VraH Is the Third Component of the *Staphylococcus aureus* VraDEH System Involved in Gallidermin and Daptomycin Resistance and Pathogenicity

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In bacteria, extracellular signals are transduced into the cell predominantly by two-component systems (TCSs) comprising a regulatory unit triggered by a specific signal. Some of the TCSs control executing units such as ABC transporters involved in antibiotic resistance. For instance, in *Staphylococcus aureus*, activation of BraSR leads to the upregulation of *vraDE* expression that encodes an ABC transporter playing a role in bacitracin and nisin resistance. In this study, we show that the small staphylococcal transmembrane protein VraH forms, together with VraDE, a three-component system. Although the expression of *vraH* in the absence of *vraDE* was sufficient to mediate low-level resistance, only this VraDEH entity conferred high-level resistance against daptomycin and gallidermin. In most staphylococcal genomes, *vraH* is located immediately downstream of *vraDE*, forming an operon, whereas in some species it is localized differently. In an invertebrate infection model, VraDEH significantly enhanced *S. aureus* pathogenicity. In analogy to the TCS connectors, VraH can be regarded as an ABC connector that modulates the activity of ABC transporters involved in antibiotic resistance.

Bacteria are challenged by different factors during colonization or infection of the human host. These are, for example, components of the complement system, antibodies, macrophages, or antimicrobial peptides (AMPs), such as defensins and cathelicidins (1). Furthermore, bacteria with the same ecological niche compete for the available resources by producing secondary metabolites with antimicrobial activity. *Staphylococcus aureus*, as a major cause of a variety of infectious diseases in humans (2), possesses a set of up to 17 two-component systems (TCSs) (3), which transduce extracellular signals into the cell. TCSs are comprised of a membrane-bound sensor histidine kinase (HK) that undergoes autophosphorylation either upon interaction with appropriate ligands or by a certain stress stimulus and further transfers the phosphoryl group to a cognate response regulator (RR) (4). In this activated state, the RR controls a set of target genes allowing the cells to adapt to an altered environment or to cope with stress factors such as antibiotics. One of the prominent systems in regard to antibiotic resistance of *S. aureus* is the glycopeptide resistance-associated system (gra), initially only composed of the sensor histidine kinase GraS and the response regulator GraR (5). However, a third accessory component, GraX, was later shown to be required to confer full resistance (6). GraX belongs to an emerging class of proteins termed “TCS connectors” that modulate the output of TCSs by affecting the phosphorylation state of response regulators. TCS connectors use different mechanisms of action for signal integration, as well as in the coordination and fine-tuning of cellular processes (7). Furthermore, sensing of AMPs by GraXRS is dependent on the ABC transporter VraFG, forming the GraXRS/VraFG five-component system. Originally believed to confer resistance against AMPs *per se* (8), VraFG is currently thought to play a major role in sensing AMPs and transferring the signal to GraS (6, 9). GraS-mediated activation of GraR results in upregulation of a number of resistance factors, like the multiple peptide resistance factor (MprF) and the DltABDCX proteins. Both MprF and DltABDCX enhance the positive charge of the cell envelope by tethering the anionic phosphatidylglycerol of the cytoplasmic

membrane with L-lysine (10, 11) and by D-alanination of teichoic acids (12–14), respectively. Another TCS system of *S. aureus* related to resistance is the bacitracin resistance-associated system (bra) (15), also described as the nisin susceptibility-associated system (nsa) (16). The ABC transporter BraDE is able to sense the presence of bacitracin and, in conjunction with BraS, leads to the activation of BraR. By binding to an imperfect palindrome in the promoter region (15), BraR boosts expression of BraDE and the two-component ABC transporter VraDE. Thus far, VraDE has been reported to be involved in resistance against bacitracin and nisin but showed hardly an effect against daptomycin (17–19). The exact mechanism of VraDE is unknown; however, since it is an ABC transporter, the resistance is most likely based on an ATP-driven efflux mechanism.

In this study, we provide evidence that full activity of VraDE in *S. aureus* is dependent on the small transmembrane (TM) protein VraH that contributes to high-level resistance against gallidermin. Furthermore, both VraH and VraDE are required to confer high-level resistance against daptomycin. By using a bacterial two-hybrid system, we show that VraDEH represents a three-component system (3-CS). This system is important for *S. aureus* in order to cope with AMPs and to survive in an infection model.

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>Escherichia coli</i> DC10B	Cloning host	20
<i>Staphylococcus aureus</i> JE2	Derivative of <i>S. aureus</i> USA 300 FPR375, cured of three plasmids	22
JE2Δ <i>vraH</i>	<i>vraH</i> deletion mutant	This study
JE2Δ <i>vraDE</i>	<i>vraDE</i> deletion mutant	This study
JE2Δ <i>vraDEH</i>	<i>vraDEH</i> deletion mutant	This study
<i>Staphylococcus carnosus</i> TM300		57
Plasmids		
pBASE6	Plasmid for construction of deletion mutants, allows ATc-induced counterselection	25
pBASE6-Δ <i>vraH</i>		This study
pBASE6-Δ <i>vraDE</i>		This study
pBASE6-Δ <i>vraDEH</i>		This study
pPTX	Derivative of pTX15, shuttle vector, <i>E. coli</i> origin of replication ColE1 and ampicillin resistance cassette inserted into the PstI site of pTX15	58; this study
pPT-tuf- <i>vraH</i>	Derivative of pPTX, xylose-inducible promoter replaced by the constitutive <i>tufA</i> promoter	This study
pRAB11	Shuttle vector, ATc-inducible promoter	30
pRAB11-sfGFP- <i>vraH</i>	N-terminal fusion of superfolder GFP to <i>vraH</i>	This study
pRAB11- <i>vraH</i> -sfGFP	C-terminal fusion of superfolder GFP to <i>vraH</i>	This study
pCX-sfGFP	Superfolder GFP under the control of a xylose-inducible promoter	59
pRB473	Shuttle vector for complementation of KO mutants	27
pRB473- <i>vraH</i>	Complementation vector for <i>S. aureus</i> JE2Δ <i>vraH</i>	This study
pRB473- <i>vraDE</i>	Complementation vector for <i>S. aureus</i> JE2Δ <i>vraDE</i>	This study
pRB473- <i>vraDEH</i>	Complementation vector for <i>S. aureus</i> JE2Δ <i>vraDEH</i>	This study

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids are listed in Table 1. Unless stated otherwise, bacteria were grown aerobically in basic medium (1% soy peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, and 0.1% K₂HPO₄ [pH 7.4]) at 37°C under continuous shaking. Antibiotics were added when appropriate in the following concentrations: 100 μg ml⁻¹ ampicillin or 30 μg ml⁻¹ kanamycin in the case of *Escherichia coli*, and 10 μg ml⁻¹ chloramphenicol or 25 μg ml⁻¹ tetracycline in the case of staphylococcal species. *E. coli* DC10B was used as a cloning host for general cloning shuttle vectors and BACTH vectors (20). *E. coli* BTH101 was used as a final host for BACTH experiments (21). *S. aureus* USA300 LAC JE2, simply referred to as *S. aureus* JE2 for ease of annotation, was used as the model *Staphylococcus* strain (22).

Molecular cloning. Oligonucleotides used in the present study were synthesized by Integrated DNA Technologies, San Jose, CA (Table 2). Plasmid DNA was isolated using a GeneJet plasmid miniprep kit (Thermo Scientific, Waltham, MA), genomic DNA of bacteria was isolated using a Quick-gDNA miniprep kit (Zymo Research, Irvine, CA) according to the manufacturer’s protocol. For cleanup of PCR products, Illustra GFX PCR DNA and a gel band purification kit (GE Healthcare Life Sciences, Chalfont St Giles, United Kingdom) St Giles, United Kingdom) were used. Q5 High-Fidelity DNA polymerase, PCR reagents, restriction enzymes, NEBuilder HiFi DNA Assembly master mix (New England BioLabs), and a T4 rapid ligation kit (Thermo Fisher) were used according to the manufacturer’s protocols. Transformation of chemical-competent *E. coli* cells and electroporation of *S. aureus* and *S. carnosus* were done as described elsewhere (23, 24).

Construction of plasmids and knockout mutants. Knockout mutants of *S. aureus* JE2 were constructed as in-frame deletions, with a left-over of three codon triplets at the 5’ and the 3’ ends of the open reading frame. We used the temperature-sensitive shuttle vector pBASE6, which allows ATc-induced counterselection for the highly effective creation of deletion mutants by homologous recombination (25). For this, ~1 kb of the up- and downstream regions of the relevant genes was amplified from chromosomal DNA of *S. aureus* JE2 by PCR (the primers are listed in

Table 2) and joined into the EcoRV restriction site of pBASE6 using NEBuilder HiFi DNA Assembly master mix (New England BioLabs). The mixture was used for transformation of chemical-competent *E. coli* DC10B cells. Colonies harboring the correct plasmid were picked, and the respective plasmids were transformed into electrocompetent *S. aureus* JE2 cells. Mutagenesis of the respective transformants was achieved as reported elsewhere (26). Mutants were checked by PCR for successful deletion of the genes.

For complementation of the knockout mutants, the plasmid pRB473 (27) was used. The respective DNA fragments harboring *vraDE* or *vraDEH* and the native *braR*-regulated promoter were amplified from genomic DNA of *S. aureus* JE2 and joined into the multiple cloning site of pBR473 (AvaI/EcoRI) using NEBuilder HiFi DNA Assembly master mix (New England BioLabs). *E. coli* transformants were picked and checked by restriction digestion and sequencing. Plasmids with the correct sequences were used for transformation of the respective mutant strains.

For expression in *S. carnosus*, *vraH* was amplified by PCR from genomic DNA of *S. aureus* JE2 with the primers *vraH* fwd BamHI and *vraH* rev AvaI and subsequently ligated into the plasmid pPTX (derivative of pTX30 harboring the *E. coli* origin of replication ColE1 and an ampicillin resistance cassette [unpublished data]) via the BamHI/AvaI restriction sites, yielding the plasmid pPTX-*vraH*. For the construction of the shuttle vector pPT-tuf-*vraH*, a 7.3-kb PCR product of the backbone of pPTX-*vraH* was amplified with the primers pPTX-*vraH* fwd and pPTX-*vraH* rev and joined with a PCR product of the *tufA* promoter of the plasmid pC-tuf-gfp (28) using NEBuilder HiFi DNA Assembly master mix. pPT-tuf-*vraH* was also used for the complementation of *S. aureus* JE2Δ*vraH*. SCA_2035 and SE_2402 were amplified with the respective primers and cloned via the AvaI restriction site into pPT-tuf using NEBuilder HiFi DNA Assembly master mix (New England BioLabs).

RNA isolation and RT-PCR. An overnight culture of *S. aureus* JE2 was grown with a sub-MIC of gallidermin (1 μg/ml) to induce the antibiotic stress response. Fresh B medium supplemented with 1 μg/ml gallidermin was inoculated with the overnight culture to an optical density at 578 nm (OD₅₇₈) of 0.05 and grown to end-exponential phase (OD₅₇₈ = 7.0). Cells

TABLE 2 Primers used in this study

Function and primer	Sequence (5'–3') ^a
Cloning	
vraH KO up fwd	cgcgcagatctgtcgacgatATTGCAATTATTTCTGCAGTTAC
vraH KO up rev	atttacctttAAATTTTCATGATTCAATCTCTCC
vraH KO down fwd	catgaaatttCCCGGGTACCGTTTCGTATAATG
vraH KO down rev	tgcaggcatgcaagcttgatAACACAACAAATACATTTTGATTAG
vraDE KO up fwd	ggcatgcaagcttgatAATGCCACAATCATTTGG
vraDE KO up rev	aaatggttttTATCGTCATAGTCTCACTCC
vraDE KO down fwd	actatgacgataAAAACCATTTAACTTACGATTAAAAAG
vraDE KO down rev	cagatctgtcgacgatCATTAGCAATGCTGTTATAGATATC
vraDE-vraH KO up fwd	ggcatgcaagcttgatAATGCCACAATCATTTGG
vraDE-vraH KO up rev	atttacctttTATCGTCATAGTCTCACTCC
vraDE-vraH KO down fwd	atgacgataAAAGGTAATAAATAAGCCTTATGTG
vraDE-vraH KO down rev	cagatctgtcgacgatCAAAATTTCTAATATTATTAATGTTATATTAA TAACCAATC
vraDE comp fwd	cgactctagaggatccccgggATAAACAGAATCCAATTTTGTTTAC
vraDE comp rev	gctcatcgagtcgcaattcTAAAAATAAATATCTAATGAAGCATC
vraDEH comp fwd	cgactctagaggatccccgggATAAACAGAATCCAATTTTGTTTAC
vraDEH comp rev	gctcatcgagtcgcaattcTAGCACATAAGGCTTATTTATTTAC
vraH fwd BamHI	ATATTAGGATCCTTAAGAAAAGGAGAGATTGAA
vraH rev Aval	ATATTACCCGGGTAAAGAAAAGGAGAGATTGAA
pPTX-vraH fwd	TTAAGAAAAGGAGAGATTGAATC
pPTX-vraH rev	AGCTTTTAATCCTTCTGCTC
tuf fwd	gcagaaggattaaaagctGTTTCGGTTATGCAACATC
tuf rev	caatctctcttttcttaaCTCTCATGATAGTTTCTCACC
vraH pUT18C fwd	caggctgactctagagAAATTTAGAGAATTAGTAAAAAATCATATG
vraH pUT18C rev	ttacttagttatctgatTTATTTACCTTTTTCTTCACGAC
vraD pKT25 fwd	gggtcgactctagagACGATATTATCAGTGCAACATG
vraD pKT25 rev	gtaaaacgacggccg TTAATGTCAATTTGAGACACC
vraE pKT25 fwd	gggtcgactctagagACATTTAACCATATCGTTTTCAAAAAAC
vraE pKT25 rev	gtaaaacgacggccgTTAAATGGTTTTCTTAAATCAATTTGTTTG
vraD pUT18C fwd	caggctgactctagagACGATATTATCAGTGCAACATG
vraD pUT18C rev	ttacttagttatctgatTTAAATGTCAATTTGAGACACC
vraH pUT18 fwd	caggctgactctagagATGAAATTTAGAGAATTAGTAAAAAATC
vraH pUT18 rev	cctcgctggcgctgATTTACCTTTTTCTTCACGAC
pKT25-vraD fwd	TTAAATGTCAATTTGAGACACCAC
pKT25-vraD rev	TCGTGACTGGGAAAACCC
vraE fwd pKT25-vraD	tgtctcaaatgacatttaaCCATATCGTTTTCAAAAACTTAC
vraE rev pKT25-vraD	gggtttcccgatcaCGAGTAAGTGTTAAATGGTTTTCTTAATC
N-term vraH fwd	tgatggtaccgttaacaGATTTAAGAAAAGGAGAGATTGAATC
N-term vraH rev	ttttgatgcTTTACCTTTTTCTTCACGAC
C-term vraH fwd	aaaaggtaaaGCATCAAAAGGTGAAGAATTATTTAC
C-term sfGFP rev	cagtgaattcgagctcattaTTTATATAATTCATCCATACCATGTG
C-term vraH fwd	tgatggtaccgttaacaATGGCATCAAAAGGTGAAG
C-term vraH rev	ctctaaatttTTTATATAATTCATCCATACCATGTG
N-term sfGFP rev	gaattatataaaaAAATTTAGAGAATTAGTAAAAAATCATATG
N-term sfGFP rev	cagtgaattcgagctcaTTATTTACCTTTTTCTTCACGAC
SCA_2035 fwd	gaaactatcatgagagccgggATAAATTGCTAGGAGGGAATATTATG
SCA_2035 rev	ttcgagcctcggtaccGGGGATTATTTGCAATTACAG
SE_2402 fwd	gaaactatcatgagagccgggAATAAAGGACGGTTTTTATTATGAAATTC
SE_2402 rev	ttcgagcctcggtaccgggCTTTTGTATTATGATTATTTATTTTCTCTTC
RT-PCR	
vraD-H fwd	AACAAAGAACTGCAGCAGCG
vraD-H rev	ACATATTGAGTTTAGCACATAAGGC
vraE-H2 rev	TACATGCAGTATTCGCCGCA
vraE-H2 rev	TGTTCTATACCGATGGAATGTGCT

^a Overhang regions used for cloning by Gibson assembly are indicated by lowercase letters. Restriction sites are underlined.

were harvested by centrifugation, washed with ice-cold phosphate-buffered saline (PBS), and lysed using FastPrep-24 (MP Biomedicals, Illkirch-Graffenstaden, France). RNA was isolated from the cell lysate with the SV total RNA isolation system kit (Promega, Madison, WI) in accordance with the protocol of the manufacturer. Reverse transcrip-

tion-PCR (RT-PCR) was performed using a One-Taq one-step RT-PCR kit (New England BioLabs) with 20 ng of RNA as the template in each reaction. Primers used for RT-PCR are listed in Table 2. One-Taq Hot-Start DNA polymerase (New England BioLabs) was used for no-RT and DNA template controls. RT-PCR products were sent to

GATC Biotech (Constance, Germany) for sequencing to validate the amplified sequences.

Localization of *VraH*. *VraH* was localized by N- and C-terminal fusion to superfolder green fluorescent protein (sfGFP) (29). *vraH* was amplified from genomic DNA of *S. aureus* JE2 and sfGFP was amplified from the plasmid pCX-sfGFP by PCR with the appropriate primers (Table 2). The shuttle vector pRAB11 allows controlled gene expression by addition of anhydrotetracycline (ATc) (30). pRAB11 was digested with BglI and joined with the respective *vraH* and sfGFP PCR products. *E. coli* transformants were verified by restriction digestion and sequencing. Vectors with the correct sequence were used for transformation of *S. aureus* JE2Δ*vraH* competent cells. For both strains, 20 ml of BM supplied with 0.5 μg of gallidermin ml⁻¹ for the induction of *vraDE* expression were inoculated to an OD₆₀₀ of 0.1 with an overnight culture of *S. aureus* JE2/pRAB11-*vraH*-sfGFP or *S. aureus* JE2/pRAB11-sfGFP-*vraH* and grown until an OD₆₀₀ of 0.4 was reached. Expression of the fusion proteins was induced by the addition of 50 ng ml⁻¹ ATc, and the cells were grown until an OD₆₀₀ of 1.0 was reached. Then, 5 μl of each culture was pipetted onto poly-L-lysine-coated glass slides (Sigma, Munich, Germany) and monitored with a Leica DM5500 B fluorescence microscope (Leica, Wetzlar, Germany). Images were captured by Leica DFC360 FX high-sensitivity monochrome digital camera.

BACTH system. We used a BACTH (bacterial adenylate cyclase two-hybrid) system kit from Euromedex, France. The adenylate cyclase (CyaA) of *Bordetella pertussis* can be divided into T18 and T25 fragments (31). Each fragment alone is not able to synthesize cAMP from ATP, but as soon as both fragments enter the spatial vicinity, the adenylate cyclase activity is restored. This effect can be exploited to monitor the interaction of different proteins in a *cyaA*-deficient *E. coli* strain by fusing the T18 and T25 fragments to the respective test proteins. Upon the interaction of the test proteins, the proximity of the T18-to-T25 fragment restores adenylate cyclase activity and thus cyclic AMP (cAMP) production, which further leads to the activation of several genes, e.g., the lactose and maltose operons. *VraD* and *VraE* were each fused at the N terminus with the T18 or T25 fragment by cloning the respective sequences into the vectors pUT18C or pKT25. Since the orientation of *VraH* in the membrane is only based on bioinformatic predictions, fusion proteins of *VraH* were constructed with the T18 fragment at the N terminus, as well as at the C terminus by cloning *vraH* into the vectors pUT18C or pUT18, respectively. The genes *vraD*, *vraE*, and *vraH* were amplified from genomic DNA of *S. aureus* JE2 with the respective primers (see Table 2), leaving out the stop codon in the case of an N-terminal fusion to the T18 domain, and cloned into the vector pUT18C, pUT18, or pKT25 via the BamHI/EcoRI restriction sites using an NEBuilder HiFi DNA Assembly master mix. For bicistronic expression of a T25-*vraD* fusion and *vraE*, the vector pKT25-*vraD* was amplified by PCR with the primers pKT25-*vraD* fwd and pKT25-*vraD* rev and joined with a PCR product of *vraE* (primers *vraE* fwd pKT25-*vraD* and *vraE* rev pKT25-*vraD*) using NEBuilder HiFi DNA Assembly master mix. Plasmids with the correct sequence were used for cotransformation of chemical-competent *E. coli* BTH101 cells and plated on MacConkey plates supplemented with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), 1% maltose, 100 μg ml⁻¹ streptomycin, 100 μg ml⁻¹ ampicillin, and 30 μg ml⁻¹ kanamycin. The plates were incubated for 30 h at 30°C. Interaction between the hybrid proteins was quantified by measuring the β-galactosidase activity. For each interaction tested, five colonies were inoculated in a 96-well deepwell block (Greiner) into 1 ml of Luria broth supplemented with 0.5 mM IPTG, 100 μg ml⁻¹ streptomycin, 100 μg ml⁻¹ ampicillin, and 30 μg ml⁻¹ kanamycin and then grown overnight at 30°C with shaking. Portions (50 μl) of each well were taken from the deepwell block and diluted 1:4 in a 96-well plate (Greiner) with water, and the OD₆₀₀ was measured with a Tecan infinite M200 plate reader (Tecan). Then, 100 μl of each diluted culture was mixed with 1 ml of PM2 buffer, 7 μl of β-mercaptoethanol, 20 μl of 0.1% sodium dodecyl sulfate (SDS), and 40 μl of chloroform. Each well was mixed 15 times with a multichannel pipette, and the resulting cell debris was allowed to settle at the bottom of the wells

by incubation at room temperature for 15 min. Next, 100 μl of the lysed cells was transferred to a 96-well plate (Greiner) preloaded with 20 μl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. After incubation at room temperature for 10 min, the reactions were stopped by the addition of 50 μl of 1 M Na₂CO₃ to each well. The OD₄₂₀ was measured by using a Tecan infinite M200 plate reader. Calculation of the enzymatic activity was performed according to the following formula: $A = [200 \times (OD_{420} - OD_{420} \text{ in the control well}) / 10] \times 11.6$. Units per mg dry weight = $A / (OD_{600} - OD_{600} \text{ in the control well})$.

MIC determination. The MICs of all strains were determined using the broth microdilution method as described by the Clinical and Laboratory Standards Institute (32). Polymyxin B, nisin, gramicidin S, bacitracin, vancomycin, and ramoplanin were bought from Sigma, Munich, Germany. Gallidermin was isolated from the producer strain *Staphylococcus gallinarum* as described elsewhere (33). NAI-107 was obtained from Naiscons, Saronno, Italy. The antibiotics were diluted with Mueller-Hinton broth (MHB) to the specific test concentration range in 96-well plates to a volume of 50 μl. *S. aureus* JE2 and the three mutant strains were grown overnight (37°C, 150 rpm) and diluted with MHB to 10⁶ CFU/ml. Then, 50 μl was used as an inoculum to obtain a volume of 100 μl in each well and a final bacterial concentration of 5×10^5 CFU/ml. The MIC was determined as the lowest concentration completely inhibiting visible growth of the test strains after 18 h of incubation (37°C, 150 rpm). MIC strips were used for the daptomycin (Liofilchem, Roseto degli Abruzzi, Italy) MIC determination according to the protocol of the manufacturer. Mueller-Hinton agar plates were supplemented with 50 μg/ml CaCl₂ in the case of daptomycin, respectively. All experiments were performed independently in triplicate, with a triple replication for each antibiotic within each experiment. The experiment was only taken into account when all three replicates showed the same MIC.

***Galleria mellonella* infection model.** Larvae of *Galleria mellonella* in the final stage were infected with *S. aureus* JE2 wild-type and mutant strains as described previously (34, 35). Larvae were purchased from R. J. Mous Live Bait, Balk, Netherlands. Ten larvae weighing between 300 and 700 mg were infected with the respective strains to be tested. Bacteria were grown overnight in basic medium at 37°C with shaking, washed two times with PBS (140 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄), and adjusted to an OD₅₇₈ of 0.1 in PBS. Each larva was injected with 10 μl of the bacterial suspension in the last left proleg, corresponding to a dose of 10⁶ CFU. We used a 500-μl Hamilton high-pressure liquid chromatography syringe and a Hamilton PB600 repeating dispenser for injection. Larvae were incubated at 37°C for 5 days after infection. Surviving larvae were counted each day starting from 24 h after infection. Each experiment was conducted three times independently, and data collected from the experiments were pooled for statistical analysis, giving $n = 30$ for every strain. Larvae injected with PBS and untreated larvae served as control groups.

RESULTS

Genetic organization of *vraDEH* in staphylococci. In most staphylococcal species, *vraH* (SAUSA300_2635) is localized downstream of *vraE* (Fig. 1). In *S. aureus* JE2, the end of *vraE* and the start of *vraH* are separated by only 91 bp that contain no conspicuous promoter or terminator structure, suggesting that *vraH* is part of the *vraDE* operon. We performed RT-PCR to amplify a 2.5-kb part of the putative *vraDEH* mRNA, spanning from the middle of *vraD* to the 3' region of *vraH*. Indeed, all three genes are cotranscribed and under regulation of the BraR-controlled *vraD* promoter, thus forming an operon (Fig. 2A and B). The next rho-independent transcription terminator is located further downstream after SAUSA300_2636. Interestingly, a few genes downstream of *vraH*, there is a duplicated version, *vraH2* (SAUSA300_2638), which shares 98% identity with *VraH* at the amino acid level; the only difference is the substitution of alanine

FIG 1 (A to C) Schematic overview of the genomic organization of *vraDE* and *vraH* in various staphylococcal species: *S. aureus* USA300 LAC JE2 (A), *S. epidermidis* ATCC 12228 (B), and *S. carnosus* TM300 (C). In *S. aureus* and *S. epidermidis*, *vraH* is part of the *vraDEH* operon, whereas in *S. carnosus* it is located at a separate spot 1,700 genes downstream of *vraDE*. In *S. aureus* there exists a duplicated version (*vraH2*).

by serine at position 52. We detected a very faint 2.2-kb RT-PCR amplicon from *vraE* to *vraH2*, suggesting that the terminator can be overread to some extent, which is frequently observed (Fig. 2C); therefore, we do not regard *vraH2* to be a formal part of the *vraDEH* operon. Not only are *vraH* and *vraH2* repeated but the 5'-truncated integrase homolog (SAUSA300_2636) is also partly repeated (Fig. 1A, repeats A and B). Since the phage tail protein-encoding gene (SAUSA300_2637) is found between the BA–AB repeat regions, we assume that the gene organization is due to a

prophage excision event. In the *S. epidermidis* ATCC 12228 genome, there is only one *vraH* gene as part of the *vraDEH* operon and, in *S. carnosus* TM300, the *vraH* homolog is located at a separate spot 1,700 genes downstream of the *vraDE* genes (Fig. 1BC).

Similarity of VraH among *Staphylococcus* and other bacteria. In the 22 analyzed *S. aureus* strains, VraH possesses a similarity in the range of 94 to 100% (Fig. 3A). While the N and C termini are conserved, some minor amino acid exchanges are present in

FIG 2 *vraDE* and *vraH* are transcribed as an operon. (A) Sketch of the RT-PCR amplicons. Green dotted line, 2.5-kb amplicon spanning *vraD*–*vraH*; red dotted line, 2.2-kb amplicon of *vraE*–*vraH2*. (B and C) A strong band of the *vraD*–*vraH* amplicon can be detected (B), but only a very faint *vraE*–*vraH2* band is visible (C). The No-RT controls show no sign of genomic DNA contamination, while DNA template controls show the effectiveness of these PCR conditions.

FIG 3 Comparison of the VraH homologues of *Staphylococcus aureus* and other major *Staphylococcus* species. The similarity at the protein level is indicated as a percentage. (A) VraH is found in all *Staphylococcus aureus* strains with a high similarity of 94%. (B) In all *Staphylococcus* species with a similarity of $>47.82\%$, *vraH* is part of the *vraDEH* operon. In all other *Staphylococcus* species with a similarity of $\leq 47.82\%$, *vraH* is located in a different genomic locus.

FIG 4 Predicted topology of VraH. (A) WebLogo image (60) of the VraH alignment of the 22 analyzed *S. aureus* strains. Conserved amino acids are in black, and variable amino acids are colored. (B) The N terminus is facing the cytoplasmic space (residues 1 to 13), followed by TM1 (14–35), TM2 (36–57), and the cytoplasmic C terminus (58–64).

the putative TM domains 1 and 2 (Fig. 4A). The *vraH* gene is also present in the genomes of the other 22 analyzed staphylococcal species. VraH of *S. epidermidis* has the highest sequence similarity with that of *S. aureus* JE2 (86.50%), followed by *S. lugdunensis* (85.50%) and *S. warneri* (82.60%). In strains belonging to the *S. saprophyticus*, *S. simulans*, and *S. intermedius* group, the *vraH* homologs are localized in a different genomic context than the *vraDE* genes, and they are least conserved. These strains are also phylogenetically more distant from *S. aureus* than the *S. epidermidis* group (36, 37). The most distant homologue is found in *S. xyloso* (43.47%) (Fig. 3B).

We used the basic local alignment search tool (BLAST) to search the nonredundant protein sequence database for related proteins in other bacterial genera. The only protein sequences related to VraH with an E value below 0.1 and homologous genetic organization with *VraDE* were found in the Gram-negative coccus *Veillonella* sp. DORA_B_18_19_24 (100% query cover/70% identity), in *Streptococcus pneumoniae* (96%/46%) and in the Gram-positive coccus *Xylanimonas cellulolytica* (96%/51%). VraH is therefore not unique to the genus *Staphylococcus*, although the prevalence in every tested staphylococcal species suggests that it is

FIG 5 Demonstration of membrane localization of VraH by fluorescence microscopy. The sfGFP was fused to the N terminus (A) and to the C terminus (B) of VraH. The fusion proteins were expressed from the plasmid pRAB11 in JE2Δ*vraH* cultivated in the presence of sub-MIC gallidermin (0.5 μg/ml) for induction of the antibiotic stress response. Both fusion constructs led to a bright green fluorescence in the membrane region.

of staphylococcal origin and might have spread to a limited number of other coccoid bacteria.

VraH is a TM protein. The TOPCONS web server (38) predicts VraH to be composed of two transmembrane (TM) regions, with TM1 spanning residues 14 to 35 and TM2 spanning residues 36 to 57. There is no signal peptide. The N and C termini are supposed to extend into the cytoplasm (Fig. 4B). To verify the proposed localization, we created fusion proteins in which superfolder GFP (sfGFP) is fused to either the N or the C terminus of VraH. Both *S. aureus* JE2 clones expressing the respective fusion proteins on plasmids pRAB11-sfGFP-*vraH* and pRAB11-*vraH*-sfGFP showed a bright green fluorescence at the cytoplasmic membrane area upon induction of expression (Fig. 5).

VraH interacts with VraDE, forming a three-component system. The genetic organization of *vraDEH* in one operon suggests a possible interaction of the three proteins. We used a bacterial adenylate cyclase two-hybrid (BACTH) system kit to analyze the interaction of VraD, VraE, and VraH. Protein-protein interactions were quantified by measuring the β-galactosidase activity (Fig. 6; see also Table S1 in the supplemental material). Here, fusion proteins with the adenylate cyclase fragment attached to the N terminus of the respective protein are termed T18/25-*vraD*/E/H and VraD/E/H-T18/25 in the case of a C-terminal attachment. The interaction of T18-VraD with T25-VraE exhibited 819 U/mg (dry weight), an 8-fold increase in comparison to the negative control (110 U/mg [dry weight]). We were not able to monitor interaction between VraH and the cytoplasmic VraD in any of the combinations. However, VraH and the membrane-bound VraE interaction between VraH-T18 and T25-VraE resulted in a β-galactosidase activity of 1,496 U/mg (dry weight), and between T18-VraH and T25-VraE resulted in 405 U/mg (dry weight), confirming the assumed interaction. We expressed T25-VraD and VraE bicistronically from the plasmid pKT25 for the full reconstitution of the putative VraDEH complex in *E. coli* and measured the interaction with T18-VraH or VraH-T18. As in the case of VraH-VraE, the interaction of T25-VraD with the C-terminal fusion of the T18 fragment to VraH resulted in a higher β-galactosidase activity (1,496 U/mg [dry weight]) than with the N-terminal fusion (579 U/mg [dry weight]). The full reconstitution of VraD and VraE is therefore necessary for an interaction of VraH with VraD.

VraDEH represents a functional entity that mediates resistance to specific antibiotics. To investigate the cooperation between VraH and VraDE, we created a single deletion mutant of *vraH* and a double deletion mutant of *vraDE* in *S. aureus* JE2. To

FIG 6 Diagram of the β-galactosidase activity from the BACTH interaction test in units/mg of bacterial dry weight. The plasmids pKT25-*zip* and pUT18C-*zip*, which harbor the leucine zipper of GCN4 (21), were used as positive controls (black bar). Empty pKT25 and pUT18C vectors were negative controls. Five colonies were tested in the assay for every interaction. The standard deviation for each interaction within the five tested colonies was always below 15%. Colonies yielding a β-galactosidase activity three times higher than that of the negative control (dark gray bars) were regarded as proof of a positive interaction. Colonies yielding a β-galactosidase activity below that of the negative control were regarded as proof of a negative interaction (light gray bars).

avoid downstream effects in the putative *vraDEH* operon, each mutant was created without the insertion of an antibiotic resistance cassette. MICs are shown in Table 3. We used gallidermin as a model cationic peptide antibiotic (39, 40). Gallidermin possesses the N-terminal double-ring structure also found in other lantibiotics such as nisin, which binds to lipid II thereby blocking cell wall biosynthesis (41, 42). Furthermore, gallidermin features a characteristic C-terminal S-[(Z)-2-aminovinyl]-D-cysteine (Avi-Cys), which can also be found in the recently characterized lantibiotic NAI-107 (43, 44). In contrast to the cationic gallidermin, NAI-107 is neutral. Gallidermin is a potent inhibitor of Gram-positive bacteria and exhibits an MIC of 8 μg/ml against *S. aureus* JE2. In our experiments, gallidermin was active against *S. aureus* JE2Δ*vraH* at a concentration of 2 μg/ml, reducing the MIC 4-fold in comparison to the wild-type strain. The lipopeptide daptomycin possesses a negatively charged core structure *per se*. By complex formation with divalent Ca²⁺ ions, however, daptomycin exhibits a positive net charge. In our study, daptomycin is even 10-fold more effective against *S. aureus* JE2Δ*vraH* (0.047 μg/ml) than against the wild-type strain (0.5 μg/ml). The MICs of nisin and bacitracin were not altered in *S. aureus* JE2Δ*vraH* compared to the wild type. *S. aureus* JE2Δ*vraDE* is as susceptible to gallidermin and daptomycin as *S. aureus* JE2Δ*vraH*, exhibiting MICs of 2 and 0.047 μg/ml, respectively. However, a 4-fold decrease in the MIC was observed for bacitracin and nisin (Table 3). The MIC of NAI-107 (0.5 μg/ml) was not altered in the mutants compared to wild-type *S. aureus* JE2.

We further constructed a *vraDEH* mutant to deplete the whole putative 3-CS. The MICs of bacitracin and nisin were essentially the same as for *S. aureus* JE2Δ*vraDE*. In the case of daptomycin, however, we observed a slight reduction in the MIC from 0.047 to 0.032 μg/ml. This decrease in resistance was

TABLE 3 MIC values of the tested deletion mutants and complemented strains

Peptide antibiotic	Antibiotic MIC ($\mu\text{g/ml}$) for strain:						
	JE2 wild type	JE2 Δ <i>vraH</i>	JE2 Δ <i>vraDE</i>	JE2 Δ <i>vraDEH</i>	JE2 Δ <i>vraH</i> /pRB473- <i>vraH</i>	JE2 Δ <i>vraDE</i> /pRB473- <i>vraDE</i>	JE2 Δ <i>vraDEH</i> /pRB473- <i>vraDEH</i>
Cationic							
Gallidermin	8	2	2	0.25	8	8	8
Daptomycin	0.5	0.047	0.047	0.032	0.5	0.5	0.5
Nisin	16	16	4	4	16	16	16
Gramicidin S	4	4	4	4	4	4	4
Polymyxin B	64	64	64	64	64	64	64
Neutral							
NAI-107	0.5	0.5	0.5	0.25	0.5	0.5	0.5
Bacitracin	32	32	8	8	32	32	32
Gramicidin A	>32	>32	>32	>32	>32	>32	>32
Ramoplanin	2	2	2	2	2	2	2
Vancomycin	1	1	1	1	1	1	1

more pronounced for gallidermin, showing a 32-fold reduction in the MIC from 8 to 0.25 $\mu\text{g/ml}$ compared to the wild type. *S. aureus* JE2 Δ *vraDEH* was also 2-fold more susceptible to NAI-107 (0.25 $\mu\text{g/ml}$) than the wild type and each of the *vraDE* and *vraH* mutants (0.5 $\mu\text{g/ml}$). No change in MIC in either of the mutants was observed for polymyxin B (+5 net charge), gramicidin S (+2 net charge), gramicidin A, ramoplanin, or vancomycin (net charge 0).

Expression of *S. aureus* *vraH* in *S. carnosus* leads to increased resistance to gallidermin. In *S. carnosus*, *vraH* is located in a different genomic locus than the *vraDE* genes and shares only a low similarity (48%) with that of *S. aureus* (Fig. 1C). Because of the different location and the absence of a BraR binding site upstream of *vraH* (SCA_2035), coexpression of *vraDE* and *vraH* in *S. carnosus* in response to peptide antibiotic stress is highly unlikely. We therefore asked whether the cloning of *S. aureus* *vraH* into *S. carnosus* has an effect on gallidermin resistance. Indeed, *S. carnosus* TM300 expressing *VraH* of *S. aureus* constitutively (pPT-tuf-*vraH*) showed an 8-fold increase in the MIC (from 0.5 to 4 $\mu\text{g/ml}$).

To test whether *VraH* homologues from other staphylococcal species are able to complement the function of *vraH*, we expressed the *VraH* homologues of *S. carnosus* SCA_2035 and of *S. epidermidis* ATCC 12228 SE_2402 (87% similarity) (Fig. 3B) in *S. aureus*

JE2 Δ *vraH*. *VraH* homologues were constitutively expressed via the plasmid pPT-tuf. Heterologous expression of both *vraH* genes increased the MIC for gallidermin at least 2-fold (from 2 to 4 $\mu\text{g/ml}$), indicating the functionality of the heterologous *vraH* genes.

***S. aureus* JE2 Δ *vraDEH* is less virulent in a *Galleria mellonella* infection model.** We used *Galleria mellonella* larvae as an invertebrate infection model to compare the virulence of *S. aureus* JE2 and *S. aureus* JE2 Δ *vraDEH*. *Galleria mellonella* is especially well suited, since the innate immune system relies heavily on a wide variety of membrane-active antimicrobial peptides, such as cecropin A and cecropin D homologues (45, 46) and *Galleria* defensin (47), as well as a set of other proline-rich and anionic peptides (48). At the end of the 5-day test period, 11 of the 30 larvae infected with *S. aureus* JE2 Δ *vraDEH* were alive, compared to no surviving larvae in the case of the wild type (Fig. 7). Infection with the complemented mutant led to slightly faster killing of the infected larvae, with the last larva dying at day 4, compared to day 5 in the case of the wild type.

DISCUSSION

S. aureus possesses numerous systems to cope with antimicrobial compounds. With regard to tolerance toward antimicrobial peptides (AMPs), two-component systems (TCSs) and ABC transporters play a major role (49, 50). Although TCSs are well studied, there are still many questions open as to the number of components involved and the precise pattern of regulation. In the GraRS system, for example, GraX has been discovered as a third important component (6). In this study, we provide evidence that the thus-far-hypothetical small transmembrane protein *VraH* is the third component of the *VraDE* complex forming a functional unit of *VraDEH*.

VraH of *S. aureus* JE2 possesses a positively charged C terminus with a YYKRREEKGK motif, which is present in all *S. aureus* strains. However, in *S. carnosus* TM300, the C terminus is altered (YYLEKNGK) and possesses a net charge of only +1. Therefore, our first assumption regarding the function of *VraH* was that the N terminus is oriented inward, whereas the C terminus with the positively charged tail is oriented outward. Because of the increased positive charge outside, cAMPs should be electrostatically repelled. Such an electrostatic repulsion is a well-known defense

FIG 7 Kaplan-Meier plot of the survival of *Galleria mellonella* larvae infected with *S. aureus* JE2, JE2 Δ *vraDEH*, and JE2 Δ *vraDEH*(pRB473-*vraDEH*). Larvae injected with PBS were used as the control group. A total of 30 larvae were used for each strain in three independent experiments. The larvae infected with the deletion mutant show a significantly improved survival compared to larvae infected with the wild type. The *P* value is <0.0001 as determined by log rank and Gehan-Breslow-Wilcoxon tests.

FIG 8 Putative model of the interaction and function of the VraDEH three-component system. Experimentally proven interactions and mechanisms are displayed as solid and putative interactions as dotted arrows. VraH interacts with VraD and VraE (solid, black arrows) to confer resistance against gallidermin, NAI-107, and daptomycin. VraD and VraE confer resistance against nisin, bacitracin, and gallidermin. VraH confers resistance to gallidermin, either on its own or by interacting with other components controlled by the antibiotic stress response.

mechanism of *S. aureus* that has been described for MprF (10) and the DltXABCD (13, 14). However, heterologous expression experiments in *S. carnosus* TM300 with VraH derivatives bearing alterations in the C-terminal amino acids, thereby altering the net charge, did not cause any major changes in MIC compared to wild-type VraH (see Fig. S2 in the supplemental material). These findings, as well as the TOPCONS prediction, support the model that both the N and the C terminus are inward oriented (Fig. 4B). Experimental evidence for this model is also provided by the BACTH experiments. We observed β -galactosidase activity of the fusion proteins with the T18 fragment fused to both the N terminus and the C terminus of VraH. In the case of an extracellular C terminus, no β -galactosidase activity would have been observable. What is striking is the finding that the C-terminal fusion of the T18 fragment to VraH led to higher β -galactosidase activity in all tested interactions, which might suggest that the N terminus of VraH is important for the interaction with its partner proteins. The T18 fragment, which is relatively large compared to VraH, might therefore lead to a steric hindrance when VraH is establishing contact with its interaction partners, resulting in lower β -galactosidase activity. Furthermore, interaction of VraH and VraD takes place only in the presence of VraE, suggesting a central role for VraE as a docking protein for cytoplasmic VraD and the trans-membrane protein VraH. A putative model for 3-CS VraDEH is shown in Fig. 8.

VraDEH is not specific for a structurally defined group of antibiotics. The individual function of VraDE and VraH in this three-component system (3-CS) is still unclear. We showed that *vraH* is important for resistance to gallidermin but not to nisin. This is surprising since these two lantibiotics share structural and mechanistic similarities. Additionally, VraH does not play a role in resistance to bacitracin, which is positively charged but structurally unrelated to both gallidermin and nisin. In contrast to nisin, gallidermin does not usually form pores in the membranes of most bacteria, so an additional mechanism must therefore be responsible for its high effectiveness (51). This mechanism might provoke membrane perturbations, as has been described for NAI-107, as well as other cAMPs (52–54). From a structural point of view, NAI-107 shares the AviCys of gallidermin and also the lipid

II binding pyrophosphate cage structure but is neutrally charged. Therefore, we assume that the low activity against NAI-107 is mainly due to its uncharged character; only the full deletion of *vraDEH* made *S. aureus* a bit more susceptible. We observed no effect in *S. aureus* JE2 Δ *vraDEH* when it was challenged with gramicidin S, which like NAI-107 causes membrane perturbations but is positively charged (55).

Particularly interesting is the severely reduced MIC of daptomycin in all mutants, which is surprising, since in previous works almost no effect of daptomycin was observed in either *vraE* or *vraDE* mutants (17, 19). Since daptomycin is dependent on Ca^{2+} ion to exhibit its full bactericidal activity (56), we investigated it both alone and in combination with Ca^{2+} ions. When the medium was not supplemented with Ca^{2+} ions, the *vraDE* double and *vraDEH* triple mutants were as resistant against daptomycin as the wild type (see Fig. S3 in the supplemental material). These findings suggest that probably in the previous studies the medium was not sufficiently or not at all supplemented with additional Ca^{2+} ions. Since Ca^{2+} ions are readily available in the human body, this supplementation brings the action environment closer to the natural system and is also recommended by the Clinical and Laboratory Standards Institute (32). These results lead us to the conclusion that *vraDEH* plays a crucial role in the daptomycin resistance of *S. aureus*.

Previously, it has been shown that VraDE causes resistance to human beta-defensin 3 (19). To investigate the role of VraDEH in virulence, we tested JE2 and the Δ *vraDEH* mutant in a *Galleria mellonella* invertebrate infection model. The innate immune system of *G. mellonella* induces a diverse set of antimicrobial defensins against bacterial pathogens. We found that *S. aureus* JE2 killed 60% of the larvae within 2 days after infection and killed 100% after 5 days, whereas the killing rate of the *vraDEH* mutant was significantly decreased. This suggests that VraDEH is crucial for *S. aureus* to cope with antimicrobial defensins of *G. mellonella*.

VraH completes the 3-CS VraDEH to its full functionality. This 3-CS is a major factor in causing resistance to daptomycin and the cationic antimicrobial peptide gallidermin. We assume that it also causes resistance to insect defensins, since it significantly contributes to virulence of *S. aureus* in the *G. mellonella* infection model. VraDEH is a promising target in combating the emerging multiple-drug-resistant bacterial pathogens.

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