Molecular and ecological aspects of MRSA ST398 colonization in pigs

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Moleculaire en ecologische aspecten
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Il y a plus de philosophie dans une bouteille de vin que dans tous les livres
Louis Pasteur

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Chapter 1

General introduction

Multidrug-resistant bacteria (MRB) are an emerging problem in public health worldwide as they have become difficult to combat [1]. The best known example of these MRB is methicillin–resistant *Staphylococcus aureus* (MRSA) [2].

S. aureus belongs to the bacterial family *Staphylococcaceae*, which is a vast group of bacteria that includes more than 40 species [3]. Most of them frequently colonize the skin and upper respiratory tracts of animals and humans, although they can also be found in soil microflora [3]. *S. aureus* that have developed resistance to beta-lactam antibiotics, which include methicillin, are known as MRSA. MRSA is an opportunistic pathogen of animals and humans and can cause various infections in both humans and animals. It has been shown that various animals may serve as potential reservoirs to transmit MRSA to other animals as well as to humans [4]. Livestock has also been recognized as a reservoir of MRSA for humans [5].

There is limited understanding of how *S. aureus* becomes MRSA and what molecular mechanisms are behind MRSA colonization of different hosts therefore making this an interesting and important area to study. The role of other staphylococci in MRSA colonization is also unknown.

Staphylococci

Staphylococci are Gram-positive, facultative anaerobic, catalase positive, non-motile bacteria and do not form spores. The name is derived from the Greek staphylē [σταφυλή], "grape" and kókkos [κόκκος], "granule or berry". Under the microscope, they appear round (cocci), and form irregular grape-like clusters. The genus *Staphylococcus* includes at least 40 species. Based on the presence of coagulase, staphylococci are divided into two groups: coagulase-negative and -positive staphylococci. The bacterial ability to produce coagulase correlates with pathogenicity. Coagulase-negative staphylococci (CNS) are less virulent, but occasionally cause infections in humans and animals [3]. *Staphylococcus* species occur as commensals on the skin and the mucous membrane of the upper respiratory tract of animals and humans. Some of them may act as opportunistic pathogens causing pyogenic infections [6].

CNS are part of the normal microflora of the skin and mucosa membranes in animals and humans. In general, animals are a common reservoir of most CNS, which can then be transmitted to humans [7]. CNS can cause a variety of infections some of which include mastitis, wound infections and skin abscesses found in animals, bacteremia, endocarditis, skin and wound infections in human. In addition CNS can colonize medical implants in humans [8,9]. It has been shown that CNS causing infections are more frequently resistant to several antibiotics than *S. aureus* [10,11]. They also have the capability for biofilm

formation thus making CNS infections challenging to treat. As with MRSA, methicillin-resistant CNS (MRCNS) have also been isolated worldwide from livestock including pigs, cows and chickens [8,12-14]. Moreover, the presence of additional antibiotic resistance, such as resistance against erythromycin, tetracycline, clindamycin, ciprofloxacin, trimethoprim/sulfamethoxazole, is also very common in MRCNS [14,15]. A recent study of MRCNS from livestock showed a high prevalence of CNS resistance to non-beta-lactam antimicrobials [14]. Additionally, most of the antimicrobial resistance determinants (e.g. against tetracycline, aminoglycosides) are located on mobile genetic elements (MGEs) such as conjugative plasmids and transposons [16]. For this reason, CNS are an important reservoir of antimicrobial resistance genes, which have the potential to transfer antibiotic resistance genes to other bacteria and may generate new multidrug-resistant bacteria. This suggests that multidrug-resistant CNS may pose a problem for veterinary medicine and public health [17].

Staphylococcus aureus

S. aureus is an opportunistic pathogen for animals and humans. A wide variety of infections can be caused by *S. aureus*, such as superficial skin and soft tissue infections and life-threatening septicemia. *S. aureus* isolates have been intensively studied using various molecular techniques like: multilocus enzyme electrophoresis (MLEE), pulsed-field gel electrophoresis (PFGE), *spa*-typing, and multilocus sequence typing (MLST) [18]. From these studies we have learned that the population of *S. aureus* is highly clonal and can be grouped into many different lineages, often referred to by their clonal complex (CC numbers). It has been shown that most MRSA can be grouped into six main lineages: CC1, CC8, CC5, CC22, CC30, and CC45 [19]. Cattle are usually colonized and infected with *S. aureus* lineages that are rarely found in humans [20] while pigs can be colonized by a unique clonal complex, CC398, of which ST398 is the most common representative. The vast majority of CC398 isolates are MRSA and are capable of colonizing humans which can lead to infections [21]. *S. aureus* isolated from companion animals usually belong to lineages typically found in humans [22]. *S. aureus* infections are causing economic losses in dairy and poultry industries [5].

Genome structure

The size of the *S. aureus* genome is approximately 2.8 Mb and contains approximately 2800 genes. When comparing completely sequenced genomes of various *S. aureus* strains, the genome content can be divided into three groups: the core genome, the core variable

(CV) genome and the mobile genetic elements (MGEs) [23]. The core genome (~75% of the genome) contains housekeeping genes and is highly conserved amongst all *S. aureus* isolates. Genes that belong to the CV genome (~10% of the genome) show substantial variation in their presence. Most of the CV genes are known to be lineage specific and usually encode surface proteins, secreted proteins and their regulators. Microarray studies of the *S. aureus* genome have shown that each *S. aureus* clonal complex possesses a unique combination of CV genes [20]. The MGEs (~15% of the genome) are originally foreign DNA fragments that can be transferred between bacteria. MGEs generally carry antimicrobial resistance, heavy metal resistance, host adaptation and virulence genes [23-26].

Mobile genetic elements of *Staphylococcus aureus*

Many different types of MGEs can be present in *S. aureus*: plasmids, Staphylococcal Cassette Chromosomes (SCCs), pathogenicity islands, transposons (Tn), insertion sequences (IS), and bacteriophages. It is remarkable that most genes encoded by MGEs remain under the control of global regulators located within the core genome [27]. MGEs are transferred via horizontal gene transfer (HGT), also called lateral gene transfer. HGT between bacteria or between the surrounding environment and bacterial cells can occur in three ways: transformation- by free DNA from the environment, transduction- by bacteriophages between bacterial cells, and conjugation- by direct contact between bacterial cells [28].

Exogenous DNA elements like MGEs play a crucial role in the plasticity of the bacterial genome, thus bacteria can more easily adapt to a new niche or adjust to environmental changes. For example, antibiotic usage creates a selective pressure that results in the survival of only those bacteria that harbor the relevant antibiotic resistance gene. Additionally, it has been suggested that acquisition and loss of MGEs is a frequent event in the evolution of *S. aureus* [26].

a) Staphylococcal Cassette Chromosome (SCC)

SCCs are large fragments of DNA that always insert into the *orfX* gene, close to the origin of replication on the *S. aureus* chromosome [29,30]. SCCs can encode antibiotic resistance and/or virulence determinants. Many SCCs encode the methicillin resistance gene (*mecA*) and are classified as SCC*mec* [31].

The *mecA* gene encodes an additional penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for all beta-lactam antibiotics [32]. SCC*mec* elements are highly diverse in their structural organization and genetic content and have been classified into types and subtypes. SCC*mec* elements have two main parts: the *mecA* complex, which is responsible for methicillin resistance and the ccr gene complex, which is responsible for mobilization / integration of SCC*mec* in the genome. The *mecA* complex is composed of *mecA*, its regulatory genes and insertion sequences. There are five different mec class gene complexes (A, B, C, D, and E). The *ccr* gene complex is composed of the *ccr* gene(s) (*ccrAB* and/or *ccrC*) and surrounding open reading frames [31]. Besides the *mec* and *ccr* gene complexes, the SCC*mec* element also contains three J-regions (joining regions). These regions constitute nonessential components of the cassette and may contain additional antimicrobial resistance or virulence determinants. J1 is the region between the right chromosomal junction and the *ccr* complex, J2 is between the *ccr* gene complex and the *mec* gene complex, and J3 is between the *mec* complex and the left chromosomal junction. The main SCC*mec* types are classified based on the combination of the *ccr* gene complex and *mecA* complex (Figure 1). Further, variations in the J-regions within the same *mec-ccr* complex are used for defining SCC*mec* subtypes [31].

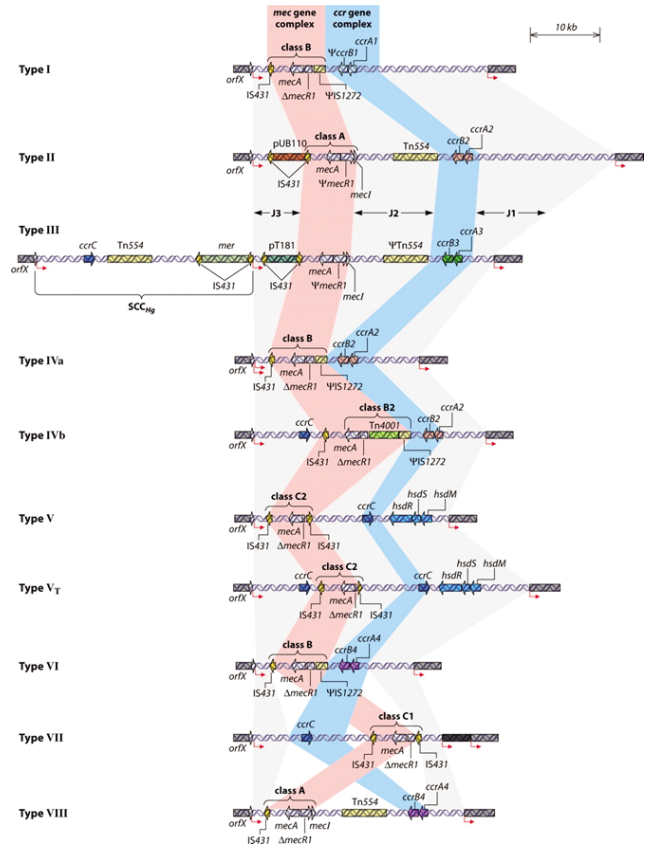

Figure 1. Structure of the eight main SCC*mec* types.
Nowadays up to eleven types have been described. The *mecA* complex is indicated in red, *ccr* complex in blue and J-regions in grey [33].

The origin of SCC*mec* remains unknown, but it is believed that the *mecA* gene itself originated from one common precursor. Homologues of the *mecA* gene have been found in coagulase-negative staphylococci (CNS): *Staphylococcus sciuri* [34] and *Staphylococcus vitulinus* [35]. However, these *mecA* genes have only ~ 80% similarity to the sequence of the gene that is present in MRSA. They are also not located in a *mecA* complex like in SCC*mec*. Recently, Tsubakishita *et al.* presented evidence that a direct precursor of the methicillin resistance determinant for MRSA is present in *Staphylococcus fleurettii* [36]. The investigators showed that this determinant has 99-100% sequence homology with the *mecA* gene present in the MRSA strain N315, and it is organized in an almost similar structure of the *mecA* complex [36]. *S. fleurettii* is a commensal bacterium of animals and the potential ancestor of the *mecA* gene present in animal-born staphylococcal species. This indicates that SCC*mec* elements may be generated in an animal-born *Staphylococcus* species. This finding is in agreement with the hypothesis that MRSA most likely acquired SCC*mec* from CNS [37]. This hypothesis is further supported by the fact that methicillin resistance among human clinical isolates is more prevalent in CNS than in *S. aureus* [10,11]. Furthermore, two observations of *in vivo* transfer of SCC*mec* from *Staphylococcus epidermidis* [38] and from *S. haemolyticus* [39] to *S. aureus* suggest that CNS may act as a source for SCC*mec* acquisition by *S. aureus*.

The presence of SCCs in the staphylococcal genome is not limited to those encoding methicillin resistances. For example, the MSSA476 strain contains SCC476 which contains a fusidic acid resistance gene [40]. Another example is SCCmercury which encodes resistance to mercury chloride. Moreover, it has been shown that some of *S. aureus* strains produce capsular polysaccharide 1, located on a SCC element named SCC*cap1*, which confers resistance to phagocytosis [41].

b) Plasmids

Plasmids are self-replicating DNA molecules. Most plasmids in nature carry genes, which are beneficial for bacterial survival (e.g. antibiotic resistance genes). Staphylococci typically carry one or more plasmids per cell. When plasmid DNA enters the staphylococcal cell it remains as free circularized DNA or integrates into the chromosome. In addition to genes encoding antibiotic resistance and molecules involved in metabolism, staphylococcal plasmids can encode resistance to a variety of organic and inorganic ions, such as cadmium, mercury, arsenate, silver, etc., which are harmful for living cells [42]. Staphylococcal plasmids may also encode toxin genes like exfoliative toxin B and bacteriocin [16].

c) Bacteriophages

Bacteriophages also called bacterial viruses are believed to have the most important impact on staphylococcal diversity and evolution. A vast majority of *S. aureus* bacteriophages belong to the *Caudovirales* order and are mainly members of *Siphoviridae* family [43]. Bacteriophages can be divided into two groups. The first group infects the bacterial cell, replicates and lyses the cell. These are the lytic phages. In the second group the bacteriophage DNA integrates into the staphylococcal genome as a prophage [16,44]. These bacteriophages can influence expression of bacterial genes by lysogenic conversion. Integration of prophages into protein-encoding genes is linked to the loss of a protein function and expression of bacteriophage's genes [44,45]. Moreover, the bacteriophage may carry genes beneficial during bacterial infection such as genes encoding immune-modulator proteins (e.g. staphylokinase (Sak), staphylococcal inhibitor of complement (SCIN), and chemotaxis inhibitory protein of *S. aureus* (CHIPS)) [45,46]. Other *S. aureus* prophages encode virulence factors such as enterotoxins and Panton-Valentine leukocidin (PVL) [47].

Some prophages can create mobility for some staphylococcal pathogenicity islands. The most common example is the ability of helper bacteriophage 80α to mediate excision and transfer of SaPI1 to other staphylococci [48,49]. Some bacteriophages also have the ability to transfer antibiotic resistance genes between staphylococci by transduction of chromosomally incorporated MGEs [50].

d) Genomic islands

Bacterial genomes also can contain clusters of genes that were acquired by HGT, called genomic islands (GIs). GIs are frequently associated with microbial adaptations to a niche and they have a substantial impact on bacterial evolution [51]. GIs associated with pathogenesis are often called a pathogenicity island (PAIs), while GIs that contain many antibiotic resistance genes are referred to as antibiotic resistance islands. The main characteristic of GIs is a different G +C content in comparison to the rest of the chromosome [52]. In the *S. aureus* genome three types of GIs, apart from PAIs, have been described: vSAα, vSAβ, and vSAγ [23,53]. The GIs are flanked by a truncated transposase gene upstream and a partial restriction-modification system (RM) type I downstream. Both flanking DNA segments contribute to the stability of the GIs within the *S. aureus* chromosome [16]. The lipoprotein gene cluster (*lpl*) and staphylococcal superantigen-like genes (*ssl*) are located on vSAα [54]; bacteriocin, enterotoxins, hyaluronate lyase, and a serine protease gene cluster are usually encoded on vSAβ [40,55,56];

β-type phenol-soluble modulins and a cluster of ssl genes are generally located on vSAγ [53].

Staphylococcal pathogenicity islands (SaPIs) are large GIs of 14–17 kb in size associated with pathogenicity. Until now more than 16 SaPIs have been reported and sequenced [16]. SaPIs form a coherent family with highly conserved core genes [48,57]. Core genes include two open reading frames encoding transcriptional regulatory proteins and a region encoding integrase, Rep protein and terminase [16]. In addition to core genes, almost all SaPIs encode enterotoxins or toxic shock syndrome toxin (TSST) [58]. Staphylococcal pathogenicity islands are integrated in specific sites in the chromosome (*att*)[48].

e) Other transposable elements

Additional MGEs present in the staphylococcal genome are: insertion sequences (IS) and transposons (Tn). They are present as a single copy or in multiple copies in the chromosome or in association with other MGEs. The IS can exist independently in the *S. aureus* genome. They can be present as pairs constituting a composite transposon [59]. IS insert into various loci and may inactivate genes by direct insertion or by having a polar effect on the transcription of nearby genes. However, polar effects can also lead to increases in gene expression [60,61].

The Tn predominantly harbor antibiotic resistance genes in *S. aureus*. The small transposons can be present in multiple copies in the staphylococcal genome and they are usually integrated into the chromosome or into MGEs, such as SCCs or plasmids [16]. For example *Tn*554 and *Tn*552, which encode resistance to macrolide-lincosamide-streptogramin B (MLSB) antibiotics and spectinomycin or penicillin, respectively [16]. Large transposons (>18 kbp) are usually present in single copies in the chromosome and may contain additional resistance genes encoding tetracycline, trimethoprim, aminoglycosides, or vancomycin resistance [16].

In summary, MGEs constitute ~15% of the staphylococcal genome [20,23,25], they can harbor numerous putative virulence factors, antibiotic resistance determinants and genes that encode factors responsible for bacterial adaptation and survival in different hosts.

Methicillin-resistant *Staphylococcus aureus*

In the past, *S. aureus* was susceptible to beta-lactams antibiotics. However, since the discovery of penicillin and its introduction as a therapeutic into medicine in 1940, *S. aureus* became rapidly penicillin resistant [2]. In 1960, methicillin was introduced as a beta-lactamase resistant, alternative beta-lactam antibiotic, to treat *S. aureus* infections and it

was only one year later that the first MRSA was reported [62]. At that time MRSA infections were infrequent. However since the mid 1970's MRSA has emerged as a major pathogen causing hospital infections. These MRSA were referred to as healthcare-associated MRSA (HA-MRSA) [2]. In the 1990's MRSA infections have increasingly been reported in people in the community who had never been in contact with a health care facility. These cases were referred to as community–associated MRSA (CA-MRSA) [63]. Genetic analyses have shown that MRSA belonging to HA-MRSA are distinct clones in comparison to CA-MRSA. Furthermore, MRSA has also been found in animals and these are defined as livestock-associated MRSA (LA-MRSA) and may act as a reservoir of MRSA for humans [21]. A much broader description of LA-MRSA can be found below. Overall, MRSA represents a serious public health concern in both hospital and community settings.

Livestock-associated MRSA (LA-MRSA)

Typically LA-MRSA refers to MRSA ST398 in Europe and North America, whereas MRSA ST9 is predominant in Asia. LA-MRSA colonizes different livestock species like pigs, cattle, and poultry, and may cause infections in humans [64-68]. Livestock and companion animals, on the other hand, may be colonized with a variety of MRSA strains. The first case of MRSA in livestock was reported in the beginning of the 1970's [69]. Further reports of MRSA in animals were sporadic and isolated strains were believed to be of human origin as shown by typing. Until 2000 it was thought that the prevalence of MRSA in animals was low and a potential MRSA reservoir in animals was of little relevance to MRSA infections in humans. It was also proposed that the presence of MRSA in animals was related to antimicrobial use in human medicine [70]. Nevertheless, with increasing numbers of reports related to MRSA in animals, the situation has changed since the beginning of the 21st century, resulting in increased interest in LA-MRSA.

The first *S. aureus* ST398 was reported in a human whom had close contact to a pig farm in France [71]. Since then there have been reports of spread MRSA ST398 in pig farms in The Netherlands [66]. MRSA ST398 has been frequently reported in different livestock, particularly in pigs and to a lesser degree in veal calves and chickens. Presently, MRSA ST398 has been identified throughout the world, including countries in Europe, North America and Asia [21,71,72]. The EU-baseline study on MRSA ST398 prevalence in pig production showed that in most European countries MRSA can be found on pig farms [73]. The colonization of livestock caused by MRSA has become a major issue as an occupational risk for humans working with pigs and veal calves [64,74].

It is known that LA-MRSA spreads mainly through the pig production chain [75,76] and the herd-size and trade contacts were recognized as risk factors for LA-MRSA

transmission [73,77]. MRSA ST398 has also been isolated from dust [78] and from rodents living on farms [79]. In addition, people working or living in close contact with pigs or cattle are at increased risk of becoming colonized and infected with MRSA ST398 [68,80,81] although it has been shown that human infection with ST398 may occur without any contact with livestock or livestock workers [82].

The origin and evolution of *S. aureus* ST398 remain unknown. Two studies have examined the gene content of ST398 strains from pig and human sources using a microarray assay [83,84]. These studies explored the genetic background of MRSA ST398 and showed that strains belonging to ST398 represent a homogenous lineage distinct from HA- and CA-MRSA strains. Human-associated virulence genes were not present among LA-MRSA ST398 strains isolated from various animals.

McCarthy *et al.* have shown that the MGE content of MRSA ST398 from different hosts is highly variable [83]. The MGEs that they found encode genes for proteins which are functional only in specific mammalian hosts. It is suggested that MGEs play a role in the adaptation to different hosts. However, there is no evidence that these genes are essential for colonization or adaptation to the host [83,85]. Detection of the φSa3 prophage (involved in human-specific innate immune evasion) in some human derived MRSA ST398 isolates suggests that this is an adaptation to a human host by MRSA ST398 [83]. Price *et al.* also came to this conclusion based on whole genome sequencing of 89 isolates [90]. Additionally, several MRSA ST398 strains that caused infection in humans were shown to contain PVL genes which were prophage encoded [86,87]. Furthermore, ST398 strains isolated from livestock usually are more resistant to antibiotics than those isolated from humans [5]. For example, the tetracycline resistance gene has universal presence in MRSA ST398 strains isolated from livestock. It was proposed that the usage of tetracycline in animal production is likely to select for LA-MRSA ST398 and maintenance in livestock. Additionally, some metals (like zinc) are frequently used in animal food and together with antibiotic usage in animal food production may co-select for colonization of MRSA ST398 in livestock, because a zinc resistance gene is present within SCC*mec* elements in MRSA ST398 [88,89]. Nevertheless, these observations do not clarify the evolution and global distribution of MRSA ST398 strains.

Price *et al.* applied whole-genome sequence typing to 89 *S. aureus* ST398 strains in order to study the evolution of MRSA ST398 [90]. This study strongly suggests that ST398, was present in humans as MSSA and subsequently spread to livestock. This implies that antibiotic usage in the livestock industry is the most likely factor to promote the development of methicillin resistance among ST398 strains. A similar route was described for poultry-associated *S. aureus* ST5, which appears to have been introduced from humans into the chicken-breeding system and disseminated worldwide [91].

Genomic analyses together with epidemiological data, suggest that the jump from humans to animals was followed by a decreased capacity for human colonization, transmission, and virulence of ST398 strains.

Nowadays, the largest threat of MRSA ST398 is further adaptation or re-adaptation to the human host and acquisition of new virulence for humans, which might facilitate a spread in the human population. Nevertheless, the genetic changes associated with the jump from humans to livestock and the successful colonization in livestock has only been hypothesized [90].

Staphylococcus aureus colonization

In humans the anterior nares of the nose are considered to be the primary ecological niche of colonizing *S. aureus*. In the human population three carriage patterns occur in healthy individuals: non-carriage, intermittent carriage and persistent carriage [92]. Carriage of *S. aureus* has been identified as a risk factor for infection in humans [93-95]. Animal nasal carriage of MRSA is recognized as reservoir for MRSA transmission to humans. Nevertheless, current knowledge about *S. aureus* in pigs is limited and mainly based on epidemiological studies. Also, it is unknown whether different carriage patterns exist in pigs. The prevalence of MRSA ST398 in pigs is high and represents successful colonization and spread of this clone among livestock. Although the knowledge about the molecular mechanisms behind *S. aureus* colonization is limited, there are a number of studies that shed light on the factors that are involved in the attachment of *S. aureus* to nasal epithelium. Additionally, models have been established to study MRSA colonization in pigs [76,96,97]. However, these models have some weaknesses such as unstable colonization [96,97], the minimum number of bacteria that can be detected [96] and experimental inoculation that may result in death of the animal [76]. Using murine [98] and rat models [99] several crucial factors have been identified and well characterized. Application of these models to investigate molecular mechanisms of *S. aureus* colonization in pigs is only suggestive.

Colonization of mucosa by *S. aureus*, as well as by other bacteria is a complex process and involves a number of different factors. First, *S. aureus* must be in close contact with the tissues and then adhere to specific cell surface receptors. *S. aureus* must also compete with other microbes, escape host immune factors to finally be able to colonize its new niche [95,100-102].

Staphylococcus aureus adherence to epithelial cells

The best characterized factor for *S. aureus* adherence to the epithelial cells in anterior nares is Clumping factor B (ClfB). ClfB is a *S. aureus* surface protein that binds to the cytokeratin 10 which is present on the surface of nasal epithelial cells [103]. It was demonstrated that a *S. aureus* mutant deficient in ClfB had a reduced ability to attach to the squamous nasal epithelial cells *in vitro* [103] and was defective in colonization of the nares of rodents [104,105]. Additionally, a ClfB mutant was weakened in the ability to colonize the nares of human volunteers when compared to wild type (WT) bacteria, indicating that ClfB is an important *S. aureus* factor in human colonization [93]. Another important factor in *S. aureus* colonization is the iron-regulated surface determinant protein A (IsdA). IsdA can bind to different substrates like: fibronectin, fibrinogen, loricrin, involucrin, and cytokeratin 10 [106,107]. It was demonstrated that an IsdA deficient *S. aureus* is defective in colonizing cotton rats compared with the WT strain [105].
Other *S. aureus* surface proteins like: Ser-Asp rich fibrinogen-binding protein (SdrC, SdrD) and surface protein G (SasG) have been shown to play a role in adhesion of bacteria to squamous cells, however, their exact interaction with the epithelial surface remains unknown [108].

Supplementary to adhesins, it has been demonstrated that a *S. aureus* wall surface polyanionic polymer-like teichoic acid (WTA) plays an important role in nasal colonization of *S. aureus*. WTA is composed of approximately 40 ribitolphosphate repeating units that are modified with D-alanine and N-acetylglucosamine and is covalently linked to the peptidoglycan [109]. Using a cotton rat model, a WTA-deficient *S. aureus* (*ΔtagO*) was unable to colonize nasal cavities [110]. Disruption of the WTA biosynthesis may have profound effects on the cell-surface architecture, but the *ΔtagO* mutant was not deficient in fibronectin or fibrinogen binding [111].

Although it appears that ClfB, IsdA and WTA are required for colonization; several other adhesins are involved in colonization underlining the multi-factorial nature of *S. aureus*–host interactions [108,112]. To understand the molecular mechanism of action, gene expression analyses based on direct RT-PCR were performed on nasal swabs from colonized rats and humans [113,114]. These results showed that different adhesins are expressed at different times during a 10-day experimental colonization in rats. More specifically, genes responsible for WTA synthesis were important during the early phase of colonization, whereas clfB and isdA were upregulated later on. It was concluded that WTA is important for initial host–bacteria interactions, whereas ClfB and IsdA are important for maintaining attachment and colonization of *S. aureus* [113,114].

Staphylococcus aureus interference with other organisms

During nasal colonization *S. aureus* must also compete with other bacterial species, which are natural residents of the nasal cavity. This bacterial interaction remains an unexplored field of study. Nevertheless, several studies have described bacterial interference of *S. aureus* with other species. One of the best known negative associations is inhibition of *S. aureus* by *Streptococcus pneumoniae* [115,116]. It was proposed that *S. pneumonia* produces H_2O_2 which is negatively associated with *S. aureus* colonization [117]. Additionally, a negative interaction between *Corynebacterium sp.* and *S. aureus* was documented, but the mechanism is still unknown [118]. In addition to single species interactions, studies on the nasal microbiome were performed to determine the correlation between the presence of natural flora and *S. aureus* [101,119,120]. For example, it was documented that there is also a negative correlation between *Staphylococcaceae* and *Actinobacteria* in the nostril [120]. A recent study described the mechanism of inhibition of *S. aureus* colonization by *S. epidermidis* [121]. Some *S. epidermidis* strains secrete a serine protease (Esp) which inhibits nasal colonization by *S. aureus*. It is suggested that the protease expressed by competing S. epidermidis removes surface protein adhesins and/or immune evasion factors of *S. aureus* that are essential for colonization [121].

It was shown that colonization with a methicillin-sensitive *S. aureus* protects the host against acquisition of MRSA [122]. It is suggested that *S. aureus* strains may interfere due to polymorphisms in the regulatory *agr* system [123-125]. This system up-regulates toxin production and down-regulates adhesion expression in the post-exponential phase. The *agr* system in *S. aureus* is divided into four groups (I–IV) and is defined on the basis of the secreted auto-inducer molecule AgrD and its receptor AgrC, resulting in cross-activation of the system within a group and cross-inhibition between different groups [109]. This form of bacterial interference does not result in growth inhibition but rather in blocking gene expression of virulence factors. Subsequently, interaction between different *S. aureus* strains may result in inhibition of one *S. aureus* strain by the other one.

S. aureus colonization is a complex, multifactorial process and understanding of this process is crucial for the development of novel strategies for intervention in humans and animals to control transmission of MRSA.

Aim of the thesis

The aim of this thesis was to understand the successful colonization of MRSA ST398 in pigs and the factors that may contribute to this colonization.

The first part of this thesis focuses on unraveling the factors that can explain the successful colonization of MRSA ST398 in pigs. The current knowledge about general molecular mechanisms of *S. aureus* colonization is mainly derived from experiments using human cell cultures [103] as well as rodent models [98,99]. Albeit, understanding of MRSA ST398 colonization in pigs is poor and is mostly based on epidemiological studies. Moreover, studies based on *in vivo S. aureus* colonization are difficult to control due to the presence of undefined local microbial and environmental factors. There are no alternative models available to study pig nasal colonization. Therefore the need for development of such a model is crucial to determine factors involved in successful colonization of MRSA ST398 in pigs and further application of this model to test potential MRSA eradication treatments in pigs. Chapter 2 describes the successful establishment of porcine nasal mucosa explants to study the interaction of *S. aureus* strains with nasal porcine tissue.

Furthermore, to gain a better understanding of nasal colonization of MRSA ST398 in pigs, changes in global gene expression of a MRSA ST398 isolate during colonization was studied using the developed *ex vivo* model. The results of this study are presented in Chapter 3. After successful establishment of growing *S. aureus* on pig nasal epithelium, the model was used to study MRSA ST398 decolonization using bacteriophage treatment (Chapter 4).

The second part of this thesis focuses on unanswered questions about the ecology of MRSA ST398, especially the relationship between staphylococcal flora with respect to MRSA ST398 generation in the farm environment and the influence of organic husbandry of MRSA ST398 on the prevalence in pig farms. Knowledge about co-existing MRCNS together with MRSA is limited. In chapter 5 the presence of a potential *mecA* reservoir among CNS recovered from 10 pig farms and the role of antibiotic usage in pig farming is described. Chapter 6 describes the first study of MRSA prevalence in organic farms in The Netherlands.

Finally, in chapter 7, studies presented in this thesis are discussed and put into perspective.

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Chapter 2

An *ex vivo* porcine nasal mucosa explants model to study MRSA colonization

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Abstract

Staphylococcus aureus is an opportunistic pathogen able to colonize the upper respiratory tract and skin surfaces in mammals. Methicillin-resistant *S. aureus* ST398 is prevalent in pigs in Europe and North America. However, the mechanism of successful pig colonization by MRSA ST398 is poorly understood. To study MRSA colonization in pigs, an *ex vivo* model consisting of porcine nasal mucosa explants cultured at an air-liquid interface was evaluated. In cultured mucosa explants from the surfaces of the ventral turbinates and septum of the pig nose no changes in cell morphology and viability were observed up to 72 h. MRSA colonization on the explants was evaluated followed for three MRSA ST398 isolates for 180 minutes. The explants were incubated with $3x10^8$ CFU/ml in PBS for 2h to allow bacteria to adhere to the explants surface. Next the explants were washed and in the first 30 minutes post adhering time, a decline in the number of CFU was observed for all MRSA. Subsequently, the isolates showed either: bacterial growth, no growth, or a further reduction in bacterial numbers. The MRSA were either localized as clusters between the cilia or as single bacteria on the cilia surface. No morphological changes in the epithelium layer were observed during the incubation with MRSA. We conclude that porcine nasal mucosa explants are a valuable *ex vivo* model to unravel the interaction of MRSA with nasal tissue.

Introduction

Staphylococcus aureus is an opportunistic pathogen colonizing the upper respiratory tract and skin surfaces of humans as well other mammalian species. The nose is considered to be the primary ecological niche of *S. aureus* colonization in humans [1]. Nasal carriage of *S. aureus* has been identified as a risk factor for the development of various infections in humans [1].

In 2004 a new distinct clone of methicillin-resistant *S. aureus* (MRSA) ST398 has been found in pigs in The Netherlands [2]. Since then, MRSA ST398 has been detected in pigs, veal calves and poultry around the world [3,4]. The transmission of MRSA ST398 from livestock to humans has been reported in many countries [5,6] and contact with livestock is recognized as a risk factor for human colonization [4,7]. Additionally, ST398 isolates may cause infections in humans [8]. However, the mechanisms underlying successful colonization of pigs are poorly understood. Determination of the essential bacterial colonization factors is crucial to develop new treatment strategies to prevent colonization and consequently reduce MRSA ST398 interspecies transmission. Animal models are useful to study MRSA colonization. Murine [9] and rat models [10] have been developed specifically for studying *S. aureus* colonization in humans.

However, the study of Gonzalez-Zorn showed that the murine nasal cavity is not a natural habitat of *S. aureus* and that this model may not be optimal to study *S. aureus* colonization [11]. Recently, *in vivo* pig colonization models have been applied [12-14]. Inoculation of pigs however, yielded variable results possibly due to unstable colonization [13,14] or too low numbers of bacteria to detect with the sampling and/or isolation method used [13]. *In vivo S. aureus* colonization may be further difficult to control due to the presence of undefined local microbial and environmental factors. A suitable alternative system to gain better understanding of nasal colonization may be the use of porcine nasal mucosa explants in which bacterial and host factors can be evaluated under controlled conditions. At present there is no *ex vivo* model to study pig nasal colonization although some models based on the nasal primary tissue culture are used in virological studies [15-17]. In the present study we for the first time established porcine nasal mucosa explants as a novel tool to study MRSA ST398 colonization in pigs using bacterial observation of CFU changes in time or maintenance on the explants as indicators of colonization.

Material and Methods

Isolation and cultivation of the nasal mucosa explants

Animals (Landrace, 6 months old sows, 70-75 kg) were MRSA negative and came from van Beek SPF pig farm B.V. (Lelystad, The Netherlands). Pigs were euthanized and exsanguinated following experimental / teaching surgery at the UMCU (Utrecht, The Netherlands). The pig head, as medical waste, was removed from the carcass, and immediately used for isolation of mucosa tissue.

Isolation of the nasal mucosa explants was performed according to the protocol of Glorieux *at al.* 2007 with some modifications [17]. Briefly, after removal of the head of the sow, the nose was sawed off the skull at a level just distal of the eyes. The mucosa membrane was carefully stripped from the surfaces of the ventral turbinates and septum using surgical blades (Swann-Morton No. 24), and placed in Dulbecco's phosphate buffered saline with calcium and magnesium (DPBS) (Gibco, The Netherlands) supplemented with 1 mg/ml streptomycin (Invitrogen, The Netherlands), 1000 U/ml penicillin (Invitrogen), 1 mg/ml kanamycin (Invitrogen) and 5 µg/ml fungizone (Invitrogen)

The stripped mucosa of each tissue was divided into equal explant pieces of 1 cm^2 using 12 mm biopsy punches (AcuDerm Inc, USA). The epithelium was placed upwards on fine-meshed gauze for culture at an air-liquid interface. The explants were cultured in serum-free medium (50% RPMI GlutaMAX™/50% DMEM GlutaMAX™ (Gibco, The Netherlands)) supplemented with 100 µg/ml gentamicin (Invitrogen), 0.1 mg/ml streptomycin and 100 U/ml penicillin (Invitrogen). The medium was added to half the height of the explant thickness to create an air liquid interface [17]. A schematic

presentation of the model is shown in Supplementary figure S1. Culture was at 37°C in a 5% CO_2 atmosphere. Ciliary beating was checked on a daily basis (each 24 h) using light microscopy as described before [17].

Morphometric analysis

The nasal mucosa explants were evaluated by light microscopy and by SEM after 0, 24, 48 and 72 h of cultivation. For light microscopy the explants were fixed in a phosphate-buffered 3.7% formaldehyde solution for 24 h. After fixation, the samples were embedded in paraffin. Sections (4 µm thick) were cut, deparaffinised in xylene, rehydrated in descending grades of alcohol, stained, and dehydrated in ascending grades of alcohol and xylene.

Haematoxylin–eosin staining was used to estimate the epithelial thickness. Using Soft Imaging System Leica LAS AF Lite software (Leica Microsystems, Germany) the effect of *ex vivo* culture of nasal mucosa explants on the epithelial morphometry was evaluated by measuring the epithelial thickness at five randomly selected places in five random fields.

Viability was analyzed using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Germany) which determines DNA fragmentation. Detection is based on the terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL assay). Detection was performed according to the manufacturer's instructions. TUNEL-positive cells were counted from in five randomly chosen fields of 100 cells in epithelium as well as in underlying connective tissue.

A microscope (Olympus microscope BX60) was used to analyze all samples at 200x magnification.

For SEM analysis explants were fixed in HEPES-buffer with 2.5% glutaraldehyde for 24 h. Afterwards, the samples were post-fixed in 1% osmium tetroxide for 2 h at room temperature. Next, the fixed samples were dehydrated in an increasing gradient of alcohol and transferred to a critical point drier. The dried samples were followed by Pt/Pd sputter coating. Cells were viewed in a field emission scanning electron microscope at 5 kV (Philips XL30S SEM FEG, Germany) at 2500x magnification.

Bacterial strains

Three different MRSA ST398 isolates were used to assess the ability to maintain on the nasal mucosa explants: MRSA S0462 (*spa*-type: t011, SCC*mec* IV) was isolated from a carrier pig, MRSA S0385-1 (*spa*-type: t011, SCC*mec* V) was derived from an endocarditis patient [18], whereas S0385-2 was a laboratory variant with a phage integrated in the beta-toxin gene (*hlb*), lacking the lyses of red blood cells. Additionally, human derived MRSA strain Mu50 (*spa*-type: t002, SCC*mec* II) was used. All strains were obtained from the UMCU collection (Utrecht, The Netherlands).

Bacterial colonization

The explants were inoculated with MRSA isolates as described previously [19]. Briefly, *S. aureus* strains were grown overnight in BHI at 37°C. A 2% aliquot was inoculated into fresh 10 ml broth and grown at 37°C under shaking (200 rpm) to mid-exponential phase (approximately 4 h). Bacteria were harvested by centrifugation at 3,750 x g for 5 min, washed 3 times in DPBS, and suspended to an OD_{600} of 0.6 (approximately $3x10^8$ CFU/ml) in DPBS. Explants were washed with the cultivation medium without antibiotics and kept without antibiotics for at least 18 h prior inoculation. Explants were taken from the gauze and placed into a 24-well plate with the epithelial surface upwards. Next, explants were inoculated with 1 ml of bacterial suspension and allowed to adhere to the explants for 2 h at 37°C and 5% CO_2. After incubation explants were washed three times with 1 ml of PBS and placed onto a 6-cell culture insert with 0.4 µm pores (Falcon, Becton Dickinson, The Netherlands), to prevent bacterial migration and growth in the lower chamber. To the lower chamber 3 ml of fresh cultivation medium without antibiotics was added, to the top chamber the medium was added until just a thin film of medium covered the explants (approximately 500 µl). Next, explants were cultivated for up to 3 h at 37°C and 5% CO_2. At different post adhering time points (0, 30, 60, 90, and 180 min) of the assay the explants were washed three times in 1 ml of PBS by pipetting. Bacteria were isolated from the explants by scraping the epithelium surface using cell scrapers (Falcon, Becton Dickinson, The Netherlands), and resuspended in 1 ml of DPBS with 0.1% Triton X-100. Bacterial suspensions were plated in serial dilution in PBS on Blood Agar Plates (Oxoid, UK). The plates were incubated overnight at 37°C and CFU were enumerated after 24 h. The colonization assay for each strain was repeated independently five times. Bacterial localization on the explants was determined by immunohistochemistry using mouse anti-*Staphylococcus aureus* protein A monoclonal antibody (Sigma–Aldrich, USA). At time 0 and 180 min after inoculation, explants were fixed in a phosphate-buffered 3.7% formaldehyde solution for 24 h. After fixation, the samples were embedded in paraffin. Sections (4 µm thick) were cut, deparaffinised in xylene and rehydrated in descending grades of alcohol. Next, sections were subjected to antigen retrieval by boiling in 10 mM sodium-citrate buffer (pH 6.0) and blocking of endogenous peroxidase. After rinsing in PBS/Tween20, primary antibodies (dilution 600x) were added and incubated overnight at 4°C. Sections were washed with PBS/Tween20 at RT and incubated with Powervision Goat-anti-Mouse/Rabbit/Rat IgG (Immunologic, The Netherlands) for 30 min at RT. After washing with PBS, sections were

incubated with 3',3'-diaminobenzidine (DAB) for 10 min at RT. Sections were washed with water, stained with heamatoxylin, and dehydrated in ascending grades of alcohol and xylene.

Results

Viability and morphology of the explants

The influence of cultivation on the viability and morphology of porcine nasal mucosa explants was first investigated. Viability was estimated by evaluating ciliary beating on the epithelial cells using a light microscope and by quantification of the number of apoptotic cells using the terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL) assay.

Cultivation of the mucosa explants for up to 72 h did not show any biological difference in the number of apoptotic cells (TUNEL-positive cells) in the epithelium (less than 1% of apoptotic cells) and basal body (less than 5% of apoptotic cells) of the explants (Figure 1A, Table 1). Morphometric analyses indicated that the explants also did not show changes in epithelial thickness after 72 h of *ex vivo* cultivation (Figures 1B and 2).

Table 1. Percentage of apoptotic cells as a parameter for the effect of cultivation.

Cell type	% TUNEL - possitive cells at hours post sampling and cultivation			
	0	24 hours	48 hours	72 hours
Epithelium	0.0 ±0.0	0.0 ±0.0	0.4 ± 0.2	0.6 ±0.5
Basal body	0.8 ±0.4	2.8 ±2.3	3.6 ±1.9	4.6 ±2.8

Scanning electron microscopy revealed the presence of both ciliated and non-ciliated cells at the surface of the explant. Representative scanning electron micrographs (SEM) of explants at 0 and 72 h of *ex vivo* culture illustrate ciliary cells and non-ciliary cells (Figure 3). During *ex vivo* culture, no morphological changes of the epithelium layer were observed. The cilia of the epithelial cells continued to beat up to 72 h after the start of cultivation.

Persistence of MRSA on the porcine mucosa explants

Next we investigated as to whether porcine mucosa explants could be exploited to study MRSA ST398 colonization. The ability of *S. aureus* to colonize the porcine mucosa explants was defined as persistence or outgrowth of MRSA on the explants. Three MRSA ST398 isolates were tested. One strain was isolated from a carrier pig (S0462), one human isolate originated from an endocarditis patient (S0385-1) and S0385-2 was a laboratory variant showing a different hemolysis pattern.

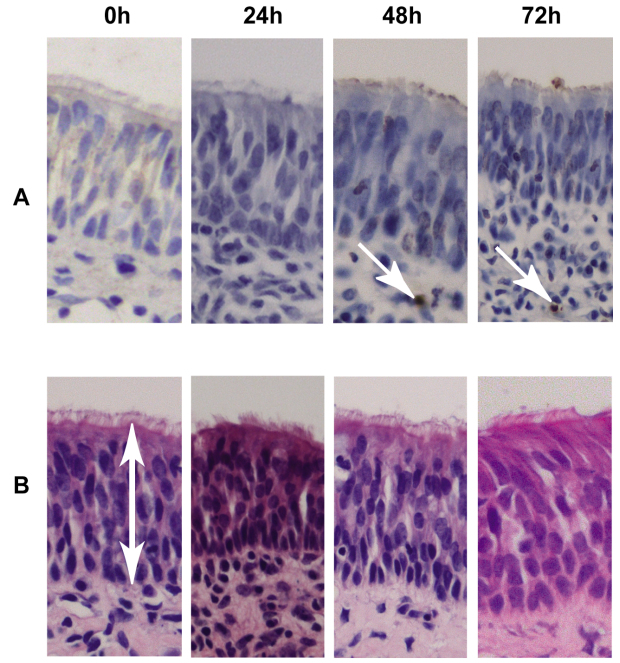

Figure 1 Evaluation of porcine mucosa explants after cultivation by means of light microscopy.
Sections of 4 mm thickness were stained by immunohistochemistry to evaluate the apoptosis of cells. TUNEL-positive cells in the epithelium are indicated with white arrows (panel A). Panel B shows the thickness of the epithelium after staining with haematoxylin-eosin (indicated with a white arrow).

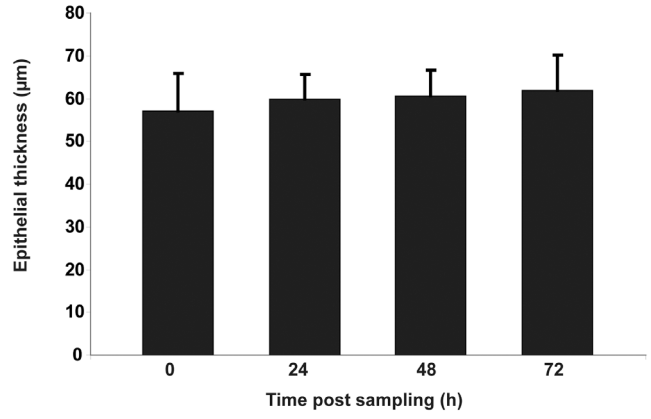

Figure 2. Average epithelial thickness of the porcine mucosa explants at different time points.
Data are presented as mean ± standard deviation (error bars) of five independent experiment.

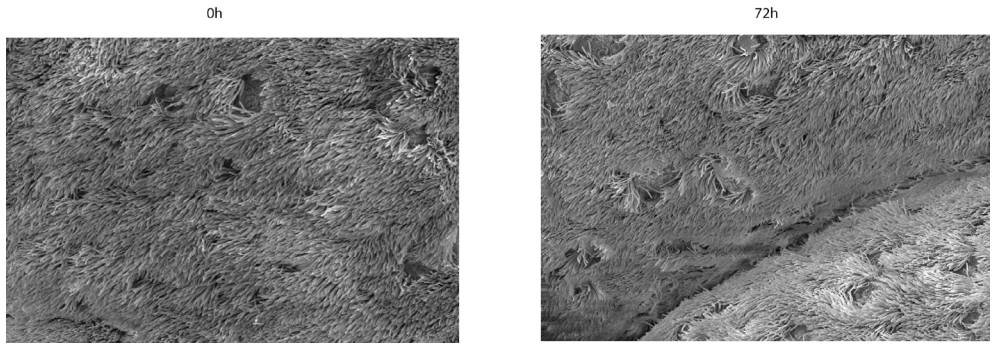

Figure 3. Scanning electron micrographs of porcine nasal epithelium.
Epithelial cells at 0 h and after 72 h of *ex vivo* cultivation.

Initial inoculation of the explants (1 cm^2) was performed with $3x10^8$ colony forming units (CFU)/ml. After 2 h of incubation and washing of the explants, approximately $8x10^6$ CFU/cm^2 (5%) adhered to the explants. The presence of the isolates on the mucosa explants was followed for an additional 180 min. During the first 30 min, S0462, S0385-1 and S0385-2 showed an initial decline in the number of CFU to approximately $3x10^6$, $2x10^5$ and $1.5x10^5$ CFU/cm^2 respectively (Figure 4). Then, the number of recovered bacteria from isolate S0385-1 remained almost stable until the end of the experiment at approximately $4x10^5$ CFU/cm^2. Bacterial adhesion for isolate S0462 remained stable until 90 min of the experiment. Then a significant increase to approximately $4x10^7$ CFU/cm^2 was observed. Bacterial recovery for MRSA S0385-2 showed a gradual decline during the experiment up to approximately $2x10^2$ CFU/cm^2 at the end of the incubation period.

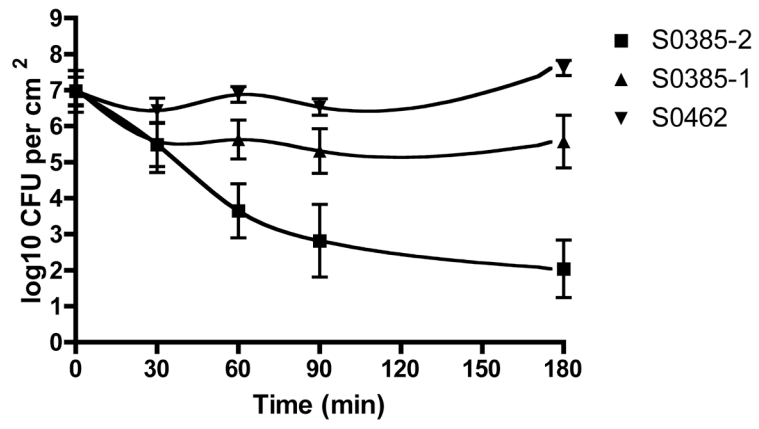

Figure 4. MRSA colonization of the porcine mucosa explants.
Log scale presence of MRSA isolates S0462 (), S0385-1 () and S0385-2 () on the porcine nasal mucosa explants. Data are presented is the mean CFU ± standard deviation (error bars) of five different pig experiment.

As a control, growth of the strains in culture medium alone without antibiotics at 37^0C in 5% CO_2 without shaking for 3 h did not show any inhibition (data not shown). These results were reproducible in five independent experiments. To verify whether the porcine nasal mucosa explants *ex vivo* model can be used to study colonization by other MRSA strains, MRSA Mu50 of human origin was used. This strain showed a similar colonization pattern as S0385-1 on the explants. The growth curve is shown in Supplementary figure S2.

To visualize MRSA ST398 on the explants during the colonization assay, immunohistochemistry using anti-*Staphylococcus aureus* protein A monoclonal antibody was used (Figure 5).

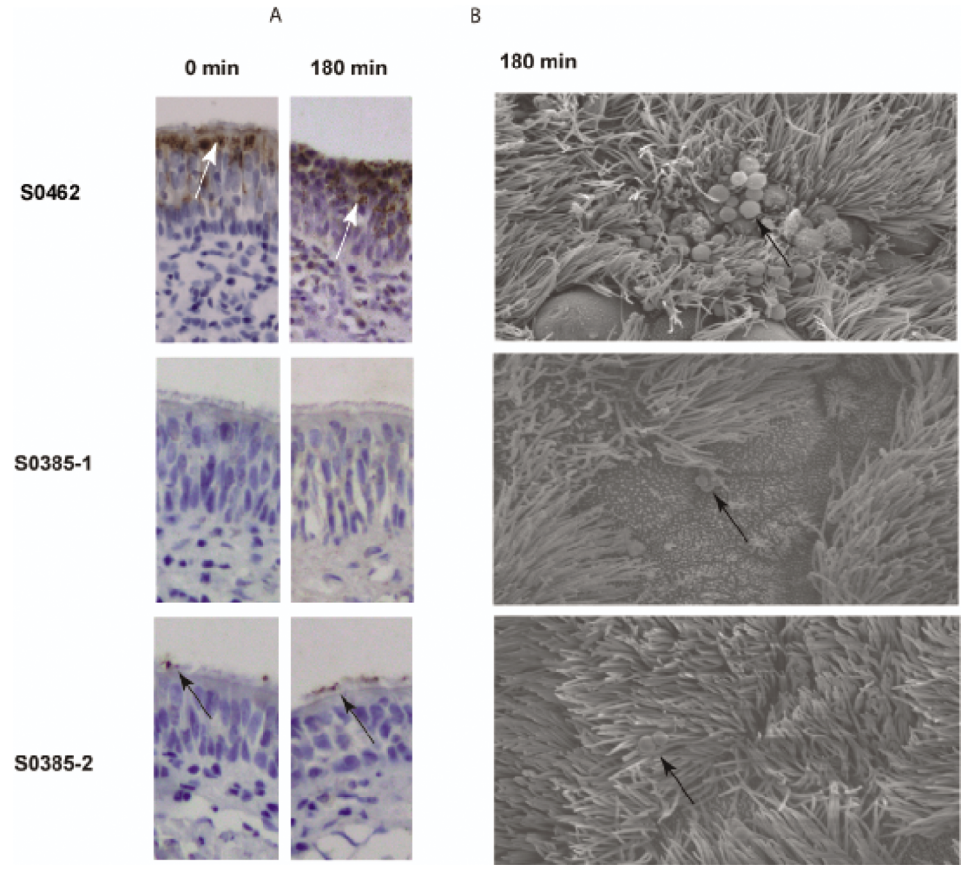

Figure 5. Bacterial localization on the porcine nose mucosa
Immunohistochemistry microscopy (panel A) of the porcine mucosa explants was used to determine the bacterial localization of MRSA S0462, S0385-1 and S0385-2 on explants at 0 and 180 min of colonization. Panel B shows the scanning electron micrographs of the surface of the porcine mucosa explants after 180 min colonization. Bacteria are indicated with arrows.

We were able to determine the localization of strain S0462. Isolate S0462 adhered to the surface under cilia and between epithelial cells of the top part of mucosa explants. After 180 min of incubation, the isolate S0462 formed clusters of colonies on the surface of the explants, and migration of the bacterial cells to the bottom part of the epithelium layer was observed. The immunochemistry of the explants inoculated with strains S0385-1 and S0385-2 yielded either no or a weak signal, respectively. Nevertheless, these strains showed comparable numbers of bacteria as S0462 at post adhering time 0 min. Isolate S0385-2 adhered and remained on to the surface of the cilia itself. After 180 min of incubation with bacteria cells, isolate S0462 showed clustering of bacteria on the surface of the explants and further migration of the bacteria in the epithelium layer. Isolate S0385-2 remained on the surface of the cilia.

To visualize the interaction of the bacteria with the epithelium in more detail, SEM was performed on the explants at 180 min of the incubation. Bacteria of the strain S0462 were present as clusters on the epithelial surface between the cilia (Figure 5, panel B). Strain S0385-2 remained on the cilial surface and did not appear in clusters. SEM on bacteria of the S0385-1 showed bacteria located on the epithelial surface. Interaction of MRSA ST398 isolates with the tissue did not result in visible changes in morphology of the inoculated epithelium.

Discussion

We evaluated porcine nasal mucosa explants as a model system to study *S. aureus* colonization in pigs. The model was adapted from a study on interaction of viruses with the respiratory tract [17]. *In vitro* adhesion of *S. aureus* to monolayer cell cultures have been used, especially to study bacterial interaction with human cells [19]. The limitation of this system is the lack of diversity in cell types and often the lack of the presence of mucus [20]. The nasal environment contains different types of epithelial cells [21]. The *ex vivo* explants model system applied here was designed to overcome these limitations and to better display the many characteristics and cell types of the porcine nasal mucosa cells *in vivo*.

The porcine nasal mucosa explants were cultivated at an air-liquid interface which creates a physiological environment corresponding to natural conditions. As described earlier [17], serum-free medium was used to cultivate the explants. It has been reported that use of fetal calf serum results in enlarged epithelial cells, loss of cell–cell contacts and a loose epithelium. With the conditions employed in our hands, we successfully maintained porcine nasal respiratory explants for at least 72 h without any signs of gross changes in cell viability as measured by the presence of apoptotic cells (more than 5%).

Similarly, morphometric analyses of the mucosa explants showed no major changes during *ex vivo* cultivation. Furthermore, ciliary beating was observed during the entire cultivation and SEM showed that cell-cell contact and three-dimensional structures of the tissue were preserved. Together, these results indicate that the porcine nasal mucosa explants preserved their integrity and viability in *ex vivo* conditions for up to 72 h under the conditions employed.

To evaluate porcine nasal mucosa explants as a new tool to study MRSA colonization in pigs, we used three MRSA ST398 strains as inoculum. All showed reproducible adherence to the epithelial layer of the mucosa explants. However, we observed differences in persistence of the isolates. One isolate was from a carrier pig (S0462), the two other strains were from a patient with endocarditis (S0385-1 and S0385-2). All three isolates showed an initial decline in the number of CFU during the first 30 min after inoculation which might indicate bacteria adaptation to the explants. After 30 min of post adhering period, bacterial recovery from the experiments showed a significant increase of the number of CFU for isolate S0462, unaltered bacterial number for isolate S0385-1, and a loss bacteria for isolate S0385-2, suggesting differences in interaction of the different isolates with the tissue. Additionally, the colonization assay using a human derived MRSA strain Mu50 showed a similar pattern to MRSA S0385-1. We conclude that this model can be use to study MRSA colonization belonging to different clonal complexes and human origin.

Attempts to visualize the bacteria on the tissue was performed using anti-*Staphylococcus aureus* protein A monoclonal antibodies were only partially successful. Isolates S0385-1 and S0385-2 were probably poorly visible due to the presence of low numbers of bacteria and/or insufficient expression of protein A. It has previously been documented that some *S. aureus* strains show no or very low expression of protein A *in vivo* [22]. Additionally, Western blotting of stationary grown bacteria in BHI medium confirmed poor expression of protein A in these strains (data not shown). SEM of tissue carrying isolate strain S0462 revealed clusters of bacteria located between the cilia. For the two isolates S0385-1 and S0385-2 only single bacteria were observed. The absence of bacterial clusters may be caused by the lower number of bacteria recovered for these strains. However, we cannot exclude the alternative possibility that the lack of bacterial cluster formation contributed to the poor bacterial recovery of these isolates. Our observations do suggest that different MRSA isolates display variable qualities in colonizing mucosa explants, which perhaps mimics natural host colonization. Due to the fact that adhesion of the bacteria to the tissue was performed in DPBS, which is not reflecting the *in vivo* situation, small changes comparing with *in vivo* situation may occur.

A major advantage of the *ex vivo* porcine nasal mucosa explants model is that different bacterial strains can be tested under controlled conditions.

From one animal around 20 explants (1 cm^2) can be obtained and multiple strains can be tested simultaneously eliminating genetic variation of the host. In addition, the model can be readily adapted for other bacterial species or the introduction of multiple species. The successful establishment of porcine nasal mucosa explants to study of the interaction of *S. aureus* isolates with nasal tissue enables studies to better understand the mechanisms of colonization of MRSA in pigs and may aid future assessment of the effects of potential inhibitory compounds on this process.

Acknowledgements

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Supplement

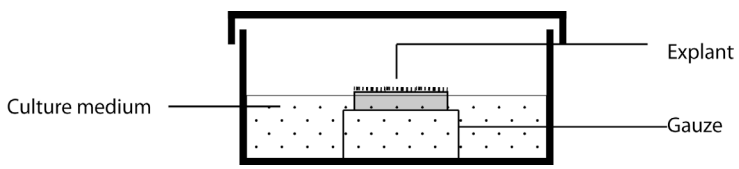

Figure S1. Schematic cross-section of a culture system using nasal mucosa explant with an air-liquid interface.

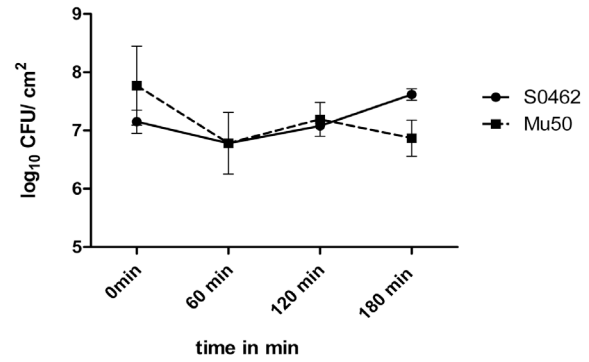

Figure S2. Log scale presence of pig origin MRSA S0462 and the human derived strain Mu50 on the porcine nasal mucosa explants.
Data are presented as mean CFU ± standard deviation (error bar) of five different pig experiments. MRSA S0462 belongs to ST398 *spa*-type: t011 SCC*mec* V. MRSA Mu50 belongs to CC5 *spa*-type t002 SCC*mec* II. Strain Mu50 shows successful colonization on the porcine nasal mucosa explants, although variation between experiments was bigger with Mu50 compared to S0462.

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Chapter 3

Staphylococcus aureus ST398 gene expression profiling during *ex vivo* colonization of porcine nasal epithelium

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Submitted for publication

Abstract

Staphylococcus aureus is a common human and animal opportunistic pathogen. In humans nasal carriage of *S. aureus* is a risk factor for various infections. Methicillin-resistant *S. aureus* ST398 is highly prevalent in pigs in Europe and North America. The mechanism of successful pig colonization by MRSA ST398 is poorly understood. Previously, we developed a nasal colonization model of porcine nasal mucosa explants to identify molecular traits involved in nasal MRSA colonization of pigs. Here, we report the analysis of the transcriptome of MRSA ST398 strain S0462 during colonization on the explant epithelium. Major regulated genes were encoding metabolic processes and regulation of these genes represents metabolic adaptation to nasal mucosa explants. Colonization was not accompanied by significant changes in transcripts of main virulence associated genes or known human colonization factors. Here, we document regulation of two genes which have potential influence on *S. aureus* colonization; cysteine extracellular proteinase (*scpA*) and von Willebrand factor-binding protein (*vwbp*, located on SaPIbov5). Colonization with isogenic-deletion strains (*ΔvwbpΔscpA*) did not alter the nasal *S. aureus* colonization compared to wild type. Our results suggest that nasal colonization with MRSA ST398 is a complex event that is accompanied with changes in bacterial gene expression regulation and metabolic adaptation.

Introduction

Staphylococcus aureus is an opportunistic pathogen colonizing the upper respiratory tract and skin of humans and other mammalian species. The nose is considered to be the primary ecological niche of *S. aureus* in humans [1]. Nasal carriage of *S. aureus* has been identified as a risk factor for the development of various infections in humans [1].

In 2004 a new distinct sequence type (ST398) of methicillin-resistant *S. aureus* (MRSA) has been isolated from pigs in The Netherlands [2]. Since then, MRSA ST398 has been detected in pigs, veal calves and poultry around the world [3-5]. The transmission of MRSA ST398 from livestock to humans has been reported in many countries [6,7] and contact with livestock is recognized as a risk factor for human colonization [4,8]. Additionally, ST398 isolates may cause infections in humans [9]. However, the mechanisms underlying successful colonization of pigs and other livestock are incompletely understood.

The molecular mechanisms involved in *S. aureus* colonization have been mainly studied using human cell cultures [10] as well as rodent models [11] [12]. *S. aureus* colonization involves many factors [13]. A crucial step of colonization is attachment to eukaryotic cells which involves several essential factors such as: clumping factor B (ClfB), iron-regulated surface determinant protein A (IsdA) and wall teichoic acid (WTA) [13]. Mutants deficient

in one of the genes responsible for expressing these components display reduced cell-attachment properties *in vitro* [10] and show reduced colonization in animal models [14-16]. Additionally, a ClfB mutant shows weakened colonization in the nares of human volunteers when compared to wild type bacteria, suggesting that ClfB is one of the main *S. aureus* factors involved in human colonization [17]. In addition to these factors, several other proteins (e.g., SdrC, SdrD, SasG, and FnbpA) appear to be involved in colonization by binding to desquamated nasal epithelial cells which confirms the multi-factorial nature of *S. aureus*–host interactions [18,19].

The natural occurrence of *S. aureus* in pigs may involve similar colonization factors as assumed for humans. On the other hand, it has been shown that the MRSA ST398 prevalence in pigs is high and that pigs are very rarely colonized by other *S. aureus* lineages. These observations suggest that additional factors may be involved in successful host adaptation and maintenance of colonization of *S. aureus* ST398 in livestock. A study of Viana *et al.* suggests that the presence of an additional von Willebrand binding factor protein (*vwbp*), located on a pathogenicity island (SaPI), represents a host adaptation factor of *S. aureus* for animals [20]. Moreover, it has been suggested that mobile genetic elements (MGEs) play a central role in the adaptation of bacteria to different host species [21].

To study *S. aureus* colonization in pigs, we have developed an *ex vivo* porcine nasal mucosa explants model [22]. Our aim was to identify bacterial factors involved in maintenance of *S. aureus* colonization in pigs by analyzing and documenting global gene expression changes in *S. aureus* during *ex vivo* colonization. This is the first study to examine the complete *S. aureus* transcriptome during experimental colonization which mimics natural MRSA ST398 colonization in pigs.

Materials and Methods

Bacterial strain and *ex vivo* colonization assay

MRSA ST398 S0462 strain (*spa*-type: t011, SCC*mec* IV) was isolated from a colonized pig. The preparation of nasal mucosa explants from pigs was performed as previously described [22]. The explants were inoculated with MRSA isolates as described previously [22]. Briefly, *S. aureus* strains were grown overnight in BHI at 37°C. A 2% aliquot was inoculated into fresh 10 ml broth and grown at 37°C under shaking (200 rpm) to mid-exponential phase (approximately 4 h). Bacteria were harvested by centrifugation at 3,750×g for 5 min, washed 3 times in Dulbecco's Phosphate-Buffered Saline (DPBS), and suspended at an OD_{600} of 0.6 (approximately 3×10^8 CFU/ml) in DPBS. The explants were inoculated with 1 ml of bacterial suspension in DPBS (approximately $3x10^8$ colony forming units (CFU)/ml in DPBS) for 2 h to allow the bacteria to adhere to the tissue (adhering time). Next, bacteria

were washed with DPBS to remove unbound bacteria and explants were cultivated for up to 3 h at 37°C in a 5% CO_2 atmosphere. At different post adhering time points (0, 30, 60, 90, and 180 min, which were defined as the time point after 2 h of inoculation) the explants were washed three times with 1 ml DPBS, before bacterial recovery for RNA isolation to remove bacterial cells not bound to the epithelium tissue. Bacteria were isolated from the explants by scraping the epithelium surface using cell scrapers (Falcon, Becton Dickinson, The Netherlands) and resuspended in 1 ml of DPBS with 0.1% Triton X-100. Nine hundred µl of the bacterial suspension was immediately centrifuged at 20,000 x g for 2 min (room temperature) and the resulting pellet was frozen at -80°C before RNA isolation. The remaining 100 µl of bacterial suspension was serially diluted in DPBS and plated on blood agar plates (Oxoid, UK). The plates were incubated overnight at 37°C and CFU were enumerated after 24 h. The colonization assay was repeated independently four times. At each time-point bacteria were harvested and stored at -80°C until further RNA isolation and microarray analyses.

RNA extraction

RNA was purified using the NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Germany) according to manufacturer's protocol with some adjustments as described [23].

Microarray design

The microarray was specifically developed for multiple *S. aureus* strains. The complete design was performed in a two-step procedure. First, 60-mer oligonucleotides were designed each 40 base pairs on alternating strands for the first sequence, ST398. Second, for each next sequence oligonucleotides were only designed for regions that were not probed by previously designed oligonucleotides. Oligonucleotides that match a sequence with a bitscore over 80 where considered as usable for probing. Using these parameters 121,901 probes were generated and manufactured as a microarray by NimbleGen (Roche, The Netherlands) in a 12x 135K format.

Labelling of total RNA, hybridization and scanning

Total RNA was labeled with fluorescent dyes by an amplification procedure and direct labeling. A total of 100 ng RNA was used as input for the Ovation Pico WTA System according to manufacturer's instructions (Nugen Technologies, Inc, USA). Two µg of purified and amplified cDNA was used as input for labeling by randomly priming with Superscript II reverse transcriptase (Invitrogen, The Netherlands), random octamers (100 ng/ µl) and actinomycine D, in a total volume of 10 µl, for 2 h at 42°C with the incorporation of Cy5- or Cy3-dUTP (Amersham, USA) with a ratio dUTP/dTTP of 3/1. Labeled cDNA was purified using Qia-quick PCR purification kit (Qiagen, USA). Incorporation of Cy3 or Cy5 was determined using a NanoDrop ND-1000.

The common reference was created by pooling Cy5-labeled RNA samples. Labeled cDNA was hybridized according to manufacturer's protocol (Roche NimbleGen, The Netherlands). A total of 1.1 µg Cy3-labeled cDNA and 1.1 µg Cy5-labeled common reference was mixed in 7.2 µl of Nimblegen hybridization cocktail. The mixture was heated to 65°C for 5 min, and a total of 6 µl was loaded onto the custom made *S. aureus* array and hybridized for 18 h at 42°C in a dedicated hybridization chamber (Roche NimbleGen, The Netherlands).

After the hybridization the arrays were dismantled at 42°C and washed in buffer 1 for 2 min at room temperature, then 1 min in wash buffer 2 at room temperature and finally 15 sec in wash buffer 3 at room temperature (Roche NimbleGen, The Netherlands). Slides were spun for 30 sec at 300 rpm to dry and scanned immediately in an Agilent DNA MicroArray Scanner. Data was extracted and processed using NimbleScantm software (version 2.6, Roche NimbleGen, The Netherlands).

Data analysis and statistical analyses

Processing of the data was performed using R (version 2.7.0) and the Bioconductor MAANOVA package (version 1.10.0). All slides were subjected to a set of quality control checks, which consisted of visual inspection of the scans, examination of the consistency among the replicated samples by principal component analysis (PCA), testing against criteria for signal to noise ratios, testing for consistent performance of the labeling dyes and visual inspection of pre- and post-normalized data with box and ratio-intensity plots. When the data was checked for effects of (random) experimental factors, slide and sample effects were observed. Slide effects were detected because eight arrays were printed on one glass slide and sample effects occurred as a consequence of the repeated measure design. After $\log_2$ transformation, the data were normalized by a LOWESS smoothing procedure to correct for dye bias effects. The resulting data were analyzed using a two-stage mixed ANOVA model [24,25]. The gene specific model included terms for Array, Slide and Sample effects (random), and Time and Reference (fixed). Genes that were differentially expressed between any of the time points were identified by a permutation test. Resulting P-values were corrected for multiple testing by calculating the false discovery rate (FDR) [26]. The significance threshold was set at 0.05 FDR. A complete set of the microarray data has been deposited at the GEO database.

Real-Time Quantitative Reverse Transcriptase PCR

RNA samples used in the microarray experiments were also analyzed by quantitative reverse-transcriptase real-time PCR (qRT-PCR). Purified *S. aureus* RNA was converted

using the Bacterial H-TR cDNA synthesis kit (AmpTech, Germany). The cDNA products were subsequently used in qRT-PCR using SYBR Green Master Mix for qRT-PCR (Takara Bio Inc, Japan) according to the manufacturer's instruction and the qRT-PCR reaction was performed in a LightCycler 4.80 (Roche, The Netherlands). The transcripts for *vwbp*, *scpA*, *hly* and *agr* were amplified using primers listed in Table S1. All signals were normalized to aroE and gmk gene transcripts (both housekeeping genes) [27]. Relative quantification of gene expression was calculated using the comparative cycle threshold method as described previously by Livak and Schmittgen [28]. Data obtained are expressed as the mean $\log_2$ fold-change in transcript during colonization *ex vivo* for selected post adhering times points (30, 60, 90, and 180 min) relative to the t = 0 control sample. Results from all time-points were analyzed in triplicate.

Construction of an isogenic *vwbp* and *scpA* deletion strain (*Δvwbp* and *ΔscpA*)

Isogenic *vwbp* and *scpA* deletions in the S0462 strain were generated by allelic replacement as described [29] with a slight modification. Briefly, regions around 1000 bp flanking the *vwbp* and *scpA* locus were amplified by PCR using primers listed in Table S1. The resulting PCR fragments were used as a template to create an insertion fragment by PCR overlap. Next, the PCR fragment was digested using *Eco*RI and *Not*I restriction endonucleases (Fermentas, Lithuania) and cloned into pKOR1 vector. The ligation product was transformed into competent *Escherichia coli* DC10b cells [30] and grown on LB agar containing 100 µg/ml ampicillin. Purified plasmid (500 ng) containing the correct insert (confirmed by sequencing) was electroporated into the target strain, *S. aureus* S0462, using the settings: 200 Ω, 25 µF and 1.5 kV. After electroshock 200 µl of TSB was added and cells were incubated at 37°C for 1 h with shaking, to allow recovery. The cells were plated on TSA plates containing 7.5 µg/ml chloramphenicol and grown overnight at 30°C. Single colonies were grown in TSB overnight at 30°C with vigorous shaking. Plasmid integration was checked by overnight culture at 43°C on TSA with 7.5 µg/ml chloramphenicol. Colonies were screened for single cross-over by PCR. Single cross-over mutants were grown in TSB without antibiotic at 30°C overnight, diluted 1:100,000 in sterile water and 100 µl was spread on TSA plate containing 50 µg/ml anhydrotetracycline and incubated at 37°C overnight. Large colonies were picked and cultured overnight at 37°C on TSA with 10 µg/ml chloramphenicol and plain TSA. Colonies growing only on plain TSA were assumed to be knock-out mutants. Putative mutants were validated by PCR amplification and sequencing of genomic DNA flanking the deletion. Confirmed knock-out strains were used in subsequent experiments.

Results

Persistence of MRSA ST398 S0462 on porcine mucosa explants

The ability of *S. aureus* to colonize porcine mucosa explants was defined as persistence or outgrowth of MRSA S0462 on the explants. The explants (1 cm^2) were inoculated with $3x10^8$ CFU/ml. After 2 h of incubation (37°C) and washing of the explants, approximately $8x10^6$ CFU/cm^2 (3%) adhered to the explants. The presence of *S. aureus* S0462 on the mucosa explants was followed for an additional 180 min. During the first 30 min of this latter period, MRSA S0462 showed an initial decline in the number of CFU to approximately $3x10^6$. Bacterial presence remained stable until 90 min into the experiment. In the following 90 min a significant increase of tissue-associated bacteria to approximately $4x10^7$ CFU/cm^2 was observed (Figure 1).

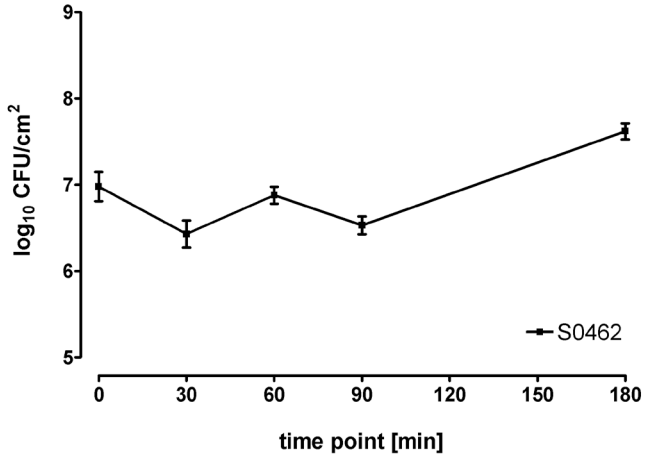

Figure 1. MRSA S0462 colonization of porcine mucosa explants.
Presence of MRSA S0462 on porcine nasal mucosa explants expressed as CFU on a log scale. Data are presented as the mean CFU ± standard deviation (error bars) of five different experiments with tissue from different pigs.

***S. aureus* transcriptome dynamics during *ex vivo* colonization**

To identify genes that possibly contributed to the successful colonization of *S. aureus* ST398 in pigs we analyzed the global changes in MRSA S0462 gene expression during *ex vivo* colonization on porcine nasal mucosa explants. From MRSA S0462, recovered at different time-points from the explants, RNA was isolated, converted to cDNA and hybridized with the *S. aureus* microarray. Expression levels were calculated as fold changes with respect to t=0 which was defined as the time point after 2 h of inoculation.

In total we documented significant regulation of the expression of 166 genes. Transcripts that were significantly up- or down-regulated (adjusted p=0.05) were visualized in a hierarchical cluster (Figure 2).

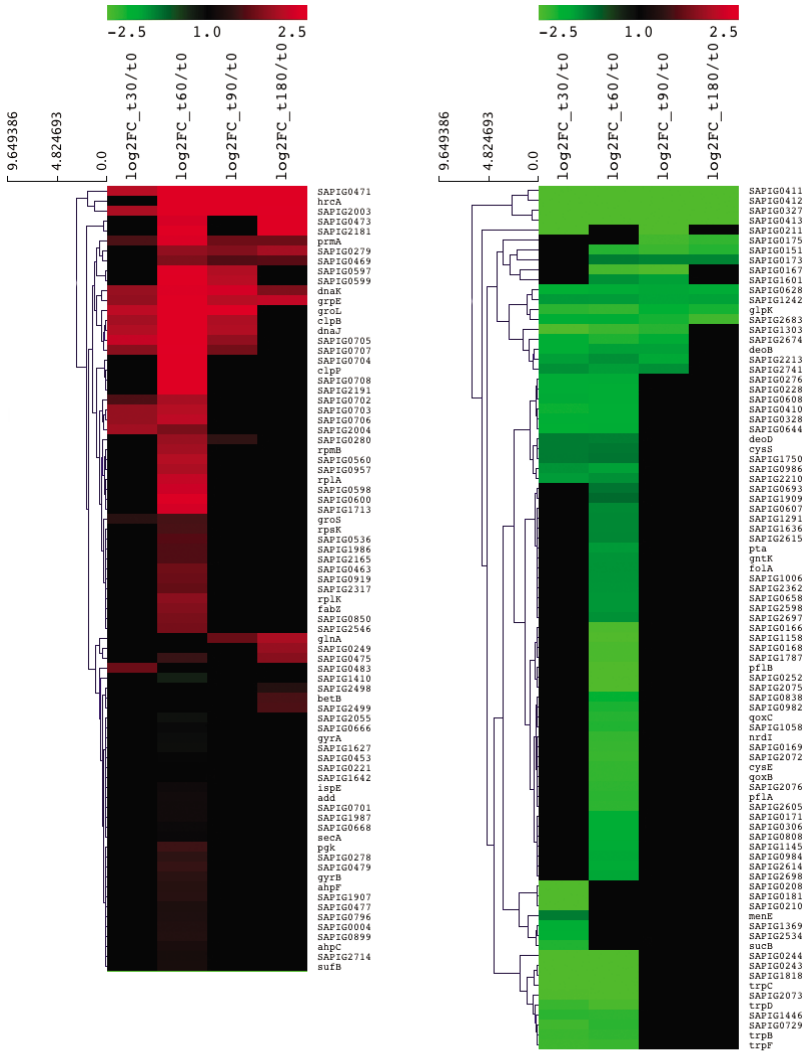

Figure 2. Heatmap of MRSA S0462 during ex vivo colonization on porcine nasal mucosa explants.
Gene expression profiles of all significantly ($p<0.05$) up- and down- regulated gene transcripts in MRSA S0462 during *ex vivo* colonization. Significantly regulated genes are indicated in red (up-regulated) or green (down-regulated). Results are presented as $\log_2$ fold-changes compared to time-point t=0.

The visualization of the significantly regulated genes shows that transcripts were either up- or down regulated over time. There were no transcripts that changed in expression direction during the *ex vivo* colonization assay. Moreover, most of the gene regulation was observed at the first two post adhering times points (t=30 and 60 min).

The regulated genes were mapped to available KEGG pathways based on the gene IDs. Most regulated genes were involved in metabolic processes, probably reflecting metabolic adaptation to nasal mucosa explants, of fatty acid biosynthesis, oxidative phosphorylation, and of phenylalanine, tyrosine and tryptophan biosynthesis (Table S1). In addition, regulation of the expression of genes that are part of the *agr* two-component regulatory system and a number of virulence genes was observed.

The genome of MRSA S0462 contains a pathogenicity island, SaPIbov5. Only a few genes located on this island were subject to regulation (*int*, *vwbp* and some of the hypothetical proteins) in our model. It has been suggested that presence of the gene encoding an additional von Willebrand binding factor protein (*vwbp*) located on the pathogenicity island may play a role in bacterial adaptation to the animal host [20]. During *ex vivo* colonization *vwbp* gene expression changed only at t=30 min (fold change 1.70). Most of the other genes located on the SaPIbov5 showed a change in expression at t=60 min.

MRSA S0462 *ex vivo* colonization was also accompanied by changes in a number of putative virulence genes. The analysis showed down-regulation of expression of the *cap* operon encoding capsular biosynthesis (mainly at t=60, fold change varied -0.78 to -2.47) and of the *hla* gene conferring alpha-hemolysis (at t=60 min fold change = -2.52). Up-regulation was observed for the cysteine proteinase cluster (*scpAB*). The expression of the *scpA* gene encoding the cysteine proteinase was up-regulated during the entire experiment (fold change varied from 2.07 to 3.52), while *scpB* encoding the cysteine proteinase cellular inhibitor was up-regulated only at t=30 and 60 min (fold change of - 2.07 and -1.78, respectively). Moreover, the expression of the genes that constitute the *agr* locus, which encodes a quorum sensing system that controls the expression of virulence genes was also down-regulated mainly at t=60 min. However, regulation of *agrD* transcription was not detected.

To verify the microarray results, four genes of interest (*vwbp, scpA, agrA,* and *hla*) were subjected to qRT-PCR (Figure 3) using the same RNA samples. The qRT-PCR results confirmed the microarray data (*vwbp* r = 0.98, *scpA* r = 0.93, *agrA* r = 0.95, and *hla* r = 0.63), although the fold change values determined by qRT-PCR were higher compared to the microarray data.

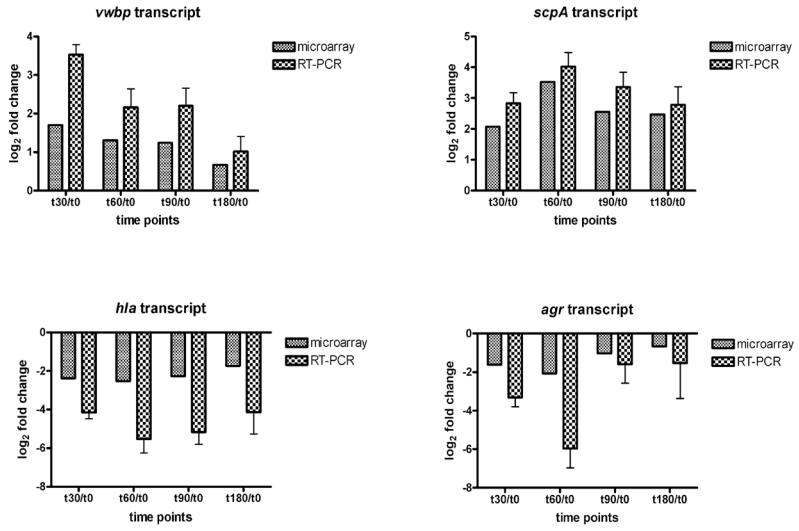

Figure 3. Changes in expression of four transcripts during *ex vivo* colonization.
Validation of microarray data by real-time qRT-PCR. Results are expressed as the average $\log_2$ fold-change in transcript during *ex vivo* colonization.

S. aureus ex vivo colonization did not show regulation of genes encoding surface proteins responsible for attachment of bacteria to the epithelium (*clfB, isdA,* and *fnbA*). To determine whether these genes were expressed during *ex vivo* colonization qRT-PCR analysis was performed. The results showed that the three genes important for human colonization: *clfB, isdA,* and *fnbA* were expressed during the colonization of the pig tissue (Figure S1).

Contribution of the *vwbp* and *scpA* to *ex vivo* colonization

Next, we generated isogenic *vwbp* and *scpA* deletion mutants of MRSA S0462 (*Δvwbp* and *ΔscpA*) to investigate the potential role of these genes in MRSA S0462 colonization. The *vwbp* and *scpA* mutants were tested in the *ex vivo* colonization assay (Figure 2S A and B). Surprisingly, the colonization pattern of the wild-type, *Δvwbp* and *ΔscpA* mutants did not show significant differences (Figure 2S C). These findings indicate that investigated genes are not crucial for colonization *ex vivo*, but may reflect adaptive changes during the colonization event.

Discussion

The nostrils are the primary reservoir of *S. aureus* both in humans and pigs. Nasal carriage of *S. aureus* has been identified as a risk factor for the development of various infections in humans [1]. The last decade, swine have appeared as the major reservoir of MRSA *S. aureus* ST398 which also emerged in other livestock animals. Currently, contact with livestock is recognized as main risk factor for MRSA ST398 colonization in humans. However, the factors which play a role in the colonization and maintenance of MRSA ST398 in livestock are unclear. *S. aureus* colonization in humans and rodent animal models is a multifactorial process which includes steps like bacterial attachment to the cells, immune escape as well as competition between *S. aureus* and natural flora. Identification of essential factors involved in *S. aureus* maintenance of colonization and adaptation in livestock are difficult to perform in an *in vivo* setting. Previously, we reported the successful establishment of an *ex vivo* model to study MRSA colonization [22]. Using this model we were able to mimic the natural situation in pig's noses under controlled conditions. During the *ex vivo* colonization, an initial decline in the number of CFU of *S. aureus* S0462 during the first 30 min after inoculation was observed. Later, a significant increase in the number of bacteria was detected, which indicates bacterial adaptation to the explants. From these data we conclude that our model mimics nasal colonization.

Data on gene expression of *S. aureus* during colonization is limited to the direct transcript analysis of a few genes [31,32]. Global changes in gene expression during colonization have not been studied before. The major aim of our study was to identify bacterial factors involved in maintenance of colonization by determining and documenting the changes in *S. aureus* gene expression during *ex vivo* colonization.

The expression of 166 genes was observed to be significantly regulated. The expression of the majority of genes was regulated between 30 and 60 min after inoculation. Only 42 genes were regulated between 90 min and 180 min of colonization although we observed increasing numbers of bacteria on the explants. This growth apparently followed changes in regulation of metabolic pathways like: fatty acid biosynthesis, oxidative phosphorylation, and phenylalanine, tyrosine and tryptophan biosynthesis and regulation in the *agr* system which were mainly up-regulated at t= 60 min. Genes involved in the main amino acid biosynthesis pathways were not regulated in our system, except for phenylalanine, tyrosine and tryptophan biosynthesis pathways. The active protein biosynthesis, together with lack of regulation of the de novo amino acid biosynthesis pathways indicates that the bacterial cells have access to these amino acids. The *ex vivo* colonization was performed at an air-liquid interface, where bacteria may have contact with the cultivation medium (RPMI and DMEM) containing free amino

acids which may have served as a source of amino acids for bacteria, although we carefully avoided the presence of culture medium on top of the explants.

A crucial step of *S. aureus* nasal colonization is adhesion to epithelial cells. It has been shown that ClfB, IsdA proteins and WTA are essential factors for *S. aureus* adherence to human cell lines *in vitro* [10,19], nasal colonization of rodents [14,16,33], and for nasal colonization of humans [17]. After initial adherence, during the first 30 min MRSA S0462 showed an initial decline in the number of CFU and significant increase of tissue-associated bacteria in later phase of the experiment. Moreover, in the pig nasal *ex vivo* system we do not observe regulation of expression of the adhesions genes, however the genes are expressed. In our settings, bacteria used for tissue inoculation were harvested in the mid-log phage, where adhesion factors are well expressed. Later, contact with the nasal epithelium did not result in regulation of the expression of genes encoding adherence factors such as *clfB, isdA,* and *fnbA*.

At least part of the adhesin expression is under the regulation of the *agr* system [31]. The *agr* system has a dual action in the global gene regulation in *S. aureus*. This system positively regulates toxins, extracellular proteases, immunomodulation factors and capsule biosynthesis, but represses the expression of some of the surface proteins such as protein A, coagulase and fibrinogen binding protein past the exponentional phase. During *ex vivo* colonization the *agr* locus was down-regulated during the first stage of colonization. However, the *agrD* gene, part of the *agr* locus, is not significantly regulated. This gene may be expressed at a basal level without a change in regulation. Additionally, we observed down-regulation of hla and the cap operon, which are controlled by the *agr* system (down-regulated). Burian *et al.* had similar findings showing that during *S. aureus* colonization of the human nose and in cotton rats the *agr* system is weakly expressed [31,32].

Interestingly, the cysteine proteinase operon (*scpAB*) was up-regulated during the whole experiment. ScpA is known to cleave a number of extracellular matrix components and it has been suggested to play a role in bacterial migration from the sites of initial colonization [34,35], but it may also play a role in the acquisition of nutrients [36]. It has been suggested that the extracellular proteases can promote *S. aureus* skin and nares colonization by degradation of some *S. aureus* virulence factors like toxins [37]. Moreover, it has been shown that *S. aureus* ScpA protease is associated with diseases such as Staphylococcal Scalded Skin Syndrome [38] and is involved in vascular leakage causing sepsis [34]. The up-regulation of the extracellular proteinase ScpA indicates that this protein may be involved in establishment of colonization *ex vivo*. However, a single knockout mutant did not show any phenotypic difference in colonization pattern *ex vivo*, which indicates that ScpA does not play a crucial role in colonization / adaptation *ex vivo*.

Also the up-regulation of some genes located on SaPIbov5 was observed. This mobile element encodes an additional von Willebrand factor binding protein. It has been shown that *S. aureus* harboring a pathogenicity island with the additional *vwbp* are widely distributed in ruminants and it has been suggested that this protein is one of the adaptation factors for *S. aureus* to animal hosts [20]. Our study showed that during *S. aureus* ST398 interaction with porcine nasal epithelium the *vwbp* is strongly up-regulated during the first phase of the experiment (t=30 min), where the initial decline of CFU was observed. This may indicate that *vwbp* may be important in *S. aureus* adaptation to the porcine nasal epithelium and may promote *S. aureus* colonization *ex vivo*. However, the single knockout mutant did not show any phenotypic difference in colonization pattern *ex vivo*, which indicates that *vwbp* also does not play a crucial role in colonization / adaptation *ex vivo*.

A limitation of our model is that adhesion of the bacteria onto the tissue was performed in DPBS. Additionally, the host immune response will be absent in our model and may influence *S. aureus* gene expression.

In summary, we could for the first time establish the global gene expression pattern of *S. aureus* during *ex vivo* colonization. Additionally, this study shows that *S. aureus* colonization is a complex process that has to be further explored in order to fully understand the molecular mechanism responsible for successful colonization of MRSA ST398 in livestock.

Acknowledgments

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Table S1. Primers, plasmids and strains used in this study.

Primer	Sequence / description / characteristic	Function	Reference
vwb_F	GTGACTGGAGAAGCAAAC	qRT-PCR	This study
vwb_R	GCGTGCCTATTTACAGAG	qRT-PCR	This study
hla90	AGAAAATGGCATGCACAAAAA	qRT-PCR	[1]
hla488	TATCAGTTGGGCTCTCTAAAA	qRT-PCR	[1]
scpA_ F:	CACCCAAACTTACAAGGACAAC	qRT-PCR	This study
scpA_R:	CGCCATTGGATACTGGAATAAC	qRT-PCR	This study
agr-1189	CGATGTTGTTTACGATAGC	qRT-PCR	[2]
agr-1436	CGACACAGTGAACAAATTC	qRT-PCR	[2]
gmk_F	AAGGTGCAAAGCAAGTTAGAA	qRT-PCR	[3]
gmk_R	CTTTACGCGCTTCGTTAATAC	qRT-PCR	[3]
aroE_F	CTATCCACTTGCCATCTTTTAT	qRT-PCR	[3]
aroE_R	ATGGCTTTAATATCACAATTC	qRT-PCR	[3]
clfB-136	ATAGGCAATCATCAAGCA	qRT-PCR	[2]
clfB-270	TGTATCATTAGCCGTTGTAT	qRT-PCR	[2]
fnbA-160	GGAGCAGCATCAGTATTCTT	qRT-PCR	[2]
fnbA-308	AGTTGCAGTTGTTTGTGTTT	qRT-PCR	[2]
isdA-2665	GCAGTTACAGCAGGTTTA	qRT-PCR	[2]
isdA-2782	CAGCAAAACCAAACAATG	qRT-PCR	[2]
UP-F_vwb_EcoRI	GAGAATTCGCAATAAAATGCTTGGAGG	Cloning	This study
UP-R_vwb_overlap	aagtaattatatttagtatCTGTAAATTCTCCTTAATTTAAACG	Cloning	This study
DN-F_vwb_overlap	aaattaaggagaatttacAGATACTAAATATAATTACTTTGCTTC	Cloning	This study
DN-R_vwb_NotI	ATCGCGGCCGCTCACATCGCTTAACAATTAACG	Cloning	This study
UP_FS600vwb	GCTACAAGCAGCTTAGATCC	Sequencing	This study
UP_RS200vwb	TTCTGACTTGTCGATAAATG	Sequencing	This study
UP_RS700vwb	GATTCCAACTCTTGATAAC	Sequencing	This study
vwb1100DOWN_R	ATTCCCACTCAATGAAATAAG	Cloning	This study
vwb1100UP_F	TGTACGATTCTGTGGAATTG	Cloning	This study
vwb_50R	ATCGCTATTCCAAAGTTGTG	Cloning	This study
vwb_50F	ATACTCTAGGCGAAGCTC	Cloning	This study
DN_FS300vwb	ATGGATTGTATTGGCATAAG	Sequencing	This study
DN_FS800vwb	GAGGCATCTTCCTTGTCATC	Sequencing	This study
DN_RS400vwb	TATTATGAAAGCCCAGTGAC	Sequencing	This study
UP_F600scpA	CACTTTACTTCTCCTATTG	Sequencing	This study
UP_R200scpA	TAAACTGTGAAGAGTAGAG	Sequencing	This study
UP_R700scpA	ATGCCATACCTATGCAATTC	Sequencing	This study
UP_R_scpA_overlap	TAAATCTAGATAGTCTTACTTTTCATATAAAAACTCCTTTC	Cloning	This study
UP_F_scpA-EcoRI	GAGAATTCGTTTTGACGAATTTTTTGTACG	Cloning	This study
scpA1100UP_F	CCATAATGTACCCACTTACG	Cloning	This study
DN_F300scpA	AACCTTTCCTCTTCAAATAG	Sequencing	This study
DN_F800scpA	GACATAACCAAACCTAAAC	Sequencing	This study
DN_F_scpA_overlap	AGGAGTTTTTATATGAAAAGTAAGACTATCTAGATTTATTTAAG	Cloning	This study
DN_R400scpA	AAGTATCACATCAAGAAATC	Sequencing	This study
DN_R_scpA_NotI	TAGCGGCCGCCCTTATGTCATTCGTAACAG	Cloning	This study
scpA1100DOWN_R	GGTTAGTTTGAAGCAGTTTG	Cloning	This study
scpA_50R	TCTCAGCGTTTGCAATAGGG	Cloning	This study
scpA_50F	ATGAACACAGAAGCTTTAGG	Cloning	This study
pKOR1 rev	CACACAGGAAACAGCTATGACATAGTC	Sequencing	[4]
pKOR1F outside	CAGCTCCAGATCCATATCCTTC	Sequencing	[4]
PLASMID			
pKOR1	*E. coli* (AmpR) - *S. aureus* (ChmR)		
	Shuttle vector for allelic replacement in staphylococci.		[4]
pKOR1Δvwb	Construction deletion of *vwbp* for *S. aureus* S0462		This study
pKOR1ΔscpA	Construction deletion of *scpA* for *S. aureus* S0462		This study
STRAINS			
S. aureus S0462	Pig carrier isolate		
S. aureus S0462 *Δvwb*	*vwbp* deletion		This study
S. aureus S0462 *ΔscpA*	*scpA* deletion		This study
E. coli DC10B	*dcm* deficient *E. coli* DH10B,	staphylococcal cloning host allowing transformation of plasmids directly into *S. aureus*	[5]

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3. Hirschhausen N, Schlesier T, Peters G, Heilmann C. (2012) Characterization of the modular design of the autolysin/adhesin *aaa* from *Staphylococcus aureus*. PLoS One 7: e40353.
4. Bae T, Schneewind O. (2006) Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. Plasmid 55: 58-63.
5. Monk IR, Shah IM, Xu M, Tan MW, Foster TJ. (2012) Transforming the untransformable: Application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. MBio 3: e00277-11

Table S2. Changes in the MRSA S0462 transcriptome during ex vivo colonization.

Microarray results are presented as the mean fold-change from six separate experiments. Only transcripts with significant fold-change (p value<0.05 -fold change in transcriptome) are included. The results are expressed as the average $\log_2$ fold-change in transcript during *ex vivo* colonization compared to t=0.

locus ST398	*symbol*	Description / function	t30/t0	t60/t0	t90/t0	t180/t0
SAPIG0004	*recF*	DNA replication and repair protein recF		1,28		
SAPIG0005	*gyrB*	DNA gyrase, B subunit		1,35		
SAPIG0006	*gyrA*	DNA gyrase, A subunit		0,90		
SAPIG0150	*deoD*	purine nucleoside phosphorylase	-0,76	-0,81		
SAPIG0151	*tet38*	MFS family major facilitator transporter, tetracycline:cation symporter		-1,97	-2,22	-1,95
SAPIG0153	*deoB*	phosphopentomutase	-1,62	-1,26	-1,27	
SAPIG0166	*capD*	polysaccharide biosynthesis protein CapD		-2,47		
SAPIG0167	*galE2*	UDP-glucose 4-epimerase		-2,36	-2,55	
SAPIG0168	*cap5F*	capsular polysaccharide biosynthesis protein Cap5F		-2,41		
SAPIG0169		UDP-N-acetylglucosamine 2-epimerase		-2,16		
SAPIG0171	*cap5I*	capsular polysaccharide biosynthesis protein Cap5I		-1,62		
SAPIG0173	*cap5K*	capsular polysaccharide biosynthesis protein Cap5K		-0,78	-0,87	-0,88
SAPIG0175	*cap5M*	capsular polysaccharide biosynthesis galactosyltransferase Cap5M			-2,32	-2,12
SAPIG0181		putative aldehyde dehydrogenase AldA	-3,15			
SAPIG0208		conserved hypothetical protein	-3,30			
SAPIG0210	*sacX*	PTS system EIIBC component SAR0193	-3,19			
SAPIG0211		rpiR family transcriptional regulator	-3,97		-3,31	
SAPIG0221	*azoR*	FMN-dependent NADH-azoreductase				
SAPIG0228	*mviM*	NADH-dependent dehydrogenase	-1,45	-1,55		
SAPIG0235	*pflB*	formate acetyltransferase		-2,88		
SAPIG0236	*pflA*	pyruvate formate-lyase 1-activating enzyme		-2,01		
SAPIG0243	*acx4*	acyl-CoA dehydrogenase	-3,47	-3,20		
SAPIG0244	*menE2*	acyl-CoA synthetase	-4,22	-3,95		
SAPIG0249		marR family transcriptional regulator				1,94
SAPIG0252		L-lactate dehydrogenase		-2,89		
SAPIG0276	*lrgB*	antiholin-like protein LrgB	-1,46	-1,43		
SAPIG0278		gntR family transcriptional regulator		1,38		
SAPIG0279		putative PTS transport system protein		1,90	1,82	2,03
SAPIG0280	*bglA*	6-phospho-beta-glucosidase		1,95	1,39	
SAPIG0306		lipoprotein, putative		-1,60		
SAPIG0327		putative sodium/glucose cotransporter	-2,73	-3,08	-2,78	-2,57
SAPIG0328	*nanA*	N-acetylneuraminate lyase	-1,59	-1,63		
SAPIG0410	*sgaT*	PTS family porter component IIC	-1,82	-1,60		
SAPIG0411	*sgaB2*	PTS family porter component IIB	-4,66	-4,67	-3,93	-4,39
SAPIG0412	*pts34A*	PTS family porter component IIA	-3,91	-4,32	-3,88	-3,67
SAPIG0413		transcriptional antiterminator, BglG family / PTS system, mannitol/fructose-specific IIA component	-3,08	-3,06	-2,96	-3,08
SAPIG0453		putative phosphoglycerate mutase		0,98		
SAPIG0457	*ahpF*	alkyl hydroperoxide reductase, F subunit		1,32		
SAPIG0458	*ahpC*	peroxiredoxin		1,21		
SAPIG0463		conserved hypothetical protein		1,72		
SAPIG0469	*int*	integrase		1,80	1,56	1,59
SAPIG0471		conserved hypothetical protein	2,15	3,55	3,58	3,99
SAPIG0473		hypothetical protein		2,34		2,78
SAPIG0475	*ri06*	saPI1 ORF18-like protein		1,43		1,85
SAPIG0477		pathogenicity island protein		1,24		
SAPIG0479		conserved hypothetical protein		1,42		
SAPIG0481	*add*	adenosine deaminase		1,12		
SAPIG0483	*vwbp*	von Willebrand binding protein	1,70			
SAPIG0536	*gltC*	transcriptional activator of glutamate synthase operon		1,57		
SAPIG0560	*veg*	protein veg		2,12		
SAPIG0561	*ispE*	4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase		1,10		
SAPIG0597	*ctsR*	transcriptional regulator CtsR		2,43	2,11	
SAPIG0598		conserved hypothetical protein		2,27		
SAPIG0599		putative ATP:guanido phosphotransferase LL3_00087		2,53	2,15	
SAPIG0600	*clpC*	ATP-dependent Clp protease ATP-binding subunit ClpC		2,39		
SAPIG0604	*cysE*	serine O-acetyltransferase		-2,11		
SAPIG0605	*cysS*	cysteine--tRNA ligase	-0,77	-0,71		
SAPIG0607	*rlmB*	trmH family tRNA/rRNA methyltransferase		-0,95		
SAPIG0608		conserved hypothetical protein	-1,39	-1,50		
SAPIG0612	*rplK*	ribosomal protein L11		1,88		
SAPIG0613	*rplA*	ribosomal protein L1		2,22		
SAPIG0628		conserved hypothetical protein	-1,74	-1,44	-1,35	-1,43
SAPIG0644	*nagB*	glucosamine-6-phosphate deaminase	-1,68	-1,55		
SAPIG0658		amino acid transporter		-1,11		
SAPIG0662	*pta*	phosphate acetyltransferase		-1,16		
SAPIG0666		phosphomevalonate kinase		0,94		
SAPIG0668	*merA*	pyridine nucleotide-disulfide oxidoreductase		1,06		
SAPIG0693		hydrolase		-0,66		
SAPIG0701		phage integrase		1,11		
SAPIG0702		putative antiporter subunit mnhA2	1,54	2,05		
SAPIG0703		putative antiporter subunit mnhB2	1,93	2,13		
SAPIG0704		putative antiporter subunit mnhC2		2,91		
SAPIG0705		putative monovalent cation/H+ antiporter subunit D	2,23	2,60	1,90	
SAPIG0706		putative antiporter subunit mnhE2	1,92	2,19		
SAPIG0707		putative antiporter subunit mnhF2	1,86	2,48	1,75	
SAPIG0708		putative antiporter subunit mnhG2		2,70		
SAPIG0729	*dhaL*	dihydroxyacetone kinase, L subunit	-2,29	-2,02		
SAPIG0796	*ltaS*	lipoteichoic acid synthase		1,25		

SAPIG0807	*nrdI*	nrdI protein		-2,14		
SAPIG0808		ribonucleoside-diphosphate reductase, alpha subunit		-1,56		
SAPIG0829	*secA*	preprotein translocase, SecA subunit		1,05		
SAPIG0838		putative acetyltransferase SAR0816		-1,78		
SAPIG0846	*clpP*	ATP-dependent Clp endopeptidase, proteolytic subunit ClpP		2,53		
SAPIG0850	*gapR*	central glycolytic gene regulator		1,77		
SAPIG0852	*pgk*	phosphoglycerate kinase		1,46		
SAPIG0899	*csd*	cysteine desulfurase		1,28		
SAPIG0901	*sufB*	feS assembly protein SufB		1,19		
SAPIG0919	*nifU*	nitrogen fixation		1,70		
SAPIG0957	*tetM*	GTP-binding domain protein		2,07		
SAPIG0974	*clpB*	ATP-dependent chaperone protein ClpB	2,02	2,53	2,08	
SAPIG0982	*oppB*	oligopeptide transport system permease protein		-1,87		
SAPIG0984	*oppD*	oligopeptide ABC superfamily ATP binding cassette transporter, ABC protein		-1,39		
SAPIG0986	*oppA*	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein	-1,07	-1,27		
SAPIG1006	*fabI*	enoyl-[acyl-carrier-protein] reductase [NADPH]		-1,08		
SAPIG1056	*qoxC*	cytochrome aa3 quinol oxidase, subunit III		-1,97		
SAPIG1057	*qoxB*	cytochrome aa3 quinol oxidase, subunit I		-2,11		
SAPIG1058	*qoxA*	cytochrome aa3 quinol oxidase, subunit II		-1,92		
SAPIG1145	*murI*	glutamate racemase		-1,47		
SAPIG1158	*hla*	alpha-hemolysin		-2,52		
SAPIG1223	*rpmB*	ribosomal protein L28		2,01		
SAPIG1242		putative membrane protein YfhO	-1,16	-1,19	-1,34	-1,28
SAPIG1291		putative pyruvate flavodoxin/ferredoxin oxidoreductase		-0,92		
SAPIG1302	*glpK*	glycerol kinase	-2,02	-2,11	-1,70	-1,86
SAPIG1303	*glpD*	aerobic glycerol-3-phosphate dehydrogenase	-2,55	-2,23	-1,99	
SAPIG1311	*glnA*	glutamine synthetase, type I			1,68	2,06
SAPIG1369	*trpG*	anthranilate synthase component II	-1,62			
SAPIG1370	*trpD*	anthranilate phosphoribosyltransferase	-2,18	-2,43		
SAPIG1371	*trpC*	indole-3-glycerol phosphate synthase	-3,04	-3,09		
SAPIG1372	*trpF*	N-(5'-phosphoribosyl)anthranilate isomerase	-2,25	-2,19		
SAPIG1373	*trpB*	tryptophan synthase, beta subunit	-2,20	-2,10		
SAPIG1410	*norD*	nitric oxide reductase activation protein NorD		0,50		
SAPIG1414	*sucB*	dihydrolipoyllysine-residue succinyltransferase, E2 component of oxoglutarate dehydrogenase (succinyl-transferring) complex	-1,93			
SAPIG1427	*folA*	dihydrofolate reductase		-1,07		
SAPIG1446		conserved hypothetical protein	-2,02	-2,07		
SAPIG1601		putative glycine dehydrogenase [decarboxylating] subunit 1		-1,06	-1,27	
SAPIG1627	*dnaG*	DNA primase		0,88		
SAPIG1636	*phoH*	phosphate starvation-inducible ATPase		-0,92		
SAPIG1642		conserved hypothetical protein		1,01		
SAPIG1644	*prmA*	ribosomal protein L11 methyltransferase	1,53	2,37	1,71	1,70
SAPIG1645	*dnaJ*	chaperone protein DnaJ	2,11	2,56	2,11	
SAPIG1646	*dnaK*	chaperone protein DnaK	1,94	2,38	2,32	1,80
SAPIG1647	*grpE*	protein grpE	1,91	2,69	2,14	2,21

SAPIG1648	*hrcA*	heat-inducible transcription repressor HrcA		3,07	2,73	2,74
SAPIG1713		conserved hypothetical protein		2,33		
SAPIG1750	*cycA2*	APC family amino acid-polyamine-organocation transporter	-0,75	-0,67		
SAPIG1787	*acuC*	acetoin utilization protein AcuC		-2,41		
SAPIG1818	*fadM*	proline dehydrogenase 1	-3,42	-3,40		
SAPIG1844	*menE*	O-succinylbenzoate-CoA ligase	-0,77			
SAPIG1907	*prsA*	foldase protein prsA		1,33		
SAPIG1909		DNA double-strand break repair ATPase		-0,53		
SAPIG1986	*ftnA*	ferritin		1,56		
SAPIG1987	*dnaQ*	DNA-directed DNA polymerase III epsilon subunit		1,12		
SAPIG2003	*scpA*	staphopain A	2,07	3,52	2,55	2,47
SAPIG2004	*scpB*	staphostatin A	2,01	1,78		
SAPIG2055	*gntR*	transcriptional regulator, gntR family		0,81		
SAPIG2066	*groL*	chaperonin GroL	2,18	2,67	2,42	
SAPIG2067	*groS*	10 kDa chaperonin	1,34	1,50		
SAPIG2072	*hld*	delta-hemolysin domain protein		-2,18		
SAPIG2073	*agrB*	agrB protein	-2,50	-2,71		
SAPIG2075	*agrC*	accessory gene regulator C		-2,78		
SAPIG2076	*agrA*	accessory gene regulator protein A		-2,07		
SAPIG2141	*fabZ*	beta-hydroxyacyl-(acyl-carrier-protein) dehydratase FabZ		1,82		
SAPIG2165	*yodB*	yodB		1,56		
SAPIG2181	*dps1*	DNA protection during starvation protein 1		2,71		2,92
SAPIG2191		conserved hypothetical protein		2,70		
SAPIG2210	*mtlA*	PTS system mannitol-specific EIICB component	-1,19	-1,02		
SAPIG2213	*mtlF*	mannitol-specific phosphotransferase enzyme IIA component	-1,25	-1,01	-1,38	
SAPIG2278	*rpsK*	30S ribosomal protein S11		1,51		
SAPIG2317		permease of the major facilitator superfamily		1,66		
SAPIG2362	*suhB*	inositol monophosphatase		-1,09		
SAPIG2498	*fni*	isopentenyl-diphosphate delta-isomerase, type 2				1,31
SAPIG2499	*corA*	magnesium and cobalt transport protein CorA				1,53
SAPIG2534		conserved hypothetical protein	-1,66			
SAPIG2546	*scrFIAM*	modification methylase ScrFIA		1,75		
SAPIG2556	*gntK*	gluconate kinase		-1,08		
SAPIG2598	*clpC*	ATP-dependent Clp protease, ATP-binding subunit		-1,11		
SAPIG2605		putative delta-1-pyrroline-5-carboxylate dehydrogenase		-2,03		
SAPIG2614	*crtQ*	4,4'-diaponeurosporenoate glycosyltransferase		-1,42		
SAPIG2615	*crtI*	phytoene desaturase		-0,90		
SAPIG2660	*betB*	betaine aldehyde dehydrogenase				1,52
SAPIG2674	*graR*	response regulator	-1,61	-1,92	-1,47	
SAPIG2683	*arcD*	arginine-ornithine antiporter	-1,63	-1,55	-1,84	-2,30
SAPIG2697	*gtfB*	accessory Sec system glycosyltransferase GtfB		-1,03		
SAPIG2698	*gtfA*	accessory Sec system glycosylation protein GtfA		-1,35		
SAPIG2714		putative capsule synthesis protein		1,18		
SAPIG2741	*citT*	DASS family divalent anion:sodium (Na+) symporter	-1,02	-1,22	-1,01	

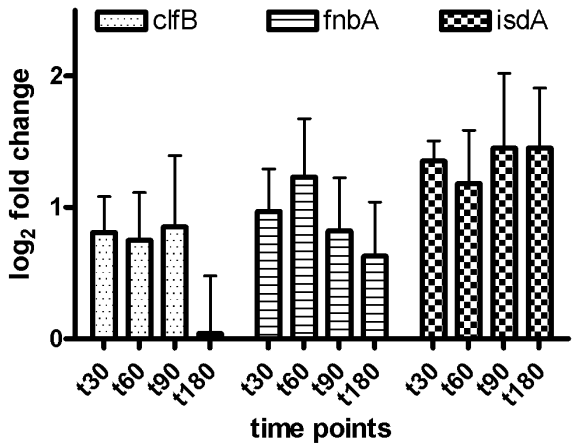

Figure S1. Expression of three important colonization genes: *clfB*, *isdA*, and *fnbA* during *ex vivo* colonization.
The qRT-PCR results are expressed as the average $\log_2$ fold-change in transcript during *ex vivo* colonization compared to t=0.

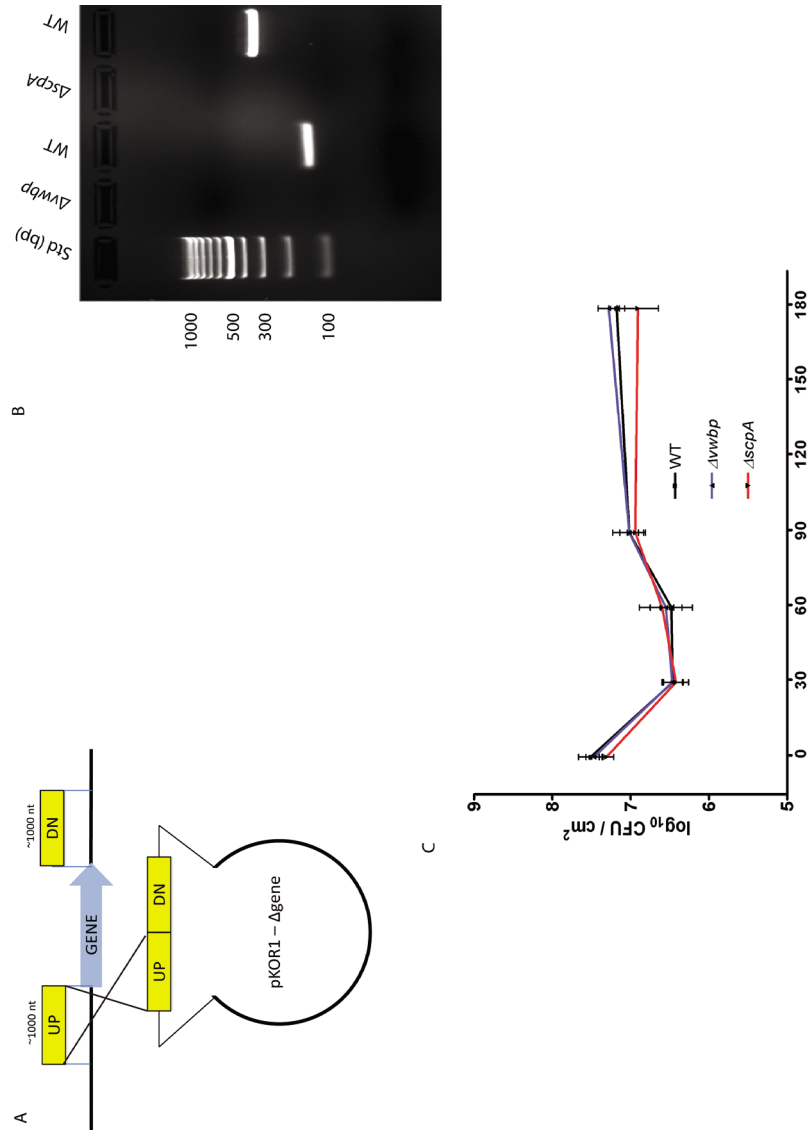

Figure S2. Construction and characterization of a knockout *vwb* and *scpA* strains in MRSA S0462.
A) Schematic representation of the deletion *vwb* or *scpA* genes in MRSA S0462 strain. B) PCR confirmation of *vwb* and *scpA* deletion mutagenesis. C) MRSA S0462 wild-type, Δ*vwb*, and Δ*scpA* colonization of porcine mucosa explants. Data are presented is the mean log CFU ± standard deviation (error bars) of three different pig experiments.

Chapter 4

The effectiveness of bacteriophages against *Staphylococcus aureus* nasal colonization in pigs

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is widespread among animals and humans in contact with pigs and veal calves. It is the cause of a variety of infections. Bacteriophages are specific for subsets of strains within a species and would be an excellent candidate for the control of colonization by MRSA. The aim of this study was to compare the efficacy of bacteriophage treatment on porcine nasal colonization with MRSA *in vitro*, *ex vivo*, and *in vivo*.

The effectiveness of bacteriophages Fred and Felix was assessed *in vitro* by incubating them with MRSA V0608892/1 (ST398) measuring the OD_{600} hourly. To study the *in vivo* effect bacteriophages were administered in a gel containing 10^9 plaque-forming units (PFU)/ml (Fred and Felix in a 19.25:1 ratio) for 5 days to piglets (N=8) that were experimentally colonized with the MRSA strain. The strain was also used to colonize porcine nasal mucosa explants and bacteriophages were applied to assess the *ex vivo* efficacy of treatment.

Bacteriophages were effective *in vitro*. Sixteen piglets were colonized with MRSA (approximately 10^5 CFU/swab), but the number of CFU recovered after the application of the bacteriophages was not reduced. In the *ex vivo* model, 10^8 CFU were used to establish colonization with MRSA, but a reduction of colonization was not observed after application of bacteriophages. However, application of muporicin in both *in vivo* and *ex vivo* resulted in an almost complete reduction of MRSA.

In conclusion: i) The MRSA strain was killed in the presence of the Fred and Felix bacteriophages *in vitro*. ii) Bacteriophages did not reduce porcine nasal colonization *in vivo*. Physiological *in vivo* and *ex vivo* conditions rather than lack of efficacy of the bacteriophages, may explain these observations.

Introduction

Worldwide, methicillin-resistant *Staphylococcus aureus* (MRSA) is an important colonizer in animals and is an opportunistic pathogen in humans. During the last decade Livestock-Associated (LA-) MRSA of sequence type (ST) 398 has emerged in Europe and North America [1-3]. The transmission of MRSA ST398 from livestock to humans has been reported in many countries [4,5] and contact with livestock is recognized as a risk factor for the presence in humans [6,7]. Although this type of MRSA is believed to have adapted to livestock, ST398 is able to colonize and cause infections in humans [8,9]. Reduction of prevalence of colonized livestock or reduction of the shedding by positive animals will reduce the exposure, and therewith presence of LA-MRSA in humans. Non-antimicrobial treatment options should be explored for efficacy to reduce LA-MRSA in animals because antimicrobial use in animals for the purpose of decolonization is a highly unwanted situation.

Bacteriophage therapy offers a possible alternative to antibiotic treatment for bacterial colonization [10]. Bacteriophages are able to infect bacteria and enter either a lysogenic or a lytic cycle. The main advantage of bacteriophages is their specificity. Whereas therapeutic treatment with antimicrobials affects many different organisms in the patient's body (e.g. the gut microbiota), causing a change in the microbial composition and inducing antimicrobial resistance in a spectrum of bacterial species, bacteriophages specifically target only the organism or even the strain that is causing the infection. If resistance against bacteriophages does develop it is restricted to the target species. The *in vitro* lytic effect of bacteriophages can be easily tested. However, the use of bacteriophages for therapy (e.g. to treat infections or reduce colonization) poses additional challenges like the accessibility of bacteria and *in vivo* inactivation of bacteriophages.

The aim of this study was to assess the effectiveness of bacteriophage treatment on porcine nasal colonization with *S. aureus* using *in vitro*, *ex vivo* and *in vivo* models.

Materials and Methods

Bacteria and bacteriophages

MRSA strain V0608892/1 (ST398, spa type t011, SCC*mec* type V) was used for these experiments. The strain was isolated from a healthy pig upon diagnostic screening.

A bacteriophage solution in a proprietary gel formulation developed by Novolytics (UK), containing bacteriophages Fred and Felix in a 19.25:1 ratio with a final concentration of 10^9 plaque-forming units (PFU)/ml was used. Fred and Felix were originally isolated from human strains of *S. aureus* and were propagated on MRSA strain SAI653. These bacteriophages were proven to be effective against several MRSA isolates of ST398 from pigs in The Netherlands and Denmark (data not shown). The gel formulation containing no bacteriophages was used as a placebo control.

***In vitro*: growth curves**

To evaluate the *in vitro* effectiveness of the bacteriophages the MRSA strain was grown overnight in BHI (Oxoid, The Netherlands) at 37°C. A 1:50 dilution was prepared in fresh BHI and cells were grown for approximately 3 h to mid-exponential phase at 37°C under shaking at 200 rpm. The cell suspension was diluted in BHI to a concentration of approximately 10^7 CFU/ml based on optical density at 600 nm (OD_{600}). A volume of 380 µL was transferred to an optical multi-well plate in duplicate and 20 µL bacteriophage containing gel (multiplicity of infection, MOI 5.3) or 20 µl placebo was added. The plate was incubated in a BioScanner C (Labsystem France SA, France) at 37°C for 20 h; absorbance at 600 nm was measured every hour and the experiment was performed three times.

***In vivo*: piglets**

Sixteen crossbred, caesarean derived colostrum deprived (CD/CD) crossbred piglets (obtained from two different sows) were equally divided over 4 isolators (A-D). Animals were obtained, housed and fed as described by Dekker *et al.* [11]. Colonization with MRSA was established according to described quantitative *in vivo* model [Unpublished data]: at the age of 5 days the animals were screened for the absence of MRSA by enriched selective culture [12] and at the age of 6 days the animals received intranasal inoculation of 10^9 CFU/animal with strain V0608892/1 (500 µl volume). Concentrations were monitored the following days by nasal sampling at days 7, 8, 9, 11 and 12; samples were quantitatively analysed for the presence of MRSA as described earlier. In brief, a swab was suspended in 1 ml of Phosphate Buffered Saline (PBS) (Lonza, Belgium) and a serial 1:10 dilution of the suspension was prepared to a final concentration of 10^{-3} of the original suspension. Fifty µl of the undiluted and of the 10^{-3} suspension were plated on Brilliance 2 MRSA agar (Oxoid, The Netherlands) using an Eddy-Jet spiral plater (IUL Instruments, Spain) in E-mode and enumerated as described by the manufacturer after the plates had been incubated overnight at 37°C. The remainder was enriched by culturing in Mueller Hinton broth supplemented with 6.5% NaCl (Oxoid, The Netherlands), which was also incubated overnight. The enrichment was plated on Brilliance 2 MRSA agar in case direct plating showed no growth. Numbers of MRSA are reported as CFU/swab.

500 µl of gel with bacteriophages per nostril was administered to the animals in isolators A and C at the age of 12 days using a syringe. The animals in isolators B and D received a placebo (same gel without the bacteriophages). These treatments were performed daily for a total of 5 days. Nasal samples were obtained before the administration of bacteriophage or placebo gel on the respective day to monitor colonization. Monitoring continued for one week after the last administration. All samples were processed within 2 hours after collection and MRSA was enumerated by quantitative plating as described elsewhere [13].

At t=19 nasal samples of bacteriophage-administered piglets were also analysed for the presence of bacteriophages. After bacterial enumeration samples were suspended in 1 ml PBS and passed through a 0.45 µm filter (Pall, The Netherlands). The filtrate was plated onto Tryptone Soy agar (Oxoid, The Netherlands) containing MRSA. Plates were incubated and assessed for the presence of plaques.

In order to show that a reduction of colonization could be achieved the animals in isolators A and B at the age of 22 days were treated with a mupirocin ointment (Bactroban 2%) for 5 days, 2 daily doses; groups C and D remained untreated (no placebo was administered). During the following 4 days nasal swabs were obtained for enumeration of MRSA to study the effect of mupirocin treatment.

***Ex vivo*: nasal mucosa colonization**

To evaluate the effectiveness of the bacteriophage containing gel on mucosal explants, porcine tissue was obtained and the colonization assay was performed as described previously with some modifications [13]. All incubation steps of the explants were performed at 37°C and 5% CO_2 atmosphere, incubation of bacteria was performed at 37°C under aerobic conditions. In brief, after nasal mucosa membrane isolation the stripped mucosa tissue was divided into explant pieces of 0.5 cm^2 using 8 mm biopsy punches (AcuDerm Inc, USA) and cultivated at an air-liquid interface. The explants were colonized with a bacterial inoculum (approximately $2x10^8$ CFU in 1 ml Dulbecco's PBS (DPBS) (Gibco, The Netherlands)) for 2 h to allow the bacterial adhesion to the tissue. Next, explants were washed three times with 1 ml DPBS. A time point is defined for this post-adhering time as t=0. The inoculated explants were incubated for 1 h [13]. Then, bacteria were isolated from the explants by scraping the epithelium surface using cell scrapers (Falcon, Becton Dickinson, The Netherlands), and cells were suspended in 1 ml DPBS with 0.1% Triton X-100 (t=1; measurement of initial attachment of bacteria). Bacterial suspensions were plated on Colombia agar with sheep blood (Oxoid, The Netherlands) in serial dilutions (1:10) in DPBS. The plates were incubated overnight and bacteria were enumerated.

To evaluate the effectiveness of the bacteriophages, 50 µl bacteriophage containing gel or placebo gel was applied after 1 h of colonization (t=1). Additionally, 50 µl mupirocin (Bactroban 2%) solution (1:1 in DPBS) was applied to another set of explants. To control for the growth of *S. aureus* on the explants a few were not treated with gel. MRSA was enumerated 4 h (t=4) and 24 h (t=24) post-adhering as described above. The experiment was performed 3 times independently.

Statistics

Mean concentrations and standard deviations were calculated from replicates (*in vitro* and *ex vivo* experiments) and pigs that were housed in the same isolator (*in vivo* experiment) because one isolator was considered one experimental unit.

The concentrations of MRSA on nasal mucosa explants were compared using an analysis of variance (ANOVA) to evaluate the effectiveness of the bacteriophage containing gel and mupirocin application. The concentrations of MRSA in nasal samples were compared between isolators by an ANOVA to see the effect of the bacteriophage treatment (days 13-22) and the mupirocin ointment (days 27-30).

Ethics

These experiments were approved by the Animal Ethical Committee of Utrecht University and were registered under 2011.II.11.180 and 2012.II.08.127.

Results

In vitro

An *in vitro* setup was used to evaluate the effectiveness of the bacteriophage containing gel on MRSA growth in BHI medium (Figure 1). Bacterial growth was monitored by hourly OD_{600} readings for 20 h in the presence of bacteriophages, placebo, and in the control samples. The results showed that the bacteriophage containing gel prevented bacterial growth, as growth was only observed in the control and placebo samples, where the bacteria reached stationary phase with an OD_{600} of 1.2 within 5 h. Additionally, no significant differences between placebo and control samples were observed (Figure 1).

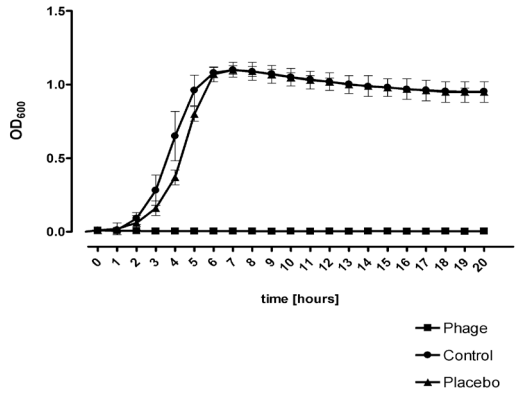

Figure 1: Effect of bacteriophage solution on the growth of MRSA V0608892/1.
OD values reflect bacterial concentration in presence of the bacteriophage solution, placebo and no-treated control. The results are presented as the mean OD ± standard deviation of 3 different experiments in duplicate.

In vivo

The bacteriophage containing gel prevented bacterial growth *in vitro*. Therefore, the bacteriophages were subsequently tested in an *in vivo* experimental setup. CD/CD piglets were inoculated with MRSA to establish nasal colonization *in vivo*. After inoculation with bacteria the MRSA strain could be retrieved from the pigs at numbers varying between 10^4 and10^6 CFU/swab and numbers of MRSA continued to vary between these values throughout the experiment (average of $5.7x10^5$ CFU/swab), see Figure 2 for observations.

During and after the application of bacteriophages the numbers of MRSA continued to oscillate between 10^4-10^6 CFU/swab in the bacteriophage-treated and placebo-treated pigs. No statistically significant effect was observed during or after bacteriophage treatment (P>0.05, ANOVA).

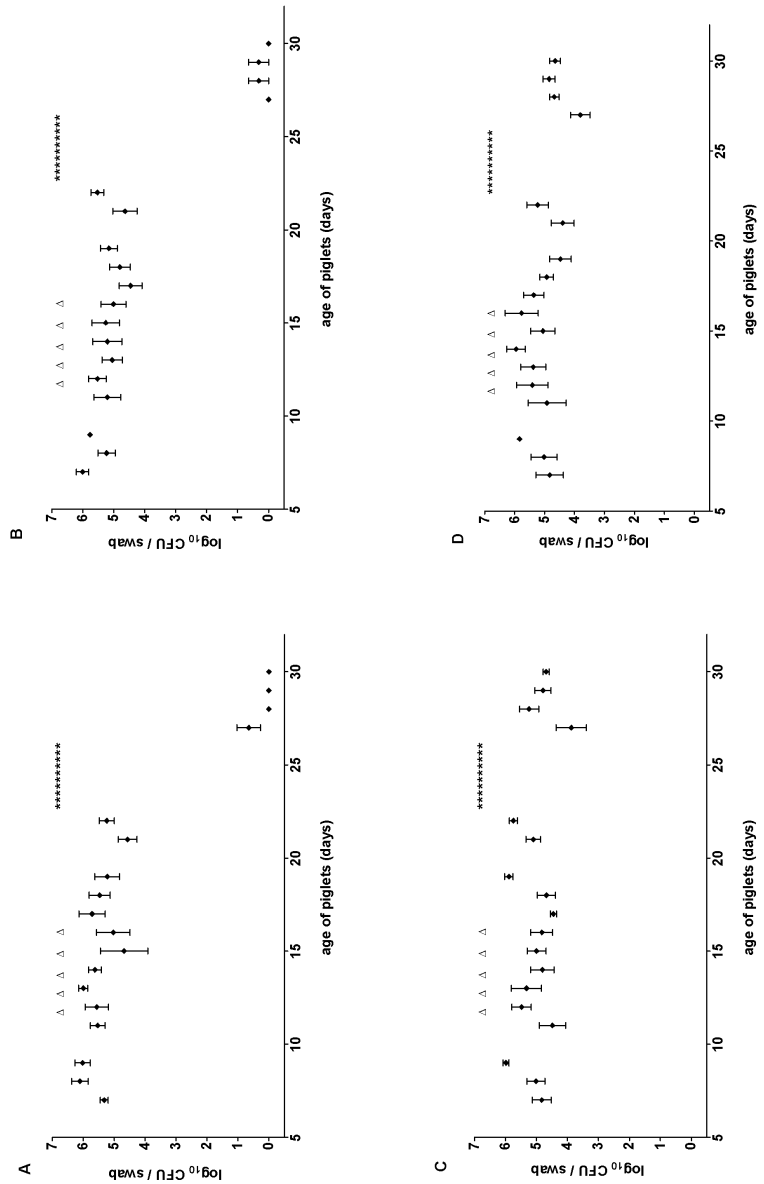

Figure 2: MRSA recovery in the *in vivo* experiment.
Culture results for nasal samples in the *in vivo* experiment. Numbers of MRSA are displayed in CFU/swab. Each group consisted of 4 piglets. Group A) piglets that were treated with bacteriophage solution and received mupirocin ointment; B) piglets that received a placebo without bacteriophage and were treated with mupirocin ointment; group C) received a bacteriophage treatment, but no mupirocin and group D) was administered a placebo without bacteriophage or placebo treatment; *) indicate ointment with mupirocin. If a sample was obtained on the same day as treatment took place the sample was taken before treatment was applied.

Bacteriophages were re-isolated at t=19 (3 days after the final phage treatment) from 5/8 piglets that received bacteriophage treatment.

Mupirocin treatment reduced the recovery of the inoculated strain. In most samples the inoculated strain could not be detected ($P<0.01$, ANOVA). Only in three samples from animals in both isolators it was possible to isolate MRSA after enrichment culture (isolator A, t=27; isolator B, t=28 and 29).

Ex vivo

Using porcine nasal mucosa explants it was possible to investigate the activity of the bacteriophage containing gel in a controlled setting that mimicked the *in vivo* situation.

After explant inoculation the bacteriophage containing gel or placebo gel was applied to the explants and the bacterial concentration on these explants was followed until 24 h post-adhering time. As a control bacterial growth was observed on untreated explants. Additionally, explants were treated with mupirocin solution as a bacterial eradication control. Similar to the animal study application of the bacteriophage containing gel showed no statistically significant differences between control, bacteriophage and placebo treatment ($P>0.05$, ANOVA). A reduction of colonization of MRSA V0608892/1 from the explants was observed only when mupirocin was applied ($P<0.01$, ANOVA) (Figure 3).

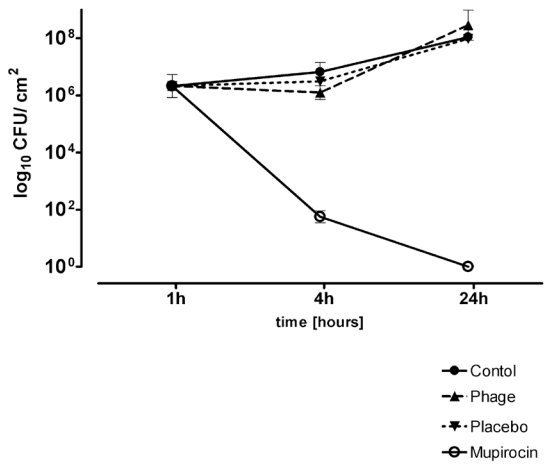

Figure 3: Eradication of MRSA V0608892/1 from the explants after application of bacteriophage solution and mupirocin.
Data are presented as the mean CFU ± standard deviation of three experiments.

Discussion

As human MRSA carriers have an increased risk for MRSA infection, control of MRSA among livestock is important to reduce the exposure and risk for humans [14]. The use of antimicrobials to reach this goal is not desirable because it would select for antimicrobial resistance. An alternative would be the use of bacteriophages. In this study the possibility of bacteriophage treatment to reduce levels of nasal colonization was investigated.

Bacteriophages were able to prevent bacterial growth *in vitro*. However, an effect of the bacteriophages in the *in vivo* and *ex vivo* experiments could not be observed. Bacteriophages were re-isolated from 5/8 piglets that received bacteriophage treatment. Moreover, these bacteriophages were found to be ineffective against MRSA isolates from the same sample (data not shown). When bacteriophages and bacterial isolates were stored at -80°C and the experiment was repeated one week later the bacteriophages were found to be effective. A likely explanation is that during initial isolation bacteriophages are still attached to bacterial cell debris. A freeze step could further degrade the cell debris and result in release of bacteriophages.

It is known that mammalian host-proteins cover the surface of bacteria [15,16]. The presence of host-proteins can hamper the adherence of bacteriophages because of steric hindrance. However, only small amounts of mucus were observed in the scanning electron microscopic images that were taken of the nasal mucosa explants (data not shown). Also, the expression of bacterial proteins *in vitro* differs significantly from the expression *ex vivo* [17]. Either there is a lack of expression of the receptor or the receptor is masked by a surface component. Nonetheless, bacteriophages have already been successfully applied to treat infections of the intestines and caeca [18,19], which are also covered with mucus, but where the contents are mixed due to peristaltic action which would enhance the chances of bacteriophages reaching target cells.

It has been reported that an optimal MOI for *in vivo* application of bacteriophages is between 1 and 10 [20]. The MOI in the *ex vivo* experiment was in fact 8.1, were in agreement with the suggested MOI. The bacterial numbers that were obtained in the *in vivo* experiment are a relative enumeration of bacteria in the nasal cavity of each pig. Therefore, it is not possible to calculate the MOI for the *in vivo* experiment. However, the average number of MRSA in the bacteriophage-treated animals at t=7 enumerated from the nasal swabs, was $2.45x10^5$. This indicates the MOI could not have been more than $4x10^3$.

In this study we observed a correlation between *ex vivo* and *in vivo* experiments. This shows that the nasal mucosa explant model can be applied as a preliminary screening

for the effectiveness of bacteriophages before animal trials are commenced.

In conclusion, the MRSA strain was killed by the bacteriophages in the gel *in vitro*. However, MRSA reduction was not observed in the pig model or in the nasal mucosa explant model in contrast to mupirocin treatment. This may be due to differences in the experimental models and/or protein expression and binding *in vivo* and *ex vivo* rather than lack of efficacy of the bacteriophages.

Acknowledgements

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Chapter 5

Methicillin-resistant coagulase-negative staphylococci isolated from pig farms are a potential reservoir of *mecA* for *Staphylococcus aureus*

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) likely originated by acquisition of the SCC*mec* from coagulase-negative staphylococci (CNS). However, it is unknown whether the same SCC*mec* types are present in MRSA and CNS that reside in the same niche. Here we report for the first time a study to determine the presence of a potential *mecA* reservoir among CNS recovered from 10 pig farms. The 44 strains belonged to 10 different *Staphylococcus* species. All *S. aureus* belonged to ST398, with the dominant SCC*mec* types V and IVa. Type IVc as well as type III, VI and novel subtypes of type IV and not-typeable types were found in CNS. *S. aureus*, *S. epidermidis* and *S. haemolyticus* shared SCC*mec* type V. Noteworthy is the presence of SCC*mec* type IVc in several staphylococcal species isolated from one pig farm that suggests exchange of this SCC*mec* type in CNS, but the general distribution of this SCC*mec* type still has to be established. In conclusion, this study shows that a SCC*mec* types among staphylococcal species on pig farms are heterogeneous. On two farms more than one recovered staphylococcal species harbored the same SCC*mec* type. We conclude that staphylococci on pig farms act as a reservoir of heterogeneous SCC*mec* elements. These staphylococci may act as source for transfer of SCC*mec* to *S. aureus*.

Introduction

Globally, methicillin-resistant *Staphylococcus aureus* (MRSA), an important pathogen in humans and animals, is responsible for considerable mortality, morbidity and health-care expenditure in both hospitals and the community[1]. Methicillin resistance is associated with the presence of the *mecA* gene which encodes an additional penicillin-binding protein (PBP2a or PBP2'). This protein has a lower affinity for all beta-lactam antibiotics [2]. The *mecA* gene is located on a mobile genetic element called Staphylococcal Cassette Chromosome *mec* (SCC*mec*) [3].

The origin of SCC*mec* remains unknown, but it is believed that the *mecA* gene itself originated from one common precursor. Homologues of a *mecA* gene have been found in *Staphylococcus sciuri* [4] and *Staphylococcus vitulinus* [5]. However, these *mecA* gene homologues are not located in a *mecA* complex as in SCC*mec*. Tsubakishita *et al.* [6] showed that a *mecA* gene homologue is present in *Staphylococcus fleurettii* that showed 99-100% sequence homology with the *mecA* gene present in MRSA strain N315. Additional sequence analysis showed the presence of an almost identical structure of the *mecA* complex. This result indicates that a direct precursor of the methicillin resistance determinant for MRSA is present in *S. fleurettii*, which is a member of the *S. sciuri* group within the staphylococci [6].

S. fleurettii is a commensal bacterium of animals and having the ancestor of the *mecA* gene present in animal-born staphylococcal species suggests that SCC*mec* elements may be generated in a *Staphylococcus* species that has an animal as its normal host. The possibility that *mecA* may have originated from *S. fleurettii* strengthens the hypothesis that MRSA probably acquired SCC*mec* from coagulase-negative staphylococci (CNS) [7]. This hypothesis is further supported by the fact that methicillin resistance among human clinical isolates is more prevalent in CNS than in *S. aureus* [8,9]. Furthermore, the observation of *in vivo* transfer of SCC*mec* from *Staphylococcus epidermidis* to *S. aureus* [10] suggests that CNS may act as a source for SCC*mec* acquisition by *S. aureus*. This would be consistent with the finding that SCC*mec* types present in CNS are more heterogeneous than in MRSA [7,11,12].

Worldwide methicillin-resistant CNS (MRCNS) have been isolated from a number of animals like pigs, horses, cows, dogs, cats [1,13,14]. Recently, MRSA belonging to sequence type (ST) 398 emerged in livestock (pigs, veal calves and poultry) in Europe and North America [14-16] whereas in Asia livestock-associated MRSA belonging to ST9 emerged [17]. Contact with animals colonized with MRSA has been recognized as risk factor for human colonization [18,19]. The increasing number of MRSA ST398 transmissions and infections illustrates that this is a public health concern [1].

Although, it has been proposed that methicillin-susceptible *S. aureus* (MSSA) ST398, highly prevalent in pigs, acquired *mecA* from coexisting staphylococci [14], the presence of *mecA*-positive staphylococci in pigs has not been studied and the source of SCC*mec* in MRSA ST398 is only speculative. Therefore it was the aim of this study to detect *Staphylococcus* species harboring SCC*mec* on pig farms. We demonstrate that a reservoir of *mecA*-positive CNS coincides with *S. aureus* on pig farms, and that the presence of the same SCC*mec* types among CNS and *S. aureus* supports the hypothesis that on pig farms CNS may act as a reservoir for exchange of SCC*mec*.

Material and methods

Study design

This study included 10 pig farms in The Netherlands with different levels of antibiotic usage that is expressed in Animal Daily Dosages per year (ADD/y). Nasal samples were taken from 5 randomly selected pigs using dry cotton swabs. In addition, on each farm 5 dust samples were taken using dry cotton swabs. The swabs were immediately transported to the laboratory and processed within 24 hours after collection.

The collection of swabs from pigs was approved by the Animal Experimental Committee of Utrecht University, according to the Dutch Law on Animal Health and Welfare.

Staphylococcal isolation

The swabs from each farm were pooled in two samples, one with the nasal swabs and one with the dust swabs. Material was eluted in 5 ml PBS by vigorous shaking and vortexing for 1 min. One hundred µl of the obtained sample suspension was inoculated on mannitol-salt agar (bioTRADING, The Netherlands) using ten-fold serial dilutions up to 10^{-4} and incubated for 72 h at 30^{0}C. An additional 100 µl of undiluted sample suspension was inoculated on Brilliance Staph 24 Agar (Oxoid, United Kingdom), a MRSA selective plate, and incubated for 24 h at 37^{0}C. From each mannitol-salt agar plate colonies with typical but divers staphylococcal morphology were selected and cultivated on blood agar with 5% sheep blood (bioTRADING, The Netherlands) for further analysis (to a maximum of 5 isolates that displayed the same morphology). Moreover, from the Brilliance Staph 24 Agar up to 5 typical MRSA colonies were selected and subcultured for further analysis. After incubation for 24 h at 37^{0}C on blood agar with 5% sheep blood (bioTRADING, The Netherlands) staphylococcal strains were verified by colony morphology, Gram staining and catalase reaction.

DNA isolation

From each isolate, crude DNA was isolated using InstaGene matrix (Bio-Rad, The Netherlands) according to the protocol of the manufacturer.

Identification of *mecA*-positive staphylococci

Detection of the *mecA* gene was carried out by multiplex PCR using primers for *mecA* [20], and the 16S rRNA gene (27F and 556R) [21], using amplification of 16S rRNA gene as a positive control for DNA extraction. PCR was performed in 20 µl containing 1x PCR Master Mix (MBI Fermentas, Lithuania) with 6 µM of the *mecA* primers and 3 µM of the 16S rRNA gene primers and 2 µl of crude DNA. The thermal cycling conditions used were 95^{0}C for 5 min followed by 35 cycles of 94^{0}C for 30 sec, 50^{0}C for 30 sec and 72^{0}C for 1 min, and final extension was at 72^{0}C for 5 min. PCR products were detected on 1.5% agarose gel stained with ethidium bromide.

The diversity of *mecA*-positive isolates was assessed by GTG-fingerprinting PCR analysis according to the protocol of Braem *et al.*, with modifications [22]. Briefly, the amplification mixture consisted of a total volume of 25 µl containing 1x PCR buffer (MBI Fermentas, Lithuania), 1.25 mM $MgCl_2$, 0.4 mM dNTPs, 10% dimethylsulfoxide (DMSO), 200 µg bovine serum albumin (BSA), 50 pmol primer and 2 U Taq polymerase (MBI Fermentas, Lithuania), and 2 µl DNA solution. Amplifications were performed using reported amplification conditions [22]. The resulting fingerprints were analyzed using the BioNumerics V 6.0 software package (Applied Maths, Ghent, Belgium). The similarity among digitized

profiles was calculated using the Pearson's correlation, and the unweighted pair group method with arithmetic means (UPGMA) with 2% optimization. Isolates recovered from the same source exhibiting distinct GTG-fingerprinting profiles (similarities less than 95%) were considered to be genetically unrelated and were included for further analysis.

Species identification of selected isolates was performed using MALDI-TOF, according to protocol of the manufacturer (Bruker, Bremen, Germany). Raw spectra were analyzed by MALDI Biotyper 2.0 software (Bruker Daltonics) with default settings. An internal control (*Escherichia coli* DH5α) was used for calibration before each experiment. Identification scores above 2 or between 1.8 and 2 for duplicate samples were considered to be reliable [23]. In isolates with unreliable identification (below 1.8), species determination was performed by sequencing of 16S rDNA [21] and tuf genes [24]. Sequences were analyzed against all available sequences using the BLAST algorithm. The species was identified when gene sequences yielded ≥98% sequence similarity with the closest bacterial species sequence in GenBank.

SCC*mec* typing

The SCC*mec* elements were typed by a recommended hierarchical system [25]. This included four multiplex PCRs (M-PCRs) according to a protocol reported previously by Kondo *et al.* [26]. In addition, where a non-interpretable type was obtained by M-PCR, a single PCR of each gene was performed. When the *ccr* complex and *mecA* complex could not be amplified no SCC*mec* type was assigned. For subtyping of the SCC*mec* type IVa isolates the *ccrB* allotype was determined by *ccrB* sequence typing [27]. Positive controls for the M-PCR were the MRSA strains COL/SCC*mec* type I, N315/SCC*mec* type II, ANS46c/SCC*mec* type III, MW2/SCC*mec* type IVa, S217/SCC*mec* type V (clinical isolate from the University Medical Center Utrecht, The Netherlands).

Rapid *S. aureus* ST398 identification

All *S. aureus* were screened for ST398 using a real-time assay based on the detection of the C01 AFLP region [28]. PCR was performed in 20 µl containing 1x LightCycler 480 probe master (Roche, Germany) with 0.9 µM of the CO1 primers [28] and 0.2 µM of the CO1 probe (5'Yakima Yellow-ATTGTCAGTATGAATTGCGGT-MGB 3') and 2 µl of DNA matrix. The real-time PCR cycling conditions used were 95^{0}C for 10 min followed by 45 cycles of 95^{0}C for 10 sec, 58^{0}C for 20 sec and 72^{0}C for 20 sec, and cooling was at 40^{0}C for 10 sec. Real-time PCR amplification was performed with a LightCycler480 (Roche, Germany).

Results

Staphylococci recovered from pig farms

A total of 65 *mecA*-positive staphylococci were isolated from 7 of the 10 pig farms. From farms 1, 2 and 6 no *mecA*-positive staphylococci were isolated (Table 1). All analyzed staphylococcal isolates were typeable using GTG-fingerprinting (Fig. 1).

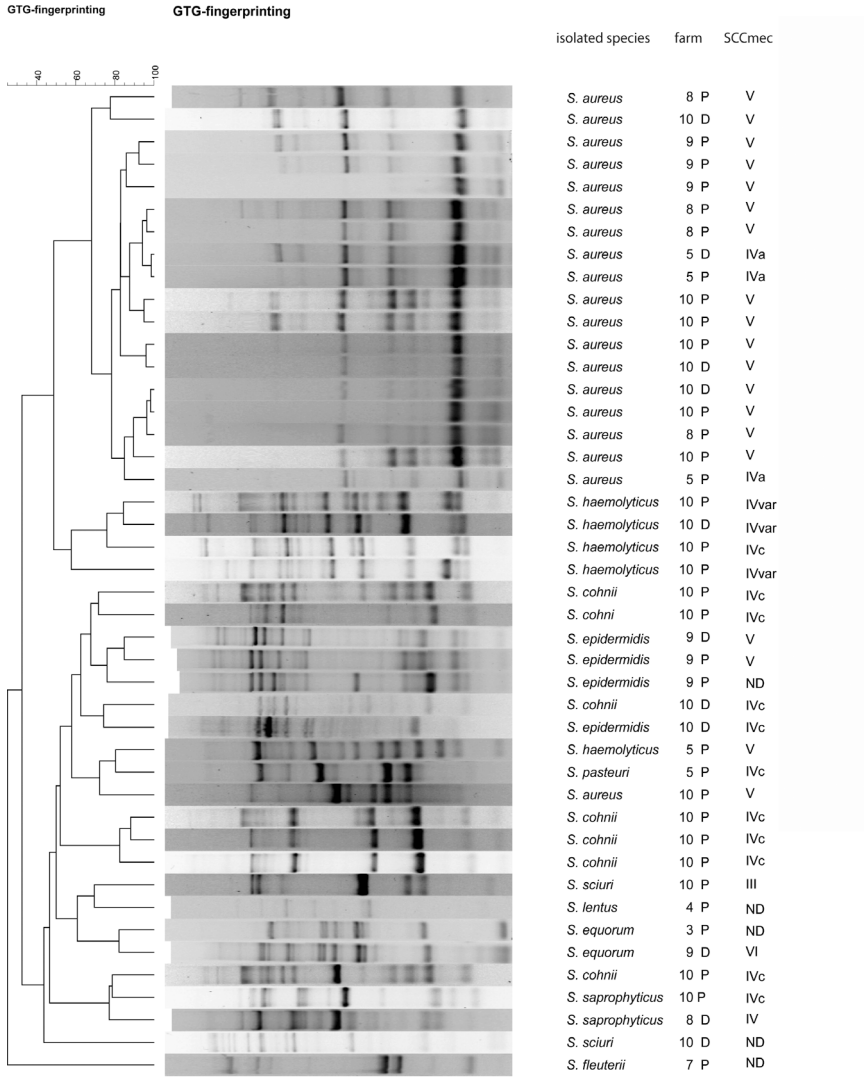

Figure 1.
Dendrogram based on the cluster analysis of the (GTG)5-PCR fingerprinting profiles of recovered *mecA*-positive staphylococci in this study using UPGMA clustering methods of Pearson's correlation coefficients. P: isolate recovered from pig nose; D: isolate recovered from dust.

Isolates recovered from the same source exhibiting distinct GTG-fingerprinting profiles (similarities less than 95%) were considered to be genetically unrelated and were included for further analysis in total 44 isolates. Of these 44 *mecA*-positive staphylococci, 33 (75%) were isolated from nose swabs and 11 (25%) from dust samples. For each isolate the species could be determined. The most common species recovered from the nasal samples were: *S. aureus* (n= 15, isolated from pigs on 4 farms), *Staphylococcus cohnii* (n=6, isolated from pigs on 1 farm), *Staphylococcus haemolyticus* (n=4, isolated from pigs on 2 farms), and *S. epidermidis* (n=3 from pigs on 1 farm). Single isolates of *S. sciuri, Staphylococcus pasteuri*, *Staphylococcus equorum*, *Staphylococcus saprophyticus*, *Staphylococcus lentus* and *S. fleurettii* were recovered from nasal swabs. The staphylococci isolated from the dust on two farms belonged to *S. aureus* (n=4 from 2 farms), and S. epidermidis (n=2 from 2 farms). Also single isolates of *S. haemolyticus*, *S. saprophyticus*, *S. cohnii, S. sciuri* and *S. equorum* were isolated from the dust samples.

Species distribution differed between farms. In general a higher number of methicillin-resistant isolates were recovered from farms with high antibiotic usage (Table 1). In addition, on farms with a high antibiotic usage also a larger number of methicillin-resistant different *Staphylococcus* species were isolated (Table 1).

GTG-PCR fingerprinting

All analyzed staphylococcal isolates were typeable using the GTG-fingerprinting. The generated PCR amplicons ranged in size from 200 bp to 8 kb and the number of PCR products from 4 to 24 bands. GTG fingerprinting showed a high diversity of GTG patterns among the recovered staphylococcal isolates (Fig. 1). The GTG-fingerprints grouped the individual species (*S. aureus*, *S. cohnii, S. haemolyticus, S. epidermidis* and *S. equorum*) in separate clusters, except for one *S. aureus* isolate. Isolates of *S. cohnii*, which were all isolated from farm 10, were located on 3 different positions in the dendrogram. The single isolates obtained for *S. fleuretti, S. pasteuri* and *S. lentus* clustered separately. The GTG-fingerprints of isolates recovered from dust samples and the pigs from the same farm clustered together.

Table 1. Characterization of the *mecA*-positive staphylococci

FARM	Staphylococcal species isolated from pig nostril (number of isolates)	SCC*mec* type	Staphylococcal species isolated from dust (number of isolates)	SCC*mec* type	ADD/y	TOTAL number
1	none		none		<5	0
2	none		none		<5	0
3	*S. equorum* (1)	ND	none		<5	1
4	*S. lentus* (1)	ND	none		<5	1
5	*S. aureus* (2) *S. pasteuri* (1) *S. haemolyticus* (1)	IVa IVc V	*S. aureus* (1)	IVa	11.2	5
6	none		none		13.1	0
7	*S. fleurettii* (1)	ND	none		21	1
8	*S. aureus* (4)	V	*S. saprophyticus* (1)		30	5
9	*S. aureus* (3) *S. epidermidis* (2)	V V, ND	*S. equorum* (1) *S. epidermidis* (1)	VI V	32	7
10	*S. aureus* (6) *S. cohnii* (6) *S. sciuri* (1) *S. haemolyticus* (3) *S. saprophyticus* (1)	V IVc III IVc, IVvar IVc	*S. aureus* (3) *S. cohnii* (1) *S. sciuri* (1) *S. haemolyticus* (1) *S. epidermidis* (1)	V IVc ND IVvar IVc	35	24
TOTAL number	33		11			44

Note: ADD/y - Animal Daily Dosages per year, ND – not determined, var – unknown subtype variant.

Diversity of SCC*mec* types in staphylococci

All selected isolates were screened for SCC*mec* types using a multiplex strategy. In 36 of 44 isolates known SCC*mec* types were detected: V (n=19), IVc (n=12), IVa (n=3), III (n=1) and VI (n=1). In the remaining 8 isolates we were not able to detect the exact SCC*mec* types according to m-PCR strategy. In 3 isolates SCC*mec* types belonged to a new variant of type IV, which could not be subtyped using Kondo m-PCR strategy. In 5 isolates the SCC*mec* types were non-typeable. Table 1 lists the detected SCC*mec* types in recovered isolates and Table 2 shows the non-typeable SCC*mec* elements.

SCC*mec* type V was predominant on the investigated farms. This type was mostly harbored by *S. aureus* (n=16/19), which were present on 3 farms. Additionally, SCC*mec* type V was found in two *S. epidermidis* and one *S. haemolyticus* isolates. Furthermore, only on farm 9 SCC*mec* type V was present in two different species: *S. epidermidis* and *S. aureus*. SCC*mec* type IVc, the second predominant type, was defined in 12 isolates recovered from 2 farms (5 and 10). On farm 10, 4 different species harbored SCC*mec* IVc (n=11): *S. cohnii* (n=7, pig and dust samples), *S. haemolyticus* (n=1, pig), *S. pasteuri* (n=1, pig), *S. saprophyticus* (n=1, pig), and *S. epidermidis* (n=1, dust). SCC*mec* type IVa was identified only in *S. aureus* isolates recovered from farm 5, and SCC*mec* types III and VI were detected in single isolates.

In 3 *S. haemolyticus* isolates recovered from farm X the SCC*mec* type was defined as type IV, based on the presence of the *ccrA2B2* genes and *mecA* complex B. However, sub-typing based on amplification of the J1 region showed presence of ORF E007 in two isolates, which corresponds to the SCC*mec* type I, and for the other *S. haemolyticus* sub-typing was unsuccessful. These isolates, we concluded, were carrying a new variant of SCC*mec* type IV (IVvar) (Table 1).

The SCC*mec* type of 5 isolates, recovered from 5 different farms, could not be determined (Table 2). In 4 isolates only the presence of a *mecA* complex B or A could be determined. Moreover, in one *mecA*-positive *S. epidermidis* isolate none of the *ccr* genes and *mecA* complex could be determined.

To exclude possible transfer of *S. aureus* SCC*mec* type IVa from the community, we performed *ccrB* sequence typing. From two *S. aureus* strains (from dust and pig) the *ccrB* sequences were identical. The other *S. aureus* strain isolated from a pig contained multiple nucleotide changes compared to type IVa. The *ccrB* sequences of the isolates were closely related to the *ccrB2* allele 401 from a human *S. aureus* isolate in the database. This indicates the presence of new *ccrB2* alleles which until now have not been describedin humans, but were associated with the pig farm environment.

Table 2. Characterization of non-typeable SCC*mec* carried by recovered CNS

Staphylococcal species (farm number / pig (P) or dust D)	*mecA*	*ccr** genes	*mecA* complex*
S. equorum (3 / P)	+	-	B
S. lentus (4 / P)	+	-	B
S. fleurettii (7 / P)	+	-	B
S. sciuri (10 / D)	+	-	A
S. epidermidis (9 / P)	+	-	-

* as determined with Kondo PCR (16)

Discussion

Horizontal gene transfer of the SCC*mec* element is considered to contribute to the generation of new methicillin-resistant staphylococci including MRSA. To investigate a potential site of such exchange of SCC*mec*, our study focused on the presence of SCC*mec* among the staphylococcal flora present on 10 selected pig farms.

In this study 10 different methicillin-resistant *Staphylococcus* species were recovered from 7 pig farms. The most common species was *S. aureus*, but the majority of the isolates were CNS. Typing of SCC*mec* from MRCNS has been described in only one other study conducted in pigs. However, in that study only carriage of *S. sciuri* with SCC*mec* type III was described and a methicillin-resistant *S. lentus* and *S. xylosus* were isolated but their SCC*mec* elements were not typed [12].

From the pig nose and the dust samples the same *Staphylococcus* species were obtained. Typing of the isolates from pig nose and dust samples showed that they were genetically related and suggests transmission of staphylococci between dust and the pig nose. The fact that multiple species are isolated from the pig nose suggests that colonization of methicillin-resistant *Staphylococcus* species occurs in the nose that may create the environment for potential horizontal gene transfer.

All *S. aureus* belonged to ST398 [15,29]. Also GTG-fingerprinting could not differentiate the isolates and supports the clonal spread of MRSA ST398 in pig farms. Remarkably, the species distribution and number of recovered *mecA*-positive staphylococci were different on the investigated farms. In general a higher number of methicillin-resistant isolates were recovered from farms with high antibiotic usage and also a larger number of different methicillin-resistant *Staphylococcus* species were isolated, which may suggest that antibiotic usage plays a role.

In the recovered staphylococci, SCC*mec* typing showed the carriage of known types (III, IVa, IVc, V and VI). In 3 isolates new subtypes of SCC*mec* type IV were found and in 5 isolates we were unable to identify the SCC*mec* element. In the recovered MRSA we found only SCC*mec* type V and IVa, which are a commonly identified SCC*mec* type in MRSA ST398 [15]; the presence of two different SCC*mec* types in *S. aureus* ST398 with the same *spa*-type (data not shown) suggests, as has been shown previously [30], that different SCC*mec* types have been transferred to an isogenic MSSA isolate. How often this transfer occurs is unknown but the farm environment where multiple *mecA*-positive staphylococci reside may provide the environment for potential lateral transfer. Another finding of a possible reservoir in pig farms is the finding of *S. aureus* harboring novel SCC*mec* variants as defined by *ccrB* sequencing. The detection of novel *ccrB2* alleles that not have been described in human isolates until now, suggest the possibility that these *S. aureus* strains were not introduced by human contact.

Our results indicate a large diversity in the J1 region in type IV of SCC*mec* in CNS. For example, *S. cohnii*, *S. haemolyticus*, *S. saprophyticus* and *S. pasteuri* SCC*mec* type IVc was detected, in two *S. haemolyticus* isolates sequences associated with SCC*mec* type I in SCC*mec* subtype IV were found, and in one *S. haemolyticus* isolate the SCC*mec* type IV could not be sub-typed indicating the presence of novel SCC*mec* subtypes. This is consistent with the findings of Berglund *et al*. [31], who reported new types of SCC*mec* type IV, based on variation in the J1 region in MRSA. Our results indicate, that on pig farms type IV, together with type V are the major SCC*mec* types in the staphylococcal population. SCC*mec* type VI harbored by one isolate of *S. equorum* has not been detected in this species before. We detected SCC*mec* type III only in one isolate of *S. sciuri*, which was previously reported by Zhang *et al*. [12]. A SCC*mec* non-typeable with the Kondo PCRs was found in one *S. sciuri* isolate. SCC*mec* typing was furthermore impossible, in single isolates of *S. epidermidis*, *S. equorum*, *S. lentus* and *S. fleurettii*. These findings indicate the presence of novel SCC*mec* elements in CNS isolates are in agreement with other studies [7,11,12], and show that diversity of SCC*mec* types in CNS is considerably larger than in MRSA recovered from the same environment.

MRCNS are considered to be a source for horizontal gene transfer of SCC*mec* elements to *S. aureus*. The diversity of SCC*mec* types among CNS is larger than among *S. aureus* [8,9] and interspecies horizontal transfer of SCC*mec* from *S. epidermidis* to *S. aureus* has recently been documented [10]. Moreover, as the study by Nübel *et al.* indicated, transfer of SCC*mec* to *S. aureus* is not a rare event [32]. All these observations support the hypothesis that CNS may act as the SCC*mec* reservoir for interspecies exchange of this element among *Staphylococcus* species. Our results are also consistent with this hypothesis, since the *S. aureus* and *S. epidermidis* isolates shared the SCC*mec* V element, and the presence

of SCC*mec* type IVc in four staphylococcal species. Our study showed a large diversity of SCC*mec* among the staphylococci present in pig farms which may be a risk for interspecies lateral transfer of SCC*mec* between CNS and *S. aureus*. Detailed genetic characterization of the SCC*mec* types in these strains is necessary to confirm that exact the same SCC*mec* elements are present in these *Staphylococcus* species.

The novel SCC*mec* types found in this study as well as type IVc have not previously been reported in MRSA ST398. Transfer of these subtypes to *S. aureus*, generating novel MRSA strains, cannot be excluded, and longitudinal surveillance will be required to follow dissemination of novel SCC*mec* types in *S. aureus* ST398 or other sequence types.

In conclusion, our data show that SCC*mec* elements present in staphylococci on pig farms are highly heterogeneous. These staphylococci may act as a source for transfer of SCC*mec* to *S. aureus*. Although direct proof of transfer was not obtained in this study, SCC*mec* type V was shared in *S. aureus* and S. epidermidis and SCC*mec* type IVc was present in 4 MRCNS and indicates the possibility of interspecies transfer of SCC*mec* elements in pig farms.

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Chapter 6

Prevalence and molecular characteristics of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in organic pig herds in The Netherlands

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Abstract

The prevalence of the methicillin-resistant *Staphylococcus aureus* (MRSA) among conventional pig herds in The Netherlands is approximately 71%. Nevertheless, information about the prevalence of MRSA among organic pig herds is lacking. Here we report a study on 24 out of the 49 organic pig herds in The Netherlands. The prevalence of MRSA-positive herds was 21%. The genetic characteristics of the MRSA isolates were similar to MRSA CC398 described in conventional pigs except one exceptional HA-MRSA CC30 found in one herd, which was presumably caused by human to animal transmission. This resulted in a prevalence of MRSA CC398 in the organic herds of 16.7%.

Introduction

In 2004 a methicillin-resistant *Staphylococcus aureus* (MRSA) that belongs to clonal complex 398 (CC398) was detected in pigs in The Netherlands [1]. The prevalence of MRSA CC398 is high in Dutch pig herds [2]. The transmission of MRSA CC398 from livestock to humans has been reported in many countries [3,4] and it has been shown that CC398 isolates may cause infections in humans [5]. Contact with livestock is recognized as a major risk factor for human colonization [6,7]. In The Netherlands, the prevalence of MRSA CC398 has been studied in conventional pig herds in detail [1,2,8], but information on organic herds is lacking.

Organic pig production in The Netherlands is governed by the EU regulation for organic production and the 'Landbouwkwaliteitsbesluit 2007 (agricultural quality decree) and is checked by the Dutch governmental inspection and certifying body Skal (www.skal.nl, 2012). In The Netherlands, most organic farms, in total 49 (25 closed, 19 finishing and 5 breeding farms), are member of the Dutch Association for Organic Pig Farms (VBV). The main differences between organic and conventional pig herds based on organic regulations are housing (lower animal density, enriched housing with straw and access to outdoor areas), feeding (at least 95% organic feed including roughage), and the ability to express natural behaviour (e.g. nest building and foraging) is encouraged. Piglets on organic farms must be suckled at least for 42 days, and tail-docking, teeth-clipping and tethering are prohibited. In relation to infectious diseases, prevention should be based on good husbandry, optimal housing conditions, high quality feed and suitable breeds. In case an infectious disease does occur, medication, including the use of antibiotics is allowed; however, under strict conditions (www.skal.nl). E.g. finishing pigs raised on the organic farms are allowed to receive one dose of antibiotics during their lifetime.

The aim of this study was to determine the prevalence and genetic characteristics of MRSA in organic pig herds in The Netherlands.

Materials and methods

Study design and sampling

For this study farms were randomly chosen based on the proportional distribution of the different farm's type: closed, fattening and breeding farms. After farmers' consent to participate in this study, 24 organic pig herds (almost 50% of all organic pig herds in The Netherlands) were included in this study. Thirteen were closed herds, nine finishing herds and two breeding herds. A herd is defined as a pig holding at one location held by one owner [9]. The herds were distributed throughout The Netherlands, with the majority of the herds (19 out of 24), in three provinces in The Netherlands (Noord-Brabant, N= 7; Gelderland, N = 6; and Overijssel, N = 6) where conventional pig farming is also concentrated. The study was conducted from November 2010 to July 2011.

Sampling procedures were similar to those used in the large Dutch national prevalence study in conventional pig herds [9]. Nasal swabs (dry cotton swabs, 155 C Rayon Plastic, Copan, The Netherlands) were collected from 60 animals (weaned piglets and /or finishing pigs) equally distributed over the stables. All sampled animals were in the herd for a minimum of one week, to exclude the reported possibility of MRSA transmission by transportation [10].

Swabs from six pigs housed in the same pen were combined into a pooled sample.

Ten pooled samples per herd were collected, for a total of 60 nasal swabs samples per farm. Additionally, five dust samples were taken from horizontal surfaces (e.g. top of fences, steel bars and ventilators) in the housing facility of the sampled pigs. Dust samples were taken by means of wipes moistened with buffered peptone water (Sodibox, France). A herd was classified MRSA positive if one or more of the pooled samples or dust samples were positive.

The study protocol was in accordance with the Dutch Law on Animal Health and Welfare and discussed with the Animal Welfare Officer of Wageningen University. The distress was considered below the European injection criterium and therefore no further approval of the Animal Welfare Commission was needed. Informed consent was obtained from each participating farmer.

Antibiotic consumption

The annual antimicrobial consumption of the pig herds was calculated using the ADD approach [11], as practically implemented in the annual MARAN reports [12], expressed as animal daily doses per year (ADD/Y). Briefly, the number of ADD/Y for the average present livestock per year was determined by calculating the total number of kilograms of animal that can be treated with the amount of active ingredient in each of the used antibiotic preparations: the treatable weight. This was divided by the total weight of the

average present livestock on the farm, assuming that the average treatment is administered to animals with an average weight (i.e. the average live weight of fattening pigs is 70 kg, sows 220 kg, maiden gilts 107.5 kg, piglets (< 25 kg) 12.5 kg, and breeding boars 350 kg; www.maran.wur.nl). All antibiotic usage data were registered by the veterinarians working on the farms.

MRSA isolation and identification

All samples were analyzed as described previously [9]. Briefly, swabs were inoculated in a non-selective pre-enrichment Mueller Hinton broth (BioMérieux, France) with 6.5% NaCl. After overnight aerobic incubation at 37°C, 1 ml of pre-enrichment culture was transferred into 9 ml phenol red mannitol broth supplemented with 75 mg/l aztreonam and 5 mg/lceftizoxim (BioMérieux, France). After overnight incubation, 10 µl of this selective enrichment broth was inoculated onto sheep blood agar (Biotrading, The Netherlands) and Brilliance MRSA AgarTM (Oxoid, The Netherlands). All suspected colonies were identified as *S. aureus* using standard techniques: colony morphology and coagulase assay (slide) and verified by *S. aureus*-specific PCR amplification [13]. The presence of the methicillin resistance gene (*mecA*) was additionally confirmed by PCR [14].

MRSA typing

Genotyping of isolated MRSA was performed using StaphyType microarrays (Alere Technologies GmbH, Germany). The arrays carried probes for the identification of *S. aureus* species control, *agr* (accessory gene regulator) groups, antibiotic resistance genes, virulence associated exotoxins of *S. aureus*, probes for *set/ssl* genes (exotoxin-like and staphylococcal superantigen-like genes) and SCC*mec* typing. Details on primers, probes and protocols have been published previously [15,16]. Briefly, DNA samples were used as templates for linear primer elongation using one primer per target. The array covers 334 target sequences corresponding to approximately 170 genes. Within this step, biotin-dUTP was incorporated into the resulting amplicons. These amplicons were hybridized to the microarray followed by washing and blocking steps. Then, horseradish–peroxidase–streptavidin conjugate was added. After further incubation and washing steps, hybridizations were visualized by adding a precipitating dye. Finally, an image of the microarray was made and analyzed using the Arraymate reader and software (Alere Technologies GmbH, Germany). Results were classified as negative if the normalized intensity of a given spot was below 25% of the median of the species markers (ribosomal probe, *gapA*, *katA*, *coa*, *spa*, *sbi*, *eno*, *nuc*, *fnbA*, *sarA* and *vraS*) and a biotin staining control spot. The results were regarded positive if the normalized intensity of a given spot was above 50%, and if it was between 25% and 50%, as ambiguous. The affiliation of isolates to clonal complexes (CC) was determined by an automated comparison of hybridization profiles to previously multi-locus sequence-typed reference strains [15]. Additionally, to confirm the CC398 assignment, a real-time assay as described previously was used [17]. Further, the polymorphic X-region of the protein A gene (*spa*) was amplified and analyzed according to the protocol [18].

Results

Farm characteristics

The characteristics of the organic herds are presented in Table 1. The 24 herds studied included 13 closed herds, with both breeding and finishing pigs, two breeding herds and nine finishing herds. On average, the herds were operated according to the organic legislation for 8.8 years (SD 3.4). There was a considerable difference in herd size, with on average 137 sows per herd (SD 73) and on average 595 finishing pigs per herd (SD 265). On average the distance between the sampled herds and conventional pig herds was one km (SD 0.9), whereas the distance to another organic pig farm was on average 12.4 km (SD 7.6).

Table 1. Characteristics of the organic pig herds (N=24)

Characteristics			
Farm type	closed (N)	13	
	breeding (N)	2	
	finishing (N)	9	
Farm size		mean	Range
	closed +breeding farms (N=15)*	137	50 - 280
	finishing farms (N=9)**	595	145 - 1080
organic production since (years)		8.8	2.4 – 15.8
distance to nearby pig farm (km)		1.0	0.05 – 3.0
distance to nearby organic pig farm (km)		12.5	1.0 – 30.0
Antibiotic use	Breeding pigs (ADD/Y, N=15)	5.3	0.03 – 21.9
	Finishing pigs (ADD/Y, N=22)	2.4	0.00 – 16.3

* The farm size is expressed as the number of sows on the farm
** The farm size is expressed as the number of finishing pigs on the farm

A considerable variation was observed in antibiotic use in 2010 between the organic herds, with an average of 5.3 ADD/Y varying from 0.03 to 21.9 ADD/Y in the breeding herds and 2.4 ADD/Y varying from 0 to 16.3 ADD/Y in the finishing herds.

Nevertheless, the total antimicrobial use on these organic farms was lower compared to data reported from conventional farms; i.e. in fattening pigs the average usage was 2.4 vs. 11.5 ADD/Y and in the breeder herds 5.3 vs. 19.2 ADD/Y in organic compared with conventional farms, respectively [12]. The major differences in antibiotic usage between organic pig farms and conventional farms were observed for tetracyclines and macrolides. In organic breeding pigs, less penicillins and trimethoprim-sulfonamide combinations were used. Additionally, in both husbandry systems the use of third- and fourth-generation cephalosporins and fluoroquinolones appeared to be negligible (Figure 1).

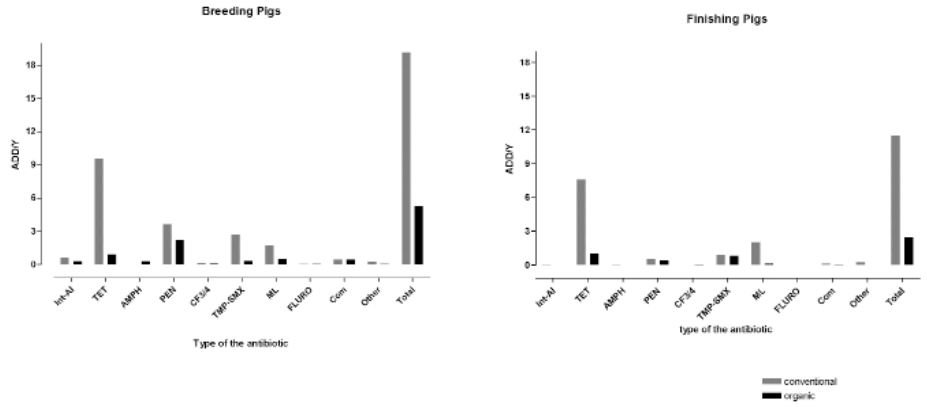

Figure 1. Amounts used (ADD/Y) of the main antibiotic classes on conventional [12] and organic pig farm in The Netherlands in 2010.
Int-AI - Intestinal anti-infectives; TET – Tetracyclines; AMPH –Amphenicols; PEN –Penicillins; CF3/4 - Cephalosporins 3/4 generation; TMP-SMX -Trimethoprim/sulphonamides; ML - Macrolides/lincosamides; FLURO – Fluoroquinolones; Com – Combinations; Other (polymyxins/pleuromutilins)

MRSA prevalence

In this study at least one positive nasal and/or dust sample was found in five positive herds (21% of all sampled herds), three were closed herds, one was a breeding herd and one was a finishing herd (Table 2). In Table 3 the number of positive pig and dust samples related to the total number of respectively pig and dust samples is presented. Of all samples collected, 3.8% of the pooled pig samples were positive for MRSA, whereas 0.8% of the dust samples tested positive (Table 3).

MRSA genetic characteristics

A total of 10 MRSA isolates were obtained from five pig farms from animal samples (farm 12, 14, 15, 16, and 19) and a dust sample (farm 15). Of these MRSA isolates, nine were from pig nasal swabs and one was isolated from a dust sample (Table 2).

Using DNA microarray-based analysis, it was possible to assign the ten MRSA isolates

to two clonal complexes. All strains characteristics and typing results are listed in Table S1. Nine MRSA isolates were assigned to CC398 and one MRSA isolate found on farm 16 was assigned to CC30 based on the microarray profiles (Table S1).

Table 2. Characteristics of MRSA positive farms

Farm no.	Farm type* / ADD/Y	MRSA pooled herd samples (Pos** / Total) Pig	Dust	MRSA characterization CC, *spa*-type; SCC*mec* (numbers of isolates)
12	C / 12.68	3/10	0/5	CC398; *spa*:t011 ;SCC*mec* V (3)
14	B / 2.99	1/10	0/5	CC398; *spa*:t011 ;SCC*mec* V (1)
15	C / 0.03	2/10	1/5	CC398; *spa*: t108 ;SCC*mec* V (3)
16	C / 0.28	1/10	0/5	CC30; *spa* t007; SCC*mec* II (1)
19	F / 1.22	2/10	0/5	CC398; *spa*: t011 ;SCC*mec* V (2)

* B- Breeding farm, C – closed farm, F – Finishing farm
** Pos – Number of MRSA-positive samples

Table 3. Positive MRSA samples in relation to the number of total collected samples in organic pig herds

	Nasal (pool)-Samples	Dust samples	Positive herd
Positive sample	9	1	5
total number of samples collected	240	120	24
percentage positive samples	3.8	0.8	21.0

All isolates of MRSA CC398 carried a SCC*mec* type V. One MRSA CC30 strain carried SCC*mec* type II. All CC398 strains belonged to *agr* group I and the CC30 strain to *agr* group III.

Spa typing was performed for all MRSA isolates. In total three *spa*-types were detected. Two *spa*-types were found in CC398: t011 (6 isolates), t108 (3 isolates). The MRSA CC30 isolate from farm 16 belonged to *spa*-type t007. MRSA CC398 isolates from the herd and dust samples from farm 15 belonged to *spa*-type t108.

The DNA microarray-based analysis identified the presence of antimicrobial resistance genes in the MRSA isolates (Table 4). All MRSA isolates carried the following antibiotic resistance genes: *mecA, blaZ* (beta-lactam resistance) and tetEfflux (tetracycline resistance). All isolates belonging to MRSA CC398 carried additional tetracycline resistance genes (*tetM* and *tetK*). All MRSA CC30 isolates harbored four other genes encoding erythromycin (*ermA*), aminoglycoside (*aacA_aphD, aadD*) and fosfomycin (*fosB*) resistance. The MRSA CC30 isolate harbored quaternary ammonium derivative resistance genes (*qacC*).

Table 4. Microarray-hybridization results for antibiotic resistance genes present in the genomes of MRSA isolates (•) positive; (○) ambiguous, blank -negative.

MRSA isolate	(origin) *	*blaZ*	*blaZ*	*blaI*	*blaR*	*ermA*	*ermB*	*ermC*	*lnuA*	*msrA*	*mefA*	*mpbBM*	*vatA*	*vatB*	*vga*	*vgaA*	*vgb*	*aacA_aphD*	*aadD*	*aphA_3*	*sat*	*dfrA*	*far1*	*Q6GD50*	*mupR*	*tetK*	*tetM*	*tetEfflux*	*cat-pC221*	*cat-pC223*	*cat-pMC524*	*cat-pSBK203R*	*cfr*	*fexA*	*fosB*	*fosB-plasmid*	*qacA*	*qacC*	*qacC-equine*	*qacC-SA5*	*qacC-Ssap*	*qacC-ST94*	*vanA*	*vanB*	*vanZ*
12.03P		•	•	•	•	•																		○		•	•	•								○									
12.04P		•	•	•	•	•																				•	•	•								○									
12.05P		•	•	•	•	•																				•	•	•																	
14.01 P		•	•	•	•																					•	•	•								•									
15.03P		•	•	•	•	•																				•	•	•																	
15.07P		•	•	•	•	•																				•	•	•																	
15.12D		•	•	•	•	•																				•	•	•																	
16.01P		•	•	•	•	•														•	•																								
19.01P		•	•	•	•															•	•					•	•	•																	
19.02P		•	•	•	•																																								

* P – pig nasal swab, D – dust

Analysis of virulence markers showed that all MRSA isolates were Panton-Valentine leukocidin (PVL) negative. Additionally, none of the MRSA CC398 isolates were able to hybridize with probes for superantigen genes, however one isolate (MRSA 14.01) harbored the enterotoxin G (*entG*) encoding gene. MRSA CC30 isolate harbored several enterotoxin encoding genes (*entC, G, L, M, O* and *U*) and toxic shock syndrome toxin 1 gene (*tst1*).

All MRSA isolates carried the genes encodingalpha, delta and gamma haemolysis (*hla, hld, hlg*-locus comprised of l*ukS*, *lukF* and *hlgA*). The genes for beta-haemolysin (*hlb*) was detected only in the MRSA CC30 isolate, as well as the genes for staphylokinase (*sak*) and staphylococcal complement inhibitor (*scn*). All MRSA CC398 isolates harbored type 5 capsule encoding genes and MRSA CC30 isolate harbored type 8 capsule encoding genes (Table S1).

Discussion

In this study, we assessed the previously unknown prevalence of MRSA on organic pig farms in The Netherlands. We tested half of the Dutch organic pig herds associated with the Dutch Association of Organic Pig Farmers (VBV), which is affiliated with most closed, breeding and finishing organic pig farms in The Netherlands. Because of the high proportion of organic farms included, the willingness of the farmers to participate and the selection of herds proportional to farm type, these results are expected to reflect the prevalence of MRSA in the Dutch organic pig farming industry. We found that the prevalence of MRSA positive organic pig herds was 21%.

The genotype analysis of MRSA isolates showed that isolates belonged to two CC groups: CC398 and CC30. Isolates belonging to CC398 group were found in nine out of ten investigated MRSA isolates. *Spa*-types detected in the MRSA CC398 isolates were all known types commonly present in The Netherlands [9]. All MRSA CC398 isolates harbored SCC*mec* type V. Detected SCC*mec* type V in MRSA CC398 is commonly present in this linage [19].

The DNA microarray-based analysis showed the presence of the several genes encoding for antimicrobial resistance to antibiotic classes such as beta–lactams and tetracycline, which are commonly found in the pig-related MRSA CC398 clone [20]. Specifically, the presence of tetracycline resistance in MRSA CC398 is linked to tetracycline usage in swine production [21]. Moreover, the lack of virulence associated PVL and *scn* genes in MRSA CC398 isolates are typical for pig isolates [22].

Surprisingly, on one farm we found one MRSA isolate that belongs to CC30. This isolate harbored SCC*mec* type II and *spa*-type: t007. Additionally, the CC30 isolate harbored genes for different virulence factors than CC398, which included genes for

enterotoxins, toxic shock syndrome toxin 1 gene (*tst1*) and staphylococcal complement inhibitor (*scn*). This MRSA isolate belonging to CC30 is a Hospital Associated (HA)-MRSA variant [23]. We hypothesize that the presence of a HA-MRSA in the pigs' sample was the result of MRSA transmission from human to animal. This observation indicates that humans may incidentally be a source for introduction of non-CC398 MRSA into livestock, as was reported in Canada and The Netherlands, where human-related MRSA was also found in pigs [3,9,24-26]. Moreover, the study of Price *et al.* strongly suggests that also MRSA ST398 has an evolutionary human origin. [27].

In The Netherlands a large scale study in conventional pig farms was performed in 2007 and 2008, using the same method of sampling and analysis [9]. Compared to the data collected in the conventional study, our data suggested that the prevalence of MRSA in organic pigs is lower. We found that 21% of the herds tested positive, of which 16.6% were positive for MRSA CC398. In the conventional systems, 71% of the herds were positive [9]. The percentage of positive samples within farms was also substantially lower (3.4%) than those found in conventional herds (38%) [9]. Recently, the absence of MRSA was reported in a German study in 25 alternative pig farms from the "Neuland" association, which also practices restrictions of antibiotic use [25]. In that study MRSA was absent in 178 nasal samples collected from 25 farms (average seven pig samples per farm). However, the MRSA detection methods used in the German study was less sensitive. The sampling strategy used in the present study can detect MRSA when the within-herd prevalence exceeds 5%, assuming that pooling of nasal swabs has a negligible effect on detection sensitivity [28]. These studies show that measurement of the prevalence of MRSA on non-conventional pig farms with an estimated low prevalence should be performed with a most sensitive detection method and sampling strategy.

In the study of Broens *et al.* [9], the herd size was the main risk factor for becoming MRSA positive. Eighty percent of the large farms (more than 500 sows) were positive, whereas only 40% of the small farms (less than 250 sows) were positive. In our study most of the organic farms can be classified as small farms, according to the criteria mentioned above. Albeit, the MRSA prevalence on the organic farms was lower compared with conventional farms of the same size (21% vs. 40%), which may suggest influence of additional factors such as antimicrobial usage, outdoor access and type of feed. Moreover, it has been shown that MRSA can be air-born transmitted between farms up to 300 m from positive pig barns [29]. In our study average distance between organic farms and conventional was one kilometer. In our study we found MRSA negative farms that were located very close to conventional farms (between 50 m and 300 m). This suggests that airborne transmission is a relative risk and colonization depends on more factors but this route of transmission cannot be excluded. Additionally, it has been reported

that people in contact with livestock are more frequent colonized by MRSA ST398 [30]. Farmers, veterinarians or employees, who have contact with conventional farms, could also be a source of MRSA introduction in to organic farms. However, this route has to be further investigated.

The association between antibiotic use and the occurrence of MRSA in pig farms was suggested before [9]. In comparison to conventional farms in The Netherlands, the average antibiotic use on the organic farms was on average 70% lower than in conventional pig farms [12]. However, in our study no association was found between antibiotic use and MRSA prevalence in herds. This may be explained by the surprisingly large variation in usage observed in this study as well as small size of the samples investigated in this study. Both the organic breeder and finisher herds included outlying herds with higher antibiotic usage than the average reported for conventional farms. In spite of the outliers, our study and the German study of Cuny *et al.* [25] showed a low prevalence of MRSA on organic farms. Organic husbandry has a restricted regulation of antibiotic use and also a different approach for health control. The farmer's management is strongly based on prevention of diseases, which is reached by a good quality of the feed, infection control measures, lower numbers of animals per group, more space per animal and access to outside areas, thus trying to reduce stress and the chance of diseases.

Additionally, animals living on the organic farms are encouraged to express natural behavior. These observations suggest also that different farm management may correlate with lower prevalence of MRSA on organic farms.

In conclusion, this study shows that the prevalence of MRSA is lower in organic than conventional pig herds in The Netherlands. The differences with conventional farms regarding pig herd's management and antibiotic use may contribute to the lower MRSA prevalence. The genetic characteristics of the MRSA isolates found were similar to those described in conventional pigs except one HA-MRSA found, which was presumably caused by human to animal transmission.

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Table S1. Complete hybridization results for MRSA isolates examined in this study

can be download via:

http://goo.gl/cW7E3

Chapter 7

General discussion

The scope of this thesis is to extend our knowledge about methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 colonization in pigs and to focus on the ecology of MRSA ST398 on pig farms. For this purpose the presented studies have been divided into two parts. The first part describes the development and application of a new *ex vivo* model to study MRSA colonization. The second part focuses on unanswered questions about the ecology of MRSA ST398, especially with respect to the possible relationship between staphylococcal flora and the generation of MRSA ST398 in the farm environment, and the presence of MRSA ST398 on organic pig farms.

Ex vivo model

Animal models are useful to study MRSA colonization. More specifically, murine [1] and rat models [2] have been readily studied and data have been extrapolated for studying *S. aureus* colonization in humans. However, these models do not reflect the natural colonization of *S. aureus* in humans. It has been reported that the murine nasal cavity is not a natural habitat of *S. aureus* and the reliability of this model to study *S. aureus* colonization is questionable [3]. Recently, *in vivo* pig colonization models have been used [4-6]. However, disadvantages of these models are, for example, unstable colonization [5,6], detection of minimal numbers of bacteria with sampling and/or isolation methods [5] and bacterial inoculation that may result in death of the animal [4]. In addition, undefined local microbial flora and other environmental factors influence the results making research that is aimed to identify essential factors involved in *S. aureus* colonization and adaptation in livestock, difficult to perform in an *in vivo* setting.

In vitro, molecular mechanisms of *S. aureus* colonization have been studied using human monolayer cell cultures [7]. Thus far, there is no established porcine cell culture system to study bacterial adhesion. Furthermore, *in vitro* systems lack various cell types and mucus [8] which are naturally present in the nostril environment [9]. A model based on isolation and cultivation of nasal mucosa membranes was successfully applied to study the interaction of viral respiratory pathogens with porcine [10] and equine [11] epithelium on respiratory tissue that was maintained for several days without significant changes in morphometry and viability [10,11]. It has been shown that these models represent an ideal tool to study interactions between viruses and respiratory nasal mucosa. **Chapter 2** describes porcine nasal mucosa explants as a novel tool to study MRSA ST398 colonization in pigs. The cultivation of the nasal mucosa explants resulted in a similar lack of changes in morphology and viability of the tissue as previously reported [10]. The nasal mucosa explants were inoculated with three MRSA ST398 strains isolated from a pig carrier (S0462) and a human patient (S0385-1 and S0385-2). MRSA S0385-2 strain was a laboratory variant of S0835-1 with a phage integrated in the beta-hemolysin

gene (*hlb*), that resulted in a lack of lysis of red blood cells. The colonization experiment resulted in three different patterns, suggesting differences in interaction of the different isolates with the tissue. All three strains showed an initial decline in the number of CFU during the first 30 min after inoculation possibly representing bacterial adaptation to the explants. After the 30 minute post adhering period, bacterial recovery from the tissues showed a significant increase of the number of CFU for isolate S0462 and an unaltered bacterial count for isolate S0385-1. Interestingly, one MRSA ST398 strain (S0385-2) was not able to colonize the explants when compared with wild type strain, S0385-1.

It was recently reported that beta-hemolysin in a mouse model plays an important role in skin colonization by damaging keratinocytes [12]. Since keratin is also expressed in the human nose [13], these observations suggest that active beta-hemolysin may also contribute to nasal colonization in pigs. However, visualization of the explants during MRSA colonization showed no tissue damage during MRSA colonization and transcriptome analysis of MRSA S0462 during colonization (**Chapter 3**) showed down-regulation of the *hlb* gene.

We also verified whether other *S. aureus* strains were able to colonize nasal mucosa *ex vivo*. Using human strains (MRSA Mu50 – reported in **Chapter 2**, USA300, MW2 and MRSA252), bovine-associated MSSA RF122 and three CNS strains isolated from pig nostrils (*S. chromogenes*, *S. fleurettii* and *S. sciuri*), we observed variation in the colonization patterns of different MRSA isolates. For example, bovine isolate RF122 and *S. chromogenes* had similar colonization patterns as the reference pig strain, MRSA S0462. With the human strains we observed variation in the number of CFU during colonization and their colonization was less when compared to MRSA S0462 (data not shown). This observation suggests that there are differences in the interaction between diverse *S. aureus* isolates with pig nasal epithelial tissue. Noteworthy is the fact that all staphylococcal strains initially adhered to the epithelium with similar numbers of cells (around 10^6 CFU/cm^2). We hypothesized that initial adherence of bacterial cells to the epithelium is not crucial for maintenance of colonization.

We then applied the *ex vivo* model to study the factors essential for colonization of MRSA ST398 on the explants. Over the past three decades, *S. aureus* adherence to human and animal nostrils has been intensively studied [14] and the essential factors for initial colonization have been very well identified and characterized [7,14,15]. However, the bacterial factors responsible for maintenance of colonization are less understood. We investigated the global gene changes during *ex vivo* colonization of MRSA ST398. The study presented in **Chapter 3** investigated the global gene expression pattern of MRSA ST398 after initial adherence. The microarray data showed that genes involved mainly in metabolic processes were up- or down-regulated during experimental colonization

while genes encoding virulence factors were down-regulated during *ex vivo* colonization.

Based on a literature search and their up-regulation in the transcriptome experiment the potential contribution to maintenance of colonization of the genes encoding a second von Willebrand factor binding protein (*vwbp*) and extracellular cysteine proteinase (*scpA*) were studied. The second vWbp is widely distributed in ruminants and it has been suggested that this protein plays a role in adaptation of *S. aureus* to animal hosts [16]. ScpA is known to cleave a number of extracellular matrix components and it has been suggested to play a role in bacterial migration from the sites of initial colonization [17,18]. Unfortunately, single knockout mutant strains did not show any phenotypic differences in the colonization pattern *ex vivo*, which indicates that these selected genes do not play a crucial role in maintenance of colonization *ex vivo*. This study showed that mechanisms responsible for successful colonization of MRSA ST398 in pigs are multifactorial and we hypothesized that this type of colonization process reflects *S. aureus* ST398 host specificity. McCarthy and Lindsay analyzed variation in surface proteins and immune evasion factors between different *S. aureus* lineages [19]. They showed that variation in genes encoding surface proteins and those involved in immune response are lineage specific with variation that mainly occurs in functional domains. Some *S. aureus* lineages appear to be host specific, while others have a broad host range [20]. Moreover, each host would have different ligands on the cell surfaces which are recognized by bacterial proteins. For example, it has been demonstrated that human non-carriers could not be colonized when experimentally challenged with *S. aureus* indicating that host genetics determines the ability to be a *S. aureus* carrier [21]. These observations also suggest that the molecular process responsible for successful colonization of MRSA ST398 in livestock is most likely a result of multiple global interactions between *S. aureus* ST398 surface proteins with host ligands and possibly certain metabolic processes. More extensive studies, including those based on proteome analysis, are needed to fully understand the molecular mechanism responsible for successful colonization of MRSA ST398 in livestock. It would be interesting to compare gene expression profiles of MRSA ST398 on nasal mucosa membranes isolated from different hosts like human, bovine and horse. It would also be interesting to study differences in metabolic adaptation to different hosts which may influence MRSA ST398 colonization. The full understanding of the process behind successful colonization of MRSA ST398 may lead to new strategies to reduce MRSA ST398 from livestock.

It has been shown that swine as well as other livestock are a potential MRSA reservoir for humans [22,23]. One of the solutions to limit MRSA ST398 transmission from pigs to humans is to reduce colonization of pigs or even eradicate MRSA from pig farms. Until now there is no such method available. In human medicine, mupirocin is an effective

treatment to eradicate MRSA from patients before hospitalization [24]. However, similar to antibiotics, MRSA resistance to mupirocin has been documented and indicates a limitation of treatment [25]. Consequently, new solutions must be tested in order to limit MRSA prevalence in livestock. Phage therapy is a possible alternative treatment [26] that can be applied to livestock. **Chapter 4** illustrates the first example to phage therapy in pigs. Bacteriophages were able to kill bacteria *in vitro*, but this effect could not be observed in the animal experiment. Similar results were obtained in an *ex vivo* experiment using the *ex vivo* nasal explants model. Moreover, application of muporicin in both *in vivo* and *ex vivo* resulted in an almost complete reduction of MRSA. However, the reason why bacteriophages were not able to limit MRSA colonization in animals and *ex vivo* is unclear. We speculated that one of the reasons may be differences in *S. aureus* surface protein expression which may limit the accessibility for bacteriophage to adhere to the bacterial cell surface. In **Chapter 3** it has been shown that the *ex vivo* model is suitable to study *S. aureus* global gene expression profiling during colonization. Comparison of transcriptome data from *ex vivo* and *in vitro* settings may be conducive to the assumption, that differences in surface expression may limit bacteriophages access to *S. aureus*. Moreover, further proteomic research may be helpful to characterize whether bacteriophage receptors are presented on the bacterial surface. This information would be helpful to improve bacteriophage therapy. In the future, new phage therapy strategies can be tested using an *ex vivo* model as a preliminary screening method before animal trials.

Research described in **Chapter 4** indicates that the *ex vivo* model is a powerful tool not only to study *S. aureus* colonization in pigs, but can also be applied for intervention studies. Moreover, the use of the explants model in the preliminary screening of new decolonization treatments would help reduce the number of experimental animals required.

Recently, a model was published to study *S. aureus* biofilm formation in humans [27]. In this study human sinonasal mucosa explants were isolated from patients undergoing endoscopic surgery for resection of a pituitary tumor and were cultivated on an air-liquid interface. This research describes the reaction of human tissue to the presence of a *S. aureus* biofilm by production and secretion of IL-6. This study showed that the sinonasal mucosal explant model is also suitable to study some aspects of innate immunity of the tissue challenged with *S. aureus*. It should be noted, that isolated nasal mucosa membrane does not have active blood vessels and an active immune response in those systems is absent. However, the authors suggest that initial steps of the adaptive immune response could be evaluated during *ex vivo* explants cultivation [27]. The human nasal explant culture model has also been used to investigate the interaction between Herpes simplex virus 1

in modulation of the *S. aureus* invasion of airway mucosa [28]. This study demonstrated that HSV1 infection significantly damaged the nasal epithelium and enhanced attachment of *S. aureus*, hence the invasion of *S. aureus* into the nasal mucosa. It has been suggested that nasal colonization with a methicillin-susceptible *S. aureus* protects the host against acquisition of MRSA [29] in which polymorphisms in the regulatory *agr* system may play a role [30-32]. We believe that an *ex vivo* model can also be applied to study this interaction between different *S. aureus* strains, between *S. aureus* and CNS, and possibly the *S. aureus* interaction with other nasal flora during colonization of the nasal mucosa explants.

All these reports show that a model based on nasal mucosa explants is a powerful tool |to study nasal colonization and is even suitable to study the interaction between different *S. aureus*, and *S. aureus* and other flora. Nasal mucosa explants can be isolated from various animals and humans [10,11,27]. Additionally, in the *ex vivo* models the normal cell-cell contacts, the cell-extracellular matrix contacts and the three-dimensional structure of the tissue are preserved. The nasal mucosa explant model can be further applied to various studies in order to investigate the complexity of bacterial colonization in pigs as well as in other animals. Furthermore, the *ex vivo* model can be used as a platform to screen new treatment strategies both in veterinary and public health settings. In conclusion, nasal explant models can be very powerful tools to study *S. aureus* colonization and the various factors involved in this process.

Ecology of MRSA ST398

It has been suggested that CNS found in the environment could serve as a source for SCC*mec* in the development of novel MRSA strains [33]. Nevertheless, this observation was obtained from SCC*mec* typing in CNS isolated from various animals and it was not investigated in staphylococci isolated from the same niche. **Chapter 5** presents the first study to detect *Staphylococcus* species harboring SCC*mec* on pig farms. A total of 4 SCC*mec* types (III, IV, V and ND) and 3 subtypes of SCC*mec* type IV (IVa, IVc and IVvar) were identified. This demonstrates that a reservoir of *mecA*-positive CNS coincides with *S. aureus* on pig farms. The presence of SCC*mec* type V among both CNS and *S. aureus* supports the hypothesis that on pig farms CNS may act as a reservoir for the exchange of SCC*mec*. SCC*mec* type IVa and V are commonly identified in MRSA ST398 [34], which shows that SCC*mec* variation between MRSA ST398 isolates is lower when compared with CNS isolated from this same niche and it indicates that uptake of SCC*mec* type IVa and V to MSSA ST398 is preferred by this lineage. Non-ST398 MRSA can also be occasionally detected in pigs [22,35,36] and can harbor other SCC*mec* types e.g. type IVc [36].

Similar observations have been reported in pigs and cows in Belgium [37,38]. These studies showed that related SCC*mec* types are present in MRSA ST398 and *mecA*-positive CNS on these farms. *mecA*-positive staphylococci have also been isolated

from turkey flocks in Germany [39] but unfortunately, SCC*mec* typing was not performed in this study. These data indicate that CNS could be an important reservoir of SCC*mec* which may aid the generation of new MRSA strains in the same environment. These studies revealed the importance of monitoring not only the prevalence of MRSA, but also the companion staphylococcal flora as a reservoir of antibiotic resistance genes, which may be transferred between staphylococcal species.

Data presented in **Chapter 5,** along with what was reported in the Belgian study [37], showed that SCC*mec* type IVc can be shared between staphylococcal species isolated from the pig farms. Recently, a Swiss study reported detection of SCC*mec* type IVc in MRSA ST1 isolated from slaughter pigs [36]. We hypothesize that CNS on pig farms can also serve as reservoir of SCC*mec* elements for non-ST398 lineages.

The direction of horizontal gene transfer (HGT) of SCC*mec* between staphylococcal species present in the same niche is speculative and it is suggested that transfer could occur in both directions. Until now, two reports documented *in vivo* transfer of SCC*mec* from *Staphylococcus epidermidis* [40] and from *Staphylococcus haemolyticus* [41] to *S. aureus*. Unfortunately, there is no report describing the mechanism of transfer of SCC*mec* between staphylococcal species. It was suggested that SCC elements less than 45 kbp in size may be transferred between staphylococci by bacteriophages [42]. Recently, it has been proposed that large SCC*mec* elements (more than 45 kbp like SCC*mec* type II) can be transferred to competent MRSA by transformation [43]. It is worthy to mention that for the latter study N315ex cells, which had lost the SCC*mec* element, were used as the recipient [43]. The contribution of transformation in transfer of SCC*mec* into other MSSA strains as well as other staphylococcal species needs to be further investigated. The *S. aureus* and *S. epidermidis* strains that showed *in vivo* SCC*mec* transfer [40] were subjected to analysis of SCC*mec* transfer *in vitro*. Nevertheless, we were not able to induce horizontal gene transfer of SCC*mec* using various techniques: during filter mating, during biofilm formation and in the *ex vivo* model described in this thesis (data not shown).

All these observations emphasize that better characterization of staphylococcal flora is required to estimate the prevalence of methicillin-resistant staphylococci in order to evaluate their clinical and public health relevance. Moreover, molecular typing of CNS strains from diverse sources including non-SCC*mec* typing based on whole genome sequencing would help us to understand the generation of new MRSA strains.

Another interesting subject in the ecology of MRSA ST398 is the possible relation between antibiotic usage and prevalence of MRSA ST398 on pig farms. It has been suggested that antibiotic consumption in livestock is associated with a high prevalence of MRSA ST398 in livestock. For example, most of the pig isolates of ST398 are resistant to tetracycline, an antibiotic commonly used in swine farming [44]. However, a risk factor analysis reported by Broens *et al.* did not find a significant association between antibiotic

consumption on the farm and MRSA prevalence [22]. Additionally, prevalence of MRSA ST398 on different pig farm systems such as organic farms has not been studied before. Chapter 6 describes the prevalence of MRSA on organic pig farms in The Netherlands. In comparison to conventional farms in The Netherlands, the antibiotic usage on the organic farms is on average 70% lower, but this is only one of the differences between organic and conventional farming [45] The data presented in **Chapter 6** suggest that different farm management can be responsible for a lower MRSA prevalence on organic farms when compared with conventional farms.

In summary, the second part of this thesis (**Chapter 5** and **6**) manifests the beginning of our understanding of the ecology of MRSA ST398 on pig farms, the presence of staphylococci on pig farms and the potential influence of different types of farm management on the development of novel MRSA ST398 in livestock. The global understanding of the ecology of MRSA ST398 may be helpful to develop and implement new strategies to limit possible transfer of MRSA from livestock to humans. Especially since MRSA ST398 can further acquire new virulence factors that empower the adaptation and infection in humans. Identification of the molecular mechanisms behind HGT of SCC*mec* between staphylococci needs to be unraveled and this remains a challenge.

Conclusion remarks

The research presented in this thesis describes the development of a new *ex vivo* model to study MRSA colonization.

Presented findings provide important insights that enrich our understanding of the potential generation of MRSA strains on pig farms, as well as the differences between farm management and the prevalence of MRSA in pigs.

The main conclusions that can be drawn from this thesis are:

- *Ex vivo* nasal mucosa explants are a powerful tool to study *S. aureus* colonization in pigs, and reflects *in vivo* conditions
- The *ex vivo* model is suitable for study colonization of MRSA strains from different origins
- The *ex vivo* model may be used as a screening platform to test new treatment strategies in order to limit MRSA prevalence in livestock
- SCC*mec* elements present in staphylococci on pig farms are highly heterogeneous and are shared between staphylococcal species
- MRSA prevalence on organic pigs farms in The Netherlands is lower than is reported on conventional farms

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Nederlandse samenvatting

Moleculaire en ecologische aspecten
van MRSA ST398 kolonisatie bij varkens

Het doel van dit proefschrift is de kennis over MRSA ST398 kolonisatie in varkens te vergroten en inzicht te krijgen in de ecologie van MRSA ST398 kolonisatie op varkensbedrijven. Het proefschrift bestaat uit twee onderdelen. Het eerste beschrijft de ontwikkeling en toepassing van een *ex vivo* model om MRSA kolonisatie in de varkensneus te bestuderen. Het tweede onderdeel richt zich op het bestuderen van de ecologie van MRSA ST398 in de varkenshouderij waarbij in het bijzonder wordt ingegaan op de mogelijke relatie tussen verschillende stafylokokken species en het ontstaan van MRSA ST398 op boerderijen en de kolonisatie met MRSA ST398 op biologische varkensbedrijven.

***Ex vivo* model**

De kolonisatie van varkens is bestudeerd in varkens diermodellen. Deze modellen hebben echter nadelen zoals instabiele kolonisatie, onbetrouwbare detectie van bacteriën door aanwezigheid van lage aantallen, en complicaties na inoculatie met de dood van het dier tot gevolg. Een bruikbaar alternatief systeem om een beter begrip te krijgen van nasale kolonisatie zou het gebruik kunnen zijn van varkensneusslijmvlies (*ex vivo* explantaat model) waarmee de interactie tussen bacteriën en slijmvlies, en de bijdrage van gastheerfactoren bestudeerd onder gecontroleerde omstandigheden kunnen worden. **Hoofdstuk 2** beschrijft de ontwikkeling van het explantaat model waarin varkens neusslijmvliesweefsel in kweek gehouden wordt (explantaat) voor bestudering van de MRSA ST398 kolonisatie in varkens. Tijdens de incubatie van de varkensneusslijmvlies explantaten traden er gedurende een periode van 72 uur geen veranderingen in de morfologie op en was geen weefselbeschadiging waarneembaar. Na inoculatie van de explanaten met drie verschillende MRSA ST398 stammen (één stam geïsoleerd bij dragerschap in een varken (S0462) en twee stammen geïsoleerd van een humane patiënt (S0385-1 en S0385-2) werden verschillende kolonisatie patronen waargenomen. MRSA S0385-2 was een laboratorium variant van S0835-1 met een faag geïntegreerd in het beta-hemolysine gen (*hlb*), wat resulteerde in remming van de lysis van rode bloedcellen. Deze stam liet een afname zien van het aantal kolonie vormende bacteriën (KVE). Stam S0462 gaf een aanzienlijke toename van het aantal KVE, en van stam S3085-1 bleef het aantal bacteriën constant. Daarnaast werd aangetoond dat gedurende de MRSA kolonisatie geen weefselbeschadiging optrad. Deze observaties doen vermoeden dat verschillende stammen gebruik maken van andere mechanismen tijdens de interactie met het neusslijmvliesweefsel. Opmerkelijk is het gegeven dat alle MRSA ST398 stammen starten met binding van een gelijk aantal cellen (ongeveer 10^6 KVE/cm^2) aan het epitheel. Op basis hiervan veronderstellen we dat de eerste binding van bacteriële cellen met het neusepitheel niet bepalend is voor het latere verloop en het behouden van MRSA

kolonisatie. Vervolgens werd de globale verandering in expressie van genen van MRSA ST398 gedurende *ex vivo* kolonisatie onderzocht. **Hoofdstuk 3** beschrijft het totale genexpressie patroon van MRSA ST398 gedurende *ex vivo* kolonisatie. De microarray data geven aan dat de expressie van sommige genen geassocieerd met stofwisseling werden geremd terwijl anderen juist werden geactiveerd gedurende experimentele kolonisatie. Genen die coderen voor virulentie factoren werden geremd gedurende *ex vivo* kolonisatie. Twee genen werden geselecteerd voor nadere studievanwege hun mogelijke bijdrage aan kolonisatie: *vwbp* en *scpA*. Stammen met de geïnactiveerde genen lieten echter geen enkel fenotypisch verschil zien in het *ex vivo* kolonisatiepatroon, wat aangeeft dat deze genen geen rol van belang spelen in het onderhouden van kolonisatie *ex vivo*. Deze studie geeft aan dat de mechanismen verantwoordelijk voor succesvolle kolonisatie van MRSA ST398 in varkens multifactorieel zijn. Meer uitgebreide studies, bijvoorbeeld gebaseerd op proteoom analyse, zijn noodzakelijk om een volledig begrip te krijgen van de moleculaire mechanismen die verantwoordelijk zijn voor succesvolle kolonisatie van MRSA ST398 in varkens.

De laatste jaren is aangetoond dat varkensbedrijven en vleeskalverenbedrijven een potentieel MRSA reservoir vormen voor MRSA transmissie naar de mens. Verondersteld kan worden dat een afname van MRSA ST398 in landbouwhuisdieren de transmissie van MRSA van dieren naar de mens zal verminderen. Tot op heden is er geen methode beschikbaar om MRSA kolonisatie in varkensbedrijven te beperken. Faagtherapie is een mogelijke behandeling welke toegepast zou kunnen worden bij dieren. In **hoofdstuk 4** is onderzocht of faagtherapie toegepast kan worden voor bestrijding van MRSA ST398 kolonisatie in varkens. De bacteriofagen waren in staat om MRSA ST398 *in vitro* te doden, maar desondanks resulteerde een behandeling van met MRSA gekoloniseerde varkens niet tot afname van kolonisatie. Vergelijkbare resultaten werden behaald in een *ex vivo* experiment met gebruik van het neusslijmvlies explantaat. Daarentegen resulteerde de toepassing van mupirocine zowel *in vivo* als *ex vivo* in een bijna volledige reductie van MRSA. De reden waarom bacteriofagen niet in staat waren de MRSA kolonisatie te verminderen in varkens en *ex vivo* is niet duidelijk. Eén van de redenen zou verschillen in de expressie van *S. aureus* oppervlakte eiwitten kunnen zijn waardoor de receptor voor faag binding afgeschermd kan zijn. Daarnaast kunnen ook gastheer eiwitten door sterische hindering de binding van bacteriofagen aan de bacterie cel blokkeren. Samenvattend kan gesteld worden dat het *ex vivo* model, beschreven in **hoofdstuk 4** een goed model is, niet alleen om de *S. aureus* kolonisatie in varkens te bestuderen, maar ook voor de evaluatie van de effectiviteit van interventiestudies. Bovendien zou het gebruik van het explantaat model in de eerste screening van een nieuwe interventie behandeling helpen om het aantal vereiste proefdieren te verminderen. Op basis van de resultaten met explantaten

van varkens lijkt het ook mogelijk om explantaten van andere dieren te gebruiken zodat ook daar kolonisatie op een eenvoudigere manier bestudeerd kan worden.
Samengevat toonde deze studie aan dat in het *ex vivo* model de cellen, de normale cel-cel contacten, de extracellulaire matrixcontacten en de driedimensionale structuur van het weefsel intact zijn. Het *ex vivo* model kan gebruikt worden om de complexiteit van de bacteriële kolonisatie in varkens te bestuderen. Bovendien kan het *ex vivo* model gebruikt worden als een platform om nieuwe interventie of behandelingsstrategieën te onderzoeken die toegepast kunnen worden in de in dier of mens.

Ecologie van MRSA ST398

Algemeen wordt aangenomen dat coagulase-negative stafylokokken (CNS) die aanwezig zijn in het milieu, kunnen dienen als bron voor transmissie van SCC*mec* en zo kunnen bijdragen aan de ontwikkeling van nieuwe MRSA stammen. Deze aanname is gebaseerd op het veelvuldig voorkomen van SCC*mec* types in CNS stammen. Verder is er sterk indirect bewijs dat twee MRSA stammen kon ontstaan uit een *S. aureus* stam door opname van een SCC*mec* element uit een CNS. Over het mogelijk ontstaan van MRSA op boerderijen was aan het begin van dit onderzoek weinig bekend. Er was alleen informatie over het voorkomen van SCC*mec* types bij CNS die geïsoleerd werden uit verschillende dieren zonder deze te vergelijken met MRSA stammen die geïsoleerd werden uit dezelfde niche. Daarom wordt in **Hoofdstuk 5** beschreven welke stafylokokken species en welke SCC*mec* types voorkomen op varkensbedrijven evenals de mogelijke transmissie van SCC*mec*. In totaal werden 4 SCC*mec* types (III, IV, V en tenminste één nieuw type) en drie subtypes van SCC*mec* type IV (IVa, IVc en IVvar) geïdentificeerd in 10 stafylokokken species. Deze studie toonde aan dat er een reservoir van *mecA*-positieve CNS voorkomt op varkensbedrijven. Bovendien ondersteunde de aanwezigheid van dezelfde SCC*mec* types bij CNS en *S. aureus* stammen de hypothese dat op varkensbedrijven CNS kan optreden als een reservoir voor de uitwisseling van SCC*mec*. Deze studie toont verder het belang aan van monitoring van niet alleen de aanwezigheid van MRSA maar ook van de overige aanwezige stafylokokken flora aangezien antibiotica resistentie genen kunnen worden uitgewisseld tussen stafylokokken soorten.

Een volgende stap om inzicht te krijgen in de verspreiding van MRSA ST398 in varkensbedrijven richtte zich op bestudering van de ecologie van MRSA ST398 op biologische varkensbedrijven en bestudering van de mogelijke relatie tussen het gebruik van antibiotica en andere managementfactoren, en aanwezigheid van MRSA. Er wordt gesuggereerd dat het gebruik van antibiotica op varkensbedrijven geassocieerd is met hoge prevalentie van MRSA ST398. Een onderzoek naar risicofactoren

uitgevoerd op conventionele varkensbedrijven in Nederland gaf echter geen significante relatie aan tussen het gebruik van antibiotica en de aanwezigheid van MRSA. De studie in **hoofdstuk 6** beschrijft de aanwezigheid van MRSA in biologische varkensbedrijven in Nederland. In vergelijking met conventionele bedrijven in Nederland is het gemiddelde antibioticagebruik op de biologische varkensbedrijven gemiddeld 70% lager. De aanwezigheid van MRSA op biologische bedrijven was ook lager in vergelijking met conventionele bedrijven (21% versus 71%). Desondanks werd een mogelijke correlatie tussen antibioticagebruik op de boerderijen en de aanwezigheid van MRSA werd niet aangetoond in ons onderzoek. De gegevens gepresenteerd in **hoofdstuk 6** suggereren dat een andere manier van management van een varkensbedrijf kan leiden tot lagere MRSA aanwezigheid op biologische varkensbedrijven.

Samengevat vergroot het tweede deel van dit proefschrift het begrip van de ecologie van MRSA ST398 op varkensbedrijven. De moleculaire mechanismen achter de horizontale uitwisseling van SCC*mec* tussen stafylokokken blijven echter nog onbekend. Verder inzicht in de ecologie van de MRSA ST398 kan helpen bij de ontwikkeling van nieuwe strategieën om de mogelijke overdracht van MRSA van dier op mens te beperken.

Streszczenie po polsku

Molekularne i ekologiczne aspekty kolonizacji świń
przez szczep gronkowca złocistego MRSA ST398

Celem niniejszej pracy jest poszerzenie wiedzy na temat kolonizacji gronkowca złocistego MRSA ST398 u świń, jak również zrozumienie ekologii MRSA ST398 występującego na fermach trzody chlewnej. Prezentowana praca składa się z dwóch części. Pierwsza, opisuje zastosowanie modelu *ex vivo* do badania kolonizacji MRSA. Natomiast druga część, skupia się na nie do końca zrozumiałych aspektach ekologii MRSA ST398, a w szczególności, na związku między różnymi gatunkami bakterii wchodzącymi w skład naturalnej flory bakteryjnej z generacją MRSA ST398 w środowisku. Ponadto, zwrócono szczególną uwagę na związek pomiędzy hodowlą świń na farmach ekologicznych, a występowaniem MRSA ST398 u trzody chlewnej.

Model *ex vivo*

Do tej pory nieznane są molekularne mechanizmy kolonizacji świń przez MRSA ST398. W celu wyjaśnienia tych mechanizmów są obecnie stosowane modele zwierzęce, które na bieżąco są udoskonalane. Jednak modele opierające się na eksperymentalnej kolonizacji świń posiadają pewne wady. Kiedy kolonizacja jest niestabilna, to wykrywalność bakterii jest niewystarczająca, a eksperymentalna kolonizacja może wywołać śmierć zwierzęcia. Alternatywnym systemem stosowanym do badania kolonizacji MRSA jest system *ex vivo*, który polega na hodowli wycinków śluzówki nosa świń (zwanych eksplantami) w warunkach laboratoryjnych. System ten, pozwala również na pracę w warunkach, w których różne czynniki środowiskowe mogą być łatwiej weryfikowane. W pracy opisano system hodowli eksplantów, jako nowego narzędzia stosowanego do badania kolonizacji MRSA ST398 u świń. Hodowane przez 72 godziny wycinki śluzówki nosa nie wykazują zmian w morfologii i żywotności tkanki. W celu weryfikacji, czy *ex vivo* system można wykorzystać w badaniach kolonizacji bakteryjnej, eksplanty zaszczepiono trzema szczepami MRSA ST398 wyizolowanymi od świni (S0462) i dwoma od pacjenta z zapaleniem wsierdzia (S0385-1, S0385-2). W wyniku eksperymentalnej kolonizacji stwierdzono trzy różne jej typy. Dla szczepu S0462 zaobserwowano znaczny wzrost liczby bakterii (CFU) na tkance, dla szczepu S0385-1 liczba bakterii była stała, a dla kolonizacji eksplantu szczepem S0385-2 zaobserwowano znaczny spadek liczby bakterii. Otrzymane typy kolonizacji sugerują, że różne szczepy MRSA ST398 różnią się interakcją z hodowaną tkanką. Przeprowadzone dodatkowo badania mikroskopowe skolonizowanych eksplantów nie wykazały uszkodzeń tkanki.

W celu zrozumienia molekularnych mechanizmów kolonizacji MRSA ST398 u świni, przeprowadzono analizę zmian w regulacji ekspresji genów u gronkowca złocistego MRSA ST398 S0462, podczas eksperymentalnej kolonizacji *ex vivo*. Analiza danych z mikromacierzy wykazała, że większość regulowanych genów, podczas kolonizacji, była odpowiedzialna za główne procesy metaboliczne. Geny kodujące czynniki wirulencji

były regulowane negatywnie podczas kolonizacji *ex vivo*. Ponadto, zaobserwowano dodatnią regulację dwóch genów: *vwbp* i *scpA*, które w literaturze naukowej opisywane są, jako potencjalne czynniki ułatwiające kolonizację gronkowca złocistego. W celu zbadania ich wpływu na kolonizację, przeprowadzono mutację badanego szczepu i wycięto odpowiednie geny (single knockout) z genomu bakteryjnego. Niestety, w porównaniu ze szczepem dzikim, szczepy nieposiadające opisanych wyżej genów, nie wykazały żadnych różnic w fenotypie podczas kolonizacji *ex vivo*. Wskazuje to na to, że analizowane geny, nie odgrywają kluczowej roli w kolonizacji *ex vivo*. Dowodzi to, że mechanizmy odpowiedzialne za kolonizację MRSA ST398 u świń są wieloczynnikowe. W przyszłości, bardziej obszerne badania molekularne są potrzebne w celu pełnego zrozumienia molekularnych mechanizmów warunkujących kolonizację MRSA ST398 u świń.

Zwierzęta gospodarskie (takie jak trzoda chlewna, bydło, drób, itp.) są potencjalnym rezerwuarem MRSA dla ludzi. Na przestrzeni ostatniego dziesięciolecia zostało udokumentowanych kilka przypadków transmisji MRSA od zwierząt do ludzi. Wydaje się, że ograniczenie występowania MRSA ST398 u zwierząt jest jednym ze sposobów na wyeliminowanie źródła transmisji MRSA ST398 ze zwierząt na ludzi. Do tej pory, nie ma opracowanych skutecznych metod na redukcję MRSA u zwierząt hodowlanych. Jedną z proponowanych metod jest terapia fagowa, która może być skutecznie stosowana u zwierząt. W niniejszej pracy przedstawiono pierwsze badania dotyczące wykorzystania terapii fagowej u świń. Nasze badania wykazały, że fagi są zdolne do zabijania bakterii w systemie *in vitro*, jednak wyniku nie potwierdzono w badaniach przeprowadzonych na zwierzętach. W doświadczeniu przeprowadzonym *ex vivo*, z wykorzystaniem hodowli eksplantów opisanych wyżej, również uzyskano wynik negatywny. Do ograniczenia występowania MRSA u zwierząt i w systemie *ex vivo*, użyto mupirocyny, leku stosowanego w medycynie. Zarówno w przypadku zwierząt, jak i eksplantów, stosowanie mupirocyny doprowadziło do całkowitej redukcji MRSA. Przyczyna, z powodu której fagi nie były w stanie zabić MRSA u zwierząt i w modelu *ex vivo*, nie jest do końca zrozumiała. Jednym z wyjaśnień może być różnica w ekspresji białek powierzchniowych u gronkowca złocistego, w warunkach *in vitro* i *ex vivo*/*in vivo*. Przyczyną może być ograniczony dostęp fagów do specyficznych receptorów obecnych na powierzchni komórki, co jest wynikiem braku przylegania fagów do powierzchni komórki bakteryjnej. Ponadto, model *ex vivo* może być stosowany do badań przesiewowych nowych terapii u zwierząt, w celu dekolonizacji zwierząt przez MRSA.

Ekologia MRSA ST398

Wiedza na temat powstawania nowych szczepów MRSA w środowisku jest ograniczona. Obecnie przyjmuje się, że gronkowce koagulazoujemne występujące

w środowisku mogą być źródłem oporności na metycilinę, która występuje na kasecie chromosomowej zwanej SCC*mec* dla gronkowca złocistego, a tym samym generacji szczepów MRSA. W pracy opisano pierwsze badania charakteryzujące florę gronkowców posiadających w genomie SCC*mec*, które zostały wyizolowane z ferm trzody chlewnej. Badania te potwierdziły hipotezę, że flora gronkowcowa występująca na fermach trzody chlewnej może być rezerwuarem SCC*mec* dla gronkowca złocistego i przyczyniać się do powstawania nowych szczepów MRSA w środowisku. Zatem, monitorowanie występowania MRSA na fermach nie jest wystarczające i badanie towarzyszącej flory gronkowcowej, jako potencjonalnego rezerwuaru genów oporności na antybiotyki dla gronkowca złocistego, jest uzasadnione i potrzebne.

Następnym interesującym zagadnieniem w ekologii MRSA ST398 jest związek pomiędzy stosowaniem antybiotyków na farmach trzody chlewnej, a występowaniem MRSA ST398. Obecnie sugeruje się, że stosowanie antybiotyków u zwierząt hodowlanych jest przyczyną obecności i rozpowszechniania się MRSA ST398. Jednak badania przeprowadzone na konwencjonalnych farmach trzody chlewnej w Holandii nie wykazały wprost związku między konsumpcją antybiotyków w gospodarstwie rolnym, a występowaniem MRSA. W pracy przedstawiono pierwsze przeprowadzone badania nad występowaniem MRSA w ekologicznych gospodarstwach trzody chlewnej w Holandii. W tych badaniach wykazano, że w porównaniu do tradycyjnych gospodarstw rolnych, średnie zużycie antybiotyków w gospodarstwach ekologicznych jest średnio o 70% niższe. Co więcej, obecność MRSA w ekologicznych stadach trzody chlewnej była znacznie niższa w porównaniu ze stadami konwencjonalnymi (obecność MRSA kształtowała się odpowiednio na poziomie 21% i 71%). Jednakże, nie znaleziono bezwzględnych zależności pomiędzy stosowaniem antybiotyków w gospodarstwach rolnych a występowania MRSA. Przedstawione wyniki wskazują, że typ gospodarstwa może mieć większy wpływ na obecność MRSA u zwierząt hodowlanych, niż konsumpcja antybiotyków.

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Paweł

Curriculum Vitae

Paweł Tuliński was born on 3rd October 1982 and raised in Zielona Góra, Poland. After graduating from high school in 2001, he then went on to study Environmental Protection at the University of Zielona Góra. In 2004 he was awarded a fellowship to continue his studies in Biotechnology at Jagiellonian University in Kraków, Poland. Here he studied the diversity of the *Escherichia coli* population in various animals and in June 2005 he was awarded his Master of Science degree. After graduation he remained at Jagiellonian University where he continued his research on extracellular proteinases in *Staphylococcus aureus*, conducted lectures and developed practical courses in molecular and environmental microbiology, and supervised Bachelor and Master Students. In 2007 he moved to Warsaw, Poland and continued his work as a consultant in a medical technology company, where he translated research for healthcare personnel. In June 2008 he started his one year fellowship on The Mina and Everard Goodman Faculty of Life Sciences at Bar-Ilan University, Israel, where he studied transcription regulation in *Tryponosoma brucei*.

In August 2009 Pawel began his PhD. research in the Department of Infectious Diseases and Immunology at the Faculty of Veterinary Medicine, at Utrecht University, The Netherlands. Under supervision of Prof. Dr. Jaap A. Wagenaar, Dr. Birgitta Duim and Dr. Ad C. Fluit, he studied molecular mechanisms of pig colonization by *S. aureus*. Moreover, his studies have focused on the relationship between *S. aureus* and staphylococci that have arisen from the same niche and how this relates to methicillin-resistant *S. aureus* generation. The research was part of the European Seventh Framework Programme FP7 under the name of CONCORD. The results of his PhD. project have been described in this thesis and have also been published in scientific journals. He also had the pleasure of presenting his results at several national and international conferences and meetings. During his PhD. he supervised two Bachelor students Diënty Hazenbrink, from Hogeschool Utrecht and Marry Goud from Avans Hogeschool in Breda.

Pawel has enjoyed his academic experiences thus far and looks forward to the next steps in his career.

Publications list

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Establishment of an *in vitro* trans-splicing system in *Trypanosoma brucei* that requires endogenous spliced leader RNA.
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Tulinski P. Molecular bases of antibiotic resistance in MRSA – selected issues.
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