Development of bacterial resistance to biocides and antimicrobial agents as a consequence of biocide usage

PhD Thesis

by

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Preface

The work of this PhD thesis was carried out at the Division for Epidemiology and Microbial Genomics (former Division of Microbiology and Risk Assessment), National Food Institute, Technical University of Denmark, from December 2009 to May 2013. Part of the research was carried out at the Department of Microbial Diseases, UCL Eastman Dental Institute, University College London, London, UK, constituting seven weeks (June 2011 and in October and November 2011). This project was funded by grant 2101-08-0030 from the program Committee for Food, Health and Welfare under the Danish Council for Strategic Research.

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Abstract

Biocides are chemical compounds with antimicrobial properties and they are widely used for disinfection, antiseptic and preservation purposes. Biocides have been applied for centuries due to early empirical approaches, such as cleansing of wounds with wine, vinegar and honey and salting of fish and meat. Today, large amounts of biocides are used for disinfection to achieve a satisfactory level of hygiene in various settings and use of biocides has become an integrated part of the industrialized world.

Despite the widespread use and application of biocides knowledge about their exact mechanisms of action, especially at sub-inhibitory concentrations, and the bacterial response to such exposure, is relatively limited. The increasing use of biocides has within recent years lead to concerns about development and emergence of biocide resistant microorganisms that might make the task of eradication of pathogenic bacteria more difficult. Furthermore, it has been suggested that use of biocides may contribute to the development of resistance in bacteria to antimicrobial agents used in human and animal therapy. So far, it is evident that cross- and co-resistance mechanisms to antimicrobials agents and biocides exist. However, much less is known about the potential effect of biocides on development of antimicrobial resistance in bacteria by promoting the horizontal transfer of resistance genes or by inducing the mutation rate. Even though biocides are commonly used at working concentrations way above the lethal bacterial dose, the efficacy of these compounds can be significantly reduced by incorrect use or the presence of residual concentrations hence, bacterial exposure to sub-inhibitory concentrations of biocides is likely to occur.

The overall objective of this study was to examine if natural bacterial isolates become less susceptible to biocides used in their environment and if this can lead to spread of antimicrobial resistant clones due to co-selection. Furthermore, the objective was to examine if exposure to sub-inhibitory concentrations of biocides induce development of resistance to antimicrobial agents.

So far, only few studies have investigated the susceptibility of livestock-associated isolates to biocides used in their environment. Pigs are increasingly recognised as a potential reservoir of community-acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA), especially clones belonging to clonal complex (CC) 398. Recently, methicillin resistant *S. aureus* (MRSA) isolates belonging to CC30 was for the first time detected among Danish pigs. The susceptibility of 79 porcine *S. aureus* isolates belonging to CC398 or CC30 to commonly used biocides in pig farming was therefore determined (Manuscript III). The biocides comprised benzalkonium chloride (BC), hydrogen peroxide (HP), sodium hypochlorite (SH), formaldehyde (FH), and caustic soda (NaOH). *S. aureus* isolates did in general not show reduced susceptibility to the biocides tested. However, a quaternary ammonium compound (QAC) resistance gene, *qacG*, was detected in MRSA CC30 isolates. The presence of *qacG* in MRSA CC30 is worrying, since use of QACs may contribute to the selection and spread of these isolates. MRSA CC30 is often associated with MRSA types giving rise to clinical infections in Denmark and porcine MRSA CC30 may be prone to adapt to humans.

Residues or inaccurate use of biocides may lead to bacterial exposure to sub-inhibitory concentrations. The bacterial response to such exposure is however unclear. It has been suggested that the SOS response contribute to antimicrobial resistance development in bacteria by inducing mutagenesis. Therefore, the effect of sub-inhibitory concentrations of the five common biocides; BC, CHX, HP, PAA, and SH on the SOS response, indicated by the use of a *rec*A-lacZ expression assay, and mutagenesis in *S. aureus* isolates was studied (Manuscript II). BC, CHX, and HP was found to induce the SOS response. In addition, HP and PAA were found to significantly ($p \leq 0.05$) increase the mutation rate by 5-15 and 3-8 fold, respectively. These results suggest that exposure to sub-inhibitory concentrations of HP and PAA may contribute to emergence of antimicrobial resistance in *S. aureus*. This may be of potential risk for human health, since these disinfectants are widely used at hospitals and in the food industry.

Mobile genetic elements such as conjugative transposons are important vectors in the dissemination of antibiotic resistance determinants. Tn*916* including the tetracycline resistance gene *tet(M)* is a conjugative transposon and the prototype of a large family of related elements. They have an extremely broad host range and have been found in both pathogenic and commensal bacteria. In the study of Manuscript I, the effect of sub-inhibitory concentrations of ETOH, HP, CHX, and SH on the conjugative transposition of the mobile genetic element Tn*916* was investigated. ETOH was found to significantly ($p < 0.05$) increase transfer of Tn*916* by an average of 5-fold, whereas an increase of 4-fold on Tn*916* conjugation frequency was observed ($p = 0.12$) when donors were exposed to hydrogen peroxide. These results suggest that exposure to sub-inhibitory concentrations of ETOH and HP may induce the spread of Tn*916*-like elements and their resistance genes, which is clinically important since these biocides are frequently used in hospitals.

In conclusion, no widespread selection for reduced susceptibility to commonly used disinfectants in pig farming was detected in porcine *S. aureus* isolates. However, a biocide resistance gene, *qacG*, was identified in several of the MRSA isolates, which has also been found in other animal related staphylococci. Surveillance of the occurrence and emergence of reduced susceptibility to biocides in bacteria are however, still encouraged, since this will provide important data to determine if decreased susceptibility to biocides happen over time. Importantly, the data from this thesis demonstrated a potential of certain biocides to contribute to antimicrobial resistance development and emergence in bacteria through increased mutagenesis and transfer of the antimicrobial resistance gene *tet(M)*. On the short term these results emphasises that correct use of biocides are of outmost importance and should not be compromised. On the long term, more studies are needed to elucidate the actual risk of biocide use on generating antimicrobial resistant bacteria in practice.

Dansk sammendrag

Biocider er kemiske stoffer med antimikrobielle egenskaber og bruges i vid udstrækning til desinfektion, antisepsis og til konservering. Biocider er blevet anvendt i århundreder til eksempelvis rensning af sår med vin, eddike eller honning og til saltning af fisk og kød på grund af tidlige empiriske tilgange. I dag, bliver store mængder af biocider anvendt til desinfektion for at opnå et tilfredsstillende niveau af hygiejne i forskellige miljøer og anvendelsen af biocider er blevet en integreret del af den industrialiserede verden.

Selvom biocider anvendes i vid udstrækning er viden omkring deres nøjagtige virkningsmekanisme relativt begrænset især ved sub-inhiberende koncentrationer. Det øget forbrug af biocider har inden for de seneste år ledt til bekymring om udviklingen og spredningen af biocid resistente bakterier, som muligvis kan besværliggøre bekæmpelsen af patogene bakterier. Endvidere er det blevet foreslået, at anvendelse af biocider kan bidrage til udviklingen af resistens hos bakterier over for antimikrobielle stoffer, der anvendes til behandling af mennesker og dyr. Indtil videre er det tydeligt at kryds- og co-resistens resistensmekanismer over for antimikrobielle stoffer og biocider findes, derimod er viden omkring biociders potentielle effekt på udviklingen af resistens overfor antimikrobielle stoffer i bakterier, forårsaget af den horisontale overførsel af resistensgener eller ved at øge mutations rate meget mindre kendt.

Selvom biocider almindeligvis anvendes ved koncentrationer, som langt overstiger den dødelige dosis for bakterier, kan virkningsgraden af disse stoffer betydeligt blive reduceret ved forkert brug eller ved tilstedeværelsen af restkoncentrationer og dermed kan bakterier blive eksponeret til lave ikke dødelige koncentrationer.

Det overordnet formål med dette projekt er at undersøge om naturlige bakterie isolater bliver mindre følsomme over for biocidstoffer, der anvendes i deres miljø og om dette kan lede til spredning af resistens overfor antimikrobielle stoffer i bakterier pga. co-selektion. Derudover, er formålet at undersøges effekten af eksponeringen af bakterier overfor sub-inhiberende koncentrationer på udviklingen af resistens overfor antimikrobielle stoffer.

Hidtil har kun få studier undersøgt følsomheden af husdyr-relaterede bakterie isolaters overfor biocider, som anvendes i deres miljø. Svin bliver i stigende grad anerkendt som et potentielt reservoir for samfundserhvervet methicillin resistente *Staphylococcus aureus* (MRSA), specielt kloner, som hører til det klonale komplex (CC) 398. For nyligt blev den første MRSA tilhørende CC30 identificeret i danske svin, som desuden også blev fundet i landmænd. Følsomheden i 79 svine *S. aureus* isolater overfor almindelige anvendte biocider i svine produktionen blev bestemt (Manuscript III). Biociderne bestod af benzalkonium klorid (BC), hydrogen peroxid (HP), natrium hypoklorit (SH), formaldehyd (FH) og kaustisk natron NaOH). *S. aureus* isolater udviste i almindelighed ikke nedsat følsomhed over for de testede biocider. Imidlertid blev et kvaternær ammoniumforbindelse (QAC) resistensgen, *qac*G, påvist i MRSA CC30 isolater. Tilstedeværelsen af *qac*G i MRSA CC30 er bekymrende, da brugen af QAC'er muligvis kan bidrage til selektionen og spredningen af disse isolater. MRSA CC30 er ofte forbundet med MRSA typer, der giver

anledning til kliniske infektioner i mennesker i Danmark og svine MRSA CC30 kan derfor muligvis være tilbøjelig til at tilpasse sig til mennesker.

Biocidrester eller ukorrekt anvendelse af biocider kan føre til at bakterier bliver eksponeret overfor sub-inhiberende koncentrationer. Cellens reaktion på en sådan eksponering er uklar. Det er blevet foreslået, at SOS responset i bakterier kan bidrage til udviklingen af resistens overfor antimikrobielle stoffer ved at øge forekomsten af mutationer i bakterien. Derfor blev effekten af sub-inhiberende koncentrationer af de fem almindelige biocider BC, CHX, HP, PAA og SH på SOS responset, indikeret ved brug af et *recA-lacZ* ekspressions assay, samt mutationsfrekvensen i *S. aureus* isolater undersøgt (Manuscript II). BC, CHX og HP øgede SOS responset. Desuden blev det påvist at HP og PAA øgede mutationsfrekvensen signifikant ($p \leq 0,05$) med 5-15 og 3-8 gange. Disse resultater tyder på, at eksponeringen overfor sub-inhiberende koncentrationer af HP og PAA kan bidrage til udviklingen af antimikrobiel resistens i *S. aureus*. Dette kan være af potentiel risiko for menneskers sundhed, da disse desinfektionsmidler i vidt omfang anvendes på hospitaler og i fødevareindustrien.

Mobile genetiske elementer såsom konjugative transposoner er vigtige vektorer i udbredelsen af antimikrobielle resistensdeterminanter. Tn*916*, som indeholder tetracyklinresistensgenet *tet*(M) er et konjugativt transposon og prototypen på en stor familie af beslægtede elementer. De har et ekstremt bredt spektrum af bakterieværter og er blevet fundet i både sygdomsfremkaldende og kommensale bakterier. I studiet, som udgør Manuskript I blev effekten af sub-inhiberende koncentrationer af ETOH, HP, CHX og SH på overførslen af Tn*916* undersøgt. ETOH blev fundet til signifikant ($p < 0,05$) at øge overførsel af Tn*916* 5 gange i gennemsnit, hvorimod en gennemsnitlig øgning i overførslen af Tn*916* på 4 blev observeret ($p = 0,12$), når donor bakterier blev udsat for HP. Disse resultater tyder på, at eksponering overfor sub-inhiberende koncentrationer af ETOH og HP kan fremkalde udbredelsen af Tn*916*-lignende elementer og deres resistensgener, hvilket er af klinisk betydning da disse biocider ofte anvendes på hospitaler.

Afslutningsvis blev der ikke fundet udbredt nedsat følsomhed over for dedinfektionsmidler, som oftest anvendes i svineproduktionen i *S. aureus* svine isolater. Imidlertid blev biocidresistensgenet, *qacG*, konstateret i flere af MRSA CC30 isolaterne. Dette gen er også fundet i andre dyre-relaterede stafylokokker. Overvågning af forekomst af nedsat følsomhed over for biocider i bakterier opmuntres dog stadig, da dette vil give vigtige data til at afgøre, om nedsat følsomhed overfor biocider sker over tid. Vigtigere er det, at data fra denne afhandling har vist at visse biocider har potential til at bidrage til udviklingen af resistens overfor antimikrobielle stoffer i bakterier via øget mutationsfrekvens og ved øget overførsel af resistensgenet *tet(M)*. På kort sigt understreger disse resultater, at korrekt anvendelse af biocider er af allerstørste betydning. På lang sigt er flere undersøgelser nødvendige for at belyse den faktiske risiko ved anvendelse af biocider på udviklingen af antibiotikaresistente bakterier i praksis.

List of abbreviations

$AgNO_3$	Silver nitrate
BC	Benzalkonium chloride
CA-MRSA	Community acquired methicillin resistant *Staphylococcus aureus*
CHX	Chlorhexidine digluconate
CIP	Ciprofloxacin
CRAs	Chlorine releasing agents
CTAB	Cetyltrimethylammonium bromide
CTM	Cetrimide
$CuSO_4$	Copper sulphate
EFSA	European Food Safety Authority
EtBr	Ethidium bromide
EtOH	Ethanol
EU	European Union
FH	Formaldehyde
HP	Hydrogen peroxide
MBC	Minimum bactericidal concentration
MIC	Minimal inhibitory concentration
MRSA	Methicillin resistant *S. aureus*
MSSA	Methicillin sensitive *S. aureus*
NaOH	Caustic soda
PAA	Peracetic acid
QACs	Quaternary ammonium compounds
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SH	Sodium hypochlorite
TET	Tetracycline
TRI	Triclosan

Objective of study and outline of thesis

Biocides are chemical compounds with antimicrobial properties and they are widely used for disinfection, antiseptic and preservation purposes [1]. Biocides have been applied for centuries due to early empirical approaches, such as cleansing of wounds with wine, vinegar and honey and salting of fish and meat [2]. Today, large amounts of biocides are used for disinfection to achieve a satisfactory level of hygiene in various settings and use of biocides has become an integrated part of the industrialized world [1]. At hospitals, biocides are widely used for disinfection of inventory and equipments and for hand disinfection to prevent transmission of pathogens and they have become an important part of infection control strategies [3]. In the food industry, biocides are extensively used in animal husbandries to prevent emergence and dissemination of disease among livestock animals and in food processing to prevent food contamination [4].

Despite the widespread use and application of biocides knowledge about their exact mechanisms of action, especially at sub-inhibitory concentrations, and the bacterial response to such exposure, is relatively limited [5]. The increasing use of biocides has within recent years lead to concerns about development and emergence of biocide resistant microorganisms that might make the task of eradication of pathogenic bacteria more difficult. Furthermore, it has been suggested that use of biocides may contribute to the development of resistance in bacteria to antimicrobial agents used in human and animal therapy. So far, it is evident that cross- and co-resistance mechanisms to antimicrobials agents and biocides exist [5]. However, much less is known about the potential effect of biocides on development of antimicrobial resistance in bacteria by promoting the horizontal transfer of resistance genes or by inducing the mutation rate. Recently, stress in bacteria caused by sub-inhibitory concentrations of antimicrobial agents has been shown to induce transfer of resistance genes [6] and to induce the mutation rate in bacteria [7–10]. Even though biocides are commonly used at working concentrations way above the lethal dose of bacteria, the efficacy of these compounds can be significantly reduced by e.g. the presence of organic matters, insufficient exposure time, and inappropriate dilution. In addition, low concentrations may be obtained in niches with large number of bacterial species, such as human and animal skin and on production lines after some time. Hence, bacterial exposure to sub-inhibitory concentrations of biocides is likely to occur. Knowledge is therefore urgently needed to shed light on the potential unintended consequences of the widespread use of biocides.

Such knowledge may contribute with information on which compounds that are more likely to contribute to unwanted traits in bacteria such as antimicrobial resistance development. On the long term this will ensure that the use of biocides remains competent of retaining the high level of hygiene developed in the medical and industrial settings, which is of outmost importance for patient and consumer safety.

The overall objective of this PhD study was to investigate whether different selected biocides may have unwanted effects. The objective, with specific research hypotheses and specific studies conducted is outlined in Figure 1.

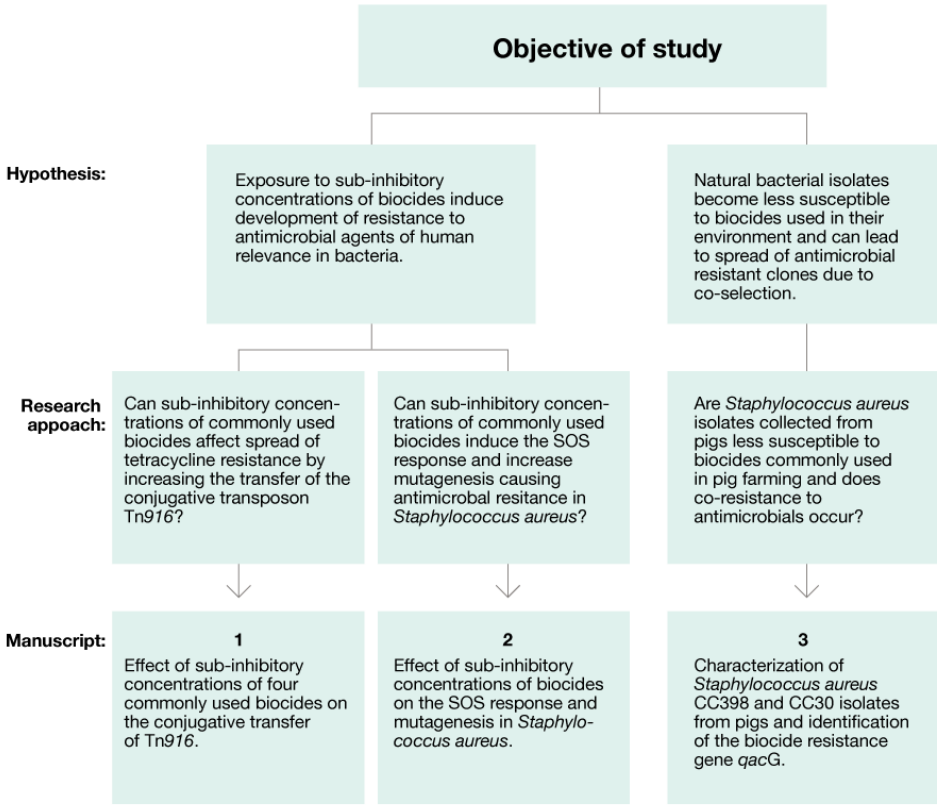

Figure 1: Objective of PhD study with specific research hypotheses and specific studies conducted.

Research approach and limitations

It was not possible to cover all potential biocides or biological systems in this project. Thus, a number of biocides and model systems have been selected. Biocides prioritized in this PhD study constitute biocidal active substances of disinfectants commonly used in the medical setting and/or in the food industry.

Staphylococcus aureus isolates of porcine origin was selected for the study of reduced susceptibility to and detection of known biocide resistance genes in natural isolates because research primarily has been focused on human clinical isolates and only few studies have been carried out on livestock associated isolates. Furthermore, pigs are increasingly recognised as a potential reservoir of community-acquired methicillin resistant *S. aureus* (CA-MRSA) [11] . Biocides selected for that study comprised benzalkonium chloride, hydrogen peroxide, sodium hypochlorite, formaldehyde, and caustic soda. The selection of biocides was based on a report published by the Danish Zoonosis Centre, National Food Institute, where the most used biocidal groups for disinfection in stables of finisher herds were presented [12]. Compounds representing the various biocidal groups were selected based on prior working experience with these compounds within the research group.

S. aureus isolates from human and porcine origin were selected for the SOS response/mutagenesis study. This bacterial species constitute a growing and important human pathogen in which increasing resistance to multiple antimicrobial agents cause increased morbidity and mortality in humans. Porcine *S. aureus* isolates were selected based on the above mentioned reasons and to include *S. aureus* from different reservoirs. Biocides selected for this study comprised ethanol and chlorhexidine digluconate, because they are the most widely used antisepsis compounds at hospitals [1,4]. Furthermore, peroxygens (peracetic acid and hydrogen peroxide), and sodium hypochlorite are widely used for disinfection in animal husbandries and in food processing in addition to hard surface disinfection at hospitals [4,12].

The conjugative transposon Tn*916* was included as a model system for transfer of the tetracycline resistance gene *tet*(M) because it has an extremely broad host range [13]. Furthermore, a regulatory mechanism for the transfer of this element has been suggested, which could be used in the set-up of the Tn*916* transfer protocol [13]. Biocides selected for this study comprised chlorhexidine digluconate, ethanol, hydrogen peroxide, and sodium hypochlorite due to their usage in the health care setting where increasing resistance in human pathogens constitute a major problem [1,3,4].

Outline of thesis

The thesis is divided into three main chapters. Chapter I gives an overview of the different groups of biocides, their mechanism of action and their application areas. In addition, current knowledge on mechanisms giving rise to reduced susceptibility to biocides in bacteria and co- and cross-resistance mechanisms to antimicrobial agents are presented. In Chapter II a summary of own results are presented and discussed. Chapter III includes the three manuscripts produced during this study:

I. **M. A. Seier-Petersen**, A. Jasni, F. M. Aarestrup, H. Vigre, P. Mullany, A. P. Roberts, Y. Agersø. Effect of sub-inhibitory concentrations of four commonly used biocides on the conjugative transfer of Tn*916*. *Manuscript submitted to Journal of Antimicrobial Chemotherapy.*

II. **M. A. Seier-Petersen**, A. S. Haugaard, F. M. Aarestrup, and Y. Agersø. Effect of sub-inhibitory concentrations of biocides on the SOS response and mutagenesis in *Staphylococcus aureus*. *Manuscript in preparation.*

III. **M. A. Seier-Petersen**, L. N. Nielsen, H. Ingmer, F. A. Aarestrup, Y. Agersø. Characterization of *Staphylococcus aureus* CC398 and CC30 isolates from pigs and identification of the biocide resistance gene *qacG*. *Manuscript submitted to Journal of Antimicrobial Chemotherapy.*

CHAPTER I

1 Introduction to biocides

Biocides are chemical compounds capable of killing or inhibiting microorganism [1]. They are defined as active substances and preparations containing one or more active substances intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means [14]. Biocidal compounds have been used for centuries due to early empirical approaches. For instance, natron, oils and balsams were used by the Egyptians in mummification of the dead and Persians used copper or silver vessels for storage of potable water. In addition, salting and natural spices have been used for preservation of foodstuffs and vinegar, wine, honey and mercuric chloride have been used in wound dressings [2,15]. Along with the industrial revolution a range of new compounds became available and the antibacterial effect of a number of compounds began to be utilized [16]. By the mid-1900s, many biocidal compounds were in common use both as industrial preservatives and in the medical field [15,16]. Since then, the number of biocidal compounds, their use and applications have increased dramatically [1615]. Today, biocides have become an integrated part of the industrialized world and are invaluable compounds for the control of human and animal pathogens and for management of unwanted microorganisms causing damage to various products, such as foods and cosmetics [14]. Large amounts of biocides are therefore consumed within the medical, industrial and domestic environment where they are used for disinfection, antisepsis, preservative, and cleaning purposes [1] (the different use practices of biocides are defined in Box 1).

Box 1 | Definition of use practices of biocides [1,3,130]:

Cleaning is the physical process by which dirt, dust, and microorganisms are removed from surfaces. In this case microorganisms are removed to inhibit propagation.

In *disinfection*, microorganisms are inhibited or destroyed to a safe level on inanimate objects; a load that will not give rise to infection.

The purpose of *sterilization* is to completely eradicate any form of living microorganism, including spores.

In *antisepsis* biocide compounds are used in or on living tissue to inhibit or destroy growth of microorganisms.

Biocides are used as *preservatives* to inhibit growth of microorganisms in various formulated products such as foods, cosmetics, toothpaste, and pharmaceuticals.

The consumption of pesticides used in agriculture has been stagnant over the last 10 years representing an annual market value of 27 billion euros within the European Union (EU). In comparison, biocides represent nearly 11 billion euros in EU and the market has been growing by 5 % per annum in the last 15 years and the market of biocides has been predicted to expand during the next years [17]. The use of biocides is much less restricted in comparison to the use of therapeutically antimicrobials for which surveillance of the consumption in some EU countries exists and the consumption of biocides in various fields is therefore largely unknown. However, a Directive (98/8/EC) of the European Parliament and of the Council on placing biocidal products on the market was adopted in 1998. The purpose of this Directive was to harmonize the European market

for biocidal products and their active substances in addition to provide a high level of protection for humans, animals and the environment [18]. In relation to the Biocide Directive the yearly consumption of biocidal product in Denmark was estimated and found to comprise up to 5,500 tonnes per year [4]. This was without including biocide used for e.g. cosmetics, human hygiene products, toys and medical products that are regulated by other directives [4]. In comparison less than 160 tonnes are annually consumed for antimicrobial agents used for therapy in humans and animals [19]. Table 1 illustrates some of the largest application areas within the estimated consumption in Denmark.

Tabel 1: Estimated yearly consumption of biocides (active compounds) in Denmark (4).

Application		Consumption (up to tonnes pr. year)
Human hygiene biocidal products	Skin disinfectants/antiseptics.	100
Private area and public health area biocidal products	»Disinfection of air, surfaces, materials, equipment and furnitures, which are not used for direct food or feed contact in private, public, industrial areas, including hospitals.«	2,863
Veterinary hygiene biocidal products	»Disinfectants applied directly to domestic animals, for areas in which animals are housed, kept or transported. Disinfections of milking equipment.«	97
Food and feed area disinfectants	»Disinfection of equipment, containers, consumption utentiles, surfaces or pipe work associated with production, transport, storage of food, feed and drinks for humans and animals.«	620
Wood preservatives	E.g. industrial wood preservatives.	474
Antifouling products	E.g. antifouling paints for vessels.	403
Others	E.g. in-can preservatives, slimicides, embalming fluids for humans and animals, film preservatives, preservatives for textiles, leather and rubber.	973
Total:		**5,530**

As can be seen from the table, biocides are used in a range of application. A short description of the application of biocides in the health care setting, in the food industry, in animal husbandry, and in consumer care products is giving below.

1.1 Application of biocides in the health care setting

In the 1860s it was demonstrated that implementation of simple hand washing among physicians delivering babies reduced the post delivery infection rate in women. It was subsequent shown that application of carboxylic acid spray (a biocidal agent) to the skin of patients and to surgical instruments lead to a dramatic decrease in the rate of post operative infections [20]. The role of microorganisms in the transmission of infectious diseases was increasingly realized and lead to the introduction of official procedures for sterilization of medical and surgical materials [21]. In the mid twentieth century several biocidal compounds were common use in clinical practice. Today, the use

of biocides has become an essential part of infectious control strategies in preventing and controlling healthcare-associated infections [3]. The worldwide increase in antimicrobial resistance in bacterial pathogens leading to increased mortality and morbidity in humans emphasizes the significance of infection control practices and the important role of biocides in the health care [5]. Biocides are widely used for antisepsis in hand disinfection to avoid transmission of pathogens via the hands of the personnel thereby preventing and controlling spread of disease among patients [3,5]. Biocides are used for chemical sterilization or disinfection of heat sensitive devices where sterilization by steam under high pressure is not possible [5]. Sterilization is particular important for devices used for penetration of the skin, such as surgical instruments, urinary catheters, and implantable devices were the risk of infection is greatest. In addition, biocides are used for chemical disinfection of "non-critical" devices, such as blood pressure cuffs and stethoscopes that only come into contact with intact skin and environmental surfaces, which includes floors, walls, tables, and bedrails [5]. Furthermore, medical devices, such as catheters and sutures, are available with biocidal compounds to reduce the risk of infections and biofilm formation [22].

1.2 Applications of biocides in the food production industry

The high-tech agriculture and the increasing demands for foods of animal origin have contributed to the development of intensive live-stock production. The increase in animal concentration has led to challenges within and demands for more effective disease control programs to withhold a satisfactory level of hygiene [23]. Disinfection is an essential part of preventing and controlling transmission of disease among animals. Biocides are widely used for cleaning and disinfection of areas associated with live-stock animals including farm buildings, beddings, equipments and transportation vehicles. In addition, biocides are used directly on animals e.g. in hoof baths to control infectious skin disease and for cleaning of udder and teats [4,5,23]. Large amount of biocides are also used in areas of food production and processing including disinfection of equipment, machinery, containers, floors, walls, and decontamination of carcasses to eradicate and prevent spread of unwanted microorganisms. Biocides are also used for preservative purposes to prolong shelf-life of products and to inhibit food contamination or spoilage [4,5].

1.3 Applications of biocides in consumer products

Biocides are used as preservatives in a range of products including personal care products; cosmetics, to prevent growth of microorganisms. Cosmetics include products that are intended for use on the human body to cleanse, perfume, protect, or to correct body odour [22]. Biocidal compounds are added to cosmetics to prevent contamination of the product by the consumer during use. The demand for preservatives in cosmetics and improvement of manufacturing practices became apparent in the 1960s and 1970s where up to 24% of unopened and up to 49% of used products were contaminated with microorganisms. The application of contaminated products including pathogens or opportunistic pathogens can result in infections at sites such as the eye or in wounds [22].

In addition, to the different areas of application mentioned above, biocides are used for a variety of other purposes contributing to human health and welfare, such as disinfection of waste water, swimming pools and as preservatives in paints and textiles [4]. Furthermore, with the public awareness of hygiene and transmission of bacteria a number of consumer products have become available with an "antimicrobial effect" and comprise chopping boards, clothing, linen, carpets, computer keypads and many more [24].

1.4 Chemical groups of biocides and mode of action

Biocides are generally broad-spectrum in activity and they are often applied in various settings. They are believed to have multiple and unspecific target sites within microorganisms constituting the cytoplasmic membrane, proteins, DNA, RNA and other cytosolic components (Figure 2).

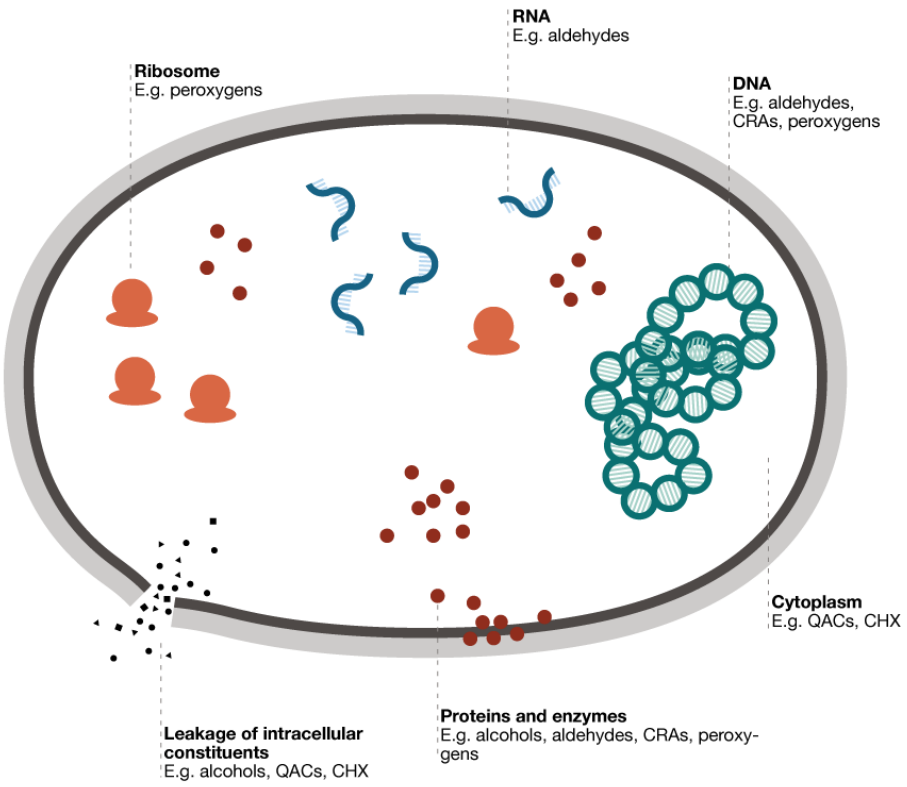

Figure 2: A simplified illustration of a bacterial cell and target sites of biocides. Abbreviations: QAC; quaternary ammonium compound, CHX; chlorhexidine dighonate, CRA; chlorine releasing agent.

In this section, an overview of commonly used biocides of different chemical groups and their general field of application is presented with focus on their use within the health care setting, food production and animal husbandry. Furthermore, the mode of action of each compound on bacteria is described. Biocides included in this PhD study are denoted in bold.

1.4.1 Alcohols

In the group of alcohols **ethanol (EtOH)**, isopropanol, and *n*-propanol are the most frequently used compounds [4]. The activity of alcohols is rapid and broad-spectrum; they are effective against bacteria, viruses, and fungi; however they are in-effective toward spores [1,25]. Alcohols are widely used for hand, skin, and hard surface disinfection in the health care setting [3].

Knowledge about the specific mode of actions of alcohols is limited however, the primary mode of action is considered to be denaturation and coagulation of proteins. This affects membrane structure and function resulting in release of intracellular constituents, interference with cell metabolism and cellular functions, and subsequent cell lysis [1,25].

1.4.2 Aldehydes

The major aldehydes used as biocides are **formaldehyde (FH)** (monoaldehyde) and glutaraldehyde (dialdehyde). They are very active compounds and have a broad spectrum of activity including bacteria, bacterial spores, fungi, and viruses [1]. At hospitals they are used for disinfection of equipment such as endoscopes. Formaldehyde is also used in veterinary hygiene for disinfection of areas where animals are housed and kept e.g. stables and for disinfection of animal transportation vehicles [4].

Formaldehyde can interact with DNA, RNA, and proteins by cross-linking of free amino groups. Glutaraldehyde also causes cross-linking of amino groups in proteins, and inhibits transport processes into the cell [1].

1.4.3 Chlorine and iodine compounds

1.4.3.1 Chlorine releasing agents (CRAs)

The most important compounds within the group of CRAs constitute **sodium hypochlorite (SH)**, chlorine dioxide, and to a lesser extent chloramine-T. These compounds are highly oxidative and have a broad-spectrum of activity including bacteria, viruses and spores. Their primary use is in hard surface disinfection [1]. Of these compounds, SH is one of the most widely used chemical disinfectants and is extensively applied in the food industry as well as for disinfection of surfaces and instruments at hospitals [4,26].

The specific antimicrobial action of CRAs are not well understood however, due to their strong oxidizing potential these compounds are believed to react with proteins and enzymes (thiol groups are especially regarded as targets), and DNA causing disruption of the cytoplasmic membrane and interference with cell metabolisms [2,26].

1.4.3.2 Chlorhexidine

Chlorhexidine is a divalent cationic compound belonging to the biguanides. Because of its low solubility in water this compound is often produced as an inorganic salt e.g. **chlorhexidine digluconate (CHX)**, to increase the solubility of the compound. Chlorhexidine is bactericidal, fungicidal, and active against some viruses, but not effective against spores [27]. Chlorhexidine constitutes one of the most widely used biocides for skin and mucosa disinfection in the health care sector [1,4]. It is also used in agriculture for direct application on domestic animals [4]. Chlorhexidine

and alcohols are often used together for skin disinfection due to the rapid effect of alcohols and the residual effect of chlorhexidine [4].

The mode of action of chlorhexidine on bacteria has been well studied and a sequence of event for the lethal effect has been described. This include a rapid attraction of the cationic compound to the negatively charged bacterial cell, followed by uptake of the compound and subsequently attack on the cytoplasmic membrane resulting in inhibition of membrane-bound enzymes and leakage of intracellular components. At high concentrations, chlorhexidine also causes precipitation of the cytoplasm [27].

1.4.3.3 Iodine and iodophors

Iodine in water solutions (I_2) have been widely used for antiseptic purposes for many years however, they have been replaced by the iodophors (iodine-releasing agents), because of the tissue irritant and excessive staining adverse effect of iodine. The most widely used iodophors are povidone-iodine and poloxamer-iodine that are used for antisepsis and disinfection. Iodine is bactericidal, fungicidal, virucidal, and sporicidal [1].

The effect of iodine is rapid and is believed to interact with proteins and enzymes (in particular the thiol groups), nucleotides and fatty acids however, the exact mechanism of action is unknown [1].

1.4.4 Metals

Copper sulphate ($CuSO_4$) is the most widely used copper compound and is used for disinfection of hoofs of domestic animals. The most important silver compounds are silver nitrate ($AgNO_3$) and silver sulfadiazine (AgSD) that are used for prevention of infections in burns [14] (T2, T3). Cu^{2+} is believed to interact with the thiol group of enzymes and proteins and cause a general coagulation of cytoplasmic constituents [28]. Ag^{+} can also interact with thiol groups in enzymes and proteins, and react with nucleic acids. The cytoplamsmic membrane, which includes many important enzymes, is thought to be an important target site for Ag^{+} [1].

1.4.5 Peroxygen compounds

The most important compounds belonging to the group of peroxygens are **hydrogen peroxide (HP)** and **peracetic acid (PAA)**.

HP is a strong oxidizing compound with a broad spectrum of activity against microorganisms including bacteria, viruses, yeast and bacterial spores. It is widely used for disinfection, antisepsis, and sterilization purposes [1]. In Denmark, HP is the most consumed biocide for disinfection of food handling areas and equipment in the food-processing industry [4]. HP is also used for veterinary hygiene purposes including disinfection of areas in which animals are housed (including entire stables), kept or transported to reduce the load of microorganisms and prevent accumulation and spread of disease [4].

HP attacks cells by formation of free hydroxyl radicals (·OH) that are highly reactive molecules due to their strong oxidizing properties [1]. They can react with and destroy important cellular constituents such as membrane lipids, DNA, ribosomes, enzymes, and proteins [1,2]. It has been proposed that sulphydryl groups and double bonds are particular targets [29].

Peracetic acid is used for disinfection and sterilizing purposes and is regarded as a more potent antimicrobial agent than HP due to its lethal effect on microorganisms at lower concentrations [29]. It is widely used at hospitals for surface disinfection and sterilization of medical equipment [1,4] and PAA is also one of the most applied compounds for disinfection of food handlings areas in Denmark [4].

Peracetic acid like HP also attack cells by formation of hydroxyl radicals and are therefore likely to cause similar reactions and damages to microorganism as HP [29].

1.4.6 Phenols and bis-phenols

Phenolic-type compounds are broad-spectrum antimicrobials having antibacterial, antifungal, and antiviral properties. They have long been used for antiseptic, disinfectant, and preservative purposes. The most important bis-phenol compounds are triclosan and hexachlorophene that are widely used for antiseptic soaps and hand-rinses [1]. Triclosan is also used in the industry and is incorporated in diverse product including toys, tooth paste, cosmetic and deodorants [30].

The mode of action of these compounds is believed to be the cytoplasmic membrane causing a rapid release of intracellular constituents [1]. However, a specific target has been identified for triclosan, the enoyl-ACP reductase in bacteria, encoded by the *fabI* gene, involved in fatty acid synthesis [30].

1.4.7 Quaternary ammonium compounds (QACs)

Some of the most important compounds within the group of QACs are **benzalkonium chloride (BC)** and cetrimide. Quaternary ammonium compounds work primarily as antimicrobials against bacteria but are also effective against enveloped viruses and fungus [1]. They are used for disinfection of areas where animals are housed, kept, and transported e.g. floors, walls, and transportation vehicles. They are also used for disinfection of food handling areas including floors, walls and inventory [4] (T3).

Quaternary ammonium compounds are membrane active compounds; their main target is the cytoplasmic membrane. The mode of action of these compounds are believed to follow a sequence of event; adsorption to and diffusion through the cell wall followed by interaction with and disruption of the cytoplasmic membrane causing release of cellular constituents and precipitation of cell content and death [31].

1.4.8 Bases and acids

1.4.8.1 Bases

Caustic soda (NaOH), caustic potash (KOH), soda (Na_2CO_3) and hydrated lime ($Ca(OH)_2$) have disinfecting properties against some bacteria and especially to some viruses. Caustic soda and caustic potash have been extensively used for cleaning of surfaces, and are specifically used for cleaning of stables [23,32].

1.4.8.2 Acids

Acids can be divided into mineral and organic acids. The most important mineral acids are hydrochloric acid (HCl) and sulphuric acid (H_2SO_4) that are extensively used in the food processing

industry for cleaning of surfaces and inventory such as pipes and milking machines [23]. Organic acids are primarily used as preservatives (e.g. lactic, acetic and citric acids). Other applications include; acetic acids can be used for treatment of wounds, and lactic acid for cleaning of udder and teats of domestic animals [4,23]. Acids can inhibit growth of bacteria, but only the strong acids (e.g. HCl) are bactericidal [23,30].

Acids are believed to cause pH-mediated coagulation of proteins [30].

1.4.9 Factors affecting biocide activity

Several intrinsic and extrinsic factors can affect the efficacy of biocides; intrinsic factors depend on the biocidal compound and its application, whereas extrinsic factors are defined by the environment during application. Regarding the intrinsic factors, the concentration of the biocidal agent and contact time between the compound and microorganism are essential parameters [5,33]. Generally, biocides are used at very high concentrations, way above the lethal dose of microorganisms. However, there are several factors that can affect the final concentration of the biocidal agent that come into contact with the microorganism. Commercial biocide products may have to be diluted before they are used for disinfection and incorrect dilution might occur. Also, insufficient cleaning before disinfection might lead to inactivation of the biocidal compound due to the presence of organic matter (e.g. blood, serum, pus, dirt or food debris), which can interact with the biocide. Furthermore, the presence of dust might hamper contact between cells and the biocidal agent [33,34]. Important extrinsic factors that can affect the activity of biocides are the temperature and pH [5,33,34]. Consequently, incorrect use of biocides can reduce the efficacy of biocides and cause bacterial exposure to non-lethal/sub-inhibitory concentrations of these compounds.

1.4.10 Biocides vs. antimicrobials used for treatment

Even though antimicrobials agents for human and animal therapy are able to inhibit or destroy bacteria, or other microorganisms, they are different from that of biocides. This is due to some very general differences in the application and mode of action between these two groups of compounds. An antimicrobial can be defined as "a drug that at low concentrations exerts an action against microbial pathogens and exhibits selective toxicity towards them" [35]. Thus, antimicrobial agents typically have one specific target site within the microbial cell interfering with a vital physiological process that generally can be grouped into the following actions; inhibition of peptidoglycan synthesis (e.g. β-lactams, glycopeptides), protein synthesis (e.g. tetracyclines, chloramphenicol, macrolides), and nucleic acid synthesis by interrupting nucleotide metabolism (e.g. sulphonamides), inhibition of RNA polymerase (rifamycins), or inhibition of topoisomerase IV / DNA gyrase (quinolones) [36]. In contrast, biocides are generally broad-spectrum and believed to have multiple targets within the cell, interfering with multiple cellular components e.g. the cellular membrane, proteins, enzymes, DNA, and RNA through physiochemical interactions or chemical reactions [1]. Furthermore, therapeutic antimicrobials are generally used for *in vivo* purposes and applied in concentrations close to the minimal inhibitory concentration (MIC) at the site of infection, where after the immune system of the host can eliminate the pathogen [37]. Biocides, however, are commonly used at concentrations that greatly exceed the MIC and the minimum bactericidal concentration (MBC) and they are often used at inanimate objects or surfaces. Moreover, the action

of biocides is generally achieved within minutes or hours, whereas antimicrobial treatments last for days and up to weeks [1].

The term *resistance* of bacteria within the two groups of compounds have often very different meanings. For antimicrobial agents standardized test methods exist for estimating the susceptibility of the bacterium to the antimicrobial agent, which is expressed by the MIC-value. In addition, well defined cut-off values for dividing bacteria into susceptible or resistance isolates are available: clinical breakpoints and epidemiological cut-off values [38]. Resistance according to the clinical breakpoints is associated with a high likelihood of therapeutic failure with the given antimicrobial agent, whereas resistance defined by the epidemiological cut-off value refers to a non-wild type isolate for a species due to the presence of an acquired or mutational resistance mechanism to the antimicrobial agent in question [38]. However, in the case of biocides no well defined susceptibility testing methods or cut-off values are available. The term biocide *resistance* in the literature is used indiscriminately and does primarily refer to reduced susceptibility/tolerance to the biocide in question and not to disinfection failure.

Throughout this thesis, the term *antimicrobial* will cover compounds used for treatment of bacterial infections in humans and/or animals, whereas the term *biocide* refers to compounds used for disinfection, antisepsis and preservative purposes. Furthermore, *resistance* and *resistance mechanisms* used in the context of biocides refer to bacteria with reduced susceptibility to the biocide and cellular mechanisms leading to reduced susceptibility.

2 Concerns about biocide usage and resistance development

There is no doubt that the use of biocides plays a crucial role in providing society with a number of benefits and contributes to human health and welfare. Biocides have become invaluable compounds and it is of upmost importance that they remain effective in the control of unwanted microorganisms in various applications. However, the widespread use of biocides has led to concerns on the emergence of bacteria with reduced susceptibility to biocides and their potential role in development of antimicrobial resistance in bacteria [5]. Within recent years, several opinions from Scientific Committees and the European Food Safety Authority (EFSA) have been composed to access the risk of resistance development to biocides and cross-resistance to therapeutic antimicrobials [5,35,39,40]. The Scientific Committees provide the European Commission with scientific advice for preparation of policies and proposals that are related to consumer safety, public health and the environment. Furthermore, they draw the attention of the Commission to new and emerging problems that pose or might pose a threat. The European Commission also relies on the work of EFSA that constitute the risk assessment body in food safety [39]. In 2009, SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks) published an opinion on *Assesment of the Antibiotic Resistance Effect of Biocides* with the purpose amongst other, to review current published data on certain active substances to estimate if they are likely to contribute to the development of antibiotic resistant bacteria [5]. The Opinion concluded that there is convincing evidence for the existence of common mechanisms conferring reduced susceptibility to biocides and antimicrobials and that co- and cross-resistance to biocides and therapeutic antimicrobials exist [5].

However, knowledge on the emergence of natural isolates with reduced susceptibility to currently used biocides and the occurrence of co- and cross-resistance is needed. Furthermore, knowledge on the possible contribution of sub-inhibitory or residual concentrations of biocides on antimicrobial resistance in bacteria by promoting the horizontal transfer of resistance determinants or by inducing the mutation rate in bacteria is very limited.

The serious concern for development of resistance to biocides and/or antimicrobial agents due to biocide usage has contributed to a regulation (Regulation (EU) No 528/2012) to the current Directive (98/8/EC) that was adopted the 22th of May 2012 and will replace the current directive as of 1st of September 2013 [14]. For authorisation of a new biocidal product in the EU, data proving that the product in question is effective and does not give rise to resistance development to biocides or antimicrobial agents, have to be submitted [14]. This has led to the preparation of "Revision of the joint AFC/BIOHAZ guidance document on the submission of data for the valuation of the safety and efficacy of substances for the removal of microbial surface contamination of foods of animal origin intended for human consumption" from EFSA [41]. The objective of the document is to provide guidance to applicants for submission of necessary data for evaluating the safety for consumers and the environment, including the effect of residual concentrations of biocides, in addition to the efficacy of substances for removal/reduction of microbial surface contamination of foods of animal origin [41]. This includes the necessary information to evaluate the potential emergence of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials [41].

2.1 Mechanisms of reduced susceptibility to biocides

Biocides have multiple target sites against the microbial cell. Therefore, the emergence of reduced susceptibility to biocides caused by target site modification or by-pass of a metabolic process is rare [5], which is in contrast to common resistance mechanisms to therapeutic antimicrobials [36]. Consequently, the general mechanism by which bacteria show reduced susceptibility to biocides is by decreasing the intracellular concentration of the biocidal compound to a level that is not harmful to the cell [5]. Several mechanisms of reduced susceptibility to biocides have been described in bacteria and can either be intrinsic or acquired. Intrinsic resistance refers to a natural property of the cell, whereas acquired resistance is the result of acquisition of genetic elements (e.g. plasmid, transposon) or mutations [1]. In Figure 3 the different mechanisms that can lead to reduced susceptibility of bacteria to biocides is illustrated.

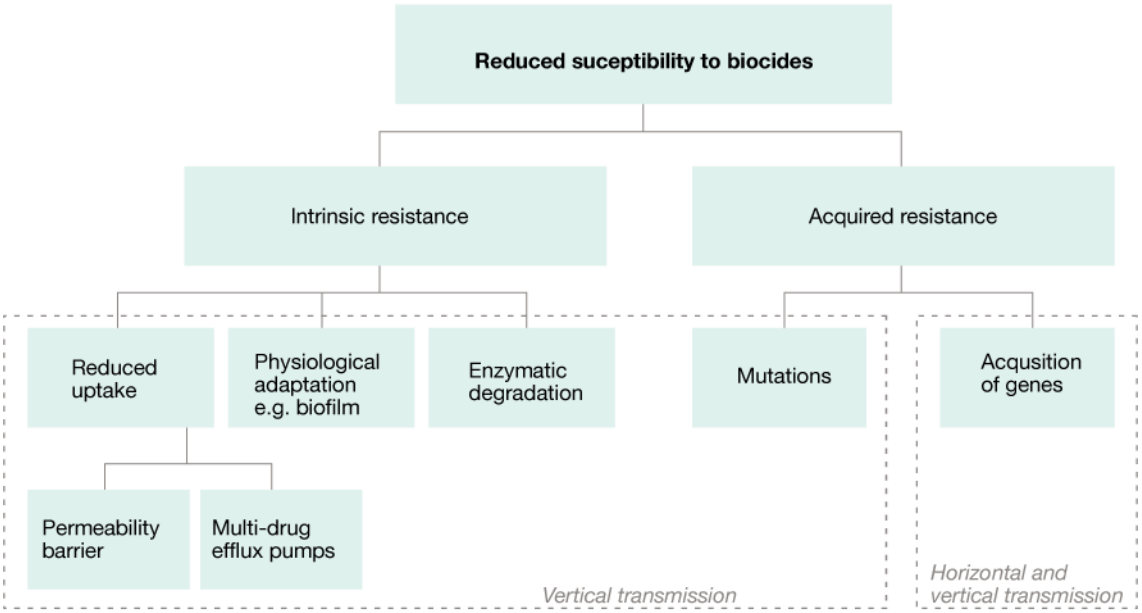

Figure 3: Illustration of intrinsic and acquired mechanisms for reduced susceptibility to biocides in bacteria.

2.1.1 Intrinsic resistance

2.1.1.1 Reduced uptake

2.1.1.1.1 Permeability barrier

The most described intrinsic mechanism of reduced susceptibility to biocides is the permeability barrier, comprised by the cell envelope, which can limit the adsorption and penetration of the biocide thereby reducing its efficacy. The susceptibility to biocides varies greatly between different microorganisms and it is generally believed that the chemical structure and composition of the outer cellular layer is an important factor (Figure 4). Mycobacteria are the least sensitive of the vegetative bacteria, which are probably due to their lipid rich and waxy cell wall that can act as a barrier and limit the uptake of many biocidal agents [34]. Gram-negative bacteria are generally less susceptible to biocides than Gram-positive bacteria. The outer membrane of Gram-negative bacteria is believed to be the major reason for this difference, since it can act as a permeability barrier and also reduce the uptake of biocides [34].

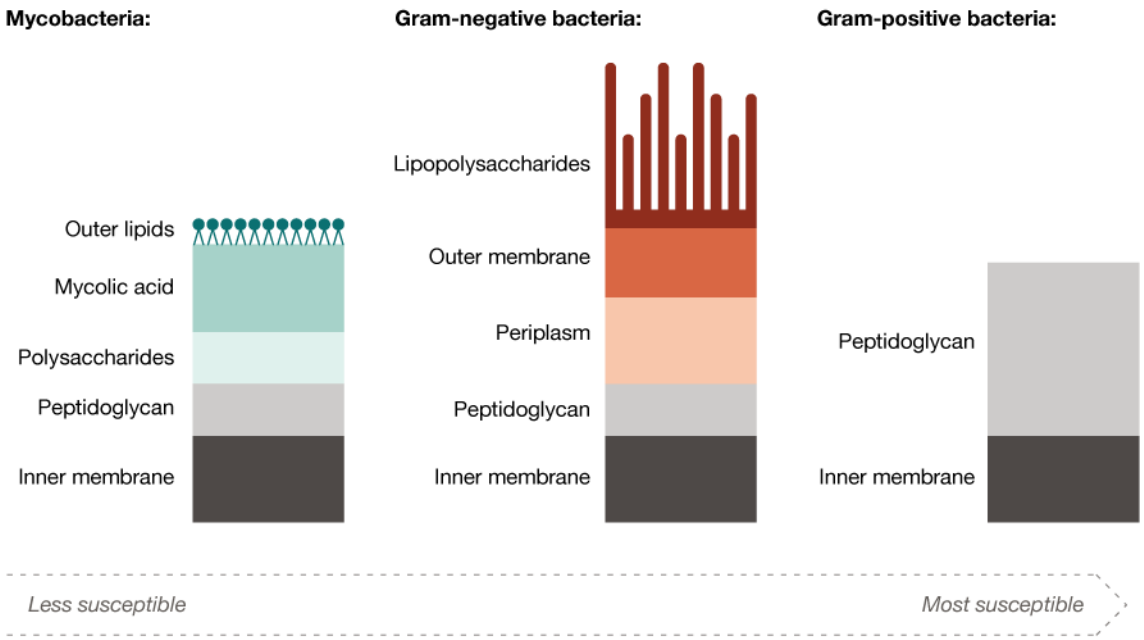

Figure 4: A simplified illustration of the outer cellular layer of mycobacteria, Gram-negative bacteria, and Gram-positive bacteria.

2.1.1.1.2 Multi-drug efflux pumps

Multidrug efflux pump systems are widespread among bacteria and five main classes of efflux pump systems capable of accommodating antimicrobials have been described; the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family (now part of the drug/metabolite transporter (DMT) superfamily), and the multidrug and toxic compound extrusion (MATE) family [42]. Several chromosomally encoded multidrug efflux pump systems, with specificity for biocides, have been described in Gram-negative bacteria. Examples of these are shown in Table 2. These systems generally accommodate QACs and/or triclosan, however the SdeXY efflux pump of *Serratia marcescens* has also been shown to transport the biguanide CHX [42]. In comparison to the Gram-negative bacteria, chromosomal efflux determinants capable of accommodating biocides are rare in Gram-positive bacteria. However, a few chromosomal encoded efflux pumps that can accommodate certain biocides have been described in *S. aureus* (Table 2) [42]; the NorA [43], NorB [44], MdeA [45] and MepA [46] efflux pumps can all accommodate several QACs and dyes (e.g. ethidium bromide (EtBr)), whereas MepA in addition can transport CHX [42]. A multidrug efflux pump homolog to the NorA transporter protein of *S. aureus* has been identified in *Enterococcus faecalis* and has been found to accommodate the dye EtBr [47]. The importance of these efflux pumps in relation to reduced susceptibility to biocides in bacteria and their clinical relevance is however uncertain, since they only give rise to a modest level of reduced susceptibility against the described biocides [48].

Table 2. Examples of chromosomally encoded efflux pumps with certain biocidal compounds as substrates (based on review 42). Abbreviations: QAC; quaternary ammonium compound, TRI; triclosan, CHX; chlorhexidine, DA; diamidine.

Efflux pump	Substrate	Organism
Gram-positive bacteria		
NorA	QAC	*S. aureus*
NorB	QAC	*S. aureus*
MdeA	QAC	*S. aureus*
MepA	QAC, CHX, DA	*S. aureus*
EmeA	QAC	*E. faecalis*
Gram-negative bacteria		
EmrE	QAC	*E. coli*
MdfA	QAC	*E. coli*
NorM	QAC	*N. gonorrhoeae, N. meningitidis*
PmpM	QAC	*Pseudomonas aeruginosa*
SugE	QAC	*E. coli*
AcrAB-TolC	QAC, TRI	*E. coli, Salmonella enterica serovar Thyphimurium*
CmeABC/CmeDEF	QAC, TRI	*C. jejuni*
Mex efflux pump systems	TRI	*P. aeruginosa*
SdeXY	QAC, CHX, TRI	*S. maltophilia*

2.1.1.2 Physiological adaptation

In addition to species differences, the physiological state of bacteria can also influence their sensitivity to biocides. Biofilms for instance comprise a serious problem both within the medical field and in the industry as bacterial cells residing within biofilms have shown to be less susceptible to both antibiotics and biocides [49]. Several mechanisms can account for this reduction in susceptibility: penetration of the biocidal compound into the biofilm might be limited, chemical interaction between constituents of the biocidal molecules and the biofilm matrix (e.g. proteins, nucleic acids or carbohydrates) that reduce the efficacy of the biocide, and the formation of micro-environments that are nutrient- and oxygen-limited. Physiological adaptation to these micro-environments can render bacteria less susceptible e.g. by modification of the cell membrane reducing accessibility of the disinfectant to the interior of the cell [49]. It has been shown that bacteria growing in biofilms can exhibit a 150 to 3000 times greater tolerance to hypochlorite compared to its corresponding planktonic cells [50].

S. aureus have the ability to produce a surrounding layer of slime giving rise to mucoid strains and it has been shown that this layer to some extent can protect the cell from the action of different

chlorine and phenol compounds [51]. The protection of this slime layer has been suggested to act as a physical barrier and also to reduce the efficacy of these compounds due to interaction with the components of the slime layer [51]. Also, it has been shown for *Escherichia coli* that starvation of this bacterium can give rise to increased tolerance to chlorine [52,53].

2.1.1.3 Enzymatic transformation

Enzymatic transformation of biocidal compounds into non-toxic forms has been described in bacteria, particularly for heavy metals, where the metal ion is reduced to a less toxic oxidation state [54]. In addition, aerobic microorganisms have enzymatic systems to relief the bacteria from the toxic side-effects (e.g. production of hydrogen peroxide and superoxide) during oxidative respiration; the enzyme superoxide dismutase can eliminate superoxide in the cell, whereas catalase handles the disposal of hydrogen peroxide [55].

2.1.2 Acquired resistance

Resistance to antimicrobial agents is a common and increasing problem among many important human pathogens. The main mechanisms by which bacteria acquire resistance to therapeutic antimicrobials involve; i) target alteration, ii) impermeability, iii) enzymatic modification or destruction of the compound, and iv) active efflux [36]. Since antimicrobial agents generally have a specific target site within the cell, acquisition of one of these mechanisms can render the bacteria resistant to the antimicrobial agents or to multiple compounds within the same class [36]. Acquired reduced susceptibility to biocides is much less common and one likely reason is that biocides appear to have multiple target sites within the cell. However, reduced susceptibility to biocides does occur and in the next section, known acquired resistance mechanisms are introduced.

2.1.2.1 Acquisition of resistance genes

2.1.2.1.1 Efflux pump systems

Efflux pumps are probably the most described resistance mechanism in terms of acquired resistance to biocides. The QAC efflux pump systems were named after one of their main substrates; the QACs. A range of various *qac* genes have so far been described and constitute the *qac*A [56], *qac*B [56], *qac*C [56,57], *qac*D [57], *qac*E [58], *qac*EΔ1 [58] (attenuated version of *qac*E), *qac*F [59], *qac*G [60], *qac*H [61], *qac*I [62], *qac*J [63], and *qac*Z gene [64]. The different genes and examples of substrate specificities are shown in Table 3.

Table 3.Overview of QAC resistance genes and the mobile genetic elements they typically are associated with or were first identified on. Abbreviations: QAC; quaternary ammonium compound, TRI; triclosan, CHX; chlorhexidine, DA; diamidine, EtBr; ethidium bromide (intercalating dye).

QAC resistance determinants	Substrates of pumps	Examples of mobile elements carrying the different *qac* genes	First identified in organism	References
qacA	QAC, CHX, EtBr, DA	pSK1 plasmid family, β-lactamase/heavy-metal resistance plasmids	*S. aureus*	56
qacB	QAC, EtBr	pSK23 (large heavy-metal resistance plasmids)	*S. aureus*	56
smr	QAC, EtBr (low-level)	Small plasmid and large conjugative multi-resistance plasmids (e.g. pSK41, pJE1)	*S. aureus*	56, 57
qacF	QAC	Class 1 integron	*E. aerogenes*	59
qacE	QAC, EtBr	Class 1 integron	*K. pneumoniae*	58
qacΔE1	QAC (low-level), EtBr (low-level)	Class 1 integron	*K. pneumoniae*	58
qacG	QAC, EtBr (low-level)	pST94	*Staphylococcus spp.*	60
qacH	QAC, EtBr (high-level)	p2H6	*S. saprophyticus*	61
qacJ	QAC	pNVH01	*Staphylococcus spp.*	63
qacI	QAC	pNLT-1	*E. coli*	62
qacZ	QAC	pTEF1	*E. faecalis*	64
qrg	QAC	Tn6087	*S. oralis*	79

The *qacA* and *qacB* genes share a high degree of homology [65] and are often referred to as the *qacA/B* gene. *qacC* and *qacD* encode the same polypeptide, however, their expression is under the control of different promoters and it has been suggested that *qacC* has evolved from *qacD* [57]. They are often referred to as the *qacC/D* or *smr* gene. The *qac* genes have been identified in various bacterial species including environmental and clinical isolates, however the individual *qac* genes are in general found to be associated with specific bacterial species. Among the Gram positive bacteria, staphylococci by far represent the species in which *qac* genes most frequently have been described and include staphylococci of human (e.g. [57,66–68]), animal (e.g. [63,69,70]) and food-related (e.g. [60,61,71]) origins. The *qacA/B* gene is often found in clinical strains of staphylococci, whereas *smr* is found to a lesser extent [72–74]. The *qacG*, *qacH*, and *qacJ* genes are rare in human staphylococci isolates [74,75], and is primarily associated with isolates of animal or food origin [60,61,63,70]. Besides the presence of *qac* genes in staphylococci the *qacA/B* gene and the newly identified *qacZ* gene have been identified in the Gram positive bacteria *E. faecalis* [64,76]. The *qacEΔ1* and *qacE* are associated with Gram-negative bacteria. Especially the *qacEΔ1* gene has been detected in a wide variety of Gram-negative bacteria, and is likely to be due to their association with the 3' conserved end of class I integrons that are widespread among Gram-negative bacteria [42,77,78].

The *qacF* and *qacI* genes have also been identified in Gram-negative bacteria, but to a much lesser extent [42]. Acquisition of one of these *qac* genes however, only results in a modest reduction in the susceptibility to the biocidal compounds. In Table 4, some examples are giving on changes in the susceptibility of bacterial isolates with and without biocide resistance genes.

Recently, a new efflux pump, *qrg*, was identified in *Streptococcus oralis* that conferred reduced susceptibility to the QAC compound cetyltrimethylammonium bromide (CTAB) [80] (Table 4).

Table 4. Examples of differences in minimal inhibitory concentrations (MIC) to different biocide compounds with and without biocide resistance genes. Abbreviations: BC; benzalkonium chloride (quaternary ammonium compound (QAC)), CTAB; cetyl trimethylammonium bromide (QAC), CTM; cetrimide (QAC), FH; formaldehyde, $CuSO_4$; copper sulphate.

Gene	Compound	MIC		Determined in organism	Ref.
		Negative of gene	Positive of gene		
qacA/B	BC	12 µg/ml	60 µg/ml	*E. coli*	79
smr	BC	0.5-1.0 µg/ml	2.5-3.0 µg/ml	*S. aureus*	69
smr	BC	2 µg/ml	8 µg/ml	*Staphylococcus spp.*	61
smr	BC	20 µg/ml	80 µg/ml	*E. coli*	58
qacE	BC	20 µg/ml	80 µg/ml	*E. coli*	58
qacEΔ1	BC	20 µg/ml	20 µg/ml	*E. coli*	58
qacF	CTAB	100 µg/ml	400 µg/ml	*E.coli*	59
qacG	BC	2 µg/ml	10 µg/ml	*Staphylococcus spp.*	61
qacH	BC	2.0-4.0 µg/ml	10 µg/ml	*Staphylococcus spp.*	61
qacI	CTAB	100 µg/ml	400 µg/ml	*E. coli*	62
qacJ	BC	1.0-1.5 µg/ml	3.5-4.5 µg/ml	*Staphylococcus spp.*	63
qacJ	CTAB	2.0-4.0 µg/ml	7.5-10.0 µg/ml	*Staphylococcus spp.*	63
qacZ	BC	4 µg/ml	16 µg/ml	*E. faecalis*	64
qrg	CTAB	4 µg/ml	8 µg/ml	*S. oralis*	80
tcrB	$CuSO_4$	4 mM	16/28 mM*	*E. faecium*	81
adhC	FH	0,009%	0,03 %	*E. coli*	82
***oqxAB* genes**	BC	2 µg/ml	16 µg/ml	*E.coli*	83
***oqxAB* genes**	CTM	2 µg/ml	8 µg/ml	*E.coli*	83

*Biphasic growth phenotype. Grew poorly in concentrations of 8, 12, 20 and 24, > 28 mM.

Many metals are essential for different enzymatic processes in the cell, however too high concentrations are toxic to the cell and bacteria have evolved different systems to keep the concentration at non-toxic levels. Plasmid-mediated copper-resistance systems have been identified

in *E. coli*, *Pseudomonas syringae* and *Xanthomonas campestris*. These systems comprise four structural genes and are essentially identical. In *E. coli* the genes comprise *pco*A, *pco*B, *pco*C, and *pco*D, whereas the determinants are denoted *cop*A, *cop*B, *cop*C, and *cop*D in Pseudomonas. In addition, the *tcr*B gene has been found to confer transferable copper resistance in *Enterococcus faecium* and *E. faecalis* and is likely to be involved in copper efflux (Table 4) [81,84]. Plasmid-mediated resistance to silver has been identified in a number of bacterial species including the Enterobacteriaceae, *Citrobacter* spp., and *Pseudomonas stutzeri*, however the mechanism of resistance is yet unknown [85]. Plasmids including resistance determinants to a wide range of other toxic heavy metal ions (e.g. Hg^{2+}, Ni^{2+}, Pb^{2+}) have also been described [86].

2.1.2.1.2 Additional target

Recently, a novel mechanism for reduced susceptibility to triclosan was discovered in clinical isolates of *S. aureus*. An additional copy of the *fabI* gene, likely to originate from *Staphylococcus haemolyticus* was identified giving rise to heterologous target duplication [87]. The authors further found, by database search, that the *S. haemolyticus* version of the *fabI* gene was present in different staphylococci, including *S. aureus* and *Staphylococcus epidermidis* in which the additional *fabI* gene was located on plasmids together with the *qacA* gene. This indicates that this novel mechanism for reduced susceptibility to triclosan is actively transferred among staphylococci [87].

2.1.2.1.3 Enzymatic degradation

Reduced susceptibility to formaldehyde has been identified amongst clinical isolates of Enterobacteriaceae and from contaminated disinfection solutions. The mechanism giving rise to reduced susceptibility has been identified as the transferable plasmid-born gene, *adhC*, encoding glutathione-dependent formaldehyde dehydrogenase that is capable of inactivating formaldehyde (Table 4) [82,88].

2.1.2.1.4 Acquisition of mutations

As previously mentioned, biocides are likely to have multiple unspecific target sites within the cell and mutation-mediated reduced susceptibility to biocides is rare. However, the enzyme enoyl-ACP reductase, encoded by the *fabI* gene, is a specific target site for triclosan [89]. Mutation-mediated alterations of the target site reducing the inhibitory action of triclosan and over-expression of *fabI* have been described as mechanisms giving rise to reduced susceptibility to triclosan [48,90,91].

2.1.2.2 Summary

Bacteria have acquired mechanisms by which they can become less susceptible to the action of different biocides. However, these mechanisms often give rise to a modest reduction in the susceptibility to these compounds and not to in-use concentrations.

In the next section, mechanisms of co- and cross-resistance to biocides and therapeutic antimicrobials are presented.

2.1.3 Mechanisms of specific and unspecific selection of antimicrobial resistance by biocides

Biocides can select for therapeutic antimicrobial resistance in bacteria through specific or unspecific mechanism that are illustrated in Figure 5. The different direct and indirect resistance mechanisms are described in the next section.

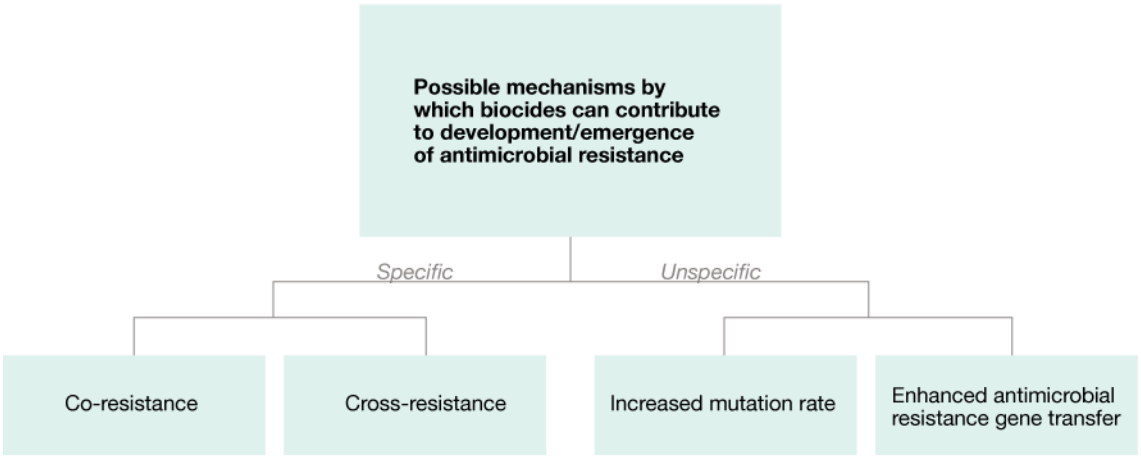

Figure 5: Possible specific and unspecific mechanisms by which biocides can contribute to antimicrobial resistance.

2.1.3.1 Biocide-antimicrobial co-resistance

Separate genes encoding reduced susceptibility to biocides and antimicrobial agents can be linked on a mobile genetic element. Selection for one resistance gene will also select for the other resistance gene(s) and multiple resistances may be transferred in a single event [35].

The various *qac* genes have been found to be associated with different mobile genetic elements including plasmids and integrons. *qacA/B* is predominantly located on the *S. aureus* pSK1 family of multi-resistance plasmids, encoding resistance to aminoglycosides and trimethoprim, and on β-lactamase/heavy metal resistance plasmids (pSK57) [92]. The *smr* genes are typically located on the small plasmid pSK89 and on large, conjugative multi-resistance plasmids, such as pSK41 and pJE1 that also encode resistance to several antimicrobial agents within the class of aminoglycosides and trimethoprim in the case of pJE1 [57,93]. The *smr* gene has also been found on a plasmid (pLW1043) isolated from a high-level vancomycin-resistant clinical isolate of *S. aureus* [68]. This plasmid included antimicrobial resistance determinants to vancomycin (located on transposon Tn*156*), β-lactams, gentamicin and trimethoprim and had homologous regions to the pSK1 and pSK41 family of multi-resistance plasmids [68].

Integrons are a group of mobile genetic elements that are widespread among Enterobacteriaceae and other Gram-negative bacteria. The most described type of integrons is the Class 1 integrons that comprise a 3'-conserved segment including the *qacEΔ1* and *sul1* (encoding sulphonamide resistance) genes. Integrons are able to incorporate single or multiple gene cassettes encoding

resistance to a wide range of antimicrobial agents and classes, including resistance to the β-lactams, aminoglycosides, trimethoprim, and chloramphenicol [94]. Furthermore, the majority of gene cassettes lack a promoter upstream of the coding region and therefore depend on expression of the integron that relies on the promoter in the 5'-conserved segment [94]. Expression of the antimicrobial resistance determinants and the *qacEΔ1* gene can therefore be co-expressed. The *qacF* gene has also been found to reside on a Class 1 integron in addition to the *qacEΔ1* [59]. *qacI* was identified on a Class 1 integron containing nine different genes encoding antimicrobial resistance to the class of β-lactams, aminoglycosides, phenicol, rifampin, and sulphonamides in addition to the QAC resistance gene. The integron was located on a transposon residing in a plasmid and might therefore be easy disseminated among enterobacterial strains [62].
The Class 1 integrons therefore constitute important mobile DNA elements in the dissemination of co-resistance to antimicrobial agents and QACs in a wide range of Gram-negative species.
The *qacZ* gene has been identified in *E. faecalis* on plasmid pTEF1 [64]. This plasmid also includes genes encoding aminoglycoside and macrolide resistance that was identified by running the sequence of pTEF1 (accession no. AE016833) through ResFinder [95] where it was found to include the *aac*(6')-*aph*(2'') gene and *erm*(B) genes with 100 % identity.

Co-localization of the *qacG*, *qacH*, and *qacJ* genes and antimicrobial agent resistance genes have so far not been described [60,61,63,70].

A new gene, *qrg*, giving rise to QAC resistance was identified on transposon Tn*6087*, a member of the Tn*916*-like family of transposons and found to co-reside with the *tet*(M) gene encoding tetracycline resistance [80]. The *tcr*B giving rise to copper resistance is genetically linked to macrolides (*erm*(B)) and glycopeptides (*van*A) resistance, since they co-reside on the plasmid isolated from *E. faecium* [81].

2.1.3.2 *Biocide-antimicrobial cross-resistance* - examples of some clinical relevant bacteria with multi-drug efflux pumps

Chromosomally encoded efflux pumps in bacteria are major contributors to antimicrobial resistance predominantly in Gram-negatives. Some types of efflux pumps give rise to intrinsic resistance to certain antimicrobial agents by expression a basal level of efflux, whereas overexpression of some efflux proteins can give rise to acquired resistance in otherwise susceptible strains [96]. Constitutive expression of efflux proteins can be acquired by mutations in the local repressor gene, in a global regulatory gene or in the promoter region. Overexpression of these efflux pumps typically has several un-structurally related molecules as substrate including antimicrobial agents and certain biocides [96]. Over expression of NorA render *S. aureus* less susceptible to a number of antimicrobial agents including chloramphenicol and the fluoroquinolones, as well as biocides within the group of QACs and dyes. The only clinical relevant antimicrobials that are substrate for NorA is the fluoroquinolones and several clinical isolates of *S. aureus* over expressing NorA have been found to be fluoroquinolone resistant [42,96]. Other efflux pumps of *S. aureus* capable of accommodating certain biocides also have antimicrobials as substrates; fluoroquinolones (NorB), lincosamides and

type A streptogramins (MdeA), and glycylcyclines (MepA). However, the clinical relevance of these efflux systems is unknown [96]. Several efflux pumps in clinical relevant Gram-negative bacteria have been described. *E. coli, Pseudomonas aeruginose* and *Salmonella enteric* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enteric* serovar Enteritidis (*S.* Enteritidis) are opportunistic pathogens. The AcrAB-TolC efflux pump system of *E. coli* and *S.* Typhimurium are similar and the substrate profile of these pumps includes several antimicrobial agents (e.g. chloramphenicol, fluoroquinolones, some β-betalactams, tetracycline, rifampicin) in addition to certain biocides (acriflavine, ethidium bromide, cetrimide, and triclosan). Overexpression of these efflux pump systems have been found in clinical isolates of *E. coli* and *S.* Thyphimurium [96]. Reduced susceptibility to the antimicrobial agent ciprofloxacin can be of clinical relevance in treatment of infections with these two species. The Mex efflux systems of *P. aeruginosa* are able to accommodate several classes of antimicrobial agents including chloramphenicol, fluoroquinolones and tetracycline, as well as certain biocides (acriflavine, EtBr, bromide and triclosan) [42,96]. Depending on the Mex efflux pump systems, clinically relevant substrates of the efflux pumps include ciprofloxacin, gentamicin, ceftazidime among other. Some of these pumps can be induced by some of their substrates [96].

In vitro studies have demonstrated the emergence of less susceptible *S.* Thyphimurium and *E. coli* isolates to antimicrobial agents after exposure to sub-inhibitory concentrations of different biocides and in some cases this was shown to be associated with over expression of efflux pumps [97–99]. Exposure of clinically *S. aureus* isolates to non-lethal concentrations of several biocides and dyes has been demonstrated to produce mutants overexpressing multi-drug efflux pumps, such as NorA, MepA and MdeA that have several antimicrobial agents as substrate [100]. It was suggested that exposure to substrates of the multi-drug efflux pumps including clinically relevant antimicrobial agents as well as disinfectants used at hospitals could result in the emergence of *S. aureus* strains adapted to these compounds [100].

Isolation of a plasmid-encoded multidrug efflux pump, OqxAB, has also been described in *E. coli*. This pump confers resistance to chloramphenicol and EtBr, however the substrate specificity was further studied by Hansen et al. (2007) and it was shown that the OqxAB pump also gave rise to reduced susceptibility to QACs (BC and to some extent cetrimide) as well as to trimethoprim and antimicrobial agents within the class of the quinolones and fluoroquinolones [83], which might be of clinically relevance for treatment of *E. coli* infections.

2.1.3.3 Summary

The above mentioned examples show the evidence of co- and cross-resistance mechanisms to biocides and antimicrobials agents. This is a potential serious problem since the use of biocides in addition to therapeutic antimicrobials at hospitals might select for strains with reduced susceptibility to antimicrobial agents. Moreover, the use of antimicrobials and biocides in veterinary practices and animal husbandries may contribute to the selection and maintenance of antimicrobial resistance determinants.

2.1.4 The SOS response

Bacteria have evolved a number of stress response systems to adapt to and survive many forms of environmental stresses. One of these systems is the SOS response that is activated by formation of single-stranded DNA (ssDNA). The SOS response is governed by the activator RecA and the repressor LexA. RecA recognises and binds to ssDNA forming nucleoprotein complexes that stimulate the self-cleavage of the LexA repressor. This result in the de-repression of the SOS regulon that include genes encoding error-prone DNA polymerases, and proteins involved in DNA repair and recombination [101]. In addition to the classical inducers of the SOS response; UV-light and mitomycin C, a variety of additional factors have been found to trigger the SOS response including exposure to a number of therapeutic antimicrobials, pH-extremes, high pressure, oxidative stress, and nutrient starvation [102].

Genes that are repressed by LexA and thereby activated upon SOS induction have been identified in *E. coli* [103,104], *Bacillus subtilis* [105], *P. aeruginosa* [106], *S. aureus* [107], and *Listeria monocytogenes* [108]. In all cases the SOS response is controlled by the expression of the positive and negative regulator, RecA and LexA respectively. Furthermore, the SOS responses also include at least one gene encoding an error-prone DNA polymerase. However, diversity among the SOS response network in the different bacterial species exists. The SOS response systems of *E. coli* [103,104] and *B. subtilis* [105] constitute 31 and 33 genes respectively that are repressed by LexA however, only eight of these are homologues to each other. Activation of the SOS response in *L. monocytogenes* has been found to up-regulate 29 genes [108]. This is in contrast to the number of genes repressed by LexA in *P. aeruginosa* [106] and *S. aureus* [107] that only constitute 15 and 16 genes, respectively. Therefore, regulation of the SOS response seems to be conserved between different bacterial species, however the genes and the number of genes constituting the SOS response differ.

2.1.4.1 Stress-induced mutagenesis

Activation of the SOS response includes activation of one or several error-prone DNA polymerases that can replicate pass DNA lesions and thus replicate damaged DNA opposed to the "normal" replicative polymerase [109]. The error-prone property of the SOS-induced polymerases acts on both damaged and undamaged DNA and can introduce mutations into the chromosome and thereby increase the spontaneous mutation rate in cells [109]. Acquired antimicrobial resistance in bacteria due to mutations in chromosomal genes have been described for several classes of antimicrobial agents, including the quinolones, rifamycins, and aminoglycosides [36]. The SOS response might therefore play an important role in the development of resistance to important antimicrobials used for human and animal treatment. Also, any compound capable of activating the SOS response might contribute to the evolution of bacterial resistance in human and animal pathogens. For instance in *S. aureus*, induction of the SOS response by UV-light was shown to increase the mutation rate in the bacterium, giving rise to increased resistance to rifampicin and streptomycin [107].

In recent years it has been demonstrated that sub-inhibitory concentrations of certain antimicrobial agents for therapy have the potential to trigger the SOS response and/or induce mutagenesis in bacteria. In *S. aureus*, exposure to ciprofloxacin and trimethoprim have been shown to induce the SOS response and a SOS-dependent increase in ciprofloxacin resistance has been demonstrated

[107,110]. In *E. coli*, exposure to several different antimicrobial agents (e.g. ciprofloxacin, rifampicin, ampicillin) at sub-inhibitory concentrations induced the mutation rate in a SOS-dependent matter and thereby resistance to these compounds [7,8]. Moreover, the SOS response and/or mutagenesis in *S.* Thyphimurium, *Mycobacterium fortuitum*, *Streptococcus pneumonia* and *Vibrio cholera* have been induced in response to exposure to sub-inhibitory concentration of certain antimicrobial agents [9,10,111,112], and the SOS response was found to be an important player in *S.* Thyphimurium [9] and *V. cholera* [10].

Activation of the SOS response can therefore result in an increased mutagenesis that in turn can confer increased antimicrobial resistance in bacteria. Sub-inhibitory concentration of antimicrobial agents used for treatment can induce this response. The SOS response in bacteria might therefore play an important role in development of resistance during treatment in a selective manner or increase resistance to antimicrobials in a non-selective manner. Inhibition of the SOS response and thereby inhibition of mutations has been suggested to be a novel therapeutic strategy to fight evolution of antimicrobial resistance to drugs [7]. However, bacterial stress caused by exposure to sub-inhibitory concentrations of biocides has also been suggested to induce the SOS response and induce antimicrobial resistance in bacteria [108]. So far, knowledge about the potential effect of sub-inhibitory concentrations of commonly used biocides on the SOS response and mutagenesis in different bacterial species is very limited. A few studies have investigated the transcriptional response to a couple of biocides in a few bacterial species.

The transcriptional response to hydrogen peroxide has been investigated in *S. aureus* [113], *E. coli* [114], *P. aeruginosa* [115], and *L. Monocytogenes* [108]. Increased expression of *rec*A indicative of SOS response induction was observed in all four bacterial species. The transcriptome response to peracetic acid has also been investigated in *S. aureus* and *P. aeruginosa* and in both bacteria, the expression of genes involved in DNA damage was induced [113,116]. Also, Ceragioli et al. (2010) investigated the genome-wide transcriptional and mutational response of *Bacillus cereus* to various biocides used in the food industry due to the frequent isolation of this bacterium in contaminated raw and processed foods. They found HP and PAA acid to induce the expression of SOS-related genes and to induce the mutation rate [117].

The bacterial response to sub-inhibitory concentrations of biocides is therefore an important area of investigation to elucidate the potential of biocides to promote antimicrobial resistance through an increase in the mutation rate.

2.1.4.2 Other stress-induced factors contributing to antimicrobial resistance

Beside its role in stress-induced mutagenesis, the SOS response has also been shown to contribute to development of antimicrobial resistance by promoting transfer of resistance genes. Transfer of the integrating conjugative element SXT, encoding resistance to chloramphenicol, sulphamethoxazole, trimethoprim, and streptomycin, has been shown to be stimulated by induction of the SOS response in *V. cholerae* and *E. coli* and ciprofloxacin was found to promote this transfer [6]. Furthermore, activation of the SOS response in *E. coli* by β-lactams has been found to compromise the lethal effect of the agents due to a SOS-induced temporarily halt in cell division protecting it from the lethal effect due to that β-lactams only act on growing cells [118]. In *E. coli*

expression of the *qnrB* gene, a plasmid-born quinolone-resistance gene, was detected and found to be induced by ciprofloxacin [119].

2.1.4.3 Summary

The SOS response can be an important player when it comes to evolution of antimicrobial resistance in bacteria and different classes of antimicrobials can induce this response. However, bacteria are constantly exposed to stressful environments and exposure to sub-inhibitory concentrations of biocides might not be an uncommon event in areas where they are widely used, such as in the food-industry and at hospitals. So far only limited knowledge is available on the possible effect of biocides on the SOS response and their possible effect on antimicrobial resistance development in bacteria.

2.1.5 Non SOS-induced transfer of resistance genes

Very few studies have investigated the contribution of biocides on dissemination of antimicrobial resistance determinants. So far, plasmid conjugation or transduction of resistance determinants in *S. aureus* has been found to be either not affected or reduced by sub-inhibitory concentrations of a number of biocides, including povidione-iodine, CHX, and different QACs [120,121]. However one QAC, cetrimide was found to cause a pronounced increase in the transducing efficiency [121]. Thus, there is a lack of results in this area in order to establish the risk of the contribution of biocides in transfer of antimicrobial resistance genes.

CHAPTER II

3 Summary of own results from the three manuscripts included and discussion

Biocides have become invaluable compounds in the industrialized world in many aspects providing society with a number of benefits. However, the large and increasing amount of biocide usage has led to concerns on development and emergence of bacteria with reduced susceptibility to biocides and the possible contribution of biocide usage on antimicrobial resistance development in bacteria.

The overall objective of this PhD study is the development of bacterial resistance to biocides and antimicrobial agents as a consequence of biocide usage and has resulted in three manuscripts (Manuscript I-III). In Manuscript III the susceptibility of porcine *S. aureus* isolates to commonly used biocides in pig farming and the presence of co-resistance to antimicrobial agents are examined. In Manuscript II and I the possible role of sub-inhibitory concentration of biocides on antimicrobial resistance development by increasing the mutation rate in *S. aureus* (Manuscript II) or by inducing the horizontal transfer of the *tet(M)* gene (Manuscript I) are investigate.

In this chapter, results obtained in Manuscript I-III are summarized and discussed in respect to the hypotheses presented in the introduction. In the first part results from Manuscript III are presented and discussed. This is followed by a presentation of results obtained from Manuscript II and Manuscript I, which will be discussed together.

3.1 The susceptibility of porcine *S. aureus* isolates belonging to CC398 and CC30 to biocides used for disinfection in pig farming

Knowledge about the occurrence of reduced susceptibility to active biocidal substances in natural bacterial isolates is limited. In the study of manuscript III the susceptibility of *S. aureus* pig isolates to active biocidal substances present in commonly used biocides for disinfection in pig farming was investigated. The selection of biocides was based on a report by the National Food Institute, Technical University of Denmark that constituted a characterization of Danish finisher herds [12]. From this report it was obvious that many different substances were used for disinfection of finisher herd stables and that the use of active biocidal substances belonging to the chemical groups of chlorine compounds, oxidizing compounds, QACs, aldehydes and strong alkaline compounds were frequent [12].

No standardized methods exist for susceptibility testing of microorganisms to biocides and it was therefore decided to use pre-existing methods for susceptibility testing of bacteria to antimicrobial agents comprising the CLSI Approved standard for MIC determinations [122] and the NCCLS approved Guideline for determination of MBCs [123]. In both cases the microdilution method was used with 2-fold increasing concentrations. MICs and MBCs were measured to explore if differences in susceptibility were present at the inhibitory or the bactericidal level.

S. aureus isolates belonging to CC398 and CC30 are the most and second most frequently identified methicillin sensitive *S. aureus* (MSSA) isolates among Danish pigs. Pigs are increasingly

recognized as a potential reservoir of community-acquired MRSA CC398 that has been found to cause infections in human [11]. Recently, *S. aureus* CC30 was for the first time described as MRSA in Danish pigs and the first cases of human nasal carriage of porcine associated MRSA CC30 has been detected [124]. MRSA and MSSA pig isolates belonging to CC398 and CC30 was therefore included in the study to examine their susceptibility to the different active biocidal agents used in pig farming. Isolates included in the study originated from diagnostic as well as colonization samples from pigs from several farms and slaughter houses.

3.1.1 No widespread selection for reduced susceptibility in porcine *S. aureus* isolates to disinfectants commonly used in pig farming

The obtained MIC- and MBC-values each gave rise to continuous distributions that were within the MICs and MBCs of the control strain. No sub-populations with reduced susceptibility to the tested active biocidal substances, based on increases in the MIC- and MBC-values, were therefore detected. The inhibitory and bactericidal concentrations were all well below working concentrations. Consequently, if proper disinfectant procedures are followed eradication of pig *S. aureus* isolates belonging to CC398 and CC30 do not seem to be an issue. Since isolates were collected from pigs originating from various farms and the biocide selection was based on a general overview of disinfectant compounds used in finisher herds, this study cannot be used for dose/response information. However, it would be interesting to compare isolates collected from farms using different biocidal products or different levels of consumption of the same compounds.

Even though the susceptibility testing of *S. aureus* pig isolates did not suggest that reduced susceptibility was present, the results do not give information about the possibility of a time-dependent development of reduced susceptibility to the compounds. This would require bacterial isolates collected before the introduction of the different biocides. In a study by Skovgaard et al. (2013) they investigated the triclosan susceptibility of Danish human clinical *Staphylococcus epidermidis* blood isolates from before (old) and after the introduction (current) of triclosan in Denmark [91]. They found that the collection of current isolates included significantly more triclosan (MIC≥0.25 µg/ml) tolerant isolates (12.5%) compared to the collection of old isolates (0%). The authors suggested that the wild-type *S. epidermidis* population structure had changed due to adaptation to the widespread use of triclosan resulting in a triclosan tolerant subpopulation present in Denmark [91].

The susceptibility testing of *S. aureus* isolates to biocides did have some limitations. In terms of disinfection procedure the actual rate of killing may have been more correct and important assay to use instead of determining the concentration at which a 99.9% killing of the final inoculums is reached (MBC-value). However, this method is too laborious to handle as many isolates that was included in the study. Furthermore, alkaline calcium compounds/lime was the most widely used compounds for disinfection of pig finisher herds. However, lime is not suitable for susceptibility testing by the methods employed in this study, and the strong alkaline calcium compound calcium hydroxide has very low water solubility. Caustic soda however is widely used for cleaning purposes in stables and has disinfecting properties [32] and was therefore included instead of alkaline calcium compounds or lime.

3.1.1.1 Detection of the QAC resistance gene, qacG, in MRSA CC30 isolates

Efflux-mediated resistance to QACs have been described in staphylococci of human [57,66–68], animal [63,69,70], and food-related [61,71,125] origin. The presence of certain *qac* genes does not necessarily give rise to an increase in the MIC toward the QAC BC. All isolates were therefore investigated in an EtBr-based efflux pump activity assay for increased activity. Four MRSA CC30 isolates showed increased EtBr efflux activity and the presence of the QAC resistance gene, *qacG*, was identified in all of the isolates. The *qacG* gene has previously been identified in staphylococci isolated from food processing plant [125], from bovine (unpasteurized milk) [70], and in porcine MRSA (CC9). The food-related staphylococci isolates were collected from different food processing plant and the *qacG*-containing isolates from bovine comprised two different coagulase-negative species (*Staphylococcus cohnii* and *Staphylococcus warneri*) from two different herds. In our study *qacG* was identified in three isolates of *S. aureus* collected from pigs at the same slaughter house, which however originated from different farms and from a *S. aureus* isolate collected from an unrelated farm. This suggests that *qacG* might be widespread "from farm to fork". In the study by Bjorland et al. (2005) they found a widespread distribution of QAC resistance genes among bovine and caprine origin. The fact that they identified *qacG* in two different staphylococci species with 100% identical gene sequences that was found to be located on highly similar plasmids suggested that interspecies dissemination of *qacG* occurs [70].

Two of the *S. aureus* pig isolates containing the *qacG* gene was found to have the highest MIC (8 µg/ml) to BC than the remaining isolates, whereas the other two *qacG* positive isolates had MICs of 4 µg/ml that was identical to several of the *qacG* negative isolates. However, the MBC-values to BC for all four isolates (8 µg/ml) were similar to several of the *qacG* negative *S. aureus* isolates. Even though the susceptibility to BC for *qacG* positive and negative isolates generally were similar, incorrect use of disinfectants or the presence of residual concentrations may result in bacteria being exposed to low concentrations of disinfectants that might give strains harbouring QAC resistance genes a selective advantage. Furthermore, Heir et al. (1999) demonstrated that *qacG* positive isolates by exposure to sub-lethal levels of BC could be adapted to grow at higher BC concentrations [60]. The presence of the QAC resistance gene, *qacG*, in MRSA isolates and exposure to sub-lethal concentration of QACs might make the task of eradication of MRSA more difficult.

3.1.1.2 Co-selection to antimicrobial agents was not detected

The various *qac* genes are widespread among Gram positive (mainly staphylococci) and Gram negative bacteria and can be associated with mobile genetic elements including resistance genes to antimicrobial agents [57,59,68,92–94]. This may lead to co-selection and maintenance of these elements due to antimicrobial treatment or disinfection with QACs.

Three of the *qac*G positive *S. aureus* pig isolates were whole genome sequenced. The *qacG* gene in these isolates was found likely to be located on the pST94 or a pST94-like plasmid on which *qacG* was detected for the first time. The presence of antimicrobial resistance genes on this plasmid has so far not been detected [60]. The presence of *qac*G in these isolates is therefore not likely to give rise to co-selection of resistance to antimicrobial agents by QACs. The presence of a QAC resistance gene on a small plasmid without any additional resistance genes however indicate that a selective

pressure from the use of QACs is presents in the environment. It is possible that pST94 can acquire additional genes, such as antimicrobial resistance determinants, from other mobile genetic elements by homologous recombination. Furthermore, it is also plausible that the small plasmid can be taken up by larger conjugative plasmids encoding multiple antimicrobial resistance genes. The QAC resistance gene *smr* are associated with the pSK41 family of staphylococci multi-resistance plasmids. Several of the antimicrobial resistance determinants carried by the pSK41 plasmid family have been found to be located on smaller co-integrated plasmids [93]. It has been suggested that the resistance genes have been collected by pre-existing conjugative plasmids via transposon insertion and insertion sequences (IS257)-mediated co-integrative capture of other plasmids [93].

One of the *qac*G positive MRSA CC30 isolates was found to be phenotypically resistant to gentamicin. This antimicrobial agent can be used for treatment of *S. aureus* infections. Exposure to low concentrations of QACs may therefore select for MRSA strains that are resistant to clinically important antimicrobial agents.

3.1.2 Porcine *S. aures* isolates are resistant to antimicrobial agent typically used in pig, however a high level of CIP resistance was observed

The phenotypic resistance profile of *S. aureus* pig isolates to antimicrobial agents in generally correlated with the use of antimicrobial agents in the Danish pig production. However, one exception was the high occurrence of CIP resistance (20 %) in *S. aureus* CC398 MRSA and MSSA isolates, since only low amount of this compound is used in pigs. Other environmental conditions may therefore contribute to the development of ciprofloxacin resistance in these strains. Ciprofloxacin resistance can arise from mutations in the chromosome including the molecular targets DNA gyrase and topoisomerase IV or in genes resulting in overexpressing of naturally occurring multi-drug efflux pumps having ciprofloxacin as a substrate [36]. The study of Manuscript II demonstrated that exposure to sub-inhibitory concentrations of HP and PAA could significantly increase the mutation rate in *S. aureus* isolates and may contribute to the development of CIP resistance. However, this is merely speculation and will require further experiments to elucidate.

3.2 The potential of biocides to increase antimicrobial resistance in bacteria

3.2.1 Certain biocides can induce the SOS response and increase mutagenesis in *S. aureus*

Within recent years is has been suggested that the SOS response system in bacteria might be an important player in development and emergence of antimicrobial resistance. Activation of the SOS system can induce mutagenesis in bacteria giving rise to mutation-mediated resistance to certain therapeutic antimicrobials. Furthermore, induction of the SOS response has been found to induce the transfer of antimicrobial resistance genes [126]. Several factors have been shown to induce the SOS response including some antimicrobial agents, pH-extremes, high pressure, and oxidative agents, however, knowledge about the stress response to various biocidal agents in bacteria are limited.

BC, CHX, HP, PAA, and SH are biocides widely used in health care facilities, food production, and/or in animal husbandry. Their ability to induce the SOS response was tested in *S. aureus* using a *rec*A-*lac*Z fusion strain. BC, CHX, and HP were shown to be inducers of the SOS response in *S.*

aureus, whereas SH was not. Sub-inhibitory concentrations of these biocides were investigated for their potential to increase the mutation rate in different *S. aureus* isolates and HP and PAA were found to significantly ($p \leq 0.05$) increase mutagenesis and the development of resistance by 5-15-fold and 3-8-fold, respectively.

Stress-induced mutagenesis is important for evolution of drug resistance in pathogens; several studies have demonstrated a SOS-dependent induction of mutagenesis resulting in increased development of resistance to the exposed antimicrobial compound or other drugs [7–10]. A study by Cirz et al. (2005) demonstrated by *in vitro* and *in vivo* (murine infection model) studies that *E. coli* was unable to develop resistance to either ciprofloxacin or rifampicin in cells by preventing induction of the SOS response [7]. Introduction of mutations into the chromosome of bacteria is in general an important factor in driving bacterial evolution. Mutations can be deleterious or neutral to the cell hence, for well adapted strains a low constant mutation rate are probably beneficial in the long run. Conversely, in stressful environments an increase in mutagenesis induces genomic instability and might result in the development of mutations that are advantageous to the cell [127]. Hence, use of and bacterial exposure to biocides that are capable of increasing mutagenesis in bacteria might potentially accelerate adaptive evolution in populations. This can subsequently result in development of bacterial persisters in their surrounding environment, such as the hospital environment, the food processing industry or in animal husbandry and potentially compromise patient and food safety.

3.2.2 Ethanol can induce transfer of Tn*916*

To investigate if bacterial exposure to sub-inhibitory concentrations of biocides can affect the spread of resistance genes the transfer of Tn*916* in *B. subtilis* was used as a model system. The conjugative transposon Tn*916* is the prototype of a large family of related elements (Tn*916*-like elements) that has an extremely broad host range; they have been found in at least 36 genera, including pathogenic and commensal bacteria [13]. Nearly all the members of the Tn*916* family of conjugative transposons have been found to encode TET resistance; however some members of this family also confer resistance to other antimicrobial agents such as macrolides and kanamycin. Furthermore, Tn*916*-like elements can be associated with non-conjugative transposons (e.g. Tn*917*), including additional antimicrobial resistance genes [13]. Tn*916* and Tn*916*-like elements therefore constitute important vectors in the dissemination of antimicrobial resistance. The conjugation experiment was carried out in *B. subtilis* because a *gus*A (encoding β-glucuronidase) reporter construct estimating transcription from the *tet*(M) promoter was available. Transcription of *tet*(M) in Tn*916* is thought to give rise to transcription of genes involved in recombination and conjugation. The reporter construct was therefore used in a β-glucuronidase assay to estimate exposure times of each biocidal compound that gave rise to changes in transcription and possibly in Tn*916* transfer. Based on the results from the β-glucuronidase assay the conjugation experiment was set-up by pre-exposing donors in sub-inhibitory concentrations of biocides for the determined times.

Ethanol was found to significantly ($p < 0.05$) increase transfer of Tn*916* by an average of 5-fold, whereas an average increase of 4-fold on Tn*916* conjugation was observed ($p = 0.12$) when donors

were pre-exposed to hydrogen peroxide. In comparison, TET was found to increase transfer by 12-fold ($p < 0.05$) in average. This is in contrast to CHX that caused a small reduction (0.7-fold) and SH that did not have an effect on Tn*916* transfer. The results of the β-glucuronidase reporter assay and the conjugative transfer of Tn*916* was in good correlation; compounds giving rise to an increase, a reduction or no response in the GusA activity was overall found to have the same effect on the transfer frequency. ETOH and CHX are both membrane active compounds; however their chemical structures differ why the overall effect on cells may be different. Also, HP and SH are both highly oxidizing compounds, nevertheless an increasing effect on the β-glucuronidase activity and an increase in the conjugative transfer of Tn*916* could only be suggested for exposure to HP. Interestingly, HP has been shown to cause reversible dissociation of ribosomes which might therefore result in fewer active ribosomes and thereby slowing down protein synthesis in a similar manner as TET. Since the efficacy of a biocidal compound is very reliant on the concentration and contact time with the microorganism it cannot be rejected that CHX and SH under different test conditions can promote the transfer of Tn*916*. Based on these results it is therefore apparent that compounds with the same overall effect on bacteria do not necessarily induce the same response in the cell.

As mentioned above transcription of *tet*(M) in Tn*916* is hypothesised to give rise to transcription of genes involved in recombination and conjugation of the Tn*916* element. Furthermore, transcription of *tet*(M) is believed to be regulated by a tetracycline dependent transcriptional attenuation mechanism; the upstream region of *tet*(M) is predicted to encode a leader-peptide (*orf*12) and transcriptional read-through of *orf*12 is believed to result in transcription of *tet*(M), which then initiate the regulatory cascade leading to increased expression of genes required for recombination and the conjugative transfer of Tn*916* [128,129]. Exposure to TET inhibits most of the ribosomes in the cell, except for those that are protected by the basal level of Tet(M) production. This will subsequently slow down translation and result in the build-up of charged-tRNA molecules leading to increased expression of *tet*(M) and the recombination and transfer genes. A very interesting aspect of this regulation process is that any compound that increase the pool of charged-tRNA molecules in the cell is likely to increase transcription of *tet*(M) and possibly increase transfer of Tn*916* [13]. Furthermore, the regulatory region of Tn*916* is conserved in nearly all the Tn*916*-like elements. ETOH may not only induce the spread of Tn*916*, but also other members of the Tn*916*-like family and their resistance genes. In theory any compound in addition to TET that leads to the build-up of charged tRNA-molecules might induce the spread of Tn*916* and members of the Tn*916*-like family [13].

3.2.3 Indirect selection of resistance to antimicrobial agents by biocides

Results from the studies of manuscript I and II demonstrate that exposure to sub-inhibitory concentrations of various biocides can induce development of antimicrobial resistance and emergence in bacteria by increasing the horizontal transfer of the TET resistance gene *tet*(M) and by increasing the mutation rate in *S. aureus*. This means that inappropriate consideration of the factors affecting the efficiency of biocides might contribute to the development of antimicrobial resistance in bacteria. Another factor to consider as a possible contributor of resistance development is the presence of residual concentrations of biocides after disinfection. It is well known that

prudent use of antimicrobial agents for treatment of humans and animals has driven the dramatically increase in antimicrobial resistance in human and animal pathogens. However, the above data indicate that not only the direct selection of resistance genes by use of antimicrobial agents occur, but also the indirect selection by biocides may contribute to resistance development.

The overall risk assessment of these findings is however difficult to determine due to several factors. The effect of biocides is investigated for sub-inhibitory concentrations of biocides because bacterial exposure to low concentrations of these compounds in natural environments might occur due to previously mentioned factors. However, the occurrence of these microenvironments in real life needs to be elucidated. Furthermore, the relevance of *in vitro* findings *in situ* is unknown. The results of *in vitro* studies nevertheless point toward what may occur in natural environments.

4 Concluding remarks and future perspective

No widespread selection for reduced susceptibility to commonly used disinfectants in pig farming in porcine *S. aureus* isolates was detected. However, a biocide resistance gene, *qac*G, was identified in several of the MRSA isolates, which has also been found in other animal related staphylococci. Surveillance of the occurrence and emergence of reduced susceptibility to biocides in bacteria are however, still encouraged, since this will provide important data to determine if decreased susceptibility to biocides happen over time. The increasing use of whole genome sequencing encourage to development of simple and easy to handle bioinformatic tools that can be used for instance to determine if the bacterial isolate has acquired biocide resistance genes and the possible linkage to antimicrobial resistance genes. In our research group a data base has been constructed that are able to identify the presence of any known acquired antimicrobial resistance gene. A similar tool could be constructed for all known biocide resistance genes. This would be very useful especially because phenotypically detection of resistance to biocides are difficult since the presence of biocide resistance genes in general give rise to modest changes in susceptibility. Within this PhD study sequences of all known *qac* genes and their variants have been collected and a database for identification of *qac* genes is currently under construction.

Importantly, the data from this thesis demonstrated a potential of certain biocides to contribute to antimicrobial resistance development and emergence in bacteria. Of the four biocides examined ethanol was found to induce transfer of the conjugative transposon, Tn*916*, that includes the tetracycline resistance gene *tet*(M). Furthermore, hydrogen peroxide and peracetic acid were shown to increase the mutation rate and antimicrobial resistance emergence in *S. aureus* isolates. On the short term these results emphasises that correct use of biocides are of outmost importance and should not be compromised. On the long term, more studies are needed to elucidate the actual risk of biocide use on generating antimicrobial resistant bacteria in practice. Moreover, the presence of residual concentrations after disinfection should be assessed, since these concentrations may give rise to bacterial exposure to low non-lethal concentrations. Furthermore, there is a need for data on quantities of biocide consumption making it possible to conduct dose / response studies. Knowledge about the consumption of the different biocidal substances in combination with data on their

potential of developing antimicrobial resistance are necessary to determine which biocides create the highest risk of contributing to antimicrobial resistance in bacteria.

Many biocidal compounds exist and the activity of biocides are very dependent on the concentration, therefore, simple and fast screening methods to investigate the effect on bacteria of a variety of biocides at multiple concentrations at one time would be very useful. For future studies there is a need for standardized methods to determine the susceptibility of bacteria to biocides and to estimate the potential contribution to antimicrobial resistance development. This will make scientific data obtained from different studies more comparable and valuable in overall risk assessment. Surveillance programmes using these standardized methods will then be able to monitor the level of biocide resistance and co- and cross-resistance to antimicrobial agents in all areas where biocides are used.

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CHAPTER III

Manuscript I-III

Title: **Effect of sub-inhibitory concentrations of four commonly used biocides on the conjugative transfer of Tn*916***

Running title: Effect of biocides on Tn*916* transfer.

Keywords: Ethanol, hydrogen peroxide, chlorhexidine digluconate, sodium hypochlorite, and resistance gene transfer

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Abstract

OBJECTIVES; large amounts of biocides are used to reduce and control bacterial growth in the health care sector, food production and agriculture. It has been suggested that bacterial exposure to sub-inhibitory concentrations of biocides may induce DNA transfer between bacteria. Here we investigated the effect of sub-inhibitory concentrations of the four commonly used biocides; ethanol, hydrogen peroxide, chlorhexidine digluconate and sodium hypochlorite on the conjugative transposition of the mobile genetic element Tn*916*.

METHODS; conjugation assays were carried out between *B. subtilis* strains where the donor containing Tn*916* was pre-exposed to sub-inhibitory concentrations of each biocide for a defined length of time.

RESULTS; ethanol was found to significantly ($p < 0.05$) increase transfer of Tn*916* by 5-fold, whereas an increase of 4-fold on Tn*916* conjugation frequency was observed ($p = 0.12$) when donors were exposed to hydrogen peroxide. Chlorhexidine digluconate and sodium hypochlorite did not induce an increase in transfer frequency.

CONCLUSIONS; these results suggest that exposure to sub-inhibitory concentrations of ethanol and hydrogen peroxide may induce the spread of Tn*916*-like elements and their resistance genes, which is clinically important since these biocides are frequently used in hospitals.

Introduction

Biocides are chemical compounds capable of inactivating microorganisms [1]. These are used for disinfection, antisepsis and preservation to inhibit or reduce bacterial loads in various settings such as the health care sector, agriculture and the food industry [1,2]. In Denmark, the yearly consumption of biocides has been estimated to comprise up to 5,000 tonnes [2], compared to less than 160 tonnes of antimicrobial agents for therapy [3]. Despite the

widespread use of these compounds, our knowledge about their mode of action, especially at sub-inhibitory concentrations, and the microbial response to exposure is relatively limited [4,5]. The working concentrations of disinfectants and antiseptics are generally much higher than the lethal dose, however the efficacy of a biocide can be significantly reduced due to the presence of organic matter (e.g. blood, serum, pus, and food debris), over-dilution or insufficient contact time with microorganisms. Also, the presence of residual concentrations might result in bacterial exposure to sub-inhibitory concentrations [6].

During recent years, it has been suggested that the use of biocides might lead to increased resistance to antimicrobial agents used for treatment of humans and animals, through increasing mutation rates in bacteria or increasing horizontal gene transfer [7,8], however evidence for such an increase is currently lacking [9]. Previously studies have shown that stress in bacteria caused by sub-inhibitory concentrations of antibiotics can promote transfer of antibiotic resistance and virulence genes [10–13]. However, the effect of different biocides has been much less studied. Plasmid conjugation or transduction in *Staphylococcus aureus* of resistance determinants has been found to be either not affected or reduced by sub-inhibitory concentrations of a number of biocides, including povidone-iodine, chlorhexidine, and different quaternary ammonium compounds [14,15]. However one compound cetrimide was found to cause a pronounced increase in the transduction efficiency [14]. Effects of biocides on transfer of other resistance determinants and in other species have however, not been studied.

Mobile genetic elements such as conjugative transposons are important vectors in the dissemination of antibiotic resistance determinants. Tn*916* is a conjugative transposon and the prototype of a large family of related elements. They have an extremely broad host range, including more than 30 bacterial genera, and have been found in both pathogenic and commensal bacteria [16]. Most of these elements contain the tetracycline resistance gene *tet*(M), but some members of this family also confer resistance to other antimicrobial agents;

macrolides, kanamycin, mercury and cetrimonium bromide (CTAB). Furthermore, Tn*916*-like elements have also been found to be associated with non-conjugative transposons (e.g. Tn*917*), which contain additional antibiotic resistance genes [16,17]. It has been hypothesised that transcription of *tet*(M) in Tn*916* leads to transcription of downstream genes involved in recombination and conjugation of the element and that transcription of *tet*(M) is regulated by a tetracycline dependant transcriptional attenuation mechanism reliant on the levels of charged-tRNA molecules within the cell [18,19]. It has subsequently been suggested that any stress that the cell encounters (other than exposure to tetracycline) that results in the build-up of charged-tRNAs is also likely to cause an increase in transcription of *tet*(M) and downstream genes and possibly an increase in transfer [16].

The aim of this study was to investigate the effect of sub-inhibitory concentrations of four commonly used biocides; ethanol, hydrogen peroxide, chlorhexidine digluconate and sodium hypochlorite on the conjugative transposition of Tn*916*.

Material and methods

Chemicals and reagents

Chloramphenicol (CHL), fusidic acid sodium salt (FUS), rifampicin (RIF), streptomycin sulphate salt (STR) and tetracycline hydrochloride (TET) were purchased from Sigma. Tetracycline discs (30 µg) were from Oxoid. BHI agar and broth were obtained from either Oxoid or Difco. BBL™ Mueller-Hinton II broth (MH II) was from Becton, Dickinson and Company, and premade tryptone soya agar plates with 5 % sheep blood (blood agar plates) were purchased from Oxoid. Biocides included in this study were: Chlorhexidine digluconate (CHX), 20 % (w/v) from Alfa Aesar; hydrogen peroxide (HP), 30 % solution from Fluka; sodium hypochlorite (SH), 10-15 % available chlorine from Sigma and absolute ethanol

(ETOH) from either BDH Prolabo or Kemetyl AB. 4-Nitrophenyl β-D-glucuronide was obtained from Sigma.

Bacterial isolates

All bacterial isolates and plasmids included in this study are listed in Table 1.

Construction of *Bacillus subtilis gus*A reporter strain

In order to determine the optimal time of exposure to sub-inhibitory concentrations of the antimicrobials prior to filter-mating a reporter construct was generated in *B. subtilis* as follows. A 450 bp fragment of Tn*916*, which includes the *tet*(M) promoter, *orf*12 and the terminator sequences was amplified by PCR using the primers P*tet*(M) For (5'-GGCGGCGGGTACCCAAAGCAACGCAGGTATCTC-3') and P*tet*(M) Rev (5'-GGCGGCGGAATTCGTGATTTTCCTCCAT-3'). The restriction sites *Kpn*I and *Eco*RI were included in the primers (underlined). Next the cwp2 promoter fragment was removed from the pUC19 based pCBR026 [22] and replaced with the *Kpn*I-*Eco*RI digested *tet*(M) promoter amplicon resulting in pUC19-P*tet*(M)-*gusA*. The fused P*tet*(M)-*gusA* was excised from pUC19-P*tet*(M)-*gusA* on a *Kpn*I-*BamH*I fragment and directionally cloned into the corresponding sites of pHCMCO5, resulting in the *B. subtilis* reporter construct, pHCMCO5-P*tet*(M)-*gusA*. The construct was then transformed into *B. subtilis* BS34A [21] using a previously described protocol [23]. BS34A contains a wildtype copy of Tn*916* therefore providing resistance to tetracycline. BS34A is the donor strain used in all of the transfer studies which is why we also used it as the reporter strain.

Minimal inhibitory concentration (MIC)-determination of biocides by the broth microdilution method

The MIC of ETOH, HP, CHX and SH was determined as recommended by CLSI guidelines [24]. Isolates were grown over night (ON) on blood agar or BHI agar plates at 37°C. Colonies were re-suspended in 0.9 % NaCl to a McFarland standard of 0.5 or an OD_{600} between 0.08 and 0.13, and then 100-fold diluted in MH II. Biocide working solutions were prepared in MH II just before two-fold dilution series were made in 96-round bottom well microtiter plates (Nunc) (50 µl per well). Then, 50 µl of cell suspension was transferred to the microtiter plate and incubated 16-20 hours at 37°C under aerobic conditions. *Escherichia coli* ATCC 25922 or *S. aureus* ATCC29213 were included as control strains for reproducibility of the susceptibility testing procedure. The MIC-value was defined as the lowest concentration of the compounds giving rise to no visible growth. MIC-determinations were done in duplicates and as a minimum repeated twice.

Effect of biocides on β-glucuronidase enzyme activity

Culture preparation for β-glucuronidase enzyme assay

B. subtilis BS34A*::* pHCMCO5-P*tet*(M)-*gusA* was grown ON at 37°C on BHI agar plates supplemented with 10 µg/ml CHL to select for the reporter plasmid construct. Cells were inoculated in 20 ml BHI (without CHL) and grown ON at 37°C with rotary shaking (200 rpm). ON cultures were diluted to an OD_{600} of approx 0.1 in 500 ml Erlenmeyer flasks containing a final volume of 100 ml BHI broth and incubated at 37˚C with shaking. After two hours of growth TET (10 µg/ml) and sub-inhibitory concentrations of biocides (MIC/4) were added to the cultures. OD_{600} was measured and 5 ml samples were collected before (1.5 h and 2.0 hours of growth) and after addition of the compounds (0.5 h, 1.0 h, 1.5 h and 2.0 h of exposure). Cells were harvested by centrifugation (3000 × *g*, 4 ˚C, 10 min) and pellets were stored at -80˚C. Cells from each of the 2.0 h samples were plated on BHI and BHI

supplemented with 10 µg/ml CHL agar to determine the stability of pHCMCO5-P*tet*(M)-*gusA*.

Measurement of β-glucuronidase enzyme activity

The β-glucuronidase activity was measured as previously described with some modifications [25]: Cell pellets were thawed at room temperature (RT) and re-suspended in 800 µl of Z-buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$; 40 mM NaH_2PO_4 H_2O; 10 mM KCl; 1 mM $MgSO_4 \cdot 7H_2O$; 50 mM 2-mercaptoethanol) adjusted to pH 7.0. Eight µl of toluene was added and the mixture was transferred to a new tube with approximately 250 µl of unwashed glass beads (150-212 µm in diameter) (Sigma) and treated in a RiboLyser (Hybaid) at a speed of 6.5 for 2 x 25 sec, with a 1 min pause in-between the two runs. Lysates were cooled on ice for 2 min and glass beads were removed by centrifugation (3000 × *g*, 4˚C , 3 min). 400µl of the supernatant was transferred to a fresh tube and 400 µl of Z-buffer was added. Samples were incubated at 37˚C for 5 min and the enzyme reactions were started by adding 160 µl of 6 mM 4-Nitrophenyl β-D-glucuronide. The enzymatic reaction was stopped after incubation at 37˚C for 5 min with 400 µl of a 1 M disodium carbonate solution and cell debris was removed by centrifugation (3000 x *g*, 25˚C, 10 min). Finally, OD_{405} of the supernatants were measured and the specific enzyme activities were calculated using the following equation: $(A_{405} \times 1000) / [OD_{600} \times t$ (min) × 1.25 × vol. (ml)] [25]. Experiments were performed on three separate occasions, except for exposure to TET, which was repeated six times.

<u>Measurement of the effect of biocides on the conjugative transposition of Tn*916*</u>

A derivative of *B. subtilis* BS34A, selected for resistance to STR (BS34ASTR) was used as the donor strain and a derivative of *B. subtilis* CU2189 selected for resistance to RIF and FUS (BSCU2189RF) was used as a recipient strain. Conjugation by filter mating was performed as

previously described [26] with some modifications. *B. subtilis* BS34ASTR and BSCU2189RF were grown ON at 37˚C on blood agar plates including a TET disc on the donor plate for selection of Tn*916*. Colonies from ON plates were inoculated in 10 ml BHI broth and incubated ON (without antibiotics) at 37˚C with shaking (150 rpm). ON cultures were diluted in pre-warmed BHI broth to an OD_{600} of approx 0.1 in 500 ml Erlenmeyer flasks to a final volume of 100 ml. Cultures were grown shaking (200 rpm) until donor cultures reached an OD_{600} between 0.5 and 0.6. Then, sub-inhibitory concentrations (MIC/4) of ETOH, HP, CHX and SH and 10 µg/ml of TET were added to separate donor cultures and further grown for 2.0, 1.5, 0.5, 1.5 and 1.0 hour, respectively. The length of exposure time of each compound was equal to the length of that expected to have the greatest effect on transcription from the promoter upstream of *tet*(M) based on the β-glucuronidase enzyme assay. Within each repetition, one culture where no compound was added served as the control donor. Then, donor and recipient cells were harvested (6000 × *g*, 5 min, 4˚C) and re-suspended in BHI broth to an OD_{600} of approx 0.5 and 5.0, respectively and mixed in a 1:1 volume resulting in an output recipient-donor ratio of approx. 1:1. 500 µl of each mixture was transferred to a sterile filter (0.45 µm, white gridded, 47 mm; Millipore) placed on a BHI agar plate. The cell mixtures were left to absorb into the filter for 0.5 hour and then incubated at 37˚C for 17.5 hours. After incubation mating filters were transferred to 10 ml 0.9% NaCl and re-suspended by vortex mixing. The numbers of donors and recipients were determined by counting on BHI agar supplemented with 10 µg/ml TET or 12.5 µg/ml RIF and 5 µg/ml FUS, respectively after 24 hours of incubation at 37˚C. Transconjugants were selected on BHI agar plates containing 10 µg/ml TET, 12.5 µg/ml RIF and 5 µg/ml FUS and counted after 48 h of incubation at 37˚C. At least ten transconjugants from each transfer experiment were verified by sub-culturing on transconjugant plates twice and once on BHI agar plates supplemented with 100 µg/ml STR on which only donor cells can grow. Transconjugants were also

screened for the presence of *tet*(M) by PCR using primer *tet*(M)-1 (5'-GTTAAATAGTGTTCTTGGAG-3') and *tet*(M)-2 (5'-CTAAGATATGGCTCTAACAA-3') [27]. Conjugation experiments were repeated five times.

The input recipient-donor ratio and the stability of Tn*916* in the control and exposed cultures were estimated in two of the conjugation experiments (expt 4 and 5) by plating donor pre-mating cultures on BHI agar plates with and without the addition of 10 µg/ml TET and recipient pre-mating cultures on antibiotic free BHI agar plates.

Data analysis

β-glucuronidase enzyme activity

Measures of the specific β-glucuronidase enzyme activities in exposed cultures (prior to and after addition of biocides) were standardized to the corresponding control sample as the percentage difference in β-glucuronidase enzyme activity. The transcriptional effect of biocides on enzyme activity was estimated as the difference in the standardized enzyme activity after addition of the compound (0.5 h, 1.0 h, 1.5 h and 2.0 h samples) relative to the enzyme activity before addition (0 h sample). The enzyme activity before addition was estimated as the average of the two samples collected before addition of the biocides.

*Conjugative transposition of Tn*916

The transfer frequencies of Tn*916* were calculated as the number of transconjugants per output donor. The significance of changes in transfer frequencies between control and treated conjugations were statistically tested using the paired, two-sided, Student's t-test, where a pair represents the transfer frequency of the control and the treated conjugations within an experimental repetition. The normality of the differences in transfer frequencies between the control and exposed conjugations were visually assessed using QQ-plots.

Results

MIC-values of *B. subtilis* strain BS34A::pHCMCO5-P*tet*(M)-*gus*A to ETOH, HP, CHX and SH are shown in Table 2 together with the corresponding sub-inhibitory concentrations (MIC/4) used in the leader peptide transcription assay and the Tn*916* conjugation experiment.

Determination of the optimum time of exposure to antimicrobials prior to filter-mating experiments

Transcription from the promoter upstream of *tet*(M) was estimated using a β-glucuronidase (*gus*A) reporter construct in *B. subtilis*. The effect of ETOH, HP, CHX, SH and TET on the β-glucuronidase enzyme activity is shown in Figure 1. The times where the greatest deviation in GusA activity from the normalised value were chosen as the times for pre-exposure to the biocides prior to filter-mating. The stability of the reporter construct pHCMC05-P*tet*(M)-*gus*A during all the experiments was found to be similar (an average of 74 – 85 %) at the end of the experiment apart from the experiment where cells were challenged with TET. In this experiment the average stability was 64% (supplementary Figure S1).

The effect of biocides on the conjugative transposition of Tn*916*

The conjugative transfer of Tn*916* was studied in *B. subtilis* where donors were pre-grown in ETOH, HP, CHX, SH and TET for 2.0, 1.5, 0.5, 1,5 and 1.0 hours, respectively prior to filter mating. Results for the effect of biocides and TET on the conjugative transposition of Tn*916* are presented in Table 3. TET and ETOH significantly ($p \leq 0.05$) enhanced transfer of Tn*916* corresponding to an average increase of 12- and 5-fold, respectively. Exposure to HP resulted in average in a 4-fold increase ($p = 0.12$) and exposure to CHX in a small (0.7-fold) decrease

(p = 0.10) in the transfer frequency. Pre-growing donor cells in SH did not affect the transfer of Tn*916* (p = 0.33).

Stability of Tn*916* in donor cells was assessed in pre-mating cultures and was not found to be significantly different between the exposed and the control cultures (supplementary Table S1). Some variation in the output recipient-donor ratio occurred between matings with pre-growth of donors in TET and ETOH and the corresponding controls. When the output recipient-donor ratios were compared to the transfer frequencies it did not suggest that differences in transfer was due to variations in this ratio. Furthermore, the input recipient-donor ratio was determined for two experiments (expt 4 and 5, data not shown). In the case of ETOH and TET these two experiments represented one of the larger and smaller differences in transfer. In both cases differences in transfer frequencies were not found to correlate with variations in the input recipient-donor ratio, since this ratio for the treated matings were within the range of the controls (supplementary Figure S2).

Discussion

The effect of sub-inhibitory concentrations of ETOH, HP, CHX, SH and TET on the conjugal transfer of Tn*916* between *B. subtilis* strains was analysed. The MIC-values of *B. subtilis* BS34A to the four biocides were comparable to MIC-values found for other Gram positive bacteria [28–33]. The sub-inhibitory concentration of each biocide used in this study was set to one quarter of the MIC.

In order to determine the optimal time of pre-exposure to the various biocides prior to filter-mating we determined the GusA activity of a plasmid based *gusA* under the expression of the Tn*916* promoter upstream of *tet*(M). BS34A:: pHCMCO5-P*tet*(M)-*gusA* was exposed to the four biocides for 2 h and the GusA activity recorded every 30 minutes. The greatest

difference in GusA activity occurred at 2h after exposure for ETOH, 1.5 h for HP, 0.5 h for CHX and 1.5 h for SH. Although a relatively crude assessment of the transcriptional activity of the *tet*(M) promoter in response to biocide exposure it provided valuable data on which to design the conjugation experiments. As a control the experiment was carried out with inhibitory concentrations of TET and the maximum response was observed at 1 h following exposure which agrees with the proposed mechanism for the transcriptional regulation of Tn*916* and previous transcriptional analysis [18].

Pre-exposing donors to TET resulted in a 12-fold increase in the transfer frequency of Tn*916*, which is in good agreement with the TET induced Tn*916* transfer study reported by Showsh and Andrews (1992) between *Bacillus subtilis* and *Bacillus thuringiensis* subsp. *israelensis* (19-fold increase calculated as transconjugants per output recipients) [13]. In our study exposure of donors to ETOH also significantly increased the transfer of Tn*916*, which corresponded to an average increase of 5-fold.

An increasing and a small decreasing effect on the conjugative transposition of Tn*916* could be seen for HP and CHX, respectively. In a previous study by Pearce *et al*. (1999) [14], CHX at the same concentration as in this study was also found not to significantly reduce ($p > 0.05$) plasmid transfer by conjugation in one mating system of *S. aureus* in the same range as in our study. Sodium hypochlorite was not found to affect the transfer frequency of Tn*916* under our test conditions.

In contrary to the specific action of tetracycline and antibiotics in general, biocides are generally believed to have multiple target sites within the cell, but knowledge about the exact mechanism of action is limited, especially at sub-inhibitory concentrations where only few target sites might be involved [4,5]. Therefore the specific mechanisms of action of sub-inhibitory concentrations of ETOH, HP, CHX and SH on cells are difficult to elucidate.

However one hypothesis which is currently being tested is that the multiple, unspecific actions of the biocides slow down protein synthesis and lead to an increase in the pool of charged-tRNAs within the cell; the rate determining step in the de-repression of transcriptional attenuation proposed for Tn*916* [18]. Since the regulatory region of Tn*916* is conserved in nearly all of the Tn*916*-like elements [16] ETOH and HP might therefore not only induce the spread of Tn*916*, but also other members of the Tn*916*-like family and their resistance genes.

An increase in horizontal transfer of Tn*916* is worrying from a clinical perspective. Ethanol is primarily used for hard surface disinfection and skin antisepsis in hospitals, but is also widely used for disinfection in the food industry, day care institutions and for preservation purposes [1,2,34]. Hydrogen peroxide is widely used for disinfection, antisepsis and sterilization purposes and is commonly used in the food industry, in hospitals and in agriculture [1,2]. Incorrect use of, or the presence of residual concentrations of these biocides may therefore promote the spread of Tn*916* and possibly other elements belonging to the Tn*916*-like family.

In summary, these results show that sub-inhibitory concentrations of ETOH and to a lesser degree HP increase the transfer frequency of Tn*916*. Current investigations are focused on determining the exact molecular mechanisms for this increase in transfer frequency with the obvious focus being on the effect on transcriptional attenuation upstream of *tet*(M). The results of this study emphasise the importance of correct use of biocides and demonstrate that transfer of Tn*916* encoded antibiotic resistance may be promoted by sub-inhibitory concentrations.

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Transparency Declaration

None to declare

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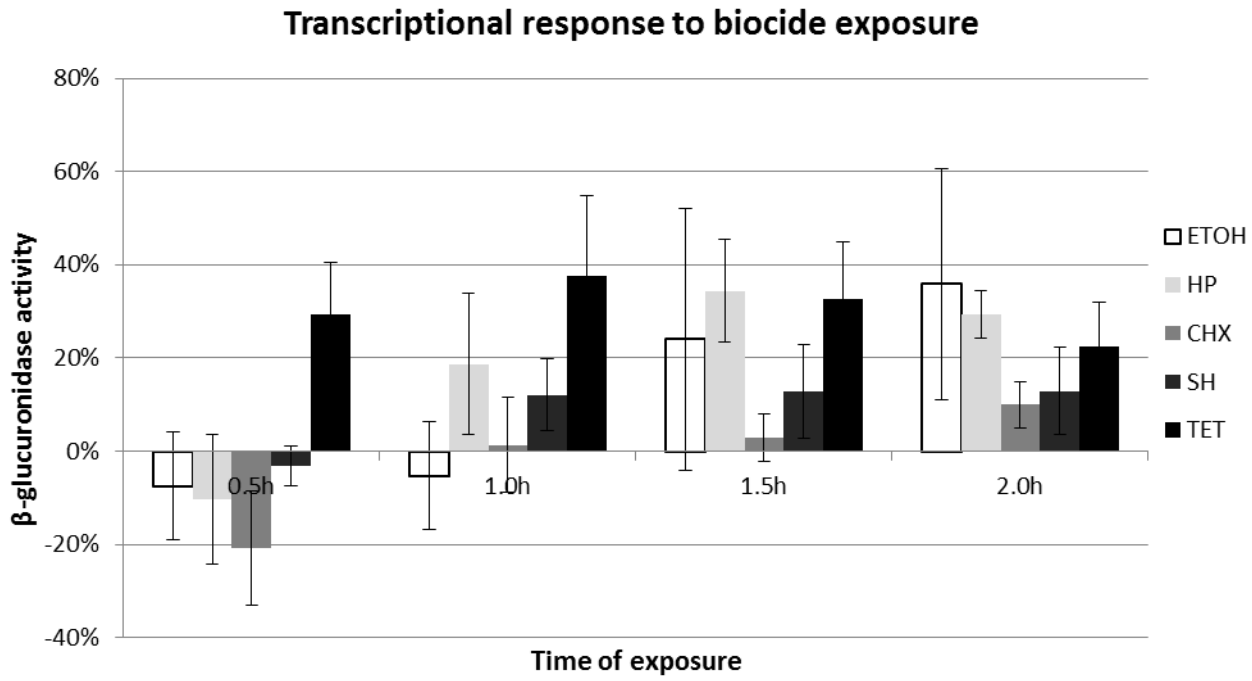

FIG. 1. The average effect of ethanol (ETOH), hydrogen peroxide (HP), chlorhexidine digluconate (CHX), sodium hypochlorite (SH), and tetracycline (TET) on β-glucuronidase enzyme activity. Data illustrate changes in enzyme activity after addition of compounds (0.5 h to 2.0 h) relatively to before addition (0.0 h). Experiments investigating the effect of TET were repeated six times, whereas the effect of biocides was repeated three times. Error bars represent ± standard deviation.

TABLE 1. Bacterial isolates and plasmids included in this study. Abbreviations: RIF, rifampicin; FUS, fusidic acid; TET, tetracycline; STR, streptomycin; CAM^R, chloramphenicol resistant.

	Relevant properties	**Reference or source**
B. subtilis		
CU2189	Recipient strain	[20]
BSCU2189RF	RIF and FUS resistant derivative of CU2189	This study
BS34A	*B. subtilis*::Tn*916* (CU2189 x FM12A). TET^R, contains a single copy of Tn*916*	[21]
BS34ASTR	STR resistant derivative of BS34A	This study
BS34A:: pHCMCO5-P*tet*(M)-*gus*A	BS34A including plasmid pHCMC05 containing a P*tet*(M)-*gus*A construct, and CAM^R marker	This study
Plasmids		
pCBR026	pUC19 containing the *gus*A reporter gene under the control of the cwp2 promoter from *Clostridium difficile*	[22]
pUC19-P*tet*(M)-*gus*A	pCBR026 with the cwp2 promoter replaced by the *tet*(M) promoter upstream of *gus*A	This study
pHCMCO5	*Escherichia coli* / *B. subtilis* shuttle vector	Bacillus Genetic Stock Centre, USA
pHCMCO5-P*tet*(M)-*gus*A	pHCMC05 containing the *tet*(M) promoter upstream of *gus*A	This study

TABLE 2. Minimal inhibitory concentrations (MICs) and sub-inhibitory concentrations (MIC/4) to ethanol (ETOH), hydrogen peroxide (HP), chlorhexidine digluconate (CHX) and sodium hypochlorite (SH) determined for *B. subtilis* containing the *gus*A reporter construct (BS34A:: pHCMCO5-P*tet*(M)-*gus*A) and *B. subtilis* used as donor in the Tn*916* transfer experiment (BS34ASTR).

	BS34A:: pHCMCO5-P*tet*(M)-*gus*A		BS34ASTR	
Biocide	**MIC**	**MIC/4**	**MIC**	**MIC/4**
ETOH (%)	16	4	8	2
HP (%)	0.008	0.002	0.004	0.001
CHX (%)	0.0002	0.00005	0.0002	0.00005
SH[a] (%)	0.5	0.125	0.5	0.125

[a]SH solution containing 14 % available chlorine.

TABLE 3. The effect of ethanol (ETOH), hydrogen peroxide (HP), chlorhexidine digluconate (CHX) sodium hypochlorite (SH) and tetracycline (TET) on the conjugative transfer of Tn*916*. Transfer frequencies were calculated as transconjugants per output donor cells. The *p*-value was determined using the paired Student's *t*-test. Abbreviations: R; recipient, D; donor, TF; transfer frequency.

	Output cells (CFU/ml)									
	Control				**Exposure to ETOH (2 %)**				**TF ratio**	
Expt.	R	D	R / D	TF	R	D	R / D	TF	**(ETOH / control)**	
1	2.8E+09	2.1E+09	1.4	4.8E-10	2.7E+09	1.0E+09	2.7	1.6E-09	3.2	
2	4.4E+09	2.3E+09	1.9	1.1E-09	4.5E+09	1.5E+09	3.0	1.9E-09	1.7	
3	2.4E+09	2.6E+09	0.9	3.5E-10	6.9E+09	1.4E+09	5.0	2.0E-09	5.9	
4	3.3E+09	5.1E+09	0.7	3.3E-10	5.2E+09	1.8E+09	2.9	3.3E-09	9.9	
5	2.1E+09	1.7E+09	1.2	1.0E-09	4.3E+09	1.8E+09	2.4	2.9E-09	2.9	
Avg.	*3.0E+09*	*2.8E+09*	*1.2*	*6.6E-10*	*4.7E+09*	*1.5E+09*	*3.2*	*2.3E-09*	*4.7*	*p-value*
SD	*9.0E+08*	*1.4E+09*	*0.5*	*3.8E-10*	*1.5E+09*	*3.4E+08*	*1.0*	*7.4E-10*	*3.3*	*0.01*

	Output cells (CFU / ml)									
	Control				**Exposure to HP (0.001 %)**				**TF ratio**	
Expt.	R	D	R / D	TF	R	D	R / D	TF	**(HP / control)**	
1	2.5E+09	2.3E+09	1.1	4.4E-10	4.4E+09	9.6E+08	4.6	4.6E-09	10.3	
2	5.2E+09	3.6E+09	1.4	1.3E-09	4.9E+09	3.5E+09	1.4	1.6E-09	1.3	
3	3.1E+09	2.6E+09	1.2	4.6E-10	3.6E+09	2.7E+09	1.3	7.7E-10	1.7	
4	3.2E+09	2.2E+09	1.4	1.8E-09	2.6E+09	2.6E+09	1.0	2.4E-09	1.3	
5	2.5E+09	2.4E+09	1.0	5.1E-10	3.1E+09	1.8E+09	1.7	2.7E-09	5.2	
Avg.	*3.3E+09*	*2.6E+09*	*1.2*	*9.1E-10*	*3.7E+09*	*2.3E+09*	*2.0*	*2.4E-09*	*4.0*	*p-value*
SD	*1.1E+09*	*5.6E+08*	*0.2*	*6.3E-10*	*9.5E+08*	*9.6E+08*	*1.5*	*1.4E-09*	*3.9*	*0.12*

	Output cells (CFU / ml)									
	Control				**Exposure to CHX (0.00005 %)**				**TF ratio**	
Expt.	R	D	R / D	TF	R	D	R / D	TF	**(CHX / control)**	
1	3.9E+09	1.9E+09	2.1	4.6E-10	5.2E+09	1.5E+09	3.6	4.8E-10	1.0	
2	5.2E+09	2.7E+09	1.9	1.3E-09	4.8E+09	2.5E+09	1.9	6.8E-10	0.5	
3	4.5E+09	2.5E+09	1.8	3.1E-10	3.1E+09	2.8E+09	1.1	2.3E-10	0.7	
4	2.4E+09	2.6E+09	0.9	6.4E-10	4.8E+09	3.3E+09	1.5	3.0E-10	0.5	
5	1.9E+09	1.0E+09	1.8	2.0E-09	3.3E+09	1.9E+09	1.7	1.8E-09	0.9	
Avg.	*3.6E+09*	*2.1E+09*	*1.7*	*9.3E-10*	*4.2E+09*	*2.4E+09*	*2.0*	*6.9E-10*	*0.7*	*p-value*
SD	*1.4E+09*	*7.0E+08*	*0.5*	*6.8E-10*	*9.7E+08*	*7.3E+08*	*1.0*	*6.3E-10*	*0.3*	*0.10*

	Output cells (CFU/ml)

	Control				**Exposure to SH[a] (0.125 %)**				**TF ratio**	
Expt.	R	D	R / D	TF	R	D	R / D	TF	**(SH / control)**	
1	2.5E+09	2.3E+09	1.1	4.4E-10	2.5E+09	2.5E+09	1.0	3.8E-09	8.6	
2	5.2E+09	3.6E+09	1.4	1.3E-09	3.7E+09	4.7E+09	0.8	1.6E-09	1.2	
3	3.1E+09	2.6E+09	1.2	4.6E-10	4.3E+09	3.0E+09	1.4	1.2E-09	2.5	
4	3.2E+09	2.2E+09	1.4	1.8E-09	3.3E+09	4.0E+09	0.8	9.3E-10	0.5	
5	2.5E+09	2.4E+09	1.0	5.1E-10	2.5E+09	1.9E+09	1.3	9.5E-10	1.9	
Avg.	*3.3E+09*	*2.6E+09*	*1.2*	*9.1E-10*	*3.3E+09*	*3.2E+09*	*1.1*	*1.7E-09*	*2.9*	*p-value*
SD.	*1.1E+09*	*5.6E+08*	*0.2*	*6.3E-10*	*8.0E+08*	*1.1E+09*	*0.3*	*1.2E-09*	*3.2*	*0.33*

[a]SH solution containing 14 % available chlorine.

Output cells (CFU / ml)

	Control				**Exposure to TET (10 µg/ml)**				**TF ratio**	
Expt.	R	D	R / D	TF	R	D	R / D	TF	**(TET / control)**	
1	3.8E+09	2.1E+09	1.8	1.8E-09	2.4E+09	2.4E+08	9.7	1.0E-08	5.8	
2	5.3E+09	2.9E+09	1.8	3.0E-09	8.0E+09	3.2E+08	25.0	2.1E-08	7.0	
3	2.8E+09	3.2E+09	0.9	6.1E-10	6.8E+09	8.1E+08	8.4	1.1E-08	18.6	
4	3.5E+09	3.7E+09	1.0	2.6E-10	7.8E+09	6.4E+08	12.2	5.3E-09	20.8	
5	2.2E+09	2.6E+09	0.9	1.1E-09	2.5E+09	9.0E+08	2.8	8.9E-09	8.2	
Avg.	*3.5E+09*	*2.9E+09*	*1.3*	*1.3E-09*	*5.5E+09*	*5.8E+08*	*11.6*	*1.1E-08*	*12.1*	*p-value*
SD	*1.2E+09*	*6.0E+08*	*0.5*	*1.1E-09*	*2.8E+09*	*2.9E+08*	*8.2*	*5.9E-09*	*7.1*	*0.01*

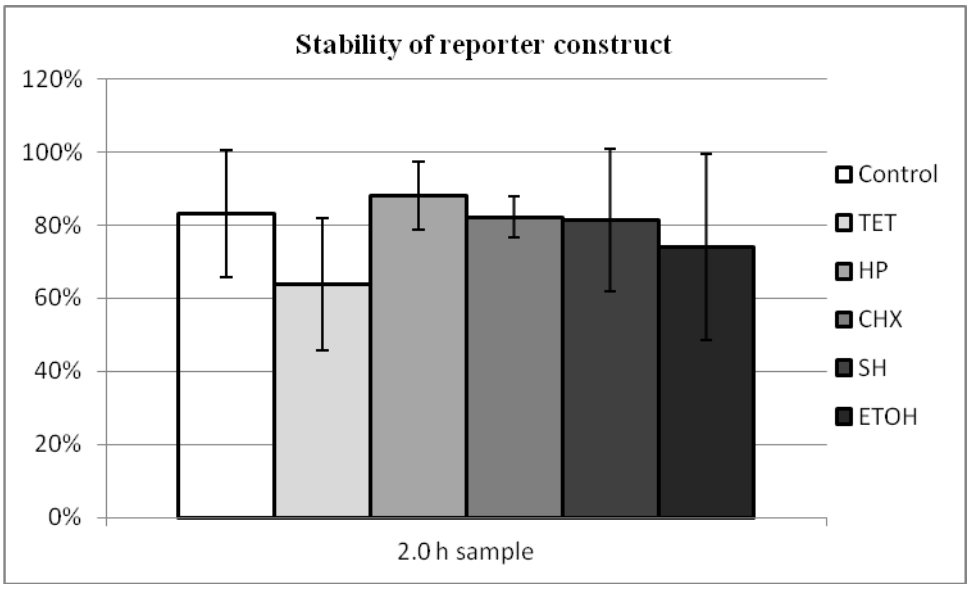

FIG S1. Stability of the reporter plasmid construct pHCMCO5-P*tet*(M)-*gus*A in *B. subtilis* in the β-glucuronidase enzyme assay was determined by plating the 2.0 h sample onto BHI agar with (+) and without (-) addition of 10 µg/ml chloramphenicol. Error bars correspond to ± 1 × standard deviation.

Table S1. Tn*916* stability in *B. subtilis* control and exposed pre-mating donor cultures. Donor cells were plated on BHI agar with and without 10 µg/ml tetracycline to estimate the stability.

		Tn*916* stability in pre- mating cultures (control / donor)
Experiment 4	ETOH	1.9
	HP	1.0
	CHX	1.0
	SH	0.3
	TET	0.3
Experiment 5	ETOH	0.8
	HP	4
	CHX	0.7
	SH	1.3
	TET	0.3

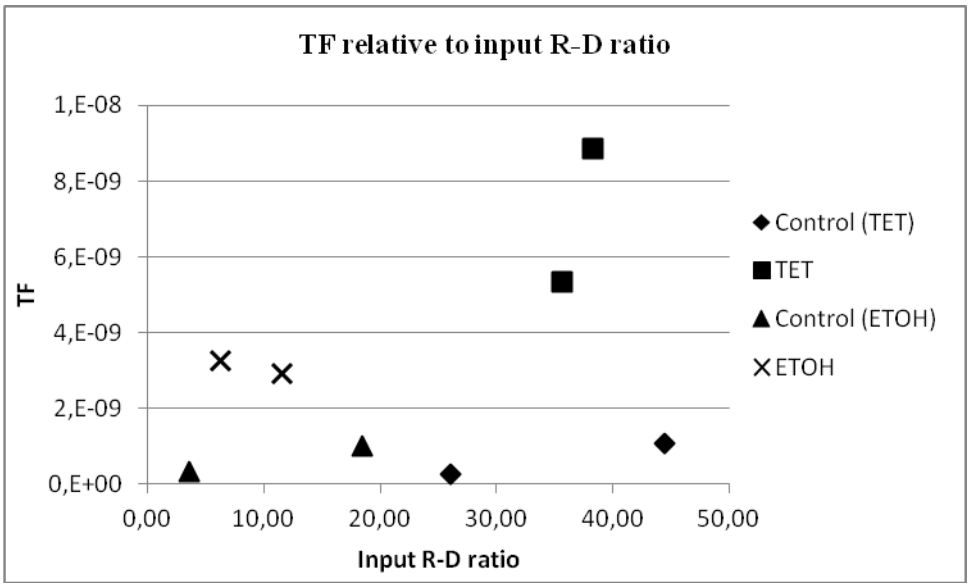

Figure S2: Transfer frequency (transconjugants per output donor) relative to input recipient–donor ratio in experiment 4 and 5. Abbreviations: TF; transfer frequency, R; recipient, D; donor, TET; tetracycline and ETOH; ethanol.

Title: **Effect of sub-inhibitory concentrations of biocides on the SOS response and mutagenesis in *Staphylococcus aureus***

Running title: Biocide effect on SOS response and mutagenesis in *Staphylococcus aureus*

Keywords: Benzalkonium chloride, chlorhexidine digluconate, hydrogen peroxide, peracetic acid, sodium hypochlorite, SOS response, and mutation rate.

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Abstract

Biocides are disinfectants widely used to reduce bacterial growth. However, residues or incorrect use may lead to the presence of sub-inhibitory concentrations. The bacterial response to sub-inhibitory concentrations of biocides is unclear. It has been suggested that the SOS response contribute to antimicrobial resistance development by inducing mutagenesis. Therefore, the effect of sub-inhibitory concentrations of five common biocides; benzalkonium chloride (BC), chlorhexidine digluconate (CHX), hydrogen peroxide (HP), peracetic acid (PAA), and sodium hypochlorite (SH) on *rec*A expression and mutagenesis in *Staphylococcus aureus* was studied. An assay based on a *S. aureus recA-lacZ* fusion strain and blue-coloring on TSA-X-gal was used to estimate *rec*A expression. Mutagenesis was assessed by counting colonies on TSA with rifampicin (100 mg/L). Benzalkonium chloride, CHX, and HP were found to increase *rec*A expression, whereas PAA had little effect and SH decreased expression. In addition, HP and PAA were found to significantly ($p \leq 0.05$) increase the mutation rate 5-15 and 3-8 fold, respectively. These results suggest that exposure to sub-inhibitory concentrations of HP and PAA may contribute to emergence of antimicrobial resistance in *S. aureus*. This may be of potential risk for human health, since these disinfectants are widely used at hospitals and in the food industry.

Introduction

Biocides are chemical compounds with antimicrobial properties. They are widely used for disinfection, antisepsis and preservation purposes in various settings to inhibit or reduce the bacterial load of microorganisms (McDonnell & A D Russell 1999). At hospitals, biocides are extensively used for disinfection of equipment and inventory to eliminate the presence of pathogens on surfaces or object that patients or personal come into contact with (Department of Antibiotic Resistance and Hospital Hygiene 2004). They are also widely applied for hand disinfection to

prevent transmissions of pathogens and thereby the spread of disease among patients via the hands of the personnel (Department of Antibiotic Resistance and Hospital Hygiene 2004). In animal husbandries biocides are used for disinfection of stable, areas transport vehicles and for direct application on animals to prevent emergence and dissemination of animal diseases (Lassen et al. 2001). In the food industry biocides are widely used for disinfection of machinery and inventory to prevent food contamination and as a preservative in foods to inhibit spoilage (Lassen et al. 2001). There is no doubt that biocides play a very important and beneficial role in human health and welfare. However, the widespread use of biocides has in recent years lead to concerns about their potential role in the development of antimicrobial resistance in bacteria through increased mutation rate (MR) or the horizontal transfer of resistance determinants (SCENIHR 2009; EFSA 2008; van der Veen & Abee 2011).

The SOS response constitutes one of the stress response systems in bacteria. It is activated upon direct or indirect DNA damage and helps bacteria to survive and adapt to environmental stress conditions (Foster 2007). The SOS response is governed by the activator RecA and the repressor LexA. DNA damage giving rise to single stranded DNA (ssDNA) is recognized by RecA that binds to ssDNA forming nucleoprotein complexes, which in turn stimulate the dissociation of the repressor LexA. Destruction of LexA causes the de-repression of SOS-related genes (Foster 2007). Genes constituting the SOS response have been identified in a number of bacteria including *Staphylococcus aureus* (Cirz et al. 2007), *Bacillus subtilis* (Au et al. 2005), *Listeria monocytogenes* (Van der Veen et al. 2010), *Escherichia coli* (Fernández De Henestrosa et al. 2000; Wade et al. 2005), and *Pseudomonas aeruginosa* (Cirz et al. 2006). The SOS response systems of the various bacterial species have in common that they are controlled by the positive and negative regulator, RecA and LexA, respectively. However, the genes and number of genes constituting the SOS response in the different bacterial species differ. In *S. aureus* and *P. aeruginose* 16 and 15 genes

have been found to be repressed by LexA, whereas the SOS responses of *E. coli* and *B. subtilis* include 31 and 33 genes, respectively, yet, only eight of these genes are homologous to each other (Cirz et al. 2007; Au et al. 2005; van der Veen et al. 2010; Fernández De Henestrosa et al. 2000; Wade et al. 2005; Cirz et al. 2006). Activation of the SOS response has been shown to induce mutagenesis in bacteria and promote the transfer of resistance and virulence determinants (Peng Wang et al. 2010; Mamber & Kolek 1993; Cirz et al. 2007; Ubeda et al. 2005; Thi et al. 2011; Maiques et al. 2006; Cirz et al. 2005). Acquired antimicrobial resistance in bacteria due to mutations in chromosomal genes have been described for several classes of antimicrobial agents including the quinolones and rifamycins (Van Hoek et al. 2011). The SOS response might therefore be an important player in the development of antimicrobial resistance in bacteria, and any compound inducing this response might contribute to the evolution of bacterial resistance in human and animal pathogens. Despite the widespread use, knowledge on the bacterial response to sub-inhibitory concentration of biocides is relatively limited. Previous studies in bacteria have shown that exposure to sub-inhibitory concentrations of hydrogen peroxide can induce expression of *rec*A (Chang, Small, et al. 2006; Palma et al. 2004; Zheng et al. 2001). Exposure of *S. aureus* to PAA has also been shown to induce the expression of SOS-related genes. A study investigating the response of *Bacillus cereus* exposure to sub-inhibitory concentrations of several disinfectants showed that HP and PAA induced expression of genes involved in the SOS response as well as increased the MR in this bacterium (Ceragioli et al. 2010). Even though the applied concentration of biocides generally is much higher than the bactericidal dose, the efficacy of biocides are greatly affected by e.g. the presence of organic matters, over-dilution, inadequate contact time between compound and bacteria, and temperature (A. D. Russell 2003). Therefore, incorrect use of biocides or the presence of residual concentrations can lead to bacterial exposure to sub-inhibitory concentrations of biocides.

The bacterial response to such exposure is therefore an important area of investigation to elucidate the potential effect of these compounds on the development of antimicrobial resistance in bacteria.

In this study we investigated the effect of sub-inhibitory concentrations of five biocidal compounds on *recA* expression, as an indicator of SOS response induction, as well as their mutagenic effect on *S. aureus* of both human and porcine origin. The biocides constitute benzalkonium chloride, chlorhexidine digluconate, hydrogen peroxide, peracetic acid, and sodium hypochlorite that are commonly used in the health care setting and/or in the food industry.

Materials and methods

Chemicals and reagents

Tryptic soya broth (TSB), tryptic soya agar (TSA), and TSA with 5 % sheep blood (blood agar plates) were from Oxoid. Spectinomycin (SPE), rifampicin (RIF), sodium hypochlorite (SH); 10-15 % available chlorine, chlorhexidine digluconate (CHX); 20 % (w/v), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and dimethyl sulfoxide (DMSO);≥ 99 % were provided from Sigma - Aldrich. Ciprofloxacin (CIP) and hydrogen peroxide (HP); 30 % solution were from Fluka, Sigma-Aldrich. Benzalkonium chloride (BC); 50 % (w/w) aqueous solution was obtained from Alfa Aesar and peracetic acid (PAA); 38 to 40 % was from Merck. Solvent used for stock solutions of CIP (25 mg/ml) was 0.1 M hydrochloric acid (HCl), whereas DMSO was used as solvent for RIF (25 mg/ml) and X-gal (20 mg/ml).

Bacterial isolates

An overview of the *S. aureus* isolates included in this study is shown in Table 1.

Effect of biocides on β-galactosidase expression

The effect of biocides on *rec*A gene expression in *S. aureus* was carried out using a previously described β-galactosidase plate assay (Anita Nielsen et al. 2010) using the *S. aureus rec*A-*lac*Z fusion strain HI2682 (Gottschalk et al. submitted). Shortly, *S. aureus* HI2682 was grown overnight (ON) in TSB supplemented with 100 µg/ml SPE at 37˚C shaking (140 rpm). One ml of the ON culture was diluted to an OD_{600} of 0.05 in TSB and was subsequently poured into petri dishes with 25 ml TSA (42 ˚C), 100 µg/ml SPE, and 150 µg/ml X-gal. All plates were dried and 6 × 5 mm wide wells were formed in plates with sterile straws. Five-fold dilutions of CIP (0.16 to 500 µg/ml), BC (0.32 to 1000 µg/ml), CHX (6.4 to 20,000 µg/ml), HP (0.001 to 4 %), PAA solution (0.006 to 20 %), and SH solution (0.032 to 100 %) were prepared and 40 µl of each dilution were transferred to five of the wells in the agar plates. Forty µl of MiliQ water was added to the sixth well as a control compound. Agar plates containing SPE and X-gal, and dilutions of biocides and CIP, but without incorporation of bacterial cells served as a sterility control of compounds and to test for unintended dissociation of X-gal caused by the compounds. Hydrochloric acid was used as a solvent for CIP and was therefore included in the assay to ensure that HCl did not affect the activity of β-galactosidase. Plates containing TSA and bacterial cells served as growth controls and plates solely containing TSA served as sterility control of the medium. All plates were incubated 18 to 20 hours at 37 ˚C in an aerobic atmosphere. Next, plates were examined for β-galactosidase activity indicated by formed blue colour in the agar due to hydrolysis of X-gal by β-galactosidase. Furthermore, the sizes of inhibition zones (concentrations greater than the minimal inhibitory concentration (MIC)) were noted. The β-galactosidase plate assay was performed in three independent experiments.

Determination of MIC to biocides

The MIC of *S. aureus* isolate 1, 2, 3, 4, and 5 were determined to BC (0.078 to 40 µg/ml), CHX (0.031 to 16 µg/ml), HP (0.0005 to 0.256 %), PAA solution (0.001 to 0.4 %), SH solution (0.004 to

2 %), and CIP (0.016 to 8 µg/ml) in TSB following the procedure described in the CLSI guideline (CLSI 2009). Briefly, isolates were grown ON on blood agar plates at 37°C. Colonies were re-suspended in 0.9 % NaCl to a McFarland standard of 0.5 (~ 10^8 cfu/ml) and further diluted 100-fold in TSB (~10^6 cfu/ml). Working concentrations of biocides were prepared in TSB just prior transferring the compounds to round-bottom 96 well micro-titre plates (Nunc). Two-fold dilutions of compounds were made in the plates (50 µl per well) followed by the addition of 50 µl diluted cell suspension (~ 5×10^5 cfu per well). One column contained a two-fold lower concentration than the lowest concentration of the test interval and served as a sterility control. One column only including TSB and bacteria served as growth control. Plates were incubated 16 to 20 h in an aerobic atmosphere at 37 °C and afterwards visually inspected. The MIC-value corresponded to the lowest concentration of compounds giving rise to no visible growth. Minimal inhibitory concentrations were determined in duplicates and as a minimum repeated twice. Only two-fold variations within duplicate determinations were accepted and the most frequent MIC-value was considered as the final MIC. *S. aureus* ATCC 29213 was included as control strain for reproducibility of the susceptibility testing procedure.

The effect of biocides on the mutation rate (MR) in *S. aureus*

Isolate 1, 2, 3, 4 and 5 were incubated ON on blood agar plates at 37°C, where after cultures of each isolate were prepared in TSB and grown ON shaking (150 rpm) at 37°C. Then, ON cultures were diluted in TSB to an OD_{600} of ~0.1 in a final volume of 100 ml in 500 ml Erlenmeyer flasks. Cells were grown shaking at 37°C until an OD_{600} of 0.5 to 0.7 was reached. Ten ml of bacterial culture was subsequently transferred to six 50 ml Falcon tubes giving a final concentration of ½ × MIC (BC, CHX, HP, and CIP) or ¼ × MIC (SH and PAA) and further incubated for 1 h. A vial with 10 ml cell culture served as the control. Control and exposed cultures originated from the same

culture flask. Then, 100 µl from each culture were serial diluted (10^{-1} to 10^{-7}) in 0.9 % NaCl and 3 × 10 µl were spotted onto TSA plates and incubated ON at 37 ˚C to estimate the number of viable cells. Two to 10 ml of each exposed culture were harvested by centrifugation (6000 × g, 4˚C, 10 min) and re-suspended in 100 µl 0.9 % NaCl. Re-suspensions were plated on big TSA plates containing 100 µg/ml of RIF. The number of mutants was counted after 48 h of incubation at 37 ˚C in an aerobic atmosphere. At least three independent experiments were carried out per compound. Mutation rates were calculated as RIF^R mutants per total viable cells. The effect (E) of biocides and CIP on the mutation rate was calculated by dividing the MR of the exposed culture with the MR of the control culture. E was assumed to follow a normal distribution and outliers in the data set were identified by determining the upper and lower fence of the data (Johnson 2005). Data point beyond the upper and lower fence values were excluded from the dataset (Johnson 2005). The significance of E was statistically tested using the paired, two-sided, Student's t-test. A pair represents the MR of the control and the treated cultures within an experimental repetition.

Results and discussion

Effect of biocides on β-galactosidase activity

The effect of BC, CHX, HP, PAA, SH, and CIP on *rec*A gene expression in *S. aureus* was estimated using a β-galactosidase (*lac*Z) reporter construct in *S. aureus*. The reporter assay indirectly measures transcription of *rec*A through expression of β-galactosidase that can be visualized by formation of a blue colour in the agar due to hydrolysis of X-gal by β-galactosidase in the medium. RecA constitute the positive regulator of the SOS response and the activity of β-galactosidase is therefore an indirect measurement of the activity of the SOS response. The β-galactosidase plate assay showed to be a simple assay to screen for the effect of several biocides on the SOS response. Since the bacterial response to compounds is dose-dependent it was an easy

method to include a large spectrum of concentrations due to diffusion of compounds into the agar. The results were generally easy to read with the exception of one compound PAA. The assay was repeated on three independent occasions and gave rise to consistent results for all compounds.

The effect of biocides and CIP on β-galactosidase activity is shown in Figure 1. To ensure that the tested concentrations affected cells only wells giving rise to an inhibition zone were included as a valid result, since the formation of blue colour on the inner site of wells containing MQ water was detected. Ciprofloxacin is known to induce the SOS response (Lili Rosana Mesak et al. 2008; Peng Wang et al. 2010; Cirz et al. 2007, 2005; Kelley 2006) and was included as a positive control in the assay. Ciprofloxacin was found to induce expression of β-galactosidase in all experimental repetitions. Benzalkonium chloride, CHX, and HP induced expression of β-galactosidase, whereas PAA had little effect and SH was found to inhibit enzyme activity. Benzalkonium chloride and CHX are both membrane acting compounds. The mode of action is believed to follow a sequence of events including adsorption to the bacterial cell surface, diffusion through the cell wall, binding to and disruption of the cytoplasmic membrane causing release of intracellular constituents (Denton 1991; Merianos 2001). Interaction and disruption of the cell membrane are likely to affect the physiological state of the cell and might interfere with DNA metabolism leading to activation of the SOS response e.g. by stalled replication forks. In a study by Ceragioli et al. (2010) the transcriptomic response to BC, HP, PAA, and SH in *B. cereus* was investigated at mild, growth-arresting, and lethal concentrations and induction of *rec*A by BC was not detected in this organism (Ceragioli et al. 2010). Locher et al. (2010) studied the effect of CHX on the SOS response in a *sul*A fusion *Escherichia coli* strain and the SOS response was not found to be induced by CHX in *E. coli* (Locher et al. 2010). Genes involved in the SOS response are expressed at different stages of the SOS induction and *sul*A represents one of the latest expressed genes, whereas *rec*A is expressed at an earlier stage of the induction (Kuzminov 1999). Furthermore, a few studies have

investigated the genotoxic potential of CHX in mammalian cells due to its application as an endodontic compound. However, these results have shown to be very ambiguous; some studies demonstrated a genotoxic effect of CHX and others did not (Ribeiro 2008). The effect of biocides are very dependent on the concentration and exposure time and can also vary between bacterial species (a D Russell & McDonnell 2000; McDonnell & A D Russell 1999; Chang, Toghrol, et al. 2006). The different responses to BC and CHX may therefore be due to variations between species or the experimental set-up. Hydrogen peroxide is a strong oxidizing compound that reacts by producing highly reactive hydroxyl radicals, which causes DNA damage (Block 2001). Hydrogen peroxide has been shown to evoke induction of the SOS response in bacteria, including *S. aureus*, which therefore is in accordance with our findings (Ceragioli et al. 2010; Bol & Yasbin 1990; Goerlich et al. 1989; Chang, Small, et al. 2006). The effect of PAA in the β-galactosidase assay was unclear; the response to PAA exposure differed between wells containing the highest and second highest concentration. The inhibition zone at the highest concentration (X_0) was preceded by a colour close to the background, which again was followed by a small increase in the blue colour. However, the well including the second highest concentration (X_1) of PAA lead to formation of a stronger blue colour just preceding the inhibition zone. Peracetic acid belongs to the chemical group of peroxygens like HP and also reacts with the bacterial cells by formation of the highly oxidative hydroxyl radical (Block 2001). It could therefore be expected that HP and PAA exposure in *S. aureus* would induce a similar response in *rec*A expression. Also, HP and PAA exposure to *B. cereus* demonstrated a large overlap in the transcriptional response to HP and PAA exposure, including up-regulation of the *rec*A gene (Ceragioli et al. 2010). Furthermore, Chang et al. (2006) studied the transcriptome response to PAA in *S. aureus* and showed that exposure to PAA up-regulated transcription of DNA repair-related genes indicating DNA damage upon PAA exposure (Chang, Toghrol, et al. 2006). It is therefore likely that PAA can induce the SOS response in *S.*

aureus even though our result was unclear. Exposure to SH gave rise to a large white zone surrounding the inhibition zone indicative of reduced activity of β-galactosidase. In water SH dissociates into Na^+ and the hypochlorite ion (OCl^-) and establishes equilibrium with hypochlorous acid (HOCl). Hypochlorous acid is believed to be the active moiety of SH, but the exact mechanism of action is not entirely understood (Dychdala 2001). Although, it has been suggested that SH produce the same reactive oxidative species as HP in the cell, the two compounds were not found to have a similar effect on β-galactosidase activity. Also, HP and SH induced different transcriptional responses in *B. cereus*; HP was found to specifically induce expression of *rec*A whereas SH was not (Ceragioli et al. 2010).

Effect of sub-inhibitory concentrations of biocides on the MR in *S. aureus*

In the β-galactosidase assay induction of enzyme activity was observed on the border of and near the inhibition zone. Bacterial exposure to sub-inhibitory concentrations of biocides corresponding to ½ × MIC was therefore included in the mutation rate experiments. However, this concentration resulted in a 5 to 6 $\log_{10}$ reduction in the viable cell counts when exposed to SH and PAA, why a concentration corresponding to ¼ × MIC of these compounds was used instead. Five different isolates of *S. aureus*, including isolates of human and pig origin, were included in the mutation rate experiment. The sub-inhibitory concentrations of BC, CHX, HP, PAA, SH, and CIP that was used in the MR experiment are shown in Table 2 and their effect on the mutation rate in the five *S. aureus* isolates are illustrated in Figure 2.

Hydrogen peroxide and PAA were found to significantly induce the MR in *S. aureus* by 5- to 15-fold and 3- to 8-fold, respectively. The effect on the MR in response to HP and PAA exposure was found to be isolate dependent. Both compounds induced the MR in two out of the five isolates, and in one of these isolates a response to both compounds was observed (isolate 3). No response in the

MR was detected upon exposure to the remaining compounds. In the study by Ceragioli et al. (2010) HP and PAA was found to significantly induce the MR in *B. cereus* (Ceragioli et al. 2010). They tested the effect of both mild (sub-inhibitory) and lethal concentrations of HP and PAA and only found lethal concentrations to induce the MR. This is in contrast to our study, where an increase in the MR was observed after exposure to sub-inhibitory concentrations. Ciprofloxacin is a well known inducer of the SOS response and has been demonstrated to have mutagenic properties in several different bacterial species (Couce & Blázquez 2009; Peng Wang et al. 2010; Drlica & Zhao 1997; Mamber & Kolek 1993; Henderson-Begg et al. 2006; Lili R Mesak & Davies 2009; Gillespie et al. 2005) and was also found to induce *rec*A expression in the β-galactosidase plate assay in our study. However, the compound at a concentration corresponding to ½ × MIC did not induce the MR in *S. aureus*. Mesak and Davies (2009) investigated the effect of three different CIP concentrations (0.1, 0.2, and 0.5 µg/ml) on three strains of *S. aureus* and only found the highest concentration to induce the MR in all strains (Lili R Mesak & Davies 2009). Therefore, the concentration included in our study (0.25 µg/ml) might have been too low to induce mutagenesis in *S. aureus*. Benzalkonium chloride and CHX was also not found to increase the MR in *S. aureus* even though they induced β-galactosidase expression. However, the effect on the MR seems to be highly concentration-dependent and it cannot be rejected that BC and CHX have mutagenic effects on *S. aureus* at higher concentrations. In addition, mutagenesis induced by the SOS response is likely to occur due to expression of error-prone DNA polymerases (Foster 2007). Exposure to BC and CHX might have an overall effect on DNA metabolism and interfere with DNA replication including the error-prone DNA polymerase inhibiting the introduction of mutations in the chromosome. Different biocides may also affect expression of genes in a compound dependent manner however, this is merely speculations and will need further investigation. Sodium hypochlorite did not induce the MR in *S. aureus*.

Recent studies have demonstrated that antibiotic-induced SOS responses can increase drug resistance in bacteria either by promoting transfer of resistance determinants or introduction of chromosomal mutation-mediated resistance (Beaber et al. 2004; Cirz et al. 2005; Nagel et al. 2011). Furthermore, antibiotic-induced SOS responses in *S. aureus* can affect virulence by promoting the transfer of virulence encoding factors (Ubeda et al. 2005; Maiques et al. 2006). *S. aureus* infections are an increasing public health issue worldwide causing considerable morbidity and mortality. We therefore found the effect of sub-inhibitory concentrations of biocides on the SOS response and mutagenesis in *S. aureus* to be a highly important area of investigation. Benzalkonium chloride, CHX, HP and likely PAA were found to induce the SOS response and sub-inhibitory concentrations of HP and PAA showed to have a mutagenic effect on *S. aureus.* Hydrogen peroxide- and PAA-induced mutagenesis in *S. aureus* is to our knowledge for the first time described in this study. Hydrogen peroxide is widely used for disinfection, antisepsis and sterilization purposes and is commonly used in the food industry, at hospitals and in animal husbandries (Lassen et al. 2001; McDonnell & A D Russell 1999). Peracetic acid is widely applied for disinfection of hospital equipment and inventory and CHX is a commonly used antiseptic especially in the healthcare setting, but also for live-stock animals (Lassen et al. 2001; McDonnell & A D Russell 1999). Quaternary ammonium compounds, such as BC, are also commonly used as an antiseptics or disinfectants in animal husbandry (Lassen et al. 2001; McDonnell & A D Russell 1999). These findings is thus of potentially major clinical relevance because exposure of *S. aureus* to sub-inhibitory concentrations of these compounds due to e.g. improper use or the presence of residual concentrations, might increase the pathogenesis of these isolates through promoting transfer of drug resistance or virulence determinants or by introducing mutations that give rise to antimicrobial resistance. Also, *S. aureus* is a frequent colonizer of live-stock animals, especially pigs, and *S. aureus* (CC398) has been shown to colonize and cause infections in humans (EFSA 2008).

Exposure of pig *S. aureus* isolates to sub-inhibitory concentrations of these biocides may contribute to the development of more pathogenic strains that might spread to humans and may compromise a potential threat to public health.

The relevance of laboratory findings to what may occur in real life is difficult to ascertain, however the likely effect of these biocides on the SOS response and mutagenesis in *S. aureus* demonstrated in the laboratory points toward the potential of what might occur in natural environments. However, further experiments including field studies are needed to evaluate the potential risks of biocides under different real-life conditions.

Conclusion

Our results showed that BC, CHX, and HP are likely to induce the SOS response and that HP and PAA are able to induce mutagenesis in *S. aureus* inducing antimicrobial resistance development. This is highly clinical relevant since CHX, HP, and PAA are frequently used in the health care settings. Also, these compounds, including BC, are commonly used in animal husbandries and *S. aureus* among live-stock animals has the potential to spread to humans. Our findings also emphasizes that incorrect use of biocides might have unintended consequences and therefore highlights the importance of correct use of these compounds and that the presence of residual concentrations might be an important issue to consider. The effect of biocides on the evolution of antimicrobial resistance in bacteria is therefore an important area of investigation and further studies, taking real-life condition into consideration are needed to evaluate the potential risk of biocides used in different settings.

Acknowledgements

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Tables and figures

Table 1. *Staphylococcus aureus* isolates included in this study. SPE^R; spectinomycin resistant.

Isolate no.	Name	Clonal complex	Relevant information	Ref.
-	HI2682	CC8	8325-4 WT with pMTC100 including a *recA-lacZ* construct and SPE^R marker, incorporated into the host chromosome	(Gottschalk et al. in preperation)
1	8325-4 WT	CC8	Derivative of NCTC 8325 devoid of bacteriophages and plasmids	(Richard Novick 1967)
2	8797	CC45	Human origin	(De Vries et al. 2009)
3	34801	CC5	Human origin	(De Vries et al. 2009)
4	2007-70-52-4	CC398	Pig origin	(Hasman et al. 2010)
5	72-12400-1	CC30	Pig origin	(Hasman et al. 2010)

Table 2. Minimal inhibitory concentrations (MICs) of *S. aureus* isolates to benzalkonium chloride (BC), chlorhexidine digluconate (CHX), hydrogen peroxide (HP), peracetic acid (PAA), sodium hypochlorite (SH), and ciprofloxacin (CIP) and concentrations used in the mutation rate experiment corresponding to ½ × MIC or ¼ × MIC.

	BC (µg/ml)		**CHX (µg/ml)**		**HP (%)**		**PAA sol.[1] (%)**		**SH sol.[2] (%)**		**CIP (µg/ml)**	
Isolate no.	MIC	½ × MIC	MIC	½ × MIC	MIC	½ × MIC	MIC	¼ × MIC	MIC	¼ × MIC	MIC	½ × MIC
1	2.5	1.25	4	2	0.032	0.016	0.2	0.05	1	0.25	0,5	0,25
2	2.5	1.25	4	2	0.032	0.016	0.2	0.05	1	0.25	0,5	0,25
3	10	5	4	2	0.032	0.016	0.2	0.05	1	0.25	0,5	0,25
4	2.5	1.25	4	2	0.032	0.016	0.2	0.05	1	0.25	0,5	0,25
5	2.5	1.25	4	2	0.032	0.016	0.2	0.05	1	0.25	0,5	0,25

[1] PAA solution containing 38 to 40 % PAA; [2] SH solution containing 10-15 % available chlorine

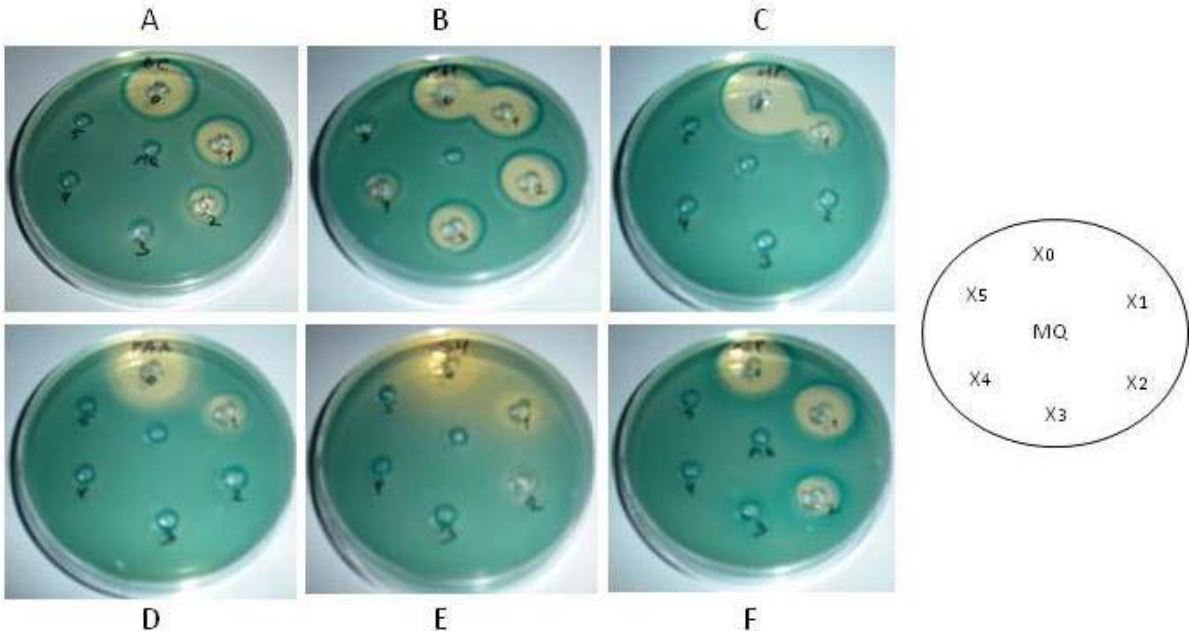

Figure 1: The effect of biocides and ciprofloxacin on β-galactosidase expression in *S. aureus.* A 5-fold dilution series of each compound was prepared and 40 µl of each dilution was transferred to wells in agar plates incorporated with the *S. aureus* HI2682 *rec*A-*lac*Z fusion strain. MQ water was included as control compound. Plates were incubated 18 to 20 h at 37˚C in an aerobic atmosphere. A; exposure to benzalkonium chloride (BC), B; exposure to chlorhexidine digluconate (CHX), C; exposure to hydrogen peroxide (HP), D; exposure to peracetic acid (PAA), E; exposure to sodium hypochlorite (SH), and F; exposure to ciprofloxacin (CIP). X0...X5; highest to lowest concentration.

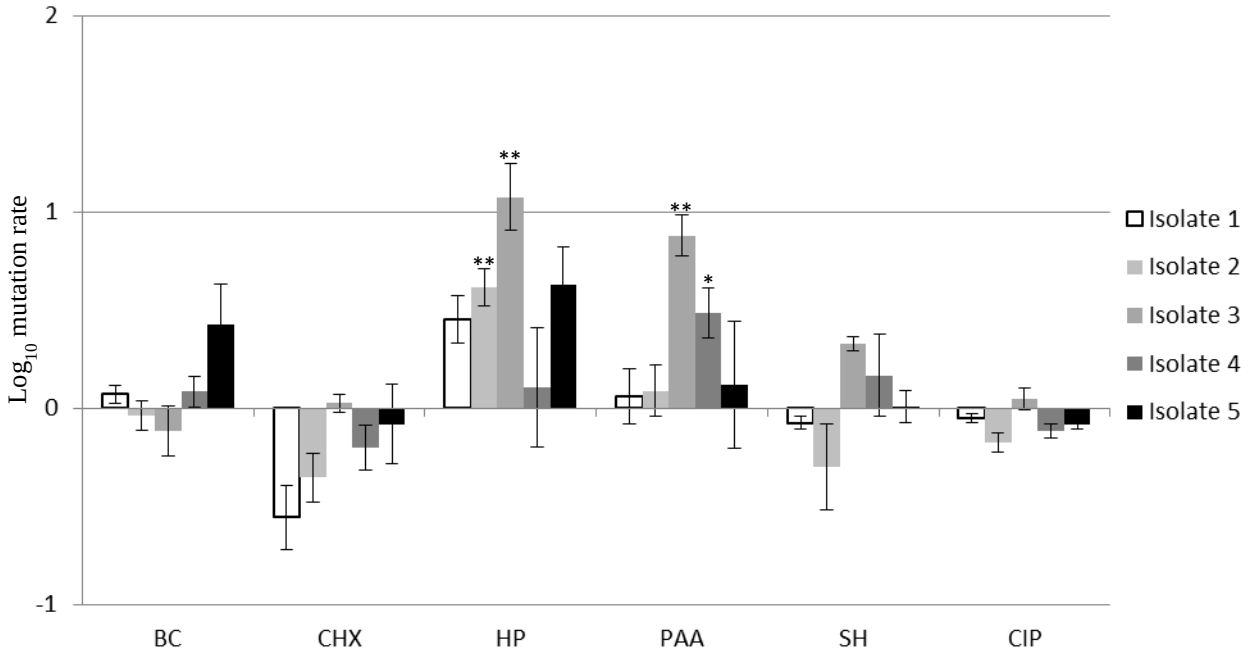

Figure 2. The average effect of benzalkonium chloride (BC), chlorhexidine digluconate (CHX), hydrogen peroxide (HP), peracetic acid (PAA), sodium hypochlorite (SH), and ciprofloxacin (CIP) on the mutation rate (MR) in *S. aureus*. Mutation rates were determined by dividing the number of rifampicin mutants with the total cell count. The effect was calculated by dividing the MR of the treated culture with the MR of the control. Experiments were repeated at least three times at different occasions. Significance of the effect of biocides and CIP on the MR was determined using the paired, two-sided, Student's t-test. A pair represents the MR of the control and the treated cultures within an experimental repetition. Asterisks (**) denote statistical significance at a 1 % level ($P \leq 0.01$) and asterisk (*) denote significance at a 5 % level ($P \leq 0.05$). Error bars represent the standard error of mean.

Characterization of *Staphylococcus aureus* CC398 and CC30 isolates from pigs and identification of the biocide resistance gene *qac*G

Running title: Characterization of *Staphylococcus aureus* CC398 and CC30

Keywords: Benzalkonium chloride, hydrogen peroxide, sodium hypochlorite, formaldehyde, caustic soda, quaternary ammonium compound resistance

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Abstract

Objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA), in particular clonal complex (CC) 398, are increasingly identified in livestock animals. Recently, MRSA within CC30 was identified in Danish pigs. Here we determined the susceptibility of porcine *S. aureus* isolates of CC398 and CC30 to disinfectants used in pig farming; benzalkonium chloride, hydrogen peroxide, formaldehyde, sodium hypochlorite, and caustic soda. Furthermore, the antimicrobial resistance profiles, hemolysis properties, and the presence of TSST-1 and PVL encoding virulence factors were investigated.

Methods: In total, 79 porcine *S. aureus* isolates were included in the study. The susceptibility to biocides and antimicrobial agents were determined by the micro-dilution method. The presence of quaternary ammonium compound (QAC) efflux pumps causing resistance was analysed using an ethidium bromide efflux pump assay. Isolates were screened for *lukPV* and *tst* genes with PCR and haemolytic activities were determined using a simple agar plate assay.

Results: *S. aureus* isolates did in general not show reduced susceptibility to the biocides tested. However, a QAC resistance gene, *qacG*, was detected in MRSA CC30 isolates. Resistance to antimicrobial agents was generally found against compounds typically used in pigs. Isolates generally had low haemolytic activity and none of the isolates included the PVL or TSST-1 encoding genes.

Conclusion: The presence of *qacG* in MRSA CC30 is worrying, since usage of QACs may contribute to the selection and spread of these isolates. MRSA CC30 is often associated with MRSA types giving rise to clinical infections in Denmark and porcine MRSA CC30 may be prone to adapt to humans.

Introduction

During the last decade, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) has been emerging throughout Europe and North America, and food production animals are increasingly recognised as a potential reservoir of MRSA [1]. The predominant clonal lineage of LA-MRSA belongs to clonal complex (CC) CC398 and pigs are particular regarded as a reservoir of this clone [1,2]. Even though, MRSA CC398 rarely causes infections in animals, this clonal lineage has attracted much attention due to its potential to colonize and cause infections in humans, making colonized/contaminated animals a potential source of community-acquired MRSA (CA-MRSA) [1]. Human acquisition of MRSA CC398 is regarded as an occupational health risk; people in close contact with colonized/contaminated animals or their environment, such as farmers and veterinarians, are at greater risk of getting infected/colonized [1]. However, carriage of MRSA CC398 has been detected in humans without known contact to livestock animals, and it has been suggested that transmissions between humans occur [3]. In Denmark, 164 new human clinical cases of MRSA CC398 were detected in Denmark in 2011, an increase of 53 and 122 cases compared to 2010 (n = 111) and 2009 (n = 42), respectively. One hundred and thirty of these isolates belonged to *spa* type t034 of which 49 caused infections in humans [4]. MRSA CC398 thus poses an increasing risk of human health in Denmark and other countries.

Denmark has an annual pig production of approximately 30 million pigs and is one of the world's largest exporters of pork [5,6]. The most prevalent methicillin-sensitive *S. aureus* (MSSA) among Danish pigs belongs to CC398 (primarily *spa* type t034) [7]. Until recently, pig associated MRSA had only been identified among CC398 in Denmark. However, a study by Agersø et al. (2012) detected the presence of MRSA CC30 (*spa* type t1333) in pigs at slaughter and it was suggested that MRSA CC30 may have the potential to spread among Danish pigs, as MSSA CC30 (primarily *spa* type t1333) is the second most common *S. aureus* in Danish pigs [8]. Recently, the first cases of human

nasal carriage of porcine associated MRSA CC30 (t1333) were detected. Isolates were identified in both pig farmers and in pigs at the farm, indicating the potential of pig to human transfer [9]. MRSA CC30 of *spa* type t019 is one of the most prevalent MRSA types giving rise to clinical infections and asymptomatic carriage in humans [10], and the presence of MRSA isolates in pigs belonging to the same clonal complex is of concern to the public health, since these strains might be prone to adapt to humans.

MRSA CC398 isolates often lack important virulence-associated factors that are often found in healthcare-associated (HA)-MRSA and CA-MRSA isolates; the toxic shock syndrome toxin (TSST-1) has not yet been reported in CC398, and carriage of the Panton-Valentine-Leucocidin (PVL) has only been identified in a few cases [1,11,12]. The virulence of *S. aureus* CC30 of porcine origin has to a much lesser extent been examined and so far there has been no reports of these virulence encoding genes in porcine *S. aureus* CC30 [13].

With the growing awareness of food safety, biocides are increasingly used in all stages of the meat production chain to reduce or inhibit growth of microorganisms and large amounts of biocides are used in controlling hygiene in the farm environment [14]. These include quaternary ammonium compounds (QACs), peroxygens, chlorine compounds and aldehydes [15]. The widespread use of biocides has led to concerns about development and emergence of microorganisms that are less-susceptible to biocides [14]. Acquired reduced susceptibility to certain biocides has been described [16]. Examples include *Staphylococcus* spp. showing less susceptibility to QACs and have been isolated from dairy cattle and goat herds [17–19]. The genetic determinants for reduced susceptibility to QACs are the *qac* genes encoding efflux pumps. Substrates of these pump systems comprise several cationic compounds, including the monovalent cations, such as the QAC benzalkonium chloride (BC) and the intercalating dye ethidium bromide (EtBr). The divalent cation chlorhexidine (CHX) that is widely used as an antiseptic compound at hospitals has also been found to be a substrate of

some QAC efflux pumps [20,21]. *qac* genes have been found to reside on mobile genetic elements, such as plasmids and integrons that also encode resistance to a range of clinical important antimicrobial agents. In *Staphylococcus* spp. *qac* genes have been reported to co-reside with genes giving rise to resistance to beta-lactams (*bla*Z), aminoglycosides (*aac*D-*aph*D), and trimethoprim (*dfr*A) [22–26] and it has been suggested that use of QACs might give a selective advantage of *qac* positive strains and lead to co-selection of antimicrobial resistance bacteria [23,27–29].

The emergence of LA-MRSA and their potential to colonize and infect humans poses an important and increasing risk for public health. The discovery of yet another clonal lineage of LA-MRSA (CC30) in Danish pigs and carried by farmers is worrying. Effective use of biocides may be important in order to reduce/eradicate MRSA in pig herds. Therefore, we investigated porcine MRSA and MSSA isolates belonging to either CC398 or CC30 for their susceptibility to active compounds of commonly used disinfectants in pig production. These disinfectants comprise benzalkonium chloride (QAC), hydrogen peroxide and sodium hypochlorite (highly oxidizing compounds), formaldehyde (aldehyde compound) and caustic soda (strong alkaline compound) [15,30]. Furthermore, the antimicrobial resistance profile, hemolysis properties, and the presence of TSST-1 and PVL encoding virulence genes were investigated.

Material and methods

Chemicals and reagents

Active disinfectant compounds included in this study were: Benzalkonium chloride (BC), 50 % (w/w) aqueous solution (VWR); Sodium hypochlorite (SH), available chlorine 10-15 % (Sigma-Aldrich); Formaldehyde solution (FA), ≥ 37 % (Merck); Hydrogen peroxide (HP), ≥ 30 % (Fluka), and Sodium hydroxide (NaOH), ≥ 98 % (Sigma-Aldrich). Premade tryptone soya agar plates with 5 % sheep blood (blood agar plates) were purchased from Oxoid. BBL™ Mueller-Hinton II broth (MHB) and agar (MHA) were from Becton, Dickinson and Company. Tryptic soy broth (TSB) and agar (TSA) were from Oxoid. Sheep blood was from Statens Serum Institute.

Strain collection

A total of 79 porcine *S. aureus* isolates were included in this study (Table S1) and comprise 21 MSSA and 40 MRSA belonging to CC398 and 13 MSSA and 5 MRSA belonging to CC30.

Twenty five of the isolates were from diagnostic submissions to The National Veterinary Institute or The National Food Institute, Technical University of Denmark [8,31]. Fifty four colonizing *S. aureus* isolates were sampled from healthy pigs (nasal swabs) at 12 different farms or from healthy pigs at seven different slaughter plants (nasal swabs before scalding) as part of the DANMAP surveillance program in 2009 [8,32,33]. The origin of two of the colonizing pig isolates was unknown. For isolates collected at slaughter plants only one sample per month was collected per Central Husbandry Register (CHR) number [8]. Isolates originating from the same farm was collected on the same day, but from different animals. In one case two isolates were collected from the same pig, however, they belonged to CC30 and CC398, respectively (isolate 53 and 81) and were therefore both included in the study.

Species identification of *S. aureus* and isolation of MRSA were performed as previously described [8,32,34,35]. The presence of the *mecA* gene encoding methicillin resistance was confirmed by multiplex PCR as previously described [35]. The *spa*-type of isolates was determined following the recommendations at SeqNet[36] using recommended primers [7,31] or primer pair 1794 (5'-AGACGATCCWTCAGTGAGC-3') and 1827 (5'-TAATCCACCAAATACAGTTGTACC-3') [8]. Based on the amplified sequence, *spa*-types were assigned to each isolate using the *spa* plugin in the BioNumerics v4.6 software (Applied Maths) [7,8,31].

Antimicrobial susceptibility testing

Determination of the Minimal Inhibitory Concetration (MIC) and Minimal Bactericidal Concentration (MBC) of biocides

Minimal inhibitory concentrations determination to BC (0.250 – 128 µg/ml), HP (0.00025 – 0.128 %), SH (0.003125 – 1.6 %), FA (0.000781 – 0.4 %), and NaOH (0.003125 – 1.6 M) were determined as recommended by the CLSI standard [37]. Selected isolates were also tested for their susceptibility to CHX (0.0625 – 32 µg/ml). Briefly, bacteria from over night (ON) blood agar plates were re-suspended in 0.9 % NaCl to a McFarland standard of 0.5 (~ 10^8 CFU per ml) and further diluted 100-fold in MHB. The size of the inoculums was determined by plating MHB cell suspensions on MHA plates and incubated ON at 37 . Working solutions of biocides were prepared in MHB and two-fold dilution series were prepared in 96-round bottom well microtiter plates (50 µl), where after 50 µl of MHB cell suspensions were transferred to the microtiter plates (Nunc) (~ 5×10^5 CFU per ml in wells). Wells with no biocides were included as positive growth controls and wells without bacteria, but containing a biocide concentration two-fold lower than the lowest concentration of the test interval, served as sterility controls. Plates were incubated at 37 for 16 to 20 hours in an aerobic atmosphere, after which cell growth was inspected by the eye. The

MIC-value was defined as the lowest concentration of the compounds giving rise to no visible growth. The minimal bactericidal concentration was determined following the NCCLS guideline [38] with minor adjustments. After the MIC-values were read, taking approximately 15 min, microtiter plates were further incubated until a total incubation time of 24 hours was reached. Then, pellets were re-suspended and 10 µl where spotted on MHA plates from wells with no visible growth, wells containing the highest concentration of biocides and where cell growth was visible, and from wells including the sterility control. Plates were incubated at 37°C and colonies were counted after 48 h of incubation. Biocide concentrations leading to a 99.9 % reduction of viable cells was regarded as the MBC-value. Minimal inhibitory concentrations and MBC determinations were performed in duplicates and only results where duplicates showed similar results or differed only by 2-fold was included. In the case of 2-fold variations the highest value was selected. *S. aureus* ATCC29213 was included as control strain for reproducibility of the susceptibility testing procedure.

Determination of the MIC of antimicrobial agents

Susceptibility to ciprofloxacin (Cip) (0.12-8 µg/ml), erythromycin (Ery) (0.12-16 µg/ml), florfenicol (Ffn) (1-64 µg/ml), chloramphenicol (Chl) (2-64 µg/ml), penicillin (Pen) (0.06-16 µg/ml), spectinomycin (Spe) (8-256 µg/ml), streptomycin (Str) (2-128 µg/ml), sulphamethoxazol (Smx) (8-512 µg/ml), tetracycline (Tet) (0.5-32 µg/ml), tiamulin (Tia) (0.25-32 µg/ml), and trimethoprim (Tmp) (1-32 µg/ml) had earlier been or were determined using the semiautomated Sensititre broth micodilution system (Trek Diagnostic Systems) as previously described [39]. Selected isolates were also tested for their susceptibility to gentamicin (Gen) (0.25-16 µg/ml). Plates were inoculated and incubated according to the CLSI guidelines (2009) [37]. *S. aureus* ATTC 29213 was used as quality control strain. The European Committee on Antimicrobial Susceptibility Testing

(EUCAST) epidemiological cut-off values (ECOFF) [40] were used to interpret MIC results as recommended by the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR) [41].

Genome sequencing

Isolate 86, 88, and 89 were whole genome sequenced (WGSed). DNA purification, sample preparation, and sequencing on an Illumina Genome Analyzer IIx (Illumina, Inc., San Diego, CA) were performed as previously described [42].

Ethidium bromide efflux pump assay and *qac*G detection

To examine for increased efflux pump activity an ethidium bromide assay was used as described previously [17]. Isolates were grown for 24 h at 37 °C on MHA containing EtBr (0.5 μg/ml), followed by inspection for fluorescence under UV light. Cells accumulating EtBr had a red fluorescence and cells that did not accumulate fluorescence were white and defined to include active efflux pumps. Strains JCM 16555 (*qacA)* and JCM 16556 (*qacB*), that overexpress efflux pumps, were included as controls.

Detection of *qac*G

Identification of QAC resistance genes in WGSed isolates showing efflux pump activity were performed by BLASTing nucleotide sequences of *qac* genes from NCBI's homepage (www.ncbi.nlm.nih.gov/) with the sequences of the isolates. Furthermore, isolates found positive in efflux pump activity were tested for the presence of *qac*G by PCR with primers: *qac*GF: TTTCGTTGGAATTTGCTT and *qac*GR: AATGGCTTTCTCCAAATACA [27]. Isolates were grown on TSA plates and DNA was extracted from bacterial colonies using a rapid boiling lysis protocol. Briefly, individual bacterial colonies were suspended in 100 μl of sterile water, incubated in an

eppendorf microcentrifuge tube for 10 min in 100 °C and then centrifugated 10 min, 8000 rpm. A 2 µl aliquot of the supernatant was used as the template for PCR. The volume of each reaction was 25 µl (12.5 µl dream taq Fermentas master mix, 1 µl (100 pmol) forward primer, 1 µl (100 pmol) reverse primer, 2 µl DNA and 8.5 µl sterile H_2O. The cycling conditions were as follows: DNA denaturation at 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. This was completed with an extension step of 72°C for 5 min. PCR products were analyzed by electrophoresis on a 1% (w/v) agarose/1× TBE gel. Primers targeting the house keeping gene *glp* were used as DNA control.

*luk*PV and *tst* detection

All isolates were screened for the virulence genes *lukPV* and *tst* using primers previously described (see list below). DNA extraction and the PCR program was the same as described above, except for the annealing temperature for *tst* that was set to 52 °C.

lukPV: Luk-PV-1 ATCATTAGGTAAAATGTCTGGACATGATCCA and
Luk-PV-2 GCATCAAGTGTATTGGATAGCAAAAGC [43].
tst: TST-1: TST-1: AGCATCTACAAACGATAATATAAAGG and
TST-2: CATTGTTATTTTCCAATAACCACCCG [44].
glp dn TGG TAA AAT CGC ATG TCC AAT TC and
glp up CTA GGA ACT GCA ATC TTA ATC C [45].

Hemolysis assay

Delta-hemolytic activities of individual bacterial isolates were assessed by cross-streaking test isolates perpendicular to the beta-hemolysin hyper producing *S. aureus* RN4220, a strain derived by multiple mutagenesis procedures from the parent strain NCTC8325 [46], on sheep blood agar plates.

Hemolysis was assayed after 24 h at 37°C and again after additional 24 h at 4°C. The latter step was performed to evoke the "hot-cold lysis" phenomenon observed with beta-hemolysis [47]. Delta-hemolytic activity was denoted by an enhanced area of hemolysis in the intersection of RN4220 and test isolate streaks.

Results and discussion

In this study we tested the susceptibility of porcine *S. aureus* CC398 and CC30 isolates to biocides that are active compounds of commonly used disinfectants in pig production. In addition susceptibility to antimicrobial agents, the presence of active efflux pumps and important *S. aureus* virulence factors was investigated.

Susceptibility to biocides and antimicrobial agents

Susceptibility to biocides

No standardized method exists for susceptibility testing of microorganism to biocides. It was therefore decided to use pre-existing methods for susceptibility testing of bacteria to antimicrobial agents; the CLSI Approved Standard [37] and the NCCLS Approved Guideline [38] for determining MIC- and MBC-values, respectively. Distributions of the MICs and MBCs of the 79 porcine *S. aureus* isolates to biocide BC, HP, SH, FH, and NaOH are shown in Figure 1. The MIC- and MBC-values can be found in Table S1. The MIC-values were generally found to be 2 or 4 µg/ml to BC, 0.001 or 0.002 % to HP, 0.05% to SH, 0.00625 % to FH, and 0.1 M to NaOH. The MBC-values were generally similar to the MIC or up to 8-fold higher. The paradoxical effect, also known as the eagle effect, was in some cases observed in the MBC determination of HP (see Table S1) namely that the number of surviving cells increased with increasing HP concentration beyond the lowest concentration giving rise to≥ 99.9 % kills [38]. The phenomenon is particular common for cell-wall

active compounds and has been described for some antimicrobial compounds (e.g. penicillin and aminoglycosides) [38] and also for a few isolates to CHX [48]. However, in our study it was only observed in MBC determinations to HP. Susceptibility testing of the control strain was reproducible within a two-fold range and the MIC- and MBC-values were within the range of the tested isolates.

The method used for MIC determination showed good agreement within duplicate determinations; duplicates were shown to have the same values in 87 to 99 % of the cases, depending on the biocide tested. A greater variance was observed for the MBC determinations in which duplicate values varied between 66 and 81 %. However, in all cases duplicate determinations did not vary more than 2-fold. The method employed to determine the susceptibility of porcine *S. aureus* isolates to biocides in this study therefore seem to be an acceptable method. Since duplicate determinations did not vary more than 2-fold it may be recommended only to include one determination in standardization of susceptibility testing of biocides to *S. aureus* isolates.

The MIC-value of *S. aureus* isolates to BC, HP, SH, and FH and MBC-values to BC, HP and SH were very similar to previous findings [28,48–52].

The porcine *S. aureus* isolates were generally distributed at two or three MIC- and MBC-values, respectively (Figure 1). In this study reduced susceptibility to a biocide was defined as a bacterial strain having a MIC- as well as a MBC-value greater than the rest of the bacterial population. Based on this criterion no isolates showed reduced susceptibility to the tested biocides and the MIC- and MBC-values were widely below the recommended working concentrations. Comparing the MIC- and MBC-values and distributions between MSSA and MRSA and between CC398 and CC30 isolates no noteworthy difference was observed. However, the distribution of the MIC-value to BC for MRSA CC30 isolates seemed to be displaced by a two-fold higher concentration (Figure 2), but this did not apply for the MBC distribution.

Susceptibility to antimicrobial agents

The phenotypic resistance profiles and distribution of resistance of the 79 *S. aureus* isolates are presented in Table 1. The resistance patterns of the *S. aureus* CC398 isolates were highly variable and represented 23 different phenotypic resistance patterns. All of the CC398 isolates were found to be Tet resistant and 84% and 56% of the isolates showed resistance to Tmp and Str, respectively. Antimicrobial resistance to Ery (46 %), Spe (38 %) and Tia (33 %) was also frequently observed, whereas resistance to Cip (20%) was found to a lesser extent. Only one isolate showed resistance to Chl-Ffn (2 %) and Smx (2 %), respectively. Thirty-one (78 %) and 16 (76 %) of the MRSA and MSSA CC398 isolates, respectively, were multi-resistant (defined as resistance to three or more classes of antimicrobial agents other than the group of β-lactams). Overall the occurrence of resistance to non-beta-lactam antimicrobials was quite similar for the MRSA and MSSA CC398 isolates, with the exception of resistance to Spe, Tmp and Str, where a higher percentage of the MRSA CC398 isolates were found to be resistant.

Multi-resistance and highly variable phenotypic resistance patterns among *S. aureus* CC398 isolates have frequently been detected [12,53–55].

The *S. aureus* CC30 isolates were arranged in 9 different phenotypic resistance patterns. None of the MSSA CC30 and one of the MRSA CC30 isolates had a multi-resistant phenotype. The group of MRSA CC30 isolates showed resistance to Pen (100 %), Tia (100 %), Ery (20 %), Tmp (20 %), and Str (100 %), whereas resistance to Pen (31 %), Tia (46 %), Ery (8 %), Tmp (8 %), Tet (15 %), Spe (8 %) and Str (8 %) was present in MSSA CC30 isolates. No more than 11 % of the total number of *S. aureus* CC30 isolates showed phenotypic resistance to Tet that is much lower than the occurrence in the CC398 isolates. The MSSA and MRSA porcine isolates belonging to CC30 have to a much lesser extent been described. However, in one study MRSA CC30 from pigs (n = 3) were found to be Tet and Ery resistant and susceptible to Chl and Cip [13]. In another study MSSA CC30

(n = 2) showed resistance to Pen or pefloxacin (a fluoroquinolone), but were susceptible to Ery [56]. In both studies susceptibilities were tested for multiple antimicrobials and therefore also showed a limited resistance phenotype.

Some of the pig isolates within the same CC were sampled from the same farm and might therefore be clonal related. Also, *S. aureus* CC398 and CC30 isolates were only sampled from the same farm in one case and the resistance profiles of *S. aureus* pig isolates belonging to CC398 and CC30 can therefore not be directly measured. However, it is worth noticing that the *S. aureus* pig isolates belonging to CC398 in general seemed more resistant than the *S. aureus* CC30 isolates. Moreover, in the one case where CC398 and CC30 isolates were sampled from the same location (Farm 1) the two *S. aureus* CC398 isolates (no. 17 and 53) showed resistance to five and eight antimicrobial agents respectively, whereas the CC30 isolate only showed resistance to one compound. Only five MRSA CC30 isolates are included in this study and drawing general assumptions to this *S. aureus* type is difficult. However, when evaluating the resistance profiles of all the *S. aureus* pigs isolates resistance seems to follow the CC; MRSA isolates were more similar to the MSSA within the same CC than the MRSA isolates from the other CC and vice versa.

Some of the most widely used groups of antimicrobial agents in pig production are tetracyclines, beta-lactamase sensitive penicillins, macrolides, pleuromutilins, penicillins with extended spectrum, and aminoglycosides [10]. The group of amphenicols and fluoroquinolones are to a much lesser extent used. The occurrence of phenotypic resistance in the porcine *S. aureus* isolates therefore correlated well with the antimicrobial agents used in pig production. One exception was the high occurrence of Cip (fluoroquinolone compound) resistance in CC398 isolates, since only a low amount of this compound is used for pigs. Other environmental conditions in pig farming might therefore contribute to the development of Cip resistance. The SOS response has been shown to be activated by sub-inhibitory concentrations of antibiotics including the quinolones and β-lactams and by HP

[57,58]. Activation of the SOS response can induce mutagenesis in bacteria leading to chromosomal mutations giving rise to Cip resistance [59]. Exposure to non-lethal doses of these compounds might therefore have the potential to contribute to development of Cip resistance in *S. aureus* pig isolates. However, this will need further studies to elucidate.

In Denmark, treatment of *S. aureus* infections may in some cases be supplemented with a macrolide (personal communication, Henrik Westh, Hvidovre Hospital, Denmark). The high level of resistance found to the macrolide Ery in the porcine *S. aureus* isolates might therefore be of clinical relevance.

Efflux pump active isolates

Disinfectants based on QACs have various applications in veterinary medicine and play an important role in the control of animal disease. Efflux-mediated resistance to QACs has been described in *S. aureus* and several QAC resistance genes have been identified [60,61]. Staphylococci including *qac*-genes do not always give rise to an increase in the MIC-value toward BC [21,28]. The porcine *S. aureus* isolates were therefore also tested for efflux pump activity, using an EtBr assay on agar plates. Four MRSA CC30 isolates (85, 86, 88 and 89) showed EtBr efflux pump activity (Figure 3) and were found positive for the *qac*G gene by WGSing and PCR (isolate no. 86, 88, and 89) and by PCR in isolate no. 85. The *qac*G gene has previously been identified in food-related staphylococci [22,28,62]. QacG has a more narrow spectrum of substrates compared to the QacA efflux pump, encoded by *qacA*, which are generally found to be associated with clinical *Staphylococcus* spp. [21]. Once before the presence of a *qac* gene has been found in MRSA from pigs in Hong Kong. However, these pig associated MRSA isolates belonged to CC8 (t899), a typical *S. aureus* clone colonizing pigs in Asia [28]. This study is therefore the first to report the presence of *qac* genes in *S. aureus* of porcine origin in Europe and in LA-MRSA belonging to CC30. Two of the strains

including the *qacG* gene were found to have higher MIC-values (8 µg/ml) compared to the remaining isolates, however two other isolates had similar MIC-values (4 µg/ml) as to some of the *qacG*-negative isolates. The MBC-values for *qacG* positive and some of the *qacG*-negative isolates were the same (8 mg/ml) and is in accordance with the study by Wong et al. (2012) [28]. The presence of *qacG* only showed small or no changes in the susceptibility to BC, and still remained lower than the recommended working concentration of BC and might therefore not be relevant in practical implications. However, a study by Otter et al. (2012) showed that use of CHX for decolonization of MRSA gave rise to a clonal-dependent increase in MRSA isolates carrying the *qacA* gene (CHX is a substrate of QacA). This was even though the level of resistance to CHX in *qacA* positive isolates was much lower than the applied concentration [63]. Incorrect use of disinfectants (e.g. over-dilution, reduced contact time with microorganisms, wrong temperature or pH), insufficient cleaning before disinfection or the presence of residual concentrations might give rise to bacterial exposure to lower level QAC concentrations and might also give strains, harbouring QAC resistance genes, a selective advantage [29]. Usage of compounds that are substrates for QAC efflux pumps might therefore select for sub-populations carrying these genes. The susceptibility to CHX of *S. aureus* isolates with and without the *qacG* was determined (Table S2). *qacG* positive isolates were found to have comparable or lower MIC/MBC-values to isolates without the gene. In the study by Wong et al. (2013), *qacG* containing isolates were found to have a mean MBC-value to CHX that was slightly higher than the *qacG* negative strains (4 µg/ml vs. 6 µg/ml) [28], and was therefore outside the detection level in our study.

In Denmark, treatment of *S. aureus* infections can be supplemented with Gen and in some cases with a macrolide as previously mentioned. Additional susceptibility testing of the *qacG* positive MRSA CC30 isolates to Gen showed that isolate 85 was also resistant to this compound. This is worrying since incorrect disinfectant procedures or the presence of residual concentrations of QACs

might select for MRSA strains that are resistant to clinical important antimicrobial agents. Also, *qac* genes have been found to co-exist on genetic elements, such as plasmids and integrons, with genes encoding resistance to important antimicrobial compounds, such as Pen, Tmp and Gen, isolated from clinical, animal or food-related staphylococci [22–24,26,64]. Use of QACs has therefore been suggested also to select for antimicrobial resistance genes located on the same genetic element [29]. The *qacG* gene was first described by Heir et al. (1999) and found to reside on a small plasmid (pST94), including two open reading frames encoding the QacG and a putative replication protein [62]. Sequence contigs including the *qac*G gene from the WGSed isolates (isolate no. 86, 88, and 89) were BLASTed against the nucleotide database at NCBI's homepage (http://www.ncbi.nlm.nih.gov/) to identify the possible location of *qac*G. Two isolates had 99% identity to the full *qacG* corresponding to position 1532 to 1855 (GenBank accession no. Y16944) and one isolate had a partial sequence on the contig corresponding to position1560 to 1855, also with more than 99% homology. The size of the contigs varied from 2093 to 2222 bp for three of the isolates and the average no. of reads were approx. 75 to 130 times higher than the average number of reads from the WGS runs of the same isolates. The size of the contigs together with the higher number of reads indicates that these plasmids are probably individual plasmids rather than integrated on the chromosome. Moreover, on the same contigs a putative replicon were identified with 92 to 93% homology to rep94 of pST94. This indicates that *qacG* in isolate 86, 88, and 89 reside on pST94 or a pST94-like plasmid. The *qacG* gene has previously been found to reside on a closely related pST94 plasmid isolated from bovine staphylococci [22] and have so far not been associated with plasmids including additional resistance genes [22,62].

Virulence of isolates

There are a number of factors that appear to play key roles in the virulence of *S. aureus.* The accessory gene regulator (*agr*) locus, a well-characterized quorum-sensing two-component regulatory system, is a principal global virulence regulator. *agr* upregulates secreted proteins, including delta-hemolysin, and delta-hemolysin is commonly used as an indicator of *agr* function [46,65]. In humans acute infections it is generally proposed that full virulence requires a functional *agr* system whilst *agr* dysfunction is associated with chronic infections, the formation of small colony variants, and biofilm associated infections [65,66]. Three of the tested isolates (40, 41 and 77) were delta-toxin producing (figure 4), three isolates were not producing any toxins and the rest either produced alpha- and/or beta-hemolysis.

Several studies have documented the frequency of delta-hemolysis positive and negative *S. aureus* strains and in human clinical isolates there are generally found more delta-toxin positive isolates than in our study [67,68]. No isolates carried the PVL genes *lukF-PV* and *lukS-PV*, or the TSST-1 encoding gene *tst*. These results are consistent with other studies of porcine *S. aureus* CC398 [12,53] and CC30 isolates [13] (only tested for the presence of PVL genes). The TSST-1 toxin has not been reported in porcine *S. aureus* CC398 isolates and the presence of PVL genes have only been identified in a few cases [69]. However, TSST-1 and PVL genes are commonly present in human-associated *S. aureus* isolates, including *S. aureus* belonging to CC30 [11,12,33,70–72].

Conclusion

In this study the presence of a *qac* gene, *qac*G, in MRSA isolates of porcine origin was for the first time described in Europe. These MRSA isolates belonged to CC30, a clonal complex that is often associated with MRSA types giving rise to clinical infections in Denmark. These findings are worrying, since incorrect use of or the presence of residual concentration of QACs, may contribute

to the spread and persistence of these isolates among Danish pigs and make the task of MRSA eradication more difficult. Furthermore, one of the MRSA CC30 isolates found positive for *qac*G were resistant to the clinical relevant antimicrobial agent gentamicin, sometimes used for treatment of *S. aureus* infections. Also, the high occurrence of erythromycin resistance in *S. aureus* CC398 was unfortunate, since macrolides can be used for treatment of *S. aureus* infections. Isolates showed a general low haemolytic activity and were all found to be negative for the virulence genes *pvl* and *tsst* that are frequently observed in HA- and CA-MRSA isolates. However, virulence genes are commonly found on MGEs and since *S. aureus* are excellent at acquiring foreign DNA material, development of more virulent strains might just be a matter of time. Surveillance of the development of increased resistance to biocides and antimicrobial agents and development of more virulent strains among porcine *S. aureus* isolates is important for the public health.

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Transparency declarations

None to declare

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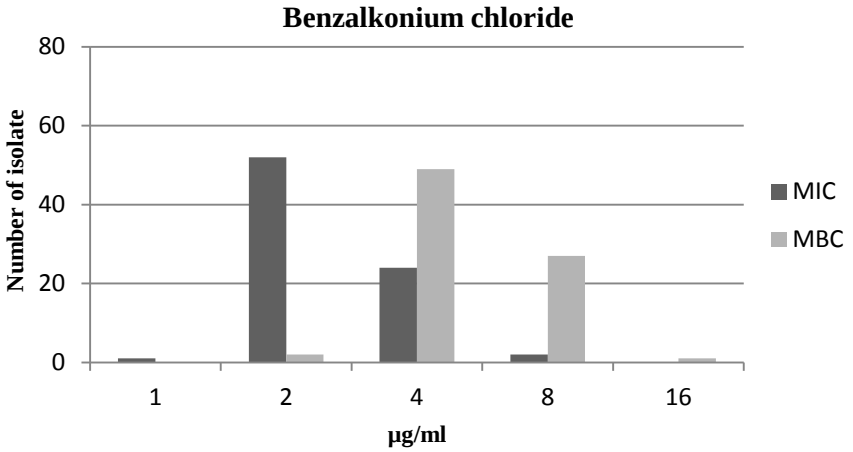
Table and figures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2
4
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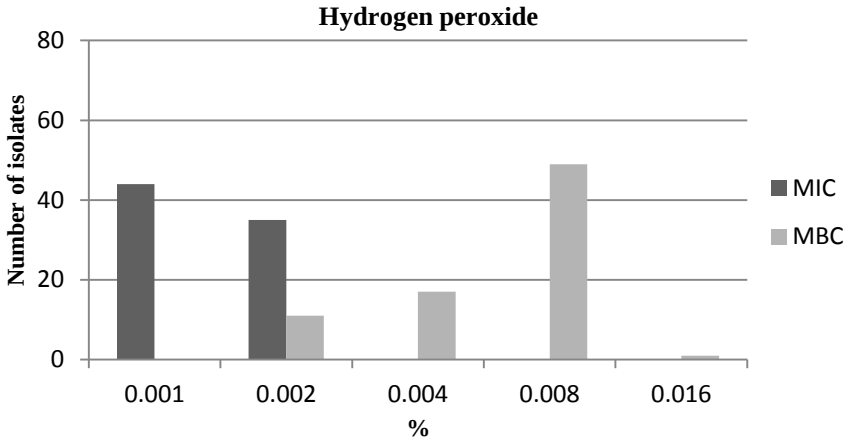
µg/ml
MIC
MBC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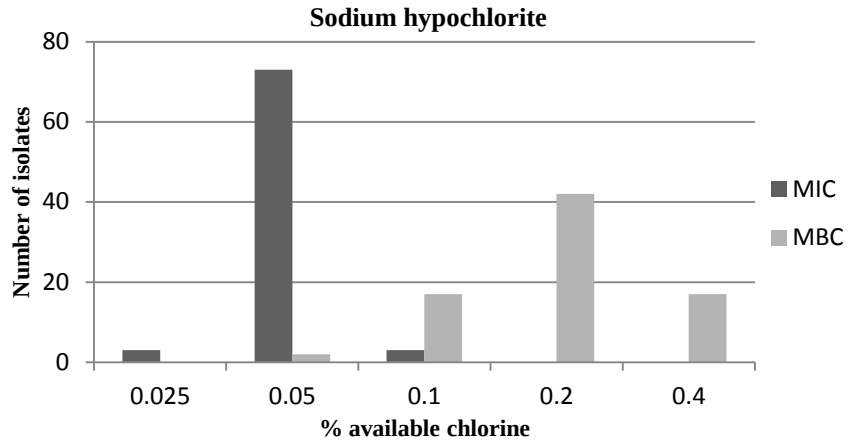
0.004
0.008
0.016
%
MIC
MBC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40
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0.2
0.4
% available chlorine
MIC
MBC

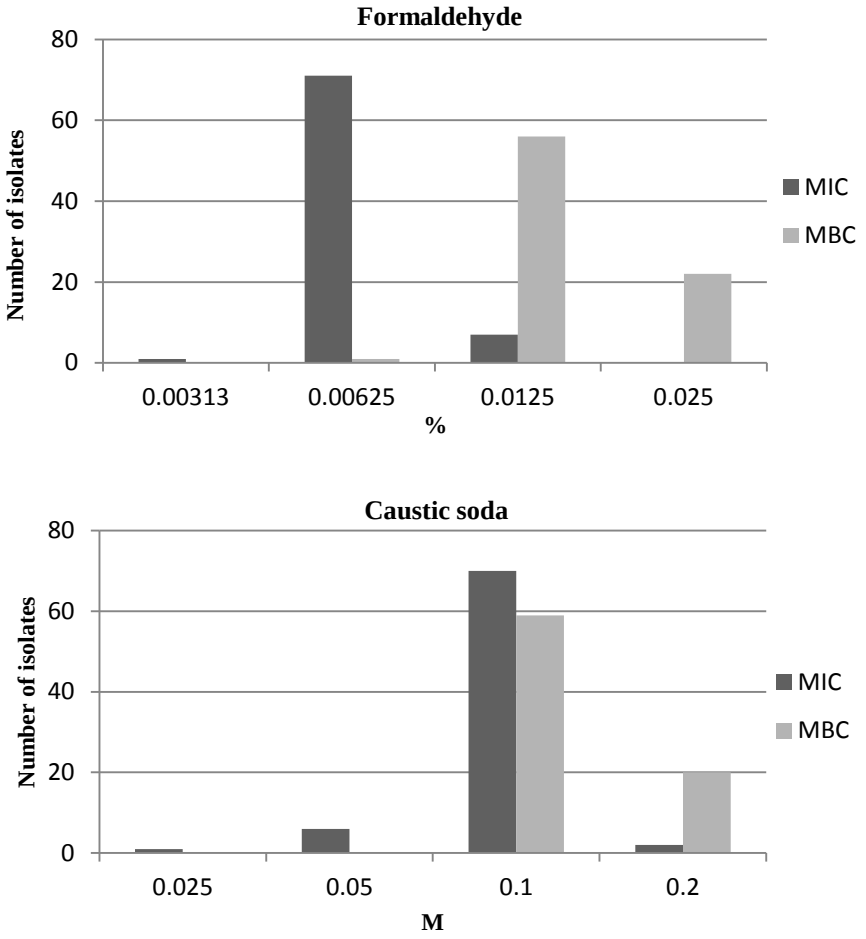
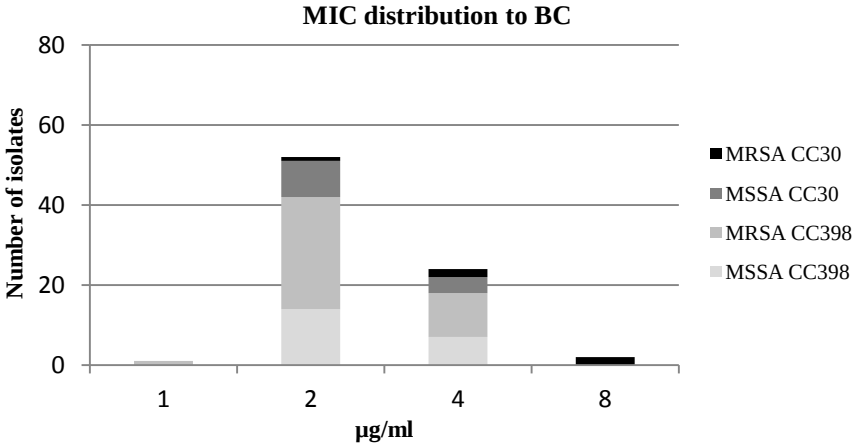


Figure 1. Distributions of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the 79 *S. aureus* pig isolates.



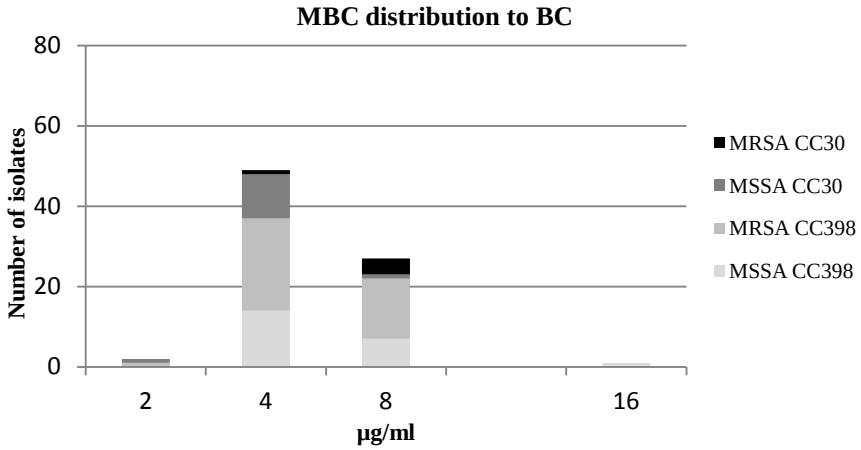


Figure 2. Distribution of the minimal inhibitory concentration (MIC) and bactericidal concentration (MBC) to benzalkonium chloride (BC) of MRSA and MSSA *S. aureus* pig isolates belonging to clonal complex (CC) 398 and 30.

Table 1. Phenotypic resistance profile (A) and distribution of resistances (B) in the 79 *S. aureus* pig isolates. CHL; chloramphenicol, CIP; ciprofloxacin, ERY; erythromycin, FFN; florfenicol, PEN; penicillin, SPE; spectinomycin, STR; streptomycin, SMX; sulphamethoxazol, TET; tetracycline, TIA; tiamulin, TMP; trimethoprim.

A)

	Isolate no.	Source	Resistance phenotype	Resistance pattern
MSSA CC398	1	D	PEN, TET, TMP, SXT, STR	R14
	2	D	PEN, TET	R9
	3	D	ERY, TET, SPE, STR	R2
	4	D	PEN, TET, TMP, SXT, STR	R14
	5	D	PEN, TET, TMP, STR	R12
	6	D	PEN, TIA, ERY, TET, TMP	R22
	7	D	PEN, TET	R10
	8	D	PEN, TET, TMP, SXT	R13
	9	D	PEN, ERY, TET	R3
	10	D	PEN, TIA, ERY, TET, STR	R21
	11	D	PEN, TIA, ERY, TET, TMP, SXT, STR	R23
	12	D	ERY, TET	R1
	13	D	PEN, TET, TMP	R11
	14	D	PEN, TIA, ERY, TET, SPE, TMP, STR	R20
	15	D	TIA, ERY, TET	R30
	16	D	PEN, TIA, TET, SPE, TMP	R27
	17	F1	PEN, TIA, TET, CIP, TMP	R26

	18	F2	PEN, TIA, TET, SPE, TMP, STR	R28
	19	F3	PEN, ERY, TET, CIP, TMP	R4
	20	F3	PEN, ERY, TET, CIP, TMP	R4
	21	F3	PEN, ERY, TET, CIP, TMP	R4
	22	S4	PEN, ERY, TET, TMP, STR	R8
	23	S4	PEN, TET, CIP, FFN, CHL, TMP, SXT	R10
	24	S3	PEN, TET	R9
	25	S5	PEN, TET, TMP,SXT	R15
	27	N	PEN, TET, TMP	R11
	28	N	PEN, TIA, TET, SPE, TMP, SXT	R29
	29	S6	PEN, ERY, TET, TMP, STR	R8
	30	S6	PEN, ERY, TET, SPE, TMP, SXT	R7
	31	S7	PEN, TIA, ERY, TET, SPE, STR	R18
	32	S5	PEN, TET	R9
	33	F4	PEN, TET, TMP, STR	R12
	34	F5	PEN, TET, TMP, STR	R12
	35	F6	PEN, TET, TMP	R11
	37	F7	PEN, ERY, TET, SPE, TMP, STR	R6
	38	F7	PEN, ERY, TET, SPE, TMP, STR	R6
	39	F7	PEN, ERY, TET, SPE, TMP, STR	R6
	40	F7	PEN, ERY, TET, TMP, STR	R8
	41	F7	PEN, ERY, TET, SPE, TMP, STR	R6
	42	F7	PEN, ERY, TET, SPE, TMP	R5
	43	F7	PEN, ERY, TET, SPE, TMP, STR	R6
	44	F4	PEN, TET, TMP	R11
	45	F4	PEN, TET, TMP	R11
	46	F4	PEN, TET, TMP, STR	R12
	47	F4	PEN, TET, TMP	R11
	48	F4	PEN, TET, TMP	R11
	49	F4	PEN, TET, TMP, STR	R12
	50	F4	PEN, TET, TMP, STR	R12
	51	F4	PEN, TET, TMP	R11
	52	F4	PEN, TET, TMP, STR	R12
	53	F1	PEN, TIA, ERY, TET, SPE, TMP, SMX, STR	R19
	54	F2	PEN, TIA, TET, SPE, TMP, STR	R28
	55	F2	PEN, TIA, ERY, TET, SPE, TMP, STR	R20
	56	F8	PEN, TET, TMP, STR	R12
	57	F9	PEN, TIA, ERY, TET, CIP, SPE, TMP, STR	R16
MRSA CC398	58	F9	PEN, TIA, ERY, TET, CIP, SPE, TMP, SXT, STR	R17
	59	F9	PEN, TIA, ERY, TET, CIP, SPE, TMP, STR	R16
	60	F11	PEN, TIA, TET, CIP, SPE, TMP, STR	R24
	61	F10	PEN, TIA, ERY, TET, CIP, SPE, TMP, SXT, STR	R17

	62	F10	PEN, TIA, TET, CIP, SPE, TMP, STR	R24
	63	F10	PEN, TIA, TET, CIP, SPE, TMP, SXT, STR	R25
	72	S1	TIA	RVI
	73	S1	TIA	RVI
	74	D	-	RIX
	75	D	PEN	RI
	76	D	-	RIX
	77	D	TIA, TMP	RVIII
	78	D	PEN	RI
	79	D	TET, STR	RV
	80	D	PEN, ERY, SPE	RII
MSSA CC30	81	F1	TIA	RVI
	82	D	TIA	RVI
	83	S2	PEN	RI
	84	D	TIA, TET	RVII
	85	F12	PEN, TIA, ERY, TMP, STR	RIII
	86	S3	PEN, TIA, STR	RIV
MRSA CC30	87	S3	PEN, TIA, STR	RIV
	88	S3	PEN, TIA, STR	RIV
	89	S3	PEN, TIA, STR	RIV

B)

		%										
		Pen	**Tia**	**Ery**	**Tet**	**Cip**	**Ffn**	**Spe**	**Chl**	**Tmp**	**Sxt**	**Smx**	**Str**
CC398	MRSA (n = 40)	100	30	43	100	20	3	48	3	93	18	3	65
	MSSA (n = 21)	86	38	52	100	19	0	19	0	67	19	0	38
	All (n = 61)	95	33	46	100	20	2	38	2	84	18	2	56
CC30	MRSA (n = 5)	100	100	20	0	0	0	0	0	20	0	0	100
	MSSA (n = 13)	31	46	8	15	0	0	8	0	8	0	0	8
	All (n = 18)	50	61	11	11	0	0	6	0	6	0	0	33

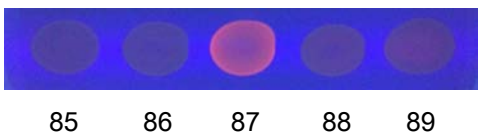


Figure 3. Ethidium bromide efflux activity assay. Isolates were grown for 24 h at 37 °C on TSA containing EtBr (1 µg/ml), followed by inspection for fluorescence under UV light. Strains JCM 16555 (*qacA)* and JCM 16556 (*qacB*), that overexpress efflux pumps, were included as controls

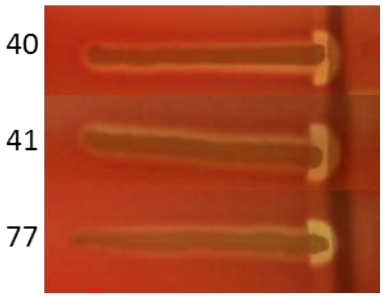


Figure 4. Hemolytic activity of *S. aureus* on sheep blood. RN4220, a strong beta-hemolysin producer, is streaked vertically and isolates horizontally. Beta-hemolysin forms a tubid zone of hemolysis surrounding the vertical streak of RN4220. Delta-hemolysis and beta-hemolysis are synergistic, producing a clear hemolysis where they intersect.

Supplementary material

Table S1. Collection of isolates included in this study. D; diagnostic submission, F; farm, N; no information, S; slaughter house.

	Isolate no.	Year	Sample	Source	*spa*-type
MSSA CC398	1	2000	Clinical	D	t034
	2	2000	Clinical	D	t034
	3	2001	Clinical	D	t034
	4	2001	Clinical	D	t034
	5	2002	Clinical	D	t034
	6	2002	Clinical	D	t034
	7	2002	Clinical	D	t011
	8	2002	Clinical	D	t034
	9	2002	Clinical	D	t034
	10	2002	Clinical	D	t034
	11	2003	Clinical	D	t034
	12	2003	Clinical	D	t034
	13	2004	Clinical	D	t011
	14	2004	Clinical	D	t034
	15	2004	Clinical	D	t571
	16	2005	Clinical	D	t034
	17	2007	Colonization	F1	t034
	18	2007	Colonization	F2	t034
	19	2007	Colonization	F3	t034
	20	2007	Colonization	F3	t034
	21	2007	Colonization	F3	t034
MRSA CC398	22	2009	Colonization	S4	t034
	23	2009	Colonization	S4	t034
	24	2009	Colonization	S3	t034
	25	2009	Colonization	S5	t034
	27	2009	Colonization	N	t034
	28	2009	Colonization	N	t034
	29	2009	Colonization	S6	t034
	30	2009	Colonization	S6	t034
	31	2009	Colonization	S7	t011
	32	2009	Colonization	S5	t011
	33	2007	Colonization	F4	t108
	34	2007	Colonization	F5	t034
	35	2007	Colonization	F6	t318
	37	2007	Colonization	F7	t034
	38	2007	Colonization	F7	t034

	39	2007	Colonization	F7	t034
	40	2007	Colonization	F7	t034
	41	2007	Colonization	F7	t034
	42	2007	Colonization	F7	t034
	43	2007	Colonization	F7	t034
	44	2007	Colonization	F4	t034
	45	2007	Colonization	F4	t034
	46	2007	Colonization	F4	t034
	47	2007	Colonization	F4	t034
	48	2007	Colonization	F4	t034
	49	2007	Colonization	F4	t034
	50	2007	Colonization	F4	t034
	51	2007	Colonization	F4	t034
	52	2007	Colonization	F4	t034
	53	2007	Colonization	F1	t034
	54	2007	Colonization	F2	t034
	55	2007	Colonization	F2	t034
	56	2007	Colonization	F8	t034
	57	2008	Colonization	F9	t034
	58	2008	Colonization	F9	t034
	59	2008	Colonization	F9	t034
	60	2008	Colonization	F11	t034
	61	2008	Colonization	F10	t034
	62	2008	Colonization	F10	t034
	63	2008	Colonization	F10	t034
MSSA CC30	72	2007	Colonization	S1	t1333
	73	2007	Colonization	S1	t1333
	74	2000	Clinical	D	t1333
	75	2000	Clinical	D	t1333
	76	2000	Clinical	D	t1333
	77	2000	Clinical	D	t1333
	78	2001	Clinical	D	t1333
	79	2002	Clinical	D	t1333
	80	2002	Clinical	D	t1333
	81	2007	Colonization	F1	t1333
	82	2002	Clinical	D	t1333
	83	2007	Colonization	S2	t1333
	84	2001	Clinical	D	t1333
MRSA CC30	85	2011	Colonization	F12	t1333
	86	2009	Colonization	S3	t1333
	87	2009	Colonization	S3	t1333
	88	2009	Colonization	S3	t1333

89	2009	Colonization	S3	t1333	

Table S2. The MIC- and MBC-values of the 79 *S. aureus* pig isolates to benzalkonium chloride (BC), hydrogen peroxide (HP), sodium hypochlorite (SH), formaldehyde (FH), caustic soda (NaOH), and chlorhexidine digluconate (CHX).

		BC (µg/ml)		HP (%)		SH (%) [a]		FH (%)		NaOH (%)		CHX (µg/ml)	
	Isolate no.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MSSA CC398	1	2	8	0.002	0.002	0.05	0.1	0.00625	0.0125	0.1	0.1	2	4
	2	2	4	0.002	0.008	0.05	0.1	0.00625	0.0125	0.1	0.1	ND	ND
	3	2	4	0.001	0.008	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	4	2	4	0.001	0.008	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	5	2	4	0.001	0.004	0.05	0.2	0.00625	0.0125	0.05	0.1	ND	ND
	6	2	4	0.001	0.008	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	7	2	4	0.002	0.004	0.05	0.1	0.00625	0.0125	0.1	0.1	ND	ND
	8	2	4	0.002	0.008*	0.05	0.2	0.00625	0.025	0.05	0.1	ND	ND
	9	2	4	0.001	0.008	0.05	0.2	0.00625	0.025	0.1	0.1	ND	ND
	10	2	4	0.001	0.008	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	11	2	4	0.001	0.008*	0.05	0.2	0.00625	0.0125	0.1	0.2	ND	ND
	12	2	4	0.001	0.008*	0.05	0.2	0.00625	0.025	0.1	0.1	ND	ND
	13	2	8	0.001	0.004	0.05	0.4	0.00625	0.0125	0.1	0.1	ND	ND
	14	4	4	0.001	0.004	0.05	0.4	0.00625	0.0125	0.1	0.1	2	8
	15	4	4	0.001	0.008	0.05	0.2	0.00625	0.0125	0.1	0.1	2	2
	16	4	8	0.001	ND	0.05	ND	0.00625	0.0125	0.1	0.2	2	4
	17	2	8	0.002	0.008*	0.05	0.2	0.0125	0.025	0.1	0.1	ND	ND
	18	4	8	0.001	0.004	0.05	0.2	0.0125	0.025	0.1	0.1	ND	ND
	19	4	8	0.001	0.008*	0.05	0.4	0.00625	0.0125	0.2	0.2	4	8
	20	4	4	0.001	0.004	0.05	0.4	0.00625	0.0125	0.1	0.2	2	4
	21	4	8	0.002	0.008	0.05	0.4	0.00625	0.0125	0.2	0.2	ND	ND
MRSA CC398	22	2	4	0.001	0.002	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	23	2	4	0.001	0.002	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	24	2	8	0.001	0.004*	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	25	4	4	0.002	0.002	0.05	0.2	0.00625	0.0125	0.1	0.2	ND	ND
	27	2	4	0.002	0.004	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	28	2	4	0.002	0.004	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	29	2	4	0.002	0.008	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	30	2	4	0.001	0.004	0.05	0.1	0.00625	0.0125	0.05	0.1	ND	ND
	31	2	4	0.002	0.008*	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	32	2	8	0.002	0.008	0.05	0.2	0.00625	0.025	0.1	0.2	ND	ND
	33	2	16	0.002	0.004*	0.05	0.1	0.00625	0.0125	0.1	0.1	ND	ND
	34	2	4	0.002	0.008	0.05	0.2	0.00625	0.0125	0.1	0.1	2	4

	35	2	4	0.002	0.004	0.05	0.1	0.00625	0.0125	0.1	0.1	ND	ND
	37	2	4	0.001	0.008*	0.05	0.4	0.00625	0.0125	0.1	0.1	ND	ND
	38	2	4	0.002	0.008	0.05	0.4	0.0125	0.0125	0.1	0.1	ND	ND
	39	2	4	0.002	0.008*	0.05	0.4	0.0125	0.025	0.1	0.2	ND	ND
	40	2	8	0.001	0.008	0.05	0.4	0.0125	0.0125	0.1	0.1	ND	ND
	41	4	8	0.002	0.008	0.05	0.4	0.00625	0.0125	0.1	0.2	2	4
	42	2	8	0.001	0.008*	0.05	0.2	0.00625	0.0125	0.1	0.2	ND	ND
	43	1	2	0.002	0.002	0.05	0.2	0.00625	0.0125	0.025	0.1	ND	ND
	44	2	8	0.002	0.008	0.1	0.4	0.00625	0.0125	0.1	0.2	ND	ND
	45	2	8	0.002	0.008	0.05	0.4	0.00625	0.025	0.1	0.2	ND	ND
	46	2	4	0.001	0.008	0.05	0.4	0.00625	0.0125	0.1	0.2	ND	ND
	47	4	4	0.001	0.008	0.05	0.4	0.00625	0.0125	0.1	0.2	ND	ND
	48	2	8	0.001	0.008	0.1	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	49	2	8	0.001	0.008	0.05	0.2	0.00625	0.025	0.1	0.1	ND	ND
	50	2	8	0.002	0.008	0.1	0.2	0.0125	0.025	0.1	0.2	ND	ND
	51	2	8	0.002	0.008	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	52	2	4	0.002	0.004	0.05	0.2	0.0125	0.025	0.1	0.1	ND	ND
	53	2	4	0.001	0.004	0.05	0.2	0.00625	0.0125	0.05	0.1	ND	ND
	54	4	4	0.001	0.008	0.05	0.4	0.00625	0.0125	0.1	0.2	4	4
	55	2	4	0.001	0.008	0.05	0.4	0.00625	0.0125	0.1	0.1	ND	ND
	56	4	4	0.002	0.004	0.025	0.1	0.00625	0.0125	0.1	0.1	ND	ND
	57	4	4	0.001	0.008	0.05	0.2	0.00625	0.025	0.1	0.1	ND	ND
	58	4	8	0.001	0.008	0.05	0.2	0.00625	0.025	0.1	0.1	ND	ND
	59	2	4	0.001	0.008*	0.05	0.2	0.00625	0.025	0.1	0.2	ND	ND
	60	4	8	0.001	0.016	0.025	0.2	0.00625	0.025	0.1	0.1	ND	ND
	61	4	4	0.001	0.008*	0.05	0.2	0.00625	0.0125	0.1	0.2	2	4
	62	4	8	0.001	0.008*	0.05	0.2	0.00625	0.025	0.1	0.2	2	4
	63	4	8	0.001	0.008	0.025	0.2	0.00625	0.025	0.1	0.1	2	4
MSSA CC30	72	2	4	0.002	0.004	0.05	0.1	0.00625	0.0125	0.1	0.1	ND	ND
	73	2	2	0.002	0.004	0.05	0.1	0.00625	0.0125	0.1	0.1	ND	ND
	74	2	4	0.002	0.008*	0.05	0.1	0.00625	0.025	0.1	0.1	ND	ND
	75	2	4	0.001	0.008	0.05	0.4	0.00625	0.0125	0.1	0.1	ND	ND
	76	2	4	0.001	0.002	0.05	0.1	0.00313	0.00625	0.05	0.1	ND	ND
	77	4	4	0.001	0.008*	0.05	0.2	0.00625	0.0125	0.05	0.1	4	4
	78	4	8	0.001	0.008*	0.05	0.2	0.00625	0.0125	0.1	0.1	ND	ND
	79	2	4	0.002	0.008*	0.05	0.2	0.00625	0.025	0.1	0.1	ND	ND
	80	2	4	0.001	0.002	0.05	0.1	0.00625	0.0125	0.1	0.1	ND	ND
	81	2	4	0.002	0.002	0.05	0.1	0.00625	0.025	0.1	0.1	ND	ND
	82	4	4	0.001	0.002	0.05	0.1	0.00625	0.0125	0.1	0.1	ND	ND
	83	4	4	0.001	0.008*	0.05	0.1	0.00625	0.0125	0.1	0.1	2	4
	84	2	4	0.001	0.002	0.05	0.05	0.00625	0.025	0.1	0.1	ND	ND

MRSA CC30	85	4	8	0.002	0.008*	0.05	0.1	0.00625	0.0125	0.1	0.1	2	4
	86	4	8	0.002	0.002	0.05	0.05	0.00625	0.0125	0.1	0.1	2	4
	87	2	4	0.002	0.008*	0.05	0.2	0.00625	0.025	0.1	0.1	2	4
	88	8	8	0.002	0.008*	0.05	0.1	0.00625	0.0125	0.1	0.1	2	2
	89	8	8	0.002	0.008	0.05	0.2	0.00625	0.0125	0.1	0.2	2	2

* Paradoxical effect observed, [a] SH: Available chlorine