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Generation of a vancomycin-intermediate *Staphylococcus aureus* (VISA) strain by two amino acid exchanges in VraS

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Objectives: *Staphylococcus aureus* is a notorious bacterial pathogen and antibiotic-resistant isolates complicate current treatment strategies. We characterized *S. aureus* VC40, a laboratory mutant that shows full resistance to glycopeptides (vancomycin and teicoplanin MICs ≥32 mg/L) and daptomycin (MIC = 4 mg/L), to gain deeper insights into the underlying resistance mechanisms.

Methods: Genomics and transcriptomics were performed to characterize changes that might contribute to development of resistance. The mutations in *vraS* were reconstituted into a closely related parental background. In addition, antimicrobial susceptibility testing, growth analyses, transmission electron microscopy, lysostaphin-induced lysis and autolysis assays were performed to characterize the phenotype of resistant strains.

Results: Genome sequencing of strain VC40 revealed 79 mutations in 75 gene loci including genes encoding the histidine kinases VraS and WalK that control cell envelope-related processes. Transcriptomics indicated the increased expression of their respective regulons. Although not reaching the measured MIC for VC40, reconstitution of the L114S and D242G exchanges in VraS(VC40) into the susceptible parental background (*S. aureus* NCTC 8325) resulted in increased resistance to glycopeptides and daptomycin. The expression of VraS(VC40) led to increased transcription of the cell wall stress stimulon, a thickened cell wall, a decreased growth rate, reduced autolytic activity and increased resistance to lysostaphin-induced lysis in the generated mutant.

Conclusions: We show that a double mutation of a single gene locus, namely *vraS*, is sufficient to convert the vancomycin-susceptible strain *S. aureus* NCTC 8325 into a vancomycin-intermediate *S. aureus*.

Keywords: glycopeptides, teicoplanin, daptomycin, antibiotic resistance, two-component regulatory systems

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is among the leading causes of mortality by any single infectious agent in the USA and the European Union.[1,2] The options for the treatment of MRSA are limited. Since the early 1990s, vancomycin and related glycopeptide antibiotics have been employed as therapy for serious MRSA infections.[3,4] However, *S. aureus* strains with intermediate or full resistance to vancomycin emerged in recent decades[5–7] and clinical MRSA strains with additional resistance to daptomycin and linezolid have been isolated.[6,8,9] Since these pathogens are able to sensitively respond to antibiotic stress, it may be predicted that the resistance situation will not substantially relax in the future.[10,11] Understanding the bacterial response to antibiotics as well as the established resistance mechanisms to clinically used drugs is mandatory to evaluate alternatives to commonly applied treatment strategies.

Here, we set out to characterize the vancomycin-resistant laboratory mutant *S. aureus* VC40,[12] which had previously been generated by 30 serial passages of strain RN4220Δ*mutS* in the presence of increasing concentrations of vancomycin.[13] *S. aureus* VC40 has a vancomycin MIC of 64 mg/L, a concentration higher than the MIC for clinical vancomycin-intermediate *S. aureus* (VISA) strains. Actually, the measured MIC for strain VC40 meets the criteria for a vancomycin-resistant *S. aureus* (VRSA) strain rather than for a VISA strain; however, it lacks the *vanA* gene

cluster,[12] which represents the common VRSA resistance determinant.[14] Comparative genomic and transcriptomic analyses of *S. aureus* VC40 revealed non-synonymous single nucleotide polymorphisms (SNPs) in the sensor histidine kinase genes *vraS* and *walK* as well as significantly altered expression levels of their respective regulons. We reconstituted VraS(VC40) into the parental background (*S. aureus* NCTC 8325), characterized the expression of the respective regulon via quantitative real-time PCR (qRT–PCR) and examined the phenotypes of the different mutant strains by transmission electron microscopy (TEM), autolysis assays and growth behaviour studies to elucidate the specific role of VraS in antibiotic resistance. Our data show that VraS is a central factor in *S. aureus* NCTC 8325 that can confer reduced susceptibilities to clinically applied drugs including the last-resort antibiotics vancomycin and daptomycin.

Materials and methods

Strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table S1 (available as Supplementary data at *JAC* Online). Unless otherwise indicated, *S. aureus* strains were grown in brain heart infusion (BHI) broth or Mueller–Hinton (MH) broth (Oxoid) and *Escherichia coli* strains were grown in lysogeny broth at 37°C or 30°C when using temperature-sensitive plasmids. For plasmid maintenance, the required media were supplemented with ampicillin (50 mg/L) or erythromycin (25 mg/L).

Growth curve analyses and antimicrobial susceptibility testing

For growth curve analyses, three parallel cultures of the desired *S. aureus* strains were grown at 37°C in BHI or MH broth with or without the addition of antibiotics in 96-well polystyrene round-bottomed microplates (Greiner). The optical density at 600 nm (OD_{600}) was monitored kinetically using the Tecan Sunrise™ plate reader equipped with an incubation chamber (Tecan Group). The microplate was read every 10 min for 8 h with shaking in between and prior to every read. The data were evaluated using Magellan™ software (Tecan Group). MIC testing was performed using the broth microdilution method in 96-well microplates with an inoculum of 1×10^5–5×10^5 cfu/mL according to CLSI standards. Glycopeptide MICs were evaluated in MH and BHI broth, since BHI is commonly used in vancomycin susceptibility testing as it was described to be more sensitive for the expression of the VISA type than MH.[15] MICs were determined after 24 h and after 48 h for the slowly growing strain VC40. For MIC determinations, $CaCl_2$ was added to the medium to a final concentration of 1.25 mM for daptomycin (Cubicin®; Novartis Pharma) and friulimicin (MerLion Pharmaceuticals) or 1 mM for mersacidin (Sanofi-Aventis).

Genome sequencing and identification of SNPs

Genome sequencing including gap closure of *S. aureus* RN4220Δ*mutS* and *S. aureus* VC40 was performed as previously described.[12] After contig assembly, the resulting genome sequences were aligned against each other as well as to the reference genome sequence of the closely related *S. aureus* NCTC 8325 (NC_007795) using Mulan software (http://mulan.dcode.org/). SNPs, insertions and deletions unique to *S. aureus* VC40 were confirmed by Sanger sequencing. The genome sequence of *S. aureus* VC40 was deposited in NCBI GenBank under accession number CP003033.[12]

Preparation of total RNA

Extraction of total RNA was done as previously described.[16] Briefly, strains were grown in BHI broth without addition of antibiotics to an OD_{600} of 1.0 and were then stabilized by incubation with RNAprotect (Qiagen) for 5 min at 37°C before harvesting. Cells were lysed in the presence of 200–400 mg/L lysostaphin (Genmedics) and total RNA was extracted using the PrestoSpin R bug Kit including DNase I treatment (Molzym). The quality and quantity of total RNA were determined by agarose gel electrophoresis and measured by using the Nanodrop spectrophotometer (Nanodrop Technologies). For each strain, the RNA of at least two independently grown cultures was analysed.

Microarray analyses

A validated *S. aureus*-specific microarray platform was employed containing 10807 60-mer oligonucleotide probes[17,18] (Agilent Technologies) covering >95% of ORFs annotated in strains N315, Mu50, MW2, COL, NCTC 8325, USA300, MRSA252 and MSSA476 including their respective plasmids. After DNase treatment of RNA samples, the absence of remaining DNA was evaluated by qRT–PCR (SDS 7700; Applied Biosystems) as previously described.[5,19] Cy3-dCTP-labelled cDNA was synthesized from 5 μg batches of total RNA using SuperScript II reverse transcriptase (Invitrogen) and was purified via QiaQuick columns (Qiagen). Microarrays were normalized using Cy5-dCTP-labelled genomic DNA from the different sequenced strains used for the microarray design as previously described.[17,20] Cy5- and Cy3-labelled cDNA mixtures were then diluted in 50 μL of Agilent hybridization buffer and were hybridized at 60°C for 17 h in a hybridization oven (Robbins Scientific). Slides were washed, dried under nitrogen flow and scanned using a microarray scanner (Agilent Technologies). The microarray data were processed and evaluated as previously described[17] and are posted in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GPL10537 and GSE46887.

qRT–PCR

Quantitative transcription data were obtained by measuring sample amplification during the log-linear phase of the PCR using the Stratagene Mx3005P instrument (Agilent Technologies). Total RNA preparations (3 μg) were transcribed into cDNA using BioScript reverse transcriptase (Bioline) and $pd(N)_6$ random hexamers (GE Healthcare) as previously described.[16] qRT–PCR was performed using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies). For all experiments, the amount of transcripts was determined from the appropriate standard curve and the target concentration was expressed in relation to the concentration of the constitutively expressed housekeeping gene *gyrB*. Each standard curve was generated by assaying gene-specific PCR products. For each strain, two different cDNA probes were synthesized employing RNA preparations from independent cultures. The PCR products were verified by melting curve analyses. All qRT–PCR runs were at least performed in duplicate.

Genetic manipulations of *S. aureus*

To reconstitute *vraS* of *S. aureus* VC40 into the susceptible parent strain NCTC 8325, *vraS*(VC40) was cloned into the temperature-sensitive shuttle vector pMAD that allows for markerless double homologous recombination.[21] To this end, a PCR product spanning the T1973900C and A1974284G mutations in *vraS*(VC40) was cloned into the pMAD vector using the primers vraS_for (5′-ACCGAATTCATGACGCAATGTATTCGAA-3′) and vraS_rev (5′-ATTGTCGACTCAATGGAAGGCGAAACAG-3′), thereby generating pMAD-vraS(VC40), which was then electro-transformed into *S. aureus* NCTC 8325 to finally yield *S. aureus* NCTC 8325 VraS(VC40) after recombination had occurred. The presence of the *vraS*(VC40) mutations after allelic exchange was confirmed by Sanger sequencing.

TEM

Strains were grown in BHI broth and cells were harvested in the exponential growth phase (OD_{600} of 1.0). Cells were fixed using 3% glutaraldehyde

and 2% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2), followed by a progressive dehydration and embedding in Epon resin (Embed-812; Electron Microscopy Sciences). Ultrathin sections of 40–60 nm were cut using an ultramicrotome (Leica) equipped with a diamond knife (*ultra* 45°; Diatome AG) and further contrasted using uranyl acetate (3%) and lead citrate as described previously.[22] The samples were visualized using an EM 900 transmission electron microscope (Carl Zeiss Microscopy) at magnifications of 30000- to 50000-fold.

S. aureus lysis assays

For Triton X-100-induced autolysis, cells were grown to the exponential growth phase (OD_{600} of 1.0) and chilled on ice before harvesting. Cells were washed once with ice-cold MilliQ ultrapure water (Merck Millipore) and then resuspended to an OD_{600} of 1.0 in 50 mM Tris-HCl buffer containing 0.05% Triton X-100. Autolytic activity was measured during incubation at 37°C as a decrease in OD_{600} over time using a spectrophotometer (UV-160; Shimadzu). For lysostaphin-induced lysis assays, strains were grown to an OD_{600} of 1.0 and were then harvested by centrifugation. Cells were washed with MilliQ water and resuspended to an OD_{600} of 0.8 in PBS including 200 ng/mL lysostaphin (Genmedics). Cell lysis was measured as described above.

Results

S. aureus VC40 shows decreased susceptibility to various cell wall biosynthesis inhibitors

S. aureus VC40 could grow on agar plates containing vancomycin at concentrations up to 40 mg/L as indicated by population analyses[13] and it exhibited increased MICs of the glycopeptides vancomycin and teicoplanin (≥32 mg/L), of the lipopeptides daptomycin and friulimicin, of the lantibiotic mersacidin as well as of several β-lactams (Table 1). Noteworthy, the MIC of daptomycin reached 4 mg/L in strain VC40 and thus clearly exceeded the MIC breakpoint for daptomycin susceptibility according to EUCAST and CLSI criteria (resistant >1 mg/L). Obviously, *S. aureus* VC40 had acquired specific mutations that affected the susceptibilities to antibiotics with different individual targets, pointing at a more universal resistance mechanism.

The *S. aureus* VC40 genome carries mutations that can be linked to antibiotic resistance

Genome sequencing of *S. aureus* VC40 revealed a total of 79 mutations in 75 different loci compared with the genomes of its parent strains RN4220Δ*mutS*, RN4220 and NCTC 8325,[12,23–25] including 52 SNPs, 14 deletions and 13 insertions. The SNPs could be divided into 34 non-synonymous SNPs, 13 synonymous SNPs and 5 intergenic SNPs. The mutated gene loci comprised regulatory genes (e.g. *vraS* and *walK*), genes related to cell envelope metabolism and transport (e.g. *lytD*/SAOUHSC_01895, *ssaA*, *mraZ*, *vraG* and *mprF*), chaperone-like genes (e.g. *prsA*, *dnaK* and *clpX*) and genes related to cell metabolism (e.g. *relP*/SAOUHSC_02811, *rpoD* and *glmM*). A complete list of the detected mutations in *S. aureus* VC40 is presented in Table S2.

Increased expression of the VraSR and WalKR regulons in *S. aureus* VC40

We compared the gene expression levels of untreated *S. aureus* VC40 with those of its parent strain RN4220Δ*mutS* during the exponential growth phase using *S. aureus* full genome microarrays. Here, strain VC40 showed an increased expression of the cell wall stress genes *vraS*, *sgtB*, *cwrA*, *fmtA*, *murZ* and *tcaA* (Table 2). Two genes that have been reported to be coregulated with the cell wall stress stimulon showed transcription levels that were opposite to those expected from published results, as the expression of *atlA* was increased and that of *prsA* was decreased, and *sle1* showed no difference in regulation between strains VC40 and RN4220Δ*mutS*. The 2-fold increased transcription of *atlA* might be explained by the result that the WalKR regulon genes are up-regulated in strain VC40 as well, e.g. *sceD* and *ssaA* showed higher transcript levels in strain VC40 (Table S3). The expression of genes controlled by VraS and/or WalK was also confirmed by qRT–PCR (Figure 1). Furthermore, various genes involved in the transport and metabolism of carbohydrates, amino acids and nucleotides were divergently expressed. For example, genes of the lactose, histidine and urease operons exhibited higher transcript levels in strain VC40. Moreover, genes encoding serine proteases (*splABCDEF*), cysteine protease (*sspBC*) as well as the ABC transporter encoding genes *vraFG* and *vraDE* were more highly expressed in strain VC40. In contrast, the *cidAB* genes, encoding positive modulators of autolysin activity, showed significantly decreased expression. The expression of genes encoding ribosomal proteins, amino-acyl-tRNA synthetase genes, F_1F_o ATP synthase genes (*atpABCDEFGH*) and other genes of the respiratory chain such as the succinate dehydrogenase genes (*sdhABC*) was also lower in strain VC40, thereby resembling the expression pattern of the stringent response of *S. aureus*.

Table 1. Susceptibility testing of *S. aureus* NCTC 8325 derivatives used in this study

	MIC (mg/L)[a]								
NCTC 8325 derivative	VAN	TEC	DAP	OXA	LEX	FOX	CTX	MER	FRI
wild-type	0.75 (1.5)	0.5 (0.5)	0.25	0.19	2	1.5	0.75	8	1.5
RN4220Δ*mutS*	0.75 (1.5)	0.5 (1)	0.25	0.25	1.5	2	1	8	1
VC40	48 (64)	32 (48)	4	1	8	8	4	64	4
VraS(VC40)	3 (4)	6 (6)	2	0.5	6	4	1	32	2

VAN, vancomycin; TEC, teicoplanin; DAP, daptomycin; OXA, oxacillin; LEX, cefalexin; FOX, cefoxitin; CTX, cefotaxime; MER, mersacidin; FRI, friulimicin.
[a]MICs in MH; numbers in parentheses represent the vancomycin and teicoplanin MICs in BHI. MICs were read after 24 and 48 h considering the slow growth of strain VC40. Noteworthy, MIC values for NCTC 8325 wild-type, RN4220Δ*mutS* and NCTC 8325 VraS(VC40) were unchanged after 24 and 48 h. MIC values represent the mean of at least four independent determinations.

Table 2. DNA microarray-based expression ratios of cell wall stress stimulon genes including those under VraSR control[26,27] of untreated *S. aureus* strains VC40 versus RN4220Δ*mutS*

ORF ID[a]	Gene	Comments	Ratio VC40/ Δ*mutS*
00427	*sle1*	*N*-acetylmuramoyl-L-alanine amidase	0.90
00994	*atlA*	bifunctional autolysin	2.09
00998	*fmtA*	autolysis and methicillin resistance-related protein	2.07
01838	*htrA*	serine protease, heat-shock protein homologue	2.02
01972	*prsA*	peptidyl-prolyl *cis/trans* isomerase	0.32
02012	*sgtB*	monofunctional glycosyltransferase	4.67
02099	*vraS*	two-component sensor histidine kinase	1.66
02100	*yvqF/vraT*	conserved hypothetical protein	1.71
02101		hypothetical protein	2.12
02112		conserved hypothetical protein	1.71
02365	*murZ*	UDP-*N*-acetylglucosamine enolpyruvyl transferase	1.92
02583		similar to *lyt* divergon expression attenuator LytR	1.90
02635	*tcaA*	teicoplanin resistance-associated protein	2.46
02723		glycerate kinase	4.09
02724		hypothetical protein	4.17
02811	*relP*	putative (p)ppGpp synthetase	5.10
02872	*cwrA*	CwrA protein, cell wall responsive for antibiotics	21.32

[a]ORF IDs correspond to 'SAOUHSC_' locus tags of *S. aureus* NCTC 8325 (NC_007795).

Mutations in vraS affect antibiotic resistance in S. aureus

The L114S and D242G exchanges in VraS(VC40) were reconstituted into the genome of *S. aureus* NCTC 8325, the parental background of strain VC40. Although not reaching the high MIC values of *S. aureus* VC40, the resulting mutant, *S. aureus* NCTC 8325 VraS(VC40), was characterized by an increased MIC of vancomycin (MIC of 3 mg/L in MH broth and 4 mg/L in BHI broth), teicoplanin (MIC of 6 mg/L in MH and BHI broths) as well as daptomycin (MIC of 2 mg/L) (Table 1). Noteworthy, the presence of VraS(VC40) also affected the susceptibilities to the cell wall-active antibiotics mersacidin and different β-lactams (Table 1). Reconstitution of the I544M exchange in WalK was also attempted; however, despite repeated experiments, the correct mutant was not obtained.

VraS(VC40) accounts for an increased expression of the VraSR regulon

To study the gene regulatory impact of VraS(VC40) in strain NCTC 8325, we measured the relative transcript quantities of the *vraS*, *sgtB* and *lytM* genes by qRT–PCR and, indeed, these genes were more highly expressed in the *S. aureus* NCTC 8325 VraS(VC40) mutant compared with its parent strain (Figure 1), indicating an increased expression of the VraSR regulon. Noteworthy, we did not observe a significant alteration of *atlA* expression in strain NCTC 8325 VraS(VC40).

VraS(VC40) contributes to an altered phenotype of S. aureus

In growth kinetic experiments, a strongly reduced growth rate of *S. aureus* VC40 was observed compared with its parent strain (Figure S1). The NCTC 8325 VraS(VC40) mutant was also characterized by a slightly lowered growth rate (Figure S1), indicating that VraS(VC40) might to some extent contribute to the low growth rate observed for strain VC40. To gain further insights into the morphologies of strain VC40 and the NCTC 8325 VraS(VC40) mutant, we performed TEM of exponentially growing cells (Figure 2). Compared with the parent strains *S. aureus* NCTC 8325 (Figure 2d) and RN4220Δ*mutS* (Figure 2a), *S. aureus* VC40 was characterized by a significantly thickened cell wall with a roughened surface and irregular appearance (Figure 2b and c). Furthermore, a pseudomulticellular phenotype probably caused by a retarded cell separation was observed in strain VC40, since new septa were formed before the cell had completed the previous cell division event. Similarly, the thickness of the cell wall was also increased in strain NCTC 8325 VraS(VC40) (Figure 2e and f). Although the cell wall of the NCTC 8325 VraS(VC40) mutant also displayed a more roughened and uneven surface, the pseudomulticellular phenotype of strain VC40 was not observed here. The measured cell wall thicknesses were 16.71 (±3.00) nm for strain NCTC 8325 wild-type, 16.97 (±3.18) nm for strain RN4220Δ*mutS*, 62.36 (±13.98) nm for strain VC40 and 38.25 (±11.31) nm for the NCTC 8325 VraS(VC40) mutant.

S. aureus strains VC40 and NCTC 8325 VraS(VC40) show significantly reduced autolytic activities and an altered susceptibility to lysostaphin

The autolytic activities of the *S. aureus* strains VC40 and NCTC 8325 VraS(VC40) were characterized by Triton X-100-induced autolysis assays and were compared with their respective parental strains. Here, strains VC40 and NCTC 8325 VraS(VC40) both exhibited a similarly severe reduction of autolytic activity (Figure S2a). In addition, we determined lysostaphin-induced lysis of the different strains over time (Figure S2b). Here, strains RN4220Δ*mutS* and NCTC 8325 showed full susceptibility to lysostaphin treatment. Strain NCTC 8325 VraS(VC40) initially displayed reduced cell lysis in the presence of lysostaphin, but the majority of the cells were lysed after 60 min. Strikingly, strain VC40 was characterized by a significantly increased resistance to lysostaphin.

Discussion

Genome sequencing of the vancomycin-resistant laboratory mutant *S. aureus* VC40 identified SNPs in the histidine kinase genes *vraS* and *walK* and we further investigated the impact of VraS(VC40) on gene regulation, phenotypic characteristics and antibiotic resistance. Mutations in regulatory genes are specifically important, since these changes may alter the expression patterns of large regulons and thus help the bacteria to adjust certain

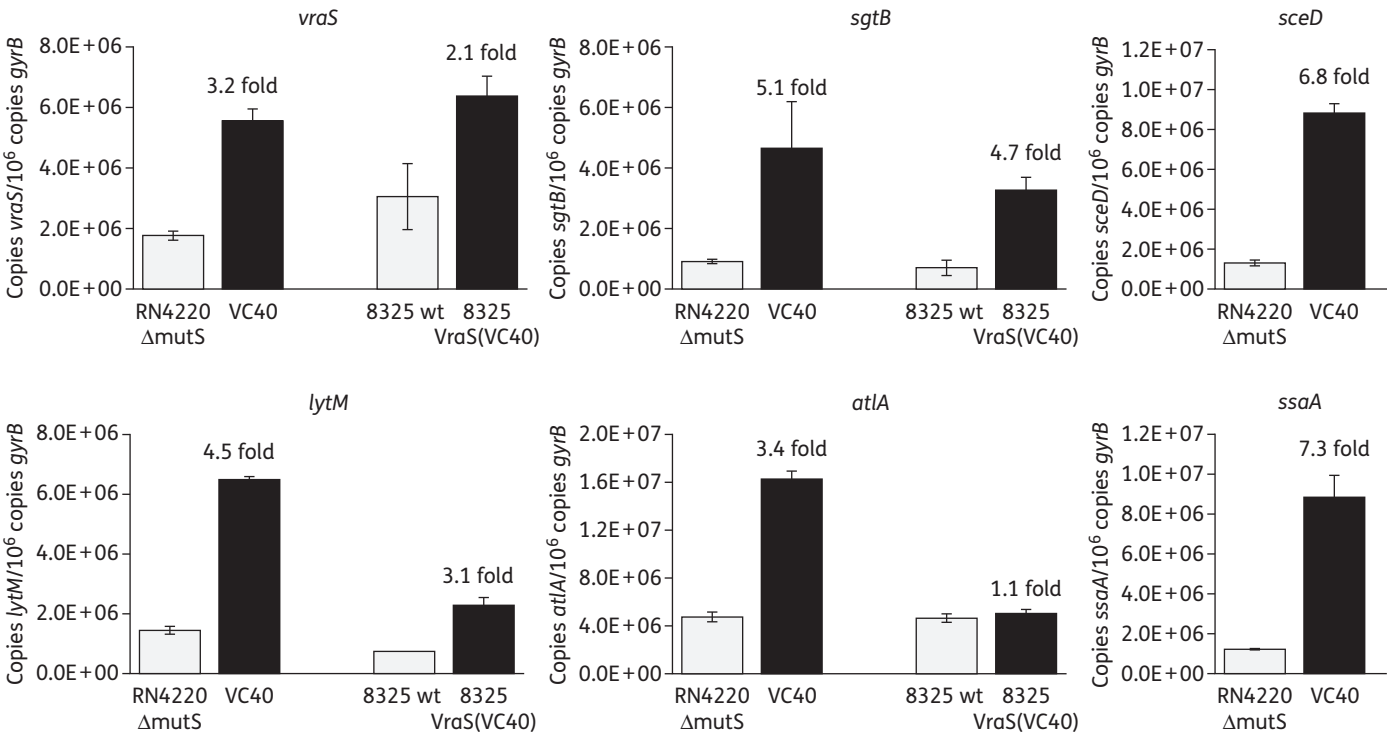

Figure 1. Gene expression analyses of VraSR and WalKR regulon members. Transcript levels of *vraS*, *sgtB*, *lytM*, *atlA*, *sceD* and *ssaA* were determined by qRT–PCR in relation to *gyrB* expression. Values represent the mean of results obtained for two independently grown cultures for each strain and are representative for at least two separate qRT–PCR experiments.

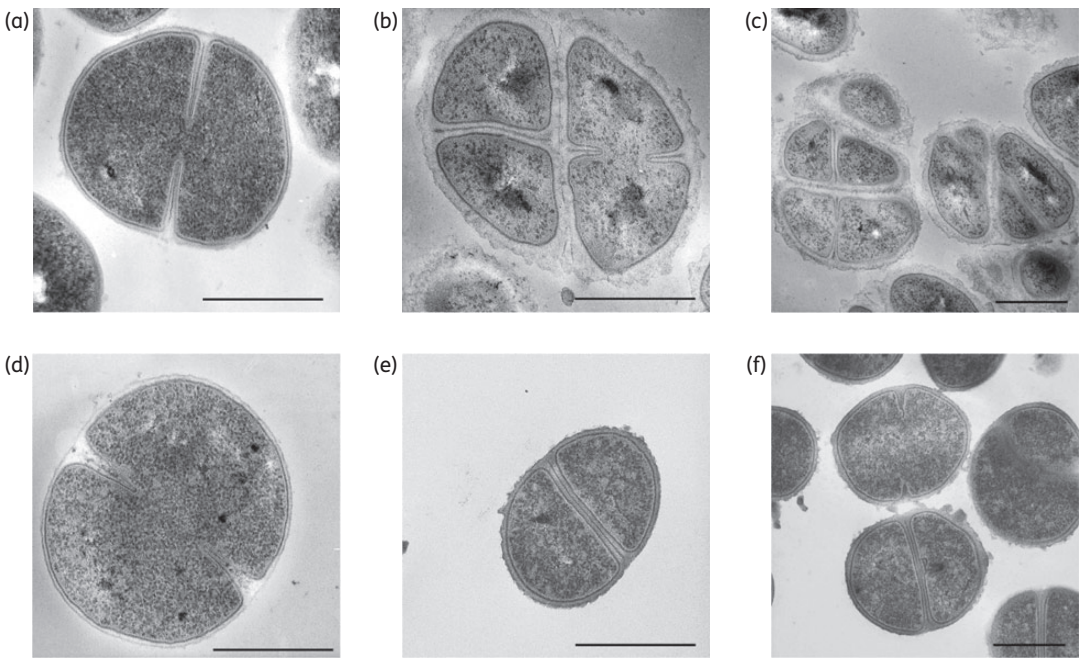

Figure 2. Morphological analyses by transmission electron microscopy. Ultrathin sections of exponentially grown *S. aureus* strains RN4220Δ*mutS* (a), VC40 (b and c), NCTC 8325 wild-type (d) and NCTC 8325 VraS(VC40) (e and f) were visualized. Scale bars represent 0.5 μm.

physiological networks that allow them to counteract antibiotic stress. In *S. aureus* VC40, the *vraS* gene harbours two non-synonymous SNPs that lead to the amino acid exchanges L114S and D242G in VraS. The introduction of these mutations into a susceptible parental background resulted in noticeable MIC increases of several cell wall-active antibiotics, including vancomycin, teicoplanin and daptomycin. VraS is part of the VraSR two-component regulatory system (TCRS) and conveys the so-called cell wall stress response, which coordinates the regulation of mostly cell wall biosynthesis-related genes such as *sgtB* or the *vraSR* genes themselves.[26–29] Various mutations in the *vraSR/yvqF*(*vraT*) locus have been reported in VISA strains[30–33] and, interestingly, the location of the SNPs in *vraSR/yvqF* in different strains is very diverse and there seems to be no specific hotspot for mutations in certain domains of these genes.

Only a few of the SNPs in *vraSR/yvqF* have been further investigated to elucidate their putative impact on gene regulation and antibiotic resistance development so far.[33,34] In our study, VraS(VC40) was sufficient to convert the vancomycin-susceptible strain *S. aureus* NCTC 8325 into a strain with a VISA type of resistance. An earlier study described the effect of the I5N amino acid exchange in VraS of strain Mu50.[34] Here, the introduction of the respective VraS(I5N) into the *S. aureus* Mu50Ω background compared with the VraS wild-type showed a lower resistance increase (MIC increase from 3.5 to 4.5 mg/L) compared with the effect that is shown by VraS(VC40) in the NCTC 8325 background (MIC increase from 0.75 to 3 mg/L).[34] Furthermore, a study by Gardete *et al.*[33] detected a Y220C amino acid exchange in YvqF in addition to mutations in *vraG*, *yycH* and *lspA* in strain SG-R, a clinical VISA isolate of the USA300 clone, and identified YvqF as a negative regulator of *vraSR*. However, a direct impact of YvqF(Y220C) on vancomycin resistance could not be determined by introducing the mutated *yvqF* gene on a plasmid into the vancomycin-susceptible strain SG-S, which still harbours a chromosomal copy of the wild-type *yvqF* gene. In contrast, the introduction of a plasmid with the wild-type version of *yvqF* into strain SG-R led to a decreased vancomycin MIC. In that study, a chromosomal reconstitution of the *yvqF* mutation into strain SG-S was not shown.[33] Noteworthy, since the mutations in *vraS* or *yvqF* mentioned above have each been reintroduced into different strain backgrounds, a strain dependency of the observed effects cannot be excluded.

In our study, the VraSR-controlled cell wall stress stimulon[26,27] was characterized by a considerably increased expression in *S. aureus* NCTC 8325 VraS(VC40) as well as in *S. aureus* VC40. Remarkably, the *lytM* gene also showed a higher transcript level in the NCTC 8325 VraS(VC40) mutant, which confirms the statement by Gardete *et al.*[33] that *lytM* appears to be an additional member of the VraSR regulon. Obviously, the increased expression of members of the VraSR regulon contributes to the observed reduced susceptibility to vancomycin, teicoplanin, daptomycin, mersacidin and oxacillin in our study. Noteworthy, these antibiotics have also been described to induce the VraSR regulon.[16,26,28,35,36] In this context, an accelerated peptidoglycan biosynthesis, which may be mediated by an activated VraSR regulon, is likely to account for this decrease in antibiotic susceptibility by supplying an increased number of cell wall precursors or by a faster incorporation of these precursors into the nascent peptidoglycan meshwork, eventually also leading to a thickening of the cell wall. Although commonly down-regulated upon vancomycin treatment, we did not observe a significant alteration of *atlA* expression in strain NCTC 8325 VraS(VC40), which is in accordance with previous observations that the regulation of *atlA* is most probably not mediated by VraSR.[26] Of note, mutations in the *vraSR/yvqF* genes are predominantly discussed regarding their impact on vancomycin resistance. However, we detected a strong influence of VraS(VC40) on daptomycin non-susceptibility, as the daptomycin MIC for the NCTC 8325 VraS(VC40) mutant reached 2 mg/L. It has to be noted that despite the effect of the *vraS*(VC40) mutations as well as the observation of Mehta *et al.*[37] concerning the contribution of deletion or up-regulation of *vraSR* in daptomycin-non-susceptible strains, which share a significant impact on daptomycin susceptibility, to our knowledge daptomycin treatment has not been described to select for mutations in the *vraSR/yvqF* operon so far.

An increased cell wall thickness as observed in strains VC40 and NCTC 8325 VraS(VC40) is likely to be a major contributor to the increased resistance to glycopeptides and daptomycin of these strains. Here, a cell wall thickness of 62 nm was reached in strain *S. aureus* VC40 and 38 nm in strain NCTC 8325 VraS(VC40). Thickening of the cell wall has been previously described as a common phenotypic feature of VISA strains[6,38,39] and has also been related to daptomycin cross-resistance;[40] however, the cell wall thickness of the typical VISA strain grown in BHI does not exceed 35 nm.[39] Noteworthy, up-regulation of the VraSR regulon and concomitant thickening of the cell wall have also been reported in daptomycin-non-susceptible *S. aureus* strains that were generated by serial daptomycin selection and that additionally showed a heterogeneous VISA phenotype.[41] Our study supports a prominent role of the VraSR regulon in the build-up of a thickened cell wall in *S. aureus*. Although the up-regulation of the cell wall biosynthesis machinery by VraSR and/or a possible deregulation of the bacterial autolytic system by the exchange present in WalKR in strain VC40 would both provide plausible explanations for the accumulation of excess cell wall material, the explicit mechanism that leads to cell wall thickening remains unclear. Interestingly, the pseudomulticellular phenotype of strain VC40 was not observed in strain NCTC 8325 VraS(VC40), indicating that VraSR is at least not solely responsible for this phenomenon and that probably more than one mutation may eventually lead to this phenotype in *S. aureus* VC40.

Although many of the autolysin genes including *atlA* were more highly expressed in strain VC40, Triton X-100-induced autolysis of strain VC40 was significantly decreased. This phenomenon has also been observed in the VISA strain JH9 and the vancomycin-resistant mutant VP32;[27,42,43] however, the reason for this remained elusive. The similarly reduced autolytic activities of strain NCTC 8325 VraS(VC40) and strain VC40 might indicate that an activated VraSR regulon generates modifications in the peptidoglycan structure of these strains, eventually leading to an increased resistance against Triton X-100-induced autolysis. In accordance with this assumption, it has previously been suggested that quantitative (or qualitative) changes in the teichoic acids of VISA strains might decrease peptidoglycan degradation by autolytic enzymes.[43] The clearly increased cell wall thickness of strain VC40 most probably accounts for the more severe decrease in lysostaphin susceptibility compared with strain NCTC 8325 VraS(VC40) and it may also play a role in the autolytic behaviour of the affected strains, but it is most likely not the sole factor in resistance against Triton X-100-induced autolysis. Since the

strains in our study and other VISA strains share the common phenotype of a reduced autolytic activity,[6,43–47] this feature is likely important for the development of resistance.

Strain NCTC 8325 VraS(VC40) did not reach the measured MICs and cell wall thickness of strain VC40, indicating that additional mutations add to the high level of resistance in the latter strain. In addition to the role of VraS(VC40) in contributing to the development of glycopeptide and daptomycin resistance described above, we identified an SNP in the *walK* gene leading to an I544M amino acid exchange located in the HATPase_c domain of the histidine kinase in strain VC40. The WalKR TCRS is an essential master regulator in cell wall metabolism and homeostasis.[48] Deregulation of this system may have important consequences for the bacteria and might enforce further adaptive changes, i.e. concerning membrane or cell wall composition. Microarray profiling revealed an increased expression of the WalKR regulon in *S. aureus* VC40 including the *ssaA*, *lytM*, *sceD* and *atlA* autolysin genes, which have been described to be under direct positive WalR control.[49,50] Mutations in the *walKR* operon have previously been linked to vancomycin or daptomycin non-susceptibility in several studies;[31,32,51–57] however, only a few of these have been analysed concerning their effect on antibiotic resistance, which might also be related to the complexity of genetic manipulation of the *walKR* locus due to its essential nature. Thus, it has remained unclear so far if an induced or a repressed WalKR regulon might finally cause a development of antibiotic resistance.[27,32,58]

Other amino acid exchanges observed in strain VC40 included MprF(H224Y) that may alter the activity of this protein and thus support daptomycin cross-resistance. In this context, *mprF* showed higher expression levels in VISA compared with vancomycin-susceptible *S. aureus* (VSSA) and decreased levels of *mprF* have also been correlated with increased vancomycin susceptibility.[27,59–61] Furthermore, increased expression or mutation of *mprF* have also been reported to occur frequently in daptomycin-non-susceptible *S. aureus* isolates[54,57,62,63] and depletion of *mprF* was described to restore daptomycin susceptibility in daptomycin-resistant *S. aureus* isolates.[64] Daptomycin cross-resistance of strain VC40 might be further supported by RpoD(D201N), which is the housekeeping sigma factor A (σ^A/σ^{70}). Noteworthy, mutations in *rpoB* and *rpoC* have been correlated with reduced vancomycin and daptomycin susceptibility in *S. aureus*[54,57,65] and the VISA strains Mu50 and JH9 also harbour SNPs in the *rpoB* and *rpoD* or *rpoB* and *rpoC* genes, respectively.[30,31]

In conclusion, our results prove the important role of the histidine kinase VraS in the physiology of the bacterial cell envelope. In our study, the amino acid exchanges in VraS led to an increased expression of the associated regulon, thereby mediating an increased thickening of the cell wall, alterations in autolytic properties and eventually resulted in significantly higher MICs of the glycopeptides vancomycin and teicoplanin as well as cross-resistance to daptomycin. Thus, VraS can act as an important switch to turn on antibiotic resistance in the *S. aureus* NCTC 8325 background.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 and Tables S1 to S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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