Functional Characterization of TcaA: Minimal Requirement for Teicoplanin Susceptibility and Role in *Caenorhabditis elegans* Virulence[∇]

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The inactivation of TcaA contributes to intrinsic teicoplanin resistance in experimental and clinical isolates of glycopeptide-intermediate resistant *Staphylococcus aureus*. PhoA fusions confirmed that TcaA is a transmembrane protein with a short intracellular N-terminal domain containing a C-4 zinc finger binding motif, a single membrane-spanning domain, and a large extracellular C-terminal domain. The region conferring teicoplanin susceptibility was narrowed down to the transmembrane part and the first third of the extracellular domain of TcaA, suggesting that neither the C-4 zinc finger binding motif nor the C terminus contributed to teicoplanin susceptibility. TcaA belongs to the cell wall stress stimulon, which comprises a set of genes universally upregulated by cell wall damage. Induction of *tcaA* was shown to be fully dependent on the two-component regulatory system VraSR. A 66-bp region upstream of the transcriptional start site, which contained an inverted repeat partially covering the promoter box, was shown to be essential for VraSR-mediated induction by cell wall stress. Interestingly, the induction or overexpression of *tcaA* did not contribute further to teicoplanin susceptibility, suggesting that small amounts of TcaA, such as those present under normal uninduced conditions, were sufficient for TcaA-mediated teicoplanin susceptibility. The strong attenuation of *tcaA* deletion mutants in a *Caenorhabditis elegans* survival assay suggested that TcaA may, in addition to affecting glycopeptide susceptibility, also play a role in virulence.

Glycopeptide antibiotics such as vancomycin and teicoplanin are currently the main first-line antibiotic therapy for multiresistant methicillin-resistant *Staphylococcus aureus* infections. *S. aureus* can acquire high-level glycopeptide resistance (vancomycin-resistant or glycopeptide-resistant *Staphylococcus aureus*) by horizontal transfer of the *vanA* resistance determinant from enterococci (9). However, clinical instances of this occurring have been rare, and the more common resistance phenotype is glycopeptide intermediate resistance (vancomycin-intermediate resistant *Staphylococcus aureus* or glycopeptide-intermediate resistant *Staphylococcus aureus* [GISA]), whereby exposure to glycopeptide antibiotics triggers intrinsic genetic changes that lead to decreased glycopeptide susceptibility (1, 16, 35).

Transcriptome profiling of GISA strains (11, 22, 26, 31) and mutagenesis studies (6, 7, 14, 19, 32, 34, 36, 37) have identified several structural and regulatory genes that can influence glycopeptide resistance levels; however, a common genetic mechanism driving the GISA phenotype has not been determined. Exposure of *S. aureus* to inhibitory concentrations of cell wall-active antibiotics, such as glycopeptides and β -lactams, triggers the induction of a group of genes collectively called the cell wall stress stimulon. The cell wall stress stimulon is thought to play an important role in GISA resistance, since several of the

components have been experimentally shown to influence glycopeptide resistance levels (26, 41). Additionally, several clinical GISA strains have shown the differential expression or induction of cell wall stress stimulon genes (26, 29), indicating that alterations in the regulation of these genes may affect resistance.

One of the members of the core *S. aureus* cell wall stress stimulon that has been linked to increased glycopeptide resistance, both experimentally and in clinical GISA isolates, is the *tcaA* gene. The deletion or disruption of *tcaA* or of the chromosomal region containing the *tcaRAB* operon in laboratory isolates was found to increase teicoplanin MICs by two- to fourfold (7). Correspondingly, resistance was reduced when a wild-type *tcaA* allele was introduced into two clinical GISA isolates with naturally occurring *tcaA* mutations (25).

TcaA is a predicted membrane protein; however, nothing is known about its cellular function or how its absence increases teicoplanin resistance. Therefore, we determined the membrane localization and topology of TcaA, the domains/regions of the protein involved in resistance, and the promoter region required for induction by the cell wall stimulus. TcaA was confirmed to be important for virulence in *Caenorhabditis elegans*, suggesting that it performs other cellular functions unrelated to glycopeptide resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. The strains were routinely cultured at 37°C in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI) and on LB or sheep blood agar and stored as frozen stocks in skim milk at –80°C. Kanamycin (50

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype ^a	Reference or source
Strains		
<i>S. aureus</i>		
RN4220	NCTC8325-4, r ⁻ m ⁺	20
COL	Ox ^r Tc ^r	13
BB1372	COL Ω 2026 Δ tcaRAB::Tn917 Em ^r	25
NM278	COL Δ tcaA	This study
BB1539	BB1372 pAW17-tcaA	25
BB1541	BB1372 pAW17	25
N315	Multiresistant clinical methicillin-resistant <i>S. aureus</i> isolate	23
KVR	N315 Δ vraSR Cm ^r	21
<i>E. coli</i>		
DH5 α	<i>supE44 ΔlacU169(ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Invitrogen
CC118	Δ (ara-leu)7679 Δ lacX74 Δ phoA20 <i>galE galK thi rpsE rpoB argE(Am) recA1</i>	24
Plasmids		
pAW17	<i>E. coli-S. aureus</i> shuttle plasmid, <i>ori</i> ColE1 <i>ori</i> pAM α 1 Km ^r	33
pAW17-tcaA	pAW17 containing <i>tcaA</i> gene from COL Km ^r	25
pKOR1	<i>E. coli-S. aureus</i> shuttle plasmid used to create markerless deletions; <i>repF</i> (Ts) <i>cat attP ccdB ori</i> ColE1 <i>bla PxyI/tetO secY570</i>	3
pHA-1	<i>E. coli</i> plasmid containing an arabinose-inducible promoter 5' of the signal sequence-less <i>phoA</i> reporter gene, Am ^r	12

^a Am^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Km^r, kanamycin resistant; Ox^r, oxacillin resistant; Tc^r, tetracycline resistant.

μg/ml), ampicillin (100 μg/ml), or erythromycin (5 μg/ml) was added to the medium when it was appropriate. Phage 80α was used for phage transductions.

Antibiotic resistance testing. Relative teicoplanin resistance levels were compared by swabbing 0.5 McFarland standard suspensions of strains across teicoplanin gradient plates. The MICs for teicoplanin were determined by an Etest method recommended to give high specificity and sensitivity for determining glycopeptide resistance levels (42). After 24 h of incubation at 35°C, Etest MICs were determined from brain heart infusion (BHI) plates containing kanamycin at 50 μg/ml that had been swabbed with 2.0 McFarland suspensions.

RNA extraction and Northern hybridization. RNA isolation and Northern blotting were performed as described previously (28). Ten micrograms of total RNA from each sample was separated through a 1.5% agarose–20 mM guanidine thiocyanate gel in 1× TBE (Tris-borate-EDTA) running buffer (15). The primers used for amplification of the digoxigenin (DIG)-labeled *tcaA*, produced by using a PCR DIG probe synthesis kit (Roche, Basel, Switzerland), have been described previously (25); and the position of the probe is shown in Fig. 1A.

Primer extension. RNA was extracted from cultures of strain BB1539 that were grown to an optical density (OD) of 1.0 and then stressed with 10 μg/ml of teicoplanin for 30 min. Primer extension was performed with 20 μg of total RNA and 3 pmol of primer PE1 (5'-GTACATATCTGTACATCATC-3') or PE2 (5'-ATACATATAACAATTGAAGAG-3') labeled at the 5' end with biotin (Fig. 1A), by using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Sequencing reactions were performed with a Thermo Sequenase cycle sequencing kit (U.S. Biochemicals, Cleveland, OH). A biotin chromogenic detection kit (Fermentas, Burlington, Ontario, Canada) was used for biotin detection.

Construction of markerless *tcaA* deletion and plasmid deletions. The in-frame markerless deletion of *tcaA* from the chromosome of *S. aureus* COL was performed by using pKOR1, as described by Bae and Schneewind (3). Deletion of the region spanning nucleotides 2411159 to 2412541 in the *S. aureus* COL genome was confirmed by PCR and sequencing across the deleted region. Portions of the *tcaA* promoter and coding regions were deleted from plasmid pAW17-tcaA by PCR. Long-range PCR amplification, performed with an Expand Long Template PCR system (Roche) and primers flanking but facing outward from the region to be deleted, was used to amplify the entire pAW17-tcaA plasmid, excluding the portion of the gene or the promoter to be removed. Recircularization of the remaining portion of the plasmid was then performed by ligation, which was facilitated by the inclusion of HindIII restriction sites at the 5' ends of both primers. All deletions were checked by sequencing across the region deleted. Deletion plasmids were first transformed into RN4220 and were then transduced into BB1372.

Membrane topology analyses. Membrane topology predictions were made by using several different web-based topology prediction algorithms, including

TMHMM (version 2.0; <http://www.cbs.dtu.dk/services/TMHMM-2.0/>), DAS (10), Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html), Split 4.0 (17), Phobius (18), and HMMTOP 2.0 (40).

PhoA fusions were constructed by cloning various regions of the *tcaA* gene downstream of the *araB* arabinose-inducible promoter and upstream of the signal sequence-less *phoA* gene in pHA-1. Regions of the *S. aureus* COL genome cloned to create the fusions spanned the following nucleotides: fusion 6, 2412539 to 2412396; fusion 5, 2412539 to 2412309; fusion 4, 2412539 to 2412012; and fusion 2, 2412539 to 2411160. The resulting plasmids were then transformed into *Escherichia coli* strain CC118. To assay PhoA activity, overnight cultures of fusion-containing strains were diluted 1:100 into fresh LB broth and grown to an OD of 600 nm (OD₆₀₀) of 0.5. The cultures were then split in two, 0.2% arabinose was added to one half, and both halves were incubated for a further 1 h. The cultures were then harvested, and PhoA activity was measured by a *p*-nitrophenylphosphate (Sigma-Aldrich, Steinheim, Germany) cleavage assay (30).

***C. elegans* survival assay.** Nematode survival assays were performed as described previously (39). *C. elegans* strain Bristol N2 was maintained at 15°C on *E. coli* OP50 grown on nematode growth medium (8). To prepare the assay plates, an overnight saturated culture of *S. aureus* in tryptic soy (TS) broth was diluted 1:10 in fresh TS broth, and 10 μl of the diluted culture was spread on a 35-mm plate containing TS agar supplemented with 5 μg/ml nalidixic acid. The plates were incubated at 37°C for 4 h and then allowed to equilibrate at room temperature before being seeded with worms. Thirty hermaphrodite nematodes at the L4 developmental stage were transferred to each assay plate (three plates for each strain tested), and survival was monitored at 25°C by light microscopy. Nematodes that died because they crawled off the plate were censored from the analysis. Survival was calculated by the Kaplan-Meier product-limit method, and survival differences were tested for significance by using the log-rank test (Graph-Pad Prism, version 4.0). *P* values <0.05 were considered statistically significant. Similar results were obtained from four independently replicated experiments.

RESULTS

***tcaA* induction.** The *tcaA* gene, a member of the *S. aureus* cell wall stress stimulon (26, 41), requires inhibitory antibiotic concentrations for induction (29). Uninduced *tcaA* transcription levels are very low, indicating weak *tcaA* expression under normal growth conditions (25). *S. aureus* strain N315 and *S. aureus* strain KVR, a Δ vraSR mutant of N315 (21), were used to determine if *tcaA* induction by cell wall antibiotics was

FIG. 1. Transcript induction and TSS mapping of *tcaA*. (A) Map of *tcaA* and the surrounding genomic region showing the position of the *tcaA* DIG-labeled probe used for Northern blots and the positions of primers PE1 and PE2 used for primer extension. (B) Northern blot showing *VraSR*-dependent induction of *tcaA* by inhibitory concentrations of glycopeptide and β -lactam antibiotics. N315 and its Δ *vraSR* deletion mutant KVR were grown to an OD_{600} of 0.5 and were either unstressed (lanes -) or stressed with teicoplanin (10 μ g/ml) (lanes T), vancomycin (10 μ g/ml) (lanes V), or oxacillin (2 $\times$ MIC) (lanes O) for 30 min before RNA extraction. Relative *tcaA* transcript levels are shown, and the corresponding ethidium bromide-stained 16S rRNA bands are shown below as an indication of RNA loading. (C) Determination of the TSSs of *tcaA* by primer extension. Two TSSs were detected and were located 2 nucleotides apart. The stronger signal detected with primer PE2 is indicated with a black arrow, and the weaker signal is indicated with a gray arrow. The same two TSSs with the same relative signal strengths were also obtained with primer PE1 (data not shown). The nucleotide sequences surrounding the TSSs are shown; the nucleotide corresponding to the stronger predicted TSS is in boldface and is indicated with an asterisk, and the nucleotide corresponding to the weaker predicted TSS is highlighted in gray. The predicted -10 box region is enclosed within a box.

dependent on the *VraSR* two-component sensor-transducer that is required for the induction of several members of the cell wall stress stimulon. Northern blots of RNA extracted from N315 and KVR unstressed and stressed with inhibitory concentrations of either teicoplanin (10 μ g/ml), vancomycin (10 μ g/ml), or oxacillin (concentrations corresponding to twice the MIC, i.e., 16 μ g/ml for N315 and 2 μ g/ml for KVR) showed strong *tcaA* induction by all three antibiotics in N315 but no induction in KVR (Fig. 1B).

Characterization of *tcaA* promoter region. Primer extension with primers PE1 and PE2 (Fig. 1A) identified two predicted transcriptional start sites (TSSs). The stronger TSS signal was 166 bp upstream of the predicted *tcaA* ATG start codon, and the second, weaker signal was a further 2 nucleotides upstream (Fig. 1C). The best potential RNA polymerase sigma factor consensus promoter sequence preceding the TSS was TTGAAC-N₁₄-TATAAT, which closely resembles the housekeeping σ^A promoter consensus sequence (TTGACA-N₁₆₋₁₈-TATAAT), although the spacer region of 14 bp is unusually short.

To identify the regions of the *tcaA* promoter required for *VraSR*-mediated induction of *tcaA* in response to cell wall stress, a series of *tcaA* promoter deletions was constructed in pAW17-*tcaA* (Fig. 2A). Plasmids containing the promoter deletions were introduced into the *tcaRAB* deletion mutant

BB1372 (7, 25). Northern blots were then used to determine if the promoter mutants had altered *tcaA* induction phenotypes when they were induced by inhibitory concentrations of teicoplanin (Fig. 2B). As expected, there was no induction in deletion 7, in which the entire promoter region had been removed. Deletion 6 was the only other deletion which abolished induction. As the induction of *tcaA* in deletion 1 was comparable to the induction in the wild-type pAW17-*tcaA* plasmid, the 27-bp region between deletions 1 and 6 must be required for induction. A notable feature of this region is that it contains half of a 13-bp inverted repeat ($\Delta G = -9.8$), the other half of which covers the -35 region of the predicted promoter consensus (Fig. 2C). The untranslated *tcaA* leader region, between the TSS and the ATG translational start codon, contained several short direct and inverted repeats (Fig. 2C), indicating that this region may be prone to genetic alterations. A BLAST search analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>), however, revealed that the entire 239-bp *tcaR-tcaA* intergenic region was highly conserved (>99% identity) in all currently available *S. aureus* genome sequences. Deletions in this region appeared to lead to increased *tcaA* transcription upon teicoplanin induction (Fig. 2B).

The same promoter deletion plasmids were also tested for their ability to restore teicoplanin susceptibility in *tcaRAB* de-

FIG. 2. *tcaA* promoter region required for induction. (A) Map of the intergenic region between *tcaR* and *tcaA* showing the positions of promoter deletions. The deletions encompass the regions between the following nucleotide coordinates from the genome of *S. aureus* COL: 1, 2412808 to 2412769; 2, 2412667 to 2412637; 3, 2412667 to 2412577; 4, 2412667 to 2412547; 6, 2412808 to 2412742; 7, 2412808 to 2412547; 9, 241269 to 2412577; and 10, 2412691 to 2412547. (B) Northern blot showing *tcaA* transcript induction from the intact *pAW17-tcaA* plasmid (BB1539) and each of the promoter deletion mutants. Strains were grown to an OD_{600} of 0.5 and were either unstressed (lanes –) or stressed with teicoplanin (10 μ g/ml) (lanes +) before RNA extraction. Ethidium bromide-stained 16S rRNA bands are shown beneath the transcripts as an indication of RNA loading. The gel at the bottom shows the lanes corresponding to promoter deletion 6 and is a longer exposure of a Northern blot showing that *tcaA* is still transcribed at a low level in deletion 6 and that transcription is not influenced by teicoplanin induction. (C) Expanded map of the region upstream of the TSS required for *tcaA* induction. The nucleotide positions marking the ends of deletion 1 and deletion 6 are indicated. The major predicted TSS is labeled, the predicted –10 and –35 promoter consensus sequences are underlined, and the arrows beneath the nucleotide sequence show the position of the inverted repeat. The positions of inverted and direct repeats present in the untranslated leader region between the TSS and the translational start site are also indicated by arrows, with each repeat differentially shaded. (D) Effect of promoter deletions on complementation of teicoplanin susceptibility. The resistance levels of BB1372 strains containing *pAW17-tcaA* promoter deletions were compared to those of BB1372 containing the intact plasmid (BB1539) and the empty *pAW17* plasmid (BB1541) by swabbing 0.5 McFarland standard suspensions of the strains across a teicoplanin gradient of 0 to 5 μ g/ml. MIC*, MIC values were determined by Etest by using 2.0 McFarland suspensions on BHI supplemented with kanamycin at 50 μ g/ml.

letion mutant BB1372. The gradient plate and the MICs in Fig. 2D show the differences in teicoplanin resistance when strain BB1372 was complemented with intact *pAW17-tcaA* (BB1539), the empty *pAW17* plasmid (BB1541), or the *pAW17-tcaA* promoter deletion mutants. Deletions 4, 7, and 10, all of which have disrupted ribosome binding sites, were the only deletions that could no longer complement teicoplanin susceptibility, presumably due to the abortive effect of the partial ribosome binding site deletion on *TcaA* translation. Deletion 6, which was defective in *tcaA* induction, was still able to complement teicoplanin susceptibility, suggesting that only small amounts of the *TcaA* protein, such as those present in uninduced cells, are sufficient to restore teicoplanin susceptibility.

***TcaA* protein structure prediction and membrane topology.** Web-based analyses of the secondary structure and topology of the *TcaA* protein sequence indicated that it is a transmembrane protein, with a short 50-amino-acid (aa) N terminus containing a C-4-type zinc finger motif, a single 20-aa membrane-spanning domain, and a large 390-aa C-terminal domain. Four of the six topology prediction programs used predicted that the N terminus was cytoplasmic and that the C terminus was extracellular.

PhoA fusions were used to confirm that *tcaA* was membrane spanning and to determine the topology. A set of plasmids containing various portions of the *tcaA* gene fused to *phoA* (Fig. 3A) were constructed in plasmid *pHA-1*, which contains a signal sequence-less *phoA* gene and the *araB* arabinose-inducible promoter, and introduced into *E. coli phoA* mutant strain CC118. The expression of *PhoA* activity requires disulfide bond formation, which occurs only in the periplasm of *E. coli*. Therefore, *PhoA* activity is detected only when the portion of the protein cloned in front of the signal sequence-less *PhoA* is directed to the periplasm during arabinose induction.

No *PhoA* activity was detected from fusion 6, which contained only the N-terminus region up until just before the transmembrane region, which indicated that this region was cytoplasmic. Fusion 5 (which contained the N terminus and the transmembrane region), fusion 4 (which contained approximately the first half of the gene), and fusion 2 (which contained the entire *tcaA* gene minus the last codon) all produced high *PhoA* activities when they were induced with arabinose (Fig. 3B), indicating that all of these fragments directed the *PhoA* reporter protein into the periplasm. Therefore, the *PhoA* fusion results were in agreement with the majority of the topology prediction results.

FIG. 3. Structure prediction and membrane topology of TcaA. (A) Map of *tcaA* showing the predicted N-terminal, transmembrane, and C-terminal domains. The position of the C-X₂-C-X₁₀-C-X₂-C sequence in the N terminus is indicated by vertical lines. The positions where *phoA* was fused to portions of the *tcaA* gene, creating fusions 2, 4, 5, and 6, are indicated by arrowheads. (B) Relative PhoA activities of fusions 2, 4, 5, and 6 with and without arabinose induction. The values shown represent the means $\pm$ standard deviations from three independent assays.

Regions of TcaA required for complementation of teicoplanin susceptibility. A series of in-frame deletions was constructed within pAW17-*tcaA* to identify the regions of TcaA needed to complement teicoplanin susceptibility in BB1372 (Fig. 4). The plasmid containing deletion A fully complemented BB1372, indicating that the N terminus of the protein, which contained the C-X₂-C-X₁₀-C-X₂-C motif, was not involved in the teicoplanin resistance phenotype. Conversely, deletion B, which encompassed the predicted transmembrane domain, abolished complementation, indicating that the membrane location of TcaA is important for teicoplanin susceptibility. Deletions C to H, sequential C-terminal deletion mutants, showed that removal of the C terminus up until the region between deletions E and F had minimal effects on complementation, while removal of the regions between deletion E and the transmembrane domain abolished complementation. Therefore, only the approximately 130-aa region indicated in Fig. 4A is needed to restore teicoplanin susceptibility in the *tcaA* mutant. Correspondingly, this essential region contained both of the previously described *tcaA* mutations in *S. aureus* clinical GISA isolates SA137/9G and MI (25).

Effect of TcaA on virulence in *C. elegans*. The nematode *C. elegans* can serve as a simple surrogate model host for the study of *S. aureus* infection (39). Recently, Bae et al. (2) listed *tcaA* and *tcaB* among 71 *bursa aurealis* transposon mutants of *S. aureus* strain Newman that were found to attenuate *C. elegans* killing. However, due to the complex transcriptional organization of the *tcaRAB* operon in the absence of antibiotic induction (25), we wanted to determine the effect of a precise *tcaA*

deletion on virulence in *C. elegans*. The vector pKOR1 was used to create an in-frame markerless deletion of the entire TcaA-coding sequence from the genome of *S. aureus* COL. The resulting *tcaA* deletion mutant, NM278, had a teicoplanin resistance level comparable to that of *tcaRAB* deletion mutant BB1372 (Fig. 5A). Teicoplanin susceptibility was restored by complementation with pAW17-*tcaA* (Fig. 5A). The markerless *tcaA* deletion in NM278 did not appear to influence the expression of the upstream *tcaR* gene or the downstream *tcaB* gene, as there were no detectable changes in the transcription of either gene in strain NM278 compared to that in strain COL under induced or uninduced conditions (data not shown). This indicated that the markerless *tcaA* deletion in NM278 was unlikely to exert polar effects on the expression of *tcaR* or *tcaB*. The relative levels of virulence of COL, NM278, and BB1372 were then examined in the *C. elegans* infection model (39). As shown in Fig. 5B, *C. elegans* killing was highly attenuated in both the in-frame *tcaA* deletion mutant NM278 and the *tcaRAB* locus mutant BB1372 compared to that in wild-type strain COL.

DISCUSSION

The coordinated induction of the cell wall stress stimulon in *S. aureus* is thought to play an essential part in mounting or increasing antibiotic resistance responses (26, 41). Like many other members of the core cell wall stress stimulon, induction of *tcaA* transcription by cell wall-active antibiotics was shown to be dependent on the two-component sensor-transducer VraSR. Currently, nothing is known about the mechanism(s) or factor(s) involved in the VraSR-dependent process of transcript induction. Here we characterized the promoter region of the VraSR-dependent *tcaA* gene, which codes for a protein influencing glycopeptide susceptibility, and determined if there was a link between its induction and its role in glycopeptide susceptibility.

Mapping of the *tcaA* TSS and construction of sequential deletions within the *tcaR-tcaA* intergenic region identified an approximately 66-bp area upstream of the predicted TSS that was essential for induction. This region contained an inverted repeat, one half of which covered the σ^A -35 box of the predicted σ^A -10 -35 binding consensus. The position of this inverted repeat, upstream of the TSS, suggests that it binds to a DNA-binding transcription factor. We hypothesize that in the absence of cell wall stress the inverted repeat forms a hairpin structure which occludes the -35 box, preventing RNA polymerase from binding, and that in response to cell wall damage a binding factor (either VraR itself or an intermediary regulator) is activated and binds to the inverted repeat region, relieving the secondary structure and allowing the access of RNA polymerase to the promoter. Alternately, a transcriptional repressor could be bound to this region under normal growth conditions and then cleaved to expose the promoter under antibiotic stress conditions. The mechanism(s) of induction of cell wall stress stimulon genes in response to cell wall damage is unknown. The DNA-binding target of VraR has not yet been determined, and no intermediary factors in the induction cascade have been identified. Experiments designed to capture the proteins binding to this region have so far proved unsuccessful but are ongoing. It seems unlikely that the mech-

FIG. 4. Determination of the TcaA region involved in glycopeptide resistance. (A) Maps showing the positions of *tcaA* deletions A to H (numbers on the right indicate the exact nucleotide positions of the deletions, according to the genome annotation of strain COL). The region of approximately 130 aa required for complementation of teicoplanin susceptibility in BB1372 is shown (*), and the positions of previously described *tcaA* mutations in clinical isolates SA137/9G and MI are indicated (25). (B) Teicoplanin gradient (0 to 5 µg/ml) plate comparing the resistance levels of BB1372 containing the intact pAW17-*tcaA* plasmid (BB1539), the empty pAW17 plasmid (BB1541), and pAW17-*tcaA* deletion plasmids A to H. MIC*, MIC values were determined by Etest by using 2.0 McFarland suspensions on BHI supplemented with kanamycin at 50 µg/ml.

anism of induction or the transcription factor(s) involved in *tcaA* induction is universally conserved among members of the cell wall stress stimulon, as in silico searches did not identify the region described above in front of other cell wall stress-induced genes or anywhere else in the *S. aureus* genome. Complementation experiments showed that when the portion of the promoter region required for induction was removed, *tcaA* was still able to complement teicoplanin susceptibility and that only mutants containing a disrupted ribosomal binding site were not able to complement teicoplanin susceptibility. This indicated that *tcaA* induction in response to cell wall stress was not linked to its effect on glycopeptide resistance, as only small, background quantities of TcaA were required to restore glycopeptide susceptibility in a *tcaA* deletion mutant.

TcaA is a predicted membrane protein of unknown function, close homologs of which are found only in staphylococci and bacilli. Interestingly, only one-third of the TcaA protein, including the transmembrane region and a directly adjacent portion of the C terminus, was required to complement teicopla-

nin susceptibility in a *tcaA* deletion mutant, thus identifying a potential glycopeptide susceptibility-determining domain within TcaA. This suggests that the membrane location of TcaA is important for its function in teicoplanin susceptibility and that the extracellular C-terminal portion, required to complement susceptibility, may be involved in sensing or interacting with membrane signals or components, extracellular signals, or the antibiotic itself. On the other hand, the N terminus containing the C-X₂-C-X₁₀-C-X₂-C motif and the terminal approximately 285 aa of the C terminus were dispensable for resistance complementation, indicating that they very likely have other cellular functions. The increased susceptibility to glycopeptides due to this glycopeptide susceptibility-determining region within TcaA suggests that it may facilitate the access of glycopeptides, in particular, that of teicoplanin, to its primary target, which is the D-Ala-D-Ala of the nascent lipid-linked peptidoglycan precursor.

In a genome-wide, transposon-based mutagenesis screen for genes influencing virulence in *S. aureus* Newman, Bae et al. (2)

FIG. 5. Survival of *C. elegans* on *tcaA* deletion mutants. (A) Teicoplanin gradient plate comparing the resistance levels of wild-type strain COL containing the empty plasmid pAW17, BB1372 containing pAW17 (BB1541) and the complementing plasmid pAW17-*tcaA* (BB1539), the in-frame, markerless *tcaA* deletion mutant NM278 containing pAW17, and NM278 complemented with pAW17-*tcaA*. MIC*, MIC values from Etests performed on BHI with 2.0 McFarland inocula are shown on the right of the gradient plate. (B) Survival of nematodes fed *S. aureus* COL (wild type; squares; $n = 90$), NM278 (COL $\Delta tcaA$; circles; $n = 90$), and BB1372 (COL *tcaRAB::ermB*; triangles; $n = 90$). P was <0.0001 by pairwise comparisons by the log-rank test of the following strain pairs: COL versus NM278 and COL versus BB1372. Data are representative of one of four independent experiments.

found that *tcaA* and *tcaB* insertion mutants were attenuated in *C. elegans* killing. To confirm these findings for *tcaA*, we constructed an in-frame deletion mutant of *tcaA* and, along with a previously characterized *tcaRAB* operon deletion mutant, examined its relative virulence in the nematode infection model. Assays showed that while wild-type COL was able to efficiently kill *C. elegans*, virulence was severely attenuated in both the *tcaA* deletion and the *tca* operon mutants. The nature of the in-frame markerless deletion of *tcaA* in NM278 minimizes the possibility of polar effects on *tcaR* or *tcaB* expression and confirms that *tcaA* is required for virulence in *C. elegans*.

Prior work has demonstrated that a diverse array of factors contribute to *S. aureus* disease in nematodes, many of which are similarly required for disease in mammalian hosts. Factors important for disease in *C. elegans* include secreted toxins, cell wall-associated products, global virulence regulatory loci, and numerous factors involved in intermediary metabolism (3–5, 38, 39). In previous work, we showed by microarray expression profiling and confirmatory Northern blot analysis that only three transcripts are significantly different in $\Delta tcaRAB$ mutant BB1372 compared to the COL parent strain: *sarS* (*sarH1*), *spa*, and *sasF*. Complementation studies showed that these transcripts are regulated by TcaR, a MarR-like transcriptional regulator, and not by TcaA (27). Furthermore, examination of NM278 has failed to reveal any obvious phenotypic abnormalities associated with reduced virulence in nematodes, including alterations in growth rate, hemolysin production, and biofilm

production (data not shown). In conclusion, we have no evidence that TcaA regulates the expression of other virulence products important for disease in nematodes.

The potential involvement of TcaA in virulence suggests that it also performs other roles in *S. aureus* that are unrelated to glycopeptide resistance. This could help to explain why only a small portion of TcaA was essential for complementing teicoplanin susceptibility and why *tcaA* is part of the cell wall stress stimulon, even though the expression of functional TcaA increases susceptibility to teicoplanin. As cell wall stress stimulon genes are induced by cell wall-damaging agents in general, it is likely that the role of TcaA in responding to cell wall stress is unrelated to its role in teicoplanin susceptibility. How TcaA contributes to the pathogenic fitness of *S. aureus* and whether it plays a similar role in pathogenesis in mammals will be the subjects of further investigations.

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