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Abstract: The *Staphylococcus aureus* cell wall stress stimulon (CWSS) is activated by cell envelope-targeting antibiotics or depletion of essential cell wall biosynthesis enzymes. The functionally uncharacterized *S. aureus* LytR-CpsA-Psr (LCP) proteins, MsrR, SA0908 and SA2103, all belong to the CWSS. Although not essential, deletion of all three LCP proteins severely impairs cell division. We show here that *VraSR*-dependent CWSS expression was up to 250-fold higher in single, double and triple LCP mutants than in wild type *S. aureus* in the absence of external stress. The LCP triple mutant was virtually depleted of wall teichoic acids (WTA), which could be restored to different degrees by any of the single LCP proteins. Subinhibitory concentrations of tunicamycin, which inhibits the first WTA synthesis enzyme TarO (TagO), could partially complement the severe growth defect of the LCP triple mutant. Both of the latter findings support a role for *S. aureus* LCP proteins in late WTA synthesis, as in *Bacillus subtilis* where LCP proteins were recently proposed to transfer WTA from lipid carriers to the cell wall peptidoglycan. Intrinsic activation of the CWSS upon LCP deletion and the fact that LCP proteins were essential for WTA-loading of the cell wall, highlight their important role(s) in *S. aureus* cell envelope biogenesis.

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Deletion of hypothetical wall teichoic acid ligases in *Staphylococcus aureus* activates the cell wall stress response

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Abstract

The *Staphylococcus aureus* cell wall stress stimulon (CWSS) is activated by cell envelope-targeting antibiotics or depletion of essential cell wall biosynthesis enzymes. The functionally uncharacterized *S. aureus* LytR-CpsA-Psr (LCP) proteins, MsrR, SA0908 and SA2103, all belong to the CWSS. Although not essential, deletion of all three LCP proteins severely impairs cell division. We show here that VraSR-dependent CWSS expression was up to 250-fold higher in single, double and triple LCP mutants than in wild type *S. aureus* in the absence of external stress. The LCP triple mutant was virtually depleted of wall teichoic acids (WTA), which could be restored to different degrees by any of the single LCP proteins. Subinhibitory concentrations of tunicamycin, which inhibits the first WTA synthesis enzyme TarO (TagO), could partially complement the severe growth defect of the LCP triple mutant. Both of the latter findings support a role for *S. aureus* LCP proteins in late WTA synthesis, as in *Bacillus subtilis* where LCP proteins were recently proposed to transfer WTA from lipid carriers to the cell wall peptidoglycan. Intrinsic activation of the CWSS upon LCP deletion and the fact that LCP proteins were essential for WTA-loading of the cell wall, highlight their important role(s) in *S. aureus* cell envelope biogenesis.

Introduction

Staphylococcus aureus mounts a general cell wall stress response in the presence of cell wall damaging agents, involving the upregulation of up to 50 genes collectively known as the cell wall stress stimulon (CWSS; Kuroda *et al.*, 2003; Utaida *et al.*, 2003; Jordan *et al.*, 2008). Induction of CWSS genes is controlled by the VraSR two-component system (Belcheva & Golemi-Kotra, 2008), which is homologous to the cell wall stress-responsive sensor-transducer systems LiaFSR of *Bacillus subtilis* (Mascher *et al.*, 2004), LiaFSR of *Streptococcus mutans* (Suntharalingam *et al.*, 2009) and CesRS of *Lactococcus lactis* (Martinez *et al.*, 2007). The sensor kinase VraS senses an unknown signal triggered by cell envelope disturbance and phosphorylates VraR, which then binds as a dimer to promoter-specific elements and facilitates

transcript induction (Martinez *et al.*, 2007; Belcheva & Golemi-Kotra, 2008; Eldholm *et al.*, 2010; Belcheva *et al.*, 2012). There is a wide variation in the fold-induction levels of different CWSS genes, which is probably linked to the specificity of VraR-binding, although the exact VraR-binding consensus and the influence of specific nucleotide differences on expression and induction of different CWSS genes has not been thoroughly analysed (Martinez *et al.*, 2007; Belcheva & Golemi-Kotra, 2008; Belcheva *et al.*, 2012).

The magnitude of CWSS induction strongly depends on the class and concentration of cell wall antibiotics (Dengler *et al.*, 2011). Disruption of wall teichoic acid (WTA) synthesis by targocil, which inhibits the WTA transporter TarG (TagG), was also shown to activate the CWSS (Campbell *et al.*, 2012). WTA are anionic glycopolymers that are attached to the peptidoglycan of

Gram-positive bacteria via a phosphodiester linkage, and they can constitute up to 60% of the total cell wall biomass. WTA of *B. subtilis* are composed of poly(glycerol phosphate) and poly(ribitol phosphate), whereas *S. aureus* contains mainly poly(ribitol phosphate) WTA. The biosynthesis of WTA is catalysed by *tag* (teichoic acid glycerol) or *tar* (teichoic acid ribitol) genes in *B. subtilis* and *S. aureus*, respectively (reviewed in Swoboda *et al.*, 2010). Besides the induction by cell wall active antibiotics, VraSR signal transduction is also triggered by internal disruption of cell wall synthesis caused by the depletion of essential cell wall biosynthesis enzymes such as MurA, MurZ, MurB (Blake *et al.*, 2009), MurF (Sobral *et al.*, 2007), PBP2 (Gardete *et al.*, 2006) or depletion of enzymes involved in mevalonate biosynthesis, the direct precursor for undecaprenyl phosphate lipid carrier synthesis (Balibar *et al.*, 2009). Induction of the CWSS enhances intrinsic resistance/tolerance to almost all cell wall damaging agents, regardless of their target or mode of action (Dengler *et al.*, 2011; McCallum *et al.*, 2011). Members of the CWSS directly linked to peptidoglycan synthesis, such as PBP2, FmtA, MurZ and SgtB, are thought to contribute to the stress response by stimulating cell wall synthesis (Cui *et al.*, 2009; Kato *et al.*, 2010; Mehta *et al.*, 2012). It is predicted that CWSS genes with unknown or poorly characterized functions are also likely to contribute to the stress response by directly or indirectly influencing cell wall synthesis.

All three *S. aureus* LytR-CpsA-Psr (LCP) genes, *msrR*, *sa0908* and *sa2103*, belong to the CWSS (Utaiida *et al.*, 2003; McAleese *et al.*, 2006; Over *et al.*, 2011). LCP proteins are unique to bacteria with Gram-positive cell walls (Hübscher *et al.*, 2008; Kawai *et al.*, 2011) and typically contain a short intracellular N-terminal region, a transmembrane domain and a large extracellular region containing the LCP domain (Hübscher *et al.*, 2008; Kawai *et al.*, 2011). Deletion of LCP proteins in *S. aureus* alters cell surface properties and decreases virulence. Phenotypes of LCP deletion mutants include defective cell separation, increased TritonX-100-induced autolysis, increased beta-lactam susceptibility, and the cell wall WTA content was reduced in an *msrR* deletion mutant (Hübscher *et al.*, 2009). Phenotypes become more pronounced in double mutants, and growth is severely impaired in the LCP triple mutant, which contains large amorphous cells with multiple septa (Over *et al.*, 2011).

Recently, the LCP proteins of *B. subtilis*, TagT (YwtF), TagU (LytR) and TagV (YvhJ) were found to be essential for the formation of a WTA-loaded cell wall. Kawai *et al.* (2011) claim that LCP proteins catalyse the final, previously uncharacterised, step in WTA synthesis, the linkage of WTA to peptidoglycan. WTA are not essential for the cell, but deletion of the first two synthesis steps, catalysed

by TarA (TagA) or TarO (TagO), leads to impaired cell division, colonization and infection *in vivo* (Weidenmaier *et al.*, 2004; Weidenmaier & Peschel, 2008; D'Elia *et al.*, 2009). However, the late-acting enzymes from TarB (TagB) onwards are conditionally essential; mutants are only viable when one of the first two steps of WTA synthesis is inhibited (Swoboda *et al.*, 2010). Blocking the flux of WTA precursors into the WTA pathway prevents the deleterious sequestration of the universal undecaprenyl phosphate lipid carrier that is also essential for peptidoglycan synthesis, and it prevents the accumulation of potentially toxic intermediates. LCP proteins in *B. subtilis* are also conditionally essential, and the LCP triple mutant is only viable when *tagO* (*tarO*) is deleted (Kawai *et al.*, 2011). Whether LCP proteins fulfil the same function in *S. aureus* has not yet been verified.

In this study, reporter gene fusions were used to analyse CWSS expression levels in LCP mutants and to identify promoter regions essential for CWSS induction of LCP genes. The effect of LCP deletion on the WTA content was determined and partial complementation of the LCP triple mutant by TarO (TagO) inhibition demonstrated, suggesting that LCP proteins play an important role in the WTA decoration of *S. aureus* peptidoglycan.

Materials and methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37 °C in Luria Bertani (LB) broth (Difco Laboratories), shaking at 180 r.p.m. with a 1 : 5 culture to air ratio or on LB agar plates. Optical density (OD) measurements were taken at 600 nm. Media were supplemented with the following antibiotics when appropriate: 10 µg mL⁻¹ tetracycline (Sigma), 10 µg mL⁻¹ chloramphenicol (Sigma), 100 µg mL⁻¹ ampicillin (Sigma) or 200 ng mL⁻¹ anhydrotetracycline (Vetranal).

Construction of Δ VraR mutants

The pKOR1 system developed by Bae & Schneewind (2006) was used to inactivate VraR in the different LCP mutant strains, by inserting an XhoI site and two stop codons in-frame into the beginning of the *vraR* coding sequence, truncating VraR after the 2nd amino acid, as previously described (McCallum *et al.*, 2011).

Northern blots

Northern blots were performed as previously described (McCallum *et al.*, 2007). To compare relative expression

Table 1. Strains, plasmids and primers

Strain/plasmid/primer name	Relevant genotype and/or phenotype (strain name) or primer sequence	Source or reference
<i>S. aureus</i>		
RN4220	Restriction-deficient derivative of NCTC 8325-4	Kreiswirth <i>et al.</i> (1983)
MSSA1112	Clinical isolate, <i>bla</i> , Mc ^s Pen ^r	Hübscher <i>et al.</i> (2009)
Δ <i>msrR</i>	MSSA1112, Δ <i>msrR</i> :: <i>ermB</i> ; Mc ^s Em ^r (JH100)	Hübscher <i>et al.</i> (2009)
Δ <i>sa0908</i>	MSSA1112, marker-less <i>sa0908</i> deletion mutant (RH53)	Over <i>et al.</i> (2011)
Δ <i>sa2103</i>	MSSA1112, marker-less <i>sa2103</i> deletion mutant (PS47)	Over <i>et al.</i> (2011)
Δ <i>sa0908/msrR</i>	MSSA1112, Δ <i>sa0908/msrR</i> double-mutant (RH72)	Over <i>et al.</i> (2011)
Δ <i>sa2103/msrR</i>	MSSA1112, Δ <i>sa2103/msrR</i> double-mutant (PS60)	Over <i>et al.</i> (2011)
Δ <i>sa2103/sa0908</i>	MSSA1112, Δ <i>sa2103/sa0908</i> double-mutant (PS109)	Over <i>et al.</i> (2011)
Δ <i>sa2103/sa0908/msrR</i>	MSSA1112, Δ <i>sa2103/sa0908/msrR</i> triple-mutant (PS111)	Over <i>et al.</i> (2011)
Δ <i>VraR</i>	MSSA1112, truncated <i>VraR</i> after the 2nd amino acid (= Δ <i>VraR</i>) (PS199)	This study
Δ <i>VraR/msrR</i>	MSSA1112, Δ <i>VraR/msrR</i> double-mutant (RH194)	This study
Δ <i>VraR/sa0908</i>	MSSA1112, Δ <i>VraR/sa0908</i> double-mutant (PS202)	This study
Δ <i>VraR/sa2103</i>	MSSA1112, Δ <i>VraR/sa2103</i> double-mutant (RH191)	This study
Δ <i>VraR/sa0908/msrR</i>	MSSA1112, Δ <i>VraR/sa0908/msrR</i> triple-mutant (NM776)	This study
Δ <i>VraR/sa2103/msrR</i>	MSSA1112, Δ <i>VraR/sa2103/msrR</i> triple-mutant (RH193)	This study
Δ <i>VraR/sa2103/sa0908</i>	MSSA1112, Δ <i>VraR/sa2103/sa0908</i> triple-mutant (RH216)	This study
SA113	Restriction-deficient derivative of NCTC 8325 (ATCC 35556)	Iordanescu & Surdeanu (1976)
SA113: Δ <i>tarO</i>	SA113, Δ <i>tarO</i> :: <i>ermB</i> ; Em ^r	Weidenmaier <i>et al.</i> (2004)
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80d/ <i>acZ</i> Δ M15 <i>recA1</i>	Invitrogen
Plasmids		
pKOR1	<i>S. aureus</i> - <i>E. coli</i> shuttle vector, <i>ori</i> pAM α 1, <i>ori</i> ColE1, <i>E. coli</i> Am ^r , <i>S. aureus</i> Cm ^r	Bae & Schneewind (2006)
pKOR1- <i>VraR</i> ::stop	pKOR1 construct containing mutant <i>vraR</i> insert with XhoI site and two inframe stop codons inserted between the 2nd and 3rd <i>vraR</i> codons.	McCallum <i>et al.</i> (2011)
pGC2	<i>E. coli</i> - <i>S. aureus</i> shuttle plasmid, <i>ori</i> ColE1- <i>ori</i> pC194 <i>bla</i> <i>cat</i> ; <i>E. coli</i> Am ^r , <i>S. aureus</i> Cm ^r	Skinner <i>et al.</i> (1988)
<i>pmsrR</i>	pGC2 containing 1.3-kb fragment comprising the <i>msrR</i> ORF and upstream flanking sequence	Hübscher <i>et al.</i> (2009)
<i>psa0908</i>	pGC2 containing 1.9-kb fragment comprising the <i>sa0908</i> ORF and upstream flanking sequence	Over <i>et al.</i> (2011)
<i>psa2103</i>	pGC2 containing 2.1-kb fragment comprising the <i>sa2103</i> ORF and upstream flanking sequence	(Over <i>et al.</i> (2011)
pBUS1	<i>S. aureus</i> – <i>E. coli</i> shuttle vector, <i>tetL</i> ; Tc ^r	Rossi <i>et al.</i> (2003)
<i>psas016_p-luc+</i>	pBUS1 containing the <i>sas016</i> promoter-luciferase reporter gene fusion	McCallum <i>et al.</i> (2011)
<i>pvr_p-luc+</i>	pBUS1 containing the <i>vraSR</i> operon promoter-luciferase reporter gene fusion	This study
<i>pmsr_p-luc+</i>	pBUS1 containing the <i>msrR</i> promoter-luciferase reporter gene fusion	Over <i>et al.</i> (2011)
<i>psa0908_p-luc+</i>	pBUS1 containing the <i>sa0908</i> promoter-luciferase reporter gene fusion	Dengler <i>et al.</i> (2011)
<i>psa2103_p-luc+</i>	pBUS1 containing the <i>sa2103</i> promoter-luciferase reporter gene fusion	Over <i>et al.</i> (2011)
<i>psas016Δ6bp_p-luc+</i>	pBUS1 containing the <i>sas016</i> promoter with 6-bp deletion fused to the luciferase gene (Fig. 2)	This study
<i>psas016Δ6Bbp_p-luc+</i>	pBUS1 containing the <i>sas016</i> promoter with 6-bp deletion variant B fused to the luciferase gene (Fig. 2)	This study
<i>pmsrΔ12bp_p-luc+</i>	pBUS1 containing the <i>msrR</i> promoter with 12-bp deletion fused to the luciferase gene (Fig. 2)	This study
<i>pmsrΔ18bp_p-luc+</i>	pBUS1 containing the <i>msrR</i> promoter with 18-bp deletion fused to the luciferase gene (Fig. 2)	This study
<i>psa0908Δ6bp_p-luc+</i>	pBUS1 containing the <i>sa0908</i> promoter with 6-bp deletion fused to the luciferase gene (Fig. 2)	This study
<i>psa2103Δ6bp_p-luc+</i>	pBUS1 containing the <i>sa2103</i> promoter with 6-bp deletion fused to the luciferase gene (Fig. 2)	This study
Primers		
<i>vra.lucF</i>	AATTTGGTACCGCACATGTAATTAATTACTT	This study
<i>vra.lucR</i>	ATTAACCATGGCTATCACCTTTTATAATAAGT	This study

Table 1. Continued

Strain/plasmid/primer name	Relevant genotype and/or phenotype (strain name) or primer sequence	Source or reference
sas016.lucF	AATTAGGTACCTGGATCACGGTGCATACAAC	McCallum et al. (2011)
sas016.lucR	AATTACCATGGCCTATATTACCTCCTTTGCT	McCallum et al. (2011)
sas016-Δ6bp.F	AAATTAAGCTTGTGGATGCACACATAAAAAAT	This study
sas016-Δ6bp.R	AAATTAAGCTTTATCAACTTTTTATCAGAC AT	This study
sas016-Δ6bpB.F	AAATTAAGCTTTCTATGTCTGATAAAAAAGTT	This study
sas016-Δ6bpB.R	AAATTAAGCTTATTACTAAGACTATTTATGT	This study
JR13 (msrR.lucF)	GGGTACCTGAGCTAAAGTTAAGTCGCC	Rossi et al. (2003)
JR14 (msrR.lucR)	TATCCATGGTTACCTACCTTATATCTTC	Rossi et al. (2003)
msrR-Δ12bp.F	AATTTAAGCTTTTATTAAGAAATCACTTGCTT	This study
msrR-Δ18bp.F	AATTTAAGCTTAGAAATCACTTGCTTTTGAA	This study
msrR-Δ12bp/Δ18bp.R	AATTTAAGCTTTCTAATGAAAGGATGTCAAA	This study
sa0908.lucF	AATTAAGTACCATAATAGTACACACGCATGT	Dengler et al. (2011)
sa0908.lucR	TTAATCCATGGTTGATGCTCTATATTAAT	Dengler et al. (2011)
sa0908-Δ6bp.R	AATTTAAGCTTTTCTTGAATTTGAATGTTT	This study
sa0908-Δ6bp.F	AATTTAAGCTTCCATAACATTTGTATTTTTAC	This study
lucF.sa2103	GGGGTACCAAATGACGACTTTAGATGGTAAG	Over et al. (2011)
lucR.sa2103	CATGCCATGGCAATCCACCCTCTTACTATTCC	Over et al. (2011)
sa2103-Δ6bp.F	AATTAGAATTCAGTATAGTAAAAAATTAT	This study
sa2103-Δ6bp.R	AATTAGAATTCACGTATAACTATTTTTATC	This study
SAS016.PErev	CTTCATGGTGATACTGTGCGATA	This study

Am, ampicillin; Cm, chloramphenicol; Em, erythromycin; Mc, methicillin; Pen, penicillin; Tc, tetracycline; r, resistant; s, susceptible. Restriction sites are underlined.

levels of *sas016* in wild type and mutant strains, overnight cultures were diluted to OD 0.05 in prewarmed LB broth and cultures grown to OD 1.5, except for the LCP triple mutant that was sampled at OD 0.5 because of its severe growth defect. Uninduced culture samples were collected, and the remainder of the culture was induced with oxacillin ($10 \mu\text{g mL}^{-1}$) for 30 min before induced samples were collected. Total RNA was extracted as described by Cheung et al. (1994). RNA samples ($9 \mu\text{g}$) were separated in a 1.5% agarose-20 mM guanidine thiocyanate gel in $1\times$ TBE buffer (Goda & Minton, 1995). The *sas016* digoxigenin (DIG)-labelled probe was amplified using the PCR DIG Probe synthesis kit (Roche) as previously described (Dengler et al., 2011).

Primer extension

The transcriptional start site of *sas016* was determined by primer extension, as previously described (McCallum et al., 2007), using primer SAS016.PErev (Table 1) and $20 \mu\text{g}$ of RNA harvested from a culture of *S. aureus* COL that had been grown to OD 0.5 and induced with $10 \mu\text{g mL}^{-1}$ of teicoplanin for 30 min.

Luciferase reporter gene fusions

The promoter region of the *vraSR* operon was PCR amplified from *S. aureus* strain COL using primer pair *vra.lucF* and *vra.lucR* (Table 1). The PCR product was

digested with Asp718 and NcoI and ligated directly upstream of the promoterless luciferase (*luc+*) gene in the vector pSP-*luc+* (Promega). Fragments containing the resulting promoter-*luc+* translational fusions were then excised with Asp718 and EcoRI and cloned into the *Escherichia coli* – *S. aureus* shuttle vector pBUS1 (Table 1). The fusion plasmids *pvr_p-luc+* and *psas016_p-luc+* (McCallum et al., 2011) were then electroporated into *S. aureus* RN4220, re-isolated and electroporated into *S. aureus* SA113, SA113Δ*tarO*, MSSA1112 and all LCP and *VraR*/LCP mutants.

Predicted *VraR*-binding sites of luciferase fusion constructs were disrupted by amplifying each promoter as two fragments, using primers listed in Table 1. Complementary fragments were digested and ligated together, to create recombinant promoters in which 6–18-bp regions were replaced by restriction sites. Promoters were then fused to the luciferase gene as described above, and the resulting plasmids were electroporated into RN4220.

Luciferase assays

To measure luciferase activities, cultures were grown from overnight cultures inoculated to an OD 0.05 in prewarmed LB broth containing tetracycline. One-millilitre culture samples were harvested by centrifugation, and the pellets frozen at $-20 \text{ }^{\circ}\text{C}$.

To determine relative light units (RLU), pellets were thawed briefly and resuspended in PBS (pH 7.4) to an

OD of either 10 or 1, depending on induction levels. Aliquots of the cell suspensions were then mixed with equal aliquots of Luciferase Assay System substrate (Promega), and luminescence was measured for 15 s after a delay of 3 s on a Turner Designs TD-20/20 luminometer (Promega) as previously described (Dengler *et al.*, 2011).

Bacitracin gradient plates and Etests

Qualitative differences in resistance levels for bacitracin (from *Bacillus licheniformis*, Sigma) were compared using antibiotic gradient plates as previously described (Hübscher *et al.*, 2009). LB medium was supplemented with ZnCl₂ (25 µg mL⁻¹), and plates were incubated at 37 °C for 48 h. Bacitracin minimum inhibitory concentrations (MIC) were detected by Etest (Bio-Mérieux) on Müller-Hinton plates swabbed with an inoculum of 0.5 McFarland and incubated at 37°C for 24 h.

Growth under subinhibitory concentrations of tunicamycin

Overnight cultures were diluted to OD 0.05 in LB media containing 0.05 µg mL⁻¹ tunicamycin (AG Scientific). OD measurements were taken hourly for 8 h.

Preparation and quantification of WTA

Cell walls and WTA were prepared as previously described (Majcherczyk *et al.*, 2003). The amount of WTA was indirectly quantified by determination of the cell wall phosphorus content (Ames & Dubin, 1960). Experiments were performed two to four times with three technical replicates per sample.

Results and discussion

Deletion of LCP proteins leads to increased *VraSR*-dependent basal expression of the CWSS

LCP proteins are essential for optimal cell separation (Over *et al.*, 2011). The severe cell division defects of double and triple LCP mutants resemble those resulting from the depletion of essential peptidoglycan biosynthesis enzymes or inhibition of WTA synthesis, which both trigger *VraSR* signal transduction and induction of the CWSS (Gardete *et al.*, 2006; Sobral *et al.*, 2007; Balibar *et al.*, 2009; Blake *et al.*, 2009; Campbell *et al.*, 2012). The most sensitive indicator of staphylococcal CWSS activation is the *sas016* gene, as demonstrated previously in Northern blot, promoter-luciferase fusion and microarray studies; however, its function is still unknown (McAleese *et al.*, 2006; Dengler *et al.*, 2011). We therefore determined the

basal CWSS transcription levels of single, double and triple LCP mutants and compared them to those of the parent strain MSSA1112 using a probe against the CWSS gene *sas016*. Northern blots showed that *sas016* transcription was detectably higher in single LCP mutants than in the wild type, with highest levels of transcription in the *Δsa0908* mutant (Fig. 1a). Transcript levels were further increased in double LCP mutants, *Δsa0908/msrR*, *Δsa2103/msrR* and *Δsa2103/sa0908*, and were extremely high in the LCP triple mutant (Fig. 1a).

To compare and quantify CWSS expression at different growth stages, a promoter-luciferase reporter construct containing the *sas016* promoter (*psas016_p-luc+*) was used as previously described (McCallum *et al.*, 2011). Figure 1b shows the luciferase activity levels measured in relative light units (RLU) in the wild type and LCP mutant strains at the time points indicated. The right graph shows the corresponding OD values of the cultures at each sampling point. To confirm patterns of CWSS upregulation, expression of the autoregulatory *vra* promoter from the *vraSR* operon was also measured, using the promoter-luciferase fusion *pvra_p-luc+* (Supporting information, Fig. S1). Both constructs, *psas016_p-luc+* and *pvra_p-luc+* displayed very similar luciferase activity profiles, with expression from the *vraSR* operon promoter being consistently lower than that from the *sas016* promoter, reflecting differences in promoter activity that were observed in previous transcriptional analyses of the CWSS (McAleese *et al.*, 2006). In all strains tested, the activity increased during exponential growth and decreased again as cells entered stationary phase, with maximum luciferase activity levels reached in late exponential growth, at around 4.5 h.

Luciferase activity profiles corresponded closely to the results from Northern blots (Fig. 1a). Expression was reproducibly higher in LCP single mutants than in the parent MSSA1112, with up to twofold increases in *Δsa2103* and *ΔmsrR* mutants and a larger, up to sixfold increase, in *Δsa0908*. The luciferase expression from the *sas016* promoter increased further in the double LCP mutants with the highest expression levels seen in *Δsa2103/sa0908* and comparable levels in *Δsa0908/msrR* and *Δsa2103/msrR*. The most dramatic increase was apparent in the triple mutant, where expression levels were up to 250-fold higher than in the wild type, similar to levels reached after antibiotic stress (Fig. 1e). Activity peaked slightly later in some mutants, possibly reflecting minor differences in growth dynamics.

To verify that increased CWSS expression was *VraSR* dependent, a *VraR* mutation was introduced into the wild type strain MSSA1112 and all single and double mutants. The *VraR* mutation could not be introduced into the triple mutant, probably due to its cell separation

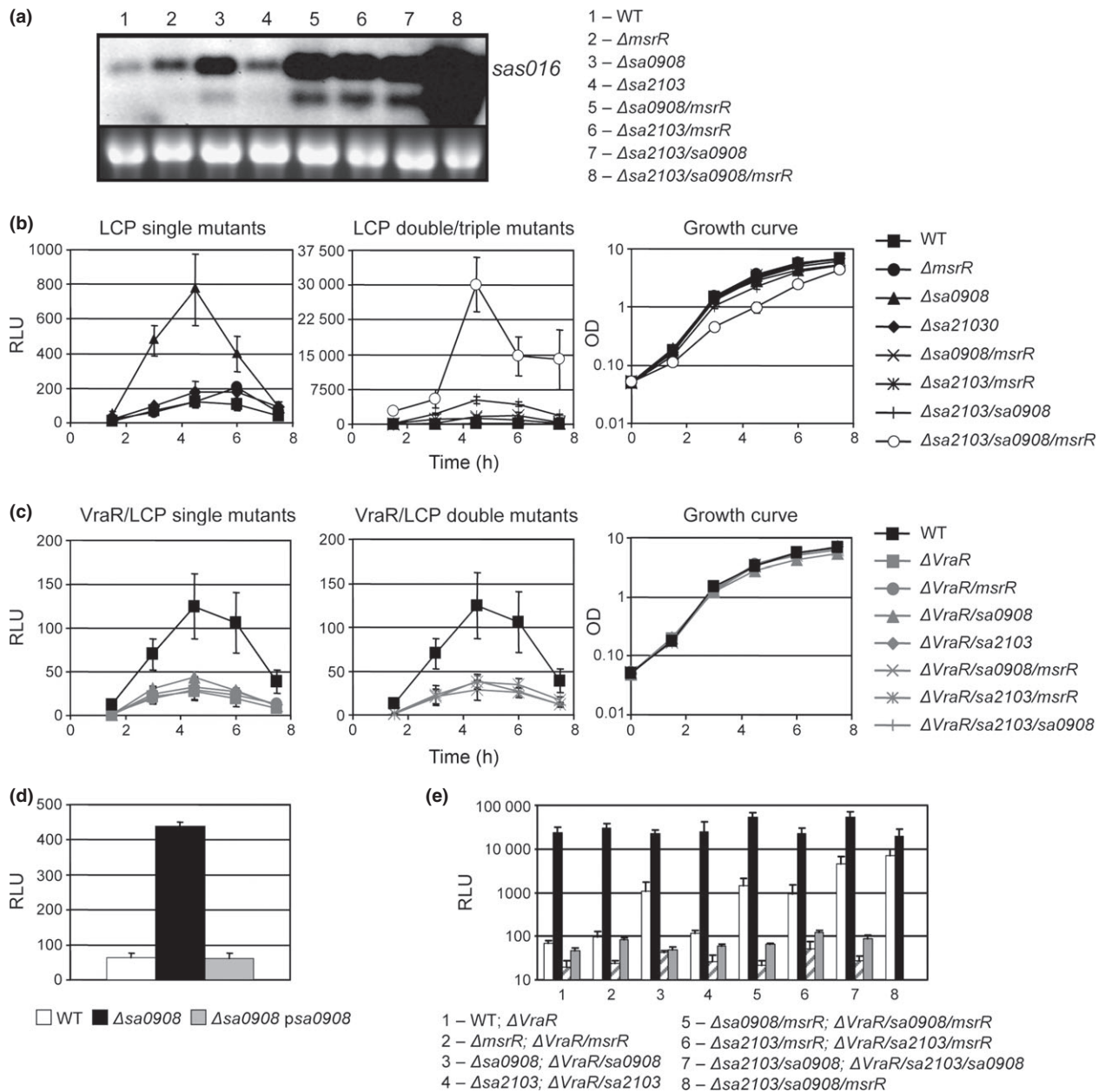


Fig. 1. CWSS expression in LCP and VraR/LCP mutant strains. (a) Northern blot analysis showing the expression of the CWSS gene *sas016* in LCP mutants. (b and c) Luciferase activities measured from reporter construct *psas016_p-luc+* in LCP mutants (b) and in VraR/LCP mutants (c). Values shown indicate the RLU measured in each of the strains at the different growth stages indicated. Left, single LCP or VraR/LCP mutants; middle row, LCP or VraR/LCP double and triple mutants; right, corresponding OD values of the cultures at each sampling point for all strains. Samples were taken at 1.5-h intervals for up to 7.5 h. The RLU scales of the different graphs were adjusted to appropriate ranges for visualizing strain-dependent differences. Average values and standard deviations from three independent experiments are shown. (d) Complementation of the $\Delta sa0908$ mutant strain by introducing *sa0908* *in trans*. RLU were measured from strains containing the reporter construct *psas016_p-luc+* that were harvested between OD 0.6 and 0.8. Values shown represent the averages and standard deviations from three independent experiments. (e) Luciferase activities of LCP and VraR/LCP mutants with and without oxacillin ($10 \mu\text{g mL}^{-1}$) induction. Cultures were grown to OD 1.5–1.8 before being split into two prewarmed flasks, one culture was induced with oxacillin and the other left uninduced, and cultures were grown for a further 30 min before samples were harvested. RLU values are shown on a logarithmic scale and represent the averages and standard deviations from three independent experiments. Untreated LCP mutants are shown in white, treated LCP mutants in black, untreated VraR/LCP mutants in grey/white hatched and treated VraR/LCP mutants in grey.

defects and temperature sensitivity (Over *et al.*, 2011). Expression of the CWSS was measured over growth in the *VraR/LCP* mutants using *psas016_p-luc+*. In all Δ *VraR* mutants, CWSS expression levels dropped clearly below wild type values (Fig. 1c). The minor differences in expression between all *VraR/LCP* mutants and *MSSA1112* Δ *VraR*, indicates that the increased basal CWSS expression levels in LCP mutants were *VraSR* dependent.

Complementation of *Asa0908*, the single mutant with the strongest effect on CWSS expression, by re-introduction of *sa0908 in trans*, reduced luciferase activity back to wild type levels (Fig. 1d), demonstrating that differences in CWSS activity were directly linked to the LCP mutations.

LCP mutants are still responsive to cell wall stress

As the CWSS was already inherently activated to varying degrees in the absence of external stress in growing LCP mutants, we tested their potential to react to an external cell wall stress. Luciferase activity from *psas016_p-luc+* was measured in exponentially growing LCP and *VraR/LCP* mutants exposed to oxacillin for 30 min (Fig. 1e). Basal transcription levels were again increased in uninduced LCP mutants. Expression was still strongly induced by oxacillin stress in the single and double LCP mutants. Expression in the untreated LCP triple mutant appeared to already be close to the maximum level, as it only increased approximately twofold upon oxacillin stress (Fig. 1e).

Identification of promoter regions involved in CWSS induction

Two *VraR*-binding sites have been identified in the promoter of the *vraSR* operon with a tail to tail tandem repeat motif ACT(X)_nAGT (X = A, C, T or G; n = 1–3; Belcheva & Golemi-Kotra, 2008; Belcheva *et al.*, 2012). They are involved in the fine tuning of the *VraR*-dependent expression of the CWSS and have different affinities for *VraR* or phosphorylated *VraR* (Belcheva & Golemi-Kotra, 2008; Belcheva *et al.*, 2012). *VraR*-binding sites in other CWSS promoters have so far only been studied *in silico*. A 16-bp palindromic sequence TCAGHCTnnAGDCTGA (H = A, T, C; D = A, T, G), deduced from the *VraR* homologue *CesR* in *L. lactis* (Martinez *et al.*, 2007) and partially overlapping the motif identified by Belcheva *et al.*, is present in the promoters of 26 *VraSR*-dependent genes of the *S. aureus* N315 genome (Martinez *et al.*, 2007). As we found the induction levels of the three LCP genes and of the highly induced CWSS gene *sas016* to

vary over a wide range, we analysed their specific *VraR*-binding motifs. The transcriptional start sites of *msrR*, *sa0908* and *sa2103* are known (Rossi *et al.*, 2003; Over *et al.*, 2011), and the transcriptional start site of *sas016* was determined by primer extension to be 29-nt upstream of the ATG (data not shown). Potential *VraR*-binding sites were predicted in all four promoters investigated in this study, based on previously published motifs (Martinez *et al.*, 2007; Belcheva & Golemi-Kotra, 2008; Belcheva *et al.*, 2012). These sequences were then disrupted and/or deleted in the promoter regions of luciferase reporter gene constructs (Fig. 2). Disruption of the predicted motifs decreased basal expression levels and largely abolished induction by oxacillin (Fig. 2). In all four promoters, the regions essential for induction were located close to the –35 boxes. The promoter of *sas016* contained a second region that was found to be essential for full induction. The presence of two *VraR*-binding sites could contribute to the extremely high induction levels of *sas016*. Alignment of the nucleotide sequences from the *VraR*-binding regions identified here revealed no obvious consensus sequence. The high-affinity *VraR*-binding region in the *vraSR* operon promoter (Belcheva *et al.*, 2012) and the *tcaA* promoter region required for induction (McCallum *et al.*, 2007) were both also in close proximity to their respective –35 box. The *msrR* promoter region needed for induction corresponded to the *CesR*-like motif identified *in silico* by Martinez *et al.* (2007; Fig. 2); however, deletion of the suggested *CesR*-binding region for *sa0908* did not affect transcription (data not shown). For the promoters of *sas016* and *sa2103*, no *CesR*-like binding sites were previously predicted (Martinez *et al.*, 2007); however, the *VraR*-binding sites identified here both contained potential *CesR*-like sequences. To create a reliable *VraR*-binding consensus for *S. aureus* CWSS gene induction, detailed promoter analysis of more *VraSR*-dependent genes is required. The trend, however, seems to involve sequences with a close proximity to the –35 box of the CWSS gene promoter.

Bacitracin hypersensitivity of the LCP triple mutant

Bacitracin inhibits the recycling of the universal undecaprenyl phosphate lipid carrier by preventing dephosphorylation of the undecaprenyl pyrophosphate (Stone & Strominger, 1971; Qi *et al.*, 2008). Kawai *et al.* (2011) recently suggested that LCP proteins transfer WTAs and other anionic polymers from the lipid carrier to the cell wall peptidoglycan in *B. subtilis*. Comparative growth of LCP mutants on bacitracin gradient plates showed that the LCP triple mutant was highly susceptible (Fig. 3a). The bacitracin MIC of the triple mutant was 4 μ g mL⁻¹

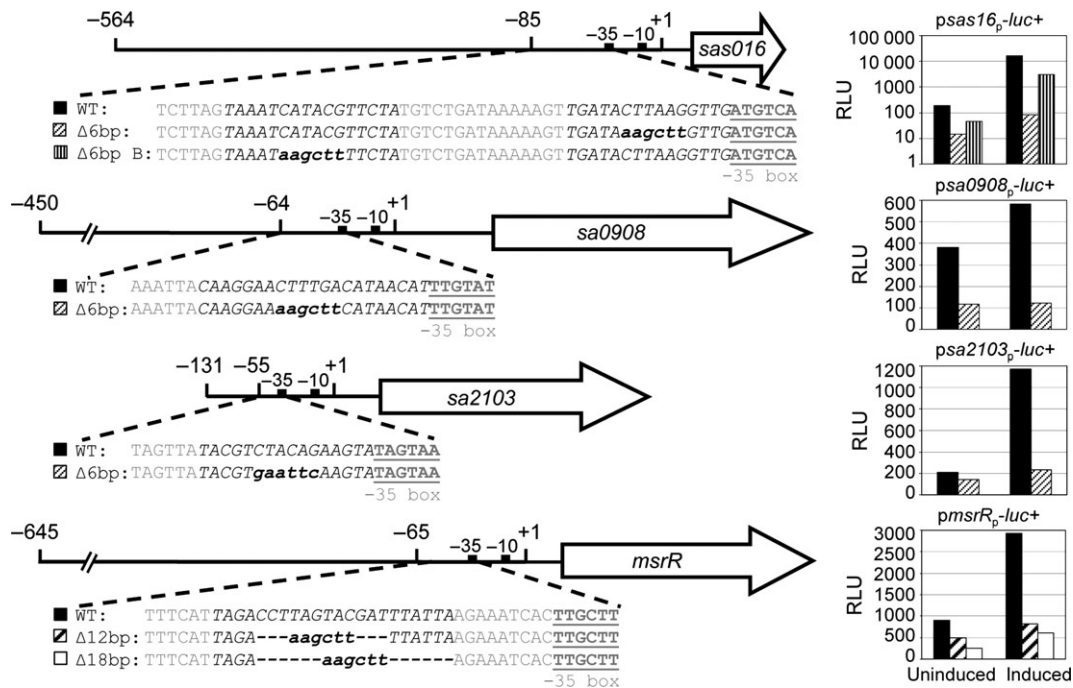


Fig. 2. Analysis of predicted VraR-binding sites in the *sas016*, *msrR*, *sa0908* and *sa2103* promoters. Nucleotide sequences of promoter regions and introduced promoter mutations are shown, together with their corresponding luciferase activities when fused to the luciferase gene and introduced into *Staphylococcus aureus* strain RN4220. RLU of the promoter constructs were measured with and without 30 min of induction with 10 $\mu\text{g mL}^{-1}$ oxacillin. Cultures were grown to OD 0.5–0.8 before splitting into two prewarmed flasks comprising the uninduced and oxacillin-induced samples. Predicted VraR-binding regions are shown in black italic capitals; –35 boxes in bold grey underlined capitals; restriction sites in bold italic lowercase letters; deleted regions are indicated by a dashed line. Representative results from three independent experiments are shown.

compared to 32 $\mu\text{g mL}^{-1}$ for wild type and all LCP single and double mutants. The hyper susceptibility of the LCP triple mutant to bacitracin could therefore be due to an additional shortage of the lipid carriers caused by the lack of the putative WTA ligase function of LCP proteins.

Deletion of all three LCP proteins in *S. aureus* depletes WTA content

In line with the proposed function of LCP proteins, previous studies showed a decrease in the WTA content of LCP mutants in different species (Hübscher *et al.*, 2008; Kawai *et al.*, 2011). Therefore, we analysed the WTA content of LCP single mutants and the triple mutant in *S. aureus*, via detection of the cell wall phosphorus content (Ames & Dubin, 1960). The previously described decrease in the WTA content of the *AmrSR* mutant (Hübscher *et al.*, 2009) could be confirmed here, and the WTA contents of the *Δsa0908* and *Δsa2103* mutants were decreased to 62% and 95% of the wild type level, respectively (Fig. 3b). An almost complete depletion of WTA was observed in the triple LCP mutant, with cell wall phosphorus content down to 2% of the wild type.

Re-introduction of single LCP genes into the triple mutant restored WTA levels to 94%, 81% and 69% of wild type levels for *sa2103*, *msrR* and *sa0908*, respectively. The capacity of all LCP proteins to restore the WTA content to a certain degree confirmed a partial functional redundancy that has been shown for other phenotypes such as growth defects, beta-lactam resistance, biofilm formation and self-agglutination (Over *et al.*, 2011). The very low WTA content of the LCP triple mutant confirmed that LCP proteins in *S. aureus* have an essential function in WTA loading of the cell wall.

TarO (TagO) inhibition can partially complement the growth defect of the *S. aureus* LCP triple mutant

The three LCP genes in *B. subtilis* are conditionally essential, meaning that an LCP triple mutant in *B. subtilis* is only viable when *tagO* (*tarO*) is also deleted, thereby preventing the flux of precursors into the WTA synthesis pathway (Kawai *et al.* 2011). The effect of TarO (TagO) inhibition on the LCP triple mutant was tested to detect a possible connection between LCP proteins with WTA

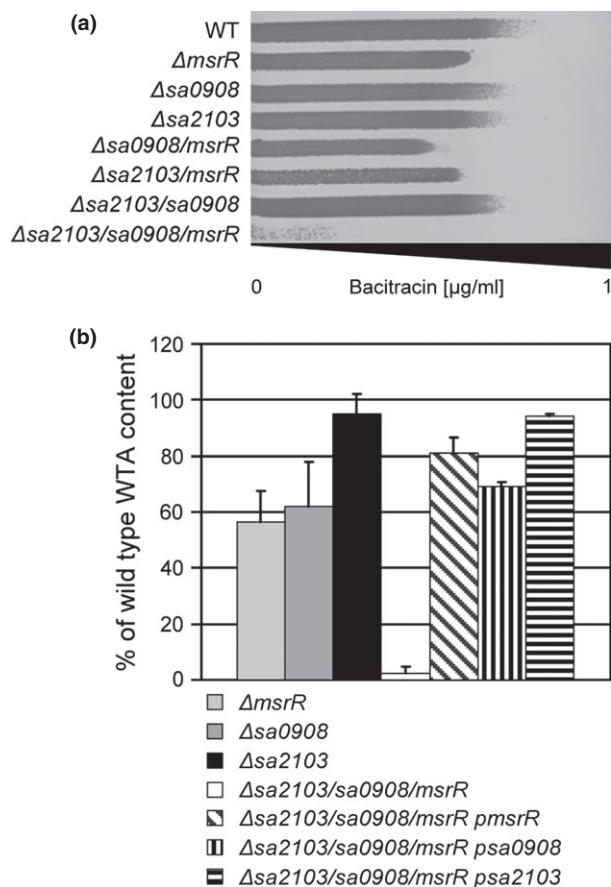


Fig. 3. Bacitracin susceptibility and phosphorus content of the cell wall (WTA content) of LCP mutants. (a) Bacitracin gradient plates of LCP mutants. MICs of bacitracin were detected by Etest (BioMérieux): wild type MSSA1112 and LCP single and double mutants all had MICs of $32 \mu\text{g mL}^{-1}$, LCP triple mutant had an MIC of $4 \mu\text{g mL}^{-1}$. (b) Relative levels of WTA contents are shown as percentages of wild type MSSA1112 content, determined indirectly by detecting the phosphorus content of the cell wall for LCP single mutants, the LCP triple mutant and complemented triple mutants. The experiment was performed two to four times with three technical replicates per sample. The average of the absolute values for the wild type was $0.81 \pm 0.04 \mu\text{mol phosphorus per mg cell wall}$.

synthesis or assembly in *S. aureus*, as found for *B. subtilis* (Kawai *et al.*, 2011). Subinhibitory concentrations of tunicamycin, which are known to inhibit TarO (TagO; Campbell *et al.*, 2011), could partially complement the growth defect of the LCP triple mutant (Fig. 4a). The minimal doubling time of the triple mutant decreased from 49 ± 2 to 34 ± 2 min upon tunicamycin treatment. Inhibition of TarO in the wild type did not significantly affect the minimal doubling time of 25 ± 0.6 min but reduced the maximal OD reached after 8 h of growth from 8.2 to 5.5. This result supports an involvement of LCP proteins in a late step of WTA synthesis in *S. aureus*.

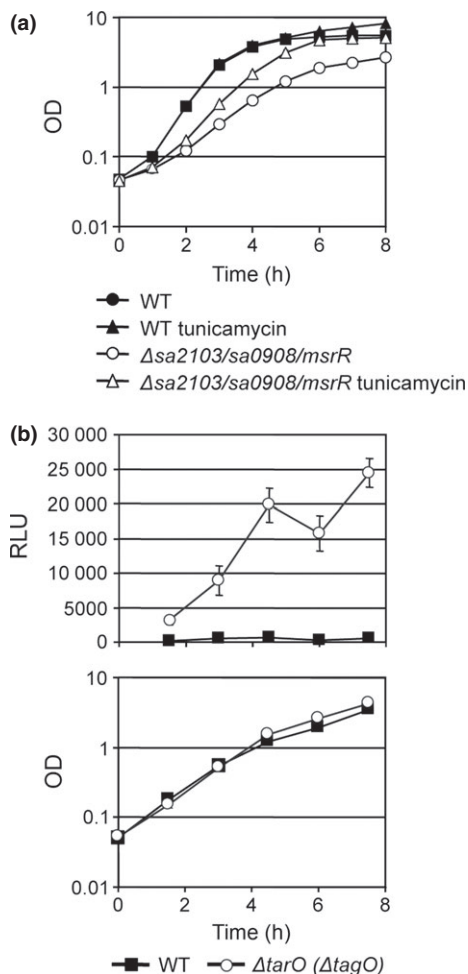


Fig. 4. Growth of the LCP triple mutant under subinhibitory concentrations of tunicamycin and CWSS expression in a *tarO* (*tagO*) mutant strain. (a) Growth of the LCP triple mutant and wild type MSSA1112 with and without tunicamycin ($0.05 \mu\text{g mL}^{-1}$). Average values and standard deviations from three independent experiments are shown. (b) Luciferase activity, in RLU, at different growth stages in the wild type strain SA113 and the SA113 $\Delta tarO$ mutant strain, measured from reporter construct *psas016_p-luc+*. Upper graph shows luciferase measurements and lower graph, the corresponding OD values of the cultures at each sampling point for all strains. Average values and standard deviations from three independent experiments are shown.

As LCP proteins in *B. subtilis* are essential, it could be that the staphylococcal LCP triple mutant is only viable because of compensatory mutations, which remains to be verified. However, it is also possible that the functions of LCP proteins in *S. aureus* are not identical to those in *B. subtilis*, because differences have been found in the WTA synthesis pathways of these closely related bacteria (Brown *et al.*, 2010). Also, in contrast to *S. aureus*, WTA-deficient strains in *B. subtilis* have significantly decreased growth rates and lost their rod shape, indicating potential

differences in the roles of WTA ligases in *B. subtilis* and *S. aureus* cell division (Weidenmaier et al., 2004; D'Elia et al., 2006).

Deletion of *tarO* (*tagO*) induces the CWSS

Measurement of CWSS expression in an *S. aureus* SA113 Δ *tarO* (Δ *tagO*) mutant (Weidenmaier et al., 2004), with the reporter plasmid *psas016_p-luc+*, revealed that inhibition of the first step of WTA synthesis induces the CWSS (Fig. 4b). This result is in conflict to the observations by Campbell et al., (2011) who showed that inhibition of TarO (TagO) by subinhibitory concentrations of tunicamycin does not induce the CWSS. They suggested that CWSS induction is triggered by the sequestration of the lipid carrier rather than WTA deficiency (Campbell et al., 2011, 2012). However, our analysis of the *tarO* (*tagO*) mutant indicates that further research is required to reveal the actual trigger of CWSS induction.

Conclusions

Deletion of LCP proteins increased basal expression levels of CWSS genes via the *VraSR* two-component system. The LCP triple mutant showed very high basal expression of the CWSS, close to levels triggered by antibiotic stress. The LCP double and single mutants, however, still responded to cell wall stress by further upregulating the CWSS.

Promoter regions required for *VraR*-dependent induction of the LCP genes and *sas016* shared low overall nucleotide similarity, but all contained fragments of the predicted *CesR*-like binding consensus or the *VraR*-binding motif of the *vraSR* operon and all were in close proximity to the -35 box of the gene's promoter.

Hyper susceptibility of the triple mutant to bacitracin, the virtual absence of WTA and partial restoration of WTA levels by complementation with each of the single LCP proteins, as well partial complementation of its growth defect by TarO (TagO) inhibition, support the hypothesis that *S. aureus* LCP proteins have WTA ligase functions, as suggested by Kawai and colleagues for *B. subtilis* (Kawai et al., 2011).

An enzymatic analysis of all three LCP proteins will be required to confirm their specific WTA ligase functions, substrates and products.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. CWSS expression in LCP mutant strains measured with *pvr_a-luc+*.

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