cwrA, a gene that specifically responds to cell wall damage in *Staphylococcus aureus*

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Transcriptional profiling data accumulated in recent years for the clinically relevant pathogen *Staphylococcus aureus* have established a cell wall stress stimulon, which comprises a coordinately regulated set of genes that are upregulated in response to blockage of cell wall biogenesis. In particular, the expression of *cwrA* (SA2343, N315 notation), which encodes a putative 63 amino acid polypeptide of unknown biological function, increases over 100-fold in response to cell wall inhibition. Herein, we seek to understand the biological role that this gene plays in *S. aureus*. *cwrA* was found to be robustly induced by all cell wall-targeting antibiotics tested – vancomycin, oxacillin, penicillin G, phosphomycin, imipenem, hymeglusins and bacitracin – but not by antibiotics with other mechanisms of action, including ciprofloxacin, erythromycin, chloramphenicol, triclosan, rifampicin, novobiocin and carbonyl cyanide 3-chlorophenylhydrazone. Although a $\Delta cwrA$ *S. aureus* strain had no appreciable shift in MICs for cell wall-targeting antibiotics, the knockout was shown to have reduced cell wall integrity in a variety of other assays. Additionally, the gene was shown to be important for virulence in a mouse sepsis model of infection.

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INTRODUCTION

Staphylococcus aureus is a facultatively aerobic Gram-positive bacterium that naturally inhabits the skin and mucous membranes of mammals. It is notorious for causing both nosocomial and community-acquired infections, resulting in a variety of human diseases ranging from mild wound infections to more serious ailments that include septic shock, endocarditis and osteomyelitis (Archer, 1998). Multidrug-resistant *S. aureus* have emerged rapidly in the clinic, and cases of community-acquired infections are on the rise worldwide (Klevens *et al.*, 2006), necessitating the need for novel drug discovery efforts.

Given its essential role in supporting cell viability and interacting with the external environment during infection (Amako *et al.*, 1982; Giesbrecht *et al.*, 1998; Navarre & Schneewind, 1999), the staphylococcal cell wall has proven to be a desirable and successful target for antibacterial intervention. In the past decade, transcriptomic studies have become increasingly utilized in the drug discovery

process, especially in elucidating the mechanism of action of novel chemical agents. Previous transcriptional profiling studies have attempted to define the staphylococcal response to treatment with cell wall-targeting antibiotics. When the transcriptomes of several strains of *S. aureus* treated with oxacillin, D-cycloserine, bacitracin or vancomycin were combined, a set of 15 upregulated and two downregulated genes were found to be common to all four studies (Kuroda *et al.*, 2003; McAleese *et al.*, 2006; McCallum *et al.*, 2006; Utaida *et al.*, 2003). These upregulated genes, *fnt*, *htrA*, *prsA*, *sgtB*, *vraS*, *murZ*, *tcaA*, SA02100, SA02101, SA02112, SA02583, SA02723, SA02724, SA02811 and SA02872, and downregulated genes, *atl* and SA00427, have been proposed to encompass a coordinately regulated group termed the ‘cell wall stimulon’.

One gene from this stimulon, SA2343 (N315) or SAOUHSC_02872 (NCTC 8325), has incited particular interest. In genetic studies that we performed on *S. aureus*, SA2343 was shown to be upregulated 100–500 fold when any of the genes in the mevalonate pathway, responsible for biosynthesis of the isoprenoid precursor isopentenyl pyrophosphate (IPP), is downregulated (Balibar *et al.*, 2009). Given that IPP is the building block for the cell wall carrier lipid undecaprenyl pyrophosphate (UPP), which is essential in peptidoglycan biosynthesis (Wilding *et al.*, 2000), this massive overexpression is presumed to be a response to cell wall damage. This upregulation of SA2343 was more than 20-fold greater than the upregulation of any

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; RLU, relative light units; TEM, transmission electron microscopy; UPP, undecaprenyl pyrophosphate.

A supplementary figure, showing a time course of lysis in response to lysostaphin, and two supplementary tables, showing the luminescence signal of RN4220/*lux1* in response to various antibiotics, and the luminescence signal of RN4220/*lux2* in response to various antibiotics, are available with the online version of this paper.

other gene in the cell wall stimulon. Further stimulating interest in SA2343 is the fact that in a separate study it was shown to be the highest-fold upregulated gene in several clinical vancomycin intermediate-resistant *S. aureus* (VISA) strains (McAleese *et al.*, 2006).

Herein, we seek to understand the role that gene SA2343, which we have renamed *cwrA* for cell wall responsive for antibiotics, plays in *S. aureus*. Using genetic and phenotypic readouts we seek to define the conditions under which this gene is expressed and the role that it plays in the physiology of *S. aureus*.

METHODS

Constructing luciferase integration vectors. For cloning purposes, *S. aureus* integration vector pCL84 (Lee *et al.*, 1991) was first double-digested with *Pst*I/*Sal*I restriction enzymes, blunt-ended with T4 DNA polymerase, and religated with T4 DNA ligase to generate pCL84ΔPstΔSal. The *luxABCDE* gene cluster was amplified from *S. aureus* strain Xen29 (Xenogen) with the primers 5'-CCAGGAT-CCTGGTCGACAAAAGGAGGATACGTATGAAATTTGGAAACTTT-TTGCTTACAT-3' and 5'-ATTCCATGGAATTCTTAACATCAA-ACGCTTCGGT-3' (restriction sites underlined), and cloned into the *Bam*HI and *Eco*RI sites in pCL84ΔPstΔSal. This resulted in pCL84/Lux, which contained an optimal ribosome-binding site (RBS) for *luxA*, and *Bam*HI and *Sal*I sites upstream of the *luxA* gene for cloning of desired promoters to generate transcriptional fusions. Either the putative promoter of *cwrA* (from positions -228 to -14 5' to the *cwrA* ORF), using the primers 5'-GGCGGATCCAATC-CTAATAACTTATTTACTTGT-3' and 5'-AAAGTCGACTTTATAT-TTCTCTTACTCACATTG-3', or the putative promoter plus the *cwrA* ORF, using the primers 5'-GGCGGATCCAATCCTAATAACT-TATTTACTTGT-3' and 5'-TAAGTCGACTTAAAGAAATCAGATGGGTTA-3', was amplified and then cloned into the *Bam*HI/*Sal*I sites in pCL84/Lux, generating pCL84/PcwrA-Lux or pCL84/PcwrA_ORFLux, respectively.

In order to generate the translational fusion vector, the region between the third codon of *cwrA* and the start of *luxA* in pCL84/PcwrA_ORFLux was deleted. Utilizing a unique *Sna*BI site adjacent to the start codon of *luxA*, the primers 5'-AATCAATTACGTATGA-AATTTGGAAACTTTTGTG-3' and 5'-ATTCAATTACGTAATATTC-TCATCGCTATCATAC-3' were used to amplify the entire plasmid outside the deleted region. The linear PCR product was then digested with *Sna*BI and ligated to yield pCL84/PcwrA_4aaLux. All plasmids were propagated in *Escherichia coli* TOP10 cells (Invitrogen) and sequenced (Agencourt Bioscience) to verify that the constructs were correct.

Integration of reporter constructs into the *S. aureus* chromosome. The integration plasmids pCL84/PcwrA-Lux, pCL84/PcwrA_ORFLux and pCL84/PcwrA_4aaLux were separately transformed into *S. aureus* strain CYL316, which is RN4220 carrying a plasmid-borne phage integrase gene that facilitates the site-specific integration of pCL84-based plasmids into the *attB* locus on the *S. aureus* chromosome (Lee *et al.*, 1991). Successful integrants were selected through tetracycline resistance conferred by the *tet*^R cassette on pCL84, and subsequently verified by PCR screening. The corresponding clones were named RN4220lux1, RN4220lux2 and RN4220lux3, respectively.

Testing of compounds on reporter constructs. In a 96-well format, overnight cultures of the *lux* reporter strains were diluted 50-fold in fresh Mueller–Hinton Broth (MHB; Becton Dickinson), and

99 μl of the diluted cells was then aliquoted to each well of a 96-well flat-bottomed white polystyrene tissue-culture plate (Costar). One microlitre of the compound to be tested, at various concentrations, was then added to each well. The cells were grown at 37 °C with shaking at 225 r.p.m. The luciferase signal in each well was determined using a PHERAstar microplate reader (BMG LABTECH) at various time points. Erythromycin, chloramphenicol, rifampicin, novobiocin, carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), oxacillin and bacitracin were purchased from Sigma. Triclosan, vancomycin, penicillin, imipenem and ciprofloxacin were purchased from USP. Daptomycin was purchased from Cubist Pharmaceuticals and hymeglusin was obtained from the Novartis Natural Products Unit (NPU).

For stress responses, hydrochloric acid (0.04–90 mM), sodium hydroxide (0.04–90 mM) or hydrogen peroxide (0.003–5.9 %, v/v) was added to cultures, which were grown and measured for luminescence as described above. Heat and cold shock were performed by growing cultures at 43 and 6 °C, respectively.

Construction of *cwrA* deletion strain. In order to delete *cwrA*, 500 bp upstream and 500 bp downstream of the gene were PCR-amplified and cloned into either side of an erythromycin-resistance cassette that was inserted into the multiple cloning site of pBAD-Kan (Guzman *et al.*, 1995) (ATCC). The upstream region was amplified using the primers 5'-AAGAATTCTTGTAATCTGTCCACCATCC-3' and 5'-AGCGGTACCGCTATCATACTCCTTTATAT-3', digested, and cloned between the *Bam*HI and *Kpn*I sites. The downstream region was amplified using the primers 5'-ACGGATCCGTCAC-CTAAGAATTGCAAATCC-3' and 5'-TTAGTCGACTAACACTTT-TTCAATACGGC-3', digested, and cloned between the *Bam*HI and *Sal*I sites. The resulting plasmid was transformed into RN4220, and successful integrants were selected by screening for resistance to erythromycin. Double-crossover deletions were then selected by restreaking single-crossover integrants and PCR screening for the replacement of *cwrA* by the erythromycin cassette, with concomitant loss of the plasmid backbone. The *cwrA* deletion was then transduced using phage 80α into SH1000 to yield SH1000Δ*cwrA*::*erm*.

RNA isolation and transcriptional profiling. Overnight cultures of SH1000 and SH1000Δ*cwrA*::*erm* *S. aureus* were diluted 5000-fold into fresh MHB. Cultures were grown with vigorous shaking at 37 °C to OD₆₀₀ 0.5, at which point 30 ml was harvested by centrifugation. The remaining culture was grown for an additional 12 h until the culture reached saturation, at which point an equivalent of 15 ml of culture at OD₆₀₀ 1.0 was harvested by centrifugation. Cells were resuspended in 4 ml H₂O and 8 ml RNeasy Protect Bacteria reagent (Qiagen), incubated for at least 30 min, pelleted by centrifugation, and then frozen at -80 °C until further use. RNA was extracted from cell pellets by using a Purescript RNA isolation kit (Gentra Systems) with an initial treatment with 0.3 μg lysostaphin μl⁻¹ (Sigma) to achieve cell lysis. A total of 100 μg total RNA was treated with 7 U RNase-free DNase I (Qiagen) and then further purified by using an RNeasy Plus Mini Kit (Qiagen). RNA quality was interrogated by reacting with formaldehyde loading dye running on a 1 % agarose denaturing gel with MOPS gel running buffer from a NorthernMax kit (Applied Biosystems). Final RNA samples were quantified by using a NanoDrop spectrophotometer.

Gene expression profiling was performed using Affymetrix GeneChips designed to interrogate over 3300 *S. aureus* ORFs and both the forward and the reverse orientation of more than 4800 intergenic regions. Fifteen micrograms of RNA was reverse-transcribed, and the cDNA was fragmented, labelled and hybridized to the GeneChip *S. aureus* genome array using the standard prokaryotic GeneChip protocol supplied by Affymetrix. GeneChip data were obtained by scanning with an Affymetrix autoloading scanner, and data were normalized and compared by using GeneSpring 7.2 analysis software

(Agilent Technologies). All experiments were done in triplicate using independent RNA samples.

Transmission electron microscopy (TEM) imaging. Overnight cultures of SH1000 and SH1000 Δ cwrA::erm were grown in MHB, harvested at stationary phase, and analysed by TEM at the W. M. Keck Microscopy Facility at the Whitehead Institute, Cambridge, MA, USA. Cells were fixed in 2.5 % glutaraldehyde (w/v), 3 % paraformaldehyde (w/v) and 5 % sucrose (w/v) in 0.1 M sodium cacodylate buffer, pH 7.4, pelleted, and post-fixed in 1 % OsO₄ (w/v) in veronal-acetate buffer. The cell pellet was stained in block overnight with 0.5 % uranyl acetate (w/v) in veronal-acetate buffer, pH 6.0, then dehydrated and embedded in Spurr's resin. Sections were cut on a Reichert Ultracut E microtome with a Diatome diamond knife at a thickness setting of 50 nm, and stained with uranyl acetate and lead citrate. The sections were examined using an FEI Tecnai Spirit transmission electron microscope at 80 kV and photographed with an AMT charge-coupled device (CCD) camera.

In vivo autolysis assays. Overnight cultures of SH1000 and SH1000 Δ cwrA::erm were diluted 1:100 into MHB + 1 M NaCl with or without 350 ng phosphomycin ml⁻¹ and grown at 37 °C for 6 h. Cells were then pelleted, washed with an equal volume of cold water, and resuspended in 50 mM Tris, pH 8, and 1 % Triton X-100 (v/v). Cultures were then incubated at 37 °C with shaking at 225 r.p.m. and OD₆₀₀ readings were taken every 20 min using a SpectraMax Plus 384 plate reader.

Preparation of autolytic extracts. Overnight cultures of SH1000 and SH1000 Δ cwrA::erm were diluted 1:100 into 1 l MHB + 1 M NaCl and grown at 37 °C for 5 h. Cells were then pelleted and washed with 500 ml cold water, and cell pellets were frozen at -80 °C until further use. Three types of autolytic extract were prepared: freeze-thaw, LiCl and SDS. For freeze-thaw extracts, cell pellets were resuspended in 4 ml 50 mM potassium phosphate buffer, pH 7.3, and subjected to three rounds of freezing at -80 °C for 1 h and thawing at 37 °C for 10 min. Cells were pelleted by centrifugation at 8000 g, and the supernatant was concentrated to ~250 µl in an Amicon Ultra-4 10 kDa molecular weight cut off (MWCO) centrifugal filter unit (Millipore). For LiCl extracts, cell pellets were resuspended in 4 ml 3 M LiCl and extracted for 1 h at 4 °C. Cells were pelleted by centrifugation at 8000 g, and the supernatant was buffer-exchanged 35 000-fold in an Amicon Ultra-15 10 kDa MWCO centrifugal filter unit (Millipore) into 500 µl 50 mM Tris, pH 7.5. For SDS extracts, cell pellets were resuspended in 1 ml 4 % SDS and extracted for 1 h at room temperature.

Crude cell wall preparation. Bacterial cell pellets remaining from autolytic extracts were boiled in 10 ml 8 % SDS for 1 h, washed five times with 50 ml H₂O until all traces of SDS were removed, incubated in 5 ml 30 mM Tris, pH 8.0, with 200 µg ml⁻¹ proteinase K (Qiagen) at 50 °C for 1.5 h, boiled in 8 % SDS again for 1 h, and then washed five times with 50 ml H₂O until all traces of SDS were removed. The remaining pellet was lyophilized to dryness.

In vitro autolysis assay. Crude cell walls were resuspended in 50 mM potassium phosphate buffer, pH 7.3, to an initial OD₆₀₀ of 0.6, and incubated with 15 µg ml⁻¹ of either freeze-thaw or LiCl autolytic extract at 37 °C in a total volume of 100 µl. Readings were taken in a 96-well plate using a SpectraMax Plus 384 plate reader.

Zymogen profiles. Crude cell walls were incorporated into a polyacrylamide resolving gel (10 % acrylamide/0.26 % bisacrylamide) at 0.2 % (w/v). Ten micrograms of total protein from SDS- and LiCl-extracted supernatants was electrophoresed for 1 h at 170 V. The gel was then washed in 1 l H₂O for 1 h and incubated overnight in 1 l 50 mM Tris, pH 7.5, 5 mM MgCl₂ and 0.1 % (v/v) Triton X-100 at

37 °C. Peptidoglycan hydrolases were visible as clearings in the opaque gel that could be imaged against a dark background.

MIC and lysostaphin susceptibility testing. For MIC testing, *S. aureus* SH1000 or SH1000 Δ cwrA::erm was inoculated into MHB and allowed to grow for 4 h. A 0.5 ml volume of culture was transferred into 2 ml of sterile saline in order to achieve the turbidity of a 0.5 McFarland standard. Subsequently, 0.5 ml of the 0.5 McFarland dilution was transferred into 4.5 ml MHB. A 1.5 µl volume of this culture and 1 µl of the compound under test at various concentrations were inoculated into 100 µl MHB in a 96-well plate, grown at 37 °C, and scored for growth the following day.

For lysostaphin susceptibility, overnight cultures of *S. aureus* SH1000 or SH1000 Δ cwrA::erm grown in MHB were diluted fivefold into 20 mM Tris, pH 8.0, 2 mM EDTA buffer. Lysostaphin was added at a concentration of 1.7 µg ml⁻¹ and lysis was monitored continuously at 600 nm while shaking at 37 °C in a SpectraMax Plus 384 plate reader.

In vivo sepsis model. A lethal sepsis model was performed by infecting female CD-1 mice (20–25 g, Charles River Laboratories) with varying infectious doses of SH1000 and SH1000 Δ cwrA::erm, ranging from 3.5 × 10⁶ to 4.2 × 10⁸ c.f.u. per mouse. Studies described in this report were approved by the Institutional Animal Care and Use Committee (IACUC) of the Novartis Institutes for BioMedical Research Inc. To prepare the inocula, 25 ml MHB was inoculated with 20 µl *S. aureus* overnight culture and incubated for 16 h at 36 °C while shaking at 150 r.p.m. These cultures were then diluted 1:5, 1:25 and 1:125 in sterile saline. The original culture and three dilutions were further diluted 1:1 in 10 % porcine stomach mucin (Sigma), and 0.3 ml was used for infection by intraperitoneal injection. Tenfold serial dilutions of the inocula were plated for c.f.u. determination. Following infection, mice were monitored for 5 days, and the LD₅₀ for both strains was calculated by probit analysis.

RESULTS

Generation and evaluation of lux reporter strains

In order to determine the conditions under which *cwrA* is expressed we generated two transcriptional fusion constructs in which the bacterial *luxABCDE* cassette (Kadurugamuwa *et al.*, 2003) was appended downstream of either the putative promoter for *cwrA* (RN4220*lux1*) or *cwrA* itself (RN4220*lux2*) (Fig. 1a). In both constructs, translation of the *lux* cassette is initiated from a strong RBS independent from the RBS for *cwrA*. A single copy was integrated into the genome of RN4220, and these reporter strains were subsequently monitored for their responses to various applied stresses.

We treated the *lux* reporter strains with a variety of antibiotics that possess different mechanisms of action, including the peptidoglycan pentapeptide binder vancomycin, the β -lactam penicillin-binding protein (PBP) inhibitors oxacillin, penicillin G and imipenem, the UPP binder bacitracin, the enoyl pyruvate transferase (MurA) inhibitor phosphomycin, the HMG-CoA synthase (MvaS) inhibitor hymeglus, the DNA gyrase inhibitors ciprofloxacin and novobiocin, the 50S ribosome inhibitors chloramphenicol and erythromycin, the enoyl-acyl carrier protein reductase (FabI) inhibitor triclosan, the lipopeptide daptomycin, the RNA polymerase inhibitor rifampicin,

Fig. 1. Reporter strains and results of monitoring *cwrA* expression in response to antibiotic treatment. (a) DNA sequence of the two transcriptional and one translational construct in which *luxABCDE* is fused to *cwrA*. (b) Luminescence signal in RLU generated by RN4220*lux2* in response to treatment with various antibiotics at $3 \mu\text{g ml}^{-1}$. The first dark-grey, second black, third light-grey, fourth mid-grey and fifth dark-grey bars represent 0, 1.5, 2.5, 3.5 and 6 h post antibiotic addition, respectively. (c) Luminescence signal in RLU generated by RN4220*lux3* in response to treatment with $3 \mu\text{g ml}^{-1}$ phosphomycin at various time point post antibiotic addition. Error bars, SD ($n=3$).

and the ionophore CCCP. Fig. 1(b) shows a summary of the luciferase signals obtained from the RN4220*lux2* strain under these various treatment conditions. Although both the RN4220*lux1* and the RN4220*lux2* strain yielded essentially identical results at the full range of antibiotic concentrations from several fold above to several fold below MIC (Supplementary Tables S1 and S2), for simplicity Fig. 1(b) shows the results from the RN4220*lux2* strain at $3 \mu\text{g ml}^{-1}$ antibiotic treatment only. The basal luciferase expression level was below the

detection limit, showing background readings of about 100 relative light units (RLU). However, when RN4220*lux2* was treated with compounds that block any aspect of cell wall biosynthesis, the resulting cultures produced luminescence readings significantly above background, without exception. In contrast, all other antibiotics with non-cell wall inhibition mechanisms, including DNA replication, RNA transcription, protein translation, fatty acid biosynthesis and membrane disruption, failed to elicit any detectable luminescence. It is interesting to note that

daptomycin, for which the mechanism of action has not been clearly defined, also triggered a significant cell wall inhibition-like response. A recent transcription profiling study indicates that daptomycin does induce the cell wall stimulon (Muthaiyan *et al.*, 2008), consistent with our findings, indicating that there is probably a cell wall component to the mechanism of action of daptomycin.

In addition to antibiotic treatment, we subjected the reporter strains to various environmental stresses including heat, cold, alkaline, acid and oxidative shock. None of these conditions was capable of inducing a luminescence signal above background (data not shown). This demonstrates that the specific and robust response of *cwrA* to cell wall-targeting agents is not due to a secondary response that can be directly produced by certain environmental stresses.

cwrA is a hypothetical protein of 63 amino acids containing an N-terminal membrane-spanning domain and an extracellular C-terminal domain, based on TOPCONS membrane protein topology prediction (Bernsel *et al.*, 2009). In order to determine whether the predicted protein was actually translated, a third *lux* fusion strain was generated, RN4220*lux3*, in which the first four amino acids of *cwrA* were fused to the N terminus of *luxA* (Fig. 1a). In this

construct, the translation of the luciferase cassette is dependent on the endogenous RBS of *cwrA*. As can be seen in Fig. 1(c), this reporter did indeed respond robustly to phosphomycin treatment, verifying that *cwrA* is not only transcribed but also translated in response to cell wall-targeting agents. Finally, to investigate whether *cwrA* is autoregulatory, a *cwrA* knockout was generated in the RN4220*lux1* background (RN4220*lux1*Δ*cwrA*::*erm*) and retested for its response to cell wall-targeting antibiotics. There was no appreciable difference between the two strains in the luciferase signal generated due to cell wall biosynthesis inhibition (data not shown), indicating that *cwrA* is not autoregulatory.

Generation of *cwrA* knockouts and transcriptional profiling

After identification of the conditions under which *cwrA* is induced, we sought to understand the biological role that *cwrA* plays in *S. aureus*. To this end, a knockout strain was initially constructed in RN4220 in which *cwrA* was deleted and replaced with the erythromycin cassette. This knockout was then transduced into an SH1000 background, in which σ^B -dependent signalling has been restored (Horsburgh *et al.*,

Table 1. Genes downregulated in SH1000 upon deletion of *cwrA* during exponential growth

SH1000 ORF (SAOUHSC no.)	Gene	Description	Fold change (Δ <i>cwrA</i> /wild-type)
01603		Conserved hypothetical protein	0.34
01297		Conserved hypothetical protein	0.48
00175	<i>msmX</i>	Multiple sugar-binding transport ATP-binding protein, putative	0.33
00176		Bacterial extracellular solute-binding protein, putative	0.40
00356		Conserved hypothetical protein	0.27
00437	<i>treP</i>	PTS enzyme II, phosphoenolpyruvate-dependent, trehalose-specific	0.06
00438	<i>treC</i>	α -Glucosidase	0.08
00439	<i>treR</i>	Trehalose operon transcriptional regulator	0.11
00690		Conserved hypothetical protein	0.49
00830		Conserved hypothetical protein	0.49
00838		Conserved hypothetical protein	0.41
01601	<i>malA</i>	α -D-1,4-Glucosidase	0.47
01602	<i>malR</i>	Maltose operon transcriptional repressor	0.41
02270	<i>nrgA</i>	Ammonium transporter	0.41
02662	<i>scrA</i>	PTS system, sucrose-specific IIBC component	0.28
02759		Conserved hypothetical protein	0.47
02827		Conserved hypothetical protein	0.40
02831		Haloacid dehalogenase-like hydrolase	0.34
02836		Conserved hypothetical protein	0.47
02853		Conserved hypothetical protein	0.39
02872	<i>cwrA</i>	Conserved hypothetical protein	0.03
02975		PTS system, fructose-specific IIBC component, putative	0.16
02976	<i>manA</i>	Mannose-6-phosphate isomerase, class I	0.21
03028		Conserved hypothetical protein	0.36
03046		Helix–turn–helix domain protein	0.26
00838		Conserved hypothetical protein	0.46
00919		Conserved hypothetical protein	0.49
01729		Conserved hypothetical protein	0.50

2002), yielding the strain SH1000 Δ cwrA::erm. The knock-out strain did not appear to have any delay in *in vitro* growth kinetics. Furthermore, co-culturing experiments, in which equal dilutions of both the wild-type and knockout strain were inoculated into the same flask and then plated after the culture grew overnight to saturation, yielded equal numbers of c.f.u., indicating no defect in growth (data not shown). The only phenotype that we could initially observe was a clumping of the knockout strain when the culture reached high saturation.

With little *in vitro* growth difference, transcriptional profiling was performed in order to ascertain whether there were any significant differences in gene expression between the wild-type and knockout strains. Transcriptional profiling was performed on cells in both exponential and stationary growth phases. The deletion of *cwrA* caused a downregulation of 28 genes (Table 1) and upregulation of 32 genes (Table 2) in exponential phase, and a downregulation

of five genes and an upregulation of 12 genes (Table 3) in stationary phase. These alterations in transcription represent a threshold of at least twofold, and have a *P* value of ≤ 0.05 as determined by one-way ANOVA. Consistent with the lack of observable difference in growth phenotype, knocking out *cwrA* had little effect on the transcriptome of *S. aureus*.

The greatest number of transcriptional changes were observed during exponential growth. The genes most prominently downregulated were for the transport and metabolism of sugars, including trehalose, maltose, sucrose and fructose. Contrarily, the entire *lac* operon, responsible for the uptake and catabolism of lactose, was upregulated in response to *cwrA* deletion. In addition to carbohydrate metabolic genes, there were several cell envelope-associated determinants that showed altered expression. A putative phosphate (Pho) regulon was upregulated in the mutant strain. Although not studied in *S. aureus*, phosphate transport systems have been shown to be important for

Table 2. Genes upregulated in SH1000 upon deletion of *cwrA* during exponential growth

SH1000 ORF (SAOUHSC no.)	Gene	Description	Fold change (Δ cwrA/wild-type)
01783		Conserved hypothetical protein	2.05
01388		Conserved hypothetical protein	3.27
00035		Conserved hypothetical protein	2.53
00206	<i>lctE</i>	L-Lactate dehydrogenase	2.46
00248	<i>lytM</i>	Peptidoglycan hydrolase	2.04
00868		Conserved hypothetical protein	2.19
00868		Conserved hypothetical protein	2.20
00869	<i>dltA</i>	D-Alanine-D-alanyl carrier protein ligase (Dcl)	2.40
00870	<i>dltB</i>	Secretion of D-alanyl Dcp	2.25
00871	<i>dltC</i>	D-Alanyl carrier protein (Dcp)	2.36
00872	<i>dltD</i>	Selection of Dcp for ligation with D-alanine	2.24
01331		Conserved hypothetical protein	2.27
01384	<i>phoU</i>	Phosphate uptake regulatory protein, putative	2.10
01385	<i>pstB</i>	Phosphate ABC transporter ATP-binding protein, putative	2.22
01386	<i>pstA</i>	Phosphate ABC transporter, permease protein, putative	2.11
01387	<i>pstC</i>	Phosphate ABC transporter, transmembrane subunit, putative	2.40
01389	<i>pstS</i>	Phosphate ABC transporter, periplasmic phosphate-binding protein, putative	2.11
01737	<i>aspS</i>	Aspartyl-tRNA synthetase	2.41
01738	<i>hisS</i>	Histidyl-tRNA synthetase	2.22
02261	<i>agrB</i>	Accessory gene regulator protein B	2.44
02333	<i>sceD</i>	Lytic transglycosylase	2.19
02449	<i>lacG</i>	6-Phospho- β -galactosidase	3.45
02450	<i>lacE</i>	PTS system, lactose-specific IIBC component	3.19
02451	<i>lacF</i>	PTS system, lactose-specific IIA component	3.39
02452	<i>lacD</i>	Tagatose-1,6-diphosphate aldolase	3.46
02453	<i>lacC</i>	Tagatose-6-phosphate kinase	3.12
02454	<i>lacB</i>	Galactose-6-phosphate isomerase	2.99
02455	<i>lacA</i>	Galactose-6-phosphate isomerase	2.82
02571	<i>ssaA</i>	Secretory antigen precursor	2.47
02932	<i>betA</i>	Choline dehydrogenase	2.35
02937	<i>cudT</i>	Choline transporter	3.09
03033		High-affinity nickel transporter, putative	2.16

Table 3. Transcriptional changes in SH1000 upon deletion of *cwrA* during stationary growth

SH1000 ORF (SAOUHSC no.)	Gene	Description	Fold change ($\Delta cwrA$ /wild-type)
Downregulated			
01554		PV83 orf 27-like protein	0.43
02021		phi ETA orf 63-like protein	0.47
02057		dUTP pyrophosphatase	0.37
02872	<i>cwrA</i>	Conserved hypothetical protein	0.02
02975		PTS system, fructose-specific IIBC component, putative	0.44
Upregulated			
01127		Conserved hypothetical protein	2.08
01366	<i>trpE</i>	Anthranilate synthase component I	6.32
01367	<i>trpG</i>	Anthranilate synthase component II	5.77
01368	<i>trpD</i>	Anthranilate phosphoribosyltransferase	6.80
01369	<i>trpC</i>	Indole-3-glycerol phosphate synthase	6.60
01370	<i>trpF</i>	N-(5-Phosphoribosyl)anthranilate isomerase	8.34
01371	<i>trpB</i>	Tryptophan synthase, beta subunit	9.37
01372	<i>trpA</i>	Tryptophan synthase, alpha subunit	9.09
02449	<i>lacG</i>	6-Phospho- β -galactosidase	2.04
02451	<i>lacF</i>	PTS system lactose-specific IIA component	2.06
02452	<i>lacD</i>	Tagatose-1,6-diphosphate aldolase	2.11
02453	<i>lacC</i>	Tagatose-6-phosphate kinase	2.05

the modification of lipopolysaccharides, outer-membrane proteins and membrane lipids, which in turn mediate resistance to different host defence elements that target the bacterial cell surface (Lamarche *et al.*, 2008). Additionally, *lytM*, encoding a gly-gly endopeptidase (Ramadurai *et al.*, 1999), and *sceD*, encoding a lytic transglycosylase (Stapleton *et al.*, 2007), both involved in cell wall turnover, were upregulated twofold in the *cwrA* knockout.

Perhaps the most interesting transcriptional change observed was the upregulation of the *dlt* operon during exponential phase. The *dlt* operon is responsible for the D-alanylation of wall teichoic acids (WTAs) and lipoteichoic acids (LTAs), components of the cell envelope in *S. aureus* (Neuhaus *et al.*, 1996). Teichoic acids represent the most abundant polyanions of the Gram-positive cell envelope (Fischer, 1994), and altering their charge properties through esterification with D-alanine has pleiotropic effects which can change autolytic enzyme activity (Wecke *et al.*, 1996), cation binding (Hughes *et al.*, 1973), susceptibility to cationic peptides (Peschel *et al.*, 1999), virulence (Collins *et al.*, 2002) and biofilm formation (Gross *et al.*, 2001). D-Alanylation also promotes coaggregation (Clemans *et al.*, 1999), which is consistent with the clumping effect observed during *in vitro* growth. It is interesting that upregulation of the *dlt* operon occurred without a significant change in expression of either the *arlSR* two-component system, which has been demonstrated to repress *dlt* operon expression (Koprivnjak *et al.*, 2006), or the *graRS* two-component system, which has been shown to activate expression of the *dlt* operon (Herbert *et al.*, 2007). Furthermore, *dlt* operon upregulation was independent of any significant change in expression of the capsular polysaccharide *cap* operon, another component of the cell envelope.

Electron microscopy imaging

Given the clumping phenotype and upregulation of the *dlt* operon in response to deletion of *cwrA*, it seemed plausible that there might be a difference in the morphology of the cell envelope between the wild-type and knockout strains. In order to investigate, TEM was employed to image the two *S. aureus* strains. TEM thin sections revealed no discernible difference between the SH1000 and SH1000 $\Delta cwrA::erm$ strains with regard to their cell wall (Fig. 2). Both strains had a cell wall visible as an amorphous matrix ~40 nm thick lying on top of the plasma membrane, which was visible as an encircling band of high density. Furthermore, there appeared to be no difference in formation of septa during cell division between the strains.

Interestingly, the wild-type SH1000 strain did differ from the SH1000 $\Delta cwrA::erm$ mutant in the presence of a white band of low density residing proximally to the intracellular side of the plasma membrane. This intracellular space is most likely an artefact of processing the sample, which included a dehydration step before embedding in resin. During this dehydration the cytoplasm most likely contracted away from the less flexible cell envelope, resulting in the formation of a low-density space. In the *cwrA* knockout, this space may not have formed because the cell wall may have been compromised, allowing it to contract along with the cytoplasm during processing.

Autolysis assays

To verify the hypothesis suggested by the TEM images that the *cwrA* knockout has compromised cell wall integrity, autolysis assays were performed on SH1000 and

Fig. 2. TEM images of SH1000 and SH1000 Δ *cwrA::erm* grown in MHB until stationary phase. Lower-panel images are a higher magnification of the upper-panel broad-field images; scale bars, 100 nm.

SH1000 Δ *cwrA::erm*. Fig. 3 demonstrates that the rate of autolysis for the knockout strain is indeed faster than that of the wild-type, supporting the role that *cwrA* plays in maintaining cell wall integrity. Several reports in the

literature have demonstrated that lysis in response to inhibitory concentrations of cell wall-targeting antibiotics involves the activity of autolytic enzymes, as mutants defective in autolysin show reduced lysis in response to

Fig. 3. Triton X-100-induced autolysis. Autolysis was monitored for SH1000 and SH1000 Δ *cwrA::erm* grown in the absence (diamonds and triangles, respectively) or presence (asterisks and circles, respectively) of 350 ng phosphomycin ml⁻¹. Error bars, SD ($n=3$).

Fig. 4. *In vitro* autolysis assays. (a) Zymographic analysis of autolysins of SH1000 and SH1000 $\Delta cwrA::erm$ extracted by either LiCl or SDS. The molecular mass ladder is Bio-Rad Precision Plus Protein Dual Color Standards. (b) *In vitro* autolysis using a freeze-thaw autolytic extract of SH1000 on cell wall extracted from SH1000 (◆) or SH1000 $\Delta cwrA::erm$ (■). (c) *In vitro* autolysis using a freeze-thaw autolytic extract of SH1000 $\Delta cwrA::erm$ on cell wall extracted from SH1000 (◆) or SH1000 $\Delta cwrA::erm$ (■). (b, c) Error bars, SD ($n=3$).

antibiotic treatment (Oshida & Tomasz, 1992; Tomasz *et al.*, 1988). However, treatment with subinhibitory concentrations of β -lactams, which yields the production of hypo-cross-linked peptidoglycan that is more susceptible to *in vitro* enzymic degradation by autolysins (Qoronfleh & Wilkinson, 1986), actually decreases the rate of autolysis in *S. aureus*. This autolytic protective effect induced by cell wall-targeting antibiotics has been shown to result from a decrease in transcription and/or the activity of several autolytic determinants, including *pbpB*, *atl*, *sleI*, SA0620 and *lytM* (Antignac *et al.*, 2007). Since *cwrA* is induced by cell wall-targeting antibiotics and its deletion resulted in a faster rate of autolysis, we investigated whether *cwrA* was involved in this autolytic suppression. The autolysis assay was repeated in the presence of subinhibitory concentrations of phosphomycin (350 ng ml^{-1}). Once again, the *cwrA* knockout had a faster rate of autolysis than the wild-type (Fig. 3). However, the partial cell wall inhibition protection still resulted in a slower rate of autolysis for both SH1000 and SH1000 $\Delta cwrA::erm$. This result indicates that despite the fact that *cwrA* is induced by cell wall-targeting antibiotics, its role is independent of the autolytic suppression induced by cell wall synthesis inhibition.

In order to confirm that increased autolysis was the result of decreased cell wall integrity and not simply increased autolysin production, as suggested by the transcriptional profiling data, peptidoglycan hydrolase profiles and *in vitro* autolysis assays were performed. As can be seen in Fig. 4(a), zymographic profiles of either LiCl or SDS autolytic extracts from SH1000 and SH1000 $\Delta cwrA::erm$ were nearly identical, indicating that there is not an altered protein expression ratio for the major autolysins. However, when comparing the susceptibility of cell walls isolated from either SH1000 or SH1000 $\Delta cwrA::erm$ with autolytic extracts from either strain, it became evident that cell walls isolated from SH1000 $\Delta cwrA::erm$ were more susceptible than cell walls isolated from SH1000 to degradation by autolytic extracts from either strain (Fig. 4b, c). This indicates that deletion of *cwrA* does indeed lead to a loss of cell wall integrity.

Antibiotic and lysostaphin susceptibility

The robust induction in response to cell wall-targeting antibiotics and the role in maintaining cell wall integrity suggest that *cwrA* is involved in protecting *S. aureus* against antibacterial agents. As such, it would suggest that loss of

cwrA would make *S. aureus* more susceptible to cell wall-targeting antibiotics. Table 4 shows MIC values for several cell wall-targeting antibiotics against SH1000 and SH1000 Δ *cwrA*::*erm*. For most compounds there was no change in MIC between the two strains, and for the few where SH1000 Δ *cwrA*::*erm* was more susceptible (ticarcillin, amoxicillin and phosphomycin), the change was only twofold. Interestingly, although the *cwrA* deletion strain showed an upregulation in the *dlt* operon, which purportedly makes the cells less susceptible to cationic peptides, there was no difference in MIC for polymyxin B, nisin and lysostaphin between the two strains. However, when a time course of lysis in response to lysostaphin was measured, there was a slight but significant increase in the rate of lysis for the *cwrA* knockout strain (Supplementary Fig. S1).

In vivo virulence

With little effect *in vitro*, we wanted to investigate whether deletion of *cwrA* had more of a consequence *in vivo*. The *S. aureus* cell wall is a major component of how the host immune system is able to detect and respond to infection. Although cell wall-targeting antibiotics may be capable of inducing *cwrA* *in vitro*, perhaps its major function lies in responding to host immune defences. A sepsis model was performed by infecting CD-1 mice with wild-type and *cwrA*-deleted strains to determine virulence. At lower inocula, neither SH1000 nor SH1000 Δ *cwrA*::*erm* was virulent, and both showed a low degree of lethality. However, at the highest inoculum investigated, wild-type *S. aureus* showed 100 % lethality, whereas the *cwrA* knockout mutant showed a reduction of virulence. Fig. 5 shows a Kaplan–Meier survival curve for the highest inoculum, demonstrating that lethality with SH1000 occurred quickly and completely within 1 day, whereas even after 5 days most of the mice infected with SH1000 Δ *cwrA*::*erm*

survived. This indicates that *cwrA* is important for virulence in an *in vivo* sepsis model of infection.

DISCUSSION

The staphylococcal cell wall is a dynamic structure that is paramount to the success of the organism. It withstands incredible turgor pressure, is intimately involved in cell division, both senses and interacts with the external environment, and is a major player in virulence (Beveridge, 1981). Since the discovery of penicillin, the bacterial cell wall has served as an effective target for antibacterial agents. Understanding the molecular and cellular response that occurs subsequent to peptidoglycan biosynthesis inhibition is of critical importance in understanding how pathogens adapt to, resist and evade killing by what are typically bactericidal agents. Herein, we studied *cwrA*, a gene that has appeared as the highest-fold upregulated in a variety of transcriptomic studies examining cell wall inhibition (Balibar *et al.*, 2009; McAleese *et al.*, 2006; Sobral *et al.*, 2007).

Using a variety of transcriptional and translation fusion constructs and utilizing the bacterial *luxABCDE* operon for a luminescence readout, *cwrA* was shown to be not only transcribed but also translated in response to treatment with cell wall-targeting antibiotics. Despite its massive transcriptional response, deletion of *cwrA* had little observable phenotype *in vitro*. Ultimately, transcriptional profiling, electron microscopy, and *in vivo* and *in vitro* autolysis assays demonstrated that deletion of *cwrA* resulted in a compromised cell wall with altered integrity. The role that *cwrA* plays in maintaining cell wall integrity could be structural or it could stem from a direct modulation of autolytic enzymes. Although *in vitro* growth

Table 4. MIC ($\mu\text{g ml}^{-1}$) of cell wall-targeting antibiotics and cationic peptides

Antibiotic or peptide	Strain	
	SH1000	SH1000 Δ <i>cwrA</i> :: <i>erm</i>
Ticarcillin	4	2
Vancomycin	1	1
Amoxicillin	0.5	0.25
Penicillin	1	1
Ampicillin	0.25	0.25
Ceftazidime	16	16
Bacitracin	16	16
Phosphomycin	2	1
Polymyxin B	512	512
Nisin	16	16
Lysostaphin	0.1	0.1

Fig. 5. Kaplan–Meier survival curve of CD-1 mice infected intraperitoneally with SH1000 (diamonds, dark grey) or SH1000 Δ *cwrA*::*erm* (squares, light grey) at 4.2×10^8 c.f.u. in a sepsis model of infection ($n=5$).

was not significantly altered by *cwrA* deletion, the virulence of *S. aureus* was significantly altered *in vivo*. Using a 5 day mouse sepsis model of infection, deletion of *cwrA* made *S. aureus* less virulent, a result that could be attributed to the loss of cell wall integrity in the knockout.

Perhaps most intriguing is that by fusing the entire gene or the putative promoter of *cwrA* to a luciferase gene operon, we successfully established *S. aureus* strains that are able to produce robust bioluminescence signals as a specific response to chemicals that block cell wall biosynthesis. Often, high-throughput screens performed during antimicrobial discovery efforts result in the identification of compounds with unknown mechanisms of action. The strains constructed here should be useful for the characterization of such compounds or could be used directly to identify antimicrobial agents with specific cell wall-directed mechanisms. These strains are responsive to a broad range of cell wall inhibitors that interfere with numerous steps in peptidoglycan biosynthesis.

Most of the cell wall inhibitors tested here triggered the luminescence signal within 2 h of the treatment. However, it is interesting that hymeglusin required more than 4 h in order to induce the expression of the reporter luciferase to a detectable level. Such a delay in response is correlated with the fact that this compound inhibits an early stage of UPP synthesis. It is also possible that the regulation that induces the cell wall stimulon is caused by the accumulation of peptidoglycan precursors, which is on a biosynthetic branch required for the generation of cell walls that is different from that inhibited by hymeglusin. Our observation that phosphomycin induces the strongest signal among all the compounds tested would be consistent with such a hypothesis. It is also intriguing that vancomycin caused only a transient signal that lasted less than 1 h.

Since these strains were constructed, similar *S. aureus* cell wall reporter strains have been described in which several promoters from other cell wall stimulon members are fused to a *lacZ* gene (Steidl *et al.*, 2008). However, the strains described here do not require the addition of an exogenous substrate, offer much lower background, and produce a more robust readout that is based on a luminescence signal.

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