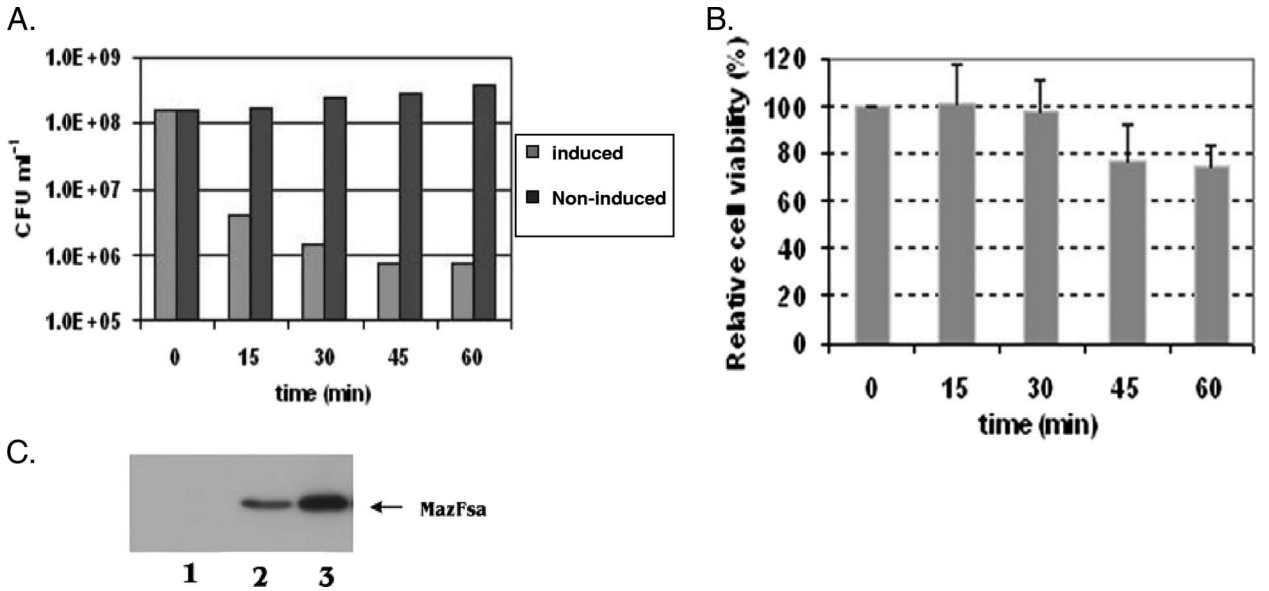

FIG. 1. Reduction of the CFU counts by ectopic overexpression of MazF$_{Sa}$. (A) Reduction of CFUs with *S. aureus* ALC6094 harboring pG164-MazF(His$_6$) under IPTG induction (1 mM) at an OD$_{650}$ of 0.4. To determine the CFUs, samples were withdrawn at various time points as indicated, diluted, spread on TSA plates supplemented with chloramphenicol (10 μg/ml), and incubated at 37°C overnight. The results are those from one typical experiment out of three similar independent experiments. (B) Relative cell viability. Cultures induced at an OD$_{650}$ of 0.4 with or without IPTG (1 mM) were assessed for percentage of cell viability with a Live/Dead *Bac*Light bacterial viability kit at indicated time points, as described in Materials and Methods. The percentage of viable cells from the induced culture at each time point was further compared to that of the noninduced culture to arrive at the relative cell viability. The results represent those of three similar independent experiments. Error bars show standard deviations. (C) Western blot analysis of MazF$_{Sa}$ expression after IPTG (1 mM) induction at an OD$_{650}$ of 0.4. Twenty micrograms of cell lysate proteins from each time point was resolved by SDS-PAGE, transferred to PVDF membrane, and probed with the anti-MazF$_{Sa}$ antibodies as described in Materials and Methods. Lanes 1 to 3 show cell lysates prepared after induction with IPTG for 0, 30, or 60 min, respectively.

in a reciprocating shaker as previously described (3). The clarified supernatant was treated with human immunoglobulin G (200 μg/ml) for 1 h with gentle mixing at 4°C to facilitate binding to protein A. Anti-SarA or anti-SigB monoclonal antibody was then added to immunoprecipitate SarA or SigB protein by gentle mixing at 4°C overnight. A 100-μl (50% slurry) amount of goat polyclonal antibody to mouse conjugated to immunoglobulin G-agarose (Abcam) was then added to pull down the immunoprecipitate by mixing the agarose beads with the reaction mixture for 1 h at 4°C. The sample was pelleted by centrifugation at 200 × *g* for 1 min and washed with TBS. Fifty microliters of sample buffer was added to the agarose beads, followed by heating at 100°C for 5 min to release the proteins. Samples were then resolved by SDS-polyacrylamide gel electrophoresis (PAGE). For the detection of resolved radiolabeled proteins, the gel was fixed with a fixing solution (isopropanol/water/acetic acid [25:65:10]) for 30 min, followed by soaking in Amplify solution (Amersham) with agitation for 15 to 30 min. The gel was then dried for autoradiography.

Northwestern blotting. Total-cell lysate from *S. aureus* was prepared by lysing cells in the lysis buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.0) with 0.1-mm silica-zirconia beads in a reciprocating shaker as previously described (3). ^{32}P-labeled mRNA probes were transcribed from BamHI-linearized pET14b-sarA, pET14b-recA, pET14b-hla, and pET14b-sigB plasmids by using a T7 large-scale transcription kit (Promega) according to the manufacturer's protocol. Northwestern blotting was carried out with a modified protocol as previously described (24). In brief, cell lysates (20 μg) were resolved on 16% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane. Proteins on the membrane were denatured in denaturing buffer (50 mM Tris-HCl, pH 8.3, 50 mM DTT, 1 mM EDTA, 1 M guanidine-HCl) for 1 h at room temperature. The membrane was then washed twice with renaturing buffer (50 mM Tris-HCl, pH 7.5, 2 mM DTT, 2 mM EDTA, 0.5 M NaCl, 0.1% Tween 20, 10% glycerol) for 1 h at room temperature, and proteins were allowed to renature in the renaturing buffer at 4°C for 24 h. The membrane was then blocked with blocking solution (0.02% bovine serum albumin, 30 mM HEPES-NaOH, pH 8.0, 20 μg/ml yeast tRNA) for 1 h at room temperature and hybridized with 2 ng/ml of ^{32}P-labeled RNA probes (5 × 10^6 cpm) in binding buffer (30 mM HEPES-NaOH, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 20 μg/ml yeast tRNA) for 3 h at room temperature. The membrane was washed three times with washing buffer (30 mM HEPES-NaOH, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA), air dried, and exposed to films to visualize bands that represented putative RNA-binding proteins.

RESULTS

Overproduction of MazF$_{Sa}$ induced cell stasis rather than cell death. To determine the extent to which ectopic overexpression of MazF$_{Sa}$ affects *S. aureus*, we examined its induction by IPTG in *S. aureus* ALC6094 harboring pG164-MazF(His$_6$) at different growth phases and the effects on CFU counts and cell viability. Cultures showed normal growth under the uninduced conditions (Fig. 1A). When MazF$_{Sa}$ was induced in early logarithmic phase (at an OD$_{650}$ of 0.4), the CFU counts decreased rapidly, with 99.5% of the original CFU lost at 60 min postinduction (Fig. 1A). To ensure that MazF$_{Sa}$ was indeed expressed in the induced culture, we monitored and detected successful expression of MazF$_{Sa}$ on immunoblots with anti-MazF$_{Sa}$ antibodies (Fig. 1C). To examine whether the cells associated with loss of CFU were indeed dead, the induced and noninduced cultures were further examined for viability with a *Bac*Light Live/Dead bacterial viability assay kit (Molecular Probes), which stains membrane-compromised dead bacteria fluorescent red due to the uptake of propidium iodide. Although there was a ~100-fold difference in CFU counts between the induced and noninduced cultures at an OD$_{650}$ of 0.4 at 60 min postinduction, the difference between the cultures in cell death was less than 27% (Fig. 1B). These results suggest that cells exposed to ectopic overexpression of MazF$_{Sa}$ are not killed in the first hour but instead are induced to undergo stasis.

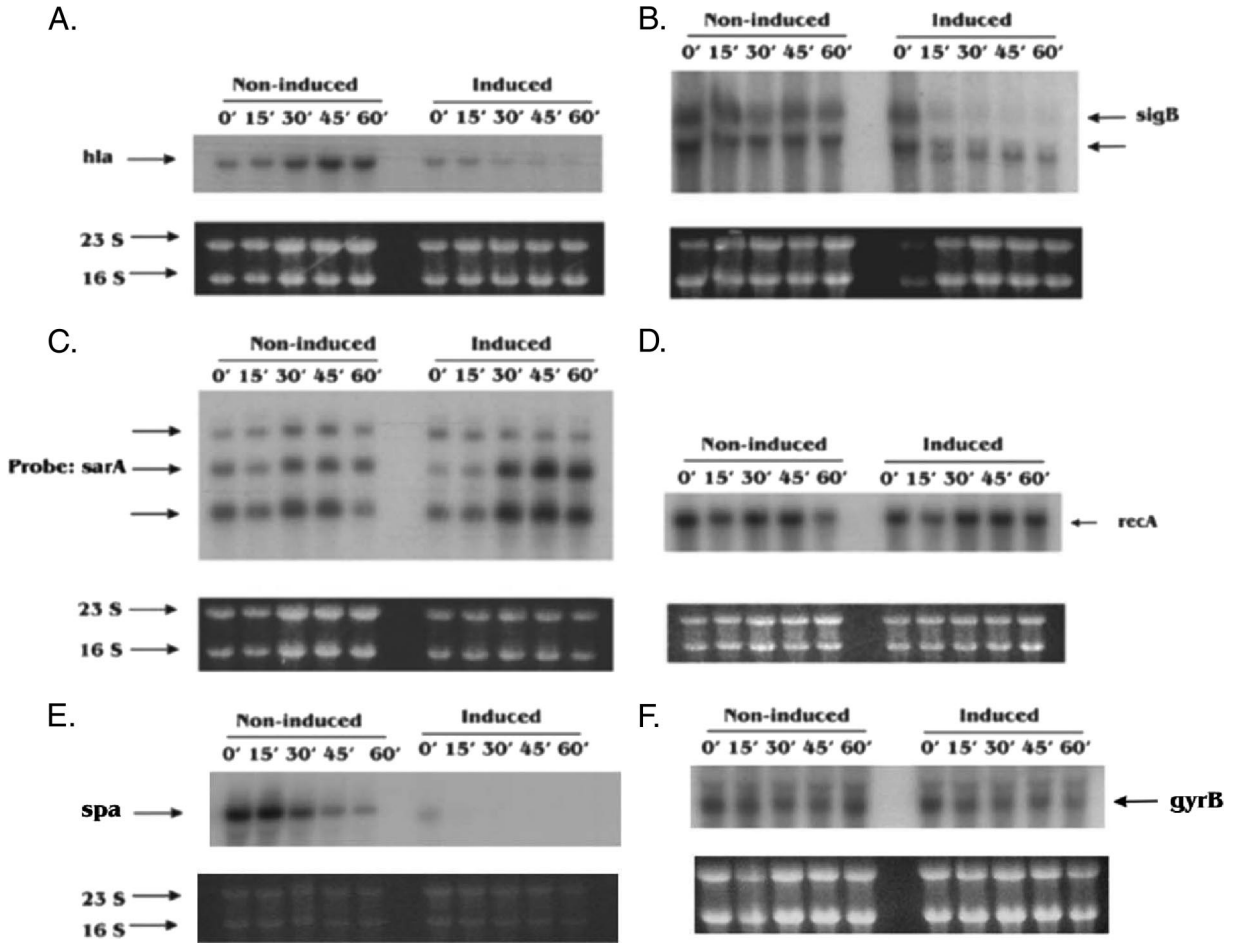

FIG. 2. Degradation of mRNAs upon ectopic overexpression of $MazF_{Sa}$. A culture of *S. aureus* ALC6094 harboring pG164-MazF(His_6) was induced at an OD_{650} of 0.4 with IPTG (1 mM), while the noninduced culture contained no IPTG. Total cellular RNAs were extracted at the indicated time points (minutes). Northern blot analysis was carried out with specific probes as described in Materials and Methods. (A to F) Transcripts were detected with *hla*, *sigB*, *sarA*, *recA*, *spa*, or *gyrB* probes. The data represent one typical experiment out of three similar independent experiments. The 23S and 16S bands served as the internal loading controls.

$MazF_{Sa}$ cleaves cellular mRNAs selectively. The overexpression of toxins from TA modules, such as *relBE*, *mazEF*, *hipAB*, and εζ, has been shown to be associated with a bacteriostatic state which can be fully reversed upon the expression of their cognate antitoxins (18, 19, 21). To examine this bacteriostatic state in *S. aureus*, we focused on the degradation of cellular mRNAs upon ectopic overexpression of $MazF_{Sa}$, which targets mRNA at the VUUV′ site. Northern blotting was carried out to analyze total cellular RNAs extracted at different time points after the induction of $MazF_{Sa}$ as described in Materials and Methods. Six transcripts representing different gene categories (e.g., virulence, stress, regulator, and essential genes) that contain a number of VUUV′ sites (where V and V′ are A, C, or G and may or may not be identical) were chosen to evaluate the degradation of mRNAs after induction of $MazF_{Sa}$ at an OD_{650} of 0.4. The transcript of the virulence gene *hla*, which has 45 VUUV′ sites, was found to degrade rapidly, being barely detectable at 60 min postinduction (Fig. 2A). The mRNA of another virulence gene, *spa*, was degraded in a manner similar to that of *hla* mRNA (Fig. 2E). The *sigB* transcript, which has 39 VUUV′ sites and encodes the stress-induced transcription factor SigB within an operon, was also cleaved after 30 min of induction with $MazF_{Sa}$ (Fig. 2B). Remarkably, the transcript of the essential gene *recA*, which has 46 VUUV′ sites, was found to be stable against $MazF_{Sa}$-mediated degradation (Fig. 2D), as was the *gyrB* transcript (Fig. 2F), even at 60 min postinduction. Additionally, the three transcripts associated with the global regulator *sarA*, which has 24 VUUV′ sites, were preserved and possibly slightly upregulated after 30 min of induction (Fig. 2C). Notably, the 16S and 23S rRNAs were quite stable against $MazF_{Sa}$ cleavage in vivo, with no appreciable changes in their band intensities on an agarose gel after exposure to $MazF_{Sa}$ for 60 min, beginning at an initial OD_{650} of 0.4 (Fig. 2, bottom panels), thus indicating that rRNAs were protected from $MazF_{Sa}$ cleavage in vivo, most likely by ribosomal proteins (30). When an *S. aureus* culture containing only the pG164 vector control (i.e., no $MazF_{Sa}$) was induced with IPTG, Northern blots with the above gene probes showed the same pattern as that of the uninduced control (data not shown), thus indicating that induction of the T7 polymerase gene with IPTG by itself did not preserve or upregulate *sarA* transcripts (e.g., by repressing a

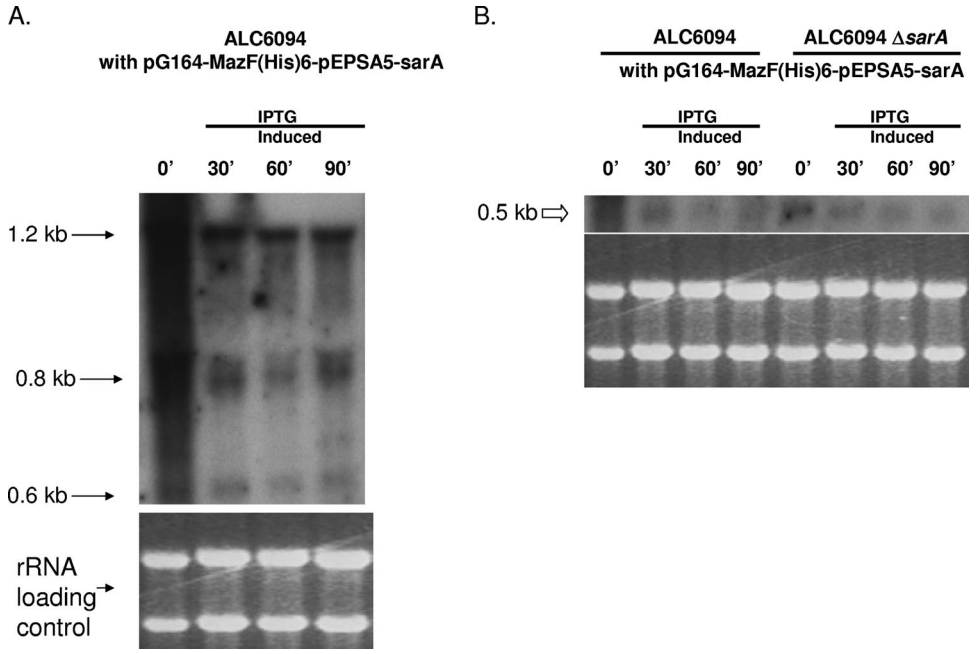

FIG. 3. Northern blot analysis of *sarA* transcripts in Newman derivative ALC6094 and its isogenic *sarA* deletion mutant carrying pG164-MazF(His$_6$)-pEPSA5-sarA. (A) The 1.2-, 0.8-, and 0.6-kb transcripts (long arrows) represent the native *sarA* transcripts from three distinct *sarA* promoters, which, as expected, were preserved upon MazF$_{Sa}$ induction. (B) The ~0.5-kb transcript (open arrow) represents the *sarA* transcript as driven from the modified pEPSA5 promoter in the plasmid pG164-MazFsa-pEPSA5-sarA. This transcript was preserved in the parental strain ALC6094 and the isogenic *sarA* mutant. Bottom, rRNA loading control. Total cellular RNAs were extracted at the indicated time points (minutes).

repressor or activating an activator), nor did it cause degradation of the mRNA transcripts we tested.

To further confirm that induced MazF$_{Sa}$ did not cleave an mRNA encoding a repressor of the *sarA* promoter, we cloned in *E. coli* the *sarA* coding region downstream of a modified pEPSA5 promoter lacking the XylR repressor (10), thus resulting in mild constitutive expression of *sarA* from the modified pEPSA5 promoter. This fragment was then cloned downstream of the *mazF* transcription terminator in pG164-MazF(His$_6$) to yield the recombinant plasmid pG164-MazF-(His$_6$)-pEPSA5-sarA containing an IPTG-inducible T7 promoter and a modified pEPSA5 promoter. The plasmid construct was then introduced into ALC6094 and its isogenic *sarA* mutant. Total cellular RNAs were then extracted from these cells before and after IPTG induction. Northern analyses of total cellular RNA with a *sarA* probe encompassing only the *sarA* coding region disclosed that the *sarA* transcripts, sized at 1.2, 0.8, and 0.6 kb and originating from the native triple promoters, were still preserved in the parental strain ALC6094 upon MazF$_{Sa}$ induction (Fig. 3A). Notably, the *sarA* transcript originating from the modified pEPSA5 promoter in the recombinant plasmid, ~0.5 kb in size (Fig. 3B), was preserved in the parental strain ALC6094 and in the isogenic *sarA* mutant. As this 0.5-kb transcript was transcribed from a heterologous promoter, this finding suggested that the preservation of this transcript upon MazF$_{Sa}$ induction could not have arisen from MazF-mediated cleavage of a transcript encoding a repressor that targets the native *sarA* promoter. Taken together, the above results suggest that overexpression of MazF$_{Sa}$ in *S. aureus* does not cleave all cellular mRNAs, since some mRNAs coding for selective virulence regulator or essential gene products were not targeted by MazF$_{Sa}$. This scenario where some gene transcripts are protected from MazF$_{Sa}$ cleavage is more compatible with cell stasis than with cell death.

De novo synthesis of SarA and SigB upon ectopic overexpression of MazF$_{Sa}$. To confirm that proteins could still be synthesized in the face of MazF$_{Sa}$ inhibition and to verify that the cleavage of selective mRNA truly leads to inhibition of de novo synthesis of the corresponding protein, we performed pulse-chase labeling of de novo-synthesized SarA and SigB with [^{35}S]methionine upon ectopic overexpression of MazF$_{Sa}$. The SarA and SigB proteins were chosen for the preservation and destruction of their mRNAs, respectively. Briefly, total-cell lysates from both induced and noninduced cultures were immunoprecipitated with specific anti-SarA and anti-SigB monoclonal antibodies and resolved on SDS-polyacrylamide gels. The radiolabeled de novo-synthesized SarA and SigB were detected as described in Materials and Methods. The expression of SigB was normal in the noninduced culture, with no significant changes in the band intensities at 30 min (Fig. 4A); in contrast, inhibition of SigB synthesis was observed at 30 min after the induction of ectopic overexpression of MazF$_{Sa}$, and after 90 min, the band could barely be discerned (Fig. 4A). In the case of SarA, the protein continued to be synthesized in the noninduced culture but was upregulated after MazF$_{Sa}$ induction (Fig. 4B). These data demonstrated that the *sarA* mRNA, being resistant to MazF$_{Sa}$ cleavage, can be continuously translated to SarA protein in the face of inhibition by MazF$_{Sa}$. These results are consistent with the Northern blot analysis shown in Fig. 2, in which the *sigB* mRNA was degraded shortly after overexpression of MazF$_{Sa}$, while *sarA*

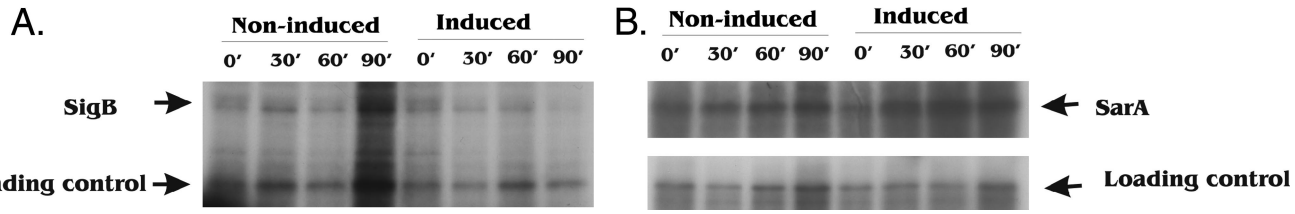

FIG. 4. Pulse-chase labeling of de novo-synthesized SarA and SigB with [^{35}S]methionine upon ectopic overexpression of $MazF_{Sa}$. A culture of *S. aureus* ALC6094 harboring pG164-MazF(His_6) was induced at an OD_{650} of 0.4 with IPTG (1 mM), while the noninduced culture received no IPTG. New protein synthesis was then monitored by isotopic labeling with [^{35}S]methionine for 15 min under induced or noninduced conditions at indicated intervals (0 to 90 min) after IPTG induction. The cells were then lysed and immunoprecipitated with anti-SigB monoclonal antibody (A) or anti-SarA monoclonal antibody (B) as described in Materials and Methods. Equivalent amounts of cell lysate, derived from equal culture volumes, were subjected to SDS-PAGE, followed by autoradiography. The labeled unknown protein that was immunoprecipitated by anti-SigB and anti-SarA antibodies served as the internal loading control.

mRNA was preserved or moderately upregulated in induced cultures. It is important to note that an unknown protein, which was pulled down nonspecifically by anti-SigB and anti-SarA antibodies, could be used as the loading control because it was constantly synthesized in noninduced and induced cultures.

***sarA* and *recA* mRNAs were degraded following rifampin treatment.** To examine if decreased degradation of *sarA* and *recA* mRNAs or increased degradation of *hla* and *sigB* transcripts was attributable to mRNA stability or instability, respectively, we performed an RNA stability assay by adding rifampin to halt de novo mRNA synthesis, as described in Materials and Methods. The decay of the mRNAs was monitored by Northern blot analysis of total cellular RNA isolated from different time points after the addition of rifampin. As shown in Fig. 5B, the *recA* mRNA disappeared within 2 min after the addition of rifampin, while the *sarA* mRNA was slightly more stable, with a half-life of >2 min (Fig. 5A). Both mRNAs were barely detectable after 10 min (Fig. 5A and B). The half-lives and decay of *hla* and *sigB* mRNAs after rifampin treatment were similar to those of *recA* mRNA (Fig. 5C and D). Interestingly, the rRNA after rifampin treatment with the above blots appeared to be relatively stable (Fig. 5A to D, lower panels). This may be due to protection by ribosomal binding proteins or the possibility that the relatively high quantity of 16S and 23S ribosomal RNAs might have taken longer to degrade than particular transcripts in lower quantities. Collectively, our data indicate that the relative stability of the *sarA*

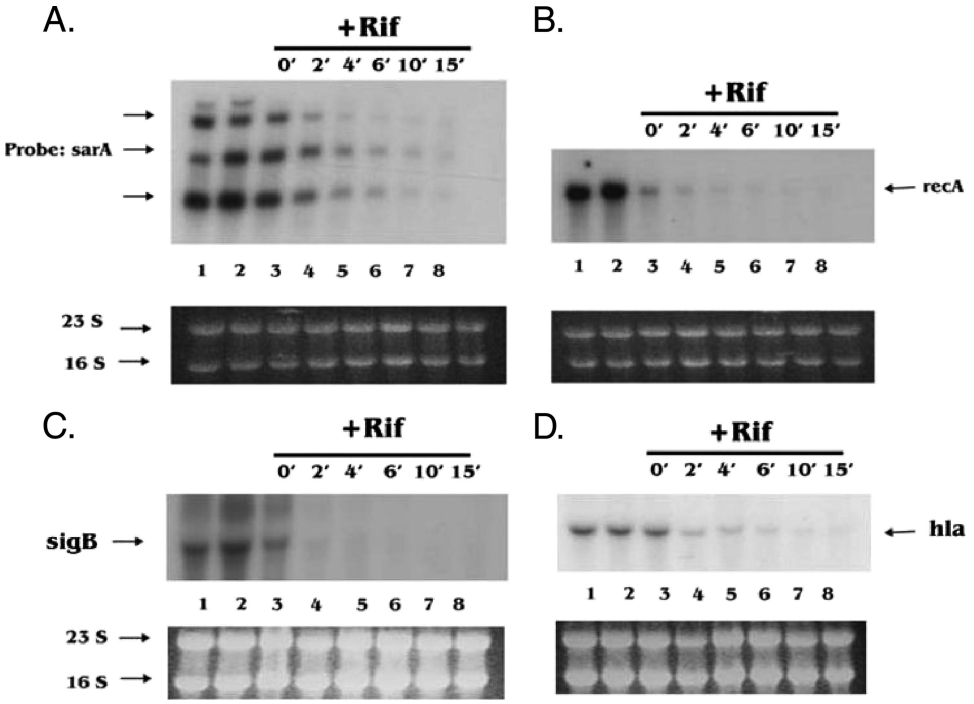

FIG. 5. Decay of RNAs upon blocking of de novo RNA synthesis with rifampin. Rifampin (200 μg/ml) was added 30 min after the culture containing *S. aureus* ALC6094 harboring pG164-MazF(His_6) reached an OD_{650} of 0.4. RNA was extracted from control samples at time zero (time when culture reached an OD_{650} of 0.4) and at 15-min time points, shown in lanes 1 and 2, respectively, in panels A to D. Lanes 3 to 8 in panels A to D show the RNA extracted after the addition of rifampin for 0, 2, 4, 6, 10, and 15 min. (A to D) Transcripts detected with *sarA*, *recA*, *hla*, and *sigB* probes. 23S and 16S rRNA served as the internal loading controls.

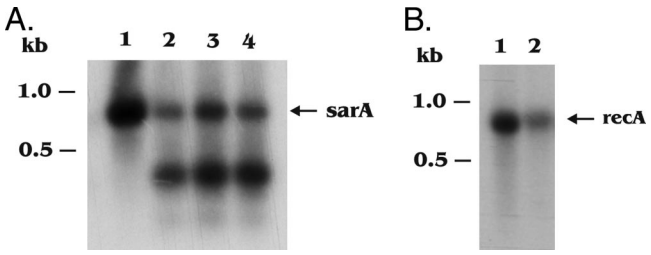

FIG. 6. Northern blot analysis of the in vitro cleavage of *sarA* and *recA* mRNAs by MazF$_{Sa}$. The *sarA* and *recA* mRNAs were transcribed from BamHI-linearized pET14b-sarA and pET14b-recA plasmids by using a T7 large-scale transcription kit (Promega) and digested with purified MazF$_{Sa}$ as described in Materials and Methods. Amounts of 5 μg of *sarA* and *recA* mRNAs were digested with 15 pmol MazF$_{Sa}$ at times indicated for panels A and B. (A) *sarA* mRNA cleavage by MazF$_{Sa}$. Lane 1, no MazF$_{Sa}$; lanes 2 to 4, digestion with MazF$_{Sa}$ for 60, 90, and 120 min, respectively. (B) *recA* mRNA cleavage by MazF$_{Sa}$. Lane 1, no MazF$_{Sa}$; lane 2, digestion with MazF$_{Sa}$ for 60 min. The cleaved fragments were detected with the ^{32}P-radiolabeled *sarA* and *recA* DNA probes in Northern blots as described in Materials and Methods.

and *recA* mRNAs in the presence of MazF$_{Sa}$ is not likely due to the stability of their structure, since their degradation by RNase in the cells upon rifampin treatment is very similar to that of *sigB* and *hla*. As a *sarA* transcript arising from a modified pEPSA5 promoter in place of the native *sarA* promoter was also protected from MazF$_{Sa}$ cleavage (Fig. 3B), we thus propose that the *sarA* and, possibly, *recA* mRNAs are likely protected by RNA-binding proteins in vivo. In contrast, the *hla* and *sigB* mRNAs were not protected from MazF$_{Sa}$-mediated cleavage in *S. aureus*.

In vitro cleavage of *sarA* and *recA* mRNAs by MazF$_{Sa}$. Since both *sarA* and *recA* mRNAs contain the VUUV′ cleavage sites for MazF$_{Sa}$, we sought to determine if these two mRNAs can be cleaved by MazF$_{Sa}$ in vitro. For this experiment, *sarA* and *recA* mRNAs generated with an in vitro transcription kit, as described in Materials and Methods, were digested by purified MazF$_{Sa}$. To enhance the sensitivity of the assay, we detected cleaved mRNA fragments by Northern blotting, using either *sarA* or *recA* DNA probes. Although there are multiple predicted VUUV′ cleavage sites in the *sarA* mRNA, only one major site was cleaved efficiently in a time-dependent manner (Fig. 6A). The remaining uncleaved sites are likely to be protected by the secondary structure of *sarA* mRNA, as MazF$_{Sa}$ has been shown to cleave duplex RNA inefficiently (11). The C terminus of the truncated *recA* mRNA contains multiple VUUV′ sequences which were cleaved more completely by MazF$_{Sa}$, yielding smaller fragments that were not readily detectable with Northern blots (Fig. 6B). These results suggest that the *sarA* and *recA* mRNAs can be cleaved by MazF$_{Sa}$ in vitro. In addition, their stability in vivo cannot be readily explained by multiple RNA duplexes or longer half-lives of the mRNA, but rather, these mRNAs may be protected from MazF$_{Sa}$ by RNA-binding proteins, similar to what has been observed with protection of rRNA by ribosomal proteins (30).

Northwestern analysis of the *sarA* and *recA* mRNA-binding proteins. To investigate if RNA-binding protein(s) exists for *sarA* and *recA* mRNAs, we performed a Northwestern blot RNA-binding assay in which total-cell lysates from cultures both induced and uninduced for MazF$_{Sa}$ were resolved on SDS-polyacrylamide gels, transferred to PVDF membranes, and probed with the radiolabeled *sarA* or *recA* mRNAs as described in Materials and Methods. The *sarA* mRNA probe detected a putative RNA-binding protein band of ~60 kDa that increased in intensity upon induction of MazF$_{Sa}$ for 60 to 90 min when compared with the amounts in noninduced controls (Fig. 7A, lanes 5 to 7). The *recA* mRNA probe also detected an mRNA-binding protein, with a pattern similar to that of the one probed with *sarA* mRNA (Fig. 7C). However, this increase in band intensity was not observed in the *hla* and *sigB* mRNA probes upon MazF$_{Sa}$ induction (Fig. 7B and D). The identification of the putative 60-kDa RNA-binding protein is ongoing. Collectively, these results suggest that RNA-binding protein(s) may be responsible for the stability of *sarA* and *recA* mRNAs in the presence of MazF$_{Sa}$.

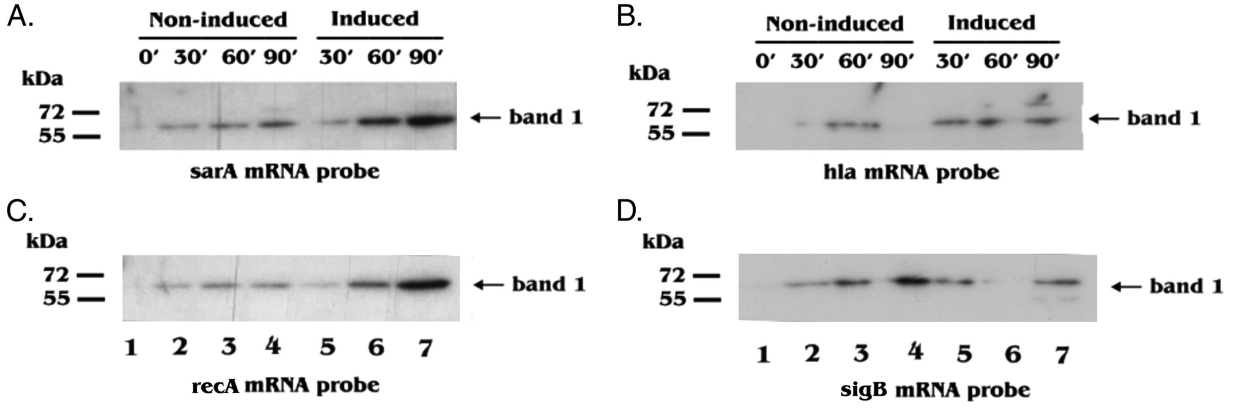

FIG. 7. Northwestern blot analysis of cell lysates with *sarA*, *recA*, *hla*, and *sigB* mRNA probes. A culture of *S. aureus* ALC6094 harboring pG164-MazF(His$_6$) was induced at an OD$_{650}$ of 0.4 with IPTG (1 mM), while the control culture contained no IPTG. Samples were taken at 0, 30, 60, and 90 min. Amounts of 20 μg of the total-cell lysates from indicated time points (minutes) were resolved on 16% SDS-polyacrylamide gels and transferred to a PVDF membrane as described in Materials and Methods. The blots were then denatured, renatured, and hybridized with the ^{32}P-labeled *sarA* mRNA (A), *hla* mRNA (B), *recA* mRNA (C), and *sigB* mRNA (D) probes. The major protein detected (band 1) is indicated on the right of each panel; the protein size markers are shown on the left.

DISCUSSION

The primary physiological role of TA systems has been the subject of much debate. One model proposes that TA loci induce bacterial PCD, while another favors the status of bacteriostasis upon toxin induction during periods of stress. Previously, we characterized *mazEF*$_{Sa}$, two genes immediately upstream of the *sigB* operon in *S. aureus*, as a TA system. The MazF$_{Sa}$ toxin is an endoribonuclease that cleaves sequence-specific mRNA at VUUV′ sites. Ectopic overproduction of MazF$_{Sa}$ inhibits cell growth (11), while the concomitant expression of MazE$_{Sa}$ can reverse this effect. In this study, we demonstrate that the growth arrest induced by MazF$_{Sa}$ is attributable to bacterial stasis. Although there is a loss of CFU, most of the bacteria exposed to MazF$_{Sa}$ for 1 h in our study are not dead, as demonstrated by the bacterial viability assay. We focused on the 1-h induction period because significant numbers of plasmid-free cells emerged after the first hour, which would affect the interpretation of the Live/Dead bacterial viability assay, as well as the total cellular RNA decay analysis.

The standard assay used to measure cell death (i.e., the inability to form colonies) cannot distinguish between cell death and bacterial stasis, wherein the cells are viable but cannot replicate to form colonies. For this reason, we deployed two assays to distinguish these two possibilities after MazF$_{Sa}$ induction. In the CFU count assay, we found that, compared with the colony-forming activities of noninduced controls, ~99.5% of the cells failed to replicate as colonies upon MazF$_{Sa}$ induction during the early logarithmic phase (at an OD_{650} of 0.4) (Fig. 1A), but the difference between the induced and noninduced cultures in the viability assay with the Live/Dead *Bac*Light kit was significantly less (<27%) (Fig. 1B).

It has been previously suggested that MazF of *E. coli* selectively degrades almost all cellular mRNAs in vivo, thus causing a precipitous drop in total protein synthesis and, hence, complete growth arrest (30). However, both 16S and 23S rRNAs of the *E. coli* cells were protected from such cleavage even though they contained a number of target cleavage sites for MazF of *E. coli*. Interestingly, both rRNAs were degraded in vitro by MazF of *E. coli* once the rRNA preparations were extracted with phenol, indicating that the resistance of rRNA to MazF cleavage in vivo may be due to protection by ribosomal proteins (30). Despite growth arrest attributable to the toxin, the cells still managed to maintain de novo synthesis of proteins from mRNAs resistant to MazF$_{Sa}$ cleavage (Fig. 4). Suzuki et al. also reported that MazF-induced cells retain the full spectrum of biosynthetic functions necessary to support mRNA transcription and translation for up to 4 days (28). These results suggest that MazF-induced cells undergo stasis rather than cell death. However, if the cells are exposed to high levels of toxin for a prolonged period of time (i.e., over 6 h with MazF in *E. coli*), the effect can be irreversible. This result can be explained by a scenario where prolonged bacteriostasis might eventually lead to cell death due to pleiotropic effects (2).

In another TA system, Lioy et al. (19) showed that overexpression of the ζ toxin triggers bacteriostasis without gross inhibition of protein translation, which can be rescued by the expression of its cognate ε antitoxin within a time frame of 4 h. However, we were puzzled by the discrepancy between the MazF-mediated degradation of almost all cellular mRNA and the preservation of the biosynthetic competence in *E. coli*. In this study, we found that induced MazF$_{Sa}$ cleaves cellular mRNAs selectively in *S. aureus*, with gene transcripts of essential genes (*recA* and *gyrB*) and also of the regulatory gene *sarA* not cleaved efficiently in vivo, while these transcripts can clearly be cleaved in vitro. The stability of the *recA* and *sarA* transcripts against MazF$_{Sa}$ is not due to the stability of their structures or their longer half-lives, as these two mRNAs were degraded rapidly by intrinsic RNases upon the halting of de novo synthesis of mRNAs by rifampin (Fig. 5A and B). Based on the precedent that 16S rRNA is stable by virtue of its protection by ribosomal proteins (30), it is likely that *recA*, *gyrB*, and *sarA* mRNAs are protected by RNA-binding proteins.

One potential caveat about our studies is that the level of MazF$_{Sa}$ expression was significantly higher than the level of MazF$_{Sa}$ achieved as a result of the proteolytic destruction of the antitoxin MazE$_{Sa}$. We took this approach because the native antitoxin MazE$_{Sa}$ was left undisturbed in our studies. Instead, we relied on the imbalance between the levels of MazF$_{Sa}$ and the antitoxin MazE$_{Sa}$ to exert the toxic effect. Thus, it is difficult to assess if our observations here will be similar to those found under physiological conditions.

The stabilities of different mRNA species in bacteria vary widely. Thus, it is not surprising that the stability of individual transcripts is often the target of regulatory processes in bacterial cells as a means of target gene modulation in vivo. Different mRNAs are protected against intracellular ribonucleases to various degrees (13). The mRNA half-lives of some genes are influenced by interaction with various RNA-binding proteins, forming ribonucleoprotein complexes. One of the RNA-binding complexes, containing the chaperon GroE, has been shown to mediate mRNA protection against RNase E in *E. coli* (13). Based on this observation, we speculate that some gene transcripts might also be protected from MazF-mediated cleavage by RNA-binding proteins. Indeed, we found a putative protein binding to both labeled *sarA* and *recA* mRNA probes in our Northwestern blot analysis (Fig. 7). However, the binding specificity of this protein to *sarA* or *recA* mRNA is not yet clear; nonetheless, the expression of this protein band was upregulated upon induction of MazF$_{Sa}$ for 60 to 90 min (Fig. 7), while this effect was much less with *hla* and *sigB* mRNA probes. Although there are other proteins that bound *hla* and *sigB* mRNA, as well as *recA* and *sarA* mRNA, it is conceivable that they might represent general RNA-binding proteins under physiological conditions or that they are nonspecific binding proteins.

Collectively, our findings suggest that MazF$_{Sa}$-induced *S. aureus* cells likely undergo stasis by protecting some essential and regulatory gene transcripts against MazF$_{Sa}$, probably by means of RNA-binding proteins. Accordingly, we predict that a strategy that blocks the activity of the RNA-binding proteins may usher *S. aureus* toward cell death rather than cell stasis.

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REFERENCES

1. **Aizenman, E., H. Engelberg-Kulka, and G. Glaser.** 1996. An *Escherichia coli* chromosomal "addiction module" regulated by guanosine [corrected] 3′,5′-bispyrophosphate: a model for programmed bacterial cell death. Proc. Natl. Acad. Sci. USA **93:**6059–6063.
2. **Amitai, S., Y. Yassin, and H. Engelberg-Kulka.** 2004. MazF-mediated cell death in *Escherichia coli*: a point of no return. J. Bacteriol. **186:**8295–8300.
3. **Cheung, A. L., K. J. Eberhardt, and V. A. Fischetti.** 1994. A method to isolate RNA from gram-positive bacteria and mycobacteria. Anal. Biochem. **222:** 511–514.
4. **Cheung, A. L., Y. Chien, and A. S. Bayer.** 1999. Hyperproduction of alpha-hemolysin in a *sigB* mutant is associated with elevated SarA expression in *Staphylococcus aureus*. Infect. Immun. **67:**1331–1337.
5. **Chien, Y., A. C. Manna, and A. L. Cheung.** 1998. SarA level is a determinant of *agr* activation in *Staphylococcus aureus*. Mol. Microbiol. **30:**991–1001.
6. **Christensen, S. K., K. Pedersen, F. G. Hansen, and K. Gerdes.** 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. J. Mol. Biol. **332:**809–819.
7. **Condon, C.** 2006. Shutdown decay of mRNA. Mol. Microbiol. **61:**573–583.
8. **D'Elia, M. A., M. P. Pereira, Y. S. Chung, W. Zhao, A. Chau, T. J. Kenney, M. C. Sulavik, T. A. Black, and E. D. Brown.** 2006. Lesions in teichoic acid biosynthesis in *Staphylococcus aureus* lead to a lethal gain of function in the otherwise dispensable pathway. J. Bacteriol. **188:**4183–4189.
9. **Engelberg-Kulka, H., R. Hazan, and S. Amitai.** 2005. *mazEF*: a chromosomal toxin-antitoxin module that triggers programmed cell death in bacteria. J. Cell Sci. **118:**4327–4332.
10. **Forsyth, R. A., R. J. Haselbeck, K. L. Ohlsen, R. T. Yamamoto, H. Xu, J. D. Trawick, D. Wall, L. Wang, V. Brown-Driver, J. M. Froelich, C. G. Kedar, P. King, M. McCarthy, C. Malone, B. Misiner, D. Robbins, Z. Tan, Z. Y. Zhu, G. Carr, D. A. Mosca, C. Zamudio, J. G. Foulkes, and J. W. Zyskind.** 2002. A genome-wide strategy for the identification of essential genes in Staphylococcus aureus. Mol. Microbiol. **43:**1387–1400.
11. **Fu, Z., N. P. Donegan, G. Memmi, and A. L. Cheung.** 2007. Characterization of $MazF_{Sa}$, an endoribonuclease from *Staphylococcus aureus*. J. Bacteriol. **189:**8871–8879.
12. **Fu, Z. B., K. L. Ng, T. L. Lam, and W. K. Wong.** 2005. Cell death caused by hyper-expression of a secretory exoglucanase in *Escherichia coli*. Protein Expr. Purif. **42:**67–77.
13. **Georgellis, D., B. Sohlberg, F. U. Hartl, and A. von Gabain.** 1995. Identification of GroEL as a constituent of an mRNA-protection complex in *Escherichia coli*. Mol. Microbiol. **16:**1259–1268.
14. **Gerdes, K., S. K. Christensen, and A. Lobner-Olesen.** 2005. Prokaryotic toxin-antitoxin stress response loci. Nat. Rev. Microbiol. **3:**371–382.
15. **Gertz, S., S. Engelmann, R. Schmid, K. Ohlsen, J. Hacker, and M. Hecker.** 1999. Regulation of sigmaB-dependent transcription of *sigB* and *asp23* in two different *Staphylococcus aureus* strains. Mol. Gen. Genet. **26:**558–566.
16. **Hazan, R., B. Sat, and H. Engelberg-Kulka.** 2004. *Escherichia coli mazEF*-mediated cell death is triggered by various stressful conditions. J. Bacteriol. **186:**3663–3669.
17. **Kolodkin-Gal, I., R. Hazan, A. Gaathon, S. Carmeli, and H. Engelberg-Kulka.** 2007. A linear pentapeptide is a quorum-sensing factor required for *mazEF*-mediated cell death in *Escherichia coli*. Science **318:**652–655.
18. **Korch, S. B., and T. M. Hill.** 2006. Ectopic overexpression of wild-type and mutant *hipA* genes in *Escherichia coli*: effects on macromolecular synthesis and persister formation. J. Bacteriol. **188:**3826–3836.
19. **Lioy, V. S., M. T. Martin, A. G. Camacho, R. Lurz, H. Antelmann, M. Hecker, E. Hitchin, Y. Ridge, J. M. Wells, and J. C. Alonso.** 2006. pSM19035-encoded zeta toxin induces stasis followed by death in a subpopulation of cells. Microbiology **152:**2365–2379.
20. **Manna, A. C., and A. L. Cheung.** 2006. Expression of SarX, a negative regulator of *agr* and exoprotein synthesis, is activated by MgrA in *Staphylococcus aureus*. J. Bacteriol. **188:**4288–4299.
21. **Pedersen, K., S. K. Christensen, and K. Gerdes.** 2002. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol. Microbiol. **45:**501–510.
22. **Pedersen, K., A. V. Zavialov, M. Y. Pavlov, J. Elf, K. Gerdes, and M. Ehrenberg.** 2003. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. Cell **112:**131–140.
23. **Pellegrini, O., N. Mathy, A. Gogos, L. Shapiro, and C. Condon.** 2005. The *Bacillus subtilis ydcDE* operon encodes an endoribonuclease of the MazF/PemK family and its inhibitor. Mol. Microbiol. **56:**1139–1148.
24. **Popovic, Z., and D. M. Templeton.** 2005. A Northwestern blotting approach for studying iron regulatory element-binding proteins. Mol. Cell. Biochem. **268:**67–74.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. **Sat, B., R. Hazan, T. Fisher, H. Khaner, G. Glaser, and H. Engelberg-Kulka.** 2001. Programmed cell death in *Escherichia coli*: some antibiotics can trigger *mazEF* lethality. J. Bacteriol. **183:**2041–2045.
27. **Schukkink, R. F., and R. H. Plasterk.** 1990. TcA, the putative transposase of the C. elegans Tc1 transposon, has an N-terminal DNA binding domain. Nucleic Acids Res. **18:**895–900.
28. **Suzuki, M., J. Zhang, M. Liu, N. A. Woychik, and M. Inouye.** 2005. Single protein production in living cells facilitated by an mRNA interferase. Mol. Cell **18:**253–261.
29. **Zhang, J., Y. Zhang, L. Zhu, M. Suzuki, and M. Inouye.** 2004. Interference of mRNA function by sequence-specific endoribonuclease PemK. J. Biol. Chem. **279:**20678–20684.
30. **Zhang, Y., J. Zhang, K. P. Hoeflich, M. Ikura, G. Qing, and M. Inouye.** 2003. MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. Mol. Cell **12:**913–923.
31. **Zhang, Y., L. Zhu, J. Zhang, and M. Inouye.** 2005. Characterization of ChpBK, an mRNA interferase from *Escherichia coli*. J. Biol. Chem. **280:** 26080–26088.
32. **Zhu, L., Y. Zhang, J. S. The, J. Zhang, N. Connell, H. Rubin, and M. Inouye.** 2006. Characterization of mRNA interferases from *Mycobacterium tuberculosis*. J. Biol. Chem. **281:**18638–18643.