Chitosan as an antimicrobial compound: Modes of action and resistance mechanisms

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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aus

Alexandria/Ägypten

Bonn 2008

“Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes“

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn.

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Tag der Promotion: 15.10.2008

Diese Dissertation ist auf dem Hochschulschriftenserver der ULB Bonn

http://hss.ulb.uni-bonn.de/diss_online elektronisch publiziert.

Erscheinungsjahr: 2008

my beloved family

ACKNOWLEDGEMENT

I would like to seize this opportunity to pay tribute to all those who have contributed, one way or another, to the completion of this work, which marks another utterly important milestone in my life. Without them and without their commitment and enthusiasm, it would not have been possible for me to enjoy this outstanding experience. It is therefore my pleasure to acknowledge the support and help of those who joined me throughout this journey, and who would forever shape my life.

First and foremost, I would like to express my profound gratitude and great indebtedness to my supervisor, Prof. Dr. Hans-Georg Sahl, for his benevolent supervision of my doctoral thesis. I am very grateful to him for giving me the opportunity to conduct this research project within his fascinating research group, for his continuous support and constructive criticism, and for encouraging and challenging me throughout the progress of the study; never accepting less than my best efforts. But most of all, I thank him for his willingness to share his fascination with microbiology during our scientific discussions.

I am indebted beyond measure to Prof. Dr. Gerd Bendas for kindly accepting to be the second referee for the present work. I would also like to express my profound thanks to Prof. Dr. Gabriele König and Prof. Dr. Albert Haas who honored me with their approval to be members of the doctoral committee.

I deeply appreciate the help and contributions received from many people during this project: I am very grateful to Prof. Dr. Albert Haas (University of Bonn, Germany), for giving me access to the transmission electron microscope, and to Sabine Spürck and Kristine von Bargen, both for their expert technical assistance. I owe deepest thanks to Prof. Dr. Andreas Peschel (University of Tübingen, Germany) for his provision of the *S. aureus* SA113 strains; to Prof. Jacques Schrenzel and Dr. Patrice François (Geneva University Hospitals, Switzerland), for their help with the differential expression profiling; and to Dr. W. Lindenthal for supplying me with some of the chitosan samples. I am greatly indebted to Mirko Weinhold (University of Bremen, Germany) for the characterization of chitosan and to Christoph Ernst (University of Tübingen, Germany) for his enthusiastic assistance with PL analysis. The help of Vera Sass with the analysis of the microarray data, and of Dr. Tanja Schneider with the *in vitro* lipid II assay is also appreciated.

At this point, I wish to repay the debt of gratitude I owe my beloved parents, my mentors and teachers, my idols and my heroes; for years of encouragement, for their strong belief in me and for their everlasting patience and understanding during the different phases of my work. I tried to follow their examples, and truly wish I was able to make them proud. I owe them great appreciation for what I have learned and what I have achieved. I also wish to acknowledge my sister and brother, my supporting pillars, whose continual back-up and loving support was of great value in accomplishing this work, and who believed in me at times, when I had the greatest doubts. I wouldn't have done it without their help. I am also very thankful to my lovely grandmother, for her constant prayers and encouraging words.

During this PhD project I deeply enjoyed the cordial atmosphere at the institute, and had the opportunity to make the acquaintance of a number of inspiring people who have motivated me during this period and for whom I am eternally grateful. I am beholden to my dear colleagues at the Pharmaceutical Microbiology Unit, in particular Kathrin Gries, Daniela Alborn, Lena Poppinga, Tim Böttiger and Dr. Beate Henrichfreise, for their encouragement and their excellent moral support; but also for the lengthy talks, good laughs, long hours spent together in the lab, and especially for all the fun we had together. Each one of them contributed to this study in many different - often very personal - ways. Their kindness and helpfulness will forever remain imprinted in my mind. Dr. Andrea Schäfer, Dr. Imke Wiedemann, Dr. Irith Wiegand and Dr. Tanja Schneider deserve sincere thanks for their help and cooperation, for many stimulating discussions and for their valuable advice. Thanks are also extended to Inge Luhmer-Becker, Bedia Bugdaci and Karina Boltersdorf, whose supportive and friendly demeanor made the last few years enjoyable; to Prof. Dr. Gabi Bierbaum for her guidance and advice; and to Michaele Josten for outstanding technical assistance in the radioactivity lab.

I am very lucky to have had so many friends beside me, who helped me a lot throughout my work. I would like to express my special gratitude to Nehal, Marwa K., Mona F., Mojdeh, Amany, Nermine, Iza, Raquel, Nihal K., Amal A., Fatemah, Heba S., Nefertiti, Mohamed B., Sherine R., Bassem and Mona; but particularly to Heba K. and Mahmoud Elg., for their genuine concern and kind support.

Last, but not least, I would like to gratefully acknowledge the German Academic Exchange Service (DAAD) for granting me a PhD scholarship, as well as for the constant support and excellent care. Particular thanks also go to the Cultural Department and Study Mission of the Egyptian Embassy for their financial support.

Dina Raafat, July 30th, 2008

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LIST OF ABBREVIATIONS

aa	**a**mino**a**cids
A_{index}	**a**bsorbance at [**index**] nm wavelength
AMP	**a**nti**m**icrobial **p**eptide
APS	**a**mmonium **p**er**s**ulfate
Aqua dest	distilled and deionized water
As	**ar**s**e**nic
ATCC	**A**merican **T**ype **C**ulture **C**ollection
ATP	**a**denosine **t**ri**p**hosphate
AU	**a**rbitrary **u**nit
BATH	**B**acterial **A**dherence **T**o **H**ydrocarbons
BHI	**b**rain-**h**eart **i**nfusion broth
BLAST	**B**asic **L**ocal **A**lignment **S**earch **T**ool
bp	**b**ase **p**air
BSA	**b**ovine **s**erum **a**lbumin
C	**c**arbon
C_{55}	undecaprenyl
$C_{55}P$	undecaprenyl**p**hosphate
CAMHB	**c**ation-**a**djusted **M**ueller-**H**inton II **b**roth
Cd	**c**a**d**mium
cDNA	**c**omplementary-**DNA**
CF	**c**arboxy**f**luorescein
CFU	**c**olony-**f**orming **u**nit
CLSI	**C**linical and **L**aboratory **S**tandards **I**nstitute
CoA	**Co**enzyme **A**
conc.	**conc**entration
cps	**c**enti**p**oi**s**e
CRV	**c**hitosan-**r**esistant **v**ariant
Cu	copper
Da	**da**lton
DD	**d**egree of **d**eacetylation
DEAE	**di****e**thyl**a**mino**e**thyl
$DiBAC_4(3)$	bis-(1,3-dibutylbarbituric acid) trimethine oxonol
DMAPP	**d**i**m**ethyl**a**llyl **p**yrophosphate
DMPC	**d**i**m**ethyl**p**ropyl **c**arbonate
DNA	**d**eoxyribo**n**ucleic **a**cid
DNase	**d**eoxyribo**n**ucle**ase**
dNTP	**d**eoxyribo**n**ucleoside **t**ri**p**hosphate
DOPC	1,2-**d**i**o**leoyl-*sn*-glycero-3-**p**hospho**c**holine
DOPG	1,2-**d**i**o**leoyl-*sn*-glycero-3-[**p**hospho-*rac*-(1-**g**lycerol)] sodium salt
DSM/DSMZ	**D**eutsche **S**ammlung von **M**ikroorganismen und **Z**ellkulturen (German Collection of Microorganisms and Cell cultures)
DTT	**d**i**t**hio**t**hreitol
EDTA	**e**thylene**d**iamine-**t**etra**a**cetic acid

erm	**er**ythro**m**ycin
et al.	**et al**teri / **et al**ii (and others)
e-value	**e**xpectation **value**
F_A	**f**raction of **a**cetylated groups
FAD	**f**lavin **a**denine **d**inucleotide
FDA	**F**ood and **D**rug **A**dministration
FDR	**f**alse **d**iscovery **r**ate
Fe	iron
FIC	**f**ractional **i**nhibitory **c**oncentration
FMN	**f**lavin **m**ono**n**ucleotide
g	**g**ram
g	**g**ravitational acceleration
GlcN	D-**gl**u**c**osami**n**e
Glc*N*Ac	***N*-ac**etyl-D-**gl**u**c**osamine
Gly	**gly**cine
GRAS	**G**enerally **R**ecognized **A**s **S**afe
h	**h**our
H_2O	water
HAc	**ac**etic acid
HCl	hydro**ch**l**l**oride / hydro**ch**l**l**oric acid
Hg	mercury
HMG-CoA	3-**h**ydroxy-3-**m**ethyl**g**lutaryl **co**enzyme **A**
HPLC	**h**igh **p**erformance **l**iquid **c**hromatography
IPP	**i**so**p**entenyl **p**yrophosphate
K	potassium
kDa	**k**ilo**D**alton
kg	**k**ilo**g**ram
l	**l**iter
LD_{50}	**l**ethal **d**ose **50**% (median lethal dose)
LMW	**l**ow **m**olecular **w**eight
LOWESS	**lo**cally **we**ighted **s**catterplot **s**moothing
LPG	**l**ysyl-**p**hosphatidyl**g**lycerol
LTA	**l**ipo**t**eichoic **a**cid
M	**m**olar
MBC	**m**inimum **b**actericidal **c**oncentration
MDa	**m**ega**Da**lton
mg	**m**illi**g**ram
MHA	**M**ueller-**H**inton **a**gar
MIC	**M**inimum **I**nhibitory **C**oncentration
min	**min**ute
ml	**m**illi**l**iter
mm	**m**illi**m**eter
mM	**m**illi**m**olar
M_n	**n**umber-average **m**olecular weight
MOPS	**mo**rpholine**p**ropane**s**ulfonic acid
mPa·s	10^{-3} Pa·s (**pa**scal-**s**econd); unit of viscosity
Mur*N*Ac	***N*-ac**etyl **mur**amic acid
mV	**m**illi**v**olt

MW	**m**olecular **w**eight
M_w	**w**eight-average **m**olecular weight
N/A	**n**ot **a**pplicable / **n**ot **a**vailable
Na	sodium
NaCl	sodium **c**h**l**oride
NAD^+/NADH	**n**icotinamide **a**denine **d**inucleotide
NADP	**n**icotinamide **a**denine **d**inucleotide **p**hosphate
NCCLS	**N**ational **C**ommittee for **C**linical **L**aboratory **S**tandards
NCTC	**N**ational **C**ollection of **T**ype **C**ultures
nm	**n**ano**m**eter
nmol	**n**ano**mol**e
OD	**o**ptical **d**ensity
OD[index]	**o**ptical **d**ensity at [**index**] nm wavelength
ORF	**o**pen **r**eading **f**rame
P	**p**hosphate
p. a.	**p**ro **a**nalysi (for analysis)
Pb	lead
PBS	**p**hosphate-**b**uffered **s**aline
PCR	**p**olymerase **c**hain **r**eaction
PEP	**p**hospho**e**nol**p**yruvate
pI	**i**soelectric **p**oint
PIA	**p**olysaccharide **i**ntercellular **a**dhesin
PL	**p**hospho**l**ipid
PMA	**p**hospho**m**olybdic **a**cid
PMF	**p**roton **m**otive **f**orce
PMT	**p**hoto**m**ultiplier **t**ube
PP	**p**entose **p**hosphate
PP	**p**enta**p**eptide
ppm	**p**arts **p**er **m**illion
PTS	**p**hospho-**t**ransferase **s**ystem
R_h	**h**ydrodynamic **r**adius
RNA	**r**ibo**n**ucleic **a**cid
RNase	**r**ibo**n**ucle**ase**
rpm	**r**ounds **p**er **m**inute
RT	**r**oom **t**emperature
SCV	**s**mall **c**olony **v**ariant
SD	**s**tandard **d**eviation
SDS	**s**odium **d**odecyl**s**ulfate
SDS-PAGE	**SDS-p**oly**a**crylamide-**g**el **e**lectrophoresis
sec	**sec**ond
SEC	**s**ize **e**xclusion **c**hromatography
Sn	tin
sp.	**sp**ecies (singular)
spec	**spec**tinomycin
spp.	**sp**ecies (plural)
SSC	**s**tandard **s**aline **c**itrate
TCA	**t**ri**c**arboxylic **a**cid
TEM	**t**ransmission **e**lectron **m**icroscope / **m**icroscopy

TEMED	N,N,N´,N´-**te**tra**me**thylethylen**d**iamine
temp.	**temp**erature
TLC	**t**hin **l**ayer **c**hromtography; 1D-TLC (one-dimensional); 2D-TLC (two-dimensional)
Tris	**tris**-(hydroxymethyl)-aminomethane
TSB	**t**ryptic **s**oy **b**roth
U	**u**nit
UDP	**u**ridine 5´-**d**i**p**hosphate
UDP-Glc*NA*c	**UDP-*N*-ac**etyl**gl**u**c**osamine
UDP-Mur*NA*c	**UDP-*N*-ac**etyl**mur**amic acid
UDP-Mur*NA*c-*PP*	**UDP-*N*-ac**etyl**mur**amyl-**p**enta**p**eptide
UV	**u**ltra**v**iolet
V	**v**olt
vol	**vol**ume
vol/vol	**vol**ume/**vol**ume
WT	**w**ild-**t**ype
wt	**w**eigh**t**
wt/vol	**w**eigh**t/vol**ume
WTA	**w**all **t**eichoic **a**cid
Zn	**z**i**n**c
[^{3}H]TPP$^+$	**[^{3}H] t**etra**p**henyl**p**hosphonium bromide
°C	degree(s) **C**elsius
$\Delta\Psi$	membrane potential
η	intrinsic viscosity
λ	wavelength
µg	**microg**ram
µl	**microl**iter
µm	**microm**eter
µM	**microm**olar

The abbreviations of the aminoacids follow the IUPAC-regulations.

1. INTRODUCTION

Polysaccharides are the most abundant of the four major classes of biomolecules, which also include proteins, lipids and nucleic acids. They are often classified on the basis of the sequences and linkages between their main monosaccharide components, as well as the anomeric configuration of linkages, the ring size (furanose or pyranose), the absolute configuration (D- or L-) and any other substituents present. Certain structural characteristics such as chain conformation and intermolecular associations influence the physicochemical properties of polysaccharides. For example, polysaccharides containing large numbers of hydroxyl-groups are often thought of as being hydrophilic. Polysaccharides fill numerous roles in living organisms, such as the storage and transport of energy (e.g. starch and glycogen) and structural components (e.g. cellulose and chitin).

1.1. CHITIN

Chitin is widely distributed in nature, mainly as the structural component of the exoskeletons of crustaceans (crab, shrimp, lobster, krill, squid, crawfish and prawn) and insect cuticles, in marine diatoms and algae, as well as in some fungal cell walls. Structurally, it is an insoluble linear mucopolysaccharide consisting of *N*-acetyl-D-glucosamine (Glc*N*Ac) repeat units, linked by *ß*-(1→4) glycosidic bonds. Technically, the structure of chitin is highly related to that of cellulose and may be regarded as cellulose where the hydroxyl [—OH] at the C-2 position is replaced by an acetamido [—$NHCOCH_3$] group [302].

Resources of chitin for industrial processing are crustacean shells and fungal mycelia; however, its commercial production is usually associated with sea food industries, such as shrimp canning. The processing of crustacean shells mainly involves the removal of proteins (“deproteinization”; in a hot basic solution, usually sodium or potassium hydroxide), and calcium carbonate (“demineralization”; with

diluted acid), both present in crustacean shells in high concentrations, encasing the chitin microfibrils [170].

Chitin has aroused great interest not only as an underutilized resource, but also as a new functional material of high potential in various fields. Several derivatives have been prepared from chitin, but none was as commonly studied, on both the academic and industrial level, as chitosan.

What probably constituted a milestone in the history of these marine polymers was the first international conference on chitin and chitosan, held in Boston, Massachusetts (U.S.A.) in 1977. It was organized by the Massachusetts Institute of Technology (MIT) Sea Grant College Program, working to promote the conservation and sustainable development of marine resources, and to find an alternate route of exploitation of these resources of high potentials in industry; an aspect which has not been fully explored up to that point. The conference focused on several aspects of these two important natural polymers, including their recovery from the various potential sources and their applications.

1.2. CHITOSAN

1.2.1. NATURE AND SOURCES

Chitosan, discovered by Rouget in 1859 [261], is a technologically important polysaccharide biopolymer. Chemically, it is a high molecular weight linear polycationic heteropolysaccharide consisting of two monosaccharides, *N*-acetyl-D-glucosamine and D-glucosamine, linked together by β-(1→4) glycosidic bonds (Figure 1). The relative amount of the two monosaccharides in chitosan may vary, giving samples of different degrees of deacetylation (75-95%), molecular weights (50-2,000 kDa), viscosities, pK_a values, etc [135,290,312]. Therefore, the term chitosan does not refer to a uniquely defined compound; it merely refers to a family of copolymers with various fractions of acetylated units.

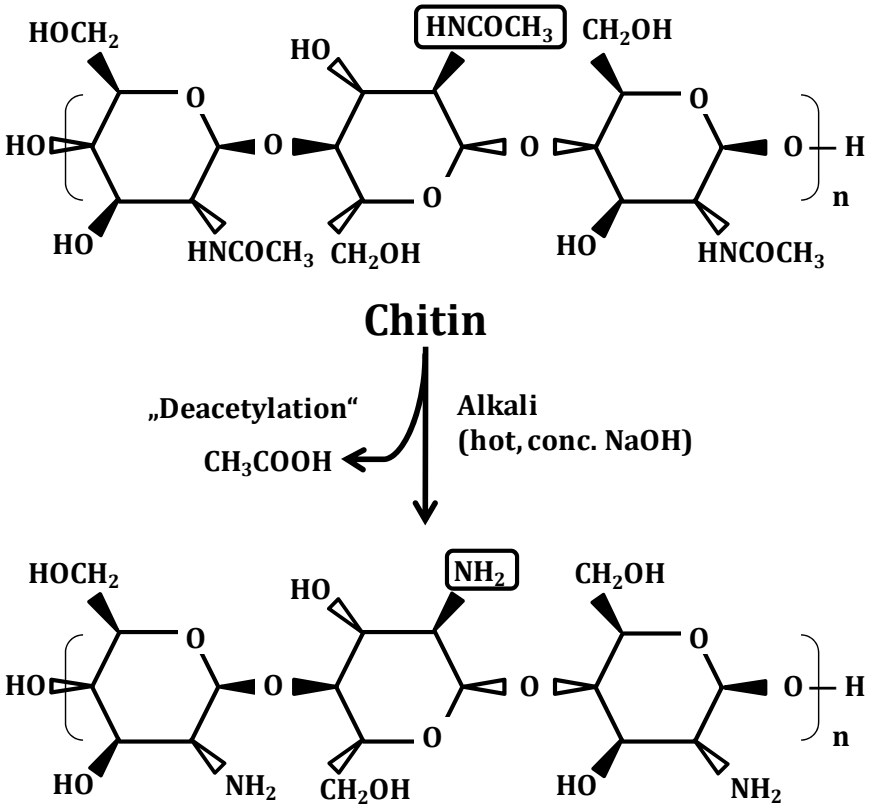

FIGURE 1: Chemical structure of chitosan, and its production from chitin.

Chitosan is a (1→4)-linked 2-amino-2-deoxy-*ß*-D-glucan, prepared from chitin through alkaline hydrolysis of the *N*-acetyl group. Upon further hydrolysis, low molecular weight oligosaccharides are produced.

Chitosan is primarily produced from chitin by exhaustive alkaline deacetylation: this involves boiling chitin in concentrated alkali for several hours (40–45% sodium hydroxide, 120°C, 1–3 h) [170]. Since this *N*-deacetylation is almost never complete, chitosan is considered as a partially *N*-deacetylated derivative of chitin. Consequently, a sharp distinction between chitin and chitosan on the basis of the degree of *N*-deacetylation cannot be drawn [170,249]. Enzymatic procedures for chitin deacetylation by chitin-modifying enzymes were also investigated in the literature.

Chitosan is also found in nature, such as in cell walls of fungi of the class *Zygomycetes* [242], in the green algae *Chlorella* sp., yeast and protozoa as well as in insect cuticles [290]. Recent advances in fermentation technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan [249]. However, chitosan from both sources differs slightly: whereas the acetyl groups in chitosan produced from crustacean chitin are uniformly distributed along the polymer chain, a chitosan of similar degree of deacetylation isolated from fungal cell walls would possess acetyl residues that are grouped into clusters.

In contrast to most of the naturally-occurring polysaccharides, e.g. cellulose, dextran, pectin, alginic acid, agar, agarose and carragenans, which are neutral or acidic in nature, chitosan is an example of a highly basic polysaccharide. Its nitrogen content varies from 5 to 8% depending on the extent of deacetylation; it is mostly in the form of primary aliphatic amino groups.

1.2.2. PHYSICOCHEMICAL ASPECTS

As mentioned above, the term "chitosan" describes a heterogenous group of polymers. Chitosan is commercially available from a number of suppliers in various grades of purity, molecular weights and molecular weight distributions, chain lengths, degrees of deacetylation, charge densities and charge distributions, salt-forms, viscosities and water retention values. These properties greatly affect its physicochemical characteristics, which in turn govern almost all of its applications.

1.2.2.1. MOLECULAR WEIGHT (MW)

Although the underlying chemical and physical effects of some of the applications of chitosan and its derivatives are still not known in detail, considerable evidence has been gathered indicating that most of their physiological activities and functional properties depend on their molecular weight [249].

The molecular weight distribution of a raw chitosan preparation is influenced by variable conditions employed in the deacetylation process, such as time, temperature, concentration and nature of starting material as well as atmospheric conditions [343]. Weight-average molecular weights of several hundreds to over one million Dalton are common, with a mean molecular mass of up to 1 MDa, corresponding to a chain length of approximately 5,000 U [255]. Because of the influence of polymer composition and molecular weight range on the various physicochemical properties of chitosan, it is very important to adequately characterize each batch of polymer produced. The molecular weight of chitosan can be determined by several methods, such as light scattering spectrophotometry, gel permeation chromatography and viscometry [170].

1.2.2.2. DEGREE OF DEACETYLATION (DD)

An important parameter to examine closely is the degree of deacetylation of chitosan, i.e. the ratio of *N*-acetyl-D-glucosamine to D-glucosamine structural units. In chitin, the acetylated units prevail, whereas the degree of deacetylation of chitosan is influenced by the preparation procedure; for example, increasing proportionally with increasing treatment time [42]. It has an impact on the extent of moisture absorption, charge distribution, intrinsic viscosity and chitosan solubility in aqueous solutions [42,67,273,290]. A number of analytical tools have been used to define the degree of deacetylation, such as FTIR spectroscopy, UV spectrophotometry, ^{1}H-NMR and ^{13}C solid-state NMR spectroscopy, various titration methods, equilibrium dye adsorption, elemental analysis, acid degradation followed by HPLC, and thermal analysis [170].

1.2.2.3. SOLUBILITY

The main difference between chitin and chitosan lies in their solubility; deacetylation transforms the insoluble chitin into the acid-soluble chitosan. Chitosan is therefore said to be chitin that has been *N*-deacetylated to such an extent that it becomes soluble in dilute aqueous acids (e.g. 0.1 M acetic acid).

Pure, native chitosan ($pK_a \cong 6.3$) is insoluble in water, in alkaline medium and even in organic solvents. However, water-soluble salts of chitosan may be formed by neutralization with organic acids (e.g. 1-10% aqueous acetic, formic, succinic, lactic, glutamic and malic acids) or inorganic acids such as hydrochloric acid [122,135,290]. The pH-dependent solubility of chitosan is attributed to its amino groups (—NH_2), which become protonated upon dissolution at pH 6 or below to form cationic amine groups (—NH_3^+), increasing intermolecular electric repulsion and resulting in a polycationic soluble polysaccharide, with a large number of charged groups on a weight basis. On the other hand, chitosan tends to lose its charge at higher pH, and may therefore precipitate from solution due to deprotonation of the amine groups [80,255,290].

1.2.2.4. VISCOSITY AND SOLUTION PROPERTIES

One of the most characteristic properties of many polymers, including chitosan, is their ability to form viscous solutions; they could therefore function as thickeners, stabilizers, or suspending agents. Chitosan solutions show pseudoplastic and viscoelastic properties; their viscosity is affected by chitosan's degree of deacetylation, molecular weight and concentration, concentration and types of solvents, the prevailing solution pH and ionic strength, as well as temperature [38,290]. The viscosity range of commercial chitosans (1% [wt/vol] in 1% acetic acid at 25°C) is from 10 to 1000 mPa·s [170].

1.2.2.5. CHEMICAL REACTIVITY AND DERIVATIZATION

Chitosan possesses three types of reactive functional groups: an amino group at the C-2 position of each deacetylated unit, as well as primary and secondary hydroxyl-groups at the C-6 and C-3 positions, respectively, of each repeat unit (Figure 1). These reactive groups are readily subjected to chemical derivatization under mild conditions, to allow for the manipulation of mechanical and physicochemical properties, for example improving chitosan's solubility at neutral pH ranges [80,290].

Furthermore, the presence of free amino groups in chitosan permits its conjugation with some drugs, as well as complexing agents (such as ethylenediamine-tetraacetic acid, EDTA) [16,80].

1.2.2.6. PROCESSABILITY

The superior solubility makes chitosan more easily manageable than chitin. It could be easily processed into a variety of useful forms such as gels, membranes, sponges, films, fibers and beads, by controlling factors such as acid solvent, degree of deacetylation and molecular weight, to address a variety of applications.

1.2.2.7. MISCELLANEOUS PROPERTIES

At pH < 6.5, chitosan is a promising cationic mucoadhesive polysaccharide. Several factors affect the mucoadhesive properties of chitosan, including its concentration, molecular weight, degree of deacetylation and cross-linking, in addition to contact time, environmental pH and ionic strength [82,116,122,177].

Chitosan and its derivatives have strong film- and gel-forming properties, with good oxygen/moisture transmission coefficients and substantivity; they are also endowed with permeation- and absorption-enhancing effects and are able to enhance the dissolution and bioavailability of poorly absorbable drugs [67,135,290,292], thus lending themselves to a variety of applications (section 1.2.4). Moreover, they are capable of strongly binding transition metals such as copper, zinc, iron *in vitro* through a chelation process, probably due to their high percentage of nitrogen (6.89%) [249,290].

1.2.3. BIOLOGICAL PROPERTIES

Much of the commercial interest in chitosan and its derivatives during the last two decades arises from the fact that they combine several favorable biological characteristics, including biodegradability, biocompatibility and non-toxicity; properties which render natural polymers superior over present-day synthetic polymers, making them valuable materials for pharmaceutical, biomedical as well as industrial applications.

1.2.3.1. BIODEGRADATION

Whereas chitosan solutions are highly stable over a long period [51], there is sometimes a need for degrading chitosan to a level suitable for a particular application, or as a means of confering solubility to chitosan at neutral pH. Several methods for producing chitosan oligomers ("chitosanolysis") have been described in literature, including radiation, chemical (acid hydrolysis or oxidative-reductive degradation) and enzymatic methods, of which enzymatic degradation is preferred, since reaction and thus product formation could be controlled by means of pH, temperature and reaction time [255].

Chitosan is susceptible to enzymatic degradation by enzymes from a variety of sources [206], including non-specific enzymes, such as lysozymes (present in tears, saliva, blood and milk) [255,331], chitinases [288], cellulases or hemicellulases, proteases (papain and pronase [169]), lipases, *ß*-1,3-1,4-glucanases, but also chitosanases [257].

Chitosanases (chitosan *N*-acetyl-glucosamino-hydrolase, EC 3.2.1.132) [75] have been generally recognized as enzymes that attack chitosan but not chitin, catalyzing the endohydrolysis of *ß*-(1→4)-glycosidic linkages between D-glucosamine (GlcN-GlcN) residues in partly acetylated chitosan [159,257] (Figure 2).

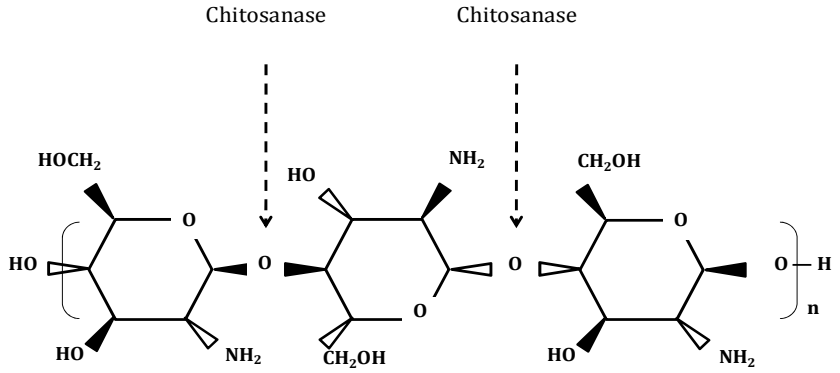

FIGURE 2: Chitosanase specificity.

Chitosan, a polymer of *ß*-(1→4)-D-glucosamine units, is specifically hydrolyzed by chitosanases.

Over the last decade, chitosanase activities with different substrate specificities have been reported in a variety of microorganisms, including bacteria (an estimated 1-7% of heterotrophic soil bacteria) and fungi as well as plants; genes encoding chitosanases have also been identified in some viruses. They have been found to belong to five glycoside hydrolase families: 5, 8, 46, 75 and 80 (Table 1). Interestingly, the majority of the sequenced chitosanases are produced by Gram-positive microorganisms. The crystal structures of *Streptomyces* sp. N174 [188] and *Bacillus circulans* MH-K1 [268] chitosanases are available.

TABLE 1: Examples of identified chitosanases

Sources	Chitosanase family*	Reference(s)
Gram-positive microorganisms		
• *Amycolatopsis* spp.	46	221
• *Bacillus* spp.	46	3,5,231,257,268,348,357,358
	8	44,139,197
	N/A	49,93,223,234,278,316
• *Nocardia* spp.	N/A	211,269
• *Nocardioides* spp.	46	190
• *Paenibacillus spp*	8	159
• *Streptomyces* spp.	46	23,92,188,191
	5	309
	N/A	220,246
Gram-negative microorganisms		
• *Acinetobacter* spp.	N/A	285
• *Burkholderia* spp.	46	282
• *Enterobacter* spp.	N/A	352
• *Matsuebacter* spp.	80	228
• *Myxobacter* spp.	N/A	118,233
• *Pseudomonas* spp.	N/A	359
• *Sphingobacterium* spp.	80	192
Fungi		
• *Aspergillus* spp.	75	39
• *Fusarium* spp.	75	283,284
• *Penicillium* spp.	N/A	84
Viruses		
• *Chlorella* virus	46	185,299,350

* N/A: not available

Fukamizo *et al.* [93] proposed the classification of chitosanases into three distinct classes according to their substrate specificities: i) class I chitosanases split the Glc*N*Ac-GlcN linkage in chitosan, e.g. *Bacillus pumilus* BN262 [93,193], *Penicillium islandicum* [84] and *Streptomyces* sp. strain N174 [92]; ii) class II chitosanases, where cleavage specificity is exclusively restricted to the GlcN-GlcN linkage, e.g. *Bacillus* sp. No.7M [139]; and iii) class III chitosanases, which can split both GlcN-GlcN and GlcN-Glc*N*Ac linkages, such as *Streptomyces griseus* HUT 6037 [309], *Bacillus circulans* MH-K1 [268], *Nocardia orientalis* [269] and *Bacillus circulans* WL-12 [197].

1.2.3.2. BIOCOMPATIBILITY

One of the most important biological properties of any implantable biomaterial is biocompatibility; i.e. it should not be affected by the host and at the same time should not elicit any undesirable local or systemic effects [227]. Chitosan is well tolerated by living tissues, including the skin, ocular membranes, as well as the nasal epithelium, and has thus been proven valuable for a wide range of biomedical applications [28,67,82,157,281].

1.2.3.3. SAFETY

The low toxicity profile of chitosan compared with other natural polysaccharides is another of its many attractive features. It has been reported that the purity of chitosan influences its toxicological profile, yet its safety in terms of inertness and low or no toxicity has been demonstrated by *in vivo* toxicity studies. Its oral LD_{50} (median lethal dose) in mice was found to be in excess of 16 g/day/kg body weight, which is higher than that of sucrose [67,290]. Nonetheless, it is contraindicated for people with shellfish allergy.

In their review article, Ylitalo *et al.* [356] reported the absence of significant side effects following chitosan ingestion in human studies (for up to 12 weeks), other than mild constipation or diarrhea in a small percentage of the participants. However, Tanaka and coworkers [310] cautioned that special care should be taken in the clinical use of chitosan over a long period of time. When chitosan was administered either

orally or parenterally to mice, their body weights decreased significantly in both cases, together with disturbances in intestinal microbial flora and several histological abnormalities. Concerns have also been raised that chitosan could cause the loss of fat-soluble vitamins, decrease mineral absorption and bone mineral content and block absorption of certain medicines [60]. No epidemiological studies or case reports investigating the association of exposure to chitosan and cancer risk in humans, no carcinogenicity studies on chitosan in animals and no *in vitro* or *in vivo* studies evaluating chitosan for mutagenic effects were identified in the available literature.

1.2.4. APPLICATIONS

Although extensive resources were involved in both research and development of processes and applications for chitosan, only the last two decades have witnessed serious developments of a variety of technologies based on the commercial utilization of chitosan and its derivatives. Chitosan, its oligomers and a number of its derivatives emerged as new biomaterials and are currently in use or under consideration in a number of applications (pharmaceutical, cosmetic, medical, food, textile, agricultural, etc.), summarized in Table 2. Due to the wide scope of applications, only a number of them will be further discussed in this section.

Introduced to the market in the 1990's, chitosan has been the subject of much research regarding its potential as a useful and promising pharmaceutical excipient in various pharmaceutical formulations [67,135,290]. Next to the more traditional formulations, chitosan has found use in novel applications such as vaccine delivery, peptide and gene delivery, in addition to its use in tissue engineering. So far, the nasal chitosan vaccine delivery system against influenza has been tested for vaccination in human subjects, and has been proven to be both effective and protective [136]. Chitosan's utility as a pharmaceutical ingredient gained more interest when a scientific understanding of at least some of the pharmacological activities of this versatile carbohydrate began to evolve [28].

TABLE 2: Applications of chitosan

Applications	Benefits / advantages	References
APPLICATIONS – PHARMACEUTICALS AND COSMETICS		
Conventional formulations		
• Tablet manufacture	binder; disintegrant; coating; lubricant; diluent	1,208,216
• Gels	sustained drug release; enhanced absorption	162,163
• Films and membranes	controlled drug release	152,353
• Emulsions	stabilizer	124
Microspheres, microcapsules	mucoadhesive; sustained delivery of drugs; penetration enhancement; increased bioavailability	62,110,226,280,362
Ophthalmic formulations	ocular tolerance; mucoadhesive; wetting and penetration-enhancing properties; antibacterial; prolonged precorneal drug residence	31,80-83,97
Transdermal delivery systems	enhancement of penetration across epithelia; controlled drug release	117,287,313,326
Colon-specific drug delivery	biodegradable by colonic bacteria	86,140,351
Targeted cancer therapy	antitumor; long systemic retention and tumor accumulation, due to enhanced permeability and retention (EPR) effect	56,70,77,105,153,293,304
Vaccine delivery		
• Mucosal vaccination	induction of mucosal and systemic immune response; penetration into intestinal and respiratory mucosae	136,327,328
• Oral vaccination	protection of antigens from gastric juice, bile acids and salts and from proteolytic enzymes of the gastrointestinal tract	329
Peptide drug delivery	improving oral bioavailability of peptides and proteins	15,33,109,168,308
Gene and nucleic acid delivery	safe, non-viral system	20,24,55,85,181
Deodorant formulations	dermatological compatibility; non-irritating; enhancing fragrance adhesion; deodorizing	129
Hair and skin care products	preservative; emulgator; thickener; moisturizer; soothing effect on skin	27,28,127,199,241

Applications	Benefits / advantages	References
APPLICATIONS – MEDICAL AND BIOMEDICAL		
Antacid and anti-ulcerogenic	demulcent and protective effect on stomach mucosa	4,138,290
Antidiabetic (hypoglycemic)	lowering of blood glucose level; increasing glucose tolerance and insulin secretion	115,175,290
Antihypertensive	—	115,175
Antioxidant	scavenging of radicals and chelation of divalent metals	36,345
Antitumor	induction of apoptosis in tumor cells	113,248,300
Anticoagulant	—	133,170,230,334
Hemostatic	biological adhesive for soft tissues	187,290
Spermicidal	strong binding to mammalian cells	281
Hypocholesterolaemic; nutritional aid for weight loss	prevention of fat absorption; reduction of blood lipid levels	132,150,151,176,290,355, 356
Wound dressings; products for wound treatment	Inhibition of fibroplasia; promotion of tissue regeneration; acceleration of wound-healing with minimal scar formation	157,183,195,196,324
Contact and bandage lenses	optical clarity; wound-healing; mechanical stability; sufficient optical correction; immunological compatibility; gas permeability; wettability; antimicrobial	80,135,170,290
Dentistry and oral medicine	bioadhesive; viscosity-enhancer; permeabilizer; antimicrobial; anti-adhesive; prolonged drug release in buccal cavity; anti-dental caries; treatment of periodontal diseases, oral candidiasis and tooth mobility; reduction of plaque formation	10,11,43,58,59,102,134,155, 270,279,311
Anti-inflammatory	—	281
Immunopotentiator	stimulation of immune system; augmenting immunogenicity of co-administered antigens; promoting resistance to systemic infections	9,222
Surgical sutures and implants	biodegradable	27,290,302
Hemodialysis membranes	—	281
Coating for prosthetics and biomedical devices	thromboresistance; compatibility with blood; anti-biofilm properties	32,281

Applications	Benefits / advantages	References
APPLICATIONS – TISSUE ENGINEERING		
Scaffold for tissue engineering applications	promoting tissue growth and differentiation	55,154,281,349
Artificial skin grafts (substratum for skin replacement)	nonantigenic; performs as a biodegradable template for synthesis of neodermal tissue	170,281
APPLICATIONS – AGRICULTURE		
Soil and plant revitalizer	preventing microbial infection and promoting growth of plants	126,202
Preservative coating and biofungicide	sprayed on seeds to extend their storage life	126
APPLICATIONS – FOOD INDUSTRIES		
Food processing	enhances safety, quality and shelf-life of food; clarification of liquids; preservative; thickener	2,61,214,219,255,258,259, 319
Coatings for vegetables, fruits and fish	improving shelf life; preventing moisture loss; delaying fungal growth	30,73,74,143
Edible antimicrobial films for food packaging	—	71
APPLICATIONS –TEXTILE INDUSTRIES		
Finishing of textiles	antimicrobial; moisture control and dye absorptive properties	72,96,207,286,307
Novel fibers for textiles	—	170
APPLICATIONS – WASTEWATER TREATMENT		
Food and beverage processing plants	coagulation and flocculating agent; resulting dried sludge used in animal feeds	40,41,95
Industrial waste	removal of heavy metal ions through adsorption and chelation	8,141,232,250
Textile effluents	sorption of dyes, due to high affinity to many classes of dyes	50,263,303
APPLICATIONS – MISCELLANEOUS		
Photographic paper	resistance to abrasion; favorable optical characteristics; film-forming ability	170
Paper finishing	imparts wet strength to paper	170

In spite of the promising use of chitosan in the pharmaceutical industry, however most of the chitosan researches are directed toward medical applications. Unfortunately, a survey of the available literature revealed that there are only relatively few specific and objective research studies to support claims, ascribing a range of rather impressive pharmacological properties to this biopolymer. Most of these studies are very difficult to take seriously, with little scientific evidence to back them up. For example, chitosan is often being heralded, and sold, as a "revolutionary" weight loss supplement, a "fat magnet", although this presumptive property is often discredited in recent studies [94]. Given the large number of proclaimed medicinal benefits of chitosan, it comes as no surprise that the literature is filled with conflicting reports about these medical potentials.

Some studies showed that chitosan, as an immune adjuvant, could effectively promote local immune response and enhance antigen presentation [22,327,346]. Porporatto *et al.* [244] propose the following mechanisms for the modulation of mucosal immune response: i) as a dietary fiber, chitosan might have an impact on the intestinal flora and mucosal microenvironment, thus influencing local immune function; ii) as a delivery agent, it might decrease the clearance rate and stimulate the uptake of antigens; and iii) as an adjuvant, it might provide "danger signals", being a component of fungal cell walls, possibly through the activation of components of the innate immune system such as macrophages. They therefore conclude that chitosan could be used to modulate the immune response to orally-administered antigens.

Probably one of the most prominent commercial applications of chitosan is its use as a hemostatic. Several chitosan-based wound dressings are available on the market for clinical use, including HemCon® Bandage and ChitoFlex wound dressings (HemCon Medical Technologies Inc., West Yorkshire, UK), as well as CELOX™ (Medtrade Products Ltd., Crewe, England); both claimed to be FDA approved.

Chitosan is implicated as a component of host-fungal interactions. It acts as a potent elicitor of plant defense responses, activating the expression of plant defensive genes and inducing the production of pathogen-related proteins, such as chitinases and other hydrolytic enzymes [66,189,243]. These enzymes can hydrolyze chitin and chitosan in fungal cell walls, consequently leading to growth inhibition and/or death. The induction of chitosanases and chitinases through genetic engineering has also been proposed [51]. In addition, chitosan oligomers exhibit fungicidal properties, which makes chitosan very promising as a biocontrol measure against plant pathogens [13]. In fact, chitosan-based plant growth stimulators found their way into the market (e.g. ChitoPlant® and SilioPlant®; ChiPro GmbH, Germany). They presumably stimulate the plant immune response against pathogens and have a growth-promoting activity.

1.2.5. Economic aspects and regulatory status

Since a large amount of the crustacean exoskeleton is readily available as a byproduct of the seafood processing industry, the raw material for chitosan production is relatively inexpensive, and thereby the production of chitosan on a large scale from this renewable bio-resource is economically feasible [249]. Chitosan is commercially produced in different parts of the world (Japan, North America, Poland, Italy, Russia, Norway and India) on a large scale [290]. It has been estimated that up to 10^9– 10^{10} tons of chitosan are annually produced in nature [240]. Another important aspect to be considered is that utilizing the shellfish waste for chitin production provides a solution for the waste disposal problem, and provides an alternative for the use of this oceanic resource.

Generally Recognized As Safe (GRAS) is a designation of the FDA (Food and Drug Administration) in the United States of America, that a chemical or substance added to foods and beverages is considered safe by experts. Chitosan has not been officially proclaimed GRAS by the FDA but one Norwegian company (Primex

Ingredients ASA), which manufactures shrimp-derived chitosan, has announced in 2001 that its purified chitosan product (ChitoClear®) has achieved a GRAS self-affirmed status in the U.S. market. On the other hand, the FDA has approved chitosan for medical uses such as bandages and drug encapsulation. Chitosan is also widely used in foods in Italy, Finland, Korea and Japan.

1.2.6. ANTIMICROBIAL ACTIVITY

The modern era of chitosan research was heralded by publications in the mid-1990s describing the antimicrobial potentials of chitosan and its derivatives, which exhibit a wide range of activities towards human pathogens as well as food-borne organisms [205,215,249,272,301,320,335]. In fact, a number of commercial applications of chitosan benefit from its antimicrobial activity, including its use in food preservation [255], in dentistry [134] and ophthalmology [81], in the manufacture of wound-dressings [324] and antimicrobial-finished textiles [307]. Therefore, investigations of the antimicrobial potential of chitosan and its derivatives have recently gained momentum.

The spectrum of antimicrobial activity of chitosan and its derivatives extends to include filamentous fungi, yeasts and bacteria, being more active against Gram-positive than Gram-negative bacteria [145,205,215,255]. More interestingly, chitosan seems to hold some promise in dentistry, since it was shown to exhibit a potent plaque-reducing action as well as *in vitro* antibacterial activity against several oral pathogens implicated in plaque formation and periodontitis, including *Actinobacillus actinomycetemcomitans, Streptococcus mutans* and *Porphyromonas gingivalis* [43,134].

After investigating the antimicrobial activity of different chitosans in lipid emulsions as well as in aqueous solutions, Jumaa *et al.* [149] suggested that chitosan could possibly be used as an antimicrobial preservative in emulsion formulations for mucosal as well as for parenteral applications. Similarly, Sagoo *et al.* [265] proposed the use of chitosan as an adjunct in the potentiation of the biocidal efficacy of antimicrobial compounds such as benzoates.

There are numerous reports on the antimicrobial activity of different chitosans and chitosan derivatives, from various sources and tested under diverse conditions. In many instances discrepancies in the results obtained were observed, which was not surprising, since chitosan's antimicrobial activity is influenced by various intrinsic and extrinsic factors. Among the intrinsic factors are the type of chitosan, its molecular weight, degree of deacetylation, viscosity, solvent and concentration; while the extrinsic factors are related to environmental conditions, such as pH, temperature, ionic strength, metal ions, EDTA, presence of organic matter, the particular bacterium and the age of a bacterial culture [51,146,149,215,281,318].

For instance, the antimicrobial activity of chitosan was found to be greatly influenced by its molecular weight, the activity decreasing with decreasing chain length; the dimer and trimer of *N*-acetylglucosamine were found to be inactive [215,255,320]. Jeon *et al.* [145] even went on to suggest that a molecular weight of more than 10 kDa is required for proper inhibition of microorganisms. Moreover, chitosans with a high degree of deacetylation were more effective than those with a low degree in inhibiting bacterial growth, probably due to the higher percentage of protonated amine groups [182,281].

The antimicrobial activity of chitosan was inversely affected by pH, with higher activity observed at lower pH value; on the other hand, it increased with increasing temperature [149,215,306,318]. However, the presence of sodium ions reduced chitosan's activity; a similar but more dramatic effect was obtained through the addition of metal ions, probably due to complex formation between chitosan and these metal ions [306,318].

1.2.7. ANTIMICROBIAL MODE OF ACTION

The antimicrobial efficacy of chitosan formulations used for a wide range of applications is generally well documented; however, the precise mechanism of its antimicrobial activity has hitherto remained only vaguely defined. There has been

much speculation as to the mechanisms underlying the antimicrobial activity of chitosan, stemming from an increased awareness of the potentials and industrial value of this biopolymer. Several theories were identified in the available literature, although it seems that this research has not received much attention, judged by the limited number of published studies.

Electron microscopical examinations of various chitosan-treated microorganisms suggest that its site of action is at the microbial cell surface [51,205,272]. It was reported that *Candida albicans* strains exposed to chitosan or its derivatives showed cell damage to various extents, due to disturbances of membrane functions; changes in intracellular structures have also been observed. On the other hand, the outer part of the staphylococcal cell wall experienced fraying and weakening; duplication was also depressed. In Gram-negative microorganisms, an abnormally expanded periplasmic space was observed, while intracellular materials appeared to be more tightly packed and lacking any organization [205].

The mode of action of cationic antibacterial agents is widely believed to be the interaction with and disruption of the cell envelope. It is generally assumed that the polycationic nature of chitosan, conveyed by the positively-charged —NH_3^+ groups of glucosamine, might be a fundamental factor contributing to its interaction with negatively-charged surface components of many fungi and bacteria, causing extensive cell surface alterations, leakage of intracellular substances, ultimately resulting in impairment of vital bacterial activities [78,121,142,169,205,272,298,311,317,318,363].

Several research groups [78,180,360] hypothesize that this electrostatic interaction takes place between chitosan and negatively-charged cell membrane components (i.e. phospholipids or proteins), affecting membrane integrity and permeability, which causes leakage of intracellular substances, and finally the death of the cell. On the other hand, Kumar *et al.* [169] believe this interaction takes place with amino acids in the Gram-positive bacterial cell wall, resulting in cell-wall distortion, exposure of cell membrane to osmotic shock and exudation of cytoplasmic contents.

As early as 1982, Young *et al.* [361] demonstrated in their study that chitosan increases the membrane permeability of plant cells, presumably by binding to polygalacturonate, a component of plant cell walls, inducing the leakage of electrolytes, UV-absorbing materials, and proteins into the medium. The basic polymers poly-L-lysine, histone, DEAE-dextran, protamine sulfate and glycol chitosan also induced leakage.

Helander *et al.* [121] studied the effects of chitosan treatment on the cell membranes of Gram-negative bacteria and found evidence for extensive cell surface alterations, marked by thickening and formation of vesicular structures on the outer membranes of both *Escherichia coli* and *Salmonella typhimurium*. They reasoned that chitosan binds to the outer membrane of Gram-negative bacteria, thereby affecting its barrier properties, probably through complex formation with various lipopolysaccharides [57]. Highly cationic mutants of *S. typhimurium* were also found to be more resistant to chitosan than the parent strains. Morimoto *et al.* [200] reported the specific binding of a chitosan derivative with a receptor on the cell surface of *Pseudomonas aeruginosa*.

More interestingly, Chung *et al.* [46] proposed that the inactivation of *E. coli* by chitosan occurs via a two-step sequential mechanism: an initial separation of the cell wall from the cell membrane, followed by destruction of the cell membrane. They came to this conclusion based on similarities between the antibacterial pattern of chitosan and those of polymyxin and EDTA.

The concept that the antimicrobial activity of chitosan is related to its cationic nature, and that free amino groups play a pivotal role in its antimicrobial activity, is backed by several research findings, including:

i) Chitosan loses its antimicrobial activity at pH 7.0, assumably due to the deprotonation of amine groups and poor solubility in water at this pH.

ii) Young *et al.* [361] observed that chitosan-induced leakage of UV-absorbing materials from *Glycine max* cells was strongly inhibited by divalent cations. The authors proposed that the cations from complexes with chitosan and, consequently, reduce the number of its available amine groups.

iii) The antimicrobial activity of chitosan was found to be directly proportional to its degree of deacetylation, which in turn is related to the number of its protonated amine groups [182,229].

iv) The *N*-acetylation of chitosan oligomers effectively destroyed their fungistatic activity, since the 2-amino groups could no longer become protonated [317].

v) Investigations of interactions of chitosan with SDS (an anionic surfactant) revealed that SDS bound strongly to chitosan via a highly exothermic electrostatic interaction, leading to the formation of an insoluble complex [314].

On the other hand, several reports postulated that other mechanisms of action, including water-binding, enzyme inactivation, selective chelation of trace metals needed by bacterial enzymes and interaction of the positively-charged chitosan with the DNA of fungi and bacteria (consequently inhibiting RNA and protein synthesis), might shed some light on chitosan's antimicrobial mode of action [182,249,311]. However, several of these studies purportedly demonstrating the existence of intracellular chitosan targets were based on indirect approaches.

Based on their findings while studying the antibacterial effects of chitooligosaccharides on *E.coli*, Liu *et al.* [182] proposed that these oligomers were taken up by the bacterial cells, causing the inhibition of DNA transcription. However, this contention has not been substantiated in the more recent literature.

Young *et al.* [360,361] suggested that chitosan caused the release of Ca^{2+} from complexes stabilizing the cell membrane of *Glycine max* cells, resulting in destabilization of the membrane and inducing the leakage of cellular components.

Sudarshan *et al.* [298] also reported evidence that leakage of intracellular components and cell permeabilization was one of the mechanisms of chitosan action at low concentrations. They attribute the antibacterial activity at higher concentrations to chitosan coating the surface of the bacteria, thereby impeding mass transfer across the cell membrane in both directions. A similar conclusion was reached by Tokura *et al.* [315], who observed that chitosan (M_W = 9300; DD = 0.51) was stacked on the cell wall and inhibited the growth of *E. coli*. They suggested that the antimicrobial action is related to the suppression of the metabolic activity of the bacteria by blocking nutrient delivery through the cell wall.

On the other hand, Tsai and Su [318] suggested that the death of *E.coli* resulted from the interaction between chitosan and the *E. coli* cell, resulting in changes in membrane permeability, and causing the leakage of intracellular components, such as glucose and lactate dehydrogenase.

Finally, Kumar *et al.* [169] propose different action patterns for chitosan based on the target organism. Aggregation and deposition of cationic chitooligomers onto the cell surface of *E. coli* due to ionic binding with the negatively-charged lipopolysaccharides in the outer membrane was deemed responsible for the observed growth inhibition and lysis, through blockage of nutrient flow. As for Gram-positive bacteria, similar binding to cell surface structures resulted in pore formation and thus permeabilization of the cell wall of *B. cereus* and spillage of cytoplasmic contents.

Clearly, these mechanisms of action are not mutually exclusive, since microbial inhibition by chitosan is thought to be a result of a number of events, that may ultimately lead to a killing process.

2. AIM OF THE WORK

Although not primarily used as an antimicrobial agent, the use of chitosan as a preservative or in products at sublethal concentrations, in which a bacteriostatic activity is required, is driving the need for a better understanding of its microbial target sites. However, published studies still leave a number of unresolved questions. Against this background, the main aim of this project was to advance our understanding of chitosan's antimicrobial activity.

This was achieved by carrying out an initial investigation of its *in vitro* antimicrobial activity, together with factors that might affect it. In this context, we tried to address some of the relevant issues of chitosan's antimicrobial activity.

Then, we conducted a systematic study of chitosan's mechanism of action, using an array of techniques, aiming at elucidating underlying molecular mechanisms involved in inhibition / killing of bacteria. Particular attention was paid to studying the antimicrobial activity of chitosan on live Gram-positive bacteria, while occasionally attempting to draw parallels – if any - to artificial model systems.

Finally, we attempted to gather information about the possible resistance mechanism(s) against this antimicrobial substance, believing that it might give some useful indications about its overall mode of action.

The possibility of a better understanding of chitosan's mode of action would help us arrive at an appreciation of the antimicrobial potential of this compound, and would open a new avenue for the introduction of new and useful applications based on this biopolymer, for example by developing antimicrobial systems which can be implemented into industrial applications such as the preservation of pharmaceutical preparations.

3. MATERIALS AND METHODS

3.1. MATERIALS

A complete list of the manufacturers and suppliers of the various materials can be found in section 6.1 (appendix).

3.1.1. CHITOSAN

Several chitosan grades were used throughout this study (Table 3). Stock chitosan solutions (1% [wt/vol]) were prepared either in 0.05 M HCl or 1% aqueous acetic acid, sterilized by autoclaving at 121°C for 20 min and stored at 4°C for subsequent use.

Table 3: Grades of chitosan

	LMW	MMW	HMW	PGC	COS	CH 200	CH 300
Product	Chitosan, low MW	Chitosan, medium MW	Chitosan, high MW	Chitosan, practical grade	Oligo-saccharide lactate§	Chitosan 85/200/A1	Chitosan 85/300/A1
Description	fine powder	small flakes	large coarse flakes	large coarse flakes	fine powder	small flakes	very fine powder
Humidity [%]	-	-	-	-	-	10.0	9.19
Particle size	-	-	-	-	-	-	< 100 µm
Ash content [%]	-	-	-	-	-	0.60	0.65
Viscosity [cps]†	20 – 200	200 - 800	800 - 2000	> 200	~ 6	170	280
DD [%]	75 - 85	75 - 85	> 75	≥ 85	> 90	85.9	86.0
MW [kDa]	50 – 190	190 – 310	>310 –375	190 – 375	Av. M_n < 5	-	-
Heavy metals [ppm]							
Pb	-	-	-	-	-	< 5	-
Cd	-	-	-	-	-	< 0.05	< 0.09
Hg	-	-	-	-	-	< 0.03	< 0.01
Supplier	Sigma-Aldrich Chemie GmbH					Heppe GmbH	

§ Prepared by enzymatic hydrolysis of chitosan, oligosaccharide content *ca.* 60%, < 15 glucosamine units/chain

† Brookfield method, 1% solution in 1% acetic acid, 20°C

Table 3: Grades of chitosan (cont.)

	CH 1011	CH N6	Vink FP	TiFi-1105	Crab 1105	CC
Product	Chitosan flake 1011	Chitosan N6‡	Vinkocos p-6N FP	TiFi-chitosan from 1105	Crab chitosan from 1105	ChitoClear® fg 95
Description	very small flakes	fine powder	very fine powder	coarse powder	large flakes	powder
Humidity [%]	< 10	7.75	7.93	-	-	-
Density [g/ml]	-	0.6	-	-	-	0.4
Particle size	-	≤ 200 µm	100 Mesh	-	-	100 Mesh
Ash content [%]	< 0.5	2.16	0.54	-	-	< 1.0
Viscosity [cps]†	400 – 500	110	100	-	-	< 500
DD [%]	> 95	86.9	96.71	90.3*	78.1*	> 95
MW [kDa]	-	30 - 1000	-	949.34	-	-
Heavy metals [ppm]	-	≤ 20	< 10	-	-	-
Pb	-	< 0,2	-	-	-	-
Cd	-	< 0.09	-	-	-	-
Hg	-	< 0.01	0.042	-	-	-
Cu	-	< 1	-	-	-	-
Sn	-	< 1	-	-	-	-
Zn	-	1	-	-	-	-
As	-	-	< 0.5	-	-	-
Total count [CFU/g]	-	< 1000	123	-	-	< 1000
Supplier	ChiPro GmbH	Kraeber GmbH & Co	Vink+CO GmbH	Institut für Umwelttechnik, FH Oldenburg/Ostfriesland/ Wilhelmshaven, Emden, Germany		Primex ehf

† Brookfield method, 1% solution in 1% acetic acid, 20°C

* calculated from the respective F_A value = fraction of acetylated groups = (100-DD)/100

‡ used as food supplement

3.1.2. ANTIMICROBIAL PEPTIDES

The following antimicrobial peptides (AMPs) were included in this study (Table 4). They were reconstituted according to the supplier's recommendations, and their stock solutions were stored at -20°C.

Table 4: Antimicrobial peptides

AMPs	Stock solution	Solvent	Supplier / Source
Lantibiotics			
Nisin	5 mg/ml	0.05% [vol/vol] HAc	Dr. I. Wiedemann
Pep5	2 mg/ml	Aqua dest	M. Josten (AG Sahl)
Gallidermin	1 mg/ml	0.05% [vol/vol] HAc	Dr. Petry Genmedics GmbH
Lipopeptides			
Daptomycin	1 mg/ml	Aqua dest	Novartis Pharma GmbH
Ca^{2+}-Friulimicin	1 mg/ml	Aqua dest	H. Labischinski, Combinature

All daptomycin and friulimicin assays were carried out in presence of 1.25 mM Ca^{2+} as recommended by the manufacturer [148].

3.1.3. ANTIBIOTICS

Antibiotics used in this work for susceptibility testing and for the preparation of selective media are listed in Table 5, together with their sources. Stock antibiotic solutions were sterilized by membrane-filtration.

Table 5: Antibiotics for susceptibility tests

Antibiotics	Stock solution	Solvent	Supplier / Source
Amikacin	10 mg/ml	Aqua dest	Bristol-Myers Squibb GmbH & Co. KGaA
Ampicillin-Na	25 mg/ml	Aqua dest	Sigma-Aldrich Chemie GmbH
Bacitracin-Zn^{2+}	2 mg/ml	10 mM HCl	Fluka
Cefotaxime	10 mg/ml	Aqua dest	Aventis Pharma Deutschland GmbH
Chloramphenicol	100 mg/ml	100% ethanol	Sigma-Aldrich Chemie GmbH
Erythromycin	10 mg/ml	100% ethanol	Sigma-Aldrich Chemie GmbH
Gentamicin sulfate	10 mg/ml	Aqua dest	Merck KGaA
Kanamycin	40 mg/ml	Aqua dest	Sigma-Aldrich Chemie GmbH
Oxacillin-Na	10 mg/ml	Aqua dest	Bayer AG
Spectinomycin $HCl \cdot 5H_2O$	50 mg/ml	Aqua dest	Upjohn GmbH
Tetracycline	25 mg/ml	100% methanol	Sigma-Aldrich Chemie GmbH
Vancomycin·HCl	10 mg/ml	Aqua dest	Ratiopharm GmbH

Bacterial susceptibility to the various antimicrobials was tested in part with the help of microtiter plates of the company MERLIN Diagnostika GmbH, which contain the antibiotics listed in Table 6 in dehydrated form.

Table 6: Antibiotics (MERLIN plates)

Class	Examples
ß-lactams	penicillin G, penicillin / sulbactam, oxacillin, ampicillin, imipenem, ertapenem, cefoxitin, ceftriaxon
Quinolones	levofloxacin, moxifloxacin, ciprofloxacin
Aminoglycosides	gentamicin, amikacin, tobramycin, streptomycin
Glycopeptides	vancomycin, teichoplanin
Miscellaneous	co-trimoxazole (trimethoprim/sulfamethoxazole), clindamycin, linezolid, erythromycin, mupirocin, synercid, doxycyclin, fosfomycin, fusidic acid, rifampicin

3.1.4. LIPIDS

Table 7 lists the phospholipids used in this study. They were purchased from Avanti Polar Lipids, Inc., and stored in chloroform in phosphate-free tubes at -20°C.

Table 7: Phospholipids

Phospholipids	Stock solution
DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; 18:1 phosphatidylcholine	25 mg/ml
DOPG: 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] sodium salt; 18:1 phosphatidylglycerol	50 mg/ml
Cardiolipin; 14:0	10 mg/ml

3.1.5. SOLVENTS

The solvents employed in this study, together with their suppliers, are denoted in Table 8.

TABLE 8: Solvents

Solvents	Supplier
Glacial acetic acid; ethanol p.A.; sodium hydroxide solution (1N)	Merck KGaA, Darmstadt, Germany
Chloroform; methanol; water (HPLC grades)	Mallinckrodt Baker B. V.
Hydrochloric acid (1 N); sulfuric acid (95-97%)	KMF Laborchemie Handels GmbH
Ammonium hydroxide	Sigma-Aldrich Chemie GmbH

3.1.6. ENZYMES

The enzymes listed in Table 9 were stored at -20°C and thawn shortly before use.

TABLE 9: Enzymes

Enzyme	Stock solution	Source
Lysostaphin [SsL-90-15], *S. simulans* biovar *staphylolyticus*, (1180 U/mg)	5 mg/ml in DMPC-treated water*; 1 mg/ml in Aqua dest	Dr. Petry Genmedics GmbH
Desoxyribonuclease, DNase I; RNase-free (10 units/µl)	—	Molzym GmbH & Co.KG
BioScript RNase H Low reverse transcriptase (200 U/µl)	—	Bioline GmbH

* DMPC is typically used to treat water to remove RNases; it reacts with amine, hydroxyl and thiol groups and thus inactivates RNases. Treatment involves adding DMPC to 0.1% [vol/vol] and incubating at 37°C for 1 h to overnight followed by autoclaving, to destroy DMPC.

3.1.7. REAGENTS AND CHEMICALS

Reagents and chemicals utilized in this work, together with their respective suppliers are listed in Table 10; they were of analytical grade or better. All other standard chemicals and reagents not listed in this table were purchased from Merck KGaA (Darmstadt, Germany).

Table 10: Reagents and chemicals

- **Amersham Biosciences:**
  radiolabeled [^{3}H] tetraphenylphosphonium bromide ([^{3}H]TPP$^+$, 30.0 Ci/mmol)
- **Amresco:**
  TEMED
- **Bioline GmbH:**
  5 × BioScript Reaction Buffer
- **bioMérieux:**
  McFarland standards
- **Carl Roth GmbH & Co:**
  SDS (sodium dodecyl sulfate); 2-mercaptoethanol
- **Fermentas GmbH:**
  PageRuler™ protein ladder [10-200 kDa]
- **Fluka:**
  bovine serum albumin (BSA); ethylene glycol
- **GE Healthcare Bio-Sciences AB:**
  Sephadex™ G-50 (medium)
- **GE Healthcare UK Limited:**
  Cy™3-dCTP [25 nmol]; Cy™5-dCTP [25 nmol]; dNTP set [100 mM solutions]; pd$(N)_6$ random hexamer
- **ICN Biomedicals Inc:**
  glycerol
- **Invitrogen:**
  RNase-Out™ recombinant ribonuclease inhibitor [25 U/ml]
- **KMF Laborchemie Handels GmbH:**
  cyclohexane
- **Merck KGaA:**
  BACTIDENT® coagulase; 1-octanol; xylene; agar-agar; sodium chloride; L-trytophane; L-leucine; L-methionine; L(+)-valine; L-serine; L(+)-glutamic acid·HCl; calcium D(+)-pantothenate; sodium hydroxide; magnesium sulfate; di-potassium hydrogen phosphate; potassium dihydrogen phosphate; sodium acetate; calcium chloride·2HCl; sodium hydrogen carbonate; D(+)-glucose·H_2O; ammonium chloride; ammonium nitrate; peptone from casein; yeast extract ; zinc sulfate; ferrous sulfate·$7H_2O$; potassium hydroxide; potassium sodium tartarate; silver nitrate; potassium chloride; Tris-(hydroxymethyl)-aminomethane "Tris"; Folin-Ciocalteu's phenol reagent; perchloric acid [70%]; bromophenol blue; coomassie brilliant blue R-250; molybdatophosphoric acid hydrate; di-sodiumhydrogenphosphate; ammonium acetate
- **MERLIN Diagnostika GmbH:**
  MICRONAUT-RPO; MICRONAUT-S plates for susceptibility testing [ES-196-100, ES-195-100 and ES-166-001]
- **Molecular Probes – Invitrogen GmbH:**
  Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$]

- **Molzym GmbH & Co.KG:**
  PrestoSpin R Bug Mini Spin Column RNA Purification Kit
- **MP Biomedicals Inc.:**
  glycine; tryptone
- **New England Biolabs GmbH:**
  Prestained broad range protein marker [6.5-175 kDa]
- **QIAGEN GmbH:**
  MinElute™ PCR Purification Kit; RNAprotect™ Bacteria Reagent
- **SCIENION AG:**
  Hybridization buffer *sciHYB*
- **Serva GmbH:**
  L-cystine, L-threonine, L-arginine, L-histidine·HCl, L-lysine·HCl, L-aspartic acid, L-phenylalanine, L-proline, glycine, L-tyrosine, p-amino benzoic acid, folic acid, pyridoxal·HCl, riboflavin, thiamin·HCl; Acrylamide-Bisacrylamide-solution (40% [wt/vol])
- **Sigma-Aldrich Chemie GmbH:**
  hemin discs; thymidine; menadione; cytochrome c [from bovine heart]; MOPS; casein enzymatic hydrolysate; manganous sulfate·H_2O; DL-alanine; L-isoleucine; L-glutamine; L-hydroxyproline; d-biotin; nicotinamide; β-nicotinamide adenine dinucleotide (β-NAD); pyridoxamine·2HCl; vitamin B_{12}; adenine; guanine·HCl; uracil; HEPES; carboxyfluorescein (CF); Triton X-100; L(+)-ascorbic acid; ammonium persulfate (APS); cerium(IV) sulfate·$4H_2O$; magnesium chloride·$6H_2O$; Tris-HCl; trichloroacetic acid sodium salt; glutaraldehyde [25%] ; choline chloride; MES hydrate; guanidine·HCl
- **Zinsser Analytic:**
  Quickszint 100

3.1.8. SOLUTIONS AND BUFFERS

Table 11 summarizes the solutions and buffers used in this work, together with their respective composition. Whenever necessary, they were sterilized either by autoclaving for 20 min at 121°C; or by membrane filtration.

Table 11: Solutions and buffers

Solutions / Buffers	Composition
Protein determination according to Lowry *et al.* [184]	
Solution A	3% Na_2CO_3 in 0.1 M NaOH
Solution B	2% $CuSO_4 \cdot 5H_2O$
Solution C	4% K-Na-Tartarate
Alkaline copper-reagent	2% solution B, 2% solution C, 96% solution A
Bovine serum albumin (BSA)	1 mg/ml Sørensen's phosphate buffer
Sørensen's phosphate buffer [0.1 M]	0.1 M Na_2HPO_4, 0.1 M KH_2PO_4; pH 7.0

Solutions / Buffers	Composition
SDS-PAGE	
Acrylamide	40% [wt/vol] Acrylamide-Bisacrylamide-solution (19 :1); Serva GmbH
Solution A	3 M Tris-HCl; pH 8.5
Solution D	0.96 M Tris, 0.8 % SDS [wt/vol]
Ammonium persulfate (APS)	0.21 % [wt/vol] (freshly prepared)
SDS	20% [wt/vol] (freshly prepared)
5× SDS-sample buffer	0.225 M Tris-HCl (pH 6.8), 50% glycerol [wt/vol], 5% SDS [wt/vol], 0.05% bromophenol blue [wt/vol], 0.25 M DTT
Electrophoresis buffer	25 mM Tris, 192 mM glycine, 0.1% [wt/vol] SDS; pH 8.5
Coomassie-staining solution	2.5 g coomassie brilliant blue R-250, 450 ml methanol, 90 ml glacial acetic acid, ad 1 l Aqua dest
Destaining solution	50 ml methanol, 75 ml glacial acetic acid, ad 1 l Aqua dest
Silver staining	
Fixing solution	400 ml ethanol, 100 ml glacial acetic acid, 500 ml Aqua dest
Sensitizing solution	75 ml ethanol, 17 g Na-acetate·$3H_2O$, 1.3 ml 25% glutardialdehyde (freshly prepared), 0.5 g Na-thiosulfate·$5H_2O$, ad 250 ml Aqua dest
Staining solution	0.125 g silver nitrate, 25 µl formaldehyde (37%, freshly prepared), ad 125 ml Aqua dest
Development solution	3.125 g Na-carbonate, 12.5 µl formaldehyde (37%, freshly prepared), ad 125 ml Aqua dest
Stop solution	14.6 g EDTA-Na_2·$2H_2O$, ad 1 l Aqua dest
Preservation solution	100 ml glycerol, ad 1 l Aqua dest
Phospholipid analysis	
Solvent 1	chloroform-methanol-water (65:25:4, by volume)
Solvent 2	chloroform-acetic acid-methanol-water (80:15:12:4, by volume)
Phosphomolybdic acid (PMA) staining reagent	2.5% [wt/vol] molybdatophosphoric acid, 1% [wt/vol] cerium(IV) sulfate in 6% [vol/vol] sulfuric acid
Preparation of liposomes	
TBS-buffer	10 mM Tris-HCl, pH 7.2, 0.85% [wt/vol] NaCl; pH adjusted to 7.2 using 1 N HCl
Choline buffer	300 mM choline chloride, 30 mM MES, 20 mM Tris; pH 6.5
Carboxyfluorescein solution	50 mM solution in TBS-buffer. CF was first completely dissolved in 1 M NaOH (25% of the final volume), and the volume was subsequently completed with TBS buffer.
KCl buffer	300 mM KCl, 30 mM MES, 20 mM Tris; pH 6.5

Solutions / Buffers	Composition
Microarray-solutions	
EDTA stock solution, RNase-free	0.5 M; pH adjusted to 8.0 with 10 M NaOH
TE-buffer, RNase-free (sterilized by autoclaving)	10 mM Tris-HCl, 1 mM EDTA (RNase-free, 0.5 M stock solution, pH 8.0), ad 100 ml DMPC-water; pH 8.0
Standard **S**aline **C**itrate, SSC	stock solution (20 × SSC): 0.3 M sodium citrate·$2H_2O$, 3.0 M NaCl; pH 7.0
SDS stock solution	20% [wt/vol] SDS
Washing solution 1	1 × SSC + 0.03% [wt/vol] SDS
Washing solution 2	0.2 × SSC
Washing solution 3	0.06 × SSC
Tris-buffer	1.0 M Tris-HCl; pH 7.5
Miscellaneous	
Sørensen's phosphate buffer with sucrose (SPS)	25.4 mM KH_2PO_4, 24.6 mM Na_2HPO_4, 0.1 M sucrose
Physiological saline solution	0.9% [wt/vol] NaCl in Aqua dest
PUM buffer	22.2 g/l $K_2HPO_4·3H_2O$, 7.26 g/l KH_2PO_4, 1.8 g/l urea, 0.2 g/l $MgSO_4·7H_2O$; pH 7.1.
Phosphate-buffered saline, PBS	8 g/l NaCl, 1.44 g/l Na_2HPO_4, 0.2 g/l KCl, 0.2 g/l KH_2PO_4; pH 7.2

3.1.9. UTENSILS AND LABORATORY EQUIPMENT

All equipment, labware and consumables used in this work, together with their respective manufacturers and suppliers, are listed in Table 12. Flasks and pipette tips were autoclaved at 121°C for 20 min. The sterilization of glassware was carried out at 200°C for 4 h in an oven, except for glassware used for RNA isolation, which was sterilized at 220°C for 8 h.

Table 12: Equipment, labware and consumables

Equipment	Manufacturers / Suppliers
1 ml syringes, OMNIFIX®	B. Braun Melsungen AG
1900 CA Tri-Carb liquid scintillation counter	Packard
Agfa scanner SnapScan™ e40	Agfa-Gevaert N.V.
Autoclave, Varioklav®	Thermo Fisher Scientific Inc.
Automatic pipette 0.5 µl, 2 µl	Eppendorf AG
Automatic pipette Gilson Pipetman P20, P100, P200, P1000, P5000	Abimed GmbH

Equipment	Manufacturers / Suppliers
Block heater	Stuart Scientific
CAMAG Flat bottom TLC chamber with glass lid	CAMAG
Cellulose acetate membrane filters, 0.2-µm, OE 66	Schleicher & Schuell BioScience GmbH
Centrifuge, Biofuge 28RS, Rotor 3751	Heraeus Sepatech GmbH
Controlled environment incubator shaker series 25	New Brunswick Scientific Co. Inc.
Deionizer, Easy Pure LF®	Barnstead International
Demineralizer, AQUADEM®	Wilhelm Werner GmbH
Desiccator	Glaswerk Wertheim
Disposable gloves, Peha-soft®	Paul Hartmann AG
DNase-/RNase free pipette tips 10-1000µl	Biozym GmbH
Electrophoresis Power Supply (PowerPac 200)	Bio-Rad Laboratories GmbH
Finnpipette® Multistepper	Labsystems
Freeze-dryer	Martin Christ GmbH,
Gel chamber Mini-Protean® III	Bio-Rad Laboratories GmbH
GELAIRE laminar air flow bench class100	Gelman Instr.
GenePix® 4000B array scanner	Axon Instruments
Heidolph® Reax top test tube shaker	Heidolph Instruments GmbH & Co.KG
Hotplate magnetic stirrer, Combimag RCT	IKA® Werke GmbH & Co. KG
Hybaid PCR Express thermal cycler	Life Sciences International GmbH
Hybridization chamber, *sciHYBCHAMBER*	SCIENION AG
Incubtors	Heraeus GmbH
Isopore™ 0.4 µm HTTP polycarbonate membrane filters	Millipore GmbH
Lint-free paper towels Kimwipes®	Kimberly-Clark Corporation
Magnetic stirrer Hellma Cuv-O-Stir® Model 333	HELLMA GmbH & Co. KG
Magnetic stirrer MR 2000	Heidolph Instruments GmbH & Co.KG
MI-409F reference and MI-442 potassium electrodes	Microelectrodes, Inc.
Micro, analytic and universal balances	Sartorius AG
MICRONAUT Scan	MERLIN Diagnostika GmbH
Microtubes (1.5 ml) and safe-seal microtubes (2.0 ml), PP	Sarstedt Aktiengesellschaft & Co.
Mini water bath Julabo MWB	Julabo Labortechnik GmbH
MiniSpin® microcentrifuge	Eppendorf AG
MS 1 Minishaker	IKA® Werke GmbH & Co. KG
MultiMate 8-channel pipette, 20-200 µl	Abimed GmbH
NanoDrop® ND-1000 spectrophotometer V3.3	NanoDrop Technologies
NanoPhotometer™ 7122 V1.6.1.	Implen GmbH
Nunc F96 MicroWell™ plates; Nunclon™ Δ, polystyrene microtiter plates	Nunc A/S
Oven and sterilizer	Memmert GmbH + Co. KG
Parafilm M® all-purpose laboratory film	Pechiney Plastic Packaging
pH 213 microprocessor pH meter	HANNA Instruments
pH indicators	Merck KGaA

Equipment	Manufacturers / Suppliers
pH-Meter CG 810	SCHOTT Instruments GmbH
Pipette tips	Sarstedt Aktiengesellschaft & Co.
Refrigerated benchtop centrifuge, Multifuge 1 S-R	Heraeus GmbH
Refrigerated microcentrifuge 2K15	Sigma
RF-5301 PC Series spectrofluorophotometer	Shimadzu Corporation
RNase Zap® wipes	Applied Biosystems/Ambion
ROTRAND filtering unit, 0.2 µm, sterile, FP 30, celluloseacetate membranes	Schleicher & Schuell Bioscience GmbH
sciTRACER S. aureus N315 full genome chips	SCIENION AG
Shaking incubator CERTOMAT® H	Sartorius AG
Shaking water bath GFL 1083	GFL
Shaking water bath Julabo SW 20	Julabo Labortechnik GmbH
Silica Gel 60 F254 high-performance TLC plates	Merck KGaA
Slide-A-Lyzer® 10K dialysis cassettes	Pierce
Spectrophotometer 150-20 (UV/Visible)	Hitachi Ltd, Tokyo, Japan
sterile 96-well round (U)-bottom polystyrene microtiter plates	Greiner Bio-One GmbH
Sterile Acrodisc® syringe filter, 0.2 µm, with Supor® PES membrane	PALL GmbH
Sterile Cellstar® PP-test tubes, 15 ml and 50 ml	Greiner Bio-One GmbH
Tray shaker	Edmund Bühler GmbH
Ultrasonic homogenizer UP50H	Hielscher Ultrasonics GmbH
Vacuum centrifuge, Speed Vac® SPD 101B	Savant
Vacuum pump Duo5	Pfeiffer Vacuum GmbH
Vortexer, Vortex Genie2	Scientific Industries Inc.

3.1.10. SOFTWARES

The following are the softwares employed in the various assays for data extraction and analysis, together with their sources.

Table 13: Softwares

Softwares	Sources
Acuity 3.1; GenePix® Pro 4.1	Axon Instruments, Inc.
SAM 1.21 software	© Trustees of Leland Stanford Junior University
HI 92000 – 3.4	HANNA Instruments
MICRONAUT MCN software	MERLIN Diagnostika GmbH
Agfa ScanWise 1.40	Agfa-Gevaert N.V.

3.1.11. CULTURE MEDIA

Table 14 includes the growth media used for the cultivation of the bacterial strains deployed in this work, together with their composition. The quantities in the table refer to a final volume of one liter. Ready-to-use culture media were prepared according to the instructions of the manufacturer. Unless otherwise indicated, the culture media were dissolved directly after being weighed in deionized distilled water, and then sterilized by autoclaving for 15 - 20 min at 121°C. Agar-agar (12-17 g/l) was used to solidify the media before autoclaving. For the preparation of selective media, the antibiotics were added to the autoclaved media, after they cooled down to 50°C.

Table 14: Culture media

Culture medium	Source / Composition
BM-broth (B-medium); BM-agar	Tryptone (10 g), yeast extract (5 g), NaCl (5 g), K_2HPO_4 (1 g), D(+) glucose (1 g) ± agar-agar (12 g)
Brain-heart infusion medium (BHI broth)	**Oxoid GmbH**: Calf brain infusion solids (12.5), beef heart infusion solids (5.0), proteose peptone (10.0), glucose (2.0), sodium chloride (5.0), di-sodium phosphate (2.5).
Columbia agar with 5% sheep blood	**Becton, Dickinson & Co (BD), BBL™**
DNase test agar	**Becton, Dickinson & Co (BD), BBL™**: Pancreatic digest of casein (15.0 g), papaic digest of soybean meal (5.0 g), sodium chloride (5.0), deoxyribonucleic acid (2.0), agar-agar (15.0).
Luria-Bertani-broth (lysogeny broth); LB agar	Tryptone (10 g), yeast extract (5 g), NaCl (10 g) ±agar-agar (17 g).
Mannitol salt agar, CM 85	**Oxoid GmbH**: 'Lab-Lemco' powder (1.0), peptone (10.0), mannitol (10.0), sodium chloride (75.0), phenol red (0.025), agar-agar (15.0).
Modified B-broth for growing *S. aureus*	Casein hydrolysate (10 g), yeast extract (5 g), K_2HPO_4 (0.5 g), glucose (10 mM); pH adjusted to 7.3 with 1N NaOH.
Mueller-Hinton II broth, cation-adjusted (CAMHB); Mueller-Hinton agar (MHA)	**Becton, Dickinson & Co (BD), BBL™**: Beef extract (3.0 g), acid hydrolysate of casein (17.5 g), starch (1.5 g) ± agar-agar (17 g)
PYG medium (**p**eptone-**y**east-**g**lucose broth), modified	Bactopeptone (2.0 g), yeast extract (2.0 g), glucose (5 mM), KPO_4-buffer (10 mM; pH 7.0)

Culture medium	Source / Composition
Standard I nutrient broth (NI broth); standard I nutrient agar (NI agar)	**Merck KGaA:** Peptones (15.0 g), yeast extract (3.0 g), sodium chloride (6.0 g), D(+)-glucose (1.0 g) ± agar-agar (12 g)
TSB broth (**T**ryptic **S**oy **B**roth), casein-peptone soymeal-peptone broth; tryptic soy agar (TSA)	**Merck KGaA:** Tryptone, peptone from casein (17.0 g), peptone from soymeal (3.0 g), D(+) glucose (2.5 g), sodium chloride (5.0 g), di-potassium hydrogen phosphate (2.5 g) ± agar-agar (12 g)
CDM (=chemically defined medium), minimal medium for *Staphylococcus aureus*	Ref. [330], with the following modifications: phosphate buffer was replaced with HEPES [13 g/l], L-cysteine and the metal ions of the first group were omitted and a lower glucose concentration [1 g/l] was used.

3.1.12. BACTERIAL STRAINS AND CULTURE CONDITIONS

Table 15 lists the bacterial strains used in this study as indicator and reference strains. *Staphylococcus simulans 22* and *Staphylococcus aureus* SG511 are well-characterized standard laboratory strains, available at the Pharmaceutical Microbiology Unit (University of Bonn, Germany), regularly used in the study of cationic antimicrobial peptides [19,225,264,267]. *Staphylococcus aureus* SA113 (ATCC 35556), together with some of its deletion mutants, were kindly supplied by Prof. Dr. Andreas Peschel (University of Tübingen, Germany).

All strains used in the current study were stored as glycerol cultures (40% [vol/vol]) at -70°C. Working cultures were additionally kept at 4°C on appropriate agar plates, which were subcultured fortnightly from the respective glycerol cultures at 37°C. Unless otherwise indicated, cultivation in liquid cultures (in a 100 ml-flask) was performed aerobically at 37°C with shaking at 150 rpm (CERTOMAT® H), using a 5% [vol/vol] inoculum from an overnight starter culture to prepare the main culture in the appropriate medium. 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. Deviant growth conditions are separately mentioned in the different experimental procedures.

CAMHB was used as the standard growth medium for all susceptibility testings. It was used for a first series of preliminary experiments and for optimization of all test systems. This medium is adjusted to the calcium (20-25 mg/l) and magnesium (10-12.5 mg/l) ion concentrations recommended in NCCLS standard M7 [212]. Since it is formulated to have a low thymine and thymidine content, it was also used for studies involving small colony variants (SCVs).

TABLE 15: Laboratory and indicator strains

BACTERIAL STRAINS	CHARACTERISTICS / APPLICATIONS	GROWTH CONDITIONS*	REFERENCE (SOURCE)
Micrococcus luteus DSM 1790	membrane preparation	TSB at 30°C with aeration	DSMZ
Staphylococcus simulans 22	indicator strain	CAMHB at 37°C with aeration	[266]
Staphylococcus aureus SG511	indicator strain, methicillin-susceptible	CAMHB at 37°C with aeration	[186]
B. subtilis 168 (*B. subtilis* subsp. *subtilis*)	laboratory strain, DSM 402, NCIB 10106	LB agar	[210]
S. epidermidis SE O-47	indicator strain for biofilm-formation	BHI broth; TSB supplemented with 0.25% glucose, 37°C	[119]
S. aureus SA NCTC 8325 SH 1000	*S. aureus* NCTC 8325-4 rsbU$^+$, indicator strain for biofilm-formation	BHI broth; TSB supplemented with 0.25% glucose, 37°C	[131]
S. aureus SA113 (ATCC 35556)	wild-type (WT) parental strain	BM-agar, 37°C	[137]
SA113Δ*tagO*::*erm*	*tagO*-mutant, completely devoid of WTA	+ 5 µg/ml erm	[338]
SA113Δ*ypfP*::*erm*	*ypfP*-mutant, lacking LTA	+ 5 µg/ml erm	[79]
SA113Δ*ypfP*::*spec*/Δ *tagO*::*erm*	double-knock-out-mutant	+ 2.5 µg/ml erm; + 150 µg/ml spec	
SA113Δ*dltA*::*spec*	*dltA*-mutant	+ 200 µg/ml spec	[238]
SA113Δ*mprF*::*erm*	*mprF*-null deletion mutant	+ 2.5 µg/ml erm	[237]

* erm, erythromycin; spec, spectinomycin.

3.2. MICROBIOLOGICAL METHODS

3.2.1. MEASUREMENT OF OPTICAL DENSITY OF LIQUID CULTURES

The cell density of bacterial suspensions was determined by measuring the optical density (OD) of appropriately diluted samples using a spectrophotometer at a wavelength of 600 nm [OD_{600}], against the respective suspending medium as a blank.

3.2.2. VIABLE COUNT ESTIMATIONS

The number of viable cells in a bacterial suspension was estimated through 10-fold serial dilutions in physiological saline (0.9% [wt/vol] NaCl). 50 µl-aliquots of the appropriate dilutions were plated onto the surface of agar plates, and incubated for 24 – 48 h at 37°C. The developed colonies were then counted, and the number of colony-forming units (CFU)/ml in the bacterial suspension was calculated based on the respective dilution factor. The detection limit of this method lies at about 20 CFU/ml. When the expected viable count would lie below this limit, then 1 ml-aliquots of the undiluted bacterial suspension were plated. In addition, 1 ml-aliquots of the undiluted bacterial suspension were filtered through 0.45 µm membrane filters, and then washed with sterile, isotonic saline. The filter was then carefully placed onto the surface of the agar plate and incubated, whereby the detection limit was lowered to 1 CFU/ml. Viable count estimations of SCVs were conducted using Mueller-Hinton agar (MHA) plates, which were incubated at 37°C for 48 - 72 h. Multiple estimations of the viable count were carried out to ensure the reproducibility of the results.

3.2.3. *IN VITRO* ANTIMICROBIAL SUSCEPTIBILITY TESTING (MIC AND MBC ESTIMATIONS)

The minimum inhibitory concentration (MIC) is a measure of the susceptibility of a bacterial strain towards a specific antimicrobial substance. Values of the MIC of the different antimicrobials were determined by a standard broth microdilution assay,

according to the guidelines of the Clinical and Laboratory Standards Institute, CLSI (formerly the National Committee for Clinical Laboratory Standards, NCCLS [212]) as follows:

Serial two-fold dilutions of the antimicrobial agent were prepared in the appropriate culture medium in sterile 96-well round-bottom polystyrene microtiter plates (Greiner Bio-One GmbH). Liquid cultures of the test strains were inoculated from an overnight culture (5% [vol/vol]), and allowed to grow in the respective broth in a rotary incubator at 37°C until reaching the early exponential growth phase [OD_{600} = 1], and subsequently diluted 1:10^4 with the same medium to a final inoculum size of around 2×10^5 CFU/ml. Each well of the microtiter plate then received 100 µl of the inoculated medium to a final volume of 200 µl per well. The inoculated microtiter plates were covered with sterile self-adhesive cellophane, placed for 10 min on a tray shaker, and then incubated at 37°C. Parallel viable count estimations were carried out to ensure an appropriate inoculum. The MIC was read as the least concentration of the antimicrobial agent resulting in the complete inhibition of visible bacterial growth after 24 - 48 h of incubation at 37°C under standard conditions that allowed confluent growth of the test strain in drug-free wells. Antibacterial activities were also expressed as the MBC (minimum bactericidal concentration), defined as the lowest concentration of the antimicrobial agent reducing the bacterial inoculum by ≥ 99.9% within 24 h. The MBC was assigned by estimating the viable count in 20-µl aliquots from each well of the microtiter plates showing absence of growth. Susceptibility tests were repeated at least three separate times to check the reproducibility of the results, and the mean of these values was taken.

In order to detect incremental changes in chitosan susceptibility during the serial passage experiment (section 3.2.9), an extended gradient of chitosan was created by combining four sets of two-fold serial dilutions in CAMHB from four different starting stock solutions (10000; 7000; 6000 and 4000 µg/ml), including chitosan concentrations of 1.95, 2.93, 3.42, 3.91, 4.88, 5.86, 6.84, 7.81, 9.77, 11.72, 13.67, 15.63, 19.53, 23.44, 27.34, 31.25, 39.06, 46.88, 54.69, 62.5, 78.13, 93.75, 109.38, 125, 156.25, 187.5, 218.75,

250, 312.5, 375, 437.5, 500, 625, 750, 875, 1000, 1250, 1500, 1750, 2000, 2500, 3000, 3500 and 5000 µg/ml, extending over four rows of the microtiter plates. Since there are no MIC guidelines established by the CLSI for chitosan susceptibility or chitosan resistance, we set an arbitrary *in vitro* breakpoint at 1000 µg/ml; strains with MIC values higher than 1000 µg/ml were thus termed "chitosan-resistant".

In case of small colony variants (SCVs), the MIC was determined in CAMHB (since its low content of menadione, thymine and thymidine fully maintains the SCV phenotype in liquid phase for several days), in absence and presence of thymidine [100 µg/ml], menadione [1 µg/ml], or glucose [5 mM]. Due to the slow growth of the SCVs, the inoculum was prepared by appropriate dilution of a 48 h-culture, and the MIC values were read after 48, 72 and 96 h of incubation at 37°C.

Bacterial susceptibility to the various antimicrobials was determined in part with the help of ready-made microtiter plates (MICRONAUT-S microtiter plates, MERLIN Diagnostika GmbH), according to the instructions of the manufacturer. These plates contain antibiotics in dehydrated form (Table 6) that are rehydrated upon the addition of the standardized bacterial suspension.

The MIC estimations for Pep5 were carried out in sterile 96-well polypropylene plates (Nunc F96 microtiter plates), to avoid the interaction of this highly cationic peptide with the anionic surface of polystyrene microtiter plates, i.e. to reduce AMP binding [99].

For membrane potential measurements, leakage and killing assays with chitosan, fixed multiples of the MICs (1- to 10-fold) were used to encompass a likely bactericidal concentration.

The MIC values of chitosan for some indicator strains were also determined under different culture conditions, including different culture media [CAMHB, PYG, CDM [330] and B-broth, Table 14], and in presence of 5, 20, 50 and 100 µM metal ions (Fe^{2+} and Zn^{2+}), glucose [10 mM] or NaCl [20 and 50 mM], respectively.

3.2.4. ANTIMICROBIAL ACTIVITY OF DOUBLE COMBINATIONS (CHECKERBOARD TITRATIONS)

The activity of double combinations of antimicrobials was studied using a two-dimensional checkerboard in 96-well Nunc plates: Eight two-fold dilutions of each of the antimicrobials tested were made in CAMHB in the grid of eight rows by eight columns in descending concentrations, starting at two-to-four times the MIC. Thus, each of the 64 wells held a unique combination of concentrations of the two antimicrobials. An inoculum of 100 µl of *S. aureus* per well was used at a concentration of about 5×10^5 CFU/ml, and the plates were incubated overnight at 37°C. Growth control wells containing only CAMHB were included in each plate, as well as controls for each antimicrobial tested. Each test was performed in duplicate. For the first clear well in each row of the microtiter plate containing both antimicrobials, the fractional inhibitory concentration (FIC) indices were calculated as follows: FIC index = $[(A)/MIC_A] + [(B)/MIC_B] = FIC_A + FIC_B$, where MIC_A and MIC_B are the MICs of drugs A and B when used alone, and (A) and (B) are the MICs of drugs A and B when used in combination. The interaction was defined as synergic if the FIC index was ≤ 0.5 and antagonistic if the FIC index was > 4.0; no interaction was observed if the FIC index was > 0.5 – 4.0 [218].

3.2.5. INVESTIGATION OF BACTERIAL BIOCHEMICAL ACTIVITIES

The investigation of the biochemical activities of the test strains was carried out using the MICRONAUT-RPO plates (MERLIN Diagnostika GmbH), which include 44 biochemical reactions, according to the manufacturer's directions. After 22 - 24 h of incubation at 37°C, the results of the tests were read both visually, and scanned photometrically using MICRONAUT Scan; data were then analyzed using MICRONAUT MCN software. The purity of the test strains was checked by inoculation onto blood agar.

Additional conventional biochemical tests included: catalase test, test for *β*-hemolysis on Columbia blood agar, tube coagulase test (BACTIDENT® coagulase),

desoxyribonuclease-test (DNase test agar) as well as mannitol fermentation (mannitol salt agar); they were carried out according to standard textbook microbiological procedures. Whenever necessary, strains *S. aureus* ATCC 29213 and *S. simulans* 22 were included as controls.

3.2.6. GROWTH CURVES AND GENERATION TIMES

Bacterial growth rates during the phase of exponential growth, under standard conditions (culture medium, temperature, pH, etc.) define the bacterium's generation time, the time interval required for the duplication of the cell number.

The growth curve of a bacterial strain was constructed by inoculating a batch culture under standard conditions, and adjusting it to a cell density of around 1×10^8 CFU/ml. This suspension was then diluted 1:10^4 in the appropriate medium to an initial count of around 1×10^4 CFU/ml. The zero-time samples were immediately taken, and then the cultures were incubated at 37°C in a shaking incubator (150 rpm). At time intervals of 30 min (90 min in case of SCVs), samples were withdrawn for viable count determination (section 3.2.2.). Values were plotted as a half-logarithmic plot (log CFU/ml against time), and the minimal generation time of each strain was calculated based on the linear region within the exponential phase, by means of the following equation: generation time = log2/m [min], where m is the slope of the regression line within the log-phase. OD_{600} measurements were also carried out alongside viable count estimations, and then plotted against time.

3.2.7. BACTERIAL KILLING ASSAYS

3.2.7.1. Determination of bacterial killing kinetics

The *in vitro* bactericidal potency (time-kill curve) of chitosan toward different test strains was assayed, by separately incubating cultures of the test strain in CAMHB (around 1×10^7 CFU/ml) in absence (control) and presence of different chitosan concentrations, for a period of 24 h at 37°C. Samples of the bacterial cultures were

removed at regular intervals to determine the OD_{600}, appropriately diluted and processed for quantitative culturing to assess the extent of killing by chitosan. The surviving $\log_{10}$ CFU/ml was plotted against time for each of the different chitosan concentrations.

In addition, killing assays were performed using bacterial cultures at different physiological states as follows: A liquid culture of the test strain in CAMHB was inoculated from an overnight culture (1% [vol/vol]), and then incubated at 37°C and 150 rpm. Growth was monitored at 30 min intervals for up to 24 h by determining the OD_{600} and by viable cell counts. Chitosan was added to the cultures at concentrations corresponding to 2× and 4× MIC as determined after 24 h. The killing was determined at OD_{600} = 0.1, 1, 6 and 14, after appropriate dilution of each of the cultures to an OD_{600} of 0.1 in CAMHB. Controls for staphylocidal activity consisted of cells in appropriate medium lacking chitosan.

3.2.7.2. Determination of extent of killing at fixed time point

Cultures of the test strains were diluted into different chitosan solutions in CAMHB to achieve an initial inoculum of around 10^7 CFU/ml and then incubated at 37°C and 150 rpm. The range of chitosan concentrations tested was 4.88 – 625 µg/ml to encompass sublethal-to-lethal chitosan levels. After 20 min of contact, samples were removed and the surviving count determined. Curves were constructed, comparing mean surviving $\log_{10}$ CFU/ml versus chitosan concentrations, using an average of 2 determinations.

3.2.8. FLOCCULATION ASSAY

The flocculation assay was essentially based on the assay described by Smit *et al.* [291], with a number of modifications. Briefly, 20 ml of CAMHB in a 100 ml-flask were inoculated from an overnight culture of each of the tested strains (3% [vol/vol]), and the culture was incubated at 37°C and 150 rpm, until reaching an OD_{600} of around 1.5. Cells were harvested by centrifugation [5,000 rpm, 5 min, 4°C], washed and resuspended in choline buffer [300 mM choline chloride, 30 mM MES, 20 mM Tris ; pH

6.5] to a final OD_{600} of 1.5 – 2.0. After 30 min of acclimatization at room temperature, 600 µl of the cell suspension were added to a 1.0-ml cuvette. With this sample volume, the light beam of the spectrophotometer monitors the optical density slightly below the surface of the cell suspension. The cell suspension was whirlmixed for 10 sec at maximum speed; this was followed by five inversions of the cuvette. Immediately thereafter, the settling profiles of the bacterial suspensions were determined spectrophotometrically, every 30 sec for 10 min. The influence of chitosan on cell flocculation was tested after it was added (at final concentrations of 0.5 – 200 µg/ml) to the buffer just before whirlmixing. A control culture (lacking chitosan) was also included. The decrease in optical density was taken as a measure of cell flocculation, and the $\%OD_{600}$ was calculated as [OD_{600} (final)/ OD_{600} (initial) × 100].

3.2.9. *IN VITRO* SELECTION FOR DECREASED SUSCEPTIBILITY TO CHITOSAN (SERIAL PASSAGE EXPERIMENT)

S. aureus SG511 was the standard strain used for the serial passage experiment, which was accompanied by a series of MIC experiments with an extended range of chitosan concentrations (section 3.2.3). On day 1 of the experiment, 10 ml of CAMHB were inoculated with an overnight culture (2% [vol/vol]), and the culture was incubated aerobically on a rotary shaker (150 rpm) at 37°C to ensure exponential growth conditions. At an OD_{600} of around 1.0, the culture was diluted $1:10^4$ in CAMHB and the MIC was determined. The original culture was left to continue growing till reaching the late exponential phase, then a 2% [vol/vol] inoculum was used to inoculate the next culture, with CAMHB containing an incrementally higher chitosan concentration, which was then incubated as before, and the process was repeated 15 times. Populations of bacteria from each serial passage were stored at -70°C as 40% [vol/vol] glycerol cultures; no changes in their initial chitosan susceptibility profiles occurred upon storage. The stability of the variant finally obtained (chitosan-resistant variant, CRV) was verified by passaging it 20 consecutive times in chitosan-free CAMHB, and checking the stability of the MIC values.

3.2.10. DETERMINATION OF AUXOTROPHY OF *S. AUREUS* SCVS

Hemin-auxotrophy was tested by plating a culture of SCVs in CAMHB (10^4 CFU/ml) onto the surface of a MHA plate, then aseptically transferring a hemin disc onto the centre of the plate. As for thymidine- and menadione-auxotrophy, a diluted culture of SCV in CAMHB was plated onto the surface of MHA plates, where cups were instilled with either thymidine (200 µg/well) or menadione (10 µg/well). The plates were incubated at 37°C for 24 - 48 h, and the growth of the SCVs on all three plates was observed.

3.2.11. BIOFILM ASSAYS

3.2.11.1.Evaluation of biofilm-forming ability of test strains

The biofilm-forming ability of several test strains was assessed using a semiquantitative microtiter plate method, which involved the determination of adhesion to 96-well tissue culture plates (Nunc, Nunclon™). Aliquots of 150 µl of the test medium appropriately inoculated from an overnight culture of each test strain (0.5% [vol/vol]) were transferred into the respective wells of the microtiter plates. The plates were then shaken for 10 min, followed by incubation at 37°C to allow biofilm formation. After 24 h of incubation, all excess medium was carefully replaced with 150 µl of fresh medium, and then reincubated, without shaking, at 37°C for another 24 h.

After visible biofilm formation at the bottom of the wells, the excess medium was again removed, and the non-adherent, loosely-associated planktonic cells were removed by gentle washing with PBS (8 g/l NaCl, 1.44 g/l Na_2HPO_4, 0.2 g/l KCl, 0.2 g/l KH_2PO_4; pH 7.2), and left to dry in the incubator. The bacterial biofilms remaining attached to the wells of the microtiter plates were stained with 0.1% [wt/vol] safranin solution for 1 minute. Unbound stain was removed by washing twice with PBS. The wells were drained, and the stained biofilms were again allowed to dry in the incubator. The plate was then examined to observe the distribution and attachment pattern of safranin on the bottom of the microtiter wells.

The biofilm-associated safranin dye was then eluted from each well using 33% acetic acid [295] and the staining of the biofilms was quantified by measuring the absorbance of the acetic acid/safranin solution in a spectrophotometer at 492 nm. The absorbance of wells incubated with 150 µl of sterile medium represented the negative control; its value was subtracted from the values for the inoculated wells.

In a parallel experiment, unstained biofilm bacteria were eluted in sterile PBS, and the biofilm-forming ability was assessed by performing quantitative bacterial counts on the resulting suspensions as well as OD_{600} measurements. Replicates of 9 (OD_{600} and viable counts) or 12 (A_{492}) wells per strain were examined for each experiment, and the consistency of measurements was evaluated on identical samples by calculating standard deviations (SD) for all repetitions of the experiment; the smaller the SD the greater the precision of the measurement.

3.2.11.2.Investigation of the effect of chitosan on biofilm formation

The effect of chitosan on biofilm formation was studied using two strains known to produce profuse biofilms, *S. epidermidis* SE O-47 and *S. aureus* SA NCTC 8325 SH 1000, in 2 different media proved to promote biofilm formation, namely BHI broth and TSB broth (supplemented with 0.25% membrane-filtered glucose). Biofilm formation in both media was compared to select the most appropriate medium for further testing.

The same biofilm assay described before (section 3.2.11.1) was applied here, and the effect of chitosan on biofilm formation was assessed using three different setups: i) effect on preformed biofilms: different chitosan concentrations were added to preformed biofilms, followed by incubation for 2 - 4 h at 37°C, washing the wells with PBS and then comparing the level of biofilm formation to the positive control (test strain alone); ii) effect of simultaneous incubation: test strains were first pretreated with different chitosan concentrations, vortexed for 10 sec, then left to stand for 10 min before transferring into the wells of the microtiter plate, and iii) inhibition of biofilm formation: the microtiter plates were first coated with different chitosan concentrations. After incubation at 37°C for 2 days, the wells were carefully washed

with Aqua dest, left to dry, and then the biofilm assay was conducted as before, to evaluate the effect of chitosan on the attachment of the test strains to the precoated microtiter plates. The percent inhibition of biofilm accumulation was then determined from the formula:

$$\% \text{ inhibition of biofilm accumulation} = \frac{(A_{492,\text{ positive}} - A_{492,\text{ test}})}{(A_{492,\text{ positive}} - A_{492,\text{ negative}})} \times 100$$

where $A_{492,\text{ positive}}$ is the absorbance value of the positive control; $A_{492,\text{ negative}}$ is the absorbance reading of the negative control (absorbance of wells incubated with sterile medium); and $A_{492,\text{ test}}$ is the absorbance reading in presence of chitosan.

3.2.12. PREPARATION AND STAINING OF STAPHYLOCOCCI FOR MICROSCOPY (TEM)

Liquid cultures of the test strains were inoculated from an overnight culture (2% [vol/vol]), and were grown in CAMHB (in a 100 ml-flask) at 37°C and 150 rpm to the early exponential phase (OD_{600} = 1.0). After incubation, 10 ml-aliquots of the bacterial cultures were harvested (in this and all subsequent steps: 1,000×*g*, 10 min, 4°C), the bacterial pellets were washed once in Sørensen's phosphate buffer with sucrose [25.4 mM KH_2PO_4, 24.6 mM Na_2HPO_4, 0.1 M sucrose, SPS] and then prefixed by suspension in SPS containing 3% [wt/vol] glutaraldehyde (4°C, 4 h).

After harvesting, the fixed cells were resuspended in SPS (12–18 h, 4°C). The collected cell pellets were washed in SPS then in cacodylate buffer [0.1 M]. Contrasting was done using 1.5 % potassium ferricyanide and 1 % [wt/vol] osmium tetroxide (2 h on ice), followed by extensive washing with double distilled water. Fixation was achieved by resuspending the pellets carefully in 5% [wt/vol] uranyl acetate (2 h on ice), followed by washing. After resuspension in 1 % tannic acid (30 min, 25°C), the pellet was dehydrated using a graded series of 70%, 90% and 100% [vol/vol] ethanol. Samples were infiltrated with Epon-propylene oxide [1:1] for 1h, transferred to Beem capsules, and then shortly centrifuged for 15 sec. The Epon-propylene oxide [1:1] mix was carefully replaced with Epon (1 ml Epon, 20 µl Epon-Accelerator DMP-30). Samples

were left in Epon (60°C, 2 - 3 days, to allow polymerization); the embedded block was subsequently cut using a microtome into ultra-thin sections, which were mounted onto formvar-coated nickel grids, post-stained with uranyl acetate followed by lead citrate, and then viewed and photographed with a Philips CM 120 transmission electron microscope under standard operating conditions.

The cell size and cell wall thickness were determined on the basis of electron micrographs taken at an amplification of 25,000 - 31,000×. The equatorial sections of an average of 4 - 5 cells per strain were measured, whereas for each cell the mean from 3 - 4 different measuring points was determined.

Electron microscopy was done at the institute for cell biology (University of Bonn, Germany), in the lab of Prof. Dr. Albert Haas.

3.2.13. AUTOLYSIS ASSAYS

Overnight cultures of each test strain in the appropriate medium were pelleted (13,400 rpm, 5 min), washed twice with cold sterile Aqua dest, then resuspended in fresh medium and grown at 37°C and 150 rpm to the early exponential phase.

After another pelleting and washing step, the cells (adjusted to a count of around 2×10^9 CFU/ml) were exposed to either 0.05% [vol/vol] Triton X-100 in Tris-HCl buffer (0.05 M; pH 7.2) [147] or lysostaphin [4 µg/ml] in Na-acetate buffer (20 mM; pH 7.5), and the bacterial suspensions were incubated at 37°C with gentle agitation. Samples were obtained at predetermined time intervals, and the OD_{600} measured. Autolysis was quantified as a per cent of the initial OD_{600} remaining at each sampling time-point.

3.2.14. INVESTIGATION OF CELL SURFACE HYDROPHOBICITY

The cell-surface hydrophobicity of the test strains was estimated using a modified Bacterial Adherence To Hydrocarbons (BATH) assay [260], based on the affinity of the strains for various liquid hydrocarbons.

Briefly, the test bacteria were grown in CAMHB at 37°C and 150 rpm and harvested (5,000 rpm, 4°C, 15 min) at early logarithmic growth phase (OD_{600} = 1.0 - 2.0), and then washed twice in PUM buffer (22.2 g/l $K_2HPO_4 \cdot 3H_2O$, 7.26 g/l KH_2PO_4, 1.8 g/l urea, 0.2 g/l $MgSO_4 \cdot 7H_2O$; pH 7.1.).

To round-bottom, 12-mm diameter, acid-washed test tubes, containing 2 ml of washed cells suspended in PUM buffer (to an OD_{600} of around 1), were added 400 µl of test hydrocarbon: 1-octanol, xylene or cyclohexane. Control test tubes (containing 2 ml of the bacterial suspension) were also included. Duplicate samples from two parallel bacterial cultures were used for each measurement. Following 10 min preincubation at room temperature, the mixtures were agitated uniformly on a vortex for 120 sec. After allowing 30 min for phase separation to occur, the loss of optical density in the lower aqueous phase (OD) relative to that of the initial cell suspension (OD_0) was determined at 600 nm using a spectrophotometer, and hydrophobicity (expressed as "fraction of adhered cells", H%), was estimated by calculating the percentage of cells adhering to the hydrocarbons using the formula $H\% = [(OD_0\text{-}OD)/OD_0] \times 100$, as suggested by Häußler *et al.* [114].

3.2.15. ESTIMATION OF CELL SURFACE CHARGE

The cytochrome c binding assay was used to estimate the bacterial surface charge and was carried out as previously described by Peschel *et al.* [238], with modifications. In brief, cells were grown overnight at 37°C and 150 rpm in CAMHB, harvested (5,300 rpm, 4°C, 10 min) and then washed twice in 20 mM MOPS buffer (pH 7.0). The cells were resuspended in the same buffer to a final OD_{578} of 7.0, and then incubated with 0.5 mg/ml cytochrome c for 10 min at room temperature. The cell pellets were collected by centrifugation twice at 13,400 rpm for 5 min and the amount of cytochrome c in the clear supernatant was determined spectrophotometrically at an OD_{410}. *S. aureus* SA113 strains (Table 14) were included as controls in this assay. The percentage of cytochrome c bound to the cell pellets of each strain was then determined, relative to

the total amount of cytochrome c added (control, containing cytochrome c in the buffer system), using the following equation:

$$\% \text{ cytochrome c bound} = \frac{(OD_{410,\,control} - OD_{410,\,sample})}{OD_{410,\,control}} \times 100$$

Data represent the means ± SD from three independent determinations, and were expressed relative to the bacterial dry weight and protein content, respectively. The bacterial dry weight was determined by lyophilization of 1 ml aliquots of the respective suspensions, and the protein content of whole bacterial cells was determined according to the method of Stickland [296], based on the biuret-reaction. An absorbance E_{555} of 1 was equivalent to 7.35 mg protein.

3.2.16. Detection of chitosan-hydrolyzing activity

The ability of various test strains to hydrolyze chitosan was detected using the chitosanase detection medium, prepared according to Beauséjour *et al.* [13]. Aliquots of the overnight cultures of the test strains were instilled into cups (6 mm in diameter) made into the chitosanase detection medium and incubated at the suitable temperature for 24 - 48 h. Since the medium is milky-white in colour, chitosan-hydrolyzing activity was detected by the formation of a clear zone of chitosan degradation around the cups. The residual chitosan-hydrolyzing activity in culture supernatants was determined after centrifugation of overnight cultures at 13,400 rpm for 5 min. An appropriate control was included (2 N HCl) to rule out a possible hydrolysis of chitosan by acidic metabolites.

3.3. Preparative and biochemical methods

3.3.1. Characterization of chitosan

The characterization of LMW chitosan in terms of MW and DD was kindly done by Mirko Weinhold (University of Bremen, Germany).

3.3.1.1. Molecular weight determination

The biopolymer analysis was performed on a TDAmax size exclusion chromatography (SEC) system (Viscotek, USA) with two serially connected ViscoGEL columns (PWXL mixed bed 6-13 μm methacrylate particles, 7.8 × 300 mm). The detection was operated by a differential refractometer at λ=660 nm and a right angle light scattering detector (RALS) with a 3 mW He/Ne laser at λ=670 nm. A dn/dc value of 0.163 (dl/g) was used. Intrinsic viscosity was measured on a 4 capillary, differential Wheatstone bridge viscometer. A degassed 0.3 M HAc/0.3 M NaAc buffer (pH 4.0) with 1% ethylene glycol was used as eluent. To ensure a low light scattering noise level the eluent was filtered through a 0.2 μm filter. LMW chitosan dissolved in the eluent at a concentration of 0.3 - 1 mg/ml was filtered through a 0.45 μm filter prior to analysis to remove aggregates. Injection volumina varied from 10 to 100 μl and the flow rate was maintained at 0.7 ml/min.

3.3.1.2. Determination of the fraction of acetylated groups (F_A)

LMW chitosan was dissolved in D_2O/DCl and its F_A was analyzed by ^{1}H-NMR. The F_A value was received after integration of the ^{1}H-NMR signals and subsequent calculation. The NMR-spectrum were recorded on a Bruker AVANCE WB-360 (8.4 Tesla) spectrometer (360 MHz). For signal integration the method proposed by Hirai *et al.* [125] was used. The DD was calculated from the F_A value as follows: $F_A = (100\text{-}DD)/100$.

3.3.1.3. Gel electrophoretic analysis of chitosan

Since chitosan samples mostly consist of mixtures of components, where an estimate of the degree of polymerization or the degree of deacetylation is only relative, we analyzed different chitosan grades using polyacrylamide gel electrophoresis according to the method of Audy and Asselin [6]. The chitosan samples (45 μg each) were dissolved in 0.5% [vol/vol] acetic acid containing 2 M urea and 15% [wt/vol] sucrose, and then boiled for 3 min prior to electrophoresis to allow for good electrophoretic separation. Electrophoresis was carried out in a 10% [wt/vol] polyacrylamide gel

containing 7 M urea and 5.5% [vol/vol] acetic acid. Electrophoresis was performed at RT using 5.5% [vol/vol] acetic acid as the electrode buffer. Chitosan migrated as a polycation from the top of the gel (anode) to the bottom (cathode). Chitosan was revealed by staining with (i) 0.2% [wt/vol] coomassie brilliant blue R-250 [106] in methanol-water-acetic acid (50:40:10; vol/vol/vol) at 60°C for 10 min, followed by 'destaining' with acetic acid/methanol, or with (ii) silver nitrate, by modification of the method of Heukeshoven and Dernick [123].

3.3.2. PREPARATION OF UNILAMELLAR VESICLES

Stable carboxyfluorescein (CF)- and potassium (K^+)-loaded unilamellar liposomes containing the zwitterionic, neutral phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), with and without the anionic phospholipid 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] sodium salt (DOPG) [Avanti Polar Lipids, Inc.] were formulated by the extrusion technique [341], for use in CF- and K^+-efflux experiments.

Briefly, lipid solutions were combined in the required molar ratio (in a total of 2 and 10 µmol total phospholipids for CF- and K^+-loaded liposomes, respectively, to overcome the low limit of detection of the potassium electrode [section 3.4.2.2.1]), and the solvent was allowed to evaporate in a desiccator. The dried lipid films were re-hydrated in the suitable marker solution [either 50 mM CF-solution in TBS-buffer (10 mM Tris-HCl, pH 7.2; 0.85% NaCl [wt/vol]; or KCl buffer (300 mM KCl, 30 mM MES, 20 mM Tris; pH 6.5), for the preparation of CF- and K^+-loaded multilamellar liposomes, respectively], followed by vigorous vortexing, to ensure the complete dissolution of the lipid film.

The multilamellar liposomes were alternately flash-frozen in liquid nitrogen and again thawed (at 30°C) “freeze-thaw method”, repeating this cycle for 8 - 10 consecutive times. Finally, unilamellar vesicles containing entrapped CF / K^+ were produced by repeated extrusion (8 - 10 times) of the multilamellar vesicles through two stacked Isopore™ 400-nm HTTP polycarbonate membrane filters. Following the extrusion,

untrapped extravesicular CF / K^+ was removed using a gel filtration column (Sephadex G-50; medium). Liposomes were finally collected and stored at 4°C until use. The phospholipid concentrations in the final liposome suspensions were quantified as inorganic phosphate after treatment with perchloric acid [262].

3.3.3. ANALYSIS OF MEMBRANE PHOSPHOLIPID COMPOSITION

3.3.3.1. Extraction of membrane phospholipids

Phospholipids (PLs) were extracted from *S. aureus* as follows: Overnight cultures of the test strains in CAMHB were harvested (5,300 rpm, 10 min, 4°C), 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 (5,000 rpm, 20 min, 4°C). The lower chloroform phase was collected and washed twice with an equal amount of distilled water to remove nonlipid contaminants and cell debris. The extracted organic layer was dried in Speedvac at 50°C, dissolved in chloroform-methanol (2:1 [vol/vol]), and stored at -20°C until analysis. The phospholipid content of the lipid extracts was determined [262], in order to be able to compare equivalent amounts of phospholipids from different extracts.

3.3.3.2. Detection and identification of membrane PLs

The major PL species were separated by one- and two-dimensional thin-layer chromatography (1D-TLC, 2D-TLC), using silica gel 60 F254 high-performance TLC plates (Merck KGaA) and subsequently developed with chloroform-methanol-water (65:25:4, by volume) in the vertical orientation and chloroform-acetic acid-methanol-water (80:15:12:4, by volume) in the horizontal orientation. PLs were visualized by phosphomolybdic acid (PMA) staining, whereas lysyl-phosphatidylglycerol (LPG) was specifically identified by ninhydrin staining [237]. The plates were subsequently scanned using Agfa Scan Wise 1.0. DOPG and cardiolipin were used as standards to determine the positions of their spots on 2D-TLC plates. All assays were performed a minimum of three times on separate days.

3.4. MODE OF ACTION EXPERIMENTS

3.4.1. INHIBITION OF THE *IN VITRO* LIPID II BIOSYNTHESIS

The lipid-bound cell wall precursor lipid II can be synthesized *in vitro* using membrane preparations from *Micrococcus luteus* DSM 1790. Inhibition of the *in vitro* synthesis of lipid II by chitosan was evaluated using the analytical lipid II synthesis assay as previously described [276] (kindly conducted by Dr. Tanja Schneider) in a total volume of 150 µl (Table 16). Chitosan was added to the reaction mixtures to achieve final concentrations of 67 and 267 µg/ml. After incubation of the reaction mixtures for 1 h at 30°C, lipids were extracted with the same volume of *n*-butanol – 6 M pyridine acetate (2:1, vol/vol; pH 4.2) and were vortexed vigorously for 1 min. The aqueous and the butanol phases were separated by centrifugation (13,000 rpm, 3 min).

TABLE 16: Composition of the analytical lipid II synthesis reaction mixture

Components of the reaction mixture	Final concentration
Undecaprenylphosphate (C_{55}-P), lipid carrier*	10 nmol
Triton X-100	0.5% [vol/vol]
Tris-HCl buffer; pH 8.0	60 mM
UDP-*N*-acetylmuramyl pentapeptide (UDP-Mur*N*Ac-*PP*)‡	100 nmol
UDP-*N*-acetylglucosamine (UDP-Glc*N*Ac)	100 nmol
Membrane preparation from *M. flavus* DSM 1790 [276]	400 µg of membrane protein
$MgCl_2$	5 mM
Aqua dest	Ad 150 µl

* The external addition of C_{55}-P is thought to increase the yield [26].

‡ The soluble cell wall precursor was accumulated in and purified from *S. simulans* 22 as previously described [165].

Lipids in the extraction mixture were separated by TLC (60 F254 silica gel plates; Merck KGaA), using chloroform-methanol-water-ammonia (88:48:10:1 [vol/vol]) as the solvent [256]. The lipid spots on the silica gel plate were then visualized by PMA staining.

3.4.2. LEAKAGE AND EFFLUX EXPERIMENTS

3.4.2.1. Carboxyfluorescein (CF)-efflux experiments

Chitosan-induced efflux of CF from liposomes was determined as follows: CF-loaded vesicles (prepared as described in section 3.3.2) were diluted in TBS buffer to a final concentration of 25 µM phospholipid on a phosphorous basis. CF leakage upon addition of various concentrations of chitosan (0.5 - 200 µg/ml), reflected by an increase in fluorescence intensity, was monitored over 5 min at 520 nm (excitation at 492 nm) on a Shimadzu RF-5301 PC Series spectrofluorophotometer. The liposome suspension in the cuvette was kept at RT and continuously stirred. The chitosan-induced CF leakage was expressed relative to the total amount of CF released (100% efflux) after disruption of the liposomes by the addition of 20 µl of 20% Triton X-100 [332].

3.4.2.2. Potassium (K^+)-efflux experiments

Potassium efflux was monitored using an MI-442 potassium electrode and MI-409F reference electrode (Microelectrodes, Inc.), connected to a microprocessor pH meter (pH 213; Hanna Instruments). In order to obtain the most stable results, the electrodes were pre-conditioned in choline-buffer for at least 1 h before starting calibration or measurements. Calibration was carried out before each determination by immersing the electrodes in fresh standard solutions containing 0.01, 0.1 or 1 mM KCl in choline buffer. The measured data were processed with the help of Microsoft Excel. Calculations of potassium-efflux in percent were performed according to the equations established by Orlov *et al*. [224].

3.4.2.2.1. Chitosan-induced K^+-leakage from KCl liposomes

Chitosan-induced potassium-leakage from K^+-loaded DOPC:DOPG (1:1) liposomes [section 3.3.2] was determined, after diluting the KCl-liposomes in choline buffer to a final concentration of 250 µM phospholipid on a phosphorous basis. Potassium efflux was monitored in presence of various chitosan concentrations [1 - 200 µg/ml], over a period of 8 min. K^+- leakage was expressed relative to the total amount of potassium

recorded after complete lysis of the liposomes through the addition of 46 µl 30% octylglycoside [289].

3.4.2.2.2. Potassium release from whole cells

The release of K^+ originally present in the cytoplasm of bacterial cells in response to exposure to different concentrations of chitosan was determined as follows: Liquid cultures of the test strains were inoculated from an overnight culture (3% [vol/vol]), and were grown in CAMHB [± 10 mM glucose] in a shaking incubator at 37°C and 150 rpm. Cells were harvested at an OD_{600} of 1.0 - 1.5 (Heraeus Biofuge 28RS; 5,300 rpm, 3 min, 4°C), washed and resuspended in cold choline buffer [± 10 mM glucose] to an OD_{600} of 30. The concentrated cell suspension was kept on ice and used within about 90 min. For each measurement, the cells were diluted in choline buffer [± 10 mM glucose, 25°C] to an OD_{600} of about 3. Measurements were carried out using 2 ml of cell suspension at RT, and the efflux of potassium was monitored over a period of 8 min, in absence or presence of different chitosan concentrations. Chitosan-induced leakage was expressed relative to the total amount of K^+-release (100% efflux) induced by the addition of nisin [1 µM].

3.4.2.3. Leakage of UV-absorbing cellular components and proteins

Bacterial cell membrane integrity was also assessed by measuring the leakage of UV-absorbing cellular components, as well as cellular proteins, upon treatment with chitosan. A culture of the test strain was inoculated from an overnight culture (2% [vol/vol]), and then grown in CAMHB in a shaking incubator at 37°C and 150 rpm to an OD_{600} of 0.5. The bacterial culture was harvested (5,300 rpm, 3 min, 4°C) then washed twice and resuspended in choline buffer. Aliquots of the bacterial suspension were treated with different chitosan concentrations and incubated at 37°C for 30 min. After pelleting the cells twice at 13,400 rpm for 3 min, the absorbance of the cell-free supernatant was measured at 260 nm [A_{260}], taking choline buffer with the respective chitosan concentration as blank.

In addition, the kinetics of cellular leakage was tested by adding chitosan to the bacterial suspension at a specified concentration, and then incubating the suspension at

37°C and 120 rpm. The A_{260} of cell-free supernatants, as well as the OD_{600} of the bacterial suspensions were measured at 5 min intervals over a period of 2 h. The % absorbance was calculated with reference to a culture run in parallel and treated with nisin [1 µM] for 2 h, whereas $\%OD_{600}$ refers to the optical density of the test culture, relative to the original optical density at the start of the experiment.

To test whether proteins leak out of the cells upon treatment with chitosan, aliquots of the cell-free supernatants were flash-frozen under liquid nitrogen, lyophilized and finally rehydrated in deionized water. The protein content of the lyophilized samples was determined in triplicate in acetate buffer (10 mM; pH 6.0), according to the method of Lowry *et al.* [184], using bovine serum albumin (BSA) as standard. In addition, an electrophoretic separation of the protein samples was conducted using common sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [173], with a 4% stacking gel, 10% and 12% resolving gels, and the broad range prestained protein marker (New England Biolabs GmbH). The SDS-PAGE was conducted in a vertical gel apparatus (Bio-Rad Mini-Protean III electrophoresis chamber) at a voltage of 90 - 120 V for 120 - 140 min; the proteins were visualized with coomassie brilliant blue R-250.

3.4.3. MEMBRANE POTENTIAL MEASUREMENTS

3.4.3.1. Estimation of membrane depolarization using [^{3}H]TPP$^+$

For the evaluation of the effect of chitosan on membrane potential ($\Delta\Psi$), a depolarization assay was conducted using [^{3}H] tetraphenylphosphonium bromide ([^{3}H]TPP$^+$), a small lipophilic cation whose equilibrium across the cytoplasmic membrane is indicative of membrane potential. Cells were grown in CAMHB with shaking at 37°C to an OD_{600} of 1.0 then harvested (10,000 rpm, 5 min, 4°C). The cell pellet was resuspended 1:3 in fresh medium, and then incubated for 15 min at 37°C under agitation.

The membrane potential was monitored by adding [^{3}H]TPP$^+$ to a final concentration of 1 µCi/ml of cell suspension. After 5 min of equilibration, the cell

culture was treated with chitosan (5×MIC), and aliquots (100 µl) were withdrawn at specific time intervals before and after chitosan addition, filtered through 0.2-µm-pore-size cellulose acetate membranes (Schleicher & Schuell BioScience GmbH) and washed twice with 50 mM phosphate buffer (pH 7.0). The filters were dried and transferred to counting vials filled with scintillation fluid (Quickszint 100, Zinsser Analytic), and the radioactivity was measured in a Packard 1900CA TRI-CARB liquid scintillation counter. Total radioactivity was determined using unfiltered culture aliquots. The counts were corrected for non-specific [^{3}H]TPP$^+$ binding to cell constituents by subtracting the radioactivity of 10% butanol-treated cell aliquots. The membrane potential was calculated as previously described by Ruhr and Sahl [264].

3.4.3.2. Fluorometric membrane depolarization assay using DiBAC$_4$(3)

The ability of chitosan to depolarize the bacterial cytoplasmic membrane, which would result in the collapse of the diffusion potential, was detected by using the negatively-charged membrane potential-sensitive fluorescent probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC$_4$(3); Molecular Probes – Invitrogen], which enters depolarized cells where it binds to intracellular proteins or membranes and exhibits enhanced fluorescence and red spectral shifts.

Cells were allowed to grow in CAMHB at 37°C with agitation until reaching an OD_{600} of 0.5. Aliquots of the cell suspension were then incubated in the dark for 25 min with 1 µM of DiBAC$_4$(3) at RT. Chitosan was then added to achieve the desired concentrations. Chitosan-induced membrane depolarization was monitored for 15 min (at 5 sec intervals), by observing the change in the intensity of fluorescence emission of DiBAC$_4$(3), using a Shimadzu RF-5301PC Series spectrofluorophotometer at an excitation and emission wavelengths of 492 and 515 nm, respectively. Controls for membrane permeabilization consisted of cells in appropriate medium lacking chitosan; nisin [1 µM] was used as positive control. Experiments were repeated independently at least three times on separate days.

3.4.4. ELECTRON MICROSCOPICAL EXAMINATION OF CELLULAR DAMAGE CAUSED BY CHITOSAN

A liquid culture of *S. simulans* 22 for microscopy was inoculated (1% [vol/vol]) from an overnight culture in CAMHB, and then grown in CAMHB (in a 100 ml-flask) at 37°C and 150 rpm to the early exponential phase (OD_{600} = 0.8 - 1.0), then split into 2 portions: one was treated with chitosan (10×MIC) and incubated at 37°C, while the other served as an untreated control, to which was added sterile medium only. The chitosan concentration was chosen, so that its effect could be seen on a greater percentage of cells. 10 ml-aliquots of the control culture (at zero time) as well as the chitosan-treated bacterial culture (collected after 5, 20 and 60 min of treatment) were harvested, and then prepared for electron microscopy as previously described (section 3.2.12).

3.5. ANALYSIS OF TRANSCRIPTIONAL RESPONSE PATTERNS

The methodological approach towards a transcription analysis in this work included the following steps: sample preparation for chips, isolation of total RNA from *S. aureus* SG511, the transcription of RNA into copyDNA (cDNA) and the concomitant fluorescence-labeling by means of reverse transcription, hybridization of the cDNA onto the *S. aureus*-chip, washing of the hybridized DNA-chips, chip-scanning and image acquisition, and the subsequent analysis of the scan-data.

3.5.1. TRANSCRIPTIONAL PROFILE ANALYSIS OF CHITOSAN-TREATED CELLS

Changes in gene expression patterns resulting from the short-term exposure of *S. aureus* SG511 to a subinhibitory chitosan concentration were detected using a genome-scale gene expression experiment. For the transcription analysis, we used the Scienion *sciTRACER S. aureus* full genome chips, which contain 2338 PCR products from all 2593 protein-coding ORFs in the annotated genome of the source organism *Staphylococcus aureus* N315 (NC_002745, contig BA000018), sequenced by Kuroda *et al.* [172].

Various conditions were first tested to optimize the assay, including different culture media, physiological states of the cultures, chitosan concentrations, as well as contact times. An outline of the procedure, with which a competitive comparison of two samples was carried out through hybridization to a DNA-microarray-chip, is depicted below (Figure 3). To minimize the technical and biological variations and to ensure that the data obtained were of good quality, each RNA preparation was used to make probes for two separate arrays for which the incorporated dye was reversed. In addition, three independent cultures were used to prepare RNA samples.

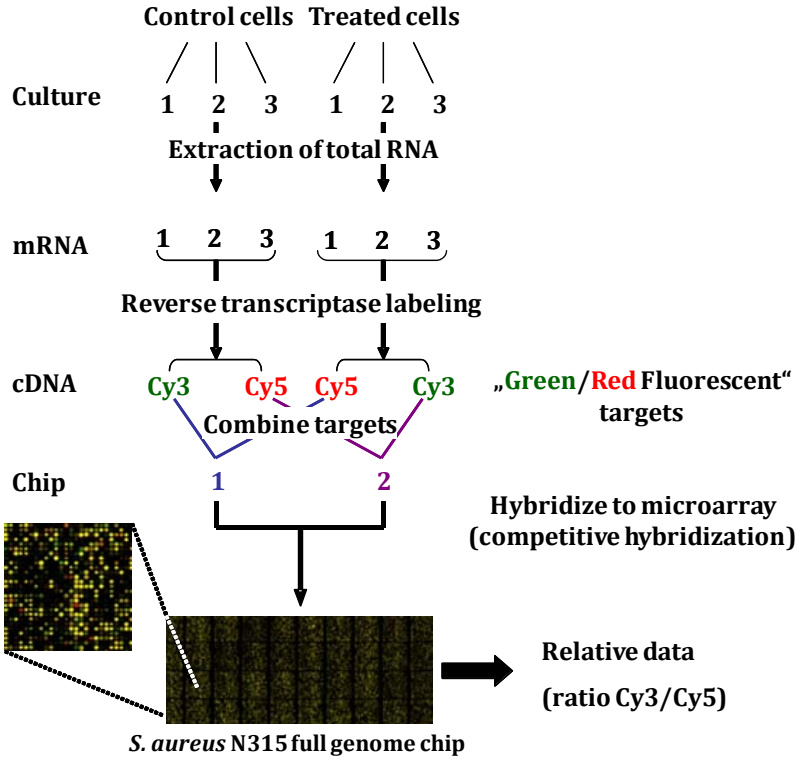

FIGURE 3: Experimental setting – microarray

A dye-swap hybridization experiment serves to compensate for signal correlation bias and thereby maximize the statistical significance of the data.

3.5.1.1. Preparation of bacterial samples

Cultivation of *S. aureus* SG511 was performed using a 2% [vol/vol] inoculum from an overnight culture, then allowing the cells to grow in CAMHB at 37°C (150 rpm) to the early log phase (OD_{600} = 0.8). Culture aliquots were either treated with chitosan

[15µg/ml] for 20 min (37°C, 150 rpm), or left untreated (control), and then collected and immediately stabilized by adding 2 volumes of RNAprotect™ Bacteria Reagent (QIAGEN GmbH), prewarmed to 37°C, to ensure reliable gene expression. The cell pellets were then stored at -70°C until processed.

3.5.1.2. Isolation of total RNA from *S. aureus* SG511

For RNA isolation, the cell pellets were lysed in 700 µl TE-buffer in presence of 300µg lysostaphin (Dr. Petry Genmedics GmbH), by incubation for 60 min at 37°C, with intermittent vortexing for 5 sec at 5 min intervals. Total RNA was extracted using the *PrestoSpin* R Bug RNA Purification Kit (Molzym GmbH & Co.KG), according to the manufacturer's instructions. DNA degradation was achieved on the spin-column prior to RNA elution by the addition of 40 U of RNase-free DNase I followed by incubation for 20 min at RT. The RNA was repeatedly eluted in 80 µl of DMPC-treated Aqua dest (70°C), and its concentration and purity were assessed photometrically, using the NanoDrop® ND-1000 spectrophotometer V3.3. The purity of the RNA samples was indicated by the ratios of sample absorbance at 260 and 280 nm [A_{260}/A_{280}] and at 260 and 230 nm [A_{260}/A_{230}], which should lie in the range of 1.9 – 2.1, and 1.8 - 2.2, respectively.

3.5.1.3. Reverse transcriptase labeling of mRNA

For the labeling reaction, around 9 µg of total RNA (from 3 different preparations) from each of the respective targets to be competitively compared were mixed with 1 µl of random hexamer primers (N_6, 3 µg/µl) on ice in a total volume of 15 µl nuclease-free water. This "annealing mix" was denatured by heating for 7 min at 70°C, and then chilled on ice for around 5 min. In the meantime the following master mix (labelling mixture) was prepared:

	1× chip	2× chip
5×first strand reaction buffer	4.4 µl	8.8 µl
dNTP-mix with low dCTP content*	4.4 µl	8.8 µl
RNase Out (40 U/µl)	1.1 µl	2.2 µl
Nuclease-free water	6.6 µl	13.2 µl
Total volume	16.5 µl	33 µl

* It consists of: dATP, dGTP, dTTP [0.5 mM] and dCTP [0.2 mM].

The annealing mix was mixed with 15.5 µl of the master mix, and used for the following reaction:

Reaction mix:

Annealing mix	15 µl
Master mix	15.5 µl
cyanine-3'-dCTP "Cy3" or cyanine-5'-dCTP "Cy5"	4 µl
BioScript reverse transcriptase (200 U/µl, Bioline)	0.5 µl
Total volume	35 µl

The reaction mix was incubated at 42 °C for 1 h, then 0.5 µl Bioscript was added and the mix was incubated for additional 1 h at 42 °C. The cDNA-synthesis reaction was stopped by the addition of 5 µl EDTA (0.5 M; pH 8.0). For alkaline hydrolysis of the RNA, 10 µl NaOH (1 N) were added and the mixtures incubated at 65°C for 15 min. The mixtures were cooled down to RT, and 25 µl Tris-HCl (1M, pH 7.5) were added for neutralization. The labeled targets were purified using the MinElute™ PCR Purification Kit (Qiagen GmbH), following the manufacturer's instructions. The concentration of cDNA in the probes, its purity ($A_{260}/A_{280} \sim 1.8$), as well as the fluorescent dye labeling density of the probes, were measured using the NanoDrop® ND-1000 spectrophotometer V3.3.

3.5.1.4. Hybridization and washing of the *S. aureus*-chips

The differentially-labeled cDNA targets to be compared were combined and competitively hybridized to the custom *sciTRACER* chips (Scienion AG) in a dye-swap hybridization experiment (Figure 3). The hybridization chamber (*sciHYBCHAMBER*) was set up for hybridization, by applying 20 µl of purified water in each of its four water reservoirs, to ensure a constant humidity in the chamber during the hybridization and prevent evaporation of hybridization liquids and subsequent drying out of the array. A coverslip (24 × 60 mm) cut in two halves in length and put on top of each other served as a spacer, when put at the bottom of the hybridization chamber. For the hybridization of a DNA-chip, 45 µl of the hybridization buffer *sciHYB* (pre-warmed to 42°C) were

combined with the labeled cDNA targets in a total volume of 55 µl. The hybridization mixture was denatured (2 min at 95°C) and then applied carefully onto the *sciTRACER* chip. The chip was placed into the prepared hybridization chamber, which was tightly closed and then carefully placed in a tempered water bath (42°C for 72 h) to allow for hybridization.

Unbound cDNA was removed from hybridized microarrays through several washing steps at RT in SSC solutions with decreasing ionic strength: washing solution 1 (1 × SSC, 0.03% [wt/vol] SDS, 30°C; 5 min), washing solution 2 (0.2 × SSC; 5 min) and washing solution 3 (0.06 × SSC; 1 min). The washed chips were immediately spun-dried at RT (Heraeus Multifuge 1 S-R, 500 rpm; 5 min). The dry hybridized chips were stored at 4°C, protected from light in a dark, dry, air-tight slide box, until scanned.

3.5.1.5. Laserscan of the DNA chips

The hybridized chips were scanned using a GenePix 4000B array scanner (Axon Instruments Inc.). Low-resolution prescans were performed on each microarray before higher resolution scanning to balance the overall fluorescence intensity of the entire microarray between the two dyes. The photomultiplier tube (PMT) gain settings were assessed and slightly adjusted for each individual microarray to achieve optimal balance with the least amount of post-scanning processing and normalization. The final settings were as follows: PMT gain [ranging from 550 to 700]; laser power [100%]; resolution [10 µm]; lines to average [2] and focus position [0], with the image balance set to minimum and maximum intensities of 500 and 65530, respectively, to give a count ratio of around 1.0. Scans were saved as 16-bit multi-image TIFF files.

3.5.1.6. Data acquisition and analysis

Image acquisition and analysis of the scan data was achieved with the help of GenePix® Pro 4.1 microarray informatics software (Axon Instruments Inc.), where the relative transcript level for each spot of the microarray was quantitated using the local background subtraction method to reduce the effect of non-specific fluorescence. To compensate for non-linear dye-bias, the data sets thus generated were corrected by

applying LOWESS (**lo**cally **we**ighted **s**catterplot **s**moothing) normalization [354], and subsequently merged using acuity 3.1 software (Axon Instruments Inc.).

Significant changes in gene expression were identified with SAM 1.21 software (significance analysis of microarrays; http://www-stat.stanford.edu/~tibs/SAM/index.html) [322], using the one class response type, generating a list of significantly up- and down-regulated genes, at a false discovery rate (FDR, percentage of genes identified by chance) < 1%. The genes were classified with regard to their function using our in-house gene annotation database, establishing regulation patterns, which were linked to the experimental setting, and compared to the results published in literature.

3.5.2. DIFFERENTIAL EXPRESSION PROFILE OF *S. AUREUS*

3.5.2.1. Sample preparation and isolation of total RNA

Cultivation was performed using a 2% [vol/vol] inoculum from an overnight culture, then allowing the cells to grow in CAMHB at 37°C and 150 rpm to the early log phase (equivalent to a viable count of around 10^9 CFU/ml). Stabilization of culture aliquots and subsequent RNA isolation were done as previously described in sections 3.5.1.1 and 3.5.1.2, respectively. The absence of remaining DNA traces was evaluated by quantitative PCR (SDS 7700; Applied Biosystems, Framing-ham, MA) with assays specific for 16s rRNA [253,274]. All the following steps were kindly carried out by Dr. Patrice François (Genomic Research Laboratory, Geneva University Hospitals, Switzerland).

3.5.2.2. Microarray design and manufacturing

The microarray was manufactured by *in situ* synthesis of 10,807 different oligonucleotide probes of 60 nucleotides length (Agilent), selected as previously described [35]. It covers approximately 96% of all ORFs annotated in strains N315 and Mu50 [172], MW2 [7], COL [100], NCTC8325 [101], MRSA252 and MSSA456 [130] and USA300 [64], including their respective plasmids. Extensive experimental validation of this array has been described previously, using CGH, mapping of deletion, specific PCR and quantitative RT-PCR [35,274].

3.5.2.3. Expression microarrays

Batches of 5 µg total *S. aureus* RNA were labelled by Cy-3 or Cy-5 dCTP using the SuperScript II (Invitrogen) following manufacturer's instructions. Labelled products were then purified onto QiaQuick columns (Qiagen) and RT 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 hours in a dedicated hybridization oven (Robbins Scientific). Slides were washed with Agilent proprietary buffers, dried under nitrogen flow, and scanned (Agilent) using 100% PMT power for both wavelengths.

3.5.2.4. Microarray analysis

Fluorescence intensities were extracted using the Feature extraction™ software (Agilent, version 8). Local background-subtracted signals were corrected for unequal dye incorporation or unequal load of labelled product. The algorithm consisted of a rank consistency filter and a curve fit using the default LOWESS method. Data consisting of two independent biological experiments were analyzed using GeneSpring 7.3 (Agilent). An additional filter was used to exclude irrelevant values. Background noise of each experiment was evaluated by computing the standard deviation of negative control intensities. 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 [161]. Fluorescence values for genes mapped by 2 probes or more were averaged. Statistical significance of differentially expressed genes was identified by variance analysis (ANOVA) [48,274], 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.

4. RESULTS AND DISCUSSIONS

4.1. CHITOSAN'S *IN VITRO* ANTIMICROBIAL ACTIVITY — A CRITICAL LOOK

4.1.1. SUMMARY

Chitosan's antimicrobial activity was more pronounced against Gram-positive than Gram-negative bacteria. Furthermore, it was related to the molecular weight of the polymer; oligosaccharides exhibited a much lower activity than the long-chained polymer. Chitosan, whose *in vitro* antimicrobial activity was greatly influenced by several factors, including the bacterial growth medium and the presence of metal ions, exhibited a dose-dependent bacteriostatic effect.

Chitosan had the ability to flocculate bacterial cells, an ability that was both strain- and dose-dependent. Using a microtiter plate assay, we could observe a marginal anti-biofilm activity of chitosan; further investigations are however warranted. No direct correlation could be found between the chitosanolytic ability of test strains, and their susceptibility to chitosan.

4.1.2. RATIONALE AND OBJECTIVES

A survey of the available literature on the antimicrobial activity of chitosan reveals wide discrepancies between the various reports, which could, at least in part, be traced back to the large number of factors which influence this activity. However, understanding these factors has become a key issue for a better usage of chitosan formulations. Therefore, in this part, we tried to tackle a number of facts and myths about chitosan, with the aim of clarifying some of the prevailing views about its antimicrobial activity as well as its interaction with bacterial systems.

Using standard microbiological methods, we investigated in this section the *in vitro* antimicrobial activity of chitosan, including spectrum of activity, potency as well as factors affecting its antimicrobial activity. In addition, other properties of chitosan, such

as flocculation and anti-biofilm properties, as well as its degradation were addressed. Our goal was also to optimize a system for studying the antimicrobial activity of chitosan, and to set guidelines to be used in the upcoming assays. The results obtained here should also contribute to an increasingly complete picture of the antimicrobial potential of this compound.

4.1.3. DEFINING CHITOSAN'S SPECTRUM OF ACTIVITY

In order to identify the most suitable indicator strains to be used for evaluating chitosan's antimicrobial activity, we performed preliminary studies, where we screened a large number of Gram-negative as well as Gram-positive organisms, clinical isolates and standard strains, in order to establish the antimicrobial spectrum of this compound.

All the tested Gram-negative bacteria (*E. coli* and *Ps. aeruginosa* strains) were insensitive to the antimicrobial activity of chitosan, with MIC values of ≥ 1000 μg/ml; on the other hand, Gram-positive strains were much more sensitive, with MIC values as low as 1 μg/ml (see below). These results are consistent with previous studies, reporting a stronger bactericidal effect of chitosan for Gram-positive bacteria [215]. This is why we decided to focus our work on Gram-positive bacteria, using a number of indicator strains belonging to several species (Table 15).

4.1.4. WORKING WITH CHITOSAN — CHOICE OF CHITOSAN GRADE

Probably the first challenge one meets when working with chitosan is to choose a product out of the large number of commercially available chitosan grades; as a matter of fact, the choice of the most suitable grade for use is mostly related to the application intended. Since our aim was to study the antimicrobial activity of chitosan, we took the MIC value as a measure of chitosan's potency, and hence compared the activity of various chitosan samples of various sources (Table 3 and Figure 4) based on this measure. These chitosan samples were of widely different MW distributions, ranging from oligosaccharides having less than 15 glucosamine units/chain (chitosan oligosaccharide lactate, COS) to polymers of up to 1000 kDa; the DD ranged from 75 - > 95% (Table 3).

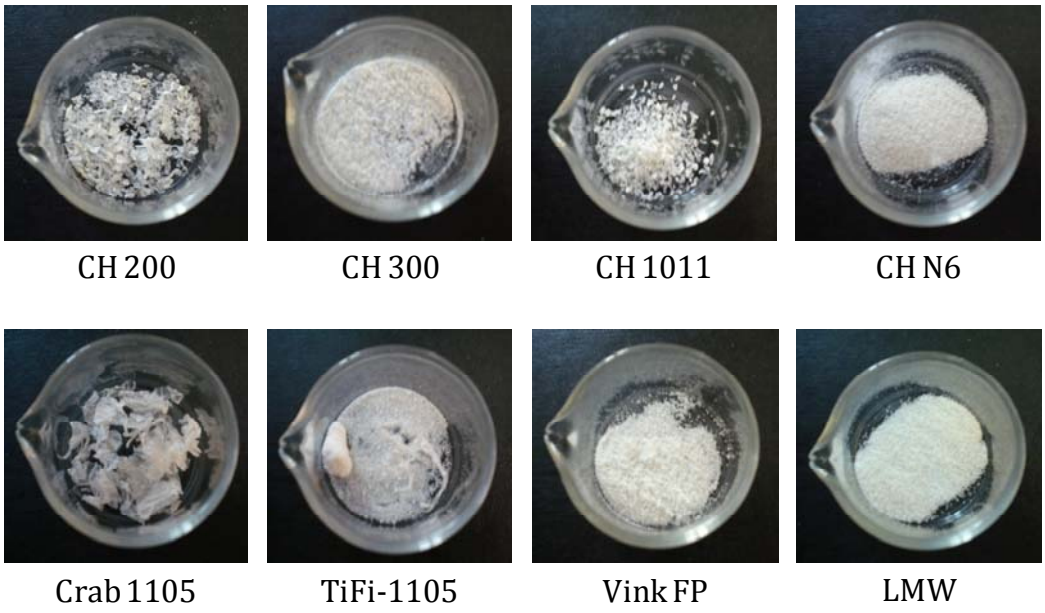

FIGURE 4: Heterogeneity of chitosan samples.

Some of the chitosan samples tested during the preliminary studies of this work are shown above to demonstrate their heterogeneity; they ranged from fine to coarse powders, small and large flakes, to mixtures thereof.

The potency of chitosan against a number of Gram-positive strains was determined through MIC estimations using the broth microdilution technique, as described in section 3.2.3. This technique was deemed most suitable for our work with chitosan, since other methods such as agar dilution resulted in a massive reduction in the antimicrobial activity of chitosan, probably due to strong binding of the polycationic biopolymer with the anionic agar, resulting in precipitation of the former and loss of its activity. However, we observed no significant difference in the respective MIC values of the various chitosan samples against the tested strains (Table 17). With the exception of oligosaccharides, which were almost completely inactive, the remaining samples were found to be more or less equally active. These data were further substantiated with the help of K^+-efflux assays (section 3.4.2.2.2) using several of these chitosan grades at a concentration of 60 µg/ml, and using *S. simulans* 22 as an indicator strain; indeed, no significant difference in potassium efflux induced by all tested samples was observed.

TABLE 17: MIC of various chitosan grades against selected indicator strains in CAMHB

Chitosan sample	*S. aureus* SG511		*B. subtilis* 168		*S. simulans* 22		*S. epidermidis* SE O-47		*S. aureus* SA NCTC 8325 SH 1000	
	MIC (µg/ml)*									
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
LMW	39.06	39.06	5.86	5.86	1.95	1.95	19.53	19.53	78.16	78.16
MMW	N/A	N/A	23.44	23.44	2.93	2.93	N/A	N/A	N/A	N/A
HMW	N/A	N/A	11.72	11.72	0.98	0.98	N/A	N/A	N/A	N/A
PGC	N/A	N/A	15.63	15.63	1.95	1.95	N/A	N/A	N/A	N/A
COS	N/A	N/A	1500	1500	1000	1000	N/A	N/A	N/A	N/A
CH 200	39.06	39.06	6.51	6.51	2.85	4.07	19.53	19.53	78.16	78.16
CH 300	39.06	39.06	6.51	6.51	2.44	3.25	19.53	19.53	78.16	78.16
CH 1011	19.53	19.53	8.14	8.14	2.85	2.85	19.53	19.53	39.06	39.06
CH N6	19.53	19.53	4.88	4.88	2.44	2.44	19.53	19.53	39.06	39.06
Vink FP	39.06	39.06	5.70	5.70	2.03	3.66	19.53	19.53	156.25	156.25
Tifi-1105	78.13	78.13	6.51	6.51	2.44	2.44	19.53	19.53	78.16	78.16
Crab 1105	78.13	78.13	11.39	11.39	3.25	3.25	19.53	19.53	78.16	78.16

Abbreviations (refer to Table 3): LMW, low MW chitosan; MMW, medium MW chitosan; HMW, high MW chitosan; PGC, practical grade chitosan; COS, chitosan oligosaccharide lactate; CH 200, chitosan 85/200/A1; CH 300, chitosan 85/300/A1; CH 1011, chitosan flake 1011; CH N6, chitosan N6 from shells of crustaceans; Vink FP, Vinkocos p-6N FP; Tifi-1105, TiFi-chitosan from 1105; Crab 1105, crab chitosan from 1105; N/A, not available.

* Average of 3 determinations.

We therefore carried out a gel electrophoretic analysis of the different chitosan samples (section 3.3.1.3). Despite the absence of specific molecular mass markers applying to the separation of chitosan, we assumed that the resulting electrophoretic profiles could shed some light on the relative importance of various chitosan fractions in a complex chitosan solution, and the relative molecular weight distribution of such a sample. The electrophoretic profiles were however almost identical, revealing the heterodispersity of the chitosan samples, which is quite expected for chitosan prepared under relatively harsh conditions (acids and bases).

Since all grades of chitosan showed similar antibacterial activities, we performed a dialysis of aliquots of each grade of chitosan against acidulated water (Slide-A-Lyzer® 10K dialysis cassette, Pierce, IL, U.S.A.) under stirring for 48 h; the dialysis solution being changed every 8 h. Our aims were as follows: i) to ascertain that the antimicrobial activity observed is due to chitosan itself, and to rule out the possibility that it is due to the presence of certain impurities in the chitosan samples, stemming from its manufacture; and ii) to check whether short-chain chitosan fragments advertently present in each sample are responsible for the observed antimicrobial activity. However, dialysis had no significant effect on the antimicrobial activity of chitosan against *S. aureus* SG511 and *S. simulans* 22 in CAMHB, indicating that molecules larger than 10 kDa were indeed responsible for the detected activity.

Because of the importance of the molecular weight range [158,215,255] and degree of deacetylation [281] on chitosan's antimicrobial properties, it was vitally important to provide an adequate characterization of the polymer sample used in the following assays. For that purpose, we subjected the LMW chitosan (Sigma Aldrich Chemie GmbH, Germany) to a detailed characterization (Table 18), including molecular weight, degree of deacetylation and viscosity determinations, with the aim of using this characterized batch of chitosan for all subsequent experiments.

Molecular weight determinations were carried out using size exclusion chromatography (SEC), which allows the separation of polydisperse polymers according to their size. Unlike monodisperse substances, no exact molecular weight is indicated, but rather a number of different means are defined, to describe the sample statistically (Table 18). The LMW chitosan sample had a degree of deacetylation of 87%± 2%, which correlated well with the reported value. On the other hand, the determined molecular weight differed from the reported one (243.17 kg/mol and 50-190 kDa, respectively), which sheds some light on the great variability in the molecular weight estimations of chitosan (depending on the method used), the inaccuracy of certain methods, and the need to unify such methods, in order to be able to correlate published data.

TABLE 18: Characteristics of LMW chitosan

	M_w (kg/mol)	**M_n** (kg/mol)	**M_w/M_n**	**[η]** (dl/g)	**R_h** (nm)	**DD** (%)
	243.17	83.55	2.91	5.0	24.59	87
RSD [%]	1.5	13.4	11.7	1.5	1.4	2

Abbreviations: M_w, weight-average molecular weight (value typically indicated as the molecular weight of polymers); M_n, number-average molecular weight; M_w/M_n, polydispersity; [η], Intrinsic viscosity; R_h, hydrodynamic radius; DD, degree of deacetylation.

In addition, the value of polydispersity, which states the relation of the weight average molecular weight (M_w) to the number average molecular weight (M_n), and is thus a measure for the width of the molar mass distribution of a polymer, indicated that this chitosan sample was fairly heterogenous, encompassing polymers of widely-ranging molecular weights. Another very important value is the hydrodynamic radius R_H. In practice, macromolecules in solution are non-spherical, dynamic (tumbling), and solvated. As such, the radius calculated from the diffusional properties of the particle is indicative of the apparent size of the dynamic hydrated/solvated particle; the hydro-dynamic radius includes both solvent (hydro) and shape (dynamic) effects.

This characterized sample of LMW chitosan was used for all the following assays; for convenience, it will be referred to as “chitosan” throughout this work.

4.1.5. WORKING WITH CHITOSAN — ACIDS AS CHITOSAN SOLVENTS

Inorganic and organic acids are commonly used for preparing chitosan solutions; in this study, we used 0.05 N HCl and 1 % acetic acid, respectively, as chitosan solvents, and compared the MIC values of both solutions for the indicator strains *B. subtlis* 168, *S. simulans 22* and *S. aureus* SG511. We found no significant difference in antimicrobial activity, and thus chose to use acetic acid as solvent, due to fear that depolymerization might take place at a higher rate in inorganic acids than in organic acids (Dr. W. Lindenthal, personal communication). The prepared stock solutions (1% [wt/vol] in 1% HAc) were stored at 4°C for further use, where they remained stable [51].

To verify that the observed activity of chitosan was not a mere "acid stress", we measured the pH of various chitosan solutions in CAMHB, and found that at the relevant concentrations (0.61 – 156.25 μg/ml), the pH was around neutrality (6.40 – 7.00). Moreover, appropriate MIC controls were carried out for some selected test strains (*S. simulans* 22, *B. subtilis* 168 and *S. aureus* SG511) in CAMHB using the acid solvent alone. Results indicated that the observed antimicrobial activity was intrinsic to chitosan, and was not conveyed by the acid solvent; cultures in wells showing growth and in those lacking visible growth had the same pH value.

4.1.6. ASSESSING CHITOSAN'S *IN VITRO* ANTIMICROBIAL ACTIVITY

Although the antimicrobial activity of chitosan is well observed on a wide variety of microorganisms, there are wide discrepancies in the reported potencies of chitosan in the available literature, possibly because it is affected by a variety of factors. We carried out several tests, to assess the influence of a number of these factors, including the culture medium, presence of metal and sodium ions, as well as the physiological state of the culture.

One factor that seemed to greatly influence the antimicrobial activity of chitosan is the bacterial growth medium. We tested the activity of chitosan against three indicator strains in 4 different media (Table 14). Judging by the MIC values, chitosan showed highest activity in CAMHB, only a residual activity in PYG and B-broth, and almost no activity in CDM (Figure 5). Apparently, chitosan activity was strongly inhibited in PYG, B-broth and CDM, probably due to their high phosphate content, and the fact that chitosan is insoluble in presence of phosphate ions [235]. Therefore, unless otherwise indicated, CAMHB was used as the standard culture medium throughout this work.

The effect of metal ions on the antimicrobial activity of chitosan was tested by determining its MIC for *S. simulans* 22 in CAMHB in presence of 4 different concentrations (5, 20, 50 and 100 μM) of Fe^{2+} and Zn^{2+}, respectively. We found that these divalent metal cations reduced chitosan's antibacterial activity in a dose-dependent manner (Figure 6).

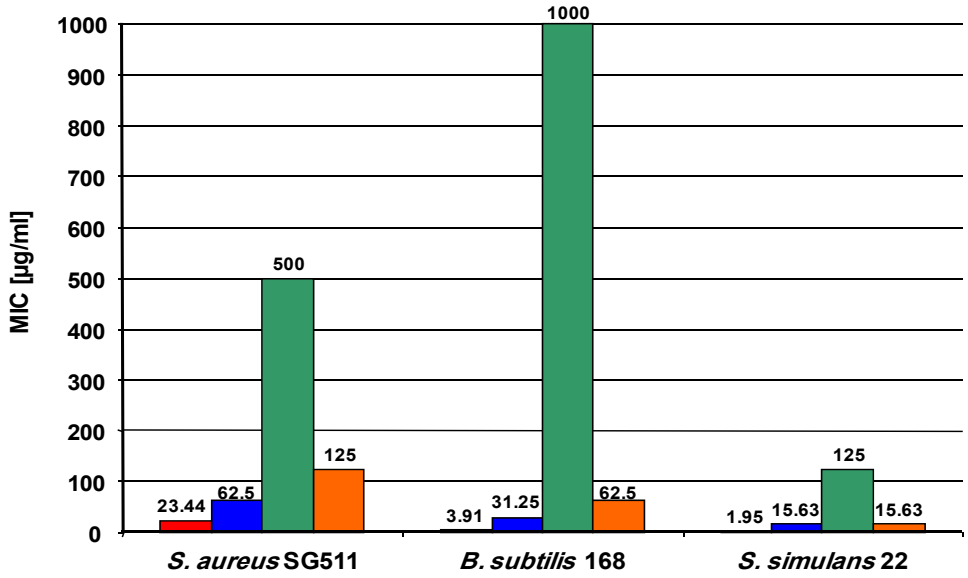

FIGURE 5: Effect of culture medium on the activity of chitosan.

Histograms indicate values of the MIC of chitosan (after 24 h) for the three indicator strains (*S. aureus* SG511, *B. subtilis* 168 and *S. simulans* 22) in different culture media: CAMHB (red), PYG (blue), CDM (green) and B-broth (orange). The highest activity was observed in CAMHB.

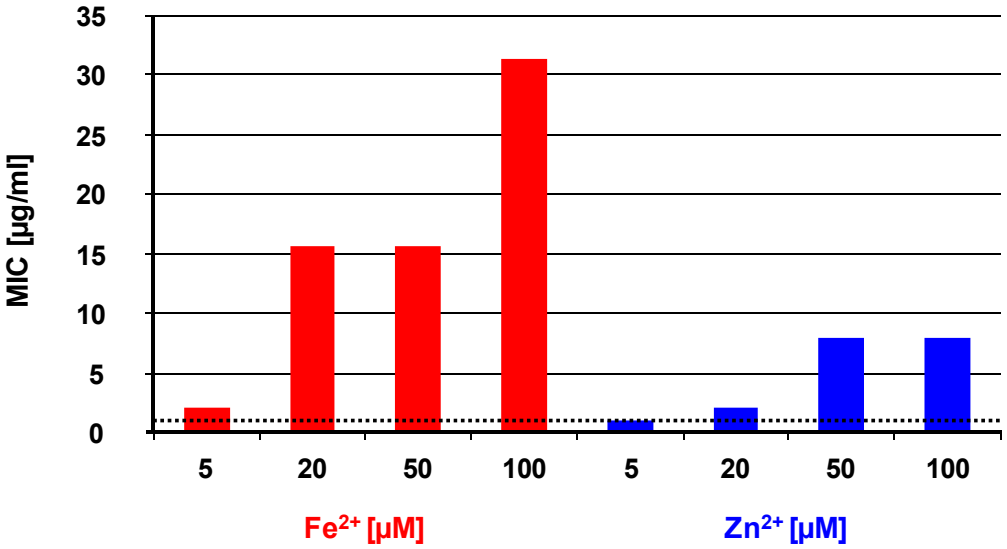

FIGURE 6: Effect of divalent metal cations on the antimicrobial activity of chitosan.

The MIC of chitosan for *S. simulans* 22 was determined in presence of divalent cations (Fe^{2+} and Zn^{2+}), each added to CAMHB at the indicated µM concentration. The dashed line indicates the control MIC of chitosan in CAMHB lacking cations.

These results were consistent with reported data [306,318], and with the *in vitro* chelating ability of chitosan and its derivatives towards transition metal salts, which is presumably due to the large number of amino groups (nitrogen content = 6.89%) which could bind to metal ions through a strong covalency [17,68], resulting in a dramatic reduction in the antibacterial activity of chitosan. These results are also in agreement with a previous observation that chitosan-induced permeability changes in plant cells (*Glycine max*) were strongly inhibited by divalent cations [361].

On the other hand, the presence of glucose [10 mM] or NaCl [10 or 25 mM], respectively, had no detectable effect on the antimicrobial activity of chitosan against the tested indicator strains. Several researchers have attempted to evaluate the effect of ionic strength on the antibacterial activity of chitosan, and have reached contradictory conclusions. Chung *et al.* [47] stated that chitosan's antibacterial activity increased with increasing ionic strength of the solution regardless of the tested strain, and reasoned that the higher ionic strength might enhance the solubility of chitosan and thus increase its antibacterial activity. On the other hand, Taha and Swailam [306] and Tsai and Su [318] claimed that the presence of sodium ions [100 mM] reduced chitosan's activity against *E. coli*.

4.1.7. CHITOSAN – A BACTERIOSTATIC OR BACTERICIDAL AGENT?

Chitosan's antimicrobial activity varied greatly against strains (Table 17); moreover, it was bacteriostatic, rather than bactericidal, since its MBC values for *S. aureus* SG511, *B. subtilis* 168 and *S. simulans* 22 in CAMHB were 62.50, 6.10 and 3.91µg/ml, respectively, and thus slightly higher than the corresponding MIC values.

The antimicrobial activity of chitosan was also investigated by means of killing assays, conducted as described in section 3.2.7.1. First, we studied the kinetics of chitosan's antimicrobial activity against two of our indicator strains, *B. subtilis* 168 and *S. simulans* 22 (Figure 7, a and b, respectively).

After an initial drop in the number of survivors observed in presence of 10× the MIC of chitosan, the culture more or less quickly resumed growth, which might be attributed to physiological adaptation of the cells to the stress exerted upon them by chitosan; another indication of the inhibitory effect of chitosan.

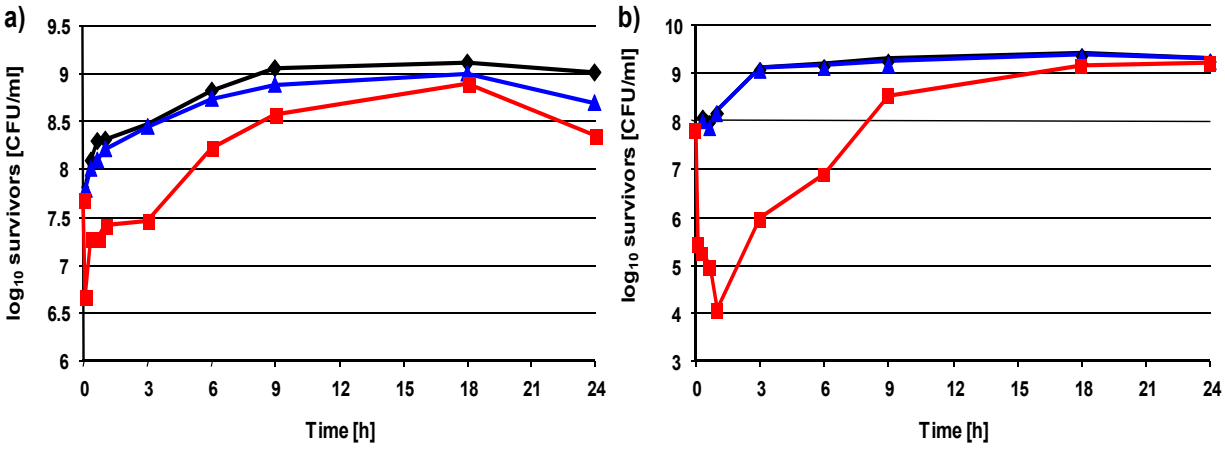

FIGURE 7: Killing of indicator strains in presence of chitosan.

Approximately 10^7 CFU/ml of *B. subtilis* 168 (a) and *S. simulans* 22 (b), both indicated by black diamonds (♦), were incubated with 2 different chitosan concentrations, equivalent to 2× (▲) and 10× MIC (■), and incubated with shaking at 37°C. Surviving counts were estimated and plotted as a semi-logarithmic growth curve.

Studying the growth of bacterial populations in batch cultures does not permit any conclusions about the growth behavior of individual cells, since in any randomly growing culture the distribution of cell age among the members of the population is completely random. In this study, we attempted however to determine at which phase in a bacterial growth cycle the population would be most susceptible to the activity of chitosan (Figure 8). Unexpectedly, the addition of chitosan to *S. simulans* 22 cells in the stationary phase yielded lower viable counts than in the late logarithmic phase (Figure 8), suggesting that, other than the inoculum size, the physiological state of the bacterial cells influences their susceptibility to chitosan. This is in contrast to the observations of Tsai and Su [318] who found that *E. coli* cells were most susceptible to the antimicrobial action of chitosan in the late exponential phase.

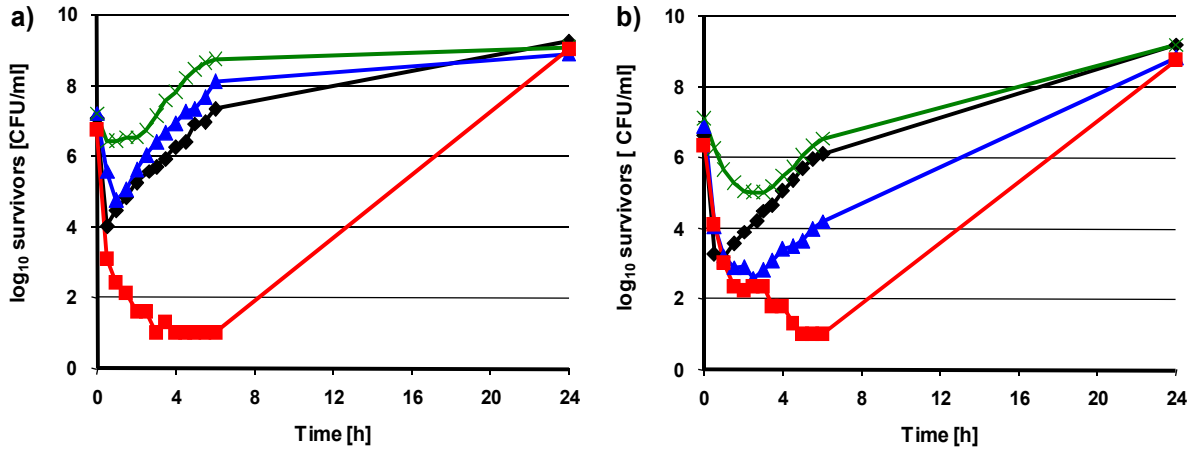

FIGURE 8: Effect of bacterial physiological state on chitosan activity.

Chitosan was added to liquid cultures of *S. simulans* 22 in CAMHB at concentrations corresponding to 2× (a) and 4× MIC (b); and the killing was determined at OD_{600} = 0.1 (♦), 1.2 (▲), 5.8 (×) and 13.9 (■), after appropriate dilution of each of the cultures to an OD_{600} of 0.1. Controls were also conducted alongside the experiment, with cells in appropriate medium lacking chitosan.

When the effect of a number of chitosan concentrations on *S. aureus* SG511 was tested, again we found that chitosan had a dose-dependent growth inhibitory effect (Figure 9a). At 10×MIC, the number of surviving cells was drastically reduced within 6 h to below the detection limit. This apparent killing phase was however followed by regrowth, which might be attributed to the emergence of small colony variants (SCVs, Figure 9b). SCVs constitute slow-growing subpopulations of some *S. aureus* strains that are generally more resistant to the action of antimicrobials than their parent strain [12]. Two intrinsic metabolic characteristics of *S. aureus* SCVs which contribute to the increased resistance to cell wall-active antibiotics and aminoglycosides [247], as well as cationic peptides [167] are their decreased metabolism, leading to slow growth [247] and their reduced uptake of drugs (such as aminoglycoside antibiotics) due to a significant reduction in the electrochemical gradient [179]. The metabolic alterations that result in the altered phenotype can be explained on the basis of defects in menadione and hemin biosynthesis resulting in interruptions in electron transport and decreased ATP production.

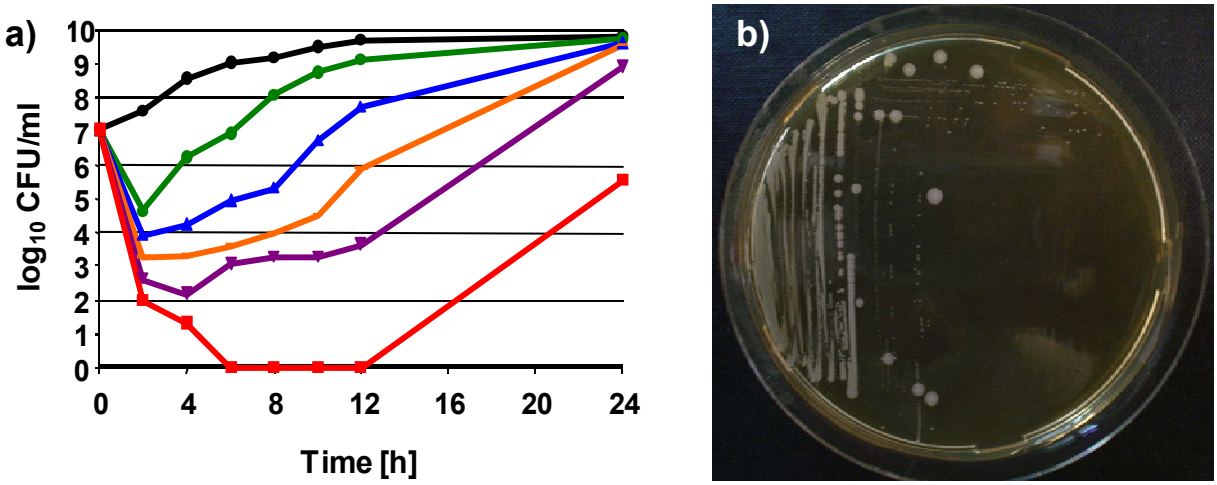

FIGURE 9: Effect of chitosan on growth kinetics of *S. aureus* SG511, and the emergence of SCVs.

a) Numbers of survivors (in log units) of *S. aureus* SG511 (starting inoculum: 1.15×10^7 CFU/ml) in CAMHB at 37°C, in presence of 0 (●), 0.5× (●), 1× (▲), 2× (—), 5× (▼) and 10× (■) MIC of chitosan as determined after 24 h. b) A MHA plate showing a mixture of the normal (large, round colonies) and the small colony variant (pin-point colonies) phenotypes of *S. aureus* SG511.

SCVs are auxotrophic for menadione, thiamine or hemin, with occasional thymidine auxotrophs. Menadione and thiamine are involved in the synthesis of menaquinone, whereas hemin is required for the synthesis of cytochromes. Supplementation with hemin, menadione or thiamine reverses the SCV phenotype [247].

The SCV investigated in this study was phenotypically stable upon multiple passages on solid media, whereas reversion to the parental phenotype was occasionally observed in liquid medium. The parental strain (*S. aureus* SG511) demonstrated a typical *S. aureus* phenotype and was haemolytic on blood agar plates, whereas the SCV colonies were notably smaller (pin-point appearance), had an unstable colony phenotype, diminished haemolytic activity, decreased pigmentation, DNase and coagulase activities (coagulase-positive by the tube test only after incubation for > 18h), delayed mannitol fermentation, impaired growth rate (doubling of generation time as compared to the wild-type) and reached conspicuously lower cell densities. Most of these characteristics can be tied together by a common thread, namely alterations in

electron transport. In addition, the SCV was auxotrophic for menadione (i.e. electron-transport-defective), since supplementation with menadione reversed the phenotype.

The SCV was less susceptible than the parent strain to chitosan, chloramphenicol, kanamycin, co-trimoxazole, rifampicin and oxacillin. Differences in MIC values ranged from 1.5- to 24-fold (after 48 h of incubation), depending on the antimicrobial agent (Table 19). On the other hand, the MIC values of vancomycin and ciprofloxacin remained unchanged, similar to previous reports [12,45].

CAMHB contains starch as well as hydrolyzed casein and meat; however, staphylococci are unable to degrade starch when growing in CAMHB (which lacks glucose), and therefore grow at the expense of aminoacids, raising the medium pH to approximately 9. Baumert *et al.* [12] therefore suggested that the addition of glucose to the medium might result in rapid degradation of glucose by the SCVs, thereby generating a membrane potential of sufficient magnitude to allow it to take up gentamicin, which in turn would enhance its killing efficiency against the SCVs. However, just like in their study, we observed no appreciable difference in the MIC values of chitosan, kanamycin and oxacillin in CAMHB supplemented with glucose (5 mM).

TABLE 19: **Susceptibility of *S. aureus* SG511 and its SCV to various antimicrobial agents**

Antimicrobial agent	***S. aureus* SG511 (WT)**			**SCV-phenotype**		
	MIC (µg/ml)*		MBC (µg/ml)*	MIC (µg/ml)*		MBC (µg/ml)*
	24 h	48 h		24 h	48 h	
Chitosan	23.44	23.44	31.25	15.63	187.5	250
Chloramphenicol	6.25	12.5	-	18.75	18.75	-
Kanamycin	0.78	0.78	-	6.25	6.25	-
Co-trimoxazole	2	2	4	2	4	> 128
Rifampicin	≤ 0.1	0.125	0.125	3	3	3
Oxacillin	0.38	0.5	0.5	0.625	1.13	2.13

*: Results are the average of 2 determinations.

4.1.8. CHITOSAN – A FLOCCULATING AGENT?

The concept behind this assay was to test the effect of chitosan on the flocculation behavior of bacteria, since chitosan is known to cause flocculation of microorganisms [291], and we did observe that cells of some indicator strains tend to flocculate upon its addition. The chitosan concentrations tested were 0.5, 1.25, 2.5, 5, 12.5, 25, 50, 100 and 200 µg/ml. Results showed that the flocculating effect of chitosan was both strain- and concentration-dependent. There was a positive correlation between chitosan concentration and the flocculation of *B. subtilis* 168 and *S. simulans* 22 cells up to a concentration of 50 and 25 µg/ml, respectively, then flocculation decreased with increasing chitosan concentration, indicating that for each strain, a concentration of maximum flocculation existed. On the other hand, the various chitosan concentrations had no appreciable flocculating effect on *S. aureus* SG511 (Figure 10).

Although chitosan is known to cause flocculation of microorganisms, a property which allows its use for example in wastewater treatment, the mechanisms by which this occurs have not been investigated; however, they apparently differ among various species.

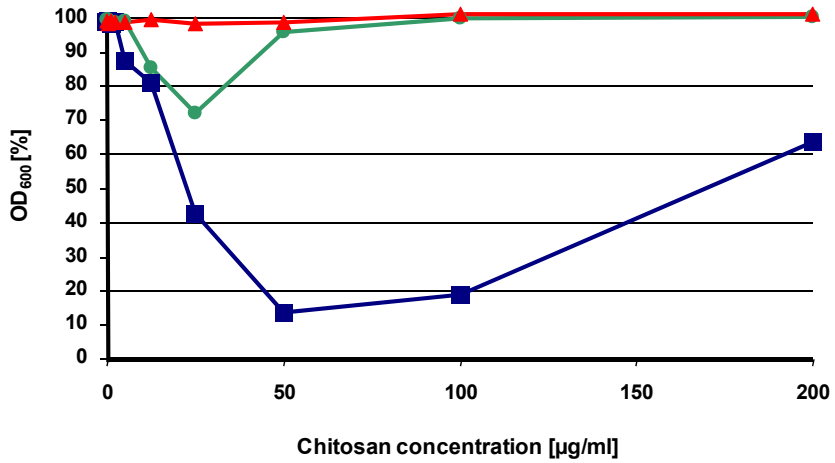

FIGURE 10: Flocculating behavior of different strains in presence of chitosan.

Comparison of the flocculating ability of various chitosan concentrations (0.5-200 µg/ml) on cells of the three indicator strains *B. subtilis* 168 (■), *S. simulans* 22 (●) and *S. aureus* SG511 (▲), as quantified by measuring the decrease in OD_{600} of a cell suspension. The % OD_{600} values after 10 min of incubation are depicted here.

4.1.9. CHITOSAN – AN ANTI-BIOFILM AGENT?

Microorganisms that attach to surfaces of implantable medical devices such as catheters, heart valves, and prosthetic joints can cause chronic infections, due to the formation of persistent biofilms, by which they manage to evade clearance by the host defenses and killing by antibiotics. The National Institute of Health (NIH) estimates that biofilms account for over 80% of human infections. Several strategies have been put in action to combat biofilm formation, including coating the surfaces of biomedical devices with agents that inhibit biofilm formation.

The use of chitosan as an anti-biofilm coating for medical applications such as implantable medical devices, wound dressings, catheters and contact lenses has been recently suggested by Carlson *et al.* [32], who claim that coating surfaces with chitosan is highly effective at retarding or preventing the formation of *S. epidermidis*, *S. aureus*, *K. pneumoniae*, *Ps. aeruginosa* and *C. albicans* biofilms under medically relevant conditions, hypothesizing that it disrupts cell membranes as microbes settle on the surface; being even superior to coatings impregnated with antimicrobial agents such as chlorhexidine.

Since such a property would indeed be profitable, helping to prevent infections due to colonialized medical devices, we chose to explore the effect of chitosan on the formation of bacterial biofilms, with the help of a semiquantitative biofilm assay (section 3.2.11.2), using *S. epidermidis* SE O-47 and *S. aureus* SA NCTC 8325 SH 1000 as biofilm-forming strains, testing them in 2 different media, BHI and TSA (supplemented with 0.25% membrane-filtered glucose). Since biofilms obtained from *S. epidermidis* SE O-47 in BHI broth were denser, we decided to use them for further testing.

No significant difference in biofilm density was observed upon adding chitosan to preformed biofilms, although viable counts in wells treated with chitosan at concentrations higher than the MIC were mostly lower than in control wells, indicating that chitosan might have acted upon surface-exposed bacteria, being unable to infiltrate the biofilms themselves. In addition, precoating of the wells prior to the biofilm assay with different concentrations of chitosan gave rise to slightly more diffuse biofilms, yet

even at suprainhibitory concentrations, precoating with chitosan was inefficient in preventing biofilm formation, compared to control wells; viable counts also revealed a slightly less populated biofilm in coated wells. On the other hand, pretreatment of the cells with chitosan showed a much reduced biofilm-forming property at concentrations above 2× MIC, which suggests that the absence of the biofilms is due to cell inhibition, rather than a specific effect on biofilm formation. Indeed, cell counts of treated biofilms were lower than in the control; the % inhibition of biofilm accumulation was 9.97%, 26.63% and 29.1%, in presence of 1×, 2× and 4×MIC, respectively, based on absorbance measurements at 492 nm.

Interestingly, Tunney *et al.* [321] discovered that the incorporation of chitosan in bone cement did not prevent or reduce bacterial adherence and biofilm formation. These contradicting results may be due to differences in the strains used, in growth conditions and chitosan concentrations tested, as well as in the experimental setting as a whole. Further investigations of this property, in particular with regard to optimization of a suitable system, are therefore warranted.

4.1.10. CHITOSANOLYSIS AND ITS RELATION TO CHITOSAN SUSCEPTIBILITY

Some microorganisms, including many marine *Vibrio* species, are capable of degrading chitin for use as a nutrient source, which constitutes an important part of their survival [156,198]. Applying the same reasoning to chitosanolytic organisms, these species would benefit from the presence of chitosan in their environment as a carbon and nitrogen source [13].

Bacillus subtilis and its close relatives are an important source of industrial enzymes, being capable of secreting these enzymes at gram-per-liter concentrations. We therefore screened a number of *Bacillus* and *Chromobacterium* strains from our culture collection at the Pharmaceutical Microbiology Unit (IMMIP, University of Bonn, Germany) for chitosan-hydrolyzing activities, to study the effect of chitosan utilization / degradation on the susceptibility of these strains to this antimicrobial biopolymer.

Several strains belonging to *Bacillus* species are listed as chitosanase producers; in addition *Chromobacterium violaceum* is known to exhibit a chitinolytic property [297], and is also believed to possess chitosanolytic activity (NCBI accession number NP_903601).

In total, 29 strains were screened, including 15 DSM strains and 6 ATCC strains (Table 20). The chitosan-hydrolyzing activities were detected as described in the experimental section (3.2.16); the formation of clear zones of degradation in chitosanase detection medium was taken as an indication of chitosan hydrolyzing activity (Figure 11).

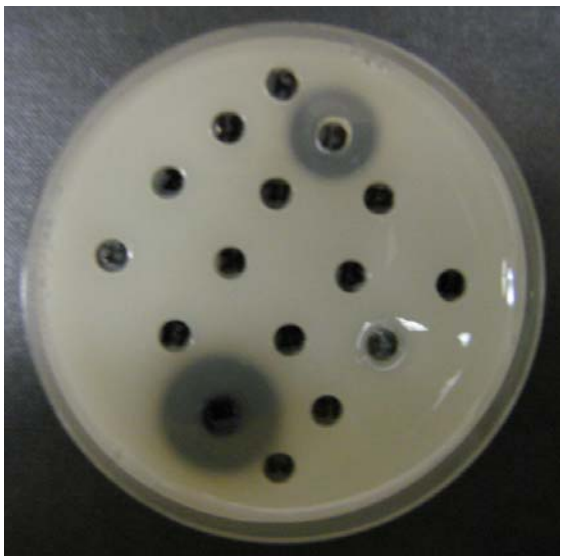

FIGURE 11: Screening for chitosan-hydrolyzing activity.

Aliquots of the test strains were instilled within cups made into chitosanase detection medium. Chitosan-hydrolyzing activity was detected by the development of a clear zone of degradation around the instilled cups, indicating that the insoluble chitosan contained in the growth medium was hydrolyzed.

Ten out of the screened bacterial strains were capable of hydrolyzing chitosan, albeit to variable extents (Table 20). To test whether chitosan hydrolysis has an effect on the susceptibility of these strains to chitosan, we determined the respective MIC and MBC values. The range of MIC values for chitosanolytic and non-chitosanolytic strains was 0.92 – 1250 µg/ml (average = 189.30 µg/ml) and 0.16 – 156.25 µg/ml (average = 41.18 µg/ml), respectively (Table 20).

TABLE 20: Strains of the culture collection screened for chitosan-hydrolyzing activity

Strain	Incubation temp.	Chitosan hydrolysis‡	MIC (µg/ml)*		MBC (µg/ml)*
			24 h	48 h	
Bacillus atrophaeus ATCC 9372	30°C	++	1.22	1.22	1.22
Bacillus cereus ATCC 11778	30°C	+	156.25	156.25	156.25
Bacillus cereus BCCR	30°C	-	58.59	58.59	58.59
Bacillus cereus BCVM	30°C	-	78.13	78.13	78.13
Bacillus cereus DSM 2302	30°C	-	156.25	156.25	156.25
Bacillus circulans DSM 11	30°C	-	9.77	9.77	9.77
Bacillus coagulans DSM 1	40°C	-	2.44	2.44	2.44
Bacillus insolitus DSM 5	26°C	+++	156.25	156.25	156.25
Bacillus lentus DSM 9	26°C	-	0.92	0.92	0.92
Bacillus licheniformis DSM 13	37°C	-	19.53	19.53	19.53
Bacillus megaterium DSM 32	30°C	-	0.16	0.16	0.16
Bacillus polymyxa NCTC 4747	30°C	-	156.25	156.25	156.25
Bacillus pumilus ATCC 14884	30°C	-	3.66	3.66	3.66
Bacillus pumilus DSM 27	30°C	-	2.44	2.44	3.66
Bacillus sphaericus DSM 28	30°C	-	3.66	3.66	3.66
Bacillus subtilis 165	37°C	+	0.92	1.83	1.83
Bacillus subtilis 168	37°C	+	1.22	1.22	1.22
Bacillus subtilis 168/1	37°C	+	2.44	2.44	2.44
Bacillus subtilis 168/2	37°C	+	4.88	4.88	4.88
Bacillus subtilis subsp. *spizizenii* DSM 347	30°C	-	9.77	9.77	9.77
Bacillus subtilis subsp. *subtilis* ATCC 6051	30°C	+	7.33	7.33	9.77
Bacillus subtilis W23Y	37°C	-	19.53	19.53	19.53
Bacillus thuringiensis DSM 2046	30°C	+	312.5	468.75	468.75
Chromobacterium violaceum ATCC 12472	26°C	+++	1250	1250	1250
Paenibacillus alvei DSM 29	30°C	-	78.13	78.13	78.13
Paenibacillus macerans DSM 24	30°C	-	19.53	19.53	29.3
Paenibacillus polymyxa ATCC 43865	30°C	-	78.13	78.13	78.13
Paenibacillus polymyxa DSM 36	30°C	-	78.13	78.13	78.13
Virgibacillus pantothenticus DSM 26	30°C	-	7.33	7.33	7.33

‡ Degradation zone diameter in chitosanase detection medium: ≤ 12 mm (+); 13 – 18 mm (++); ≥ 19 mm (+++).

* Average of 2 determinations

The highest MIC (1250 µg/ml) was observed for *Chromobacterium violaceum* ATCC 12472, which possesses a considerable chitosan hydrolytic activity; on the other hand the MIC for *B. subtilis* 165, also a chitosan-hydrolyzing strain, was below 1 µg/ml, indicating the absence of a direct correlation between hydrolysis and susceptibility to chitosan. These findings debunk the theory of Beausejour *et al.* [13], who claimed that the presence of a chitosan-hydrolyzing activity is virtually synonymous with immunity towards chitosan, and therefore suggested its use together with a biocontrol strain exhibiting chitosanolytic activity as a promising biocontrol tool.

Although previous studies have investigated the chitosanolytic properties of a number of organisms, and their possible use for chitosan-degradation, we are not aware of any reports studying the relation between chitosanolytic activity on one hand, and bacterial susceptibility to chitosan in an *in vitro* setting.

Whereas both *S. simulans* 22 and *S. aureus* SG511 have no chitosan-degrading activity, *B. subtilis* 168 demonstrated an adequate activity in chitosanase-detection medium. The highest chitosan-hydrolyzing activity of *B. subtilis* 168 was observed in cultures grown in rich media, such as TSB and BHI broth, followed by NI broth, and a residual activity in CAMHB; the activities being detected both in the culture, as well as in the cell-free supernatant.

4.1.11. DISCUSSION

With a growing demand for the rationalization of the use of chemicals, much attention has focused on the safety and efficacy of chitosan as a natural preservative, to be included in pharmaceutical and food preparations [121,149,215,265]. Yet, since many factors seem to influence the *in vitro* antimicrobial activity of chitosan, we decided to investigate some of them, with the aim of optimizing a system for testing the potency of chitosan in further assays. Where possible or feasible, we tried to judge the effectiveness of chitosan in a pharmaceutical or food system, where aspects such as antimicrobial spectrum and potency would be of practical importance.

In an *in vitro* setting, the potency of an antimicrobial agent is governed on the one hand by its properties and on the other hand by the specific environmental context, including the type of growth medium, the presence of extraneous matter and the experimental setting used to assess the potency. Underestimation of any of these factors would inevitably lead to false conclusions.

The MIC is usually taken as a measure of the susceptibility of a bacterial strain towards a specific antimicrobial substance. In this study we present evidence that a number of factors may account for large variations in the reported MIC values of chitosan in the available literature.

The discrepancies between data may result from the different degrees of deacetylation and molecular weights of chitosan. The evaluation of the dependence of the antimicrobial activity of chitosan on each of these characteristics requires keeping all other variables constant, for example by using chitosans of a wide MW range with the same DD. This is however almost impossible to achieve, since chitosan is a natural polymer; there would always be variations within product batches. Therefore, it would be rather difficult to determine the optimal MW for the maximum antimicrobial activity.

Liu *et al.* [182] studied the antimicrobial activity of chitosan against *E. coli*, and came to the conclusion that its activity increased with increasing MW, up to a MW of 91.6 kDa; above that value, there was an inverse relationship between both. We were not able to reach a similar conclusion based on our findings; however, we discovered that oligosaccharides lack the antimicrobial activity that is exhibited by large chains of the polymer. This view is shared by Uchida *et al.* [323], who observed that chitosan oligomers possessed weak or no antibacterial activity at levels as high as 0.5 –1.0%. In addition, Young *et al.* [361] demonstrated in their study that chitosan increases the membrane permeability of plant cells, a property shared by basic polymers, such as poly-L-lysine, histone, DEAE-dextran, protamine sulfate, and glycol chitosan; in contrast, monomeric D-glucosamine and L-lysine showed no effect at concentrations up to 500 µg/ml. On the other hand, Rhoades and Roller [255] theorized that a mild degradion of chitosan enhances

its antimicrobial action, whereas highly degraded chitosan displayed no antimicrobial action. Therefore, it seems reasonable to suggest that there seems to be a minimum degree of polymerization required for antimicrobial activity, above which the activity remains more or less constant.

Noteworthy of mentioning is that the reported MIC values of chitosan are usually much higher than those encountered in our current study. Apart from differences in chitosan characteristics (including the use of plain or derivatized chitosans, oligomers or high molecular weight polymers), we can conclusively demonstrate that this could be ascribed to differences in experimental settings, including both the culture medium used, as well as the method used for susceptibility assessment, which play critical roles in the antimicrobial potency of chitosan.

For instance, we chose to use the broth microdilution method for susceptibility testings, based on preliminary results that demonstrated the inferior activity of chitosan in agar media. Nonetheless, some researchers used agar-based methods (agar dilution [215] and agar diffusion [128]) to assess the antimicrobial potency of chitosan.

Moreover, the antibacterial effects of chitosan and its oligosaccharides were often studied in nutrient broth [182,335], tryptic soy broth [144,145] and phosphate buffer [318], where the activity of chitosan is only minimal, resulting in considerably higher MIC values. In addition, some of these researchers tracked the inhibitory effects of chitosan spectrophotometrically [144,182]. Based on the results of flocculation assays, we deem such a method inappropriate, due to large fluctuations in optical density measurements in presence of chitosan. Therefore, it seems that several studies ostensibly demonstrating the antimicrobial efficiency of chitosan were based on unsuitable approaches.

Among other criteria that need to be taken into consideration when evaluating the efficiency of chitosan for use as a preservative is its concentration, which is a major factor in antimicrobial activity. Most chitosan formulations contain high concentrations of chitosan to achieve an optimal, broad spectrum activity. Here, we would like to point

out that the concentrations of chitosan used in this study are far below those used in chitosan formulations or in other studies. For instance, Bae *et al.* used a 1.0% chitosan solution in a clinical trial to study the effect of chitosan on plaque formation [10], while Roller and Covill studied the antimicrobial properties of chitosan glutamate in mayonnaise and mayonnaise-based shrimp salad [259] as well as in laboratory media and apple juice [258], at a level of 3 g/l and 0.1 - 5 g/l, respectively.

In sum, our findings uniformly indicate that, while there is little doubt that chitosan could indeed be included as a preservative in certain systems; there is still much to be learned about its antimicrobial potential. However, a stage has being reached at which it is becoming possible to present a general account of the main criteria that should be closely observed while developing antimicrobial systems to be implemented into industrial applications. Therefore, at this point we would like to reiterate that applications relying solely upon *in vitro* results should be treated with caution, since results may differ considerably in a food or pharmaceutical formulation, where such factors as the complexity of the medium, the presence of organic matter, the pH of the formulation as well as the presence of other active agents, would all play an important role in the final efficiency of the formulation.

4.2. INSIGHTS INTO THE MODE OF ACTION OF CHITOSAN AS AN ANTIBACTERIAL COMPOUND

4.2.1. SUMMARY

We demonstrated here that the mode of action of chitosan is probably complex; it is thought to act in a rather non-specific way, resulting in random multiple detrimental events that lead to a killing process; the precise target sites might however be difficult to pinpoint. We believe that chitosan interferes with the function of the bacterial membrane; it rapidly but partially dissipated the membrane potential, causing a generalized destabilization and subsequent disruption of membrane-associated functions; a direct pore-forming activity is however not envisaged. However, it provoked no leakage of marker molecules from neutral and anionic model membrane systems, even at relatively high concentrations. No interference with lipid II biosynthesis was observed. Chitosan treatment of *S. simulans* 22 cells did not give rise to cell wall lysis; the cell membrane also remained intact. Analysis of transcriptional response data revealed that chitosan treatment lead to multiple changes in the expression profile of *S. aureus* SG511 genes involved in the regulation of stress and autolysis, as well as genes associated with energy metabolism.

Undoubtedly, the bactericidal activity of chitosan is based on the highly cationic nature of the molecule, and results, at least in part, from its interaction with components of the cell envelope, therefore a possible mechanism for chitosan's activity against staphylococci was postulated, based on the following sequence of events: (i) rapid interaction with negatively-charged cell-surface structures such as lipoteichoic and wall teichoic acids, coupled with a potential extraction of membrane lipids and LTA; (ii) partial disruption of normal permeability functions of the cytoplasmic membrane and damage of membrane integrity, resulting in the release of K^+ and other cellular components, and (iii) complete loss of membrane function followed by a sequence of events that ultimately lead to bacterial death.

4.2.2. RATIONALE AND OBJECTIVES

Understanding the mechanism(s) of action of chitosan has become an important issue with the emergence of a range of applications that rely on its antimicrobial activity, and the suggestion that it could be used as a food or pharmaceutical preservative.

In the work reported here we have embarked on a systematic investigation of the possible mechanisms of antibacterial action of chitosan. Two basic approaches were undertaken to probe this: The first was to demonstrate the effects of chitosan on living bacterial cells, using a combination of techniques, including cellular leakage assays, membrane potential estimations and electron microscopy, in addition to transcriptional response analysis of bacterial cells treated with chitosan. The second approach consisted of *in vitro* assays, including applying chitosan to a defined *in vitro* model of a bacterial membrane, as well as to a reaction system mimicking cell wall biosynthesis machinery, in search for the most plausible mechanism of antimicrobial activity of chitosan.

4.2.3. CELL LEAKAGE ASSAYS

In terms of susceptibility to antimicrobial agents, the cytoplasmic membrane, which regulates the transfer of solutes and metabolites in and out of the cell cytoplasm, is often considered as one of the major target sites. Damage to the membrane can take several forms: (i) physical disruption of the membrane; (ii) dissipation of the proton motive force (PMF) and (iii) inhibition of membrane-associated enzyme activities.

Leakage is best considered as a measure of the disruption of the cell permeability barrier, therefore we paid particular attention in this study to assess the effect of chitosan on membrane integrity, using several assays which help detect the leakage of intracellular components from whole cells, including potassium (K^+), materials absorbing at 260 nm and proteins.

A potassium-sensitive electrode permits a rapid as well as accurate *in situ* measurement of potassium leakage following treatment of washed microbial suspensions with membrane active antimicrobial agents. Since the leakage of potassium ions occurs rapidly after treatment, it is proposed that the efflux of this ion is one of the first indications of the disruption of the selective permeability of the cytoplasmic membrane, and is thus a primary indication of membrane damage [174].

We used a K^+-specific ion-electrode to detect membrane impairment and leakage of potassium from *S. simulans* 22 cells (section 3.4.2.2.2) in response to exposure to different chitosan concentrations (5 – 60 µg/ml). The data depicted in Figure 12a, reveal that the initial effect of chitosan upon *S. simulans* 22 under these conditions was to initiate a gradual, dose-dependent flux of K^+-ions (hitting the 70% mark within 8 min) which was rather incomplete when compared to the pore-forming lantibiotic nisin. Pre-energization of the cells in presence of 10 mM glucose resulted in an accelerated K^+-efflux from treated cells, however without greatly influencing the extent of leakage.

To test whether other larger molecules would be able to escape from cells treated with chitosan, we measured the absorbance, at 260 nm, of cell-free supernatants of bacterial suspensions treated with chitosan, in order to detect the leakage of UV-absorbing substances, likely representing nucleotide and coenzyme pools (section 3.4.2.3). Treatment of *S. simulans* 22 with chitosan (20 µg/ml, equivalent to around 10× MIC) resulted in a concentration-dependent, gradual leakage of UV-absorbing substances from bacterial cells, followed by a plateau up to 2 hours (Figure 12b). Optical density measurements of the treated culture revealed around 50% reduction in culture density after 2 h, which was attributed to aggregation and flocculation of the cells in presence of chitosan (refer to section 4.1.8). Chemical and electrophoretic analyses of cell-free supernatants of chitosan-treated cell suspensions, using both conventional protein determination and SDS-PAGE, showed that interaction of chitosan with *S. simulans* 22 either involved no release of proteins, or their amount was too small to be detected.

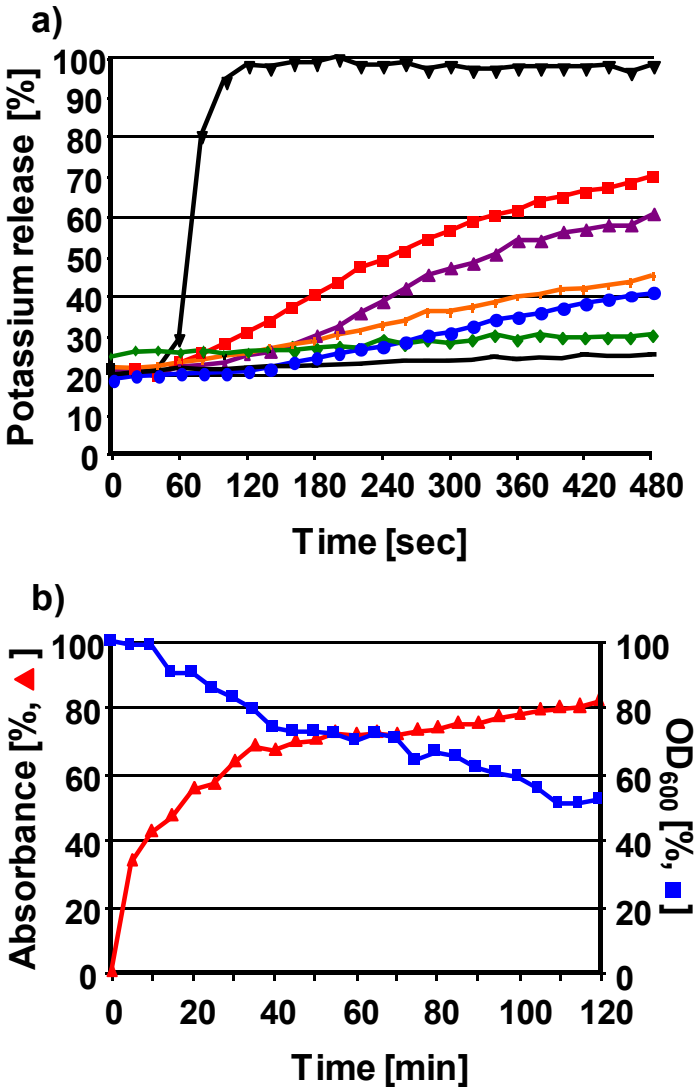

FIGURE 12: A time course for leakage of cellular components from *S. simulans* 22 upon exposure to chitosan.

a) Potassium release from *S. simulans* 22 (—) increases with increasing amounts of chitosan: 5 µg/ml (♦), 10 µg/ml (●), 20 µg/ml (+), 40 µg/ml (▲) and 60 µg/ml (■). 100% potassium leakage was achieved by the addition of 1 µM of the pore-forming lantibiotic nisin (▼). b) Leakage of UV-absorbing cellular components from *S. simulans* 22 as a function of time. 260 nm-materials were measured in the supernatant of cultures growing in CAMHB at 37°C and treated with chitosan (20µg/ml; ▲). Hundred percent of leakage was defined as the maximal value of absorbance at 260 nm obtained with nisin [1 µM]. Parallel optical density measurements were conducted and compared to the initial culture density (%OD600, ■).

In similar experiments, Young *et al.* [361] found that chitosan treatment greatly increased the membrane permeability of suspension-cultured plant cells, reflected by leakage of electrolytes and UV-absorbing materials from *Phaseolus vulgaris* cells. The low amount of soluble protein found in the medium after treatment with a chitosan concentration as high as 500 µg/ml was explained by binding of proteins to chitosan molecules attached to insoluble cell material or their precipitation by chitosan.

In sum, the leakage experiments uniformly indicated that chitosan efficiently permeabilized the plasma membranes of staphylococci for small cellular constituents. These data were in fair agreement with previous reports that described chitosan as a membrane-perturbing compound [121,142,363].

4.2.4. ASSESSMENT OF LIPOSOMAL PERMEABILIZATION.

Whole cell leakage assays confirmed that chitosan might, in some way or another, cause membrane destabilization, and we aimed at extending these observations by using model membrane systems, hoping that this would shed some light on elemental mechanisms taking place at the level of the bacterial cell membrane. Therefore, in an *in-vitro* assay, we investigated whether unilamellar liposomes (prepared as described in section 3.3.2) became damaged upon treatment with chitosan and leaked trapped marker molecules, namely carboxyfluorescein (CF) and potassium ions (K^+).

CF was used as a marker, since its fluorescence is self-quenching at high concentrations, and thus the net fluorescence detected is minimal. Therefore, intact liposomes prepared in the present study (encapsulating 50 mM CF) emit relatively low levels of fluorescence. However, permeabilization of liposomes (for example through pore formation) would cause the leakage and dilution of CF into the buffer medium, resulting in an increase in total fluorescence intensity. Thus, liposome permeabilization was detected by an increase in fluorescence over time [37]. Chitosan (10-100 µg/ml) was unable to induce an efflux of CF from unilamellar DOPC liposomes. Since DOPC liposomes carry an overall neutral charge, it was postulated that this lack of charge might be responsible for the inactivity of chitosan, due to the absence of electrostatic

interactions. Therefore, the negatively-charged lipid, DOPG, was used together with the neutral lipid DOPC, to prepare negatively-charged liposomes that would be assumed to be affected by the addition of chitosan. Still, chitosan was unable to cause permeabilization of the negatively-charged unilamellar DOPC:DOPG (1:1) liposomes to CF (Figure 13a), indicating that it has no direct pore-forming action on artificial membranes that mimick bacterial cell membranes. This assumption was confirmed by the fact that chitosan had no detectable effect on the release of K^+-ions from K^+-loaded DOPC:DOPG (1:1) liposomes (Figure 13b).

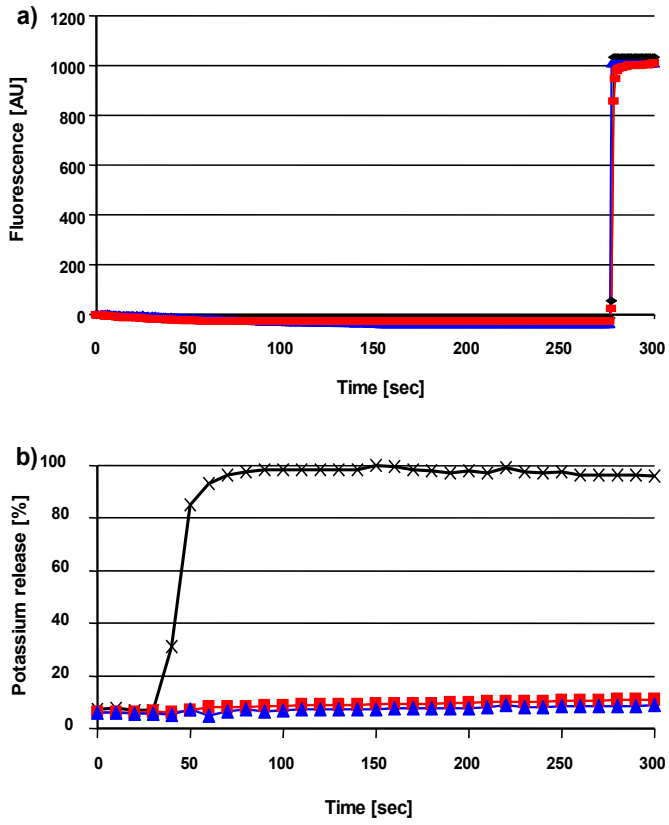

FIGURE 13: Liposomal permeabilization assays.

No leakage of carboxyfluorescein (a) or potassium (b) was observed upon treatment of unilamellar DOPC-DOPG (1:1) liposomes with chitosan (40 µg/ml, ▲ and 80 µg/ml, ■), followed over 5 min. Chitosan was added to CF-loaded liposomes (a) after 2½ min, whereas Triton X-100 was added after 4½ min to check the stability of the liposomes. Octylglycoside (0.7%, ×) was added to K^+-loaded liposomes to mark the 100% level (b).

These *in vitro* leakage assays served to illustrate that although chitosan might be endowed with considerable membrane permeabilization capacity when tested *in vivo*, it may be unable to effectively demonstrate a similar effect in an *in vitro* setting.

4.2.5. EFFECT ON PEPTIDOGLYCAN BIOSYNTHESIS

An *in vitro* lipid II synthesis assay was constructed, with the aim of detecting the ability of chitosan to inhibit lipid II biosynthesis by interfering with any of its key steps, through specific inhibition of an enzyme involved in lipid II biosynthesis or selective binding to the lipid carrier C_{55}-P. Negative and positive controls were run alongside the samples, with a reaction mixture lacking chitosan, and another treated with friulimicin (a lipopeptide known to inhibit lipid II biosynthesis [275]), respectively. No effect on lipid II biosynthesis was observed (Figure 14).

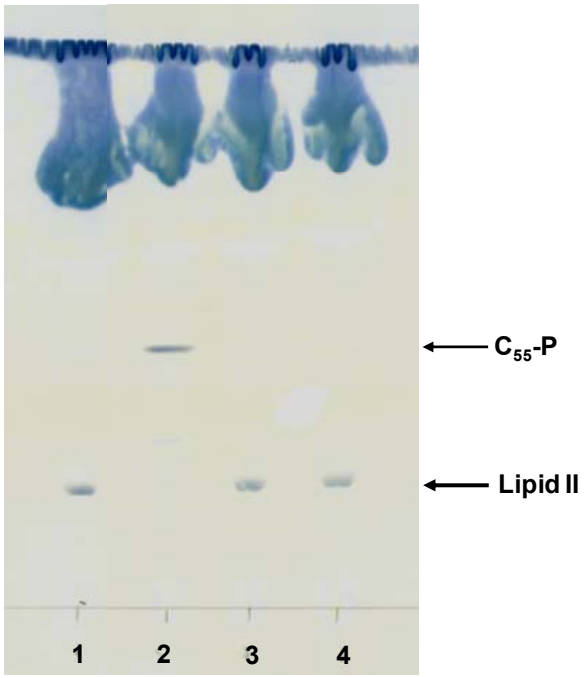

FIGURE 14: Analytical Lipid II assay.

The lipid II assay was used to test a possible inhibition of peptidoglycan biosynthesis by chitosan, added at 2 different concentrations: 67 µg/ml (lane 3) and 267 µg/ml (lane 4). A negative control (lane 1) was included, showing efficient lipid II synthesis, while the positive control (lane 2) inhibited lipid II biosynthesis, and hence lead to the accumulation of the precursor C_{55}-P.

This stands in stark contrast to the antimicrobial peptide nisin, which is known to form a defined and stable pore, using the membrane-bound bacterial cell wall precursor lipid II as anchor molecule, simultaneously resulting in inhibition of cell wall biosynthesis [340,341].

4.2.6. MEMBRANE DEPOLARIZATION ASSAYS

The membrane potential ($\Delta\Psi$) is defined as the electrochemical potential difference across a cell's membrane, which is provided by ion gradients, resulting through directed proton extrusion and the action of secondary ion transporters embedded within the membrane [25]. The generated proton motive force is involved in active transport, oxidative phosphorylation and adenosine triphosphate (ATP) synthesis in bacteria. Perturbation of membrane integrity, by antimicrobials for instance, leads to membrane depolarization and ultimately bacterial cell death. Theoretically, the membrane potential can be determined by the distribution of lipophilic ionic molecules between the cells and the suspending medium. In this study we aimed at evaluating the effect of chitosan on bacterial membrane potential, by measuring membrane depolarization using two techniques (section 3.4.3).

First, we followed the distribution of the small lipophilic charged [^{3}H]TPP$^+$ ions between cells of *S. simulans* 22 and the suspending medium, in response to treatment with 10 µg/ml of chitosan (Figure 15), and found a substantial reduction in bacterial membrane potential, indicating that subjecting *S. simulans* 22 to chitosan resulted in the dissipation of the membrane diffusion potential. The depolarization kinetics was similar to the time-course of potassium efflux (Figure 12a).

This contention was corroborated with the help of the fluorometric membrane depolarization assay using $DiBAC_4(3)$, a lipophylic and anionic fluorescent distributional probe, which accumulates only in cells in which the $\Delta\Psi$ is dissipated. Increased depolarization results in more influx of the anionic dye and thus an enhanced fluorescence, whereas hyperpolarization is indicated by a decrease in fluorescence [76].

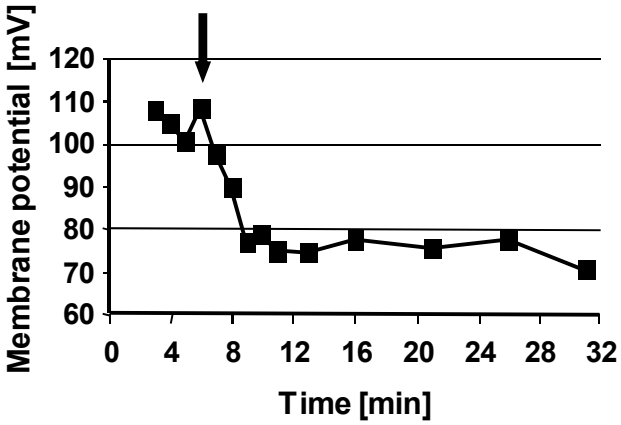

FIGURE 15: Membrane depolarization assay using [^{3}H] TPP$^+$.

Cells of *S. simulans* 22 in the late log phase were allowed to equilibrate with [^{3}H]TPP$^+$ in CAMHB. Chitosan was then added (arrow) to a final concentration of 10 µg/ml (equivalent to 5×MIC).

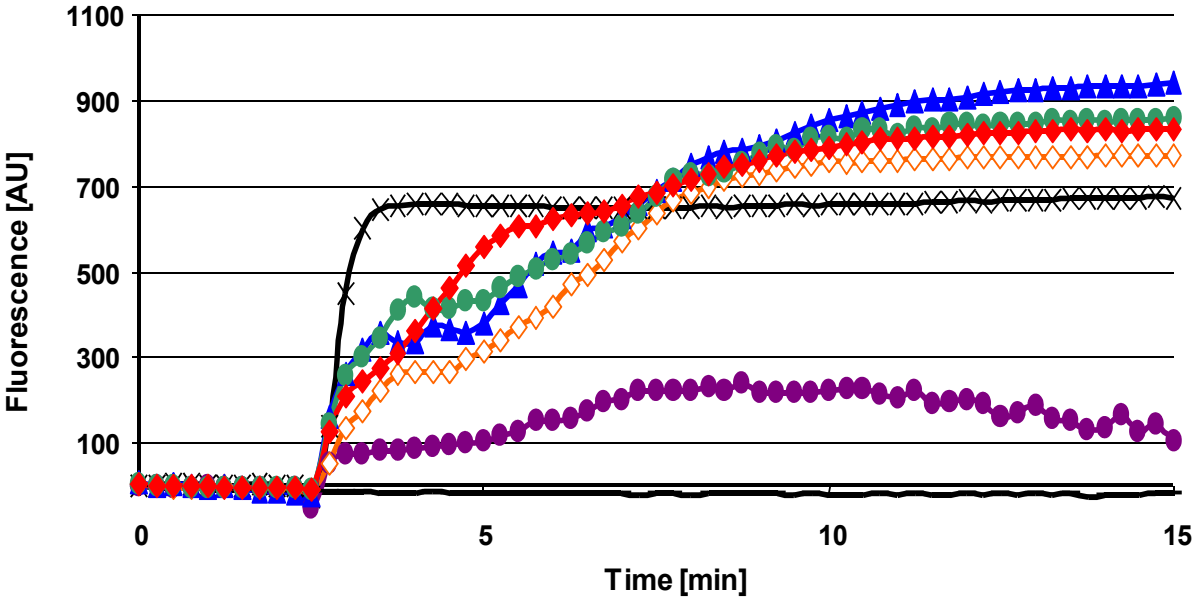

FIGURE 16: Fluorescent measurement of chitosan's ability to perturb the membrane potential.

Potential-dependent fluorescence changes generated by DiBAC$_4$(3), when *S. simulans* 22 (—) in CAMHB was subjected to different concentrations of chitosan: 10 (●); 20 (◊), 40 (▲), 60 (●) and 80 µg/ml (♦). Chitosan increased the permeability of *S. simulans* 22 to the membrane potential-sensitive dye in a concentration-dependent manner. Nisin [1 µM, ×] was used as positive control.

When the test strain *S. simulans* 22 in the logarithmic phase was subjected to different concentrations of chitosan in CAMHB, a steady loss of membrane potential was observed, manifested by a gradual increase in fluorescence units, following an increase in the uptake of $DiBAC_4(3)$ by the cells as a function of chitosan concentration (Figure 16). However, when compared to the antimicrobial peptide nisin (positive control), it was obvious that chitosan-induced depolarization was much slower and incomplete. Thus, the combination of these two approaches enabled us to document the ability of chitosan to disrupt the membrane potential.

4.2.7. EXAMINATION OF CELL DAMAGE BY TEM

To further understand chitosan's mode of action, we monitored ultrastructural changes taking place in *S. simulans* 22 upon exposure to chitosan, compared to control cells, using transmission electron microscopy (TEM), to identify the damages caused during the treatment. The chitosan concentration (10×MIC) was chosen because it was sufficiently high to induce cellular response in a great percentage of cells, without causing massive cell mortality.

Control cells showed an intact plasma membrane of high electron density and an outer cell-wall of medium electron density which was more or less uniform along the entire cell perimeter; sites of cell division were also evident (Figure 17a). On the other hand, cells treated with chitosan for as short as 5 min showed irregular structures protruding from the cell surface, which might be chitosan deposits still attached to the negatively-charged surface polymers (Figure 17b-d). Although chitosan should be largely washed from electron microscopic preparations during the various washing steps, its presence could still be detected, indicating that it binds strongly to the bacterial cell surface.

Interestingly, it seemed that the cell membrane became locally detached from the cell wall, giving rise to "vacuole-like" structures underneath the cell wall, possibly caused by the constant disturbances in membrane function that lead to ion and water efflux and decreased internal pressure.

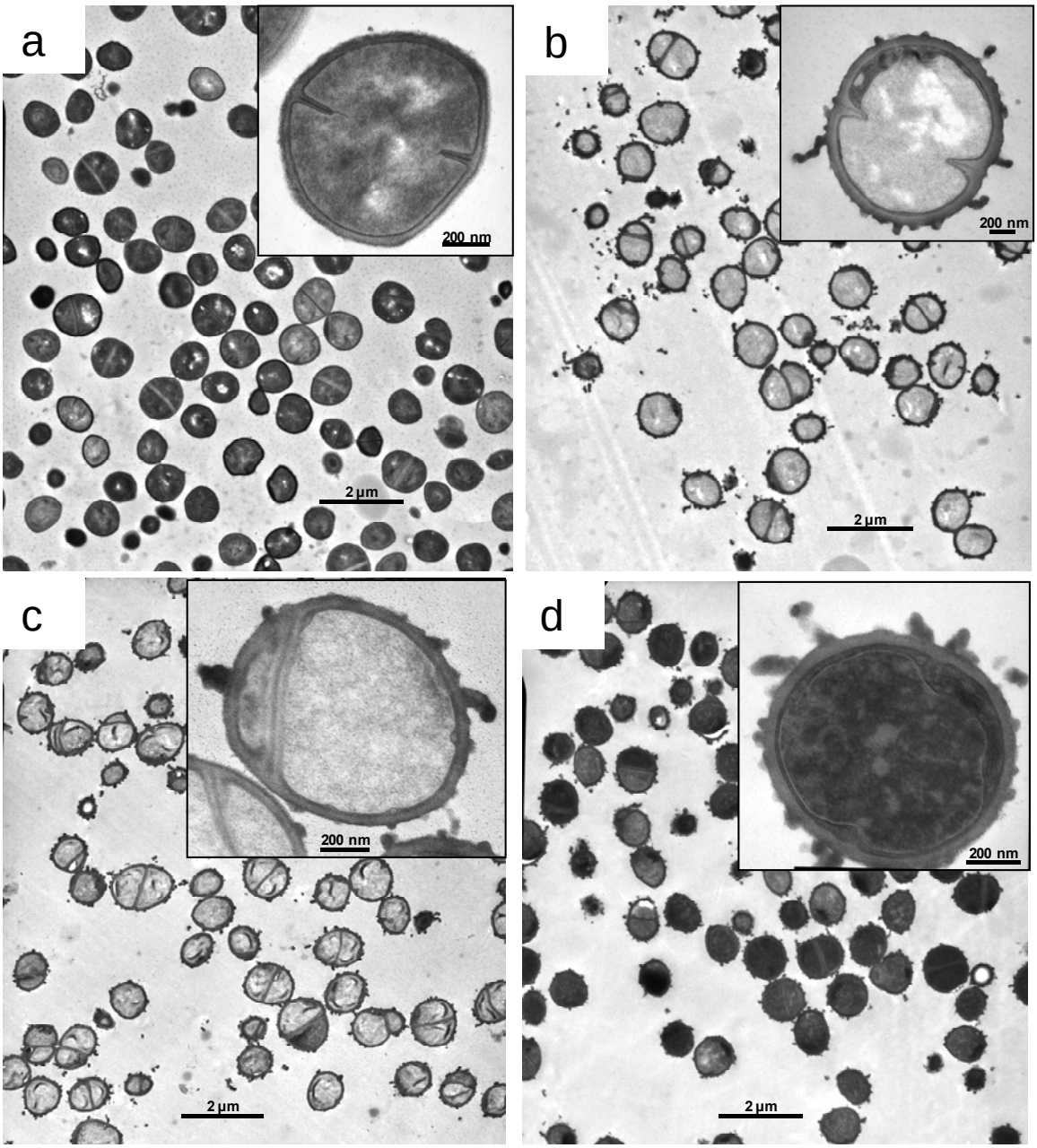

FIGURE 17: Ultrastructural changes induced by chitosan treatment as seen under a transmission electron microscope.

Shown here are electron micrographs of *S. simulans* 22 cells (control, a), treated with 10×MIC of chitosan for 5 min (b), 20 min (c) and 60 min (d), all at a magnification of 5600×. Inserts show close-ups of single cells at 53,000× (a), 31,000× (b), 25,000× (c) and 53,000× magnification (d). The microscopic observation clearly indicated that chitosan treatment brought about cytoplasmic membrane shrinkage, albeit no cell wall damage.

None the less, the membrane was well discernible in all sections, i.e. was more or less physically intact, and remarkably, there was no evidence for cell wall lysis, as described for *S. simulans* 22 treated with the cationic peptides Pep5 and nisin, which displace, and thereby activate cell wall-lytic enzymes (autolysins) from polyanionic cell wall polymers (teichoic, teichuronic and lipoteichoic acids), the latter acting as noncompetitive inhibitors of enzyme activity [19]. Electron micrographs of cells treated with cationic peptides demonstrated a degraded cell wall, particularly in the area of the septum between two daughter cells, eventually leading to disruption of the cell wall in this area and release of the cell contents [18]. Being a highly cationic compound itself, it was thought that chitosan might share this activity with these much-studied antimicrobial peptides, which was not the case. Moreover, our electron microscopical findings did not support earlier work by several research groups, which demonstrated an irregularly-structured and frayed cell wall in chitosan-treated microorganisms [205], and even the appearance of protoplasts [63].

There was a fairly conspicuous nucleoid region of low density in both the control sample and in cells treated with chitosan for 5 min (Figure 17, a and b, respectively); however this region became more disperse and ill-defined in cells treated for 20 and 60 min (Figure 17, c and d, respectively), indicating that changes of the intracellular ionic milieu after chitosan addition might affect the bacterial nucleoid organization.

4.2.8. Influence of teichoic acids on the susceptibility of *S. aureus* to chitosan

Teichoic acids are essential polyanionic polymers found in the cell wall of Gram-positive bacteria, which appear to extend to the surface of the peptidoglycan layer, thus contributing to the negative charge of the cell wall. In *S. aureus*, they can be either covalently linked to the C-6 position of *N*-acetylmuramic acid (Mur*N*Ac) of the peptidoglycan layer through a phosphoester-link (wall teichoic acids, WTA) or anchored into the outer leaflet of the cytoplasmic membrane via a glycolipid (lipoteichoic acids, LTA).

To evaluate the possible involvement of teichoic acids of *S. aureus* in chitosan's antimicrobial activity, and to analyze their role in chitosan susceptibility, we tested *S. aureus* SA113 (ATCC 35556) together with four of its mutants, lacking one or more genes involved in teichoic acids biosynthesis (Table 21). *S. aureus* SA113 Δ*tagO* is completely devoid of wall teichoic acids, due to deletion of the *tagO* homolog which codes for an enzyme catalyzing the first step in WTA synthesis [338]. In the *ypfP* deletion mutant, the gene responsible for the biosynthesis of the glycolipid anchor of LTA was absent, causing 87% reduction in LTA content compared to the wild-type [79]. A double mutant was also available, where the *tagO*-gene was substituted by an erythromycin cassette, and the *ypfP* gene was substituted by a spectinomycin cassette. The *dltABCD* mutant was constructed by insertional disruption of the *dltA* gene of *S. aureus* SA113, through substitution with a spectinomycin resistance gene [238]. The *dltABCD* operon is responsible for the addition of D-alanine to teichoic acids, thereby introducing positively-charged amino groups into the otherwise negatively-charged teichoic acids.

The Δ*tagO* mutant was the most resistant to the antimicrobial activity of chitosan (with more than 5-fold higher MIC), followed by the double-mutant and the Δ*ypfP* mutant (Table 21). The relevance of this finding is significant, since the lack of teichoic acids in staphylococci results in a less negatively-charged cell wall, further substantiating the hypothesis that the polycationic nature of chitosan is a major factor contributing to its antimicrobial activity.

TABLE 21: Comparison of the MIC of chitosan for *S. aureus* SA113 and its mutants

Strain	Description	MIC (µg/ml)*		MBC (µg/ml)*
		24 h	48 h	
S. aureus 113 (SA 113)	ATCC 35556, wild-type	84.8	84.8	84.8
SA113Δ*tagO*::*erm*	*tagO* deletion mutant	471.6	545.5	602.3
SA113Δ*ypfP*::*erm*	*ypfP* deletion mutant	224	224	232.6
SA113Δ*ypfP*::*spec*/Δ*tagO*::*erm*	double-knock-out-mutant	375	385.4	385.4
SA113Δ*dltA*::*spec*	*dltA*-mutant	0.85	0.85	0.85

* Average of at least 3 determinations.

We believe the same reasoning can be applied to the fact that the Δ*dltA* mutant, which lacks the D-alanine modification in teichoic acids, as a result of which the cells carry an increased negative net cell surface charge, was almost 100-times more susceptible to the action of chitosan, with an MIC as low as 0.85 µg/ml (Table 21). The lack of D-alanine esters probably leads to a pronounced increase in the attractive forces, thereby potentiating the activity of chitosan and leading to the observed hypersensitivity.

This is reminiscent of previous observations that the Δ*dltA* mutant was considerably more susceptible to human defensin hNP1-3, cathelicidin LL-37 and lactoferrin [338], and other cationic pore-forming antimicrobial peptides, such as nisin, α-defensins, and related peptides [238] than the wild-type strain, confirming studies on the role of teichoic acid D-alanine in the resistance to vancomycin and antimicrobial peptides [236,239].

4.2.9. Analysis of transcriptional response pattern to chitosan

The analysis of gene expression has been revolutionized by DNA-microarray technology, which allows for a quick and accurate screening for changes in bacterial gene expression patterns across the entire genome in response to antimicrobial substances, studying thousands of affected genes among multiple samples at a time.

We carried out a genome-scale microarray experiment (section 3.5.1) to detect global changes in *S. aureus* SG511 gene expression pattern induced in response to treatment with a subinhibitory chitosan concentration (15 µg/ml) for a short time (20 min), thereby identifying fine-tuned responses of bacteria to the stress induced by chitosan. SAM (significance analysis of microarrays) revealed a total of 166 ORFs that showed a statistically significant (at 0.64% FDR) change in expression level; a comparatively small number of genes, compared to cationic AMPs [225,271]. A complete list of the significant gene responses, including 84 up- and 82 down-regulated genes, is given in the appendices (sections 6.2.1 and 6.2.2, respectively).

Table 22 summarizes the regulated genes, according to their function. We may not be able to address all responses, but will try to give an outline of the major changes in gene expression observed.

TABLE 22: Genes regulated in chitosan-treated *S. aureus* SG511 cells

	Up-regulated genes*		Down-regulated genes*	
	Number	%	Number	%
Cell wall	1	1.2	1	1.2
Transport/binding proteins and lipoproteins	7	8.3	12	14.6
Membrane bioenergetics	4	4.8	0	0.0
Cell division	2	2.4	0	0.0
Metabolism-related genes	15	17.9	26	31.7
DNA recombination	0	0.0	1	1.2
RNA synthesis/regulation /termination	6	7.1	3	3.7
Protein-related genes	1	1.2	15	18.3
Adaption to atypical conditions	3	3.6	5	6.1
Detoxification	2	2.4	0	0.0
Antibiotic production	0	0.0	1	1.2
Phage-related	3	3.6	2	2.4
Pathogenic factors	3	3.6	3	3.7
Miscellaneous	3	3.6	0	0.0
Hypothetical genes	34	40.5	13	15.9
Total number of genes	**84**	**100.0**	**82**	**100.0**

* Compared to the untreated culture

Chitosan treatment reduced the bacterial growth rate and this was clearly reflected in genetic expression profiles, through the down-regulation of macromolecular biosynthesis, including a large number of genes involved in RNA and protein synthesis (14 ribosomal protein genes), as well as in metabolism of carbohydrates, amino acids, nucleotides and nucleic acids (six genes), lipids and coenzymes (section 6.2.2).

Transcriptional response data provided us with indirect evidence that chitosan treatment interferes with cellular energy metabolism. This is supported by the fact that several of the genes preferentially expressed under oxygen depletion conditions were up-regulated in this study. In bacteria, under aerobic conditions, protons are

transferred from NADH to the electron transport chain, generating H_2O and a membrane potential which is used to synthesize ATP. Under low-oxygen conditions, and in the absence of external electron acceptors such as oxygen or nitrate, NADH must be recycled by fermentation, otherwise, the NAD^+ pool is depleted in a very short time; therefore NAD^+ is regenerated by reduction of metabolic intermediates through fermentation or nitrate respiration, rather than through the respiratory chain [91]. Consistent with this view, among the proteins with the highest levels of transcription (section 6.2.1) were components of fermentative pathways, including those coding for formate acetyltransferase (*pflB*), together with *pflA* (the activating enzyme) and alcohol-acetaldehyde dehydrogenase (*adhE*). The genes *pflA* and *pflB* catalyze the nonoxidative transformation of pyruvate to acetyl coenzyme A and formate. The activation of the anaerobic stress pathway, including overexpression of these genes, corresponds to the typical response of a bacterium to oxygen-limiting conditions [91], oxidative stress [34] as well as interruption of the electron transport chain [164].

This is further substantiated by the fact that transcripts of the *nar* (narG and narK) and *nir* (SA2189) operon, involved in nitrite-reduction and anaerobic respiration, a gene encoding a putative L-lactate permease (SA2156), the regulatory gene *srrA*, already shown to be involved in oxygen regulation in *S. aureus*, together with the gene *ndhF*, encoding an NADH-dehydrogenase and linked to electron transport [91], were all found to be up-regulated during chitosan treatment. Therefore, it appears reasonable to hypothesize that the electron transport chain was uncoupled in *S. aureus* SG511 in response to chitosan treatment, resulting in impairment of oxygen consumption, which forced the bacteria to shift to anaerobic respiration.

Interestingly, both *pflA* and *pflB* were shown to be down-regulated in mild acid (pH 5.5) [339] and in response to the cell wall-active antibiotics bacitracin, D-cycloserine and oxacillin [325]. On the other hand, a decrease in the expression level of SA2156 and SA2189 was observed in response to bacitracin, d-cycloserine and oxacillin [325] as well as the *ß*-defensin hBD3 [271].

Defects in menadione biosynthesis (such as in case of SCVs, section 4.1.7) result in interruptions in electron transport and decreased ATP production, thus inducing the expression of fermentation enzymes, even under aerobic conditions. This is an indication that, other than the oxygen concentration, several factors might act as a signal for anaerobic gene regulation in *S. aureus*, such as the reduced state of component(s) of the respiratory chain, the membrane potential, and/or the increased level of NADH.

Acid stress is not likely to play a major role in chitosan's mode of action, since none of the urease genes, deemed to be an important acid-shock mechanism for *S. aureus* to counteract the acidic environment [21], was up-regulated upon chitosan treatment. In addition, at relevant concentrations, the pH of the chitosan solution used was around neutrality (section 4.1.5).

Chitosan does not seem to interfere with lipid II biosynthesis, as seen from the *in vitro* lipid II assay (section 4.2.5). This hypothesis was further confirmed by the fact that none of the major peptidoglycan biosynthesis genes was regulated upon chitosan treatment. However, up-regulated genes included *bsaA*, *prsA* (peptidyl-prolyl cis/trans isomerase homolog) and the hypothetical proteins SA1703 and SA2221, which were also identified upon vancomycin treatment and which are considered parts of the staphylococcal cell wall stress stimulon [194,325].

The *S. aureus* LytSR two-component regulatory system is known to affect murein hydrolase activity and autolysis. A LytSR-regulated operon encoding two potential membrane-associated proteins, designated LrgA and LrgB, is believed to confer negative control on extracellular murein hydrolase activity, by acting as "antiholins", thus inhibiting autolysis [107]. Whereas Weinrick *et al.* showed that both genes were down-regulated in mild acidic conditions [339], we saw that both genes were strongly up-regulated under chitosan stress, which is in chorus with our electron micrographs (section 4.2.7), where no cell lysis could be observed.

The overall transcriptional profile of chitosan-treated *S. aureus* did not coincide with other published antibiotic profiles or with our own unpublished datafile [mainly including cationic AMPS [225,271]], indicating that chitosan's mode of action is difficult to compare with that of classical antimicrobials. For instance, the ABC transporter VraDE, which was found to be highly up-regulated upon defensin stress [225,271] was not regulated with chitosan. Upon testing the susceptibility of the *S. aureus* SG511Δ*vraDE* mutant to chitosan, no appreciable difference in MIC values was seen, compared to the parent strain.

Noteworthy of mentioning is that among the 166 genes that showed a statistically significant change in expression level, 32 (19.3 %) encoded enzymes of unknown specificity, 23 (13.9 %) were associated with proteins of unknown function and 47 (28.3%) encoded hypothetical proteins, i.e. a total of 102 out of 166 genes (61.4%) were of unspecified function. This demonstrates the complexity of such an analysis and its limitations.

4.2.10. DISCUSSION

The overall mechanism(s) of action of an antimicrobial may be defined according to the bacterial structure against which it has its main activity. Thus, three levels of interaction can be described: (i) interaction with outer cellular components; (ii) interaction with the cytoplasmic membrane and (iii) interaction with cytoplasmic constituents. Different theories have been put forward to explain chitosan's antimicrobial mode of action. Several of the proposed theories will be discussed below, based on the aforementioned experimental findings.

Some of the published papers, including those of Liu *et al.* [182] and Rabea *et al.* [249] fostered the impression that chitosan might have intracellular targets, such as DNA; by interacting with DNA it would be able to inhibit RNA and protein synthesis. Because of its cationic nature, chitosan has widely been investigated for the purpose of non-viral gene delivery in the form of DNA-chitosan complexes or as nanoparticles [24]. However, the biological significance of this property is unclear, since chitosan would not normally

be able to reach a cytoplasmic target, unless it is able to circumvent the plasma membrane, to afford access to the cytoplasmic constituents. Therefore, this mechanism is rather controversial, i.e. internal cell components are most probably not primary target sites for chitosan activity.

The chelating activity of chitosan has also been often implicated as a possible mode of action, for example by depriving bacteria of essential trace elements [47]. Yet, based on our results, chelation of metals doesn't seem to be of overriding importance for the antibiotic activity of chitosan; in contrast, complex formation with metal ions appears to abrogate this activity (section 4.1.6). Interestingly, Young *et al.* [361] suggested that polycations, including chitosan, acted by displacing cations (such as Ca^{2+}) from electronegative sites on the plant membrane which require coordination with cations for dimensional stability and maintenance of membrane integrity.

The most prominent commercial use of chitosan is as a fat binder in dietary preparations [356]. Wydro *et al.* [344] demonstrated that there are significant lipid-chitosan attractive interactions, including electrostatic (formation of electrostatic complexes through interactions of the carboxylic groups of fatty acids with $-NH_3^+$ groups of chitosan) and hydrophobic interactions, as well as hydrogen bonds between hydroxylic groups. Related to this is the question of whether chitosan, being a lipid binder, might be able to extract lipids from the bacterial membrane. In view of the data we gathered so far, this notion might be plausible, should there be sites on the cell surface where chitosan might interact with lipids extending from the membrane. However, we would have expected to observe a destabilization of liposomes upon contact with chitosan, which was not the case.

At present, the prevailing contention is that chitosan acts as a membrane perturbant [121,142,363]. Although the results discussed in this work suggest that such an activity might be part of chitosan's antibiotic mechanism, there is no evidence that chitosan's antimicrobial activity is mediated by a direct action on the cell membrane, because chitosan must first gain access to the bacterial membrane. To be able to do this, chitosan must pass through the bacterial cell wall, composed of multilayers of murein,

where glycan strands of alternating *ß*-1→4-linked Glc*N*Ac-Mur*N*Ac disaccharides are cross-linked by short peptides (Figure 18).

It has been previously reported that chitosan can penetrate plant cells, being detected 15 min after its applicaton to the surface of the plant tissue within the plant cytoplasm and conspicuously detectable within the plant nucleus [111]. Furthermore, Young *et al.* [361], suggest that quite large 'pores' can be induced in the plant membrane by chitosan, as evidenced by leakage of low- and high-molecular weight proteins; arguing that large polycations such as DEAE-dextran do penetrate the cell wall to interact with the plasma membrane. They even go further to hypothesize that polyanions (polygalacturonate) on plant cell walls help protect the plasma membrane, the actual target of chitosan action, by binding to the polycationic chitosan, thus preventing the contact with the cell membrane. However, a consideration of the molecular size of chitosan would render such a notion rather unlikely.

Various models have been proposed to predict and explain the spatial arrangement of murein in the cell wall. Vollmer and Höltje [333] argue that the preponderance of evidence supports the “horizontally layered murein model”, in which the glycan strands run parallel to the cytoplasmic membranes, with pores in the murein having a mean radius of 2.06 - 3 nm, allowing the free diffusion of globular protein with a maximum molecular mass of 50-100 kDa when stretched *in vivo*. On the other hand, Dimitriev *et al.* [65] promoted the “vertical scaffold model of murein architecture”, where both the glycan and oligopeptide chains of staphylococcal murein run in a plane perpendicular to the plasma membrane; the distance between the glycan chains would then be 4.2 nm. Yet another group proposed the “threefold helical honeycomb structure of the bacterial peptidoglycan”, where it is claimed that at the highest cross-linked strand density, intact honeycomb pores were formed with an approximate width of 7 nm, with larger pores where strands are absent [88]. What they all agreed upon was that the bacterial surface, including the peptidoglycan, must be porous, to allow the controlled ingress and egress of solutes (Figure 18).

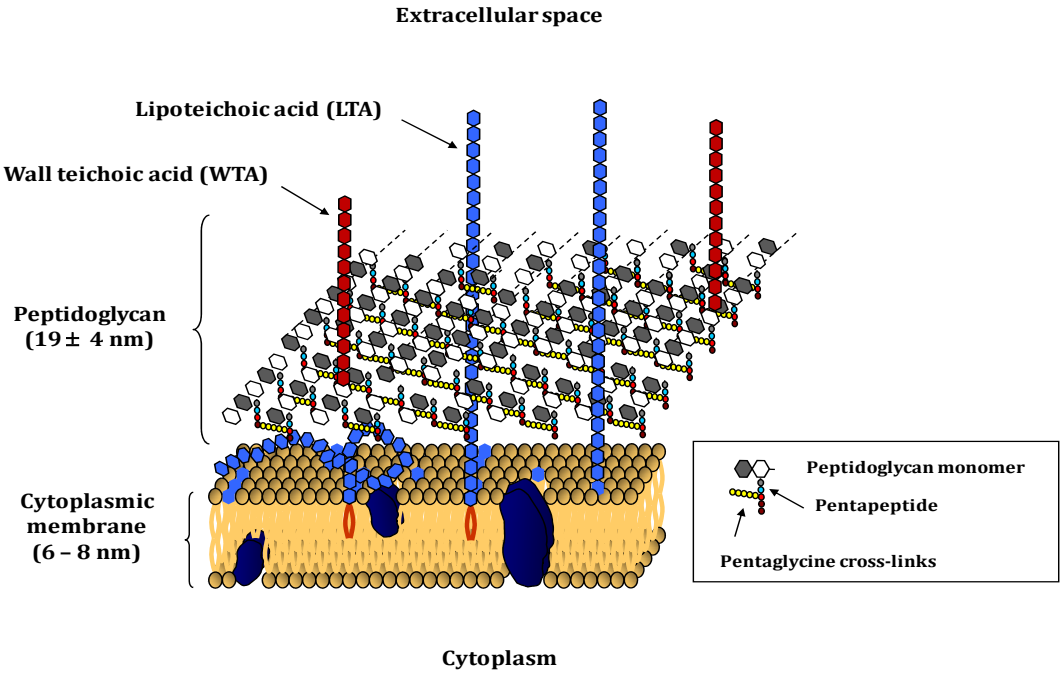

FIGURE 18: The staphylococcal cell envelope.

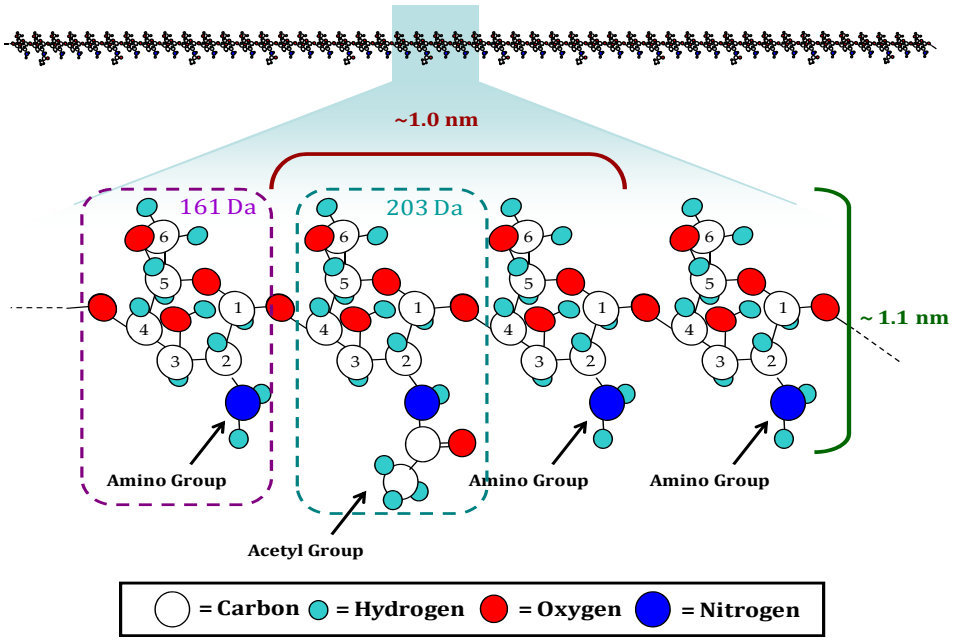

FIGURE 19: Detailed structure of a chitosan molecule.

Shown is an enlarged portion of the chitosan molecule, with relevant dimensions. For a chitosan molecule of a MW of 240 kDa and a DD of 87%, an average chain would consist of around 1400 units, with an average length of about 700 nm.

Chitosan, as already mentioned, is a linear polysaccharide. Taking into account that the average disaccharide diameter of glycan strands is around 1.1 nm [65], and that chitosan, being similar in structure, would have a diameter in the same order of magnitude in its extended conformation (Figure 19), one might hypothesize that it might be able, at least in part, to diffuse through the pores in the murein structure. However, this seems unlikely in light of the fact that chitosan most probably exists in solution in a hydrated form that is much larger. Indeed, the hydrodynamic radius R_H of chitosan, which indicates the apparent size of the dynamic hydrated particle, was 24.59 nm ± 1.4 RSD [%] (Table 18). Therefore, none of the models of peptidoglycan structure would explain how a molecule of this size might be able to cross the cell wall. Moreover, there is no evidence that chitosan is broken down by extracellular staphylococcal enzymes into active smaller fragments, which might pass the cell wall. In addition, dialyzed chitosan was fully antimicrobial (section 4.1.4), suggesting that large molecules are responsible for its activity. Notwithstanding the above evidence, the possibility that some of the chitosan molecules might penetrate through the cell wall cannot be entirely excluded.

Although chitosan and cationic AMPs share similar effects on treated cells on the cellular level, including cellular leakage and membrane perturbation, the transcriptional response patterns of both show surprisingly little similarity [225,271]. The upregulation of anaerobic pathways and the lack of interference in cell wall stress stimulon upon chitosan treatment suggest that the underlying antimicrobial mechanisms are different, which is very likely, since their chemical structures differ markedly.

TEM analysis of *S. simulans* 22 was consistent with an intact membrane, but impaired membrane function; shrinking of the membrane suggested water and ion loss from the cell. However, the addition of chitosan to the growth medium was not likely to change osmotic conditions directly; it was rather inducing the leakage of ions (potassium, for instance) by an unknown mechanism, possibly by escaping through deenergetized K^+-transporters. No gross membrane disruption or pore formation was observed. Also, it appears highly unlikely that the changes in membrane permeability

result from osmotic stress, since transcription of genes typically up-regulated under such stress conditions, e.g. those responsible for accumulating proline and betaine (PutP, BPI and BPII) [217], was not significantly altered after chitosan addition. Therefore, osmotic stress seems to be a result of chitosan's action, not its cause.

On the basis of our findings and the supporting literature, we believe that chitosan's mode of action is not confined to a single target molecule, but that the final antibiotic effect results from a sequence of rather "untargeted" molecular events, taking place simultaneously or successively, that are all probably necessary for effective cell inhibition.

Our data clearly indicate that the initial contact between the polycationic chitosan macromolecule and the negatively-charged cell wall polymers is indeed driven by electrostatic interactions, and that teichoic acids play a major role (as seen with the *dltA*-mutant, showing maximum susceptibility to chitosan), leading to a disruption of the equilibrium of cell wall dynamics. The originality of this hypothesis lies in the fact that the bacterial cell wall biogenesis is dynamic, with 40-45% of its structure released and recycled during each growth cycle [88]. Although the possibility that dealanylated teichoic acids might represent a "target" for chitosan's action might spring to mind, we can, at this stage, neither explicitly refute nor confirm this contention. However, taking into account that the concentration of LTA in the outer leaflet of the cytoplasmic membrane of *S. aureus* is 10 – 20 mol% of polar lipids [87], a possible immobilization ("cross-linking") or even extraction of LTA (extended chain around 17.5 nm in length) by chitosan may have drastic consequences on the vital lateral diffusion of proteins as well as molecular machineries located within the cell membrane; disrupting the intermolecular bonding responsible for maintaining an intact membrane, changing membrane fluidity, inhibiting normal enzymatic activity or affecting the distribution or activity of components associated with transport across the plasma membrane. This could well upset the regulation in the cytoplasmic membrane and hence alter its optimal functioning, also inhibiting important survival functions. Thus, LTA might provide a molecular link for chitosan at the cell surface, allowing it to disturb membrane functions

(Figure 18). However, the possibility that changes to the outer cell layer may occur to allow chitosan to penetrate the cell and reach internal target site(s) is highly speculative at present.

Binding of chitosan to cell wall polymers would also trigger other secondary cellular effects: destabilization and subsequent disruption of bacterial membrane function occurs, albeit via unknown mechanisms, compromising the membrane barrier function and leading to leakage of cellular components without causing distinct pore formation. In addition, membrane-bound energy generation pathways are affected, probably due to impairment of the proper functional organization of the electron transport chain, thus interfering with proper oxygen reduction and forcing the cells to shift to anaerobic energy production. This might ultimately lead to dysfunction of the whole cellular apparatus. The transition from sublethal injury, caused by disruption of the cell permeability barrier and leakage, to cell death might be mediated by metabolic imbalance and impaired ionic homeostasis following chitosan challenge. We may also tentatively speculate that the accumulation of the polymer in the membrane vicinity triggers various stress responses, due to a local low pH or other factors that remain to be identified.

Nevertheless, the precise sequence of events and the complex mechanisms by which these processes are coupled or interrelated, and their relevance to the antimicrobial activity of chitosan, have not been fully ascertained, and might indeed be rather difficult to identify.

4.3. SELECTION AND CHARACTERIZATION OF CHITOSAN-RESISTANT *S. AUREUS*

4.3.1. SUMMARY

In the present study, we investigated several aspects of the *in vitro* development of chitosan resistance, through the selection for a chitosan-resistant *S. aureus* variant (CRV), using a serial passage experiment. We demonstrated that, in comparison to the parental strain, the CRV displayed (i) modestly increased cell surface hydrophobicity; (ii) higher levels of the positively-charged phospholipid lysyl-phosphatidylglycerol (LPG); (iii) lowering of the overall negative cell surface charge (manifested by its relatively lower binding of the cationic protein cytochrome c); (iv) cross-resistance to a number of classical antibiotics as well as antimicrobial peptides; and (v) comparable response to Triton-induced lysis, but higher susceptibility to lysostaphin-mediated lysis. On the other hand, CRV was indistinguishable from the parent strain regarding colony morphology, biochemical activities, cellular ultrastructure and biofilm-forming ability. Double combinations of chitosan with friulimicin, daptomycin or Pep5 were found to be antagonistic, when tested against CRV, but not against the wild-type. Finally, transcriptional profiling suggested major alterations in the structure of the cell envelope, as well as cellular metabolism. These data suggest a close nexus between changes in cell envelope structure and charge with the *in vitro* susceptibility of *S. aureus* to the action of chitosan. Moreover, the cross-resistance to antimicrobial peptides may also indicate a more general mechanism of resistance.

4.3.2. RATIONALE AND OBJECTIVES

Increasingly frequent reports have described the *in vivo* as well as *in vitro* development of bacterial resistance to antimicrobials; the mechanisms by which this occurs are rather complex; transport across the cell membrane, enzymatic inactivation of the antibiotic and target alterations might be involved. On the other hand, investigations into the potential mechanism(s) of chitosan resistance are lacking,

prompting us to conduct a detailed investigation of the determinants of chitosan resistance in *S. aureus*. Moreover, a genetic and biochemical understanding of the adaptive mechanisms of *S. aureus* to chitosan, complex though those might be, is critical to our understanding of how this compound exerts its antimicrobial activity in the first place and, on a more general note, might help us to shed some light on the physiological adaptation of microbes to antibiotic stress.

Since the bacterial cell envelope is a principal target for initiating the staphylocidal pathway of chitosan (section 4.2.10), the present study was designed to investigate several aspects of the *in vitro* decrease in chitosan susceptibility among staphylococcal strains, especially to delineate possible changes in cell surface phenotypes relevant to the *in vitro* chitosan resistance in a well-characterized laboratory strain, *S. aureus* SG511, from which a stable variant exhibiting more than 50-fold reduction in its sensitivity towards chitosan was obtained. We then attempted to demonstrate the multifaceted phenotype of the chitosan-resistant variant (CRV), by using a number of techniques, to investigate: (i) biochemical activities; (ii) microscopic ultrastructure; (iii) membrane phospholipid (PL) composition; (iv) cell surface charge; (v) cell surface hydrophobicity; (vi) antibiotic susceptibility profiles, including both prototypic cationic antimicrobial peptides (AMPs) as well as classical antibiotics, in addition to binary combinations of antimicrobials (checkerboard titrations); and (vii) autolytic behavior. In addition, differential gene expression profiling was conducted to compare the transciption profiles of chitosan-sensitive and -resistant cells. A number of interesting observations emanated from this study.

4.3.3. *IN VITRO* SELECTION FOR DECREASED SUSCEPTIBILITY TO CHITOSAN

We selected for a chitosan-resistant *S. aureus* SG511 variant, using a serial passage experiment as described in section 3.2.9, by the use of incrementally increasing chitosan concentrations. MIC and MBC analyses of daily archived colonies from the different passages 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 (after 72 h), indicating that a critical "genetic event" probably took place at this time-point (Table 23 and Figure 20). The experiment was ended after 15 passages, when the MIC values reached a plateau. Since the final variant obtained had an MIC of 1750 µg/ml, it was termed chitosan-resistant variant (CRV). The observed adaptation to high levels of chitosan was stably inherited after 20 consecutive passages of the CRV in chitosan-free CAMHB, indicating that the adaptation was genotypic rather than phenotypic.

TABLE 23: MIC and MBC values of chitosan during the serial passage experiment

Passage number	Chitosan conc. (µg/ml)	MIC (µg/ml)		Fold increase in MIC		MBC (µg/ml)
		24 h	72 h	24 h	72 h	
0	**0**	**31.25**	**31.25**	**1**	**1**	**31.25**
1	40	46.875	46.875	1.5	1.5	46.875
2	80	78.125	78.125	2.5	2.5	78.125
3	120	187.5	187.5	6	6	187.5
4	160	218.75	218.75	7	7	218.75
5	200	312.5	312.5	10	10	312.5
6	240	375	375	12	12	437.5
7	280	375	1500	12	48	1500
8	320	437.5	1750	14	56	1750
9	375	750	1500	24	48	1750
10	437.5	500	1500	16	48	1500
11	500	1500	1750	48	56	1750
12	625	1500	1750	48	56	1750
13	1500	1750	1750	56	56	1750
14	1750	1500	1500	48	48	1750
15	2000	1500	1750	48	56	1750

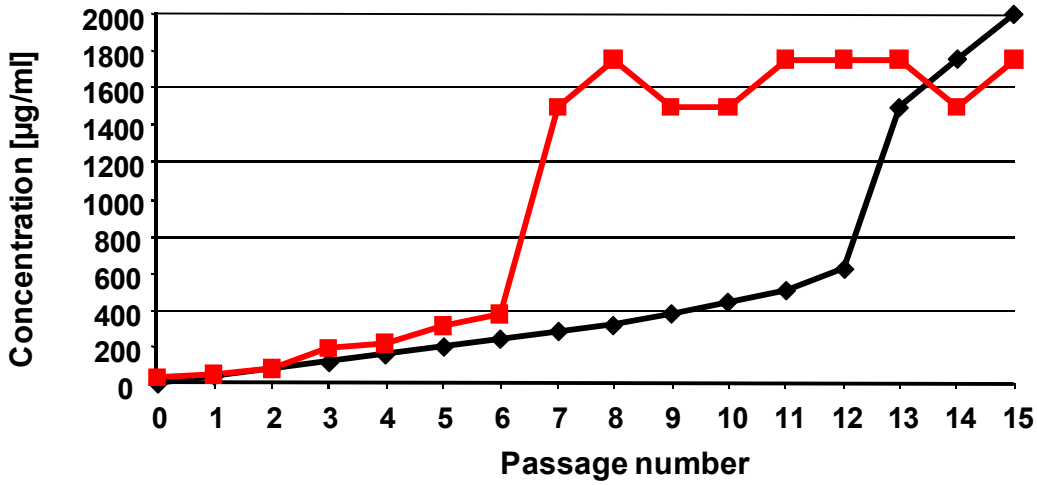

FIGURE 20: Selection for a chitosan-resistant *S. aureus* SG511 variant using the serial-passage experiment.

***S. aureus* SG511 was incubated 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 hrs of incubation (■).**

4.3.4. PHENOTYPIC STUDIES

In the next series of experiments, we carried out a detailed characterization of the CRV, with the intention of identifying key phenotypic changes that might have occurred to the parent strain, resulting in the emergence of the CRV, which would correlate to the increased resistance observed.

4.3.4.1. Biochemical activities

As a first approach, we compared the biochemical activities of this strain set using the MICRONAUT-RPO plates (section 3.2.5), which include 44 reactions specific for testing for peptidases (15), glucosidases / esterases (12), fermentation reactions (14) and decarboxylases (3); in addition to standard biochemical tests. Both variants exhibited similar biochemical activities, except for a slightly delayed coagulase reaction observed with the CRV. In addition, no difference in colony morphology was observed.

4.3.4.2. Killing assay

The number of survivors of both variants (parent strain and CRV) was compared after 20 min of exposure to varying chitosan concentrations. We have observed a marked reduction in the viable count of the parent strain with increasing chitosan concentrations, whereas the CRV was fairly unaffected by the tested concentrations, even when increasing the chitosan concentration to up to 5000 µg/ml (not illustrated). The killing assays were performed on two separate days, and the mean number of survivors was determined.

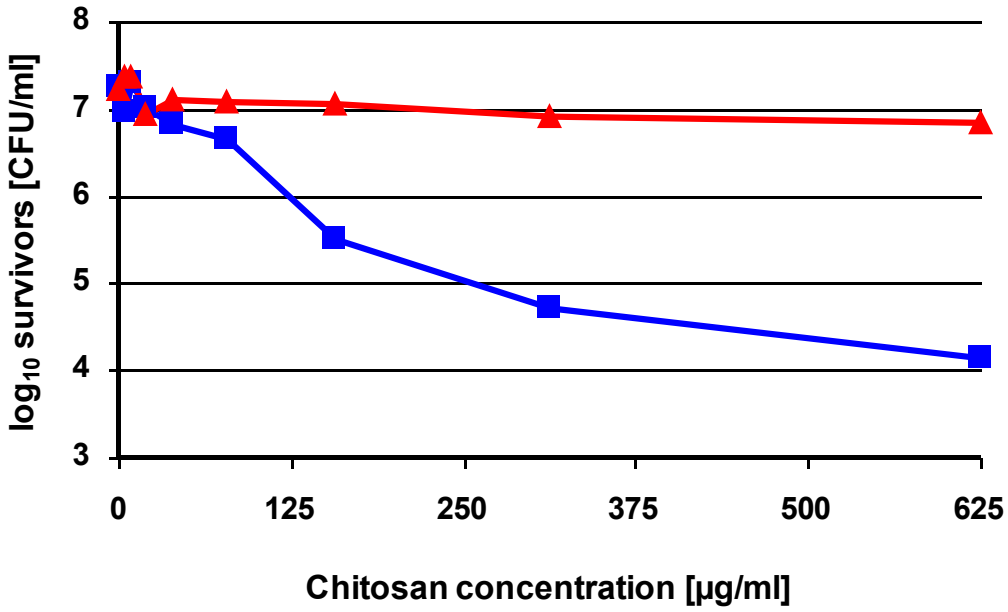

FIGURE 21: Killing assay for the isogenic strain pair.

Cultures of *S. aureus* SG511 (wild-type, ■) and its CRV (▲), at ca. 10^7 CFU/ml, were incubated with different chitosan concentrations (4.88 - 625 µg/ml) for 20 min, then the surviving count was plotted against the tested chitosan concentration.

4.3.4.3. Cross-resistance to other antimicrobials

Determining the MIC of an antimicrobial is a classical method of assessing its potency. A standard broth microdilution technique was used to determine the MIC of various selected antimicrobials from different classes, some of which currently used in clinical practice against staphylococci, for the parent strain *S. aureus* SG511 and its CRV.

Compared to the parental strain, CRV posessed higher MIC values (1.5 — 32-fold) for most of the antibiotics tested in this study (Table 24). On the other hand, CRV remained fully susceptible to a group of antimicrobials, including *ß*-lactams (ampicillin, oxacillin, cefoxitin, ceftriaxon), quinolones (moxifloxacin), synercid, teichoplanin, spectinomycin and linezolid.

TABLE 24: The MIC of various antimicrobials for *S. aureus* SG511 and CRV

Antimicrobial	*S. aureus* SG511			CRV			Fold MIC (48 h)
	MIC (µg/ml)*		MBC* (µg/ml)	MIC (µg/ml)*		MBC* (µg/ml)	
	24 h	48 h		24 h	48 h		
Chitosan	**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**

* Results are the average of 2 determinations.

The documented cross-resistance of CRV to the cationic peptides and common antimicrobials in our study should be emphasized. Since aminoglycosides, tetracycline, chloramphenicol and erythromycin are inhibitors of protein synthesis, which bind to the 30S or 50S ribosomal subunits, it seems highly unlikely that chitosan would compete with these antibiotics for their bacterial target. However, it is conceivable that this cross-resistance is due to the decreased accumulation of these antibiotics, as a result of 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; (iii) sequestering of the tested antibiotics, thus inhibiting them from reaching their target site; and (iv) shielding of the target site by competitive binding to it, as could be the case with those antimicrobials that act on the bacterial surface, such as the glycopeptide vancomycin, and the antimicrobial peptides.

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, ultimately resulting in bacterial impermeability to these compounds.

4.3.4.4. Checkerboard assay

The activity of double combinations of antimicrobials, comprising chitosan with daptomycin, friulimicin or Pep5 was studied against this strain pair, *S. aureus* SG511 (wild-type) and CRV. Fractional inhibitory concentration (FIC) analysis was carried out in microtiter plates using the two-dimensional checkerboard procedure (section 3.2.4). The MIC of chitosan for CRV was at no time affected by the presence of any of the three antimicrobials. In contrast, the coincubation of each of the three tested antimicrobials and ½ the MIC of chitosan increased the MIC values of each peptide by more than four-fold compared to those for each peptide alone (FIC index > 4.0), indicating that all three double combinations were less active against CRV. These interactions were interpreted as antagonistic, and were thought to originate from shielding of the bacterial target site. Interestingly, these antagonistic activities were not observed with the parent strain.

4.3.4.5. Growth curves

The growth rate and growth patterns of CRV were assessed by viable count estimations; the parent strain exhibited no growth advantage over CRV. However, the optical density of both cultures varied greatly (Figure 22); although the viable counts were almost identical at the various sampling intervals, the optical density of the CRV culture was mostly much higher than that of the parallel wild-type culture.

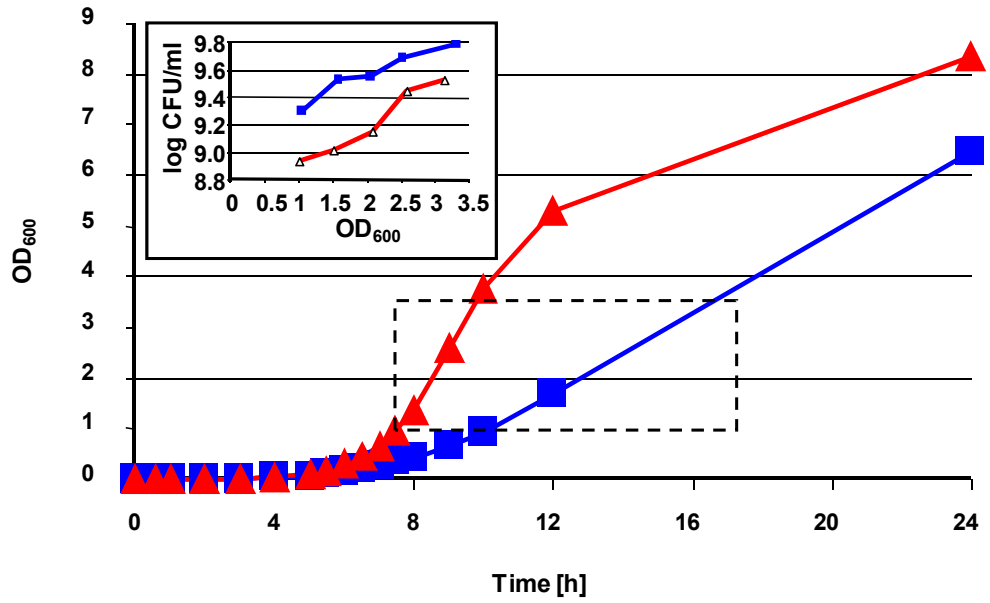

FIGURE 22: Growth curves of *S. aureus* SG511 and CRV in CAMHB.

Cultures of *S. aureus* SG511 (wild-type, ■) and its chitosan-resistant variant (CRV, ▲) were incubated in CAMHB at 37°C and 150 rpm. The OD_{600} of both cultures was determined at the selected time points. The insert shows a standard curve, where viable cell counts (CFU/ml) of both variants were plotted against OD_{600}, in an optical density range of 1.0 – 3.5.

Measurement of optical density (turbidimetry) is a common method of rapid monitoring of pure prokaryotic cultures, which is based on the principle of light scattering, where light passing through a suspension of microorganisms is scattered and the amount of light transmitted is inversely proportional to optical density (most closely related to the dry weight of cells). It could therefore be taken as an indication of the biomass present in the suspension [160].

We observed that the OD_{600} as well as the absorption spectrum (350 – 650 nm) of the supernatant of CRV cultures was more or less the same as that of the wild-type, indicating that the higher optical density measurements were associated with the bacterial cells, probably implying a change in their size, shape, or in the internal light-absorbing components of the cells, thus introducing some inaccuracy to this method of cell counting. Moreover, the dry weights of *S. aureus* SG511 (WT) and CRV were 5.87 ± 0.058 and 6.10 ± 0.1 mg/ml, respectively (average of 3 determinations).

To rule out the possibility that the higher optical density measurements were related to a change in clumping behavior of the cells, we examined cells of both cultures under an ordinary light microscope, following negative staining with India ink; no signs of difference in cellular arrangement were seen, with both cells exhibiting pairs, tetrads, individual cells as well as clusters (Figure 23).

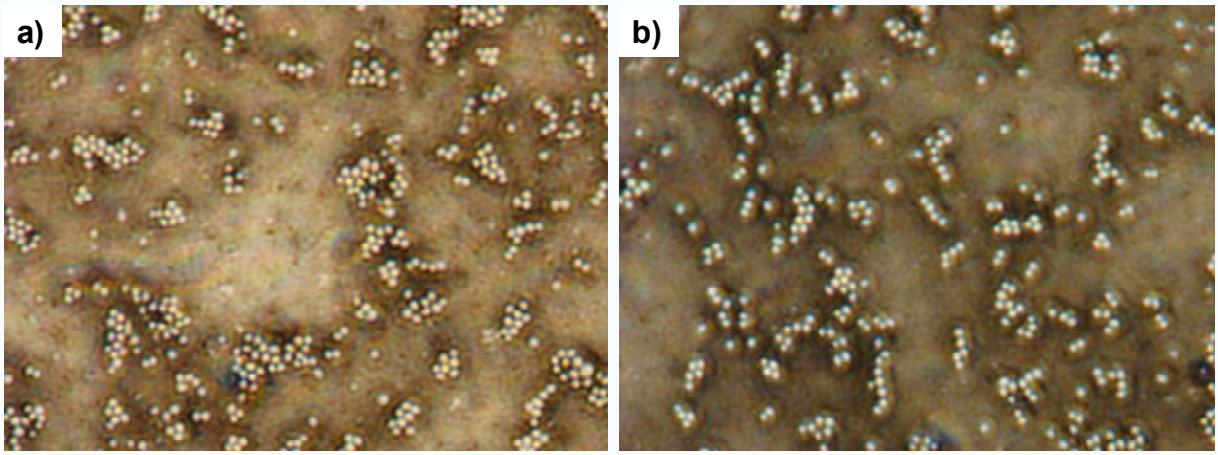

FIGURE 23: Bacterial cellular arrangement.

Shown are cells of wild-type *S. aureus* SG511 (a) and CRV (b) as seen under an ordinary light microscope after India ink negative staining.

Noteworthy of mentioning is that estimates of viable cell concentrations for all the assays were ascertained by reference to the predetermined standard curves (Figure 22, insert).

4.3.4.6. Biofilm assay

Biofilms are sessile microbial communities embedded in a self-produced extracellular polymeric matrix. They have a structurally complex and dynamic architecture and develop on many abiotic (plastic, glass, metal and minerals) and biotic surfaces (plants, animals and humans) [112,337]. Biofilm bacteria evade antimicrobial challenges (antibiotic therapy and host defenses) by 2 main mechanisms: (i) failure of the antimicrobial agent to penetrate the biofilm, due to exopolysaccharides, acting as a barrier against the penetration of antibacterial agents; and (ii) adoption of a resistant physiological state or phenotype by at least a fraction of the cells in a biofilm (such as decreased growth rate) [69,337].

We evaluated the possibility that the CRV produced biofilms or secreted some extracellular polymeric substances, which would i) accumulate outside the bacteria and act as a diffusion barrier, impairing the penetration of antibiotics, thus conferring the increased *in vitro* resistance of these cells; and ii) account for the higher optical density values. For that purpose, we compared both variants with respect to their ability to form biofilms in a static biofilm system, using the microtiter plate biofilm assay (section 3.2.11.1), which is a useful method for assessing bacterial attachment to an abiotic surface by measuring the staining of the adherent biomass.

Unlike the compact and dense biofilms produced by staphylococci in glucose-supplemented rich media such as TSB or BHI broth [108], the biofilms produced in CAMHB were much less organized, giving more or less inconsistent results in our initial optimization of the biofilm experiment (not illustrated). Safranin staining was employed, as it is an established method for staphylococcal biofilm research. Using the quantitative safranin-staining measurements, as well as OD_{600} and viable count estimations of viable biofilm bacteria, we could demonstrate no substantive quantitative difference in the biofilm-forming behavior of both variants, irrespective of the parameter examined (Figure 24). OD_{600} values (± SD) were 0.98±0.15 and 1.06±0.23, log viable counts (± SD) were 9.19±0.09 and 9.28±0.08, and A_{492} (± SD) were

3.03±0.32 and 3.56±0.79, for the wild-type and CRV respectively. Taken together with the fact that CRV lawns had no slimy mucoid nature, these findings argue against the hypothesis that the production of biofilms as a survival strategy accounts for the increased resistance of CRV.

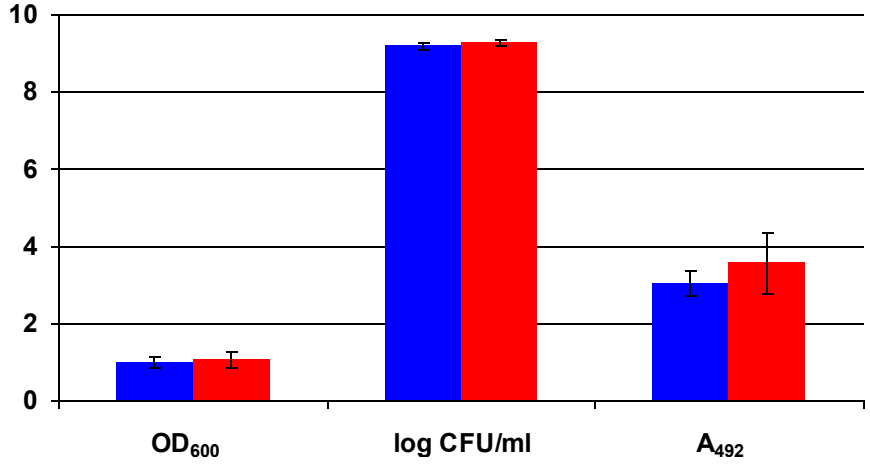

FIGURE 24: Comparison of the biofilm-forming ability of the isogenic strain pair using a semiquantitative microtiter plate method.

Biofilms of *S. aureus* SG511 (WT, blue bars) and CRV (red bars), respectively, in 96-well tissue culture plates (Nunclone™) were eluted in sterile PBS for optical density determination (OD_{600}) and estimation of viable counts (log CFU/ml). Moreover, the absorbance of biofilms stained with 0.1% safranin and eluted in 33% acetic acid was determined at 492 nm (A_{492}). Values shown here are the means of nine (OD_{600} and viable counts) and twelve (A_{492}) replicate determinations ± SD.

4.3.4.7. Transmission electron microscopy

In order to identify changes in bacterial microscopic morphology that might be associated with the decreased susceptibility of the chitosan-resistant variant, we carried out an ultrastructural examination of both variants under a transmission electron microscope. No significant difference was observed in the morphology of the cells as evidenced by TEM analysis (Figure 25). Moreover, we could not detect any difference in cell size or cell wall thickness, determined on the basis of the electron micrographs taken at an amplification of 25,000× (WT) and 31,000× (CRV); the average of 20 and 16 determinations, respectively, was determined.

Our electron microscopical findings are therefore at variance with reports that implicate cell wall thickening as a physical barrier in the resistance of bacteria to surface-active compounds, including vancomycin and daptomycin [52,53].

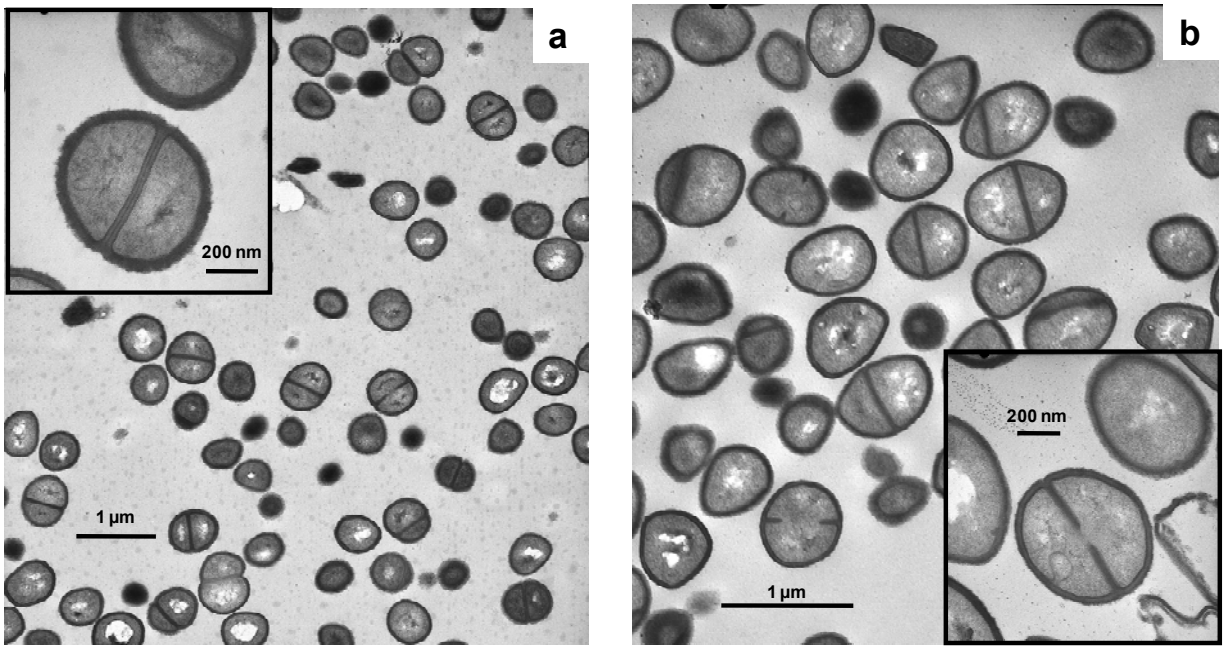

FIGURE 25: Ultrastructure of *S. aureus* SG511 and CRV, as seen under a TEM.

Pictures of *S. aureus* SG511 (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.

4.3.4.8. Autolysis

Autolysis assays were conducted in order to test the response of *S. aureus* SG511 (WT) and the CRV to the addition of Triton X-100 and lysostaphin, respectively. Triton X-100 is a non-ionic detergent with solubilizes and thus ruptures the cell membrane, as well as activates the autolysins [166,251], while lysostaphin is a glycylglycine endopeptidase, which specifically cleaves a Gly-Gly bond in the pentaglycine inter-peptide link joining staphylococcal cell wall peptidoglycans, ultimately causing lysis of bacterial walls.

The rates of Triton X-100-induced autolysis seemed to be equivalent among the isogenic strain set over the 30 hours of the assay. However, the CRV cells were more sensitized to lysosaphin-mediated autolysis, lysing more quickly than the parent strain during the first hour of exposure to lysostaphin (Figure 26); possibly suggesting different cross-linking densities of the peptidoglycan chains in both variants.

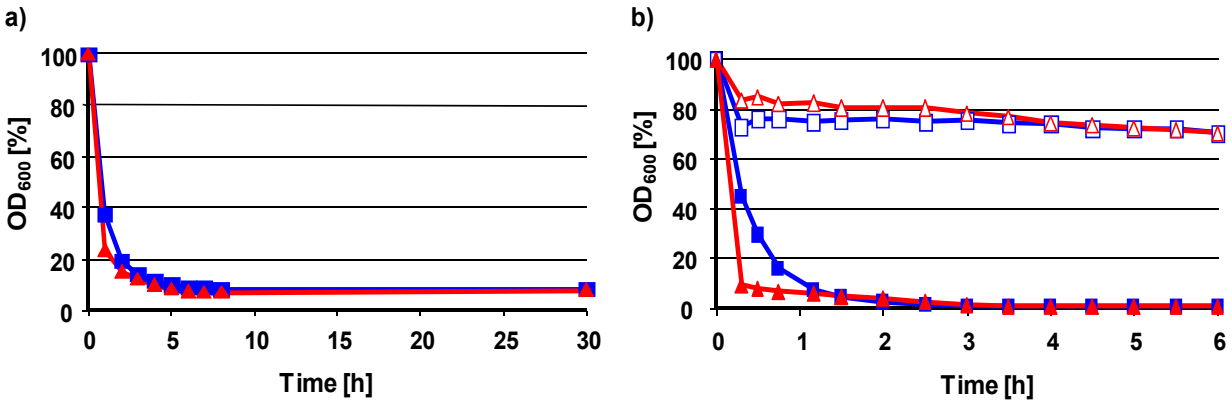

FIGURE 26: Autolysis assays of *S. aureus* SG511 and CRV.

The figure depicts the response of early logarithmic phase cultures of *S. aureus* SG511 (WT, ■) and CRV (▲) to treatment with a) 0.05% [vol/vol] Triton X-100 and b) lysostaphin (4 µg/ml), respectively. The bacterial suspensions were incubated at 37°C with gentle agitation. Autolysis was quantified as a per cent of the initial OD_{600} [%OD_{600}]. Open symbols indicate the behavior of control cultures run in parallel.

4.3.4.9. Hydrophobicity

The hydrophobic nature of the outermost surface of various microbial cells has been implicated in a number of biological phenomena. The determinant of hydrophobicity is believed to be a protein or protein-associated molecule localized at the cell surface of the organism, i.e., a component of either the cell wall, cell membrane, or both [252]. We used a simple and rapid quantitative method for estimating cell-surface hydrophobicity of CRV in comparison with the corresponding wild-type *S. aureus* SG511, based on the relative affinity of both variants to various liquid hydrocarbons following a brief period of mixing (BATH assay, section 3.2.14).

A decrease in the optical density of the respective bacterial suspension after treatment with the hydrocarbon would indicate that the cells had passed into the non-aqueous layer (= organic layer). Bearing in mind the variation in results obtained with different hydrocarbons, the BATH assay showed that the CRV exhibited a modestly higher cell surface hydrophobicity than its parent strain (Figure 27). The relative affinity (H% ± SD) of *S. aureus* SG511 and CRV to the liquid hydrocarbons 1-octanol, xylene and cyclohexane were 73.31 ± 3.05, 86.22 ± 1.07, 87.33 ± 2.30; and 74.04 ± 0.24, 92.96 ± 0.08, 95.05 ± 0.47, respectively.

An increase in cell surface hydrophobicity is apparently one of the mechanisms, by which staphylococci adapt to chitosan, as it might be implicated in increasing bacterial impermeability to this polycationic biopolymer.

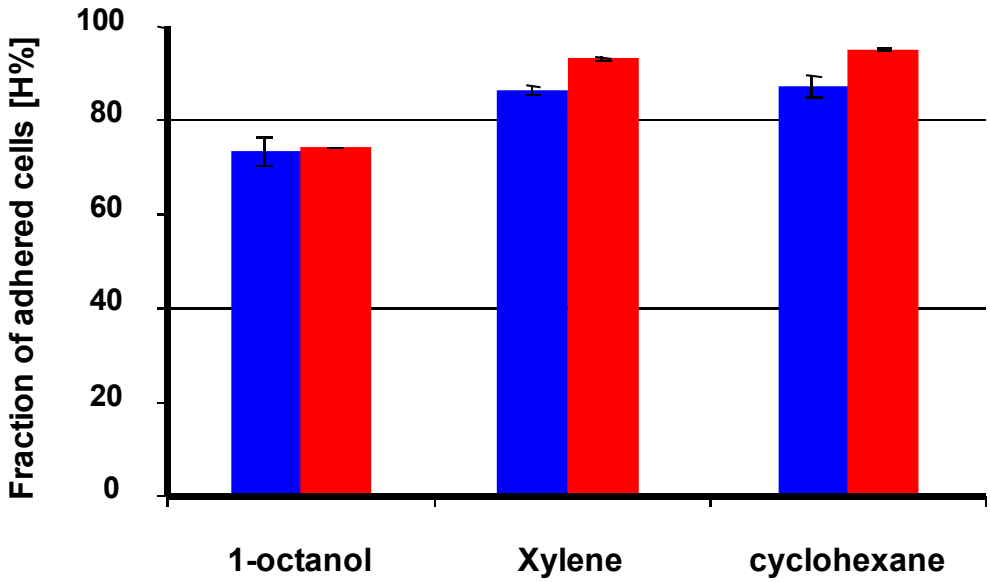

FIGURE 27: Relative affinity of *S. aureus* SG511 and CRV to liquid hydrocarbons.

Suspensions of *S. aureus* SG511 (blue bars) and CRV (red bars) were mixed for a short period of time with the indicated liquid hydrocarbons. The fraction of adhered cells [H%] was determined by calculating the percentage of cells adhering to the hydrocarbon phase, with respect to the initial optical density of the aqueous bacterial suspensions. Each histogram represents the means ± SD of two replicate experiments.

4.3.4.10. Cell surface charge

Cytochrome c is a cationic protein (pI of around 10) that has been previously used to estimate the relative surface charge of the cell envelope of isogenic *S. aureus* strain pairs [238] and to study the interactions of cationic peptides with charged bilayer membranes [120]. The lower the percentage of cytochrome c bound to the *S. aureus* pellet, the more positively-charged the *S. aureus* cell envelope. The absorbance of cell-free supernatants was determined at 410 nm (contrary to reference [238]), the absorbance maximum of cytochrome c.

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 a decrease in overall cell negative charge (Figure 28). The strains *S. aureus* SA113 WT (4.05 ± 0.11%) and *S. aureus* 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 cytochrome c bound (since less positively-charged), compared to the control.

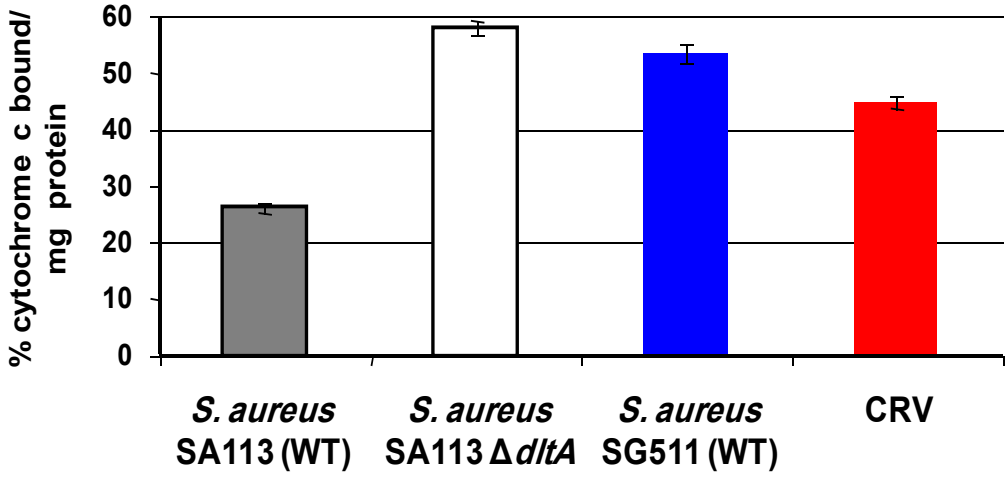

FIGURE 28: Cell surface charge of the test strains, as determined using the cytochrome c binding assay.

The % of cytochrome c bound by each of the four indicated strains was determined with reference to the bacterial dry weight. Absorbance measurements were carried out at 410 nm. The bars represent the mean values of three determinations ± SD for each strain.

The results of this assay thus mirror our previous data concerning the various mutants of *S. aureus* SA113, which showed the impact of cell surface charge on chitosan's antimicrobial activity (section 4.2.8). The bacterial cell surface charge apparently plays a pivotal role in the initial step of interaction between chitosan and the bacterial cell surface, suggesting that repulsive electrostatic forces could have a considerable impact on the resistance of CRV to chitosan, as well as to other cationic antimicrobials.

Taken together with the fact that the CRV failed to collect as a dense pellet at the bottom of polypropylene tubes upon centrifugation, forming frayed, loose and diffuse pellets; both the altered surface hydrophobicity and cell surface charge suggest that changes in cell surface compounds take place, probably involving changes in the chemical nature of the cell envelope, demonstrating that the cell surface properties play a major role in *S. aureus* resistance to the antimicrobial activity of chitosan.

4.3.4.11. Analysis of membrane phospholipid profiles

An additional mechanism by which bacteria may alter their susceptibility to antimicrobials relates to the membrane phospholipid content. The *S. aureus* cell membrane consists of three major PL species: negatively-charged phosphatidylglycerol (PG) and cardiolipin (CL) and positively-charged lysyl-phosphotidylglycerol (LPG), the latter accounting for up to 38% of the total PL content of the *S. aureus* cytoplasmic membrane, contributing to the relative positive charge characteristics of the staphylococcal cell surface [237].

Whereas *S. aureus* produces LPG in comparatively high amounts, coagulase-negative staphylococci contain only traces (*S. epidermidis*, *S. xylosus*) or no detectable amounts (*S. haemolyticus*, *S. saprophyticus*) of this unusual lipid [209]. MprF (FmtC) is the enzyme involved in the biosynthesis of LPG through esterification of PG with L-lysine, and for its translocation into the outer leaflet [178,237]. Several laboratories have shown that the overall content of this unique positively-charged membrane phospholipid species, LPG, may substantially affect the overall surface charge of the organism [201,347].

Peschel *et al.* [237,238] have shown that deletion of the genes responsible for the lysinylation of PG (*mprF*) or the D-alanylation of cell wall teichoic acid (*dltA*) yields staphylococcal cells with more negatively-charged cell envelope, and hence increased *in vitro* susceptibility to a broad variety of cationic AMPs, including PMPs, hNP-1, and gramicidins, due to the increased attraction and binding of cationic AMPs by the bacteria.

In an attempt to evaluate possible alterations in the PL membrane composition of CRV, which might correlate with its reduced sensitivity towards chitosan, we isolated membrane lipids from *S. aureus* SG511 and its CRV, and analyzed them by 2D-TLC (Figure 29a). Definable differences were observed between the membrane PL profiles of both variants, the CRV exhibiting significantly increased LPG levels (6 - 7× higher), relative to the chitosan-susceptible parent strain; this was kindly confirmed by Christoph Ernst (University of Tübingen, Germany) in a 1D-TLC, where LPG was specifically stained with ninhydrin (Figure 29b).

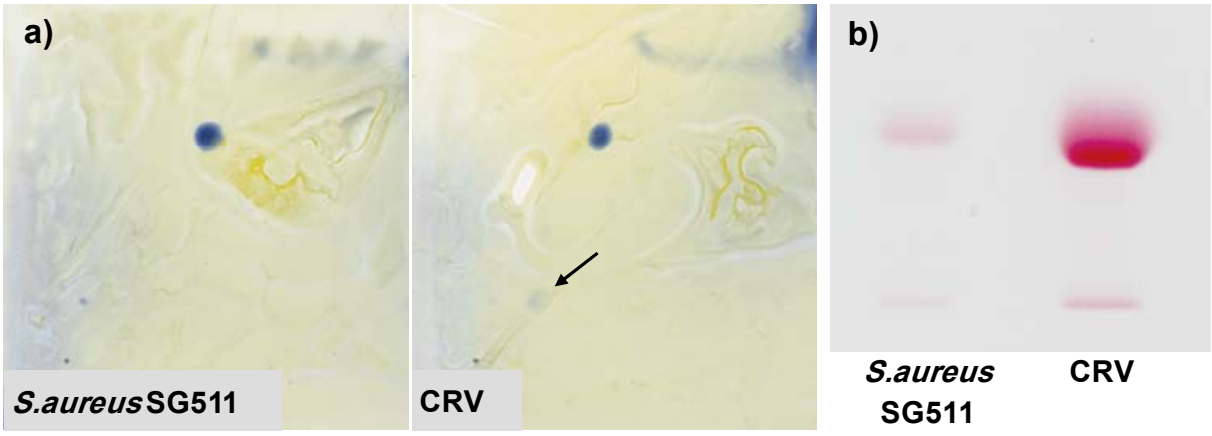

FIGURE 29: Analysis of membrane PL composition of the isogenic strain pair.

a) Membrane extracts of the strain pair separated by 2D-TLC. The arrow points out the location of the LPG spot. b) Specific staining of the LPG fraction after 1D-TLC using ninhydrin.

Our data thus support the notion that *S. aureus* cells protect themselves against cationic antimicrobial compounds by modulating the electrostatic properties of their cell envelope; i.e. 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. Interestingly, an *mprF*-null deletion mutant of *S. aureus* SA113 also showed increased *in vitro* sensitivity to chitosan (MIC = 15.63 μg/ml). Thus, it was not unexpected that this enhanced production of LPG has a significant impact on membrane surface charge and interactions with chitosan.

Overall, our results do indicate that an overall negative cell surface charge seems to be required for the antimicrobial activity of chitosan against *S. aureus*, and to this extent they are consistent with the conclusions that can be drawn from the published studies mentioned above.

4.3.5. DIFFERENTIAL GENE EXPRESSION PROFILING

While the genes of an organism are relatively fixed, the mRNA population represents how genes are expressed under any given set of conditions. Analysis of RNA by microarray analysis can thus provide a good reflection of an organism's gene expression profile in a certain biological state. Genotypic studies were therefore conducted in order to compare the gene expression profile of the parental strain *S. aureus* SG511 with that of CRV, in order to be able to draw conclusions about changes in gene expression patterns of the CRV that might account for its increased resistance.

A statistical analysis of the results, at an FDR limit of 5 %, identified a total of 333 genes differentially expressed in CRV, as compared to the wild-type strain. The detailed results of the microarray experiment, including complete lists of up- (221 genes) and down-regulated genes (112 genes) in CRV, compared to the parent strain, are given in sections 6.3.1 and 6.3.2, respectively. Figure 30 summarizes the regulated genes, according to their function.

Probably the most striking feature is that around 39% (86 out of 221) of up-regulated ORFs encode for hypothetical proteins, which did not make the analysis of the data any easier. In addition, a considerable number of genes involved in metabolism (30%), transport and binding of proteins (14%) and pathogenicity (7%) were also regulated.

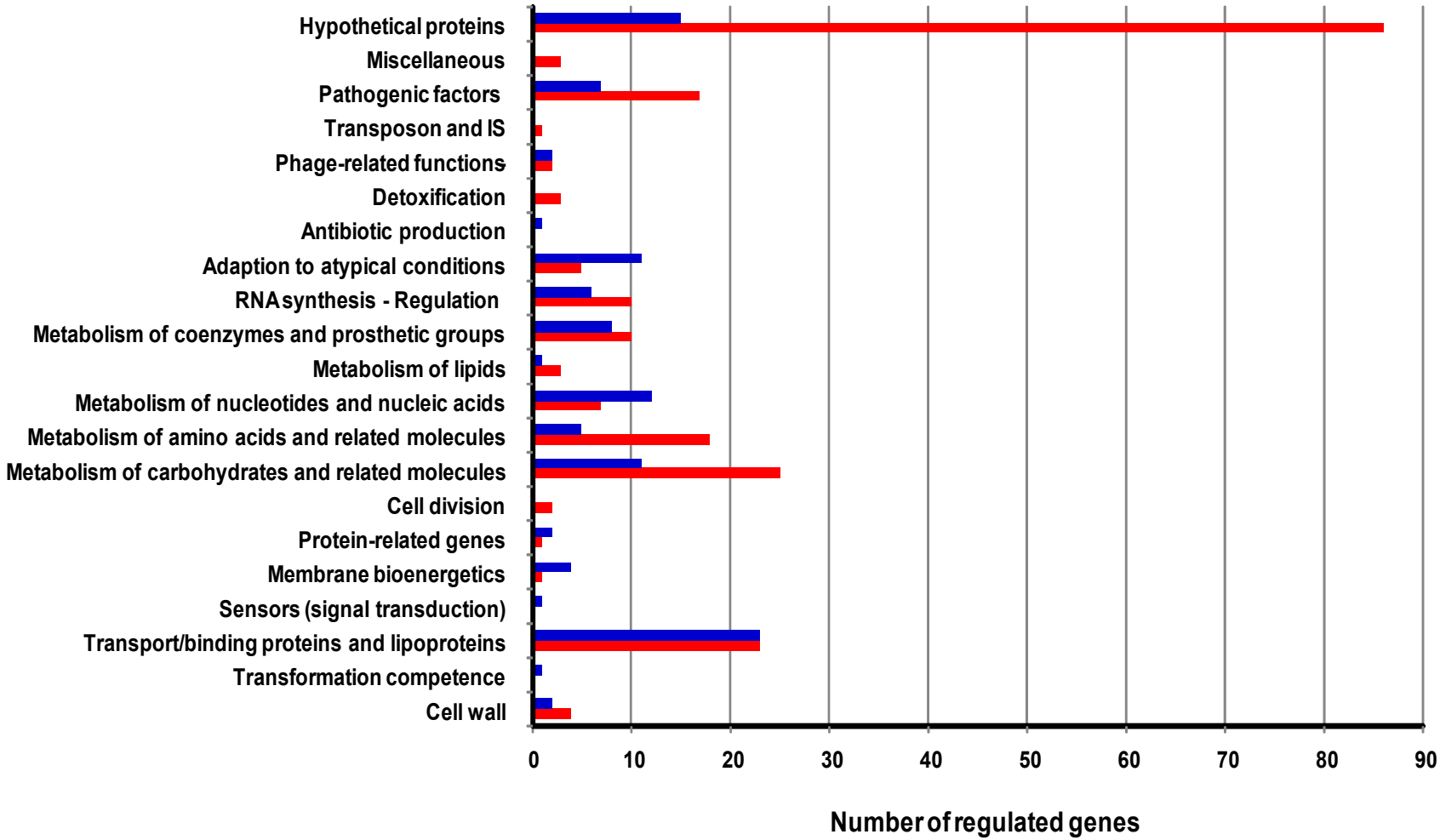

FIGURE 30: Genes regulated in CRV, compared to the parent strain.

The figure summarizes genes which were up-(red bars) and down-regulated (blue bars) in the CRV, compared to the parent strain.

The gene with the highest level of upregulation (> 240× higher levels in CRV) is SA2192, which encodes a hypothetical protein, consisting of 60 aminoacids (183 bp); with a molecular mass of 6976 and an isoelectric point at 10.57, as determined using the Pepstats software. It has the following aminoacid sequence:
MVERYIKVLILYIFTTLLSSISVTSKCVPNKVIRFILRTAIGYSIFAYGLHYFSNLKKNK

Using the program blastx (**B**asic **L**ocal **A**lignment **S**earch **T**ool; http://www.ncbi.nlm.nih.gov), a similarity search program, we compared the SA2192 sequence with previously characterized genes; 10 hits of sequences with a significant sequence similarity to SA2192 were identified (Table 25), which were then aligned using ClustalW2 software (Figure 31). Interestingly, this gene was annotated only in staphylococci.

TABLE 25: Sequences producing significant alignment to SA2192

Description*	Species	Length (aa)‡	Score (Bits)	E-Value	Similarity (%)	Positives (%)	Gaps (%)
HP SAV2404	*S. aureus* subsp. aureus Mu50	60	120	4e-26	100	100	0
HP MW2326	*S. aureus* subsp. aureus MW2	60	119	6e-26	98	100	0
HP SAR2494	*S. aureus* subsp. aureus MRSA252	60	112	9e-24	88	95	0
probable membrane protein	*S. aureus* RF122	60	111	2e-23	88	93	0
HP SH0648	*S. haemolyticus* JCSC1435	60	66.6	6e-10	50	71	0
HP SSP0496	*S. saprophyticus* subsp. saprophyticus ATCC 15305	58	57.8	3e-07	44	67	0
HP SAV0239	*S. aureus* subsp. aureus Mu50	57	41.6	0.019	36	63	8
HP SAR0232	*S. aureus* subsp. aureus MRSA252	57	41.2	0.025	36	63	8
HP SERP0326	*S. epidermidis* RP62A	72	39.7	0.072	34	58	6
HP SE0441	*S. epidermidis* ATCC 12228	61	39.7	0.072	34	58	6

* HP, hypothetical protein

‡ Number of aminoacids

```
SA2192_Staphylococcus        MVERYIKVLILYIFTTLLSSISVTSKCVPNKVIRFILRTAIGYSIFAYGLHYFSNLKKNK 60
SH0648_Staphylococcus        MKERYLKVLALFSVSTLIPTVLLNSKAIDNSAIKYVLRTALGYGIFATGLRYLSRLKAHK 60
SSP0496_Staphylococcus       MNKRYMKVLGLYSFSTLIPTVLLNEKTLSSHTFKWFLRTLAGYGIFAYGLHFLSKFKQ-- 58
SAR2494_Staphylococcus       MAQRYIKVLILYIFTTLLSSITVTSKCVPNKVVRFILRTVVGYGIFAYGLHYFSNLKKNK 60
PMP_Staphylococcus           MAQRYIKVLILYIFTTLLSSIIVTSKCVPNKVVRFILRTVVGYGIFAYGLHYFSNLKKNK 60
SAV2404_Staphylococcus       MVERYIKVLILYIFTTLLSSISVTSKCVPNKVIRFILRTAIGYSIFAYGLHYFSNLKKNK 60
MW2326_Staphylococcus        MVERYIKVLILYIFTTLLSSISVTSKCVPNKVIRFILRTAIGYSVFAYGLHYFSNLKKNK 60
SAV0239_Staphylococcus       --QRYVKVFALYFVSIVTANIIVKN----NNLIKTLIQTIAGYTVFAVGLKYLTK-RKNK 53
SAR0232_Staphylococcus       --QRYVKVFALYFASIVTANIVVKN----NNLIKTLIQTLAGYTVFAVGLKYLTK-RKNK 53
SERP0326_Staphylococcus      MNPRYVKVFFLYLVSTFISNRLTTNK----SLCKTLMQGILGYGLFALGLKYLTI--KKK 54
SE0441_Staphylococcus        MNPRYVKVFFLYLVSTFISNRLTTNK----SLCKTLMQGILGYGLFALGLKYLTI--KKK 54
                                **:**: *:  : . ..  ..       : .::  ** :** **::::
```

FIGURE 31: CLUSTAL W alignment of the sequences identified by BLAST.

Based on its protein structure, the gene product of SA2192 is certainly not an enzyme (ArchaeaFun 1.0 Server, Technical University of Denmark); it might be associated with "energy metabolism/transporter" (ProtFun 2.2 server, Technical University of Denmark).

Prediction of the transmembrane topology of SA2192 was accomplished using the Hidden Markov Model-based transmembrane domain prediction with TMHMM server v. 2.0 (Technical University of Denmark, http://www.cbs.dtu.dk/services/TMHMM/); it contains 2 possible transmembrane helices (Figure 32), between aminoacids 5 and 24 (from inside to outside) and between aminoacids 34 and 56 (from outside to inside), with an extracellular loop extending between aminoacids 25 and 33, comprising two positively-charged residues. SA2192 does not contain a putative signal peptide sequence (Signal P software, Version 3.0; hidden Markov model (HMM); Technical University of Denmark, www.cbs.dtu.dk and SIG-Pred Signal Peptide Prediction, http://bmbpcu36.leeds.ac.uk/prot_analysis/ Signal.html).

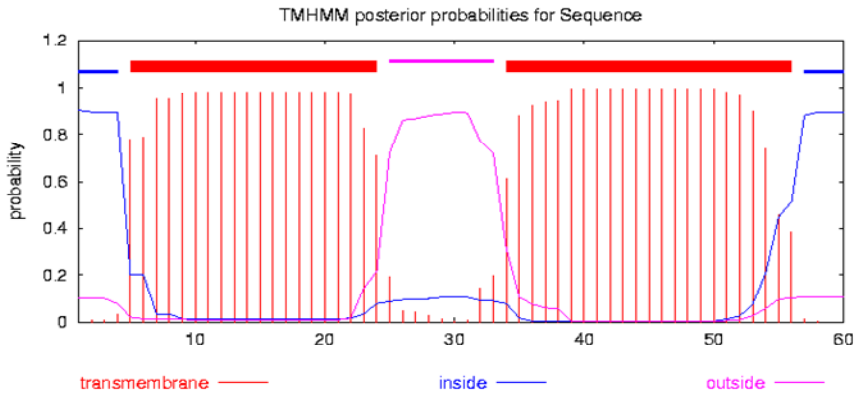

FIGURE 32: Location of transmembrane regions using the TMHMM software.

Since understanding the organization of operons in a bacterial genome provides insight into both gene function and regulation, Wang *et al.* [336] have integrated several operon prediction methods and developed a consensus approach to score the likelihood of each adjacent gene pair to be co-transcribed. Based on their data, SA2192 is likely to be co-transcribed with SA2193; however, this is rather unlikely, since SA2193 was not regulated in CRV. Therefore, further studies of the molecular background of the involvement of this peptide in the chitosan-resistance observed, for instance by constructing and characterizing a corresponding knockout mutant, seem warranted.

Transcriptional profiling suggested major alterations in cell envelope structure of CRV. For instance, another gene which was highly up-regulated in CRV was SA0035 (105-fold induction, compared to parent strain), which codes for a probable HMG-CoA synthase. The HMG-CoA sythetase is a member of the family of acyl-condensing enzymes; it is a key enzyme for the synthesis of isoprenoids, catalyzing the reaction where acetyl-CoA condenses with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) (Figure 33), the first committed step in the mevalonate pathway.

The mevalonate pathway is important for the production of dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), activated C_5-isoprene units that serve as the basis for the biosynthesis of neutral lipids, through polymerization into isoprenoids, polymers that function in processes as diverse as electron transport (quinones), sugar and oligosaccharide transfer via the membrane (undecaprenol), light absorption (carotenoids) and membrane stability (squalene and hopanoids).

Many bacteria employ the non-mevalonate pathway for synthesis of IPP; however, Gram-positive cocci and *Borrelia burgdorferi* use exclusively the mevalonate pathway, which is essential for their growth [342].

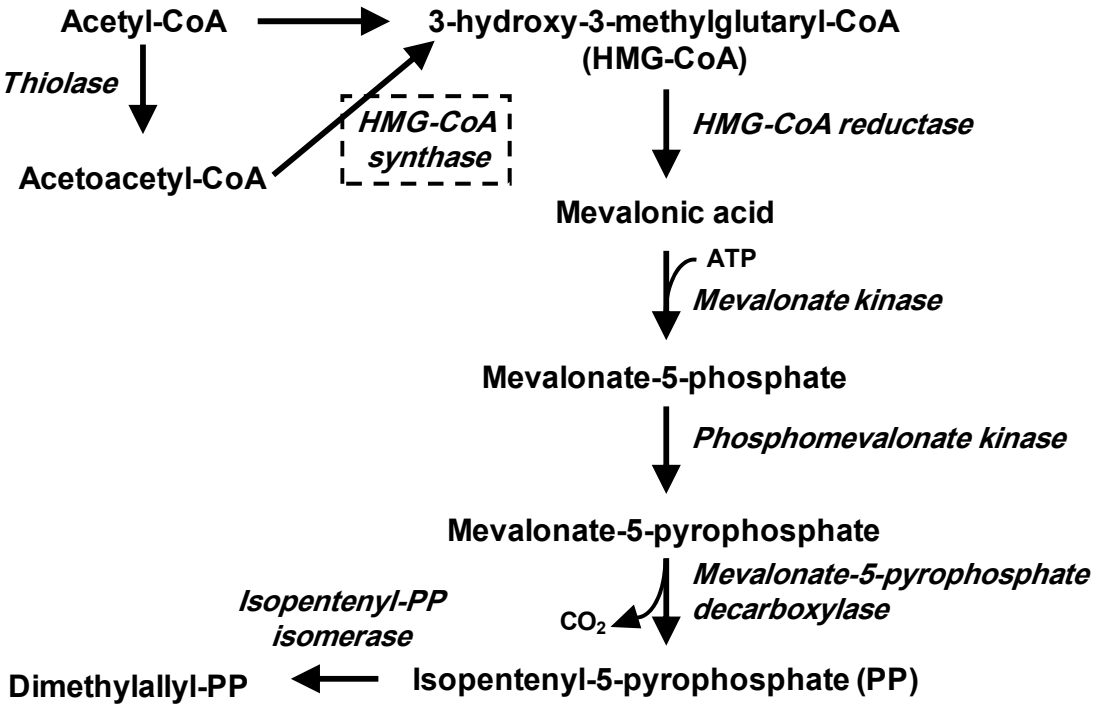

FIGURE 33: The Mevalonate pathway.

The HMG-CoA sythetase (marked by a dashed box) is a key enzyme of the mevalonate pathway, an important pathway for the production of precursors for isoprenoid biosynthesis.

In bacteria, the principal products of IPP include the lipid carrier undecaprenol which is involved in cell wall biosynthesis [254]; the Mur*N*Ac-pentapeptide portion of UDP-Mur*N*Ac pentapeptide is transported across the cytoplasmic membrane with the help of the lipid carrier, which in turn is biosynthesized by means of a prenyl transferase enzyme undecaprenyl pyrophosphate synthetase, which catalyses the stepwise addition of IPP units onto farnesyl pyrophosphate to give the C_{50} and C_{55} prenyl pyrophosphates [29]. Moreover, teichoic acid biosynthesis begins with the formation of an undecaprenyl-pyrophosphoryl disaccharide on the cytoplasmic face of the cell membrane through the successive action of proteins TarO (*N*-acetylglucosamine-1-phosphate transferase, the TagO homolog) and TarA (*N*-acetylmannosamine transferase). These polymers are synthesized in a stepwise manner on the cytoplasmic face of the cell membrane onto undecaprenyl-phosphate [54].

We therefore believe that the considerable up-regulation of SA0035 might indicate that the CRV is capable of synthesizing much more carrier for cell wall, WTA and capsule biosynthesis. Yet since almost all of the capsular genes (*capC-capG* and *capL-capP)* were markedly down-regulated, it seems that the lipid carrier would be exclusively dedicated to the production of cell wall polymers.

It is also intriguing that the amount of *spa* (staphylococcal protein A) transcripts encoding protein A was 5.5 times higher in CRV; this surface protein of *S. aureus* is reported to be covalently bound to the pentaglycine interpeptide bridge of peptidoglycans [213]. If we assume that the production of protein A must be performed in a concerted way to produce a complete cell-wall structure, it is reasonable that the regulation of *spa* gene expression is altered together with the change in the mode of cell wall synthesis presumed to accompany the acquisition of chitosan resistance.

Related to the cell envelope is the upregulation of *drp35*, *prsA* and *sarA*, previously shown to be inducible by cell wall-active antibiotics [203,325] and daptomycin [204]. However, *vraS* and *vraR*, controllers of the cell wall stress regulon, were not induced.

The proteins LrgA and LrgB act as antiholins which modulate murein hydrolase activity, and are thus involved in the peptidoglycan biosynthesis; the genes encoding both proteins 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 [204], their downregulation might indicate increased murein production.

The up-regulation of the *dltABCD* operon (section 6.3.1) contributes a net positive charge to the *S. aureus* surface envelope by alanylating teichoic acids. We can therefore conclude that D-alanylation of teichoic acids is a major factor contributing to the decreased susceptibility of chitosan. These data are consistent with the susceptibility testing results presented in Table 21.

Taken together with the fact that the expression of several cell-wall anchored surface proteins, including clumping factor B (ClfB) and Sdr proteins (SdrC, SdrD and

SdrE) [89], seemed to be affected by the treatment, all these findings suggest that the major alterations in the gene expression profiling of CRV involved cell envelope structures, again corroborating our previous results, which indicated that chitosan's antimicrobial activity is closely related to the cell surface structure of bacteria.

Among the highly up-regulated genes were *bioABD* which are involved in the synthesis of biotin (vitamin H), a carrier of activated bicarbonate. Biotin is an indispensable cofactor for a class of important metabolic enzymes, biotin carboxylases and decarboxylases, and thereby essential for the acetyl CoA carboxylase-catalyzed synthesis of malonyl CoA from acetyl CoA, the committed step of the fatty acid biosynthesis pathway. Apparently, lipid synthesis and membrane turnover needs to be differently controlled in the CRV and up-regulation occurs on both the coenzyme level and on the carbon substrate level, to help in the synthesis of new fatty acids [225].

A number of genes and operons involved in dissimilatory nitrate/nitrite reduction, which allow the use of nitrate as an alternative terminal electron donor, were down-regulated, including genes of the nitrate reductase operon (*narG*, *narH*, and *narI*), which catalyze the energy-gaining reduction of nitrate to nitrite, SA2183 and the nitrite extrusion protein NarK, which were also down-regulated upon cell wall stress [325], as well as genes involved in nitrite respiration (*nasF*, *nasE* and *nasD*). Nitrate reductases are flavoproteins, either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). The downregulation of these genes was therefore accompanied by a down-regulation (6.3 – 51.5×) of riboflavin biosynthesis gene cluster (*ribDBAH*), since riboflavin (vitamin B2) is an essential component of the basic metabolism, being a precursor of coenzymes FAD and FMN.

Moreover, lactate dehydrogenase LctE (SA0232), a hypothetical protein similar to the transcriptional regulator gene *nirR* (SA2189) which, in *S. carnosus,* is involved in nitrite-reduction and anaerobic respiration as well as *nirC* (SA0293) [225] were strongly down-regulated. Downregulation of anaerobic metabolism might be part of a general stress management strategy in an attempt to produce energy more efficiently through aerobic respiration.

On the other hand, the increased level of transcription of *pflB*, encoding formate acetyltransferase, together with its activating enzyme (*pflA*), which catalyze the non-oxidative transformation of pyruvate to acetyl coenzyme A and formate, might suggest that the cells are aiming to increase levels of acetyl CoA, which is needed for instance in lipid metabolism.

The phosphoenolpyruvate (PEP)-dependent, sugar transporting phospho-transferase system (PTS) transports and simultaneously phosphorylates its sugar substrates in a process called group translocation. Interestingly, a number of PTS and non-PTS sugar transporters were up-regulated, including: i) the gene encoding MtlA, the mannitol-specific PTS Enzyme II, which takes up exogenous mannitol, releasing the phosphate ester, mannitol-1-P, into the cell cytoplasm in preparation for oxidation to fructose-6-P by the NAD-dependent mannitol-1-phosphate dehydrogenase (MtlD); ii) the *fruAB* operon (fructose-specific PTS Enzyme II), which is involved in taking up extracellular fructose and releasing the 1-phosphate ester into the cell cytoplasm (FruA), which is further phosphorylated into fructose-1,6-diphosphate by FruB in preparation for metabolism [245]; and iii) the non-PTS transporter *uhpT* (hexose phosphate transport protein).

Activated fructose operon genes *fruAB* (both up-regulated 4.4 times) correlates well with increased peptidoglycan synthesis. Those enzymes have a critical role in the initiation of peptidoglycan synthesis by converting fructose-6-phosphate in Emden-Meyhof pathway into glucosamine-6-phosphate, from which murein monomer precursor is synthesized [171]. This effect is even more highlighted by the upregulation of the mannitol-specific PTS Enzyme II, 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.

Cells adapt to new environmental and stress conditions by regulating their metabolism, promoting the operation of certain optimal subsets of metabolic reactions

and adjusting the fluxes through the different central metabolic pathways for the establishment of a new steady state, that allows them to grow efficiently.

Staphylococci are facultatively-anaerobic microorganisms. The glycolysis and the oxidative pentose phosphate (PP) pathways are the two central routes of glucose metabolism [103]. Numerous genes encoding glycolysis pathway proteins were differentially regulated, perhaps as a response to the increased cell wall biosynthesis, and the lack of glucose in the medium (CAMHB). We have reason to believe that, in response to the high demand for sugars for peptidoglycan biosynthesis, CRV has managed to strongly alter the distribution of intracellular fluxes, switching from glycolytic to gluconeogenic substrates, rerouting the main glucose catabolism from glycolysis to the PP pathway, which was modulated by the flux through the glyceraldehyde-3-phosphate dehydrogenases, GapA and GapB.

GapA and GapB are isoenzymes with different cofactor specificities that catalyze opposite fluxes through a key reaction of the glycolytic pathway, which were differentially expressed in CRV. GapB (NADP(H)-dependent glyceraldehyde-3-P dehydrogenase) differs from GapA in being NADP(H)-dependent and in efficiently catalyzing the gluconeogenic reduction of 1,3-bis-phosphoglycerate to glyceraldehyde-3-phosphate, being much less efficient than GapA for the NAD-dependent conversion of glyceraldehyde-3-phosphate into 1,3-di-phosphoglycerate [305].

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 the tables in sections 6.3.1 and 6.3.2, 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 lead to an increased formation of NADPH and favor gluconeogenic conditions [305]. At the same time, the increased PEP carboxykinase flux limits the amount of oxaloacetate available for fueling the tricarboxylic acid (TCA) cycle, causing a shortage of TCA cycle intermediates and simultaneously avoiding ATP dissipation via

this cycle. This becomes more evident with the downregulation of *pgk* and *pgm*, which code for enzymes catalyzing sequential metabolic reactions in the lower part of glycolysis. 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 the absence of glucose (since it is lacking in CAMHB) the cells would normally have to synthesize glucose or glucose 6-P (gluconeogenesis), all the more so, if there is a higher demand for sugars, through a reversal of glycolysis. In gluconeogenesis, certain bypass steps are involved, which are carried out by enzymes encoded by *pycA*, *pckA* and *fbp*. Indeed, two of these enzymes, PckA and Fbp were found to be up-regulated in CRV. Moreover, a large number of genes involved in aminoacid metabolism were up-regulated, including *argFGH*, *arcABC*, *hisG*, *hutH*, *rocAD*, *ald* and *gudB*, in an attempt to utilize the aminoacids in the growth medium for energy generation.

Altogether, these data indicate that the CRV cells tend to redirect their metabolism towards gluconeogenesis, in an effort to meet the high demand for the sugar building blocks of the cell wall.

On the other hand, other sugar transporters were down-regulated, including *lacFE* (lactose-specific IIA and IIBC components), *scrA* (sucrose-specific IIBC component) and *ptsG* (glucose-specific IIABC component). In *S. aureus*, lactose is transported by the PTS system. Internalized lactose 6-phosphate is hydrolyzed by a phospho-*ß*-galactosidase (LacG) to yield glucose and galactose-6-phosphate, the latter is then catabolized through the tagatose-6-phosphate pathway, the key enzyme of which is tagatose-6-P kinase (encoded by *lacC*) [245]. The lactose operon of *S. aureus* contains not only the phospho-*ß*-galactosidase gene and genes involved in lactose uptake, but also the genes of the tagatose-6-P pathway. The corresponding genes, arranged in a heptacistronic operon (*lacABCDFEG*), were all down-regulated in CRV.

The majority of the genes of the pyrimidine biosynthesis gene cluster (*pyr*; SA1041-SA1049) and a potassium-specific transport system (*kdpABC*; SA1879-SA1881) were also down-regulated. The former was also down-regulated in *S. aureus* cells treated with the antimicrobial peptide hBD3 [271], while the latter was induced upon daptomycin treatment [204].

Other 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. On the other hand, vraDE, an ABC transporter implicated in the resistance to cationic AMPs in *S. aureus* SG511, was not differentially regulated [271].

4.3.6. 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, through developing concepts for reducing the development and spread of resistant bacterial strains. Moreover, it helps shed some light on the antimicrobial mode of action of new drugs, by monitoring changes that occur in a bacterial cell, in an attempt to survive this stress. More importantly, mechanisms that are sometimes used by bacteria to counteract the action of an antimicrobial might allow for a better understanding of the physiological complexity of bacterial populations.

Most bacteria have multiple routes of resistance to any given drug; the main mechanisms by which microorganisms exhibit resistance to antimicrobials are: (i) modification of an antimicrobial's target site; (ii) functional bypassing of a target, for example by altering a metabolic pathway; (iii) reduced drug accumulation, either through decreasing drug permeability or increased active efflux; and (iv) enzymatic inactivation or modification of the antimicrobial agent.

In this part, we will discuss the results of our work concerning the development of *in vitro* chitosan resistance in *S. aureus*, with regard to possible resistance mechanisms. The main and relevant findings are summarized in Figure 34, and will be discussed in detail in this context. Since chitosan is thought to act upon the microbial cell envelope, the discussion will largely evolve around the observations that emanated from this study regarding the cell surface alterations observed in CRV, in an attempt to provide a detailed analysis of correlates of *in vitro* chitosan resistance.

Although the precise mechanisms by which *S. aureus* strains adapt to the antimicrobial activities of chitosan remain incompletely defined, yet a detailed analysis of cell envelope properties of *S. aureus* SG511 and the CRV identified a cadre of phenotypes that readily distinguished the chitosan-susceptible parental strain from the resistant variant. Both phenotypic and genotypic data confirm the key association of cell envelope structural and functional alterations with *in vitro* development of chitosan resistance in *S. aureus*, including a reduced overall negative charge of both the cell wall and cell membrane, coupled with a slight enhancement of cell surface hydrophobicity, as well as mutations in genes involved in sugar metabolism. This is in fair agreement with several previous studies, which have shown an apparent association between the activity of certain antimicrobials and cell surface structures (discussed below).

The cell surface of *S. aureus*, as in most bacteria, has a moderately negative net charge at neutral pH [294], which is probably due to the fact that the teichoic acids contain fewer positively-charged D-alanine residues than negatively-charged phosphate groups [238]. In *S. aureus*, wall teichoic acids are covalently-linked to approximately every thirteenth muramic acid in the peptidoglycan by phosphodiester linkages. Moreover, 70% of the glycerophosphate units of LTA are esterified with D-alanine [104].

S. aureus and other bacterial pathogens have evolved countermeasures to limit the effectiveness of cationic AMPs, including the modification of anionic surface molecules (e.g. teichoic acids, phospholipids and lipid A), to achieve a reduction in the net negative charge of the bacterial cell envelope, thereby increasing the repulsion of cationic AMPs. For instance, although the D-alanine-esterification of teichoic acids is not essential for

growth, it is believed to be essential in the protection against human and animal defense systems [238]. Peschel *et al.* [237,238] have demonstrated that strains showing relative increases in cell surface positive charge have cell membranes and cell walls that contain high proportions of positively-charged phospholipids and teichoic acids species, respectively, rendering them relatively resistant to killing by a number of cationic AMPs, including tPMP-1. Lysinylation of phosphotidylglycerol and D-alanylation of teichoic acid appeared to be responsible for these observed phenotypes.

Results of cell surface charge and PL analyses were complementary and provided valuable insights into the nature of the chitosan resistance displayed by CRV. Our data suggest that the increased overall LPG content rendered the CRV surface envelope less negatively-charged, reflected in the reduced binding of cytochrome c compared to the parent strain, providing CRV with an enhanced repulsive cell surface toward the polycationic chitosan, contributing to the resistance observed.

MprF, which is responsible for the biosynthesis of lysyl-phosphatidylglycerol (LPG), has been implicated in the resistance of bacteria to the action of cationic AMPs [201,237,347] and daptomycin [90,147]. However, unlike with daptomycin, the regulation of *mprF* was not directly implicated in the reduced susceptibility of CRV to chitosan; although LPG was produced in 6-7 times higher amounts in this variant, indicating that the regulation of MprF occurs at a level other than the transcriptional one. The significance of these observations is underscored by our previous findings, concerning the higher susceptibility of an *mprF*-null mutant to the antimicrobial activity of chitosan, compared to the parent strain.

CRV displayed cross-resistance to a number of antimicrobials, predominantly cationic in nature. Considering that the MIC increase was not confined to the antimicrobial used for selection (i.e. to chitosan) but also extended to other antimicrobials, the involvement of transport problems becomes very likely, as can be seen from the upregulation of a number of ABC transporters. These cross-resistance studies add weight to the conclusion that changes in cell envelope structure do contribute to chitosan resistance.

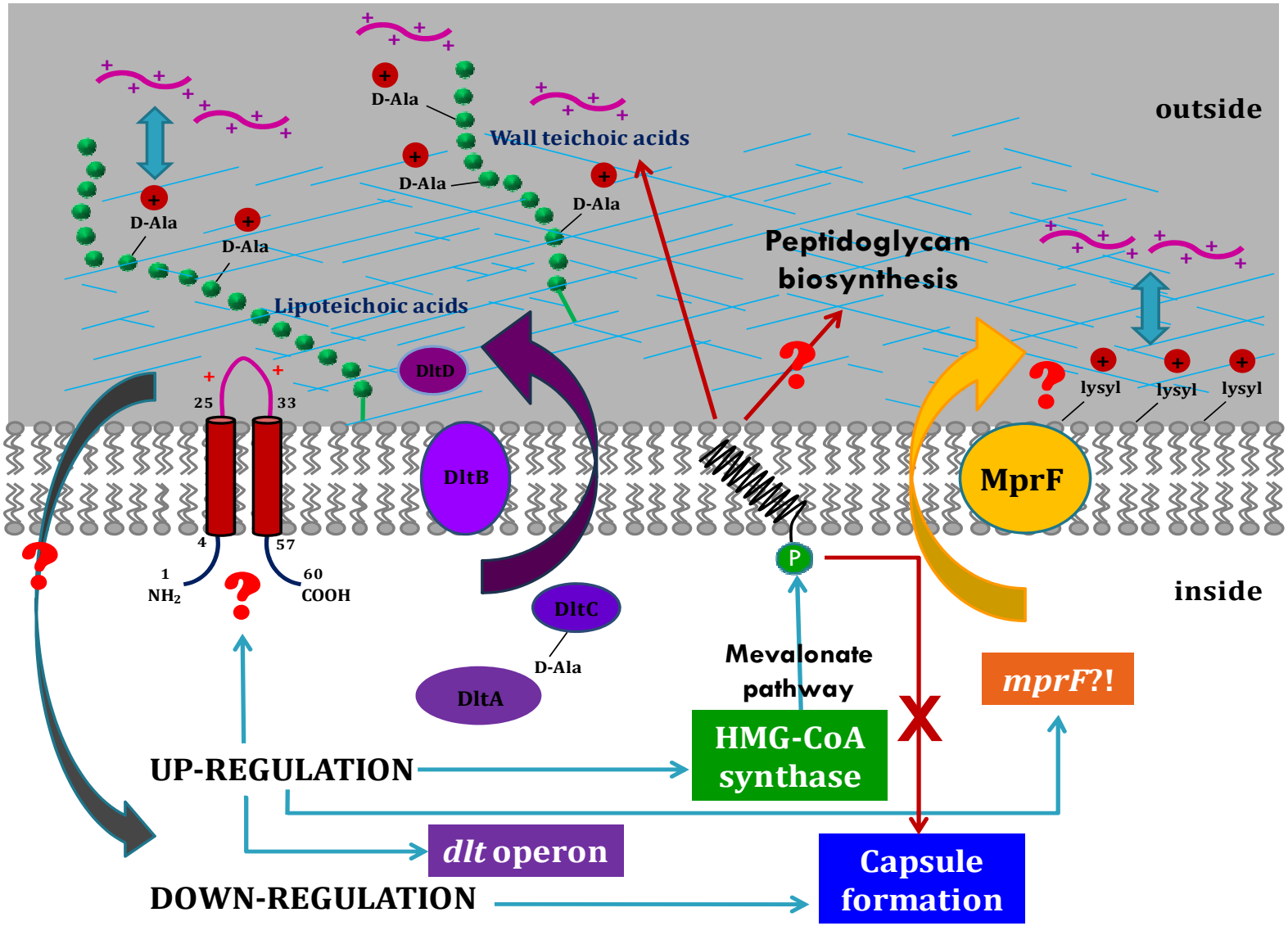

FIGURE 34: Possible resistance mechanisms of *S. aureus* against chitosan.

Coincubation of chitosan and other AMPs (friulimicin, daptomycin or Pep5, respectively) substantially affected the *in vitro* activity of these AMPs. It may therefore be reasonable to assume that the antagonism originated from a sort of competition between the tested antimicrobials for target binding sites.

Capsular polysaccharides, changes in bacterial cell surface hydrophobicity and biofilm formation are often implicated in bacterial resistance to antimicrobials. In case of CRV, almost all of the capsular genes were down-regulated, clearly indicating that these polysaccharides were not involved in the observed resistance. This finding is however not quite unexpected, since several researchers, including Seaman *et al.* [277] discovered that capsule polysaccharides did not present a permeability barrier to the antimicrobials used in their investigation, indicating that they are not involved in conferring reduced antibiotic or biocide susceptibility. On the other hand, it should be emphasized that although differences in cell surface hydrophobicity between the parental strain and CRV were not significantly high, it is likely that they contributed to the composite mechanism of chitosan resistance in this variant.

Some staphylococci are capable of producing a biofilm-associated capsular polysaccharide adhesin PIA (polysaccharide intercellular adhesin), a linear homoglycan composed of *ß*-1→6-linked Glc*N*Ac residues with partly deacetylated residues, in which the cells are embedded and protected against the host's immune defence and antibiotic treatment; it is a product of the *ica* operon (*icaADBC*) [98, 104].

Gross *et al.* suggested that the *S. aureus* Δ*dltA* mutant, which bears a stronger negative surface charge due to the lack of D-alanine esters in its teichoic acids can no longer colonize polystyrene or glass; the mutation abrogating the primary adhesion to plastic due to repulsive electrostatic forces [108]. Even though we would have expected CRV, with its increased overall positive charge, to adhere more to the polystyrene plates, this was not the case, as could be seen in the biofilm assay. Although the possibility that the limitations of the assay (CAMHB as medium, and the fact that relatively few *S. aureus* strains form biofilms under standard laboratory conditions [14])

may have masked effects; however this appears to be unlikely, since the colonies of CRV exhibited no mucoid appearance. In addition, only *icaC* (of an unknown function, but believed to be involved in the translocation of PIA across the cytoplasmic membrane) was up-regulated in CRV, suggesting that biofilm formation does not contribute to the observed resistance of CRV.

The overall peptidoglycan synthesis can be divided into three distinct stages, namely: (i) formation of the nucleotide sugar-linked precursors, UDP-Glc*N*Ac and UDP-Mur*N*Ac; (ii) transfer of the precursors to the lipophilic carrier, undecaprenyl phosphate; and (iii) transfer of the complete subunit to the growing peptidoglycan.

Although TEM pictures clearly do not argue in favor of a thickened cell wall, yet two lines of evidence reported here suggest otherwise: (i) the higher optical density measurements of CRV cultures, compared to the parent strain, could give an indication to a change in cell size or cell wall thickening; and (ii) gene expression profiles suggest an enhanced production of cell wall elements.

The most prominent change in the relative fluxes through metabolic pathways of CRV was the switch from glycolysis to PP pathway. A slowdown of the upper glycolysis elicited by a strong backflux through GapB, as well as an increased gluconeogenic PEP carboxykinase flux, is believed to result in high intracellular glucose-6-phosphate concentration, leading to higher PP pathway flux.

We believe that the cells alter their metabolism to accommodate the increased production of components of the cell envelope, through: (i) upregulation of a putative HMG-CoA synthase, thereby producing increased amounts of the lipid carrier required for peptidoglycan and teichoic acid synthesis; (ii) upregulation of gluconeogenetic pathways, which would underly a well-directed programme by which the cells are producing sugar building blocks for cell wall biosynthesis; (iii) reduced capsule production to dedicate the cell wall precursor machinery wholly to the production of cell wall components; (iv) increased expression of the *dltABCD* operon that is responsible for the D-alanylation of teichoic acids, resulting in a decrease in the relative

overall negative bacterial surface charge; (v) suppressing the action of LrgA and LrgB, thereby allowing for cell wall biosynthesis; and (vi) enhancement of fatty acid biosynthesis and increased production of membrane phospholipids (including LPG).

It was rather surprising that the CRV demonstrated an unexpected higher rate of lysostaphin-mediated autolysis, compared to the parental strain. We believe however that this could be an epiphenomenon of the increased cell wall biosynthesis, and comparatively inefficient cross-linking accompanying this process, probably due to the overloaded machinery. This phenomenon may thus be based on the observed changes in the properties of the cell envelope and supports the view that these changes are relevant for the development of resistance.

We did not analyze the CRV strain for possible changes in membrane fluidity, although it is conceivable that such changes could contribute to the resistance profile we observed. Increased membrane fluidity is consistently associated with resistance to cationic AMPs and daptomycin [90,147].

Although we have no evidence to support this hypothesis, we tentatively postulate that the conceivable production and shedding of large amounts of anionic cell surface polymers (teichoic acids) into the culture medium might allow the cells to evade the antimicrobial activity of chitosan, by scavenging this polycationic polymer, thus leading to the observed resistance. We will aim at corroborating this hypothesis, by analyzing and quantitating the teichoic acids produced by this variant.

In summary, although the underlying mechanisms are not yet fully understood, this study has collected significant evidence that resistance to chitosan can be obtained *in vitro* by exposure of the bacteria to subinhibitory concentrations of this antimicrobial.

Our observations regarding cell surface charge, coupled with the fact that the CRV is probably producing larger amounts of cell wall polymers, suggest a broad model of resistance that is closely related to both cell wall and cell membrane structures.

Moreover, although these alterations in cell envelope structures might be the major factor contributing to chitosan resistance, additional phenotypic and genotypic

events are required. These proposed resistance mechanisms are not mutually exclusive; indeed we believe that combinations of these general types of resistance work in concert. However, it remains to be determined how these complex phenomena interact and contribute to the patterns of gene expression that we have observed.

Further studies in our laboratory will be focusing on (i) relevance of the peptide (encoded by SA2192) in the development of resistance, and its possible involvement in stress sensing; (ii) investigations to define the mechanisms of enhanced resistance of CRV with regard to membrane fluidity; as well as (iii) analysis of both the total amounts and chemical compositions of the synthesized cell wall constituents (peptidoglycan and teichoic acids).

5. SUMMARY AND CONCLUDING REMARKS

Chitosan, a polysaccharide biopolymer, has a unique chemical structure as a linear polycation with a high charge density, reactive hydroxyl- and amino-groups as well as extensive hydrogen bonding. It combines a group of physicochemical and biological characteristics, which allow for a wide range of applications.

In this study, we investigated the *in vitro* antimicrobial activity of chitosan. Our goals were to (i) identify factors influencing the antimicrobial activity of chitosan and study its interaction with bacterial systems; (ii) explore possible mechanisms of its action against Gram-positive bacteria, specifically staphylococci; as well as (iii) discover potential resistance mechanisms developed by bacteria against this compound.

Chitosan exhibited an adequate strain-dependent *in vitro* antimicrobial activity against Gram-positive bacteria, and was almost inactive against Gram-negative microorganisms. It displayed a dose-dependent growth-inhibitory activity (i.e. bacteriostatic rather than –cidal), where resistant subpopulations would usually emerge, as for instance *S. aureus* small colony variants, which displayed typical phenotypic differences from the parent strain, associated with defects in electron transport and reduced metabolism.

Chitosan's activity was influenced by a number of factors, including its chain length (with oligosaccharides showing almost no activity), culture medium (optimum activity in CAMHB) and the presence of metal ions, the latter abrogating its antimicrobial activity.

Chitosan's ability to flocculate bacterial cells was clearly evidenced, and seemed to depend on both the test strain and on the chitosan concentration. On the other hand, its anti-biofilm property was only partly documented, and needs to be further investigated.

Although several bacteria were capable of degrading chitosan, possibly through the production of specific chitosanases or other non-specific enzymes, this did not influence their susceptibility to the antimicrobial activity of this biopolymer.

These results would appear to warrant further investigation into the potential value of chitosan as a preservative for pharmaceutical and food preparations, which would open a new avenue for the use of this natural product. However, the detailed application setting has to be well-defined, in order to avoid unfavorable interactions, or loss of its activity.

We carried out a systematic study of chitosan's mode of action, trying to identify the underlying molecular mechanisms of this activity, bearing in mind that such mechanisms most probably differ among various species. The results obtained with several test bacteria demonstrate the complexity of the mode of action of chitosan. Several lines of evidence suggest that its site of action is at the microbial cell envelope, but we do contend that there probably is not a single classical target that would explain chitosan's antimicrobial action; it may therefore be argued that the final effect could be the result of several independent antibiotic activites.

The cationic nature of chitosan plays a pivotal role in its antimicrobial activity, allowing its interaction with the largely anionic bacterial surface. This was verified by the use of various mutants displaying different overall cell surface charges, where we could establish that a highly anionic bacterial surface greatly enhances the antimicrobial activity of chitosan.

We speculate that interaction of chitosan with lipoteichoic and wall teichoic acids, coupled with a potential extraction of membrane lipids and lipoteichoic acid, probably triggers secondary responses in the cells which are responsible for the observed inhibition / death. Thus, the interaction of chitosan with the bacterial cell surface polymers leads to a generalized destabilization of the cytoplasmic membrane and subsequent disruption of membrane function, probably by interfering with molecular machineries located within the cytoplasmic membrane. These might include the cell

wall biosynthesis machinery, the electron transport chain, or other components that influence both membrane integrity and functionality.

A simultaneous permeabilization of the physically intact cell membrane to small cellular components was detected, coupled with a significant, yet still incomplete, membrane depolarization, probably due to uncoupling of the electron transport chain, thus affecting membrane energetics. Leakage was however not detected in model membrane systems; an indication that these might not accurately reflect *in vivo* conditions and that chitosan was unable to form discrete pores within the lipid bilayer.

A concomitant interference with lipid II biosynthesis was not observed, although this does not rule out a possible disturbance of cell wall biosynthesis. Moreover, chitosan treatment, unlike prototypic cationic AMPs, did not bring about cell lysis.

Analysis of transcriptional response data revealed that chitosan treatment lead to multiple changes in the expression profile of *S. aureus* SG511 genes involved in the regulation of stress and autolysis, as well as genes associated with energy metabolism, resulting in impairment of oxygen consumption and forcing cells to shift to anaerobic respiration.

Several pathways via which staphylococci may develop resistance against chitosan have been recognized; certainly, others await discovery. Our analyses did not identify prototypic changes in the chitosan-resistant variant which are usually associated with enhanced resistance profiles, such as enhanced biofilm and capsule formation; however, the following phenotypic cell envelope alterations correlated with the *in vitro* chitosan resistance in *S. aureus* SG511: (i) increased positive surface charge resulting in reduced chitosan binding, (ii) modest increase in hydrophobicity; (iii) enhanced production of cell wall polymers; (iv) elevated levels of positively-charged membrane lipids, thus increasing electrostatic cell surface repulsion of chitosan; and (v) reduced capsule formation; most of these phenotypes are interrelated. Other membrane events that may also contribute to the chitosan-resistant phenotype in our strain set, such as increased turnover of membrane fatty acids was also envisaged, whereas changes in membrane

fluidity remain to be investigated. Of note, we could not detect any substantial differences in colony morphology, biochemical patterns and microscopic ultrastructure in the current stain set.

Thus, our findings are consistent with the hypothesis that the polycationic nature of chitosan is likely to trigger a combination of events that contribute to the antimicrobial activity of chitosan; such an activity requires an anionic bacterial surface charge.

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 over-the-counter use of chitosan.

The findings of this study might help to unify what may have been considered disparate conclusions about the antimicrobial activity of chitosan in the literature, and they might extend our understanding of this industrially-important natural polymer. There is still much to be learned, but a stage has being reached at which it is becoming possible to present a general account of the main processes involved in chitosan's antimicrobial activity in terms of basic molecular findings.

6. APPENDICES

6.1. LIST OF MANUFACTURERS AND SUPPLIERS

The following is a complete list of the manufacturers and suppliers mentioned in this work.

- Abimed GmbH, Langenfeld, Germany
- Agfa-Gevaert N.V., Mortsel, Belgium
- Agilent, Palo Alto, CA, USA
- Amersham Biosciences (GE Healthcare), Piscataway, NJ, USA
- Amresco, Ohio, U.S.A.
- Applied Biosystems/Ambion, Austin, TX, U.S.A.
- ATCC - American Type Culture Collection, Manassas, VA, USA
- Avanti Polar Lipids, Inc., Alabaster, AL, U.S.A.
- Aventis Pharma Deutschland GmbH, Bad Soden am Taunus, Germany
- Axon Instruments, Inc., Union City, CA, U.S.A.
- B. Braun Melsungen AG, Melsungen, Germany
- Barnstead International, Dubuque, Iowa, U.S.A.
- Bayer AG, Leverkusen, Germany
- Becton, Dickinson & Co (BD), Sparks, MD, USA,
- Bioline GmbH, Luckenwalde, Germany
- bioMérieux, Marcy l'Etoile, France
- Bio-Rad Laboratories GmbH, München, Germany
- Biozym GmbH, Hamburg, Germany
- Bristol-Myers Squibb GmbH & Co. KGaA, München, Germany
- CAMAG, Muttenz, Switzerland
- Carl Roth GmbH & Co, Karlsruhe, Germany
- ChiPro GmbH, Bremen, Germany
- Dr. Petry Genmedics GmbH, Reutlingen, Germany
- DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany
- Edmund Bühler GmbH, Tübingen, Germany
- Eppendorf AG, Hamburg, Germany
- Fermentas GmbH, St.Leon-Rot, Germany
- Fluka, Neu-Ulm, Germany

- GE Healthcare Bio-Sciences AB, Uppsala, Sweden
- GE Healthcare UK Limited, Buckinghamshire, UK
- Gelman Instr., Milan, Italy
- GFL Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
- Glaswerk Wertheim, Wertheim, Germany
- Greiner Bio-One GmbH, Frickenhausen, Germany
- HANNA Instruments, Kehl am Rhein, Germany
- Heidolph Instruments GmbH & Co.KG, Schwabach , Germany
- HELLMA GmbH & Co. KG, Müllheim, Germany
- Heppe GmbH, Queis, Germany
- Heraeus Sepatech GmbH, Osterode, Germany
- Hielscher Ultrasonics GmbH, Teltow, Germany
- Hitachi Ltd, Tokyo, Japan
- ICN Biomedicals Inc, Eschwege, Germany
- IKA® Werke GmbH & Co. KG, Staufen, Germany
- Implen GmbH, München, Germany
- Invitrogen, Basel, Switzerland
- Julabo Labortechnik GmbH, Seelbach, Germany
- Kimberly-Clark Corporation, Irving, Texas, U.S.A.
- KMF Laborchemie Handels GmbH, Lohmar, Germany
- Kraeber GmbH & Co, Ellerbek, Germany
- Labsystems, Helsinki, Finnland
- Life Sciences International GmbH – ThermoQuest, Egelsbach, Germany
- Mallinckrodt Baker B. V., Deventer, Holland
- Martin Christ GmbH, Osterode, Germany
- Memmert GmbH + Co. KG, Schwabach, Germany
- Merck KGaA, Darmstadt, Germany
- MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany
- Microelectrodes, Inc., Bedford, NH, USA
- Millipore GmbH, Schwalbach/Ts., Germany
- Molecular Probes – Invitrogen GmbH, Karlsruhe, Germany
- Molzym GmbH & Co.KG, Bremen, Germany
- MP Biomedicals Inc., Ohio, U.S.A.
- NanoDrop Technologies, Inc., Wilmington, DE, USA.
- New Brunswick Scientific Co. Inc., Edison, New Jersey, USA
- New England Biolabs GmbH, Frankfurt am Main, Germany
- Novartis Pharma GmbH, Nürnberg, Germany
- Nunc A/S, Roskilde, Denmark

- Oxoid GmbH, Wesel, Germany
- Packard, Zurich, Switzerland
- PALL GmbH, Dreieich, Germany
- Paul Hartmann AG, Heidenheim, Germany
- Pechiney Plastic Packaging, Menasha, WI, U.S.A.
- Pfeiffer Vacuum GmbH, Asslar, Germany
- Pierce, IL, U.S.A.
- Primex ehf, Siglufjordur, Iceland
- QIAGEN GmbH, Hilden, Germany
- Ratiopharm GmbH, Ulm, Germany
- Robbins Scientific, Sunnyvale, CA, USA
- Sarstedt Aktiengesellschaft & Co., Nümbrecht, Germany
- Sartorius AG, Göttingen, Germany
- Savant, Minnesota, U.S.A.
- Schleicher & Schuell BioScience GmbH, Dassel, Germany
- SCHOTT Instruments GmbH, Mainz, Germany
- SCIENION AG, Berlin, Germany
- Scientific Industries Inc., Bohemia, N.Y., U.S.A.
- Serva GmbH, Heidelberg, Germany
- Shimadzu Corporation, Kyoto, Japan
- Sigma, Osterode am Harz, Germany
- Sigma-Aldrich Chemie GmbH, Steinheim, Germany
- Stuart Scientific (Bibby Scientific Limited), Staffordshire, United Kingdom
- Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.
- Upjohn GmbH, Heppenheim Germany
- Vink+CO GmbH, Kakenstorf, Germany
- Wilhelm Werner GmbH, Leverkusen, Germany
- Zinsser Analytic, Frankfurt, Germany

6.2. TRANSCRIPTIONAL PROFILE ANALYSIS OF CELLS TREATED WITH CHITOSAN

6.2.1. GENES UP-REGULATED UPON CHITOSAN TREATMENT

The following genes were up-regulated in the chitosan-treated strain, compared to the untreated culture.

ORF	Gene	Function*	Fold change
Cell wall			
SA0205		HP, similar to lysostaphin precursor	1.4
Transport/binding proteins and lipoproteins			
SA0293		HP, similar to formate transporter NirC	1.9
SA0640		HP, similar to ABC transporter required for expression of cytochrome bd	1.4
SA1270		HP, similar to amino acid permease	1.7
SA1674		glutamate ABC transporter ATP-binding protein	1.6
SA2156		L-lactate permease lctP homolog	1.7
SA2167	scrA	PTS system, sucrose-specific IIBC component	1.5
SA2176	narK	nitrite extrusion protein	1.7
Membrane bioenergetics (electron transport chain and ATP synthase)			
SA0411	ndhF	NADH dehydrogenase subunit 5	2.2
SA0937		cytochrome D ubiquinol oxidase subunit I homolog	1.4
SA0938		cytochrome D ubiquinol oxidase subunit II homolog	1.7
SA2185	narG	respiratory nitrate reductase alpha chain	2.2
Cell division			
SA0249	scdA	cell division and morphogenesis-related protein	2.0
SA1023	ftsL	cell division protein	2.8
Metabolism of carbohydrates and related molecules			
SA0143	adhE	alcohol-acetaldehyde dehydrogenase	2.0
SA0218	pflB	formate acetyltransferase	2.7
SA0219	pflA	formate acetyltransferase activating enzyme	6.4
SA1556	acuC	acetoin utilization protein	1.5
SA1599		HP, similar to transaldolase	1.6
SA2001		HP, similar to oxidoreductase, aldo/keto reductase family	1.7
SA2104		HP, similar to suppressor protein suhB	1.4
SA2294	gntK	gluconokinase	1.6
Metabolism of amino acids and related molecules			
SA0829		HP, similar to 5-oxo-1,2,5-tricarboxilic-3-penten acid decarboxylase	1.9
SA1216		HP, similar to oligoendopeptidase	1.4
SA1271		threonine deaminase IlvA homolog	1.6
SA2189		HP, similar to NirR	2.5

ORF	Gene	Function*	Fold change
Metabolism of lipids			
SA0220		HP, similar to glycerophosphodiester phosphodiesterase	1.8
SA1584		lysophospholipase homolog	1.8
Metabolism of phosphate			
SA0881		HP, similar to nucleotidase	1.5
RNA synthesis - Regulation			
SA0322		HP, similar to transcription regulator	1.6
SA1323	srrA	staphylococcal respiratory response protein SrrA	1.7
SA1947	czrA	repressor protein	2.0
SA2295	gntR	gluconate operon transcriptional repressor	1.6
SA2379		HP, similar to transcriptional regulator tetR-family	1.8
SA2433		HP, similar to transcription antiterminator BglG family	1.8
Protein folding			
SA1659	prsA	peptidyl-prolyl cis/trans isomerase homolog	2.4
Adaption to atypical conditions			
SA0723	clpP	ATP-dependent Clp protease proteolytic subunit homolog	1.6
SA1146	bsaA	glutathione peroxidase	2.4
SA2405	betA	choline dehydrogenase	2.1
Detoxification			
SA0681		HP, similar to multidrug resistance protein	1.2
SA1238		HP, similar to tellurite resistance protein	1.9
Phage-related functions			
SA0252	lrgA	murein hydrolase regulator LrgA	4.0
SA0253	lrgB	antiholin-like protein LrgB	2.6
SA0754		HP, similar to lactococcal prophage ps3 protein 05	3.9
Pathogenic factors (toxins and colonization factors)			
SA0102		67 kDa Myosin-crossreactive streptococcal antigen homolog	1.5
SA0742	clfA	fibrinogen-binding protein A, clumping factor	1.8
SA1813		HP, similar to leukocidin chain lukM precursor	1.5
Miscellaneous			
SA0231		HP, similar to flavohemoprotein	1.8
SA0914		HP, similar to chitinase B	2.4
SA2480	drp35	Drp35	1.5
Hypothetical proteins			
SA0175		conserved HP	2.0
SA0412		conserved HP	1.9
SA0725		conserved HP	1.8
SA0772		conserved HP	3.6
SA0890		conserved HP	2.0
SA1022		conserved HP	1.5
SA1293		conserved HP	1.5
SA1419		conserved HP	2.0
SA1618		conserved HP	1.4
SA1649		conserved HP	1.7

ORF	Gene	Function*	Fold change
SA1658		conserved HP	1.6
SA1692		conserved HP	1.9
SA1942		conserved HP	2.6
SA2220		conserved HP	1.6
SA2262		conserved HP	1.9
SA2378		conserved HP	1.5
SA2474		conserved HP	1.4
SA0536		HP	2.7
SA0651		HP	1.8
SA0806		HP	1.4
SA1001		HP	1.8
SA1162		HP	2.0
SA1476		HP	3.1
SA1546		HP	1.4
SA1665		HP	2.5
SA1703		HP	1.8
SA2049		HP	2.7
SA2110		HP	1.5
SA2116		HP	1.4
SA2158		HP, similar to TpgX protein	1.8
SA2491		conserved HP	2.1
SA2221		HP	2.8
SA2268		HP	2.6
SAS011		HP	1.6

* HP, hypothetical protein

6.2.2. GENES DOWN-REGULATED UPON CHITOSAN TREATMENT

The following genes were down-regulated in the chitosan-treated strain, compared to the untreated culture.

ORF	Gene	Function*	Fold change
Cell wall			
SA2354		HP, similar to acyltransferase	1.5
Transport/binding proteins and lipoproteins			
SA0209		maltose/maltodextrin transport permease homolog	1.7
SA0682		HP, similar to di-tripepride ABC transporter	1.4
SA0794	dltB	DltB membrane protein	1.5
SA0846		HP, similar to oligopeptide transport system permease protein OppC	1.3
SA0950	potA	spermidine/putrescine ABC transporter, ATP-binding protein homolog	1.7
SA0951	potB		2.0
SA0952	potC	spermidine/putrescine ABC transporter homolog	2.0
SA1183	opuD	glycine betaine transporter	1.5
SA1547	ptaA	PTS system, *N*-acetylglucosamine-specific IIABC component	1.1
SA1879	kdpC	probable potassium-transporting ATPase C chain	1.5
SA1881	kdpA	probable potassium-transporting ATPase A chain	1.6
SA1977		HP, similar to ferrichrome ABC transporter (permease)	1.7
Protein secretion			
SA2446		HP, similar to preprotein translocase secY	1.5
Metabolism of carbohydrates and related molecules			
SA0223		acetyl-CoA acetyltransferase homolog	1.9
SA1523		acetyl-CoA carboxylase transferase beta subunit	1.6
SA2129		HP, similar to aldose 1-epimerase	1.2
SA2327		HP, similar to pyruvate oxidase	1.5
SA1245	odhA	2-oxoglutarate dehydrogenase E1	1.4
Metabolism of amino acids and related molecules			
SA0344	metE	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	1.4
SA1165	thrC	threonine synthase	1.9
SA1166	thrB	homoserine kinase homolog	1.6
SA1231		HP, similar to alanine racemase	1.7
SA1297	aroA	3-phosphoshikimate 1-carboxyvinyltransferase	1.6
SA1366		glycine dehydrogenase subunit 1 (decarboxylating)	1.6
SA2427	arcB	ornithine transcarbamoylase	1.6
Metabolism of nucleotides and nucleic acids			
SA0373	xprT	xanthine phosphoribosyltransferase	1.3
SA0918	purC	phosphoribosylaminoimidazolesuccinocarboxamide synthetase homolog	1.8

ORF	Gene	Function*	Fold change
SA0920	purQ	phosphoribosylformylglycinamidine synthase I PurQ	2.3
SA0921	purL	phosphoribosylformylglycinamidine synthetase PurL	2.0
SA0923	purM	phosphoribosylformylglycinamidine cyclo-ligase PurM	1.6
SA0925	purH	bifunctional purine biosynthesis protein PurH	1.7
Metabolism of lipids			
SA0224		HP, similar to 3-hydroxyacyl-CoA dehydrogenase	2.4
SA0225		HP, similar to glutaryl-CoA dehydrogenase	2.4
SA0226		HP, similar to acid-CoA ligase	2.0
SA1072	plsX	fatty acid/phospholipid synthesis protein	1.6
SA1434		acetyl-CoA carboxylase (biotin carboxylase subunit), accC homolog	1.7
SA1435		HP, similar to acetyl-CoA carboxylase (biotin carboxyl carrier subunit), accB homolog	1.7
Metabolism of coenzymes and prosthetic groups			
SA1588	ribB	riboflavin synthase alpha chain	1.5
SA1896	thiD	phosphomethylpyrimidine kinase	1.3
DNA recombination			
SA1468	ruvA	holliday junction DNA helicase	1.5
RNA synthesis			
SA1748		HP, similar to transcription regulator, GntR family	1.5
SA1897		HP, similar to transcriptional activator TenA	1.5
SA0494	nusG	transcription antitermination protein	1.3
Protein synthesis			
SA0504	rpsG	30S ribosomal protein S7	1.7
SA2030	rpmD	50S ribosomal protein L30	1.7
SA2031	rpsE	30S ribosomal protein S5	1.7
SA2035	rplE	50S ribosomal protein L5	1.7
SA2037	rplN	50S ribosomal protein L14	1.9
SA2038	rpsQ	30S ribosomal protein S17	1.8
SA2039	rpmC	50S ribosomal protein L29	2.0
SA2041	rpsC	30S ribosomal protein S3	1.7
SA2042	rplV	50S ribosomal protein L22	1.7
SA2045	rplW	50S ribosomal protein L23	1.9
SA2046	rplD	50S ribosomal protein L4	1.9
SA2048	rpsJ	30S ribosomal protein S10	1.4
SAS079	rpsN	30S ribosomal protein S14	1.7
SA1716		glutamyl-tRNAGln amidotransferase subunit A	1.4
Adaption to atypical conditions			
SA0150	capG	capsular polysaccharide synthesis enzyme Cap5G	1.7
SA0156	capM	capsular polysaccharide synthesis enzyme Cap5M	1.3
SA0157	capN	capsular polysaccharide synthesis enzyme Cap5N	1.9
SA0158	capO	capsular polysaccharide synthesis enzyme Cap8O	1.8
SA0159	capP	capsular polysaccharide synthesis enzyme Cap5P	1.5
Antibiotic production			
SA0173		HP, similar to surfactin synthetase	1.8

ORF	Gene	Function*	Fold change
Phage-related functions			
SA1786		HP [Bacteriophage phiN315]	1.1
SA1798		HP [Bacteriophage phiN315]	1.6
Pathogenic factors (toxins and colonization factors)			
SA0091	plc	1-phosphatidylinositol phosphodiesterase precurosr	0.9
SA2430	aur	zinc metalloproteinase aureolysin	1.0
SA2447		HP, similar to streptococcal hemagglutinin protein	1.4
Hypothetical proteins			
SA0174		conserved HP	1.4
SA0212		conserved HP	1.4
SA0227		conserved HP	1.6
SA0555		conserved HP	1.5
SA0556		conserved HP	1.2
SA0770		conserved HP	1.4
SA0919		conserved HP	1.9
SA1040		conserved HP	1.3
SA1433		conserved HP	1.4
SA1436		conserved HP	1.6
SA2050		conserved HP	2.1
SA2329		conserved HP	1.5
SA0492		HP	1.4

* HP, hypothetical protein

6.3. GENE EXPRESSION PROFILE OF CRV

6.3.1. GENES UP-REGULATED IN CRV COMPARED TO THE PARENT STRAIN

ORF	Gene	Function*	Fold change‡
Cell wall			
SA0125		HP, similar to Eps(Exopolysaccharide)G	4.5
SA0793	dltA	D-alanine-D-alanyl carrier protein ligase	4.5
SA0795	dltC	D-alanine-poly(phosphoribitol) ligase subunit 2	3.8
SA1691	sgtB	HP, similar to penicillin-binding protein 1A/1B	3.1
Transport/binding proteins and lipoproteins			
SA0099		HP, similar to transmembrane efflux pump protein	7.5
SA0127		HP, similar to capsular polysaccharide synthesis protein 14L	3.5
SA0163		HP, similar to cation-efflux system membrane protein CzcD	5.0
SA0186		HP, similar to sucrose phosphotransferase enzyme II	3.9
SA0214	uhpT	sugar phosphate antiporter	15.0
SA0302		probable pyrimidine nucleoside transport protein	11.1
SA0617	vraG	ABC transporter permease	4.0
SA0655	fruA	fructose specific permease	4.4
SA0794	dltB	DltB membrane protein	4.0
SA0796	dltD	poly D-alanine transfer protein	3.6
SA1592		arsenical pump membrane protein homolog	3.4
SA1634	truncated-SA	truncated HP [Pathogenicity island SaPIn3]	4.4
SA1674		glutamate ABC transporter ATP-binding protein	3.1
SA1675		HP, similar to glutamine-binding periplasmic protein	3.5
SA1962	mtlA	PTS system, mannitol specific IIA component	3.1
SA2081		HP, similar to urea transporter	3.2
SA2135		HP, similar to sodium/glutamate symporter	3.3
SA2242		conserved HP	5.0
SA2243		HP, similar to ABC transporter (ATP-binding protein	5.0
SA2314		HP, similar to ABC transporter (ATP-binding protein)	4.2
SA2339		HP, similar to antibiotic transport-associated protein	3.5
SA2415		HP, similar to ABC transporter (permease)	3.0
SA2426	arcD	arginine/ornithine antiporter	6.9
Membrane bioenergetics (electron transport chain and ATP synthesis)			
SA0211		HP, similar to NADH-dependent dehydrogenase	3.1
Cell division			
SA0249	scdA	cell wall biosynthesis protein ScdA	4.1
SA0724		HP, similar to cell-division inhibitor	3.5

ORF	Gene	Function*	Fold change‡
Metabolism of carbohydrates and related molecules - Specific pathways			
SA0035		probable HMG-CoA synthase	105.3
SA0162	aldA	aldehyde dehydrogenase homolog	9.6
SA0182		HP, similar to indole-3-pyruvate decarboxylase	4.3
SA0218	pflB	formate acetyltransferase	3.4
SA0219	pflA	formate acetyltransferase activating enzyme	3.8
SA0299		HP, similar to carbohydrate kinase, PfkB family	6.0
SA0562	adh1	alcohol dehydrogenase I	3.1
SA0654	fruB	fructose 1-phosphate kinase	4.4
SA1141	glpK	glycerol kinase	3.4
SA1338	malA	alpha-D-1,4-glucosidase	3.8
SA1553	fhs	formyltetrahydrofolate synthetase	3.1
SA1554	acsA	acetyl-CoA synthetase	3.8
SA1924		HP, similar to aldehyde dehydrogenase	3.5
SA1963	mtlD	mannitol-1-phosphate 5-dehydrogenase	4.1
SA2119		HP, similar to dehydrogenase	3.8
SA2260		HP, similar to glucose 1-dehydrogenase	3.5
SA2327		HP, similar to pyruvate oxidase	3.2
SA2395		L-lactate dehydrogenase	4.7
SA2490		HP, similar to *N*-hydroxyarylamine *O*-acetyltransferase	3.6
Metabolism of carbohydrates and related molecules - Main glycolytic pathway			
SA1510	gapB	glyceraldehyde 3-phosphate dehydrogenase 2	7.3
SA1599		HP, similar to transaldolase	3.1
SA1609	pckA	phosphoenolpyruvate carboxykinase	4.1
SA2304	fbp	fructose-bisphosphatase	4.4
Metabolism of carbohydrates and related molecules - TCA cycle			
SA1517	citC	isocitrate dehyrogenase	3.3
SA1518	citZ	methylcitrate synthase	3.5
Metabolism of amino acids and related molecules			
SA0008	hutH	histidine ammonia-lyase	4.1
SA0346		HP, similar to cystathionine beta-lyase	3.2
SA0818	rocD	ornithine-oxo-acid transaminase	3.6
SA0819	gudB	NAD-specific glutamate dehydrogenase	3.8
SA0821	argH	argininosuccinate lyase	5.1
SA0822	argG	argininosuccinate synthase	6.2
SA1012	argF	ornithine carbamoyltransferase	8.7
SA1531	ald	alanine dehydrogenase	5.2
SA1585		proline dehydrohenase homolog	7.3
SA1814		HP, similar to succinyl-diaminopimelate desuccinylase	6.8
SA2125		HP, similar to formiminoglutamase	7.5
SA2341	rocA	1-pyrroline-5-carboxylate dehydrogenase	5.1
SA2389	truncated-SA	truncated HP, similar to metalloproteinase mpr precursor	4.5
SA2397		4-aminobutyrate aminotransferase	4.1

ORF	Gene	Function*	Fold change‡
SA2425	arcC	carbamate kinase	12.1
SA2427	arcB	ornithine carbamoyltransferase	8.9
SA2428	arcA	arginine deiminase	6.2
SA2471	hisG	ATP phosphoribosyltransferase catalytic subunit	4.8
Metabolism of nucleotides and nucleic acids			
SA0016	purA	adenylosuccinate synthase	6.3
SA0816		HP, similar to polyribonucleotide nucleotidyltransferase	3.1
SA0917	purK	phosphoribosylaminoimidazole carboxylase carbon dioxide-fixation chain PurK homolog	3.2
SA0926	purD	phosphoribosylamine--glycine ligase PurD	4.7
SA1013		HP, similar to carbamate kinase	6.7
SA1172		guanosine 5'-monophosphate oxidoreductase	3.0
SA1938	pdp	pyrimidine nucleoside phosphorylase	3.5
Metabolism of lipids			
SA0572		HP, similar to esterase/lipase	6.1
SA0820	glpQ	glycerophosphoryl diester phosphodiesterase	3.8
SA2351		HP, similar to phytoene dehydrogenase	3.1
Metabolism of coenzymes and prosthetic groups			
SA0181		HP, similar to isochorismatase	4.0
SA0915	folD	FolD bifunctional protein	4.3
SA1894	thiE	Chain B, thiamin phosphate synthase	3.4
SA1895	thiM	hydroxyethyl thiazole kinase	3.2
SA1896	thiD	phosphomethylpyrimidine kinase	3.2
SA2077		HP, similar to biotin biosynthesis protein	4.2
SA2211		HP, similar to 6-carboxyhexanoate--CoA ligase	12.6
SA2213	bioB	biotin synthase	19.6
SA2214	bioA	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	5.8
SA2215	bioD	dethiobiotin synthetase	14.2
RNA synthesis - Regulation			
SA0108	sarH1	staphylococcal accessory regulator H1	5.0
SA0187		HP, similar to transcription regulator	6.5
SA0573	sarA	staphylococcal accessory regulator A	4.4
SA0653		HP, similar to transcription repressor of fructose operon	5.4
SA1217		HP, similar to negative regulator PhoU	3.3
SA1833		HP, similar to transcription regulator [Pathogenicity island SaPIn1]	8.1
SA1897		HP, similar to transcriptional activator TenA	3.1
SA1961		HP, similar to transcription antiterminator BglG family	3.3
SA2108		HP, similar to transcription regulator, RpiR family	3.3
SA2424		HP, similar to transcription regulator Crp/Fnr family protein	14.6

ORF	Gene	Function*	Fold change‡
Protein folding			
SA1659	prsA	peptidyl-prolyl cis/trans isomerase homolog	3.1
Adaption to atypical conditions			
SA0153	capJ	capsular polysaccharide synthesis enzyme Cap5J	9.7
SA0755		HP, similar to general stress protein 170	5.8
SA1984	asp23	alkaline shock protein 23, ASP23	5.8
SA2170		HP, similar to general stress protein 26	6.0
SA2336	clpL	ATP-dependent Clp proteinase chain clpL	7.9
Detoxification			
SA0650	norA	quinolone resistance protein	5.8
SA0681		HP, similar to multidrug resistance protein	4.0
SA2124	fosB	fosfomycin resistance protein FosB	3.0
Phage-related functions			
SA1762		HP [Bacteriophage phiN315]	3.2
SA1783		HP [Bacteriophage phiN315]	3.2
Transposon and IS			
SA0034		transposase for IS-like element	11.4
Pathogenic factors (toxins and colonization factors)			
SA0107	spa	Immunoglobulin G binding protein A precursor	5.5
SA0191		conserved HP	3.3
SA0389	set13	superantigen-like protein	4.1
SA0841		HP, similar to cell surface protein Map-w	3.9
SA1004		HP, similar to fibrinogen-binding protein	4.3
SA1268	ebhB	HP, similar to streptococcal adhesin emb	3.1
SA1577		HP, similar to FmtB protein	3.9
SA1638	lukE	leukotoxin LukE [Pathogenicity island SaPIn3]	4.9
SA1758	sak	staphylokinase precursor [Bacteriophage phiN315]	6.8
SA2006		HP, similar to MHC class II analog	5.3
SA2207	hlgA	gamma-hemolysin chain II precursor	3.6
SA2323		conserved HP	3.2
SA2353		HP, similar to secretory antigen precursor SsaA	3.5
SA2430	aur	zinc metalloproteinase aureolysin	3.2
SA2462	icaC	intercellular adhesion protein C	4.2
SA2463	lip	triacylglycerol lipase precursor	3.3
SAS029		HP, similar to lactococcin 972	3.0
Miscellaneous			
SA0185	murQ	*N*-acetylmuramic acid-6-phosphate etherase	3.5
SA1452	csbD	sigmaB-controlled gene product	3.4
SA1606		plant metabolite dehydrogenase homolog	3.3
SA1709		HP, similar to ferritin	4.7
Hypothetical proteins			
SA0023	orfX	conserved HP orfX	3.5
SA0046		conserved HP	3.4

ORF	Gene	Function*	Fold change‡
SA0129		HP	3.0
SA0184		conserved HP	3.7
SA0212		conserved HP	4.4
SA0300		truncated HP	5.8
SA0301		conserved HP	7.6
SA0359		conserved HP	11.0
SA0372		HP	5.1
SA0395		HP [Pathogenicity island SaPIn2]	3.0
SA0631		HP	3.7
SA0635		conserved HP	3.3
SA0636		conserved HP	3.0
SA0637		conserved HP	4.7
SA0707		conserved HP	10.5
SA0740		HP	3.4
SA0752		HP	9.8
SA0792		HP	6.7
SA0873		conserved HP	3.5
SA0883		HP	5.6
SA0888		conserved HP	5.9
SA0889		HP	3.5
SA0976	isdB	conserved HP	3.3
SA1005		HP	4.1
SA1014		conserved HP	5.4
SA1019		conserved HP	6.3
SA1057		conserved HP	6.9
SA1208		HP	4.3
SA1280		conserved HP	3.5
SA1293		conserved HP	5.4
SA1476		HP	6.3
SA1477		HP	4.8
SA1528		conserved HP	7.7
SA1529		conserved HP	3.0
SA1532		conserved HP	6.6
SA1573		HP	3.8
SA1610		HP	3.2
SA1706		HP	3.1
SA1803		HP [Bacteriophage phiN315]	3.3
SA1825		HP [Pathogenicity island SaPIn1]	3.0
SA1829		HP [Pathogenicity island SaPIn1]	3.6
SA1937		conserved HP	3.8
SA1946		conserved HP	5.3
SA1971		HP	3.2
SA1985		HP	4.9

ORF	Gene	Function*	Fold change‡
SA1986		HP	4.6
SA2004		conserved HP	4.1
SA2011		HP	4.2
SA2049		HP	4.8
SA2076		HP	5.3
SA2101		conserved HP	3.0
SA2116		HP	3.3
SA2158		HP, similar to TpgX protein	4.2
SA2192		HP	242.7
SA2212		HP, similar to 8-amino-7-oxononanoate synthase	16.8
SA2219		conserved HP	3.9
SA2259		conserved HP	3.6
SA2262		conserved HP	14.0
SA2283		conserved HP	4.6
SA2309		conserved HP	3.9
SA2321		HP	4.6
SA2328		conserved HP	3.6
SA2331		HP	3.8
SA2343		HP	6.9
SA2350		conserved HP	3.2
SA2367		conserved HP	5.4
SA2398		HP	4.3
SA2403		conserved HP	6.7
SA2432		conserved HP	3.0
SA2451		HP	3.3
SA2480	drp35	Drp35	3.0
SA2491		conserved HP	6.6
SA2497		HP	3.0
SAS011		HP	3.9
SAS016		HP	14.6
SAS025		HP	3.4
SAS030		HP	3.8
SAS046		HP	3.8
SAS049		HP	3.7
SAS050		HP	3.5
SAS056		HP	3.3
SAS061		HP	4.4
SAS073		HP	3.4
SAS074		conserved HP	3.8
SAS083		HP	3.2

* HP, hypothetical protein

‡ Threshold ratio value set at ≥3.0-fold change

6.3.2. GENES DOWN-REGULATED IN CRV COMPARED TO THE PARENT STRAIN

GeneID	Component	Function*	Fold Change‡
Cell wall			
SA0876	murE	UDP-N-acetylmuramoylalanyl-D-glutamate--2, 6-diaminopimelate ligase	3.7
SA1458	lytH	N-acetylmuramoyl-L-alanine amidase	3.1
Transformation competence			
SA0882		HP, similar to competence transcription factor	3.1
Transport/binding proteins and lipoproteins			
SA0137		HP, similar to transport system protein	3.2
SA0166		HP, similar to nitrate transporter	4.2
SA0167		HP, similar to membrane lipoprotein SrpL	4.1
SA0168		HP, similar to probable permease of ABC transporter	4.0
SA0293		HP, similar to formate transporter NirC	3.9
SA0374	pbuX	xanthine permease	7.5
SA0531	proP	proline/betaine transporter homolog	3.5
SA0956		HP, similar to $Mn2^{+}$-transport protein	4.3
SA1042	pyrP	uracil permease	10.7
SA1519	aapA	D-serine/D-alanine/glycine transporter	3.4
SA1879	kdpC	probable potassium-transporting ATPase C chain	5.2
SA1880	kdpB	probable potassium-transporting ATPase B chain	6.4
SA1881	kdpA	probable potassium-transporting ATPase subunit A	7.0
SA1992	lacE	PTS system, lactose-specific IIBC component	5.2
SA1993	lacF	PTS system, lactose-specific IIA component	6.8
SA2167	scrA	PTS system, sucrose-specific IIBC component	3.8
SA2176	narK	nitrite extrusion protein	4.2
SA2201		HP, similar to ABC transporter, permease protein	3.4
SA2202		HP, similar to ABC transporter, periplasmic amino acid-binding protein	3.6
SA2203		HP, similar to multidrug resistance protein	3.8
SA2239		HP, similar to amino acid transporter	4.7
SA2326	ptsG	PTS system, glucose-specific IIABC component	5.9
SA2411		HP, similar to magnesium citrate secondary transporter	3.6
Sensors (signal transduction)			
SA2180		HP, similar to two component sensor histidine kinase	5.0
Membrane bioenergetics (electron transport chain and ATP synthesis)			
SA2182	narI	nitrate reductase gamma chain	4.5
SA2183		HP, similar to nitrate reductase delta chain	11.3
SA2184	narH	nitrate reductase beta chain narH	10.8
SA2185	narG	respiratory nitrate reductase alpha chain	9.5

GeneID	Component	Function*	Fold Change‡
Protein secretion			
SA2446	secY	HP, similar to preprotein translocase subunit SecY	3.9
Metabolism of carbohydrates and related molecules - Specific pathways			
SA0232	lctE	L-lactate dehydrogenase	3.0
SA1991	lacG	6-phospho-beta-galactosidase	4.8
SA1994	lacD	tagatose 1,6-diphosphate aldolase	6.0
SA1995	lacC	tagatose-6-phosphate kinase	6.8
SA1996	lacB	galactose-6-phosphate isomerase LacB subunit	4.1
SA1997	lacA	galactose-6-phosphate isomerase LacA subunit	4.3
SA2007		HP, similar to alpha-acetolactate decarboxylase	4.1
SA2008	alsS	alpha-acetolactate synthase	9.7
Metabolism of carbohydrates and related molecules - Main glycolytic pathway			
SA0727	gap	glyceraldehyde-3-phosphate dehydrogenase	10.4
SA0728	pgk	phosphoglycerate kinase	9.5
SA0730	pgm	phosphoglyceromutase	6.6
Metabolism of amino acids and related molecules			
SA1225	lysC	aspartokinase II	4.7
SA1272		alanine dehydrogenase	3.5
SA2187	nasE	assimilatory nitrite reductase	5.3
SA2188	nasD	nitrite reductase	7.6
SA2189		HP, similar to NirR	9.3
Metabolism of nucleotides and nucleic acids			
SA0373	xprT	xanthine phosphoribosyltransferase	9.6
SA0375	guaB	inositol-monophosphate dehydrogenase	5.9
SA0376	guaA	bifunctional GMP synthase/glutamine amidotransferase protein	4.0
SA0453		HP, similar to 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	5.1
SA1043	pyrB	aspartate carbamoyltransferase catalytic subunit	16.5
SA1044	pyrC	dihydroorotase	17.8
SA1045	pyrAA	carbamoyl-phosphate synthase small subunit	12.4
SA1046	carB	carbamoyl-phosphate synthase large subunit	8.6
SA1047	pyrF	orotidine-5-phosphate decarboxylase	3.2
SA1048	pyrE	orotate phosphoribosyltransferase	3.6
SA1921	tdk	thymidine kinase	3.1
SA2297		HP, similar to GTP-pyrophosphokinase	4.3
Metabolism of lipids			
SA1357	accC	acetyl-CoA carboxylase accC, biotin carboxylase subunit	3.0
Metabolism of coenzymes and prosthetic groups			
SA0472	folP	dihydropteroate synthase chain A synthetase	4.4
SA0473	folB	7,8-dihydroneopterin aldolase	3.1

GeneID	Component	Function*	Fold Change‡
SA0666		HP 6-pyruvoyl tetrahydrobipterin synthase homolog	3.6
SA1586	ribH	6,7-dimethyl-8-ribityllumazine synthase	6.3
SA1587	ribA	riboflavin biosynthesis protein	42.8
SA1588	ribB	riboflavin synthase subunit alpha	39.6
SA1589	ribD	riboflavin specific deaminase	51.5
SA2186	nasF	uroporphyrin-III C-methyl transferase	5.4
RNA synthesis - Regulation			
SA0726	gapR	glycolytic operon regulator	9.2
SA1041	pyrR	pyrimidine regulatory protein PyrR	9.4
SA1949	truncated-SA	lytic regulatory protein truncated with Tn554	3.1
SA2179		HP, similar to response regulators of two-component regulatory	4.5
SA2295	gntR	gluconate operon transcriptional repressor	3.1
SAS066	agrD	AgrD protein	5.5
Protein modification			
SA1725		staphopain, cysteine proteinase	3.9
Adaption to atypical conditions			
SA0146	capC	capsular polysaccharide synthesis enzyme Cap8C	3.3
SA0147	capD	capsular polysaccharide synthesis enzyme Cap5D	3.8
SA0148	capE	capsular polysaccharide synthesis enzyme Cap8E	3.5
SA0149	capF	capsular polysaccharide synthesis enzyme Cap5F	4.3
SA0150	capG	capsular polysaccharide synthesis enzyme Cap5G	4.7
SA0155	capL	capsular polysaccharide synthesis enzyme Cap5L	7.3
SA0156	capM	capsular polysaccharide synthesis enzyme Cap5M	7.1
SA0157	capN	capsular polysaccharide synthesis enzyme Cap5N	6.4
SA0158	capO	capsular polysaccharide synthesis enzyme Cap8O	4.7
SA0159	capP	capsular polysaccharide synthesis enzyme Cap5P	4.5
SA0462		HP, similar to low temperature requirement B protein	3.1
Antibiotic production			
SA0173		HP, similar to surfactin synthetase	6.5
Phage-related functions			
SA0252	lrgA	murein hydrolase regulator LrgA	5.6
SA0253	lrgB	antiholin-like protein LrgB	4.1
Pathogenic factors (toxins and colonization factors)			
SA0309	geh	glycerol ester hydrolase	5.7
SA0519	sdrC	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	4.0
SA0520	sdrD	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	3.2
SA0521	sdrE	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein	3.8
SA0746		staphylococcal nuclease	3.3

GeneID	Component	Function*	Fold Change‡
SA2423	clfB	clumping factor B	4.8
SA2447		HP, similar to streptococcal hemagglutinin protein	10.2
Hypothetical proteins			
SA0174		conserved HP	3.6
SA0228		HP	3.9
SA0282		conserved HP	3.7
SA0285		HP	3.4
SA0286		conserved HP	3.9
SA0287		conserved HP	3.6
SA0412		conserved HP	4.0
SA0667		conserved HP	3.6
SA0929		conserved HP	3.2
SA1307	engA	HP, similar to GTP-binding protein EngA	3.1
SA2181		HP	4.6
SA2329		conserved HP	4.0
SA2444		HP	4.9
SA2445		HP	4.8
SAS017		HP	3.1

* HP, hypothetical protein

‡ Threshold ratio value set at ≥3.0-fold change

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LIST OF PUBLICATIONS

Parts of this work were or will be shortly published in the following scientific journals:

1) ***Raafat, D.*, and *H.-G. Sahl*.** 2007. Chitosan as an antibacterial compound: Insights into its mode of action. In: Advances in Chitin Science. S. Şenel, K. M. Vårum, M. M. Şumnu, and A. A. Hıncal (Eds.), Alp Ofset, Ankara, Vol X, pp: 326 - 332.

2) ***Raafat, D.*, *K. von Bargen*, *A. Haas*, and *H.-G. Sahl*.** 2008. Insights into the mode of action of chitosan as an antibacterial compound. Applied and Environmental Microbiology 74: 3764 - 3773.

3) ***Raafat, D.*, and *H.-G. Sahl*.** Chitosan's *in vitro* antimicrobial activity — A critical look.

   (in preparation)

4) ***Raafat, D.*, *François P., Schrenzel J.,* and *H.-G. Sahl*.** Selection and characterization of chitosan-resistant *Staphylococcus aureus*.

   (in preparation)

ERKLÄRUNG

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Bonn, den 28. Juli 2008

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Dina Raafat Gouda Fouad