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Development of *in vitro* resistance to chitosan is related to changes in cell envelope structure of *Staphylococcus aureus*

Dina Raafat[a,*], Nicole Leib[a], Miriam Wilmes[a], Patrice François[b], Jacques Schrenzel[b], Hans-Georg Sahl[a]

[a] *Institute for Medical Microbiology, Immunology and Parasitology (IMMIP), Pharmaceutical Microbiology Unit, University of Bonn, D-53115 Bonn, Germany*

[b] *Genomic Research Laboratory, Division of Infectious Diseases, University of Geneva Hospitals, CH-1211 Geneva, Switzerland*

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ABSTRACT

The bacterial cell envelope is believed to be a principal target for initiating the staphylocidal pathway of chitosan. The present study was therefore designed to investigate possible changes in cell surface phenotypes related to the *in vitro* chitosan resistance development in the laboratory strain *S. aureus* SG511-Berlin.

Following a serial passage experiment, a stable chitosan-resistant variant (CRV) was identified, exhibiting >50-fold reduction in its sensitivity towards chitosan. Our analyses of the CRV identified phenotypic and genotypic features that readily distinguished it from its chitosan-susceptible parental strain, including: (i) a lower overall negative cell surface charge; (ii) cross-resistance to a number of antimicrobial agents; (iii) major alterations in cell envelope structure, cellular bioenergetics and metabolism (based on transcriptional profiling); and (iv) a repaired sensor histidine kinase GraS. Our data therefore suggest a close nexus between changes in cell envelope properties with the *in vitro* chitosan-resistant phenotype in *S. aureus* SG511-Berlin.

1. Introduction

Chitosan is a linear high molecular weight heteropolysaccharide, consisting of *N*-acetyl-D-glucosamine and D-glucosamine units, linked together by β-(1 → 4) glycosidic bonds; it is produced from chitin by exhaustive alkaline deacetylation (Kumar, 2000). Since the relative amount of the two monosaccharides may vary, the term chitosan usually refers to a family of copolymers with various fractions of acetylated units (Singla & Chawla, 2001; Tharanathan & Kittur, 2003). In contrast to most of the naturally-occurring polysaccharides, chitosan is an example of a highly basic polysaccharide with a high charge density. Its unique chemical structure, combined with its physico-chemical and biological characteristics, allow for a wide range of applications ranging from pharmaceutical, cosmetic, medical, food and textile to agricultural applications (Raafat & Sahl, 2009).

Recent developments in the field of biomaterials have led to a renewed interest in this biopolymer, especially with an increasing number of publications describing the antimicrobial potentials of chitosan and its derivatives against filamentous fungi, yeasts and bacteria (Champer et al., 2013; Galván Márquez et al., 2013). It is generally assumed that the polycationic nature of chitosan contributes to its interaction with anionic microbial cell surface components, resulting in random multiple detrimental events which may each contribute to the overall efficacy (Je & Kim, 2006; Raafat, Bargen, von Haas, & Sahl, 2008; Torr, Chittenden, Franich, & Kreber, 2005; Zakrzewska, Boorsma, Brul, Hellingwerf, & Klis, 2005). Our previous data clearly indicate that the initial contact between chitosan and the negatively-charged cell wall polymers (teichoic acids) is indeed driven by electrostatic interactions. This leads to impairment and destabilization of membrane function with subsequent leakage of cellular components, and ultimately to

Abbreviations: AMP, antimicrobial peptide; CAMHB, cation-adjusted Mueller–Hinton II broth; CL, cardiolipin; CRV, chitosan-resistant variant; FDR, False Discovery Rate; HMG-CoA, Hydroxymethylglutaryl-CoA; L-PG, lysyl-phosphotidylglycerol; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; OD, optical density; ORF, Open Reading Frame; PG, phosphatidylglycerol; PL, phospholipid; RT, room temperature; TCRS, two-component regulatory system; TEM, Transmission electron microscope; WT, wild-type.

* Corresponding author. Present address: Institute of Immunology and Transfusion Medicine, Immunology Department, University Medicine Greifswald, F. Sauerbruch-Straße DZ 7, D-17475 Greifswald, Germany.

E-mail addresses: dina.raafat@uni-greifswald.de, dina_raafat@yahoo.com (D. Raafat), Nicole.Leib1@gmx.de (N. Leib), mwilmes@uni-bonn.de (M. Wilmes), patrice.francois@genomic.ch (P. François), jacques.schrenzel@hcuge.ch (J. Schrenzel), hgsahl@uni-bonn.de (H.-G. Sahl).

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a lethal metabolic imbalance following chitosan challenge (Raafat et al., 2008).

Although the antibacterial activity of chitosan against *S. aureus* is well documented in a large number of publications, original research articles dealing with chitosan's mode of action against this notorious microbe are rather scarce. On the other hand, several articles report on the use of chitosan as a film coating for food (Kanatt, Rao, Chawla, & Sharma, 2013), as edible film (Li, Kennedy, Peng, Yie, & Xie, 2006), as chitosan embedded cotton (Gupta & Haile, 2007) or in the form of nanoparticles (Sadeghi et al., 2008). All of these reports attribute to chitosan a high antimicrobial activity against *S. aureus*.

Interestingly, investigations into the potential mechanism(s) of resistance to chitosan are lacking, prompting us to conduct a detailed investigation of the determinants of chitosan resistance in *S. aureus*. We believe that a genetic and biochemical understanding of the adaptive mechanisms of *S. aureus* to chitosan is critical to our understanding of how this compound exerts its antimicrobial activity in the first place. On a more general note, it might help us shed some light on the physiological adaptation of microbes to antibiotic stress.

In the present study, we investigated the *in vitro* development of chitosan-resistance, through the selection for a stable, chitosan-resistant *S. aureus* SG511-Berlin variant, using a serial passage experiment, and characterized the multifaceted phenotype of this variant. In addition, differential gene expression profiling was conducted to compare the transciption profiles of chitosan-susceptible and -resistant cells. A number of interesting observations emanated from this study, regarding pathways via which staphylococci may develop resistance against chitosan.

2. Materials and methods

2.1. Antimicrobial agents, reagents and chemicals

Low molecular weight (LMW) chitosan (Sigma-Aldrich Chemie GmbH) with a molecular weight of 243.17 kg/mol (±1.5% RSD) and a degree of deacetylation of 87% (±2% RSD) was used in this study (Raafat et al., 2008). Its stock solution (1% [wt/vol] in 1% acetic acid) was sterilized by autoclaving at 121 °C for 20 min and stored at 4 °C for subsequent use. All assays involving daptomycin (Cubicin®, Novartis Pharma GmbH) and friulimicin (Ca^{2+}-Friulimicin, Combinature) were carried out in presence of 1.25 mM Ca^{2+} as recommended by the manufacturer (Jorgensen, Crawford, Kelly, & Patterson, 2003). The antimicrobial peptides Pep5 and nisin were kindly provided by M. Josten and Dr. I. Wiedemann, respectively. Gallidermin was purchased from Dr. Petry Genmedics GmbH. All other standard chemicals, antibiotics, solvents and reagents were of analytical grade or better.

2.2. Bacterial strains and culture conditions

Staphylococcus aureus SG511-Berlin (Robert Koch Institute, Berlin, Germany) is a well-characterised methicillin-susceptible (MSSA) laboratory strain, regularly used in the study of cationic antimicrobial peptides (AMPs) (Maidhof, Reinicke, Blümel, Berger-Bächi, & Labischinski, 1991). *S. aureus* SA113 (ATCC 35556), *S. aureus* SA113Δ*dltA*::*spec* and *S. aureus* SA113Δ*mprF*::*erm* were kindly supplied by Prof. Dr. Andreas Peschel (University of Tübingen, Germany).

Unless otherwise indicated, cultivation in liquid cultures was performed aerobically at 37 °C and 150 rpm, in cation-adjusted Mueller–Hinton II broth, CAMHB (BBL™, Becton, Dickinson & Co, USA). Cells were then adjusted spectrophotometrically (optical density at 600 nm [OD_{600}]) to the final desired inoculum. All spectrophotometric approximations were verified by quantitative culturing.

2.3. Serial passage experiment

The *in vitro* selection for decreased susceptibility to chitosan was achieved with the help of a serial-passage experiment, using the standard laboratory strain *S. aureus* SG511-Berlin (wild-type; WT), and was accompanied by a series of MIC estimations. On day 1 of the experiment, CAMHB was inoculated (2% [vol/vol]) with an overnight culture of WT strain, and the culture was left to grow to an OD_{600} of around 1.0, where it was then diluted 1:10^4 in CAMHB to determine the MIC (see below). In addition, the original culture was used to inoculate the next culture, containing chitosan at a concentration equivalent to the MIC value of the last passage, and the process was repeated 15 times. Populations of bacteria from each serial passage were stored as glycerol cultures; no changes in their initial chitosan susceptibility profiles occurred upon storage.

Moreover, the identity of the strain pair (WT and CRV) was confirmed by biochemical methods (using MICRONAUT-RPO plates; MERLIN Diagnostika GmbH, Germany), as well as genetic tools, including PFGE- and phage-typing (Bonness, Szekat, Novak, & Bierbaum, 2008; Goering & Duensing, 1990).

2.4. Antimicrobial susceptibility testing

MIC values of the different antimicrobials tested in this study were determined by a standard broth microdilution assay (Clinical and Laboratory Standards Institute, 2012), using 96-well polystyrene microtiter plates (Greiner Bio-One GmbH, Germany), except in case of Pep5, where polypropylene plates (Nunc F96, Nunc A/S) were used, in order to avoid the interaction and binding of this highly cationic AMP with the anionic surface of polystyrene microtiter plates (Giacometti et al., 2000). Bacterial susceptibility to the various antimicrobials was determined in part with the help of ready-made MICRONAUT-S plates (ES-196-100, ES-195-100 and ES-166-001; MERLIN Diagnostika GmbH), according to the instructions of the manufacturer. The *in vitro* antimicrobial susceptibilities were expressed as MIC, as well as MBC (Minimum Bactericidal Concentration) (Raafat et al., 2008). Susceptibility tests were repeated at least three times independently to check the reproducibility of the results, and the mean of these values was taken.

2.5. Growth curves

Overnight cultures of the test strains were diluted in CAMHB to an initial count of around 1×10^4 CFU/mL, and allowed to grow. Samples were withdrawn at 30 min intervals for viable count estimations and OD_{600} measurements.

2.6. Transmission electron microscopy

Liquid cultures of the test strains were grown in CAMHB to the early exponential phase, then aliquots of the cultures were harvested. Cells were prepared and stained for transmission electron microscopy, and then examined in a Philips CM 120 transmission electron microscope (TEM) as described in a previous study (Raafat et al., 2008).

2.7. Estimation of cell surface charge

The cytochrome c binding assay was performed according to a previously described method (Peschel et al., 1999), with modifications. In brief, overnight cultures were harvested, washed twice and resuspended in 20 mM MOPS buffer (pH 7.0) to a final OD_{578}

of 7.0, and then incubated with 0.5 mg/mL cytochrome c (Sigma-Aldrich Chemie GmbH, Germany) for 10 min at room temperature (RT). The cell pellets were collected, and the amount of cytochrome c in the clear supernatant was determined spectrophotometrically at an OD_{410}. *S. aureus* SA113 strains were included as reference in this assay. Data represent the means ± standard deviation (SD) from three independent determinations. The percentage of cytochrome c bound to the cell pellets relative to the total amount of cytochrome c added was expressed relative to the bacterial dry weight.

2.8. Extraction and identification of membrane phospholipids (PLs)

Overnight cultures of the test strains in CAMHB were harvested, washed and resuspended in Na-acetate buffer (20 mM; pH 4.6). The culture lipids were extracted using methanol-chloroform (1:1:1, by volume) by vortexing for 30 min, followed by centrifugation (5000 rpm, 20 min, 4 °C). The lower chloroform phase was collected and washed twice with distilled water to remove non-lipid contaminants and cell debris. The lipid extracts were dried in a vacuum centrifuge at 50 °C, dissolved in chloroform-methanol (2:1 [vol/vol]), and the individual PLs were separated by thin-layer chromatography (TLC), using Silica Gel 60 F254 high-performance TLC plates (Merck KGaA, Germany) and subsequently developed with chloroform-methanol-water (65:25:4, by volume). L-PG was specifically identified by ninhydrin staining (Peschel et al., 2001). All assays were performed a minimum of three times on separate days.

2.9. Sample preparation for microarray

Samples for RNA isolation were prepared in triplicates. Culture aliquots were collected at the early log phase and immediately stabilized by the addition of 2 vol of RNAprotect™ Bacteria Reagent (QIAGEN GmbH, Germany), according to the manufacturer's instructions. Cell pellets were stored at −70 °C until processed.

2.10. Preparation of total RNA

Cell pellets were lysed by incubation with lysostaphin in TE-buffer for 90 min at 37 °C. Total RNA was extracted (*PrestoSpin* R Bug RNA Purification Kit), then RNA concentration and purity were assessed photometrically using NanoDrop® ND-1000. The absence of remaining DNA traces was evaluated by quantitative PCR (SDS 7700; Applied Biosystems, Framingham, MA) with assays specific for 16s rRNA (Renzoni et al., 2006; Scherl et al., 2006). The isolated RNA was then stored at −70 °C until use.

2.11. Microarray design and manufacturing

The microarray was manufactured as formerly depicted by Fischer et al. (2011). Extensive experimental validation of this array has been previously described, using CGH, mapping of deletion, specific PCR and quantitative RT-PCR (Charbonnier et al., 2005; Scherl et al., 2006).

2.12. Expression microarrays

Batches of 5 μg total *S. aureus* RNA were labelled with Cy-3 or Cy-5 dCTP using the SuperScript II (Invitrogen, Switzerland) following manufacturer's instructions. Labelled products were then purified onto QiaQuick columns (Qiagen); reverse transcription efficiency and dye incorporation were checked using NanoDrop® ND-1000. Equivalent amounts of labelled products were mixed in 50 μl Agilent hybridization buffer, and then hybridized at a temperature of 60 °C for 17 h in a dedicated hybridization oven (Robbins Scientific, USA). Slides were washed with Agilent proprietary buffers, dried under nitrogen flow, and scanned (Agilent, USA) using 100% PMT power for both wavelengths.

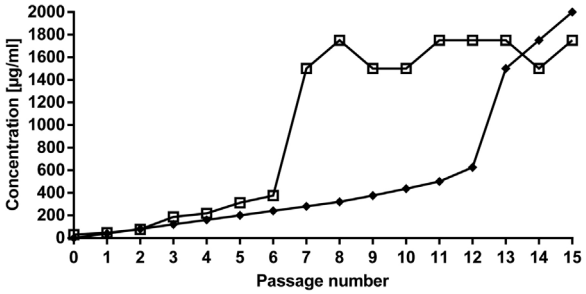

Fig. 1. Serial-passage experiment with *S. aureus* SG511-Berlin. Selection for a strain with increased resistance to chitosan was attempted by incubating the test strain with incrementally increasing chitosan concentrations. The figure depicts the chitosan concentration [μg/mL] used for selection in each passage (♦), together with the corresponding MIC value [μg/mL] after 72 h incubation (□).

Table 1
Primers used for sequencing.

Primer	Sequence (5′ - 3′)	Reference
SA0613_for	CTAACTCTACGTATAATATGGGC	Sass and Bierbaum (2009)
graS_rev	CAATTCTAAGTAACAAAACGCATG	Sass and Bierbaum (2009)
graSintern_rev	GCGCTGAGATTTGACGATATAAATAA	Sass and Bierbaum (2009)
graRint_rev	ATACCAGCAACATTAAAATCCCATT	Sass and Bierbaum (2009)
SA0613int_rev	GTGTTATTTTGGCAGAATTCTTTGT	Sass and Bierbaum (2009)
SA2192-1	CGATTAAAACACCTACAATGACCAG	this work
SA2192-2	CCAAGTCATCTTCTTAACAGAAACG	this work

2.13. Microarray analysis

Hybridization fluorescence intensities were extracted, quantified and corrected as previously described (Fischer et al., 2011). An additional filter was used to exclude irrelevant values, including background noise (Koessler et al., 2006). Fluorescence values for genes mapped by 2 probes or more were averaged. Statistical significance of differentially expressed genes was identified by variance analysis (ANOVA) (Churchill, 2004; Scherl et al., 2006), performed using GeneSpring 7.3 (Agilent). Genes with at least 2 fold induction/reduction of expression (WT as compared to CRV) were accepted as differentially expressed. The experiments were performed in triplicates. The complete microarray data set has been posted on the NCBI Gene Expression Omnibus (GEO) database (available at http://www.ncbi.nlm.nih.gov/geo/), under accession numbers GPL7137 for the platform design and GSE48912 for the original data set.

2.14. Nucleotide sequence analysis of graRS

The nucleotide sequences of *graRS* were determined from PCR fragments, which were amplified from genomic DNA using a standard PCR protocol and the primers listed in Table 1. The nucleotide sequences were obtained by Sanger sequencing including quality editing of the automatically determined sequence (Sequiserve, Germany). *In silico* analysis of sequence data was performed using online analysis tools (http://molbiol-tools.ca/).

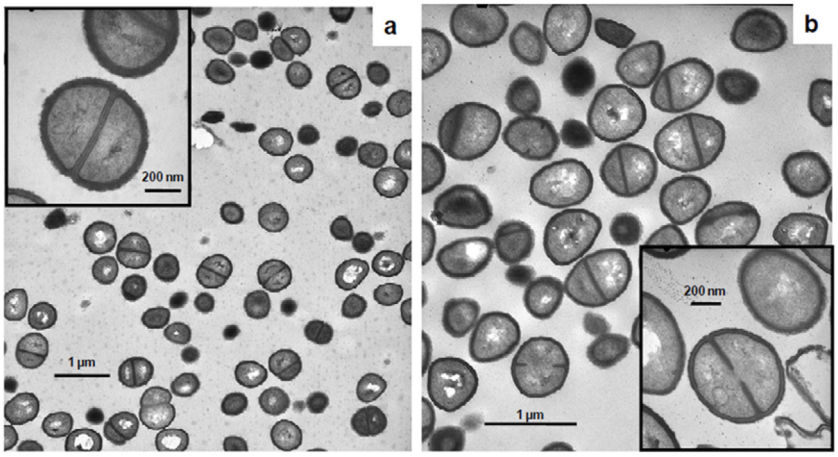

Fig. 2. Ultrastructure of *S. aureus* SG511-Berlin and CRV, as seen under a TEM. Pictures of *S. aureus* SG511-Berlin (WT, a) and CRV (b) were taken at a magnification of 8800× and 15000×, respectively. The inserts (at a magnification of 66,000× and 53,000×, respectively) show close-ups of single cells. Bars indicate 1 µm (200 nm for the inserts). Cells of both variants did not differ in their diameter (nm ± SD), with WT having a size of 605.29 ± 58.98 nm, and the CRV 623.33 ± 72.91 nm. The cell wall thickness (± SD) of both variants was 41.62 ± 6.16 nm and 41.61 ± 4.30 nm, respectively.

2.15. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 software (GraphPad Software, La Jolla, USA). Data were analyzed by Student's *t*-test for comparison between 2 groups. Significance was set at $p \leq 0.05$; and significance levels are indicated as asterisk symbols with notes in figure legends.

3. Results and discussion

The mounting prevalence of bacterial resistance to antimicrobials is a growing threat that has profoundly impacted the medical community. Understanding the bacterial defense strategies greatly helps in combating bacterial pathogens, sheds light on the antimicrobial mode of action of new drugs, and allows for a better understanding of the physiological complexity of bacterial populations.

3.1. In vitro *selection for decreased susceptibility to chitosan*

Selection for a chitosan-resistant *S. aureus* SG511-Berlin variant was achieved by serial passage, using incrementally increasing (0–2000 µg/mL) chitosan concentrations in CAMHB (Fig. 1). MIC and MBC analyses of daily archived colonies from the different passages were conducted, and the experiment was terminated after 15 passages, when the MIC values reached a plateau (Fig. 1). Since there are no MIC guidelines established by the CLSI for chitosan resistance, we termed the final variant obtained (MIC = 1750 µg/mL) as **c**hitosan-**r**esistant **v**ariant (CRV). Interestingly, MIC analyses revealed an initial gradual increase in the MIC value of chitosan during the first six passages, which was followed by a "leap" in the MIC value, indicating that a critical "genetic event" probably took place at this time-point. Indeed, sequence analysis revealed that the WT strain *S. aureus* SG511-Berlin harbours a native mutation that reverted in the CRV (discussed further in detail under Section 3.7). The observed adaptation to high levels of chitosan was stably inherited after 20 consecutive passages of the CRV in chitosan-free CAMHB. It should be noted that the growth kinetics of both the CRV and its parent strain were similar (Supplementary Fig. 1).

3.2. Examination of S. aureus *ultrastructure*

In order to identify changes in bacterial morphology that might be associated with the decreased susceptibility of the CRV, we examined the ultrastructure of both variants under a TEM. TEM analysis revealed no significant differences in cell morphology, size or cell wall thickness (Fig. 2). Our electron microscopical findings were thus at variance with reports that implicated cell wall thickening as a physical barrier in the resistance of bacteria to surface-active compounds, including vancomycin and daptomycin (Cui et al., 2003; Cui, Tominaga, Neoh, & Hiramatsu, 2006). However, the higher optical density measurements of CRV cultures, compared to the parent strain (Supplementary Fig. 1) could give an indication to a change in the biochemical composition of the cells, that would explain the increased resistance observed (further detailed discussion of cellular metabolism based on gene expression profiles under Section 3.6).

3.3. Cross-resistance to other antimicrobials

Compared to the parental strain, CRV showed higher MIC values (1.5–32-fold) for most of the cationic AMPs and common antimicrobials tested in this study (Table 2), some of which currently used in clinical practice against staphylococci. This documented cross-resistance of CRV to the cationic peptides and common antimicrobials in our study should be emphasized. It is conceivable that it is due to one or more of the following: (i) decreased antibiotic influx through mutations affecting membrane transport, impairing antibiotic diffusion and uptake; (ii) active efflux of the drug out of the bacteria through induction of membrane transport proteins; and (iii) changes in cell envelope structure resulting in decreased accumulation of these antibiotics.

Interestingly, most of the antibiotics, to which the CRV exhibited a higher resistance are cationic in nature (mostly rich in amino- and hydroxyl-groups), which might indicate a general resistance mechanism to cationic compounds. Together with the fact that chitosan's site of antimicrobial action is believed to be at the bacterial cell envelope, these results add weight to the hypothesis that changes in cell envelope structure do contribute to the observed resistance to chitosan. This is why, in the following two sections we will discuss in detail two major aspects of cell envelope structure, namely bac-

Table 2
MIC values of various antimicrobials for *S. aureus* SG511-Berlin (WT) and CRV in CAMHB.

Antimicrobial	WT			CRV			Fold MIC
	MIC[a]		MBC[a]	MIC[a]		MBC[a]	
	24 h	48 h		24 h	48 h		
LMW Chitosan[b]	31.25	31.25	31.25	1500	1750	1750	56
Erythromycin	0.063	0.125	0.125	0.125	0.25	0.25	2
Chloramphenicol	2	4	4	3	6	12	1.5
Tetracycline	0.25	0.5	0.5	0.125	1	1	2
Vancomycin	0.25	0.25	0.25	0.5	0.5	0.5	2
Kanamycin	1.5	2	2	1	3	3	1.5
Gentamicin	0.5	0.5	1	2	4	4	8
Amikacin	2	4	8	16	16	16	4
Tobramycin	0.5	0.5	0.5	1	2	2	4
Bacitracin	2.5	4	4	6	8	8	2
Daptomycin	0.02	0.02	0.02	0.313	0.313	0.313	16
Friulimicin	0.234	0.313	0.313	2.5	2.5	3.75	8
Nisin	1.25	1.25	1.25	2.5	2.5	2.5	2
Gallidermin	0.078	0.25	0.25	0.375	1	1	4
Pep5	0.5	1	3	12	32	32	32

[a] MIC and MBC values are given in μg/mL. Susceptibility tests were repeated at least three separate times to check the reproducibility of the results, and the mean of these values was taken.
[b] 1% in 1% HAc.

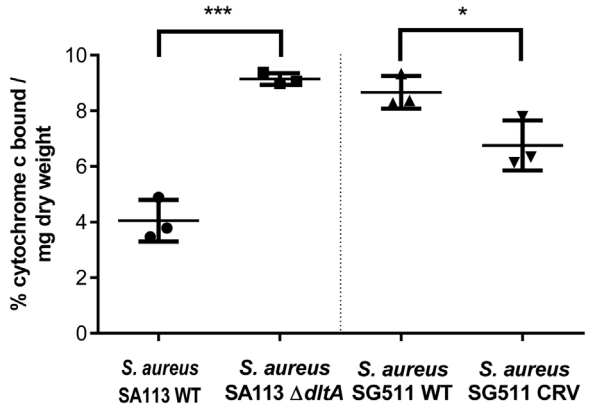

Fig. 3. Cell surface charge of the test strains, as determined using the cytochrome c binding assay. The % of cytochrome c bound by each of the four indicated strains was determined with reference to the bacterial dry weight, using the following formula: $[(OD_{410}$ of control $- OD_{410}$ of sample$)/OD_{410}$ of control$] \times 100$. The scatter plot represents the values of three independent determinations for each strain ± standard deviation (SD). $^{*}P < 0.05$; $^{***}P < 0.001$ indicate statistical significance relative to the parent strain.

terial cell surface charge (Section 3.4), and membrane phospholipid composition (Section 3.5).

3.4. Influence of cell surface charge

It is assumed that the cationic nature of chitosan, brought about by its protonated amino groups, allows its ionic interaction with negatively-charged cell surface structures, such as teichoic acids (Raafat et al., 2008). The latter are essential polyanionic polymers found in the cell wall of Gram-positive bacteria, which extend to the surface of the peptidoglycan layer, thus contributing to the negative charge of the cell wall.

Cytochrome c is a cationic protein, which has previously been used to estimate the relative surface charge of the cell envelope of isogenic *S. aureus* strain pairs (Peschel et al., 1999). CRV showed a decrease in cytochrome c binding, compared to the parent strain (6.76 ± 0.11% and 8.67 ± 0.09% bound/mg dry weight ± SD, respectively), which is consistent with an overall less negative surface charge (Fig. 3). The *S. aureus* strains SA113 WT (4.05 ± 0.11%) and SA113 Δ*dltA* (9.20 ± 0.09%) were included in this assay as reference controls, where the *dltA*-mutant (lacking the D-alanyl groups in teichoic acids) showed a higher % of bound cytochrome c (since less positively-charged), compared to the control.

3.5. Analysis of membrane PL profiles

The *S. aureus* cell membrane consists of three major PL species, the negatively-charged phosphatidylglycerol (PG), cardiolipin (CL) and the positively-charged lysyl-phosphotidylglycerol (L-PG). L-PG is unique to *S. aureus*, accounting for up to 38% of the total PL content of its cytoplasmic membrane (Peschel et al., 2001). MprF is the enzyme involved in L-PG biosynthesis through lysinylation of PG, and for its translocation to the outer leaflet (Ernst et al., 2009; Peschel et al., 2001).

In an attempt to evaluate possible alterations in the PL membrane composition of CRV, which might correlate to its reduced sensitivity towards chitosan, we isolated membrane lipids from WT and its CRV, and analyzed them by 2D-TLC (Supplementary Fig. 2). Differences were observed between the membrane PL profiles of both variants, where the CRV exhibited 6–7 × higher levels of the positively-charged L-PG, compared to the chitosan-susceptible parent strain. This was confirmed by 1D-TLC, where L-PG was specifically stained with ninhydrin (Fig. 4).

Interestingly, an *mprF*-null deletion mutant of *S. aureus* SA113, which was more susceptible to a broad variety of cationic AMPs (Peschel et al., 2001), also showed increased *in vitro* sensitivity to chitosan (MIC = 15.63 μg/mL). Our findings thus corroborate the biological importance of the charge balance of the cell surface, and support the notion that the charge-based surface repulsion and reduced binding of the polycationic chitosan by a less negatively-charged surface envelope could contribute to the chitosan resistance observed with CRV.

3.6. Differential gene expression profiling

A genome-scale gene expression experiment was conducted, in order to compare the parental strain (*S. aureus* SG511-Berlin) and CRV on the transcriptional level, in an attempt to delineate the cause for the observed phenotypic differences among both strains, and to draw conclusions about changes in the gene expression profile of CRV that might account for its increased resistance.

A statistical analysis of the results, at a FDR (**F**alse **D**iscovery **R**ate) limit of 5%, identified a total of 333 Open Reading Frames (ORFs) differentially expressed in CRV, as compared to the WT

Table 3
Summary of regulated genes in CRV, compared to the parent strain, clustered according to process.[a]

Process	Upregulated genes		Downregulated genes	
	Number	%	Number	%
Adaption to atypical conditions	5	2.3	11	9.8
Antibiotic production	0	0	1	0.9
Cell division	2	0.9	0	0.0
Cell wall	4	1.8	2	1.8
Detoxification	3	1.4	0	0.0
Hypothetical proteins	86	38.9	14	12.5
Membrane bioenergetics	1	0.5	4	3.6
Metabolism of amino acids and related molecules	18	8.1	5	4.5
Metabolism of carbohydrates and related molecules	25	11.3	11	9.8
Metabolism of coenzymes and prosthetic groups	10	4.5	8	7.1
Metabolism of lipids	3	1.4	1	0.9
Metabolism of nucleotides and nucleic acids	7	3.2	12	10.7
Miscellaneous	3	1.4	0	0.0
Pathogenic factors (toxins and colonization factors)	17	7.7	7	6.3
Phage-related functions	2	0.9	2	1.8
Protein folding	1	0.5	0	0.0
Protein modification	0	0	1	0.9
Protein secretion	0	0	1	0.9
RNA synthesis – Regulation	10	4.5	7	6.3
Sensors (signal transduction)	0	0	1	0.9
Transformation competence	0	0	1	0.9
Transport/binding proteins and lipoproteins	23	10.4	23	20.5
Transposon and IS	1	0.5	0	0.0
Total	221 genes	100	112 genes	100

[a] Data consisting of two independent biological experiments were analyzed using GeneSpring 7.3 (Agilent). Features whose intensities were smaller than the standard deviation value of the negative controls in all the measurements were considered as inefficient hybridization and discarded from further analysis. Fluorescence values for genes mapped by 2 probes or more were averaged. Statistical significance of differentially expressed genes was identified by variance analysis (ANOVA), performed using GeneSpring, including the Benjamini and Hochberg false discovery rate correction (5%). Genes with at least 2 fold induction/reduction of expression were accepted as differentially expressed. The experiments were performed in triplicates.

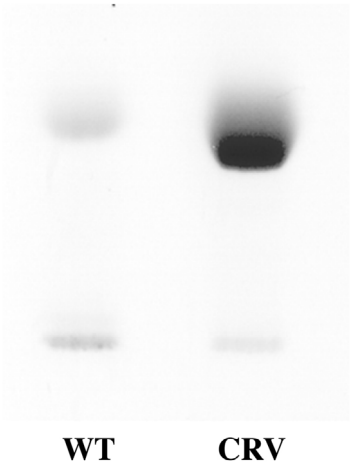

Fig. 4. Analysis of membrane PL composition of the isogenic strain pair using one-dimensional TLC. The extract (5 μl) of each strain was spotted on Silica gel plates, developed in the appropriate solvent, and the L-PG fraction was specifically stained with ninhydrin.

strain (Table 3). The detailed results of the microarray experiment, including complete lists of up- (221) and down-regulated genes (112) in CRV, compared to the parent strain, are given in the Supplementary Tables 1 and 2, respectively.

Around 39% of up-regulated ORFs encode for hypothetical proteins (Table 3). For instance, the gene with the highest level of upregulation (>240 × higher levels in CRV) was ORF SA2192 (Supplementary Table 1), a gene which was annotated only in staphylococci. It encodes a small hypothetical protein (60 aa), which contains 2 possible transmembrane helices, with an extracellular loop (TMHMM server v. 2.0, Technical University of Denmark). Interestingly, the expression of ORF SA2192 was not induced upon treatment of *S. aureus* SG511-Berlin with cationic peptides such as P19 (6|E), a synthetic α-helical peptide with a positive net charge of +6 (Pag et al., 2008), or the human β-defensin 3 (hBD3), an eukaryotic host defense peptide with a net charge of +11 (Sass, Pag, Tossi, Bierbaum, & Sahl, 2008). Since, especially in the case of hBD3, a high net charge is involved, it is questionable that the expression is solely dependent on the presence of cationic substances, and is rather due to an additional, possibly specific activity of chitosan.

In general, gene expression profiles suggest that cells alter their metabolism to accommodate the increased production of components of the cell envelope. The involvement of changes in cell envelope structure in the observed resistance of CRV to chitosan was mostly highlighted by the up-regulation of a gene (SA0035; 105-fold induction in CRV), which codes for HMG-CoA (Hydroxymethylglutaryl-CoA) synthase, a key enzyme for the synthesis of isoprenoids, catalyzing the first committed step in the mevalonate pathway. The mevalonate pathway is important for the production of activated C_5-isoprene units that serve as the basis for the biosynthesis of a number of polymers that play an important function in diverse processes within the bacterial cell, including the lipid carrier undecaprenol (involved in cell wall biosynthesis) and teichoic acids (Bugg and Walsh, 1992; D'Elia et al., 2006; Reusch, 1984). The considerable up-regulation of SA0035, coupled with the marked down-regulation of almost all of the capsular genes (*capC-capG* and *capL-capP*), might indicate that CRV is capable of synthesizing more lipid carrier (C_{55}P), which would be exclusively dedicated to the production of cell wall polymers.

Genes encoding a number of proteins closely associated with cell wall structure were also up-regulated in CRV (Supplementary Table 1), including: (i) *spa*, encoding protein A, a surface protein that is reported to be covalently bound to the pentaglycine interpeptide bridge of peptidoglycans (Navarre & Schneewind, 1999); (ii) several cell-wall anchored surface proteins, including clumping factor B (ClfB) and Sdr proteins (SdrC, SdrD and SdrE) (Foster & Höök, 1998); and (iii) the *dltABCD* operon, which is responsible for

the D-alanylation of cell wall teichoic acids, thereby introducing positively-charged amino groups into the otherwise negatively-charged teichoic acids (Peschel et al., 1999) and biochemically reflected in a less negatively-charged cell envelope, as evidenced through the reduced binding of cytochrome c by the surface envelope of CRV.

Moreover, genes encoding proteins LrgA and LrgB, which modulate murein hydrolase activity, and are thus involved in the murein sacculus and peptidoglycan biosynthesis, were down-regulated in CRV. Whereas induction of *lrgA* and *lrgB* can be viewed as a response of the cell to preserve peptidoglycan when faced with the challenge of a cell wall-active agent as daptomycin (Muthaiyan, Silverman, Jayaswal, & Wilkinson, 2008), their downregulation might indicate increased murein production.

Staphylococci are facultatively-anaerobic microorganisms, which utilize glycolysis and oxidative pentose phosphate (PP) pathway as the two central routes of their glucose metabolism (Götz, Bannerman, & Schleifer, 2006). However, all experiments throughout this study were carried out in CAMHB, a culture medium containing starch instead of glucose. Thus, staphylococci were forced to grow at the expense of amino acids, due to their inability to degrade starch. This would explain the upregulation of a large number of genes involved in amino acid metabolism, including *argFGH*, *arcABC*, *hisG*, *hutH*, *rocAD*, *ald* and *gudB*, in an attempt to utilize the amino acids in the growth medium for energy generation. In addition, the lack of glucose in the growth medium would prompt the cells to synthesize glucose or glucose 6-P (gluconeogenesis), all the more so, if there is a higher demand for sugars as a response to the increased cell wall biosynthesis, through a reversal of glycolysis. Indeed, we have reason to believe that, in an effort to meet the high demand for the sugar building blocks of the cell wall, CRV has managed to strongly alter the distribution of intracellular fluxes, rerouting the main glucose catabolism from glycolysis to the PP pathway, and thus redirecting their metabolism towards gluconeogenesis. This would explain the fact that numerous genes encoding glycolysis pathway proteins were differentially regulated.

Activated fructose operon genes *fruAB* (both up-regulated 4.4 times) correlate well with increased peptidoglycan synthesis. Those enzymes have a critical role in the initiation of peptidoglycan synthesis by converting fructose-6-phosphate in Embden-Meyerhof pathway into glucosamine-6-phosphate, from which the murein monomer precursor is synthesized (Kuroda, Kuwahara-Arai, & Hiramatsu, 2000; Postma, Lengeler, & Jacobson, 1993). This effect is even more highlighted by the upregulation of *mtlA* (responsible for taking up exogenous mannitol), as well as fructose-bisphosphatase (*fbp*), both resulting in accumulation of fructose-6-phosphate. It is therefore a reasonable assumption that the uptake of these sugars is encouraged to complement enhanced activity of HMG-CoA synthase to synthesize more peptidoglycan.

In addition, the glyceraldehyde-3-phosphate dehydrogenases GapA and GapB, which are isoenzymes catalyzing opposite fluxes through a key reaction of the glycolytic pathway, were differentially expressed in CRV. GapB differs from GapA in being NADP(H)-dependent and in efficiently catalyzing the gluconeogenic reduction of 1,3-di-phosphoglycerate to glyceraldehyde-3-phosphate (Tännler et al., 2008). Genes encoding the gluconeogenic enzymes GapB and PckA (PEP carboxykinase) were up-regulated. An increase in GapB expression, with a simultaneous decrease in *gapA* transcription (refer to Supplementary Tables 1 and 2, respectively), would lead to strongly increased intracellular concentrations of intermediates in the upper part of glycolysis (including fructose-6-phosphate), causing a metabolic jamming of this pathway and, consequently, redirecting the relative flux through the PP pathway, which in turn would favor gluconeogenic conditions (Tännler et al., 2008). Consistent with this is the strong upregulation of genes coding for proteins involved in gluconeogenesis such as *aldA* (aldehyde dehydrogenase homolog) and *acsA* (acetyl-CoA synthetase). The required energy is probably derived from the up-regulation of NADH-dependent dehydrogenases (SA0211, SA0819) and various other dehydrogenases that could generate reduction equivalents. In gluconeogenesis, certain bypass steps are involved, which are carried out by enzymes encoded by *pycA*, *pckA* and *fbp*. Indeed, two of these genes (*pckA* and *fbp*) were found to be up-regulated in CRV.

Considering that the resistance exhibited by CRV was not confined to the antimicrobial used for selection (*i.e.* chitosan) but also extended to other antimicrobials (predominantly cationic), the involvement of transport problems becomes very likely. Indeed, a number of genes coding for hypothetical ABC transporters were found up-regulated in CRV, including: *vraG*, SA1674, SA2243, SA2314 and SA2415, indicating that exporters play a role in the cellular adaptation to chitosan challenge, possibly in the detoxification of membrane environment. In addition, a gene encoding a membrane transport protein involved in the active efflux of quinolones out of the bacteria (*norA*) was 5.8-fold up-regulated in CRV.

3.7. Impact of a repaired graS gene

GraRS is a two-component regulatory system (TCRS) that has been proposed as an AMP sensing system in *S. aureus*; it consists of the transmembrane sensor histidine kinase GraS and the cytosolic response regulator GraR. The TCRS GraRS controls the expression of several resistance genes, including the ABC-transporter VraFG, the components of the *dlt*-operon (*dltABCD*), as well as the gene *mprF* (Peschel et al., 2001). The latter two regulations result in an increased positive cell surface charge, thereby reducing the affinity of the bacterial surface to cationic AMPs (Herbert et al., 2007; Kraus et al., 2008; Li, Cha et al., 2007; Li, Lai et al., 2007).

Several researchers have demonstrated that knockouts of either *mprF* or *dltA* yield staphylococcal cells with a more negatively charged cell envelope, enabling cationic AMPs to bind to the surfaces of these strains more avidly, thus ultimately increasing the *in vitro* susceptibility to a number of cationic AMPs, including PMPs, HNP-1, gramicidins, as well as daptomycin (Bayer, Schneider, & Sahl, 2013; Friedman, Alder, & Silverman, 2006; Jones et al., 2008; Mukhopadhyay et al., 2007; Peschel et al., 1999; Peschel et al., 2001; Xiong, Mukhopadhyay, Yeaman, Adler-Moore, & Bayer, 2005). This mirrors our previous data concerning the impact of cell surface charge on chitosan's antimicrobial activity, using various mutants of *S. aureus* SA113, with defects in their teichoic acid machinery (Raafat et al., 2008).

The strain *S. aureus* SG511-Berlin harbours a native mutation (thymine insertion at position 190) in *graS*, which generates a premature stop codon, thereby deleting 283 amino acids of GraS, representing the entire cytoplasmic, signal transducing part of the membrane protein (Li, Lai et al., 2007) that is responsible for the activation of the response regulator GraR (Sass & Bierbaum, 2009). A defective GraS, followed by an impaired signal transduction, is believed to be the reason for the high susceptibility of *S. aureus* SG511-Berlin to various antimicrobials, due to a less positive cell surface charge (Sass & Bierbaum, 2009).

A sequence analysis of *graRS* in CRV showed that, unlike its parent strain *S. aureus* SG511-Berlin, CRV lacks the thymine insertion at position 190 in *graS*, resulting in the translation of the entire peptide chain, and thus the production of the full-length functional GraS protein, which might explain the higher amounts of L-PG (product of MprF), the more positive cell surface charge (related to both the L-PG content and the higher expression levels of the *dlt*-operon), as well as the observed cross-resistance of CRV to cationic antimicrobials. Regardless of whether the mutation of this gene in CRV is random or through adaptive selection, it represents an enormous

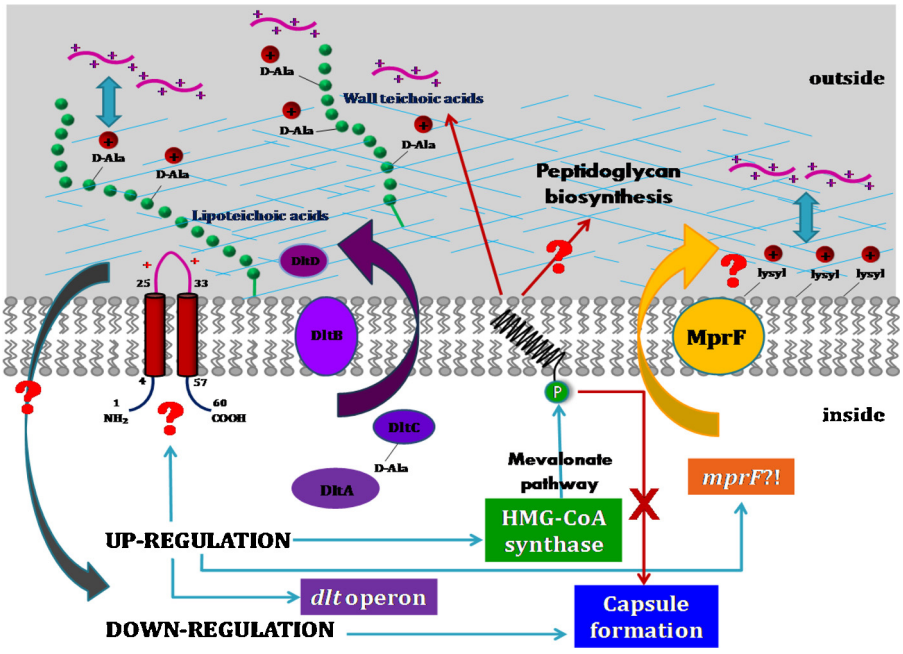

Fig. 5. Possible resistance mechanisms of *S. aureus* SG511-Berlin against chitosan. The cationic nature of chitosan plays a pivotal role in its antimicrobial activity, allowing its interaction with the largely anionic bacterial surface. Therefore, several cell envelope alterations depicted here, which are mostly interrelated, correlate with the *in vitro* chitosan resistance in *S. aureus* SG511-Berlin, including (i) increased positive surface charge due to the upregulation of the *dlt* operon, resulting in reduced chitosan binding, (ii) enhanced production of cell wall polymers; and (iii) elevated levels of positively-charged membrane lipids, thus increasing electrostatic cell surface repulsion of chitosan.

advantage of the mutant relative to the WT strain in its defense against cationic antimicrobials.

To test the impact of the mutation in *graS* on chitosan resistance, we used *S. aureus* SG511-Berlin pTX*graS*, a *graS* complemented *S. aureus* SG511-Berlin (Sass & Bierbaum, 2009), and compared MIC values of chitosan and other cationic antimicrobials (the lantibiotics nisin und Pep5, the fungal defensin plectasin, as well as the antibiotics daptomycin, friulimicin and vancomycin) for strains with a functional sensor histidine kinase GraS (CRV, *S. aureus* SG511-Berlin pTX*graS*(+); induced) with those of *graS*-mutants (WT, *S. aureus* SG511-Berlin pTX*graS*(−); not induced).

Strain SG511 pTX*graS* proved to be less sensitive to most of the tested cAMPs upon induction of the *graS* gene, compared to the non-induced strain (data not shown). The MIC profile of the *graS*-deleted *S. aureus* strains WT and SG511 pTX*graS*(−) showed only minor differences, which could be statistically attributed to standard deviations. A different picture emerged when comparing the MIC values of the two strains expressing a functional sensor histidine kinase. Compared to the induced strain SG511 pTX*graS*, the mutant exhibited up to 15-fold higher MIC values for most of the tested antibiotics. Therefore, the hypothesis that the resistance behavior observed is primarily due to the presence of a functional sensor histidine kinase could not be corroborated here. On the contrary, it was shown that a functioning kinase alone had no significant effect on the resistance behavior towards chitosan. Nevertheless, estimations of MIC values could not exclude the positive effect of the reversion of the *graS*-mutation for the CRV, but it proved to play rather a secondary role in connection with the chitosan resistance.

4. Conclusions

Although the precise mechanisms by which *S. aureus* SG511-Berlin develops resistance against the antimicrobial activities of chitosan remain incompletely defined, yet the current study provided valuable insights into the nature of the chitosan resistance displayed by CRV (summarized in Fig. 5).

Both biochemical/phenotypic and genotypic analyses were complementary and confirmed the key association of changes in cell surface properties with the *in vitro* development of chitosan resistance in *S. aureus* SG511-Berlin. This involves a reduced overall negative charge of both the cell wall and cell membrane (resulting in reduced chitosan binding), as well as changes in the expression of genes involved in sugar metabolism. This is in fair agreement with several previous studies, which have shown an association between the activity of certain antimicrobials and cell envelope structures (Friedman et al., 2006; Jones et al., 2008; Mukhopadhyay et al., 2007; Muthaiyan et al., 2008; Peschel et al., 1999; Utaida et al., 2003; Xiong et al., 2005). Furthermore, the phenotype of the current CRV (increased L-PG production, increased relative surface positive charge, and resistance to cationic AMPs) suggests a "gain in *mprF* function". However, the transcriptional analysis revealed no direct implication of the regulation of *mprF* in the reduced susceptibility of CRV to chitosan, indicating that the regulation of MprF occurs at a level other than the transcriptional one.

Interestingly, most of the observed phenotypes are interrelated, and suggest a broad model of resistance that is closely associated with both cell wall and cell membrane structures. These proposed resistance mechanisms are not mutually exclusive; indeed we believe that combinations of these general types of resistance work in concert, ultimately contributing to the patterns of *in vitro* resistance that we observed.

More importantly, the relatively quick development of stable resistance to chitosan, and the cross-resistance of the emerged isolate to other antimicrobials would warrant more caution in the indiscriminate use of chitosan as an additive to a number of pharmaceutical and biomedical formulations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.09.075.

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