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

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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.

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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}