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List of abbreviations

Abbreviation	Meaning
Δ	deletion
X g	Gravitational force
°C	Degree celcius
Mg	Microgram
µL	microliter
ATC	anhydrotetracyclin
Bp	Base pair
CA-MRSA	community-associated MRSA
CC	Clonal cluster
CFU	Colony forming units
ClfA	Clumping factor A
Cm	Chloramphenicol
coA	Coagulase A
CV	Core variable genes
DNA	Desoxyribonucleic acid
dNTPs	Deoxynucleotides
ECDC	European Centre for Disease Prevention and Control
ET	Exfoliative toxin
Egc	enterotoxin gene cluster
HCL	Hydrochloric acid
HGT	Horizontal gene transfer
Hla	α-hemolytic toxin
IEC	Immune evasion cluster
Jep	JSNZ extracellular protease
Kb	Kilo base
Kda	Kilo Dalton
MCS	Multiple cloning site
MGEs	mobile genetic elements
MLST	multiple locus gene sequencing
MRSA	methicillin-resistant *Staphylococcus aureus*
Nacl	Sodium chloride
OD	Optical Density
PCR	Polymerase chain reaction
Rpm	Rounds per minute
S. aureus	*Staphylococcus aureus*
Sag	superantigen
SaPIs	*Staphylococcus aureus* pathogenicity islands
SDS	Sodium dodecyl sulfate
SEIX	superantigen enterotoxin-like toxin X
Spa	*Staphylococcus aureus* protein A
Spls	Serine like proteases
SSSS	*staphylococcal* scalded skin syndrome
ST	Sequence type
TCA	Trichloroacetic acid
TEMED	Tetramethylethylendiamine
TSA	Tryptic Soy Broth-Agar
TSB	Tryptic Soy Broth

WT	Wild type

Gene abbreviation	**Codes for**
agr	accessory gene regulator
chp	chemotaxis inhibitory protein of S. *aureus*
egc	enterotoxin gene cluster
eta	exfoliative toxinA
hla	α-hemolytic toxin
hlb	Beta Hemolysin
IsdA	Iron-regulated surface determinant protein A
lukF PV	Panton-Valentine leucocidin
sak	staphylokinase
sar	*staphylococcal* accessory regulator
SCCmec	*Staphylococcal* cassette chromosome mec
scn	*staphylococcal* complement inhibitor
scpA	thiol protease
sea	enterotoxin A
se	*Staphylococcal* enterotoxin
selX	*Staphylococcal* enterotoxin-like toxin X
sep	*Staphylococcal* enterotoxin P
Spa	*Staphylococcus aureus* protein A
ssl	*Staphylococcus* superantigen-like gene
sspA	*Staphylococcal* serine protease A
tst	Toxic shock syndrome toxin
vwbp	von Willebrand binding protein

Chapter 1: Introduction

1.1. *Staphylococcus aureus* a frequent colonizer and lethal infectious agent

Staphylococcus aureus (S. aureus) infections are a major threat to human health and a strong burden to the health care systems. These bacteria cause a broad range of diseases, from simple skin lesions to serious, life-threatening systemic illnesses. The latter include endovascular infections, pneumonia, septic arthritis, endocarditis with subsequent heart failure or strokes, osteomyelitis, both traumatic and subsequent to bacteremia, foreign body associated infections and sepsis. *S. aureus* is the second most common cause of blood stream, cardiovascular, ear, nose and throat and eye infections [43]. It is also the number one cause of hospital acquired pneumonia [24] and surgical site infections [61],[17].

Acquiring nosocomial *S. aureus* infections results in longer hospital stays for patients, with higher total charges and higher in-hospital mortality rates. The average hospital stay for patients with an *S. aureus* infection is 3 times longer than that of other patients. Such patients also face a nearly 5-fold higher risk of mortality compared with other patients [43].

S. aureus, and more importantly methicillin-resistant *Staphylococcus aureus* (MRSA), are recognized endemic pathogens of hospitals worldwide [9]. Infections associated with health care affects an average of 1 in 20 hospitalized patients a year in Europe. MRSA is isolated in 5% of those cases, which makes it the major resistant bacterium isolated in hospitals in Europe, the Americas, North Africa and the middle and far East [52]. MRSA infections are estimated to affect more than 15000 patients in the EU, burdening EU health care systems with extra costs of 380 million Euros for in-hospital expenses [12].

Despite the large panel of diseases caused be *staphylococcus aureus*, the most common interaction of the bacterium with the human host is colonization [49]. According to the European Centre for Disease Prevention and Control (ECDC) the nasal carriage rate for S. *aureus* among humans is around 30% [12]. A population-

based study from the North-East of Germany recently reported a similar carriage rate of 27% [19]. In fact, the human body provides some major ecological niches for S. *aureus*. The nares, throat and perineum are most commonly colonized, however, colonization of other parts of the skin and the intestine are also common [52], [56]. S. aureus colonization per se usually does not cause any symptoms in healthy, immunocompetent persons. If, however, the colonized person undergoes a major surgery or is in an immunosuppressive state, the delicate balance between S. aureus and its host is disturbed and carriers are at risk of autoinfection [52]. This means that colonized patients act as an endogenous reservoir for serious infections [11]. Most importantly, S. *aureus* bacteremia is three times more common in carriers than non-carriers [56].

Carriage has another implication in health care settings: In the hospital setting colonized medical personnel can spread S. aureus, including MRSA, to hospital patients. Health care workers are often transiently colonized with MRSA, but they can turn into persistent carriers and subsequently be the cause of prolonged MRSA [2]. The prevalence of MRSA colonization is assessed to be 6% among health care workers by several studies in Europe and the United States [10], and the MRSA carriage rate among health care workers in Germany was found to be between 5 and 17% according to hospital based studies [1]. Holtfreter et al., observed a carriage rate in the general population in Northeast Germany of 0.34% [19]. Nasal decolonization of health care personnel, as well as of patients, by topical administration of the antibiotic mupirocin, which also eliminates hand carriage, is recommended to break the cycle of transmission. However, the repercussions of being a persistent MRSA nasal carrier can be severe in countries like Germany, where MRSA-positive health care workers are not allowed to have patient contact [10].

S. aureus has demonstrated enormous capacity to develop antibiotic resistances, that has kept it a step ahead of the efforts exerted by scientists and clinicians to combat it. What adds to the grimness of the health concerns associated with S. aureus and MRSA, is the spread of community-associated MRSA (CA-MRSA), which is epidemic in some countries [9] and the emergence of last resort antibiotic-resistant S. *aureus* strains worldwide [21].

Despite the gravity of the threat S. *aureus* poses and the global efforts exerted to combat it, an efficient antimicrobial treatment is still lacking and no new antibiotic

classes have been introduced over the last three decades [21]. Vaccine research endeavors so far have only met with failure, but they remain of huge relevance to the fight against S. *aureus*. This only adds emphasis on the importance of revealing the host-pathogen interaction, and for that, finding the proper infection model is of utmost importance.

1.2. Genetic composition of *S. aureus*

The success of S. *aureus* as a major human pathogen can be in part attributed to its high genetic variability. The S. *aureus* genome is composed of 2.8 Mb that code for around 2800 proteins [31]. Different S. *aureus* strains can differ in up to 20 % of the genome [20]. Its genome can be divided into three parts: the core genome, the core variable genome and the mobile genetic elements (MGEs) [35].

The core genome represents the biggest (75%) and most preserved part of the S. *aureus* genome. It codes for the house keeping genes and some conserved virulence factors like protein A (*Spa*), α-hemolytic toxin (*Hla*), the superantigen enterotoxin-like toxin X (SEIX) [57] and some exo-enzymes. The core variable genome codes for the surface proteins, regulators of virulence, resistance and iron metabolism genes. It also codes for the superantigen (SAg) genes of the enterotoxin gene cluster (*egc*), which are located on the genomic island SaPI3. The egc includes genes like *SEG*, *SEI*, *SELO* and *SEIN* which are the most prevalent superantigens among *S. aureus* strains and are causative agents of food poisoning. [53], [18], [14].

They are the most prevalent superantigens among *S. aureus* strains and are causative agents of food poisoning and sepsis [19], [18]. The core variable genome composition is strictly linked to S. aureus lineages and hence varies between different lineages.

Lastly, the MGEs comprise S. *aureus* plasmids, pathogenicity islands, bacteriophages, genomic islands, transposons and chromosomal cassettes. MGEs encode genes that can alter the resistance, virulence or the host range of *S. aureus*. The *S. aureus* resistance genes are mainly encoded on the chromosomal cassettes such as the well-known *SCCmec* that carry the *mecA* gene encoding methicillin resistance. In contrast, virulence genes are frequently encoded by bacteriophages and pathogenicity islands. Enterotoxin A and exfoliative toxin A, for example, are

carried by bacteriophages. The pathogenicity islands carry virulence genes including the genes encoding the serine-like proteases (Spls), which are encoded on the stable vSpaß pathogenicity island [44]. Several SAg genes are also encoded by pathogenicity islands, such as the toxic shock syndrome toxin gene *tst* [31],[13].

MGEs can be mobilized and exchanged between S. aureus strains. Bacteriophages play a decisive role, because they can excise and pack *S. aureus* pathogenicity islands (SaPIs) instead of phage DNA and hence enable the horizontal transfer of these elements. [38]. Another source of staphylococcal genetic innovation are the conjugative plasmids, these have the ability to carry transposons that can integrate into the *S. aureus* chromosomal DNA. Plasmids have played a pivotal role in the evolution of antibiotic resistance, which was first reported for the spread of an antibiotic resistance gene from enterococci to *S. aureus*. Plasmids belonging to the pG01/pSK41 have been recently reported to be related to the development of resistance to the linezolid and vancomycin antimicrobials, which are the most important antimicrobials used in MRSA treatment [4], [54].

Several methods are available for analyzing the population structure of the species *S. aureus.* One method is the specific protein A (*spa*) gene typing and grouping the strains according to the differences on the gene sequence. Another method, which still remains the gold standard, is the multiple locus gene sequencing (MLST). In this method, the 7 house-keeping genes are sequenced and the strains are grouped according to the variants of the 7 genes. Unique alleles of the seven loci are given an allelic number. The resulting allelic profile is then used to allocate a sequence type (ST) for each isolate. *S. aureus* isolates having at least 5 identical alleles of the 7 targeted housekeeping genes are classified into the same clonal cluster (CC) [32]. Both methods are highly concordant. In some cases, however, some discrepancies may arise as a result of *spa* locus recombination events.

Apart from sequencing, the virulence gene pattern also provides information about the underlying lineage. Isolates of each CC carry a strictly defined pattern of variants of core variable (CV) genes [31]. Moreover, each CC has a panel of MGE-encoded virulence factors that are characteristic to it, except for some variations found within each lineage. Assessing the CV genome and MGE encoded virulence factors thus provides a set of lineage markers which can be utilized to assign the S. aureus isolates to CCs. Staphylococcal array hybridization kits like the Alere Manual *S. aureus* Genotyping Kit 2.0 provide a microarray with probes for CV and MGE

genes, e.g. genes encoding exotoxins, antibiotic resistance and various enzymes. The overall pattern detected in the microarray allows the allocation of the isolated strain to a certain CC.

1.3. Host specificity and adaptation

The success of *S. aureus* in human infection and colonization is paralleled in livestock, domestic animals and wild animals. However, *S. aureus* acts highly host-specific [30],[59]. Considering MLST data, it is clear that some CCs are predominant in certain hosts [45]. Lineages causing human infections most commonly are the CC30 (at almost 20% of all isolates according to the SHIP-TREND-0 study), followed by CC45, CC15, CC8, CC22, CC7 and CC25. Lineages common in livestock animals include CC97, CC130 and CC151 [22],[59].

The underlying genetic basis for S. *aureus* host specificity remains unclear, but the broad diversity of the human *S. aureus* lineages and the limited number of animal specific lineages imply that *S. aureus* is primarily a human-adapted strain that has gone through several host jumps which were followed by genetic modifications that in turn led to adaptation to the new hosts [16].

Horizontal gene transfer (HGT) plays an important role in the ability of S. *aureus* to adapt to its host, where the principal mechanisms through which bacteria achieve host adaptation include the acquisition (or in some cases loss of MGEs). This means that the bacteria acquire resistance and virulence genes that help it resist the host defense mechanisms and adapt to different conditions. In principle, this process is mediated by transformation, which is the acquisition of free DNA from the environment, bacteriophage transduction, and conjugation, which occurs by the direct contact between cells [35]. In *S. aureus,* transformation does not occur efficiently, this is in contrast to phage mediated transduction that is therefore responsible for most of the staphylococcal HGT [38]. However, there is a general restriction of HGT in *S. aureus* mediated by the different restriction modification systems [41]. Other mechanisms through which the staphylococcal genome evolves and adapts to new hosts include the accumulation of point mutations and the differential gene expression of core and core variable genome genes [36]. All of these mechanisms arm the bacteria with genetic flexibility that holds a continuous

danger of a shift to increased pathogenicity [58].

The best characterized example for human host specificity factors are the immune evasion cluster (IEC) genes: the chemotaxis inhibitory protein of S. *aureus* (*chp)*, the staphylokinase (*sak),* enterotoxin A (*sea*), enterotoxin P (*sep*) and the staphylococcal complement inhibitor (*scn)* that are carried on Sa3int phages and have human-specific immune evasion functions. This phage is present in almost all human-specific S. *aureus* lineages and rarely found in animal lineages [37]. Whole genome sequencing also revealed some animal-specific allelic variants of virulence factors, e.g. SCIN, the product of the chromosomal gene *scn,* inhibits the human complement system but lacks the ability to produce the same effect in other species. However, a pathogenicity island-encoded allelic variant demonstrates a larger target host range enabling complement activation inhibition in animal host also [48]. Moreover, an animal specific allele of the von Willebrand binding protein (*vwbp*) gene has been reported, which possesses a unique N- terminal region, specific for the activation of ruminant and equine prothrombin [55]. Both the *vwbp* and *scn* genes are encoded on the Sa1int phage and provide good proof for host adaptation [37].

1.3.1. *S. aureus* in lab mice

For a long time, mice were thought to be no natural hosts of S. *aureus* [22, 49]. Despite this widely-accepted notion, mice were, and still are, the most commonly used animals for *S. aureus* infection models as they have a well characterized immune system with several knock-out strains. Also, they are relatively easy and inexpensive to breed. But this means that for infection and experimental colonization models, mice are inoculated with high non-physiological doses of human-adapted *S. aureus* strains, thus failing to closely mimic the human staphylococcal infections [5]. Moreover, intranasal colonization is usually only transient [26],[49].

This belief was challenged in 2008 after an outbreak of an *S. aureus* infection in the animal breeding facility of the University of Auckland, New Zealand, causing preputial gland abscesses in C57BL/6J mice. In collaboration with our research group, a novel *S. aureus* strain was isolated in this outbreak and was named JSNZ. Genotyping of the newly isolated strain revealed that it belonged to *spa* type t729 and CC88 which

is uncommon in human and animal isolates [17], [5], [49].

This outbreak prompted our research group to investigate whether laboratory mice are naturally colonized with *S. aureus*. In close collaboration with Charles River, USA, Silva Holtfreter's team analyzed the prevalence of *S. aureus* in specific-pathogen-free laboratory mice, looked for evidence of murine host adaptation and tested whether murine colonization primes the adaptive immune system. This work demonstrated that lab mice are frequently colonized with *S. aureus* and that they efficiently transmit the bacteria to their offspring, leading to persistent colonization. Genetic profiling of a total of 99 *S. aureus* isolates from laboratory mice revealed that most strains belonged to the same CC as JSNZ: CC88. Other lineages that are commonly found in humans (the CC5, 8, 12, 15, 25 and 30) represented collectively only 37.4% of the isolates. Notably, the murine isolates showed features of murine host adaptation. The murine isolates lacked MGEs that encode human-specific virulence factors. For example, only 12.9% (7/54) of murine CC88 strains carried IEC-encoding Sa3int phage compared to 100% (24/24) of the human CC88 isolates. Also 45.8% of the human CC88 isolates were SAg-positive, whereas all the 54 murine CC88 isolates were SAg-negative, and while all the murine CC88 strains were ampicillin sensitive, 16/24 human isolates showed ampicillin resistance. Data also showed that colonized mice mount a significant systemic antibody (IgG) response against a panel of S. aureus proteins like ClfA, IsdA, Hla and many other proteins, some of which are vaccine candidates from current or previous clinical trials. This means that unreported colonization or the uneven priming of laboratory mice can be a significant confounding factor for experimental studies of infection or vaccination of mice [49].

1.3.2. JSNZ

Like the later isolated murine strains, phage typing of JSNZ showed that it lacks the *hlb*-integrating Sa3int phage that codes the human-specific immune evasion factors, but harbors the Sa1int phage.

Comparing the performance of JSNZ with the human derived strain Newman, which is frequently used in mouse *S. aureus* infection models, JSNZ was found to be more virulent in intraperitoneal infection models and to be a better colonizer of mice. In

fact, the strain persisted in SPF C57BL/6J mice breeding colony for 2.5 years. This presented JSNZ as the first mouse-adapted strain identified and studied in detail. Moreover, this strain could easily be genetically modified by phage transduction and electroporation. It presents an excellent opportunity for optimization of the animal *S. aureus* infection models and understanding the host-pathogen interaction in nasal and gastrointestinal colonization [22].

1.3.3. *S. aureus* colonization in small rodents and shrews

For a long time, mice were not considered to be natural hosts of *S. aureus* because *S. aureus* colonization of wild mice had never been reported. Together with Rainer Ulrich (FLI) our research group tested whether small rodents and shrews, including *Apodemus agrarius* (striped field mouse), *Apodemus flavicollis* (yellow-necked mouse), *Apodemus sylvaticus* (wood mouse), *Arvicola scherman* (montane water vole), *Microtus agrestis* (field vole), *Microtus arvalis* (common vole), *Mus musculus* (house mouse), *Myodes glareolus* (bank vole), *Sorex araneus* (common shrew) and *Sorex coronatus* (crowned shrew) are natural hosts of *S. aureus*.
Animals were caught in remote areas (either forest or meadow) in North, Central and South Germany. 34% of the small mammals tested positive for *S. aureus.* Spa typing assigned the strains to 6 lineages: CC49, CC88, CC130, CC1956, Sequence type (ST) 890, ST3033. Similar to *S. aureus* isolates from laboratory mice, most strains lacked the Sa3int phage. One isolate, ST130, was *mecC*-positive which also implies that wild mice could act as reservoir for MRSA. This study was able to prove that wild mice are natural hosts of *S. aureus* and are colonized with unique lineages that are rarely found in the human isolates. Studying the genome of the isolated strains can help shed more light on the factors essential for host adaptation as well as on the evolution of these strains.

1.4. JSNZ extracellular protease (Jep)

Characterization of the mouse-adapted strain JSNZ revealed an unusual exoproteome, where strikingly 1/3 of the total extracellular protein production of JSNZ

was composed of a single 25 KDa protein. This protein was named Jep (JSNZ extracellular protease) (Master thesis, J. Gumz). This information points to the fact that JSNZ is dedicating a huge part of the cellular machinery to the production of a single protein.

Whole genome sequencing of JSNZ revealed that the *jep* gene is located on the Sa1int phage. The Sa1int phage is a Siphoviridae bacteriophage which is organized into six modules: lysogeny, DNA replication, regulation of transcription, packaging and head, tail, and lysis. If a Sa1int phage carries a virulence gene, it would typically lie between the lysogeny module and the DNA metabolism module or downstream of the lysis module. Normally, a functional module in one phage can be replaced in another related phage by another module that has a different sequence but still fulfils the same function [8]. The *jep* gene was found to be located downstream of the lysis module and compared to the PhiETA phage, this is the same location of the exfoliative toxin A (*eta*) which is a serine protease responsible for the pathogenesis of staphylococcal scalded skin syndrome (SSSS) (Figure 1).

Whole genome sequencing also revealed that *jep* bears 45-48% sequence identity to *Spls,* and that the *Spl* catalytic residues (His-74, Asp-113, Ser-189) are also conserved in Jep (Figure 2). These data suggested that Jep is in turn also a serine protease (Master thesis J. Gumz, P. Trübe).

1.4.1. *S. aureus* extracellular proteases

S. aureus extracellular proteases are well-known virulence factors. The ten most important *S. aureus* proteases are: Metalloprotease aureolysin, staphopain A and B (cysteine proteases), 6 serine protease-like proteins (Spls: SplA to F), the V8 protease (serine protease) and the exfoliative toxins (ETA-ETD) which are also serine proteases [27], [50]. The expression of many extracellular proteases is under the control of the global *agr,* which promotes their expression in response to increased cell density in the early stationary growth phase. The staphylococcal accessory regulator (*Sar*) has a negative regulatory effect on them [46].

Several functions are mentioned for the *S. aureus* proteases that help the bacteria modify host proteins to their benefit and regulate the exoprotein activity of the bacteria itself [46]. They aid in host damage and adaptation of the bacteria to its host

through the degradation of immunoglobulins and complement system factors, through the interaction with components of the coagulation and fibrinolysis pathways [60], thus contributing to the ability of *S. aureus* to invade tissues and also to disseminate [23], [27].

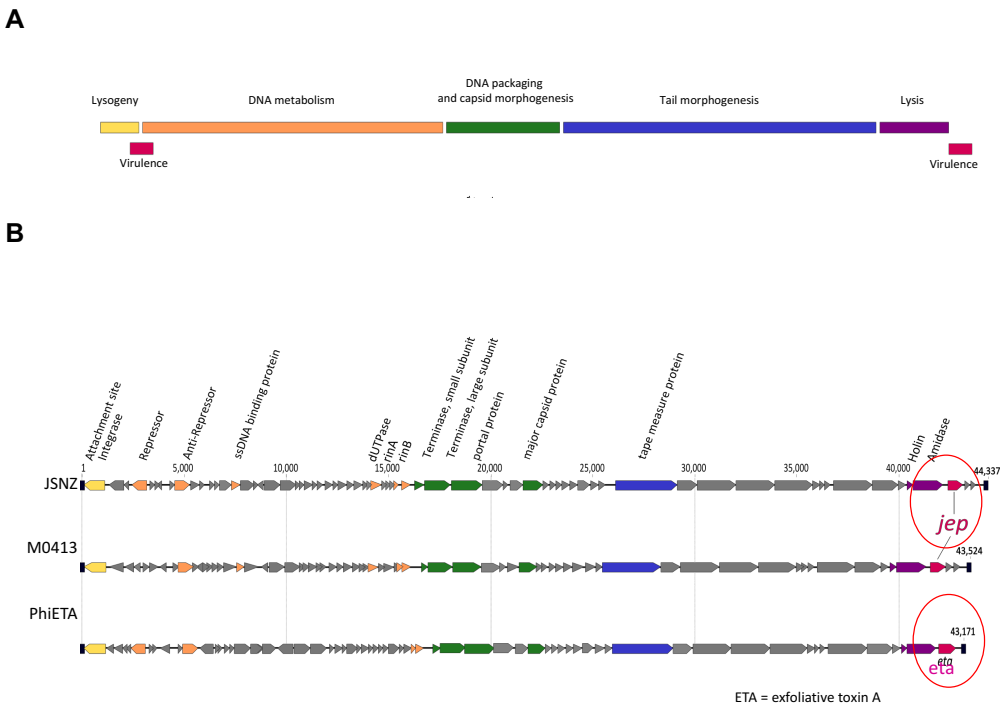


Figure 1: The *jep* gene is located at the C-terminal end of the Sa1int.
(A) General genome organization of the Siphoviridae: The five functional modules of Siphoviridae (modified according to Deghorain et al., [8]) are shown. Yellow: lysogeny, orange: DNA metabolism, green: DNA packaging and capsid morphogenesis, blue: phage tail morphogenesis, purple: lysis. If present, the virulence genes (red) are located either between the lysogeny module and the DNA metabolism module or downstream of the lysis module. (B) Comparison of the Sa1int phage genome of S. aureus JSNZ with the human CC15 isolate M0413 and the phage PhiETA. The jep gene is located on a Sa1int phage and like the eta gene, is located downstream of the lysis module. (Image was kindly provided by P. Trübe).

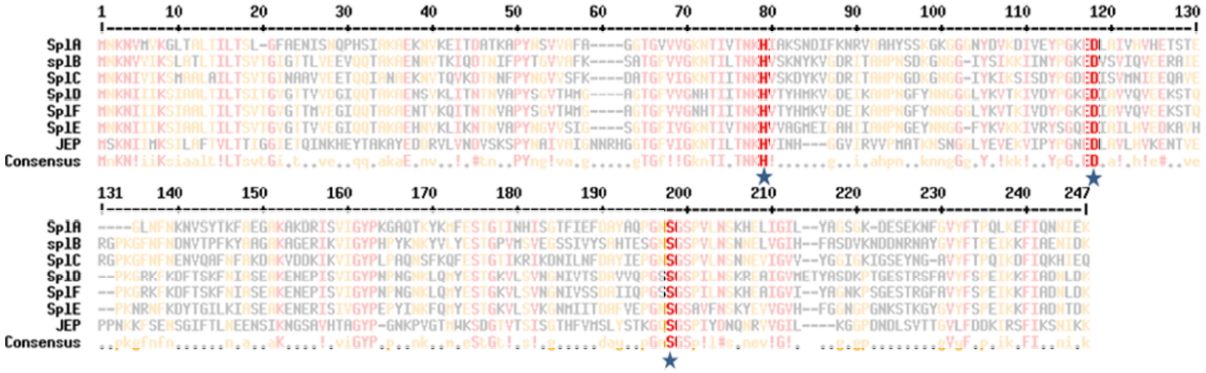


Figure 2: Spl catalytic residues are preserved in *jep*.

The amino acid sequences (AS) of SplA-F (N315) were aligned, together with Jep (JSNZ), using the online tool MultAlin. The AS of SplA-F and JEP were compared. The AS of the catalytic triad (Asterisks) are located in this alignment at position 79 (His), 118 (Asp), and 198 (Ser). red = high agreement; orange = low agreement; black = no match. (Image kindly provided by J. Gumz)

They also help the bacteria modulate its own virulence factors and adhesion molecules, thus mediating the transition from an adhesive to an invasive phenotype [34]. This is achieved through the cleavage of *S. aureus* surface proteins such as fibrinogen binding protein, Spa and ClfB. Proteases are also believed to downregulate the virulence of the bacteria in certain niches to enable a colonization state [50], such as the skin and the nares.

Kolar et al., have shed light on the role of the extracellular proteases in nutrition and virulence by comparing a wild type (WT) strain with a protease-null mutant, in which all extracellular proteases were deleted [27]. According to this study, proteases have a role in the acquisition of nutrients, particularly in a protein-based medium, the resistance against antimicrobial peptides as well as phagocytosis by neutrophils.

The *S. aureus* proteases are frequently linked to host adaptation mechanisms, whether it is the emergence of novel proteases like the thiol protease (ScpA) located on the pAvX 17 kb plasmid and implicated in the pathogenesis of poultry dermatitis [33] and the gain of the novel serine protease (BH1491) involved in epidermin leader peptide processing by ruminant-specific strains [59], or the mutations leading to loss of function of proteases like the SspA involved in blood clot formation in the poultry strain ED98 [33] and the *SplA* pseudogene found in the strain ED133. The mutations leading to genes inactivation were found to be distributed in a strain specific pattern indicating a selective pressure for the function loss [16] and showing that animal-

adapted strains repeatedly inactivate Spls.

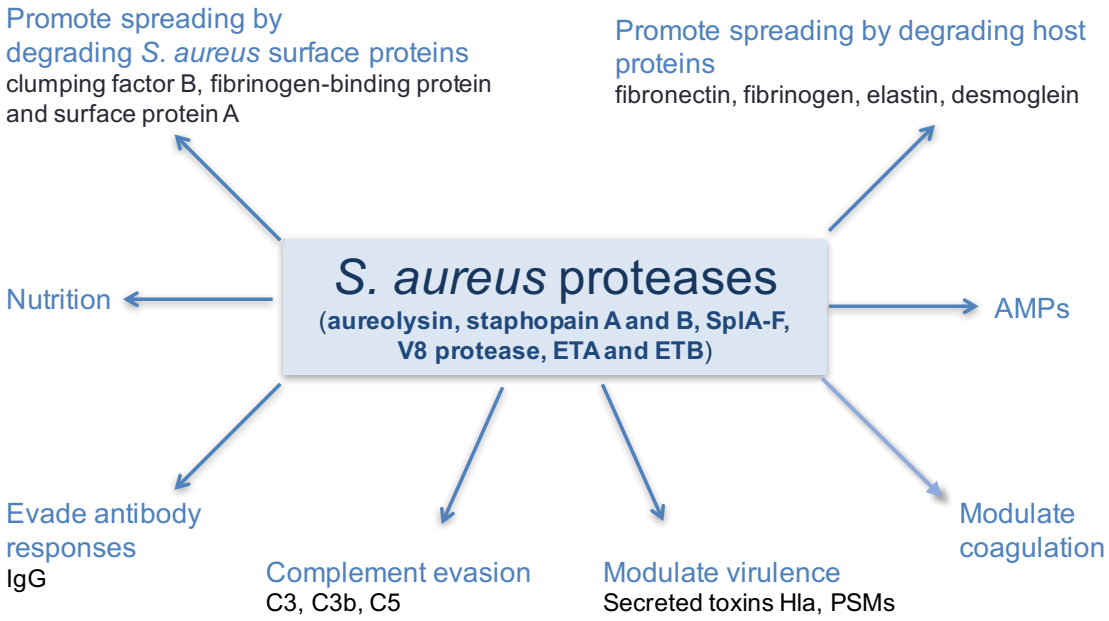


Figure 3: *S. aureus* serine proteases can contribute to nutrient acquisition, bacterial spreading, and immune evasion [28], [25].

Provided data collectively point to the importance of the S. *aureus* extracellular proteases in advancing the staphylococcal virulence and host adaptability and postulated a central role for them in the survival of S. *aureus*, presenting an intriguing research point.

Previous work of our research group has revealed some specific characteristics of the extracellular protease Jep. Examining the exoproteome of JSNZ and comparing it to the human CC88 strains (M3, M25, F25, A7 and A50) has shown the absence of Jep in the human strains. To further investigate that our research group examined more than 490 *S. aureus* isolates of human and murine origins for *jep.* The tested human isolates were negative for *jep* which was only detected in the murine strains. Of the murine strains *jep* was found mainly in lab mouse strains CC88 and CC15 and the wild mouse strains CC49 and CC130.

To reveal those characteristics the growth and survival of the JSNZ WT and the *jep* gene deletion mutant was compared in TSB, milk, serum and whole blood. The long-term growth and survival of *S. aureus* JSNZ WT and JSNS Δ*jep* in TSB was

compared. The JSNZ Δ*jep* grew somewhat slower than the WT and both had a similar OD_{595} after 7 h.

To investigate the role of the putative secreted protease Jep with respect to the nutrients release and the bacterial defense mechanisms against the host immune response, the growth of the wild type and the deletion mutant in serum was tested. JSNZΔ*jep* showed a strongly delayed growth in murine serum. After 24 h, both JSNZ strains reached a similar bacterial density, which on average constituted about 177% the initial concentration.

To validate the initially obtained results the *jep* deletion mutant strain was complemented with the *jep* gene after inducing a single nucleotide mutation of its stop codon. The success of the complementation was tested by examining the exoproteome of the complemented strain, which showed a recovered ability for Jep production. Also, by PCR which showed a *jep* gene band of the right size in the complemented strain and sequencing results of the complements gene that was free of mutations other than the induced mutation of the stop codon. The complemented strain together with the deletion mutant strain were put to the test to compare their growth and survival in Tryptic Soy Broth (TSB), minimal media and whole blood (both human and murine whole blood). Again, the WT JSNZ showed superior growth in TSB and a two-log phase higher survival and multiplication ability in the murine whole and showed no difference growth ability in human whole. However, the discrepancy was also seen for the complemented strain that showed an identical phenotype to the deletion mutant strain rather than the WT. The effect of the three strains on murine plasma coagulation was compared and again the complemented strain showed the same phenotype of the mutant strain with slower plasma coagulation.

To conclude, the ability of the WT JSNZ to withstand the murine serum or the blood defense mechanisms, which at this point denote the involvement of Jep, represent an intriguing host adaptation mechanism.

1.5. Aim of the work

We hypothesize that Jep may be a virulence factor of *S. aureus* and might be playing an important role in the adaptation to the murine host. The preliminary data showed a

difference in the growth and survival ability of the JSNZ WT versus JSNZΔ*jep* in murine serum and whole blood. However, this difference could not be seen during the growth of both strains in TSB and milk. We hypothesize that Jep could be facilitating the growth of the wildtype strain by the cleavage of the components of the immune system. To ensure that the observed effect was mediated by Jep we wanted to complement the deletion mutant with the *jep* gene and once more compare the survival of the three strains in different media like TSB, milk, minimal media, blood and serum. Defining the function of Jep is a prerequisite for the use of the mouse-adapted strain JSNZ in murine models of *S. aureus* infection or colonization and understanding the host pathogen interactions and the underlying dynamics of host adaptation.

Apart from Jep, murine host adaptation could involve the acquisition of other novel mouse-specific virulence factors, the presence of mouse-specific allelic variants, or the loss of human-specific virulence factors. The latter can be easily addressed by analyzing the virulence gene repertoire using the *S. aureus* Genotyping Kit. In this work, we aimed to genotype 25 murine and 13 lineage-matched human isolates using the Alere *S. aureus* genotyping Kit 2.0.

Chapter 2: Materials

2.1. Laboratory equipment

Agarose gel electrophoresis chamber	Thermo Scientific, Waltham (USA); Bio-Rad München
Analysis scale	Sartorius, Göttingen
Autoclave	Tuttnauer Systec, Wettenberg
Bacterial shaker	Grant, Shepreth (United Kingdom)
Centrifuges:	
Biofuge pico	Thermo Scientific, Rockford (USA)
Megafuge 16	Thermo Scientific, Rockford (USA)
Multifuge X3R	Thermo Scientific, Rockford (USA)
Fresco 17 & 21	Thermo Scientific, Rockford (USA)
MICRO 200R	Hettich Zentrifugen, Kirchlengern
VWR Galaxy Mini	VWR, Darmstadt
Digital scale	Sartoruis, Göttingen
Dissection set (Scissors, tweezers)	Aesculap, Tuttlingen
Film welding machine	Severin, Sundern
Fridges and Freezers	Liebherr, Biberach an der Riss; Heraeus Instruments, Hana
Heat block Thermomixer comfort	Eppendorf, Hamburg
Magnetic stirrer MR3001	Heidolph, Schwabach
Microwave	Severin, Sundern;Bosch, München
Mini Protean® Tetra System	Bio-Rad, München
Multichannel pipettes	Eppendorf, Hamburg; Brandt, Wertheim
NanoDrop Spectrophotometer	Thermo Scientific, Rockford (USA)
PCR-Thermocycler T1	Biometra, Göttingen
pH meter	Mettler Toledo, Gießen
Pipettes	Eppendorf, Hamburg; Brandt, Wertheim
Pipetus®	Hirschmann Laborgeräte, Eberstadt

Power PacTM	Basic Bio-Rad, München
Pressure cooker	WMF, Geislingen
Royal Intas ChemoCam Imager	Intas, Göttingen
Scanner CanoScan LiDE 110	Canon, Krefeld
Sterile work bench Safe2020	Heraeus Instruments, Hanau
Shaker KL-2	Edmund Bühler, Hechingen
TECAN infinite M200 Pro	Männedorf (Schweiz)
Ultrasound cleaning bath	VWR, Darmstadt
Vortex	Heidolph, Schwabach; Labdancer VWR, Darmstadt
Water Bath	GFL, Burgwedel

2.2. Materials

Bottle-top filter (0,22 µm)	Merck Millipore, Billerica (USA)
Cell culture plates (96°, flat base)	Sarstedt, Nümbrecht
Centrifuge tubes (15 ml)	Sarstedt, Nümbrecht
Centrifuge tubes (50 ml)	BD Biosciences, Franklin Lakes (USA)
Disposable cuvettes	Roth, Karlsruhe
Electroporation cuvettes	Roth, Karlsruhe
FACS tubes	BD Biosciences, Franklin Lakes (USA)
Inoculating loop	Nerbe Plus, Winsen/Luhe
Nunc™ MicroWell™ 96-Well Microplates	Thermo Scientific, Rockford (USA)
NunclonTM (96°, flat base)	Thermo Scientific, Rockford (USA)
NunclonTM (96°, round base)	Thermo Scientific, Rockford (USA)
Parafilm	Pechiney Plastic Packaging (USA)
PCR reaction tubes (0,2 ml)	Biozym, Hessisch Oldendorf
Petri dishes (10 cm)	Sarstedt, Nümbrecht
Pipette tips	Eppendorf, Hamburg; Sarstedt, Nümbrecht
Reaction tubes (4,5 ml)	Greiner, Frickenhausen
Serological pipettes (5 ml, 10 ml; 25 ml)	Sarstedt, Nümbrecht

Sterile filtre (0,2 µm, Ø 16 mm)	Sarstedt, Nümbrecht
Sterile filtre (0,2 µm, Ø 15 mm)	Roth, Karlsruhe

2.3. Chemicals and Reagents

2.3.1. Protein biochemical work

Acrylamide 4K solution (40 %) Mix 37,5:1	AppliChem, Darmstadt
Agar bacteriological (Agar No. 1)	Oxoid LTC, Cambridge (UK)
Agarose	Invitrogen, Karlsruhe
Ammonium persulphate (APS)	Serva, Heidelberg
Aqua bidest	Life Technologies GmbH, Darmstadt
Bromphenol Blue	Sigma-Aldrich, Steinheim
Coomassie Brillant Blue G250	Merck, Darmstadt
ELISA substrate BD OptEIA	BD Biosciences, Franklin Lakes (USA)
Ethanol (> 99,5 %)	Roth, Karlsruhe
Glycerol (87 %)	Roth, Karlsruhe
HPLC water	J.T. Baker Avantor, Center Valley (USA)
Hydrochloric acid (HCL)	Roth, Karlsruhe
Isopropanol	Merck, Darmstadt
LB Broth	Sigma Alderich, Steinheim
Methanol	Roth, Karlsruhe
PBS	Biochrom, Berlin
Protein Marker VI (10 - 245) prestained	AppliChem, Darmstadt
Sodium chloride (NaCl)	Sigma-Aldrich, Steinheim
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe
Sodium dodecyl sulfate (SDS) 20 %	Roth, Karlsruhe
Sodium hydroxide	Sigma-Aldrich, Steinheim
Tetramethylethylendiamine (TEMED)	Serva, Heidelberg
Thiourea	Sigma-Aldrich, Steinheim (UK)
Trichloroacetic acid	Roth, Karlsruhe
Tris-Base	Sigma-Aldrich, Steinheim

Tris-HCl	Sigma-Aldrich, Steinheim
Tween 20	Sigma-Aldrich, Steinheim
Urea	Sigma-Aldrich, Steinheim
Zink chloride (ZnCl2)	Merck, Darmstadt

2.3.2. Microbiology and molecular biology work

5 × Green Gotaq Flexi Buffer	Promega, Madison (USA)
Ammonium chloride ICN	Biomedicals GmbH, Eschwege
Boric acid	AppliChem, Darmstadt
Calcium chloride	Merck, Darmstadt
Calcium chloride dihydrate	Merck, Darmstadt
Cut Smart buffer	New England Biolabs
DNase/RNase free water	Life Technologies GmbH, Darmstadt
DNA size marker (GeneRulerTM 100 bp)	Thermo Scientific, Waltham (USA)
dNTPs	Thermo Scientific, Waltham (USA)
Enzymes:	
SacI_HF (20000 U/ml)	New England Biolabs (USA)
EcoRI_HF (20000 U/ml)	New England Biolabs (USA)
FastAP thermosensitive alkaline phosphatase (1 U/μl)	
Erythromycin	Roth, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Steinheim Material
GoTaq polymerase	Promega, Madison (USA)
Magnesium chloride (MgCl2)	Promega, Madison (USA)
Phusion High-Fidelity DNA Polymerase	New England Biolabs (USA)
Q5 High-Fidelity DNA Polymerase	New England Biolabs (USA)
RedSafe™ DNA gel stain	Chembio, Medford, (USA)
Tryptone	AppliChem, Darmstadt
Tryptone Soya Broth (TSB)	Oxoid LTC, Cambridge (UK)
Yeast extract	AppliChem, Darmstadt

2.4. Media, buffers and solutions

10 × SDS PAGE buffer	30 g Tris-Base 144 g Glycine 10 g SDS ad 1 l double-distilled water
Coomassie Blue stain	400 ml Methanol 100 ml Acetic acid 2.5 g Comassie blue R-250 500 ml double-distilled water
30 mM Na-Phosphate buffer pH 6.0	438.5 ml 0.2 M NaH_2PO_4 solution 61.5 ml 0.2 M Na_2HPO_4 solution 2.5 l double-distilled water
5 × SDS Loading buffer (not reduced)	0.6 ml Tris/HCl (1 M, pH 6.8) 2 ml SDS (10 % (v/v)) 5 ml Glycerin (50 %) 1.4 ml double-distilled water 1.5 ml Bromophenol blue (1 %)
Rehydrating buffer	4.8 g Urea 1.52 g Thiourea 0.2 g Chaps ad 10 ml HPLC water
Stacking gel	258 µl Acrylamide (40 %) 525 µl Tris 0.5 M, pH 6.8 (0.4 % SDS) 1.3 ml double-distilled water 7.5 µl APS (10 %)

	1.25 µl TEMED Material
SOB medium	20 g peptone 5 g yeast extract 0.5 g Nacl 1 L double-distilled water (autoclave) 10 ml 1 M $Mgcl_2$ (filter sterilized) 10 ml 1 M$MgSO_4$ (filter sterilized)
10 x TBS	24.2 g Tris-Base 80 g Nacl ad 1 l double-distilled water and set to pH 7.6
Transfer buffer	3.025 g Tris-Base 15 g Glycine 200 ml Methanol (100 %) ad 1 l double-distilled water and set to pH 8.5
Separating gel (8 %)	1 ml Acrylamide (40 %) 1. 27 ml Tris 1,5 M pH 8.8 (0.4 % SDS) 2.71 ml double-distilled water 25 µl APS (10 %) 1.25 µl TEMED
Separating gel (12 %)	1.56 ml Acrylamide (40 %) 1.27 ml Tris 1.5 M pH 8.8 (0.4 % SDS) 2.15 ml double-distilled water. 25 µl APS (10 %) 1.25 µl TEMED

Tris/HCl 1.5 M, pH 8.8	182 g Tris base ad 1 l double-distilled water pH 8.8 adjust with HCl
Tris/HCl 1.5 M, pH 6.8 (0.4 % SDS)	182 g Tris base 20 ml SDS (20 %) ad 1 l double-distilled water, set to pH 8.8 with HCl
Tris 0.5 M, pH 6.8 (0.4 % SDS)	65.07 g Tris (121 M) 20 ml SDS (20 %) ad 1 l double-distilled water, set to pH 6.8 with HCl
Agarose gel (1.5 %)	3 g Agarose 200 ml 1 × TBE 7.5 µl Red Safe
LB agar	LB-Medium with 1.3 % (w/v) Agar
LB medium	20.6 g LB broth ad 1 l double-distilled water
TSB agar (TSA)	15 g TSB (Pulver) 7.5 g Agar bacteriological ad 500 ml double-distilled water
TSB medium	ad 500 ml double-distilled water

2.5. Kits

DNeasy Blood and Tissue Kit	Qiagen, Hilden
Fast Link DNA Ligation Kit	Epicentre, Wisconsin (USA)
High Pure Plasmid Isolation Kit	Roche, California (USA)

Monarch PCR and DNA cleanup Kit	New England Biolabs (USA)
Nucleospin Gel and PCR clean-up Kit	Marchery-Nagel GmbH & Co, Düren
Q5 Site-Directed Mutagenesis Kit	New England Biolabs, USA
Rapid DNA Ligation Kit	Fermentas, Massachusetts (USA)
S. aureus Genotyping Kit 2.0	Alere Technologies GmbH, Jena

2.6. Blood and sera

Human blood	healthy donors
Mouse blood	C57BL/6 mice, males

2.7. Bacterial strains

Table 1: Bacterial strains used

Bacterial strain	Spa-Type	MLST-CC	Origin	Genetic modification	Referencce
JSNZ WT	t729	CC88	Mouse abscess	-	Holtfreter et al., 2013
JSNZ∆*jep*	t729	CC88		∆*jep*	n.p.: S. Wiles, University of Auckland
JSNZ∆*jep::jep*	t729	CC88		∆*jep::jep*	This work

n.p. = not published

2.8. PCR primers

Table 2: List of PCR primers used

Primer	Primer Sequence (5'-3')
PA-EcoR1	CTAGGAATTCCGTAAGGGACGGCTATTCAA
PD- Rsac1	CTAGGAGCTCAGTGTAAGGAGGAGCCGTCA
pIMAY MCS F	TACATGTCAAGAATAAACTGCCAAAGC
pIMAY MCS R	AATACCTGTGACGGAAGATCACTTCG
seq 160-F	CGGTAGGTACAATGTGGAAATC

Jep-Mut-F	TATAAAAAAATAAAAGTTTTAAAATAATATGTTTTTAATTG
Jep-Mut-R	TTACTTTTAATAAAACTTCTGATTTTATC
New-POUT-F	GACGCAGCAGGAGAAAATGC
NEW-POUT-R	GCGACTTGTTATGCACCACC
Jep-F	CAGGAGAGGTAGACGAGGCAG
Jep-R	GGCTTTCGGTCTAGGTAGCC

2.9. Software and data bases

Graphpad Prism 5	GraphPad Software, Inc., California (USA)
Graphpad Prism 6	GraphPad Software, Inc., California (USA)
Genome compiler	Genome Compiler Corporation, California (USA)
i-control 1.10 (TECAN)	Tecan Group Ltd., Männedorf (Schweiz)
multalin	http://multalin.toulouse.inra.fr
NCBI PubMed	http://www.ncbi.nlm.nih.gov/pubmed
Zotero	http://www.zotero.org

Chapter 3: Methods

3.1. Sera

3.1.1. Mouse sera

Sera were obtained from male C57BL/6 mice selected due to surplus animal production. Blood was provided by Dr. rer. nat. Grazyna Domanska. The mice were anaesthetized with ketamine / xylazine solution (100 mg/kg body weight ketamine, 10 mg/kg body weight xylazine). A 20 µLhaematocrit capillary tube was used for posterior orbital venous sinus bleeding. Blood was collected in 1.3 ml heparinized reaction vessels and subsequently used for *S. aureus* cultivation in whole blood (see 3.3.4. Survival in murine and human whole blood).

3.1.2. Human sera

Human sera were obtained from healthy adult human volunteers. All participants gave written informed consent in accordance with the Declaration of Helsinki, and the study was approved by the ethics board of the Medical Faculty of the University of Greifswald (BB 014/14; 24.01.2014).

3.2. Complementation of JSNZ∆*jep*

Testing the role of the *jep* gene in host adaptation and its role in survival and growth fitness of the mouse adapted strain JSNZ has been investigated by comparing different phenotypic features of JSNZ WT and the *jep* gene deletion mutant. However, to be able to reliably attribute any phenotypic discrepancies to the absence of Jep, the mutant strain was chromosomally complemented with the *jep* gene to exclude the possibility that any unspecific changes happened during the creation of the knockout strain.

3.2.1. Plasmid isolation

The pIMAY plasmid was isolated from DH10B *E. coli*. DH10B *E. coli* were stored in glycerol stocks. An inoculating loop was used to scrape a drop off the glycerol stock, which was inoculated on TSA+ 5 µg/ml Chloramphenicol (Cm) agar using streak dilution technique. The plates were incubated at 37°C overnight. A single colony was picked with a pipette tip and transferred into 10 ml LB/Cm medium. The culture was then incubated at 37°C overnight in the shaker (200 rpm, circular). For every culture a sterility control was made. Erlenmeyer flasks were always filled to one fifth with medium. The pIMAY plasmids were then isolated from the overnight cultures using the Roche "High Pure Plasmid Isolation Kit":
0.5 ml of the overnight cultures was centrifuged for 30 s at 6000 g. The supernatant was discarded and the pellet was re-suspended in 250 µL suspension buffer/RNase then 250 µL of the lysis buffer were added and mixed gently. The mixture was incubated at room temperature for 5 minutes. Then 350 µL of chilled binding buffer were added and again mixed gently and incubated for another 5 minutes on ice. After 5 minutes the mixture was centrifuged for 10 minutes at 13 000 rpm. This time the pellet was discarded and the supernatant was transferred to the high pure filter tube (provided with the kit) and centrifuged at 13 000 rpm for 30 s. The flow through was discarded and 500 µL of the wash buffer I were added and centrifuged at 13 000 g for 1 min. The flow through was discarded and 700 µL of wash buffer II were added and centrifuged at 13 000 g for 1 min and then another minute after discarding the flow through. After discarding the flow through once more, 100 µL of the elution buffer were added to the membrane of the filter tube and centrifuged at 13 000 g for 1 min. The flow through was collected and the concentration of the isolated plasma was measured by the Nanodrop spectrophotometer and found to be 491ng / µL.

3.2.2. *Jep* gene amplification

The *jep* gene of the JSNZ WT strain was amplified using *jep* gene flanking primers (PA-EcoR1 and PD- Rsac1). The primers were designed to have 30 bp homologous region to the pIMAY vector down- & up-stream of the restriction sites (Primer sequences are displayed in table 2, pages 27 and 28).

The PCR reaction was prepared as follows:

23.5	µL	PCR water
10	µL	Phusion buffer
1	µL	Template*
10	µL	dNTPs
2.5	µL	5'-Primer (10 µM)
2.5	µL	3'-Primer (10 µM)
0.5	µL	Phusion Polymerase
50	µL	total

*Template DNA = JSNZ WT isolated DNA (concentration: 89.6 ng/µL)

The cycler conditions were set as follows:

Table 3: Cycling conditions for the amplification of the JSNZ WT *jep* gene

Step	Temperature [°C]	Time (sec)	Cycles
Initial Denaturation	98	60	1
Denaturation	98	20	30
Annealing	60	30	
Elongation	72	90	
Final Extension	72	300	1
Hold	4	∞	

The PCR products were then examined by means of agarose gel electrophoresis. In this method, DNA fragments are separated by the application of an electrical voltage according to their size. 1.5% gels were used. Agarose was dissolved in 1 × TBE buffer by boiling. Once the solution cooled, RedSafe, which binds to nucleic acid, was added and the gel was poured. 10 µL of the PCR product or marker (GeneRuler™ 100 bp plus) were applied and separated at 120 V for about 45 minutes. Subsequently, the gel was read in the VWR GenoPlex. The PCR product was then purified using the Monarch PCR & DNA Clean-up Kit. The PCR product was first diluted with the DNA Clean-up Binding Buffer at a ratio of 200 µL Binding Buffer to 100 µL DNA sample. The Buffer DNA solution was mixed well by pipetting up and down. The mixture was added to the column and centrifuged at 16 000 g for 1 min at room temperature. The flow through was discarded with the collecting tube and the column was inserted into a new collection tube. 200 µL of the DNA wash buffer were added and centrifuged again for 1 min. The wash step was repeated one more time. The column was then transferred into a clean 1.5 ml micro-centrifuge tube and 10 µL of the DNA elution buffer were added to the membrane of the column and

after 1 min incubation was centrifuged for 1 min. DNA concentration was measured using the Nanodrop spectrophotometer and for the amplified *jep* gene was found to be 392 ng/µL.

3.2.3. Plasmid vector linearization and dephosphorylation

The restriction enzymes EcorI and SacI were used to restrict the pIMAY plasmid vector, where a mixture was prepared as follows:

10 µL	plasmid DNA
2 µL	10 x Cut Smart buffer
1 µL	Fast Digest restriction enzyme EcorI
1 µL	Fast Digest restriction enzyme SacI
1 µL	FastAP alkaline phosphatase
5 µL	Water
20 µL	total volume

the reaction mixture was mixed thoroughly and incubated at 37°C for 60 min. The reaction was stopped by heating at 65°C for 20 min.

3.2.4. Insert restriction

The amplified and isolated *jep* gene with the overhangs was restricted using the same enzymes. The Cut Smart buffer was diluted at a ratio of 1:10. The diluted Cut Smart buffer was then used to dilute the restriction enzymes 1:10 to reach a final concentration of 2 units/µL. The following reaction mixture was prepared and incubated as described above (3.1.3):

7.75 µL	insert DNA
2 µL	10 x Cut Smart buffer
1 µL	Fast Digest restriction enzyme EcorI
1 µL	Fast Digest restriction enzyme Sac
8.25 µL	Water
20 µL	total volume

3.2.5. Ligation of the plasmid vector and the insert

Using the Fast Link DNA Ligation Kit the ligation of the plasmid and vector was carried out according to the protocol. The molar concentrations and dilutions of the

plasmid and insert were:

Plasmid = 491 ng/µL= 131.73 nM (5.743 Kbp) x 0.1 = 131.73 nM

Insert = 392 ng/ µL= 402.67 nM (1.53 Kbp) x 0.127 = 51.13 nM

The Insert:plasmid ratio was 3.88:1

1.5 µL	10 x Fast link buffer
1.5 µL	10mM ATP
0.7 µL	vector DNA
9.3 µL	insert DNA
1 µL	Fast Link Ligase
15 µL	total volume

The reaction was incubated at room temperature for 5 min, then for 15 min in the heat block at 70°C. The mixture was then centrifuged at 11 000 rpm for 30 seconds and placed on ice for transformation.

3.2.6. Transformation of Competent DC10B *E. coli*

First competent DC10B cells were prepared according to the following protocol:
SOB medium and CCMB80 (provided by Johannes Dick) were prepared. Overnight cultures from DC10B *E. coli* were prepared and incubated at 37°C. The overnight cultures were diluted 1:100 in SOB medium. The bacteria were left to grow at 28°C to OD_{595} 0.2. The cells were pelleted by centrifugation at 4°C and 4000 rpm for 10 min in 50 ml falcon tubes. The supernatant was discarded and the cells were re-suspended in ice cold CCMB80 buffer, where 16 ml buffer were added per 50 ml culture. The bacterial suspension was incubated on ice for 20 min. The cells were once more pelleted, buffer removed and re-suspended in 2 ml ice cold CCMB80 per 50 ml of original culture. The suspension was incubated on ice for another 20 min. The now competent bacteria were aliquoted into 50 µL volumes and stored at -80°C.
50 µL vials of competent cells were thawed on ice. 1 µL and 5 µL of the ligation reaction were pipetted directly into separate 50 µL vials of competent cells and tapped gently. The vials were incubated on ice for 30 minutes. Afterwards the vials were placed in 42°C water bath for exactly 30 seconds. The vials were removed and placed on ice. 250 µL of pre-warmed SOC medium were added to each vial. The vials were placed in a micro-centrifuge rack on its side, secured with tape, in a shaking incubator for 1 hour at 37°C and 225 rpm. 20 and 200 µL from each

transformation vial were spread on separate LB/Cm agar plates, Cm was added to the agar at a concentration of 15 µg/ml to select for bacteria successfully transformed with the pIMAY plasmid which renders the transformed cells Cm resistant. The plates were incubated overnight at 37°C.

3.2.6.1. Colony PCR to confirm transformation

Colonies were picked from the overnight incubated plates to perform colony PCR in order to identify the successful transformation of plasmids containing the desired insert. A single colony was picked with a sterile inoculation loop and diluted in 10 µL PCR water. The same loop was used to streak a new LB agar plate. The suspension of bacteria in PCR water was placed in the heat block at 95°C for 5 minutes to kill the bacteria. Colony PCR was performed using primers that bind just outside the multiple cloning site (MCS) of the pIMAY plasmid (Primers: MCS-F and MCS-R). the reaction mixture was prepared as follows:

17.75 µL	PCR water
10 µL	Gotaq Green buffer
10 µL	dNTPs
5 µL	$Mgcl_2$
5 µL	Template
1 µL	5'-Primer (10 µM)
1 µL	3'-Primer (10 µM)
0.25 µL	Gotaq Polymerase
50 µL	total volume

The incubation conditions were set to:

Table 4: Cycling conditions for colony PCR for the amplification of the jep gene within the pIMAY plasmid

Step	Temperature [°C]	Time (sec)	Cycles
Initial Denaturation	94	300	1
Denaturation	94	30	
Annealing	52	30	30
Elongation	72	120	
Final Extension	72	420	1
Hold	4	∞	

The PCR products were then examined by means of agarose gel electrophoresis. Colonies showing the right sized band were cultured overnight in LB medium to which Cm was added at the same concentration of 15 µg/ml. the cultures were

incubated overnight in the shaking incubator at 37°C and 225 rpm. From the overnight cultured cells, plasmids were isolated using the Roche High Pure Plasmid Isolation Kit. The desired region containing the *jep* gene was again amplified (see above) and sequenced by chain termination sequencing (Sanger sequencing) using the MCS forward and reverse primers and the Seq160-F primer by the company GATC. The resulting sequences were aligned using Genome Compiler and multAlin software.

3.2.7. Mutation of the *jep* gene stop codon

The aim of this step was to induce a single base pair mutation of the *jep* gene stop codon to mark the complemented strain. Q5 site directed mutagenesis kit was used. Primers were designed using the NEBase changer (Jep-Mut-F and Jep-Mut-R).
The first step was the Exponential Amplification step, where the mutagenic primers induced substitution of the last nucleotide of the stop codon of the *jep* gene inserted in the plasmid vector (changing it from TAG to TAA sequence). The following reaction mixture was prepared:

12.5 µL	Q5 Hot Start High-Fidelity 2x Master Mix
1.25 µL	10 µM Forward Primer
1.25 µL	10 µM Reverse Primer
1 µL	Template DNA*
9 µL	PCR water
25 µL	total volume

*Template DNA= the isolated pIMAY plasmid containing the *jep* gene insert (concentration = 39 µl)

The cycler conditions were set as follows:

Table 5: Cycling conditions for the Q5 Site-Directed Mutagenesis reaction

Step	Temperature [°C]	Time (sec)	Cycles
Initial Denaturation	98	30	1
Denaturation	98	10	
Annealing	56	30	35
Elongation	72	215	
Final Extension	72	120	1
Hold	4	∞	

The following step was the KLD reaction which involved incubation with an enzyme

mix containing a kinase, a ligase and DpnI. Together, these enzymes allow for rapid circularization of the PCR product and removal of the template DNA. PCR product 1 µL, 2 x KLD buffer 5 µL, 10 x KLD Enzyme mix 1 µL and PCR water 3 µL were incubated at room temperature for 5 minutes.

The last step was the transformation of the pIMAY plasmid containing the modified *jep* gene (jep_A) into competent DC10B competent cells according to the previous protocol.

3.2.8. Electroporation of JSNZ∆*jep*

Plasmids were isolated from liquid culture of the transformed DC10B *E. coli*. Both, the plasmid containing the *jep* gene with the stop codon mutation (pIMAY jep_A) and the unmodified *jep* gene were ethanol precipitated, where 30 µL Na-acetate and 900 µL100% EtOH were added to 300 µL plasmid eluent. The mixture was placed at -80°C for one hour, after which the mixture was centrifuged at 13 000 rpm for 30 minutes at 4°C. The supernatant after the centrifugation was carefully pipetted out followed by washing with 800 µL70% Ethanol, repeating the centrifugation conditions. The pellet was left to dry for 15 minutes then re-suspended in 19 µL water. The final concentration of the acquired DNA was measured and found out to be 326 and 348 ng/ µL for the pIMAY *jep* and the pIMAY jep_A respectively.

Electroporation-competent JSNZ∆*jep S. aureus* were prepared (protocol: see above 3.3.6). For the electroporation step 50 µL vials of electro-competent JSNZ∆*jep S. aureus* were prepared They were placed into cold electroporation cuvettes, to which 5 µL of the plasmid suspension was added. The GenePulser was set to 100 Ω, 2500V, 25uF, 0.1 cm cuvette. Immediately 0.5M sucrose in TSB was added into the cuvette, mixed and transferred into yellow cap containers. The cells were incubated at 28°C for 2 hours. 100 µL of the mixture was plated on TSB with 5 µg/ml Cm (low concentration culture). The rest of the cells were spinned down, the supernatant was discarded and the cells re-suspended in 200 µL TSB and streaked on TSB/Cm (high concentration culture). The plates were incubated for 48 hours at 28°C.

After 48 hours 6 colonies were picked of the low and high concentration culture plates, diluted in 10 µL PCR water, re-streaked on TSA/Cm and incubated for another 48 hours at 28°C. At this point clones were screened with the MCS primers to confirm the presence of the replicating pIMAY.

To induce integration of the plasmid into the chromosomal DNA a single colony off the 28°C plate was vortexed in 200 µL of TSB and was diluted to the 10^{-3}. All dilutions were then plated on TSA/Cm and incubated at 37°C overnight. Large colonies were picked off the plates, re-streaked on TSA/Cm plates and re-incubated under the same conditions.

For the confirmation of the integration of the plasmid, colony PCR was repeated (MCS-F & R primers) only this time awaiting negative results for positive integration. The reaction mixture was prepared as follows:

10.3 µL	PCR water
5 µL	Gotaq Green buffer
5 µL	dNTPs
2.5 µL	$Mgcl_2$
1 µL	Template
0.5 µL	5'-Primer (10 µM)
0.5 µL	3'-Primer (10 µM)
0.2 µL	Gotaq Polymerase
25 µL	total volume

The incubator conditions were set as follows:

Table 6: Cycling conditions for colony PCR for the detection of the absence of the replicating pIMAY plasmid

Step	Temperature [°C]	Time (sec)	Cycles
Initial Denaturation	95	300	1
Denaturation	95	30	
Annealing	50	30	35
Elongation	72	240	
Final Extension	72	300	1
Hold	4	∞	

A PCR was done to determine the side of the integration of the plasmid backbone related to the *jep* gene. The reaction included a genomic primer and a *jep* specific primer (Primers: N-POUT forward and reverse and Jep forward and reverse). The annealing temperature of the two reactions was set to 68°C, other than that the reaction mixture and the cycler conditions were the same as those of the colony PCR (see above). For the first reaction (of primers: N-POUT-F and Jep-R) the expected product size for a positive integration was 1884 bp, while a negative insert product size was 1146 bp. For the second reaction (Primers: N-POUT-R and Jep-F) the expected positive insert size was 1852 bp and the negative product was 1114 bp. All PCR products were resolved by electrophoresis in 1.5 % agarose gels (1x TBE buffer), stained with RedSafe^{TM} and visualized under UV light in the VWR genoplex.

Single colonies from each side of integration (as determined by PCR results) were inoculated into 10 ml TSB and incubated overnight in the shaking incubator at 28°C and 200 rpm. The overnight cultures were then used to make serial dilutions. Dilutions 10^{-3}, 10^{-4} and 10^{-5} were streaked on TSA containing 1 µg/ml anhydrotetracycline (ATc) and incubated at 28°C for 48h. This step mediates the selection of the strains where the pIMAY plasmid expulsion has occurred because bacterial growth at 28°C allows pIMAY replication and induction of the secY antisense RNA which prevents the growth of strains that retain the plasmids and selects for strains that lost them.

To select for the colonies showing the correct phenotype, large colonies were picked, each diluted in 10 µL PCR water and then used to streak both TSA/Cm and TSA/ATc plates. All plates were incubated overnight at 37°C. The right phenotype for proper excision of the vector backbone was: growth on ATc and no growth on Cm, while persistence of the plasmid would have been indicated by growth on Cm and lack of growth on ATc.

Strains showing the desired phenotype were cultured in 4 ml TSB for 5h at 37°C and 225 rpm. The cultures were used for DNA isolation using the Qiagen DNeasy Blood and Tissue Kit where using 1 ml of the bacterial culture, the cells were pelleted by centrifugation (5000 G, 10 min). The supernatant was discarded. The bacterial pellet was re-suspended with 190 µL of lysis buffer and 20 µL of lysostaphin (both added directly to the pellet), mixed by pipetting and incubated for 60 min at 37°C. 200 µL of AL buffer and 25 µL of proteinase K were added and mixed by vortexing. The mixture was incubated in the heat block for 30 min at 56°C. 200 µL of ethanol (96%) were

then added and homogenized by vortexing. The mixture was incubated at room temperature for 5 min, then was pipetted into the DNeasy mini spin column placed in a 2-ml collection tube and was centrifuged at 8000 rpm for 1 min. The flow-through was discarded. 500 µL of AW1 washing buffer was added and centrifuged. The flow-through was again discarded. 500 µL of AW2 washing buffer was added and centrifuged at 13000 rpm for three minutes this time. The flow-through was once more discarded. To dry the membrane the membrane was centrifuged for 1 min at 13000 rpm. The spin column was placed in a clean 1.5 ml micro-centrifuge tube and 100 µL of DNase free water (pre-warmed to 72°C) were pipetted directly onto the DNeasy membrane. It was incubated for 5 min at 72°C and centrifuged for 1 min at 8000 rpm.

3.2.9. Confirmation of complementation

3.2.9.1. PCR of the *jep* gene

The isolated DNA was used to perform a PCR to confirm the persistence and integration of the *jep* gene after excision of the pIMAY plasmid vector.

The *jep* region flanking primers were used (N-POUT-F and N-POUT-R). The expected product size for positive results was 2436 bp, while that for a negative result was 1698 bp. The reaction mixture was prepared:

12.5 µL	Q5 High-Fidelity 2x Master Mix
1.25 µL	10 µM Forward Primer
1.25 µL	10 µM Reverse Primer
1 µL	Template DNA
9 µL	PCR water
25 µL	total volume

The cycler conditions were set as follows:

Table 7: Thermo-cycling conditions for PCR for insert detection

Step	Temperature [°C]	Time (sec)	Cycles
Initial Denaturation	98	30	1
Denaturation	98	10	
Annealing	68	20	30
Elongation	72	60	
Final Extension	72	120	1
Hold	4	∞	

products were resolved by electrophoresis in 1.5 % agarose gels (1x TBE buffer), stained with RedSafe™ and visualized under UV light in the VWR genoplex.

The PCR product was isolated using the Monarch DNA and PCR Cleanup Kit according to the manufacturer's protocol.

3.2.9.2. Sequencing

The isolated DNA was sent for sequencing. The DNA was sequenced by chain termination sequencing (Sanger sequencing) with the help of the *jep* region flanking forward and reverse primers (N-POUT-F & R), and the *jep* gene specific primers (Jep-F & R) by the company GATC.
The resulting sequences were aligned using Genome Compiler and multAlin software.
Glycerol stocks were prepared of the strains showing the right pheno- and genotypes.

3.2.9.3. Extracellular proteins electrophoresis

This step was performed to confirm the recovered ability of the complemented strain to produce Jep. For the precipitation of extracellular proteins, the overnight liquid cultures (see 3.3.2. Culture in liquid medium) were first centrifuged for 15 minutes at 8,000 × g and 4°C, and the supernatant was subsequently filter sterilized. Then ice-cold, 100% (v/v) tri-chloro-acetic acid was slowly added on ice while stirring to a ratio of 1:10 (final concentration: 10%). The proteins were precipitated overnight at 4°C and afterwards pelleted by centrifugation at 13 000 rpm and 4°C for 10 minutes. The supernatant was discarded and the pellet was washed twice with 100% (v/v) Ethanol. The supernatant was removed and the pellet was dried under the bench for 120 minutes. The dry pellet was re-suspended in 20 µL1 x SDS loading buffer. 5 µL of 5x SDS loading Buffer were also added. The suspension was heated for 5 minutes at 95°C and centrifuged afterwards to precipitate residual protein aggregates. 10 µL of each sample were loaded onto 12% SDS gels for protein electrophoresis.
By means of the SDS-PAGE proteins are separated based on their molecular weight in an electrical field. A discontinuous SDS-PAGE with 12% separating gel (see 2.4. Media, Buffers and Solutions) was prepared. To separate the protein mixtures in the SDS gel, they were first mixed with 5 × SDS loading buffer and denatured at 95°C for five minutes. 10 µL of sample or 5 µL of protein marker (protein marker VI pre-

stained) were applied and separated at 150 V until the bromophenol blue band of the loading buffer had run out of the gel.

3.3. Growth kinetics in different media

To observe the possible differences induced by Jep on growth and survival, the growth of *S. aureus* strains JSNZ WT, JSNZΔ*jep* and JSNZΔ*jep::jep* was investigated in various media, i.e. Tryptic soy broth medium (TSB) which contains no proteins, only short peptides, mostly 6 to 16 amino acids. In Iscove's Modified Dulbecco's Medium (IMDM). IMDM does not contain iron, thus depriving the bacteria from the iron induced stimulatory effect on growth. Also, in human and murine whole blood. The growth was analysed over a period of 24 h.

3.3.1. Culture on solid medium

All bacterial strains were stored in glycerol stocks. An inoculating loop was used to scrape a drop off the glycerol stock, which was inoculated on TSA agar plates using streak dilution technique. The plates were incubated at 37°C overnight.

3.3.2. Culture in liquid medium

To prepare a pre-culture, a single colony was picked with a pipette tip and transferred into 10 ml TSB. The culture was then incubated at 37°C overnight in the shaker (200 rpm, circular). For every culture a sterility control was made. The volume ratio of culture medium to Erlenmeyer flasks was maintained at 1:5 ratio.

3.3.3. Growth in TSB and IMDM

3.3.3.1. Overnight cultures

A single colony was picked for each of the JSNZ WT, JSNZ∆*jep* and JSNZ∆*jep::jep* and inoculated into 3 ml TSB in 15 ml falcons. A sterility control was also prepared and all cultures were incubated over night at 37°C overnight in the shaker (200 rpm, circular).

3.3.3.2. Washing cultures

To compare the growth of the three strains; the JSNZ WT, the deletion mutant JSNZ∆*jep* and the complemented strain JSNZ∆*jep::jep* it was first necessary to free the cultures for the growth curve cultivation from their secreted proteases and toxins, as well as from nutrients of the TSB. For this purpose, overnight cultures of the three strains were washed with sterile PBS. The overnight cultures were centrifuged (10 min, 4000 × g). The supernatant was then discarded and the pellet re-suspended in 1 mL of sterile PBS. The 50-mL tube was then filled to 30 mL with PBS and centrifuged again (10 min, 4000 × g). The supernatant was again discarded and the pellet re-suspended in 1 mL of sterile PBS and filled up to 10 ml.

3.3.3.3. Synchronization of several cultures

Also for appropriately comparing the growth performance of the three strains in TSB and IMDM, the cultures should be in the same growth phase (late logarithmic phase, OD_{595} = 0.5-1). Therefore, they were synchronized where 10 ml of TSB were inoculated to a uniform OD_{595} = 0.01 (corresponds to approximately 0.5 × 10^7 CFU / mL) for the new cultures. First the OD_{595} was measured for washed overnight cultures and then the inoculation volume was calculated for 10 ml TSB.

$$\frac{0.01}{OD595} x\ 10\ 000 = \ inoculation\ volume\ in\ \mu L$$

After cultures synchronization, they were incubated for 24 hours at 37°C. The growth of the three strains in TSB and IMDM was monitored by measuring the optical density (OD_{595}) every 30 minutes for 24 hours.

The exponential growth rate μ was calculated from measurement times t0 and t (here these were: t_0 = 0.5 h, t = 1 h) and the corresponding OD_{595} values x_0 and x. The doubling time td was then determined based on the ratio of the natural logarithm of 2 (ln2) and μ. It represents the time when the bacterial mass doubled. The growth rate and doubling time formulas were as follows:

$$\mu = \frac{In\ x - In\ x_0}{t - t_0}\ [min^{-1}] \qquad t_d = \frac{In\ 2}{\mu}\ [min]$$

3.3.4. Survival in murine and human whole blood

For the survival of the bacteria in whole blood the bacterial suspensions, overnight TSB cultures, were washed (see 3.3.3.1. Washing cultures). The OD was adjusted to OD_{595} 0.05 in 1.5 ml PBS (corresponds to approximately 2.5 × 10^7 CFU / mL).

$$\frac{0.05}{OD595} x\ 1500 = \ inoculation\ volume\ in\ \mu L$$

The culture was diluted 1:25 in PBS (50 µL bacterial suspension + 950 µL PBS) to reach a starting concentration for the cultures equal to 1 × 10^6 CFU / ml. A volume of 37.5 µL of the bacterial suspension was then added to 112.5 µL of human or murine heparinized whole blood in a 96 well plate (bringing the concentration to 2.5 × 10^5 CFU / ml). All the remaining plate wells were filled with PBS and the whole plate was covered with Parafilm to prevent evaporation of the cultures. Cultures were incubated in the shaker (200 rpm, circular) at 37°C for 24 hours.

The growth pattern in human and murine blood was investigated by plating serial dilutions (up to 10^{-8}) of the cultures on TSA plates at 0, 1, 3, 5 and 24 hours and determining the colony forming units (CFUs).

3.3.4.1. Determining the CFU/ ml

For the cultures in human and murine blood, the growth pattern of the three strains was determined by plating serial dilutions of the cultures at 0, 1, 3, 5 and 24 hours and counting the CFU. At the indicated time points 10 µL samples were removed and diluted 1:10 with sterile PBS. The samples were treated in the ultrasonic water bath 3 min to dissolve aggregates. The samples were then diluted serially with a multi-channel pipette in a 96-well plate 1:10 to a dilution of 10-8. From each dilution, 20 µL were in each case plated in triplicates. The TSA plates were incubated overnight at 37 ° C in the incubator. As a negative control, PBS only was added to blood (murine and human blood) and plated at each time point. The sterility of the media was checked by plating out the 1:10 dilution on agar. The CFU was determined by considering all three replicates of a dilution with analysable colony count, but at least ten CFUs.

3.4. Coagulation assay

To examine the possible influence of Jep on murine plasma coagulation, the JSNZ wild type, deletion mutant and the complemented strain were incubated with murine plasma and the coagulation was visually assessed based on a previously set scale. The coagulation was compared to that of the complemented strain to ensure that any effect detected could be attributed to Jep production. The *S. aureus* Newman strain was used as a positive control and sterile murine plasma as a negative control.

65 µL overnight cultures of the four strains were inoculated into 500 µL commercial murine plasma placed in a glass tube and incubated at 37°C in water bath for 24 hours. The degree of plasma coagulation was assessed after 30 minutes, 1h, 2h, 3h, 4h, 20h and 24h for the four strains and the control as previously reported (Schulz et al and Sperber et al). The degree of plasma coagulation was assessed visually, so the experiment was blinded to prevent bias where all the tubes labelling was covered. The different degrees of coagulation were marked with a score, where score 0 meant no coagulation, 1 a small clot, 2 a large clot, 3 a larger clot with a remaining amount of non-clotted plasma, 4 coagulation of the whole plasma and 5 a hard clot with possible tube inversion (see figure 1). The experiment was repeated 4

times.

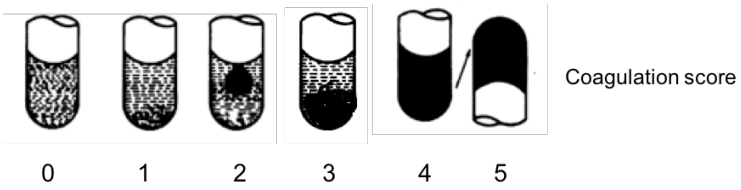


Figure 4: Scoring system of the coagulation assay.
The degree of coagulation was assessed in visually in a blind fashion. The coagulum seen was given a score from 0 to 5. 1 was given on seeing a small clot, 2 for a larger clot not reaching the bottom of the tube, 3 for a clot that includes all the tube bottom with a remaining amount of non-clotted plasma, 4 for complete clotting of plasma without possibility of tube inversion and 5 with a clot hard enough not falling upon tube inversion.

3.5. Testing Jep production at different growth phases

At this point, there were one of two explanations to the failure of the complemented strain to revert to the phenotype of the JSNZ WT, the first being the accidental creation of a mutation in the JSNZ genome during the creation of the jep gene mutation that affected JSNZ in a non Jep related manner. The second possibility was that Jep was not being produced by the complemented strain in a similar fashion or in equal amounts to the production of the original strain and this would be explained by a mutation that was also accidentally produced, but this time during the complementation process and affecting a jep regulatory gene.

To try and find out the actual cause of the unexpected finding we tested the Jep production during the different phases of a bacterial growth cycle by two complemented strains (one of them not having the stop codon mutation) and compare them to JSNZ WT, also including the *jep* deletion mutant in the experiment.

Overnight liquid cultures of the four bacterial strains were prepared in 50 ml falcons by inoculating into 10 ml TSB at 37°C and 200 rpm. The overnight cultures were then centrifuged for 10 min at 4000 g and the supernatant was discarded, pipetting the rest out. The pellets were re-suspended in 10 ml TSB and centrifuged again for 10 min at 4000 g and the supernatant was discarded. The pellet was again re-

suspended in 10 ml TSB and the OD_{595} was determined for each culture at a dilution of 1:10. The OD_{595} of all cultures was readjusted to 0.05 in 80 ml TSB:

$$\frac{0.05}{OD595} x\ 80 = \ inoculation\ volume\ in\ \text{ml}$$

The calculated amount was added to 80 ml TSB after removing corresponding volume of TSB in 250 ml flasks.

After 2.5 h the OD_{595} was again measured (was around 0.05) and was then re-adjusted to 0.05 in 600 ml TSB liquid cultures. Once more equal volume of TSB was removed before adding the bacterial suspension in 1 l flasks. The cultures were incubated in a shaking water bath at 37°C. A bacterial growth curve was plotted for the four chains by measuring the OD_{595} Every 30 min. 30 ml of the cultures were removed for protein precipitation at time points: 1.5, 2.5, 3.5, 5 and 7 h. Each of the obtained 30 ml were centrifuged for 10 min at 8000 g and 4°C. the supernatant was filtered through 0.45 µl filter into new 50 ml falcons. The new falcons with the supernatant were placed on ice and TCA (tri-chloro acetic acid) was added drop by drop while vortexing the falcons on low speed. The falcons containing the bacterial supernatant and the TCA were placed overnight at 4°C to precipitate the proteins. The next day the falcons were centrifuged for 1 h at 8000 g and 4°C, after which the supernatant was discarded. The pellet was re-suspended in 1.5 ml 70% Ethanol and the suspension was transferred into Eppendorfs. They were then centrifuged for 10 min at 16000 g and 4°C discarding the supernatant after the centrifugation. The pellet was washed 6 times with 500 µl 96% ethanol, where the pellets were repeatedly re-suspended in and centrifuged for 10 min at 13 000 rpm and 4°C. After the last washing step, the supernatant was discarded and 500 µl of 96% ethanol were added to the pellet without re-suspension. It was once more centrifuged, after which the supernatant was pipetted out completely and the pellet was left to dry for 60 to 90 min under the bench. When the pellet dried, it was re-suspended in rehydrating buffer. The amount of rehydrating buffer (see 2.4. Media, Buffers and Solutions) added was calculated according to the OD_{595} recorded at the time of obtaining the 30-ml bacterial suspension, with a starting volume calculated to the strain showing the lowest OD and between 40 and 50 µl according to the size of the pellet. After the rehydrating buffer was added the suspension was sonicated for 6 min and then put in the mixer at 23°C, 14000rpm for 10 min to dissolve the pellet. Then the suspension

was centrifuged at 16000 g and 23°C for another 10 min. The supernatant was obtained and added to a new Eppendorf. 10 µL of samples were added to 2.5 µL of 5x SDS loading Buffer. The suspension was heated for 5 minutes at 95°C and centrifuged afterwards to precipitate residual protein aggregates. 10 µL of each sample were loaded onto 12% SDS gels for protein electrophoresis.
By means of the SDS-PAGE proteins are separated based on their molecular weight in an electrical field. A discontinuous SDS-PAGE with 12% separating gel (see 2.4. Media, Buffers and Solutions) was prepared. To separate the protein mixtures in the SDS gel, they were first mixed with 5 × SDS loading buffer and denatured at 95°C for five minutes. 10 µL of sample or 5 µL of protein marker (protein marker VI pre-stained) were applied and separated at 150 V until the bromophenol blue band of the loading buffer had run out of the gel.

3.6. Genetic profiling of murine and matched human *S. aureus* isolates

To shed more light on the colonization and infection of wild mice by S. *aureus*, the most common strains and their adaptation mechanisms to their host, *S. aureus* isolates from wild mice were examined by DNA microarray. The *S. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany) is an array hybridization kit for DNA-based detection of resistance genes and pathogenicity markers of *S. aureus* and assignment of unknown *S. aureus* isolates to known strains. It is based on multiplex linear DNA amplification and hybridization of the biotin-labelled amplicons to probes on the microarray. The hybridization is then visualized by a streptavidin- horse radish-peroxidase-catalyzed dye precipitation. The Genotyping Kit 2.0 covers 333 target sequences, corresponding to 170 distinct *S. aureus* genes and their allelic variants, including species markers, resistance genes, exotoxins, adhesins, surface proteins, capsular proteins and *agr* group typing markers.
To perform the array on the isolated strains from small rodents and shrews the Alere Kit was used as follows: all the isolated strains were stored in glycerol stocks, an inoculating loop was used to scrape a drop off the glycerol stock. The recovered

bacteria ware inoculated on Colombia blood agar plates using streak dilution technique. The plates were incubated at 37°C overnight.

A single colony for each strain was picked by a pipet tip and cultured in LB medium for 5h. Genomic DNA isolation followed by the DNeasy Blood and Tissue Kit (Qiagen) according to the protocol (see 3.2.8. Electroporation of JSNZΔ*jep*).

Following successful DNA extraction (concentration A_{260} not less than 0.1 µg/ µL), a mixture was prepared for linear amplification and internal Biotin labelling:

49 µL of $B1^{ST2}$ labelling reagent (version 2.0) + 0.1 µL of B2 labelling enzyme per sample.

5 µL DNA was added to a 5 µL aliquot of the Master Mix. All vials were marked clearly for each isolate.

Amplification in the thermocycler was performed according to the following protocol:

Table 8: Incubation conditions for linear amplification and internal Biotin labelling

The lid was pre-heated to 105°C	
300 sec at 96°C	
55 cycles with:	60 sec at 96°C
	20 sec at 50°C
	40 sec at 72°C
Cool down to 4°C, hold	

The amount of Array Strips needed were removed from the pouches and inserted into the white frames (provided with the Kit). The strips were pre-washed in two steps: first with PCR-grade distilled water, 200 µL per well at 50°C, 5 min and 550 rom in the thermos-shaker. Second with C1 Hybridization buffer, 200 µL per well at 50°C, 5 min and 550 rpm. 90 µL of buffer C1 was added to each tube with 10 µL labelled amplification product and were mixed gently. The buffer was removed from the array and the mixture of C1 and labelled amplification product were added. The strips with the mixture was incubated at 50°C, 60 min and 550 rpm. The liquid was then removed and the strips washed with 200 µLC2 washing buffer, which was pipetted up and down four times, removed and discarded. Another 200 µL of the C2 buffer was added and incubated at 30°C, 10 min and 550 rpm. The conjugate was prepared by adding 1 µL conjugate 100 x HRP to 99 µLC4 conjugation buffer (ex.: for 16-20 wells: 21 µLC3 buffer + 2079 µLC4 buffer). The washing buffer was discarded

after incubation and 100 µL of the diluted conjugated was added to each well, was incubated at 30°C, 10 min and 550 rpm. The liquid was removed and the wells were washed with 200 µLC5 washing buffer, which was pipetted up and down four times, removed and discarded. Another 200 µL of the C5 washing buffer were added and incubated for 2 min at 30°C and 550 rpm. The washing buffer was after incubation removed and discarded and 100 µL of D1 substrate (precipitating dye) was added and incubated without shaking for 6 min at 25°C. The liquid was removed completely and the outside of the bottom of the array strips was cautiously cleaned with wipes. Lastly the strips the strips were scanned and processed in the ArrayMate.

Chapter 4: Results

4.1. Generation of the complemented strain by homologous recombination into the chromosome

Previous data showed that JSNZ WT and JSNZ∆*jep* differed strongly in growth and survival in murine serum. This suggests that Jep could be involved in adaptation to the murine host. However, to validate that the detected outcome, and subsequent assays results, could in fact be attributed to the effect induced by Jep, the deletion mutant was complemented with the *jep* gene by homologous chromosomal recombination. This step was done with the intention to exclude any other mutations which might have accidentally happened and would be causing the detected phenotype.

4.1.1. Generation of the plasmid construct

The *jep* deletion mutant was chromosomally complemented with the *jep* gene using the pIMAY system. Firstly, the *jep* gene was amplified by PCR from the wild type JSNZ using primers that are complementary to the *jep* gene flanking region, and designed to have 30 bp homologous region to the pIMAY vector down- and up-stream the restriction sites. Next, the PCR product was purified and ligated into the linearized and dephosphorylated pIMAY vector using the restriction enzymes EcoRI and SacI. Afterwards, the resulting pIMAY-*jep* plasmids were transformed into transformation-competent DC10B *E. coli.* DC10B *E. coli* are genetically modified DH10B strains in which the modification allows the plasmid DNA isolated from them to be transformed into *S. aureus* with high efficiency. Successful transformation was confirmed by PCR using primers that bind the MCS of the pIMAY plasmids. Sequencing of the *jep* region with MCS primers confirmed the correct integration of the *jep* gene into the plasmid and the lack of any unexpected mutations within the ORF (Figure 5).

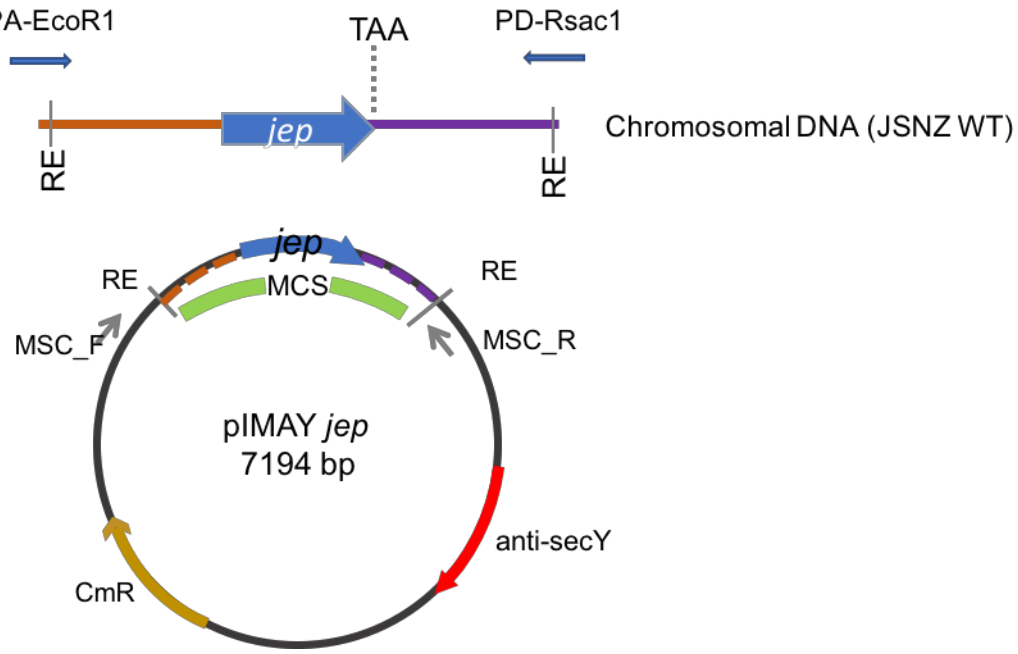


Figure 5: pIMAY *jep* plasmid construct

The *jep* gene of JSNZ WT was amplified using *jep* gene flanking primers PA-EcoR1 and PD- Rsac1. The primers were designed to have 30 bp homologous region to the pIMAY vector down- and up-stream of the restriction sites for direct cloning into pIMAY and transformation into *E. coli* DC10B. Clones were screened with primers external to the MSC (MSC primers).

4.1.2. Induction of a mutation in the *jep* gene stop codon

A single nucleotide exchange in the stop codon of the *jep* gene was induced to mark the complemented strain using the Q5 site directed mutagenesis kit. In a first step, mutagenic primers induced a single nucleotide substitution in the stop codon, changing its sequence from TAG to TAA. According to manufacturer's protocol, the following Kinase, Lipase and DpnI (KLD) reaction induced the circularization of the PCR product and the removal of the template DNA (the pIMAY *jep*) (Figure 6).
Competent DC10B cells were again transformed with the plasmids containing the modified *jep* gene (which was designated jep_A gene). The plasmids were isolated from liquid cultures of the transformed DC10B cells, and sequenced to confirm the presence of the mutation (Figure 7).

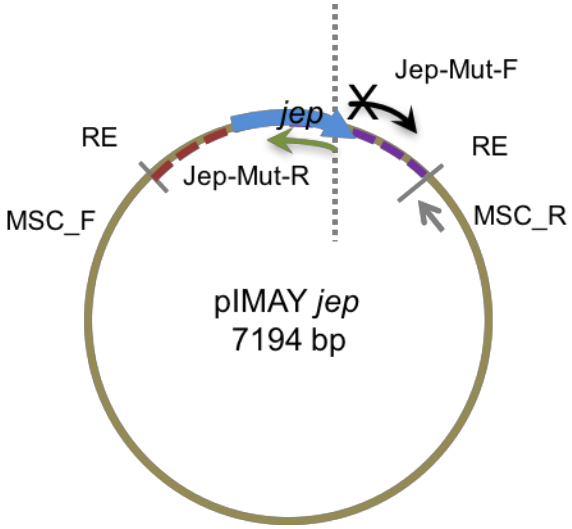
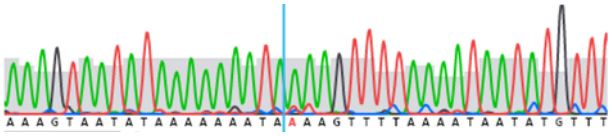


Figure 6: Single base pair substitution in the *jep* gene stop codon was induced with the help of Q5 site directed mutagenesis kit.

Mutagenic primers were designed using the NEbase changer. The mutagenic primers were designed to have the desired nucleotide to be changed in the center of the forward primer with at least 10 complementary nucleotides on the 3' end of the mutation. The reverse primer was designed so that the 5' ends of the two primers anneal back-to-back.



Isolated *jep* gene with modified stop codon (jep_A)

JSNZ WT *jep* gene sequence

Figure 7: Sequencing of the pIMAY jep_A after inducing the mutation of the last nucleotide of the stop codon showing the mutation at the desired site.

Sequencing of the plasmid revealed the desired mutation of the stop codon (at position 3915) that changed the stop codon from TAG to TAA. The mutated nucleotides are shown in red and indicated by the blue line.

4.1.3. Transformation of *S. aureus*

The isolated pIMAY jep_A plasmids, together with plasmids containing an unmodified *jep* gene, were concentrated by ethanol precipitation and electroporated into electro-competent JSNZΔ*jep*. The transformed cells were plated on TSA with

chloramphenicol to select for cells containing the plasmid, as the pIMAY plasmid confers chloramphenicol resistance. Clones were screened with the MCS primers confirming the presence of replicating pIMAY plasmids. Plasmid integration into the bacterial chromosome was induced by growth at 37°C in presence of chloramphenicol. Integration of the plasmid backbone into the bacterial chromosome could be at either side of the jep_A gene according to whether the recombination occurred through the upstream or the downstream region of *jep* (Figure 8).

The integration of the plasmid backbone together with either the jep_A gene or the non-modified *jep* gene into the bacterial chromosome was confirmed by screening several colonies by PCR using one primer that binds in the bacterial chromosome adjacent to the *jep* deletion and one primer that binds to the plasmid-encoded *jep* gene. PCR results confirmed the chromosomal integration of several colonies at both sides of integration. PCR reaction using the N-POUT-F and Jep-R primers revealed integration of the plasmid and insert into the chromosomal DNA through the upstream region of the *jep* gene producing a band of 1884 bp. Upon failure of integration on this side a band 1146 bp was detected. PCR reaction with N-POUT-R and Jep-F primers detected integration through the downstream region of the *jep* gene, positive integration was indicated by an 1852 bp band and negative by a 1114 bp one. Clone 1 and 2 had the non-modified *jep* gene, while clones 3 and 4 had the jep_A gene.

In only one colony derived from clone 1 we observed an integration at the upstream region, as reflected by the larger 1884 bp fragment using the upstream integration primers and the smaller 1114bp fragment using the downstream integration primers (Figure 9A and B). All other colonies showed an unspecific band at 3000 bp.

Clone 2 isolates all showed the band of the right size (upstream integration) paralleled by negative bands in figure B. Except for colony 2.3 the correct band size was detected also for colonies 3.1, 3.2, 4.3 and 4.4. As seen in figure B, Colony 2.3 showed a positive insert of 1853 bp meaning it was positive for downstream integration (to the right of the jep gene), as was also seen for colonies 3.3 and 4.1. (Figure 9).

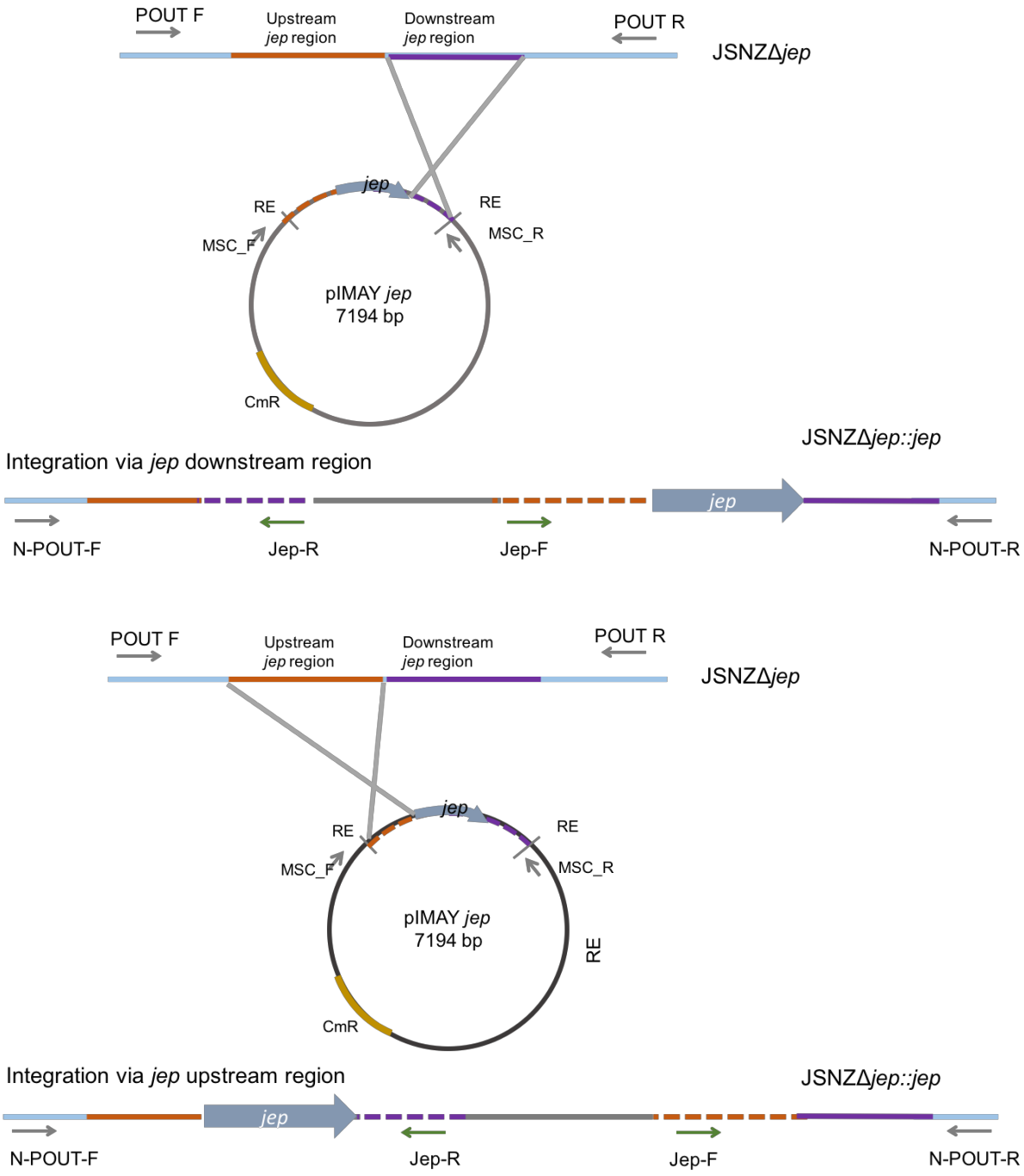
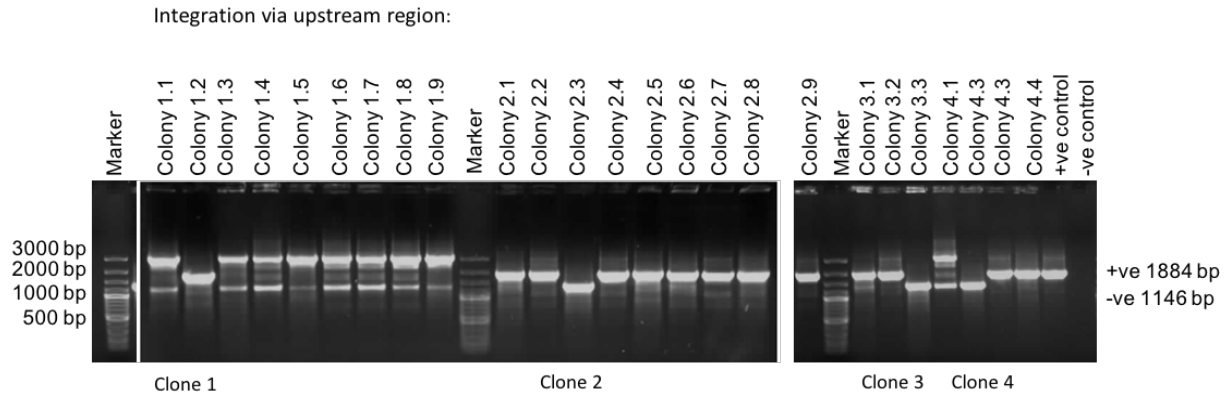


Figure 8: Side of integration of the pIMAY backbone into the JSNZΔ*jep* chromosome

Homologous recombination results in integration of the jep gene and the pIMAY plasmid into the deletion mutant chromosome at the site of the deleted *jep* gene, between its up- and down-stream regions. Integrations was defined as upstream or downstream relative to the side of integration of the plasmid backbone relative to the *jep* gene.

A



B

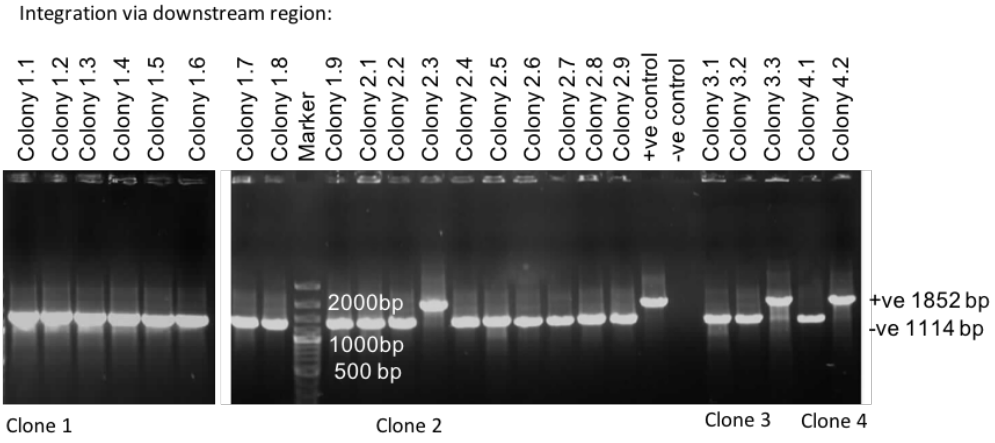


Figure 9: PCR showing side of integration of pIMAY plasmid backbone in the bacterial chromosome relative to the position of the *jep* gene.

In this PCR reaction, we were able to differentiate between integration that occurred through the upstream and downstream regions of the *jep* gene. (A) Using the N-POUT-F and Jep-R primers, integration through the upstream region was reflected by a band of 1884 bp. A lack of integration at the upstream region was reflected by a band of 1146 bp. (B) Primers N-POUT-R and Jep-F were used to detect the integration through the downstream region of the *jep* gene. Positive integration produced a band of 1852 bp and no integration a band of 1114 bp.

The next step was the plasmid excision by homologous recombination. A single colony from each side of integration was allowed to grow in broth at 28°C, which is a temperature condition permissive for rolling cycle replication (RCA), without antibiotic

selection. The cultures were plated on TSA with anhydro-tetracyclin (ATc) since Tetracycline induces the expression of *secY* antisense RNA, thus inhibiting the growth of cells maintaining the plasmids and selects for the plasmid free cells. Homologous recombination generates two outcomes at a 1:1 ratio: A) the (unwanted) excision of the plasmid via the original side of integration, thus excluding the original plasmid including the *jep* gene. B) excision via the opposite homology region, thus excising the plasmid backbone with the region of the knockout gene leaving a chromosomally integrated *jep* gene within the bacterial chromosome (Figure 10).
Identifying the colonies which had retained the plasmids, whether in the cytosol or in the bacterial chromosome, from the ones that had achieved the correct plasmid excision at this point was done by diluting each colony in 10 µl PCR water and streaking the suspension on both TSA + Cm and TSA + ATc. The right phenotype, which is growth on ATc and no growth on Cm, was displayed by several of the selected colonies. DNA was isolated from all strains showing the correct phenotype.

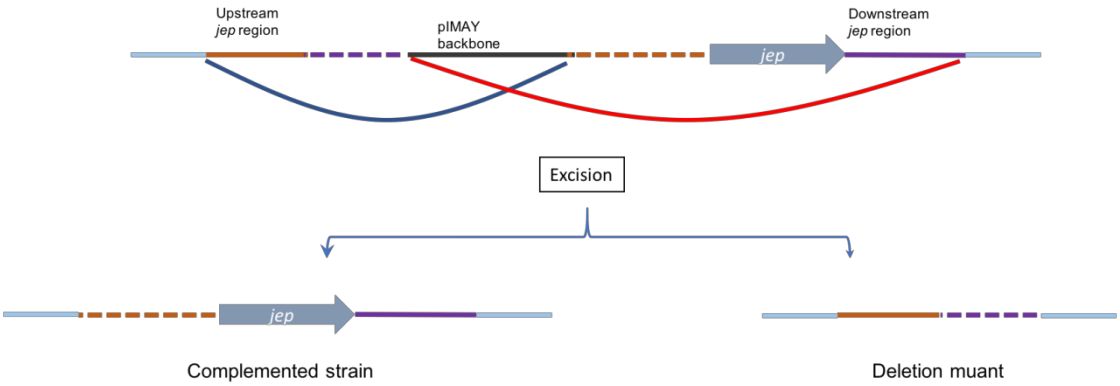


Figure 10: Homologous recombination has two possible outcomes, either the excision of the plasmid backbone leaving a chromosomally integrated jep_A gene or the excision of the plasmid alongside the jep_A gene with failure of complementation.

4.2. Confirmation of the successful complementation

4.2.1. Confirmation of integration of the *jep* gene by PCR

The putatively successfully complemented colonies were tested by PCR using the *jep* flanking primers N-POUT-F and N-POUT-R. As expected, the complemented

strains showed a larger PCR product than the deletion mutant strain (2436 bp versus 1698 bp) due to the presence of the complemented jep_A gene with a length of 738 bp (Figure 11).

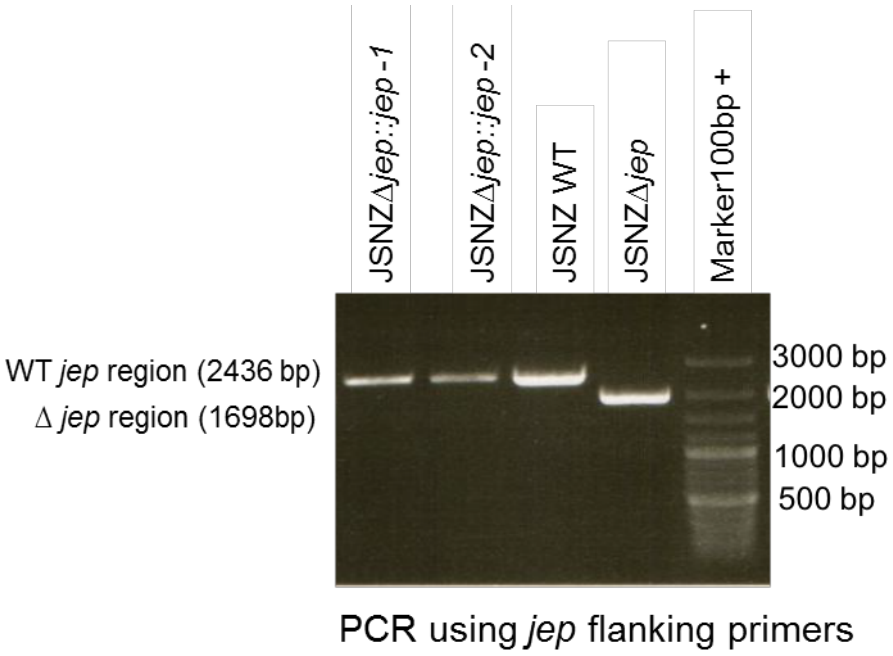


Figure 11: PCR confirmed the integration of the *jep* gene using *jep* flanking primers.

The jep_A gene region was amplified by using *jep* flanking primers. The *jep* gene region of the JSNZ WT strain was 2436 bp long, while the deletion mutant generated the expected 1698bp fragment. The correct band size was detected for the JSNZΔ*jep::jep*1 and 2 (clones 3 and 4).

4.2.2. Sequencing results confirmed successful complementation

A second confirmation of the proper chromosomal integration of the *jep* gene was obtained through Sanger sequencing. The obtained sequences were aligned to the WT *jep* gene and showed the desired sequence, also revealing the single nucleotide substitution of the stop codon (TAG → TAA). Moreover, the gene was present in the correct genomic location. Two point mutations were also detected outside the open reading frame up and downstream of the *jep* gene (Figure 12).

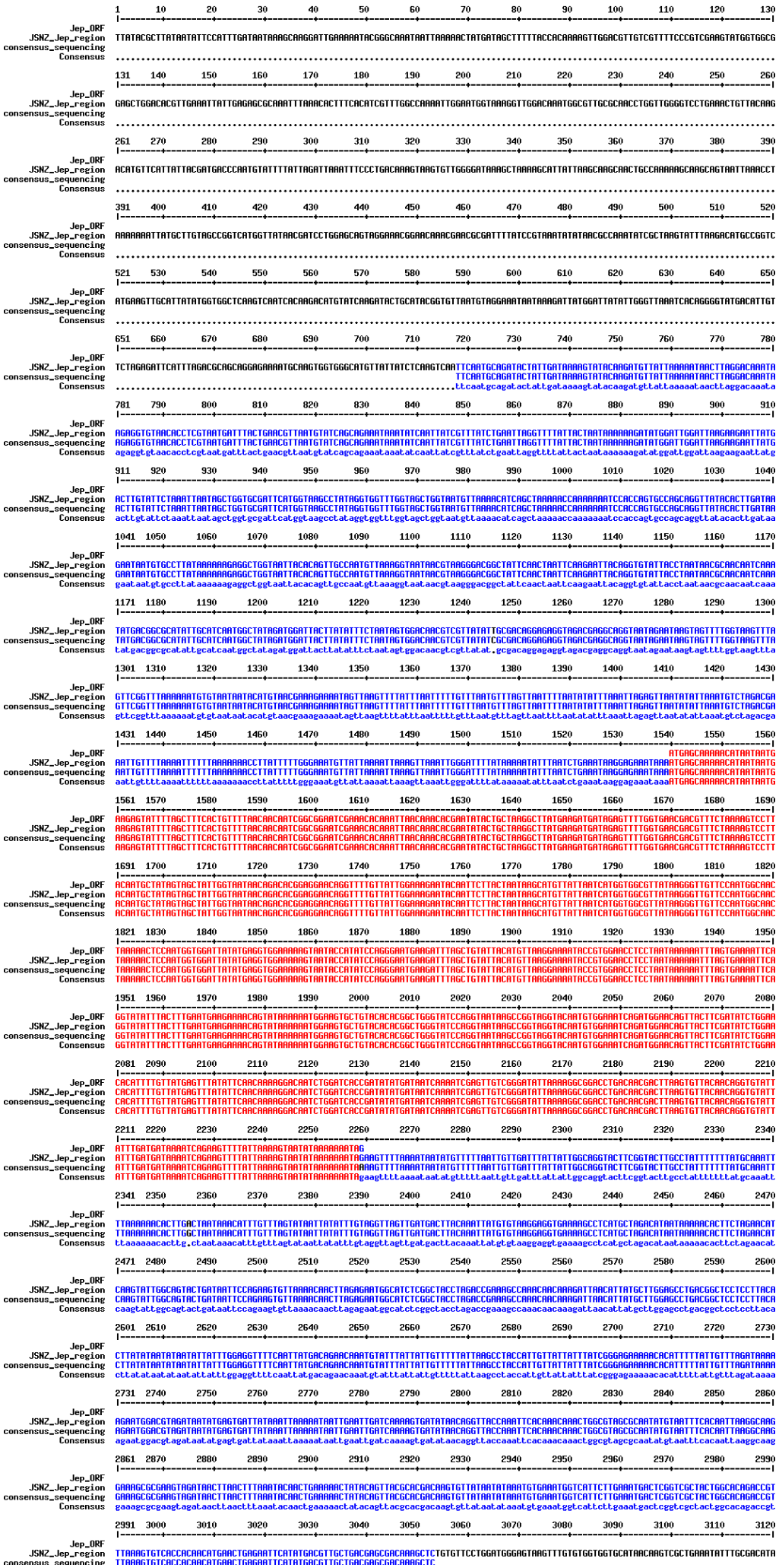

Jep_ORF
JSNZ_Jep_region
consensus_sequencing
Consensus

Figure 12: Sequencing of the *jep* region revealed correct integration of the *jep* gene
PCR products of the colonies that showed the right band size indicating successful complementation were purified and sequenced. Sequencing results showed the complementation of the *jep* gene, with the induced single base mutation of the stop codon, that changed from TAG sequence to TAA at position 2259. Two other mutations could be detected at positions 1245, 2355 outside the open reading frame of the *jep* gene.

4.2.3. Extracellular protein electrophoresis demonstrated recovered ability of the complemented strain to produce Jep

The extracellular proteomes of the JSNZ WT, the JSNZ*Δjep* and the JSNZ*Δjep::jep* were compared by SDS PAGE to test whether the complementation that was confirmed at the genomic level could also be corroborated on the protein level. The SDS gel showed a prominent 25 KDa band, which likely corresponds to Jep, in the extracellular proteins of both JSNZ WT and the complemented strain, while it was absent in the deletion mutant (Figure 13). This suggested that the strain complemented with the jep_A gene was secreting Jep in roughly the same amount produced by the WT strain.
In summary, data on both the gene and protein levels showed that the *jep* deletion mutant was successfully complemented with an intact jep_A gene by chromosomal integration. Hence, we moved on with our experiments and used the complemented strain with the jep_A gene as a control for the analysis of the role of Jep in immune evasion and host adaptation.

4.3. Characterization of the complemented strain JSNZ*Δjep::jep*.

4.3.1. Growth in TSB revealed differences between the WT and the genetically modified strains

TSB is tryptic-digested casein and soybean meal and therefore contains no proteins, but only short peptides, mostly 6 to 16 amino acids. As a first experiment, we wanted to assess the impact of Jep on bacterial viability and the ability of JSNZ to extract

nutrients by cleaving peptides into smaller fragments for growth and nutrition. JSNZ WT, JSNZΔ*jep* and JSNZΔ*jep*::*jep* were cultured in a 96-well plate in the complex medium TSB over a period of 24 h and optical densities were automatically determined every 30 minutes. The growth pattern of the strains showed a typical growth curve for this experimental setting with a lag phase of around 3 h, a log phase of about 1 hour and a subsequent stationary phase.

Figure 14 shows that the deletion mutant and the complemented strains grew slower than the WT, and also reached a lower final OD (Figure 14, table 9). This was also reflected in the doubling time of the three strains, which was roughly eight minutes longer for the mutant and the complemented strains (table 9). This difference was statistically significant for the mutant strain ($p<0.05$) but not for the complemented strain, because for this strain only 2 values for the doubling time were considered. Nevertheless, the difference is clearly visible on the growth curve.

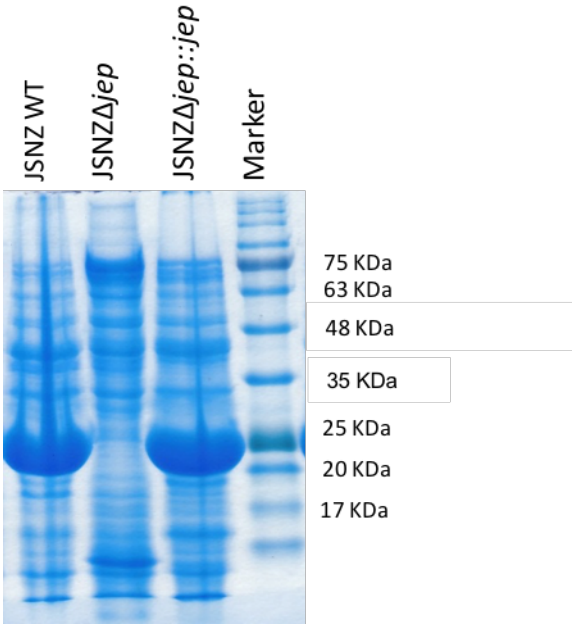


Figure 13: The complemented strain JSNZΔ*jep::jep* produced Jep in similar amounts to the JSNZ WT strain

The extracellular proteins of a TSB overnight culture of the strains JSNZ WT, the JSNZΔ*jep* and the JSNZΔ*jep*::*jep* were precipitated by 10 % (W/V) TCA and resolved by protein electrophoresis in a 12% SDS gel. The prominent 25 kDa band, likely corresponding to Jep, could be detected for JSNZ WT strain and the complemented strain JSNZΔ*jep::jep*, but was missing in the deletion mutant JSNZΔ*jep.* This suggests successful complementation, with recovered ability of the complemented strain to produce Jep.

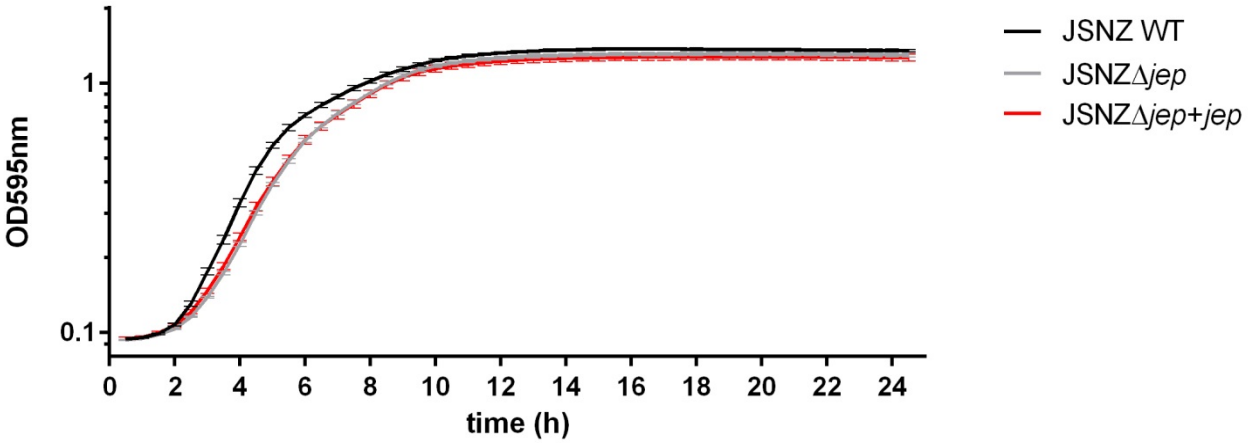


Figure 14: Both the JSNZΔ*jep* and the complemented strain showed a slightly reduced growth rate in TSB.

Three technical replicates of the three strains JSNZ WT, JSNZΔ*jep* and the JSNZΔ*jep*::*jep* were grown in 96-well flat bottom plates in TSB with a starting OD of 0.01. The OD was determined automatically at regular intervals over a period of 24 hours by means of the Tecan absorbance microplate reader. The WT strain showed a slightly higher growth ability than the genetically modified strains during the logarithmic growth phase. The mean +/-SD of three technical replicates is depicted.

Table 9: Both the JSNZΔjep and the complemented strain showed a prolonged doubling time TSB.

	JSNZ WT	**JSNZ*Δjep***	**JSNZ*Δjep::jep***
Doubling time (mean ± SD)[1]	67.9 ± 4.5 min	76.5 ± 4.6 min*	75.7 ± 1.5 min
Doubling time (median)	69.9 min	78.2 min	75.7 min
Time of entry to stationary phase	5 h	5.5 h	5 h
Final OD_{595}	1.35	1.29	1.26

1 Due to different timing in entering the logarithmic growth phase, we included four values for the doubling time for the JSNZ WT, three values for the JSNZ mutant, but only two values for the complemented strain.

*, $p<0.05$ (Kruskal-Wallis test)

4.3.2. Growth in IMDM was inconclusive

Next, we analyzed the survival of the three strains in the minimal medium IMDM. Intra-assay variation was large in this experiment, preventing any reliable conclusions about the growth behavior of the tested strains (Figure 15). The three strains displayed an even longer lag phase compared to the growth in TSB. Moreover, all three strains reached a lower final OD_{595}.

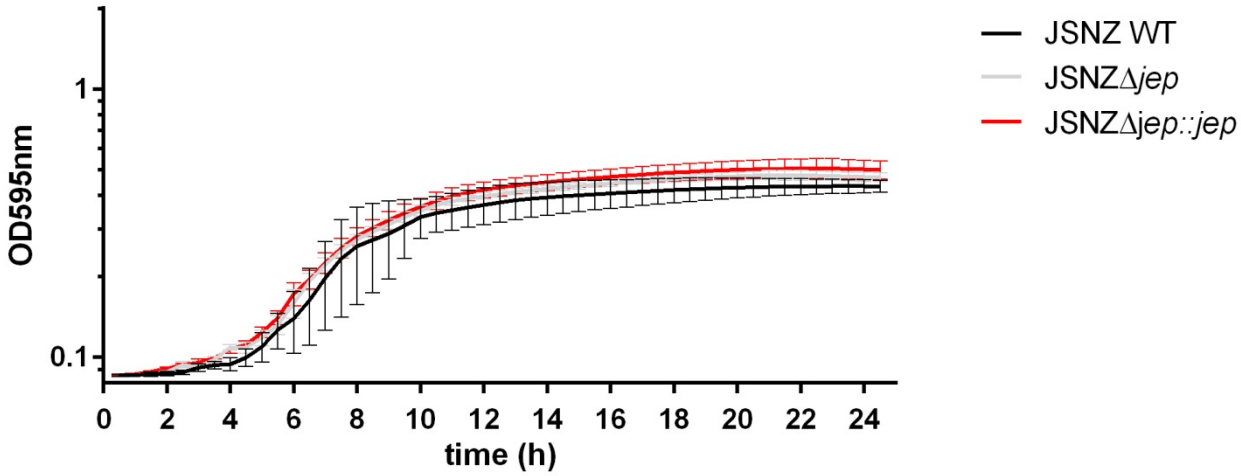


Figure 15: Both the JSNZΔjep and the complemented strain showed a slightly higher growth rate in IMDM.

Three biological replicates of the three strains JSNZ WT, JSNZΔ*jep* and the JSNZΔ*jep*::*jep* were grown in 96-well flat bottom plates with a starting OD of 0.01 and the OD was determined automatically at regular intervals over a period of 24 hours by means of the Tecan absorbance microplate reader for ELISA The mean +/-SD of three technical replicates is depicted.

4.3.3. Growth in murine whole blood revealed a 2-log difference in bacterial numbers between the WT and the genetically modified strains

The immune components in the whole blood include phagocytic cells, complement factors, AMPs and antibodies. Extracellular proteases may be important for *S. aureus* to produce nutrients in whole blood or to counteract the host immune response. In order to investigate the role of the putative secreted protease Jep with respect to the

nutrients release and the bacterial defense mechanisms against the host immune response, such as killing by neutrophils and other phagocytes the three strains were inoculated into heparinized murine whole blood and incubated at 37°C shaking for 24 h. At designated time points (0, 1, 3, 5, 7 and 24 h) the viability (live cell count) of the bacteria was measured by plating serial dilutions of each strain on TSA and determining the CFU/ml. As previously reported, JSNZ WT and the Jep deletion mutant differed strongly in their viability (Figure 14). Notably, more than 2 log difference in the viable colony counts could be detected at time points 5 and 7 h between the WT and the mutant strain. Unexpectedly, the complemented strain did not show the growth behavior of the JSNZ WT strain, but resembled closely the mutant strain (Figure 16).

This unexpected finding suggests that whatever caused the better fitness for growth and survival of the WT was not only lacking in the *jep* deletion mutant but also in the complemented strain. It also raised another question of whether this unknown factor was involved in the survival of JSNZ in its natural host - the mouse -, or whether it is a general survival tool. To test this, the same experimental conditions were repeated this time in human whole blood.

4.3.4. Neither JSNZ WT nor the genetically modified strains could grow in human whole blood

The viability of JSNZ and the mutant strains was then compared in murine and human whole blood to shed light on the possible role Jep plays in adaptation to its natural environment.

The survival of the three strains in human blood was tested in the same steps used in the murine blood survival assay. The resulting data showed no significant difference in the performance of the three strains in human blood as opposed to the picture seen in murine blood. Notably, all three strains were not able to multiply in human blood. Indeed, the bacterial counts dropped from an average of 4.6×10^5 CFU/ml to 9×10^3 CFU/ml at 24h (Figure 17). This contradicts the results of murine whole blood survival assay.

This points to a mutation, whether it is in fact *jep* or some other mutation, that caused a reduced ability of growth in murine blood and not in human blood.

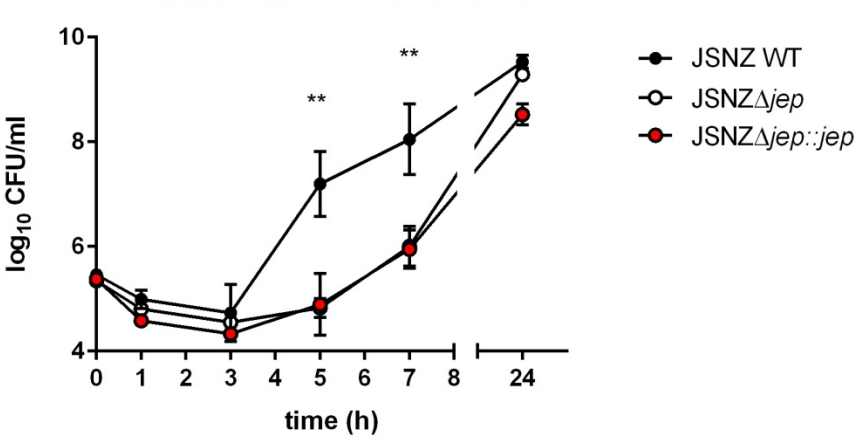


Figure 16: JSNZ WT showed 2 log higher growth in murine whole blood than the deletion mutant and the complemented strain.

The three strains JSNZ WT, JSNZΔ*jep* and JSNZΔ*jep::jep* were inoculated in triplicates into heparinized murine whole blood at a concentration of $2.5x10^5$ CFU/ml and incubated at 37°C for 24 hours. The CFU was then determined at time points 0, 1, 3, 5, 7 and 24h by plating serial dilutions of the inoculated blood on TSA. More than a two-log difference in growth and survival could be detected between the JSNZ WT and the deletion mutant. The complemented strain, however, showed the phenotype of the *jep* deletion mutant rather than a recovered ability for survival. The graph depicts the mean + SD of three biological replicates. Data were statistically evaluated with a one-way ANOVA test, with the significance level of $\alpha = 0.05$ (* = $p \leq 0{,}05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$).

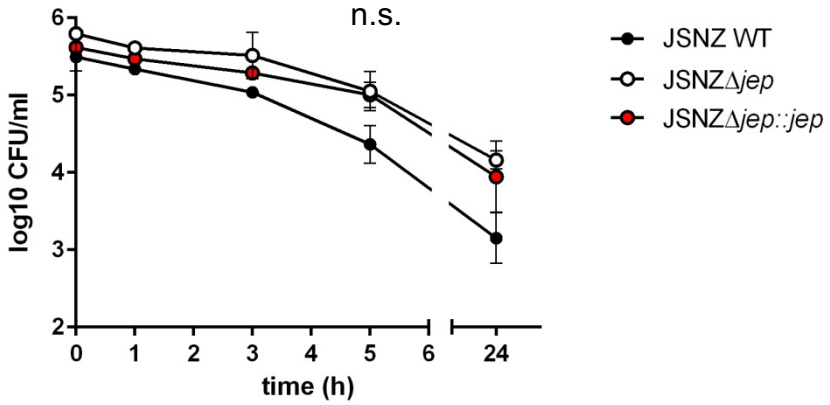


Figure 17: Neither JSNZ WT, nor JSNZΔjep and JSNZΔ*jep::jep* were able to multiply in human whole blood.

The three strains were inoculated into whole blood at a concentration of 2.5 x 10^5 CFU/ml and incubated at 37°C for 24 hours. The CFU was determined at 0, 1, 3, 5 and 24h by plating serial dilutions on TSA and determining the CFU/ml. JSNZ did not show better survival ability than the deletion mutant and the complemented strains in contrast to the picture seen in the murine blood cultures. The graph depicts the mean + SD of two biological replicates. Data were statistically evaluated with a one-way *ANOVA* test, with the significance level of α = 0.05. (n.s.= not significant).

4.3.5. The *jep* deletion mutant and complemented strain induced the same coagulation pattern of murine plasma

The murine blood survival assay and the growth curves in TSB and IMDM showed a shared phenotype by the JSNZΔ*jep* and JSNZΔ*jep::jep* that was different to that of the WT parent strain. To confirm the persistent link of the two strains phenotypically, despite the proved proper complementation, the possible effect of Jep on murine plasma coagulation was tested. A previous study on the CC88 and its prototype JSNZ has shown that it can coagulate murine plasma faster than matched human *S. aureus* isolates (Schulz et al., 2017). This effect was tested by incubating the three strains in murine plasma for 24h in a water bath and visually judging the degree of coagulation at 30 min, 1, 2,3, 4, 20 and 24h.

Once more the genetically modified strains showed an almost identical phenotype with a weaker effect on plasma coagulation than the JSNZ WT (Figure 18).

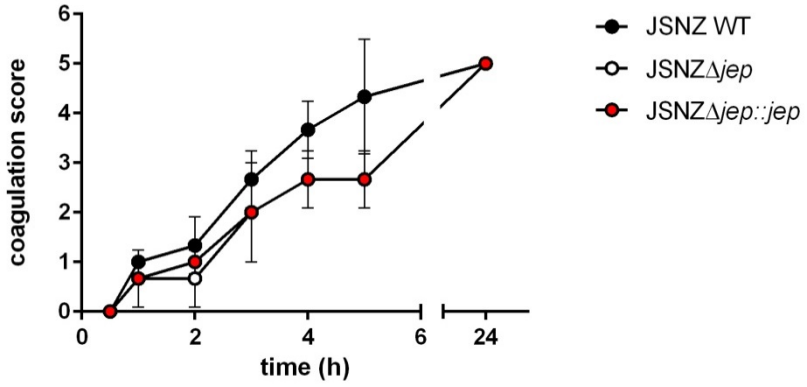


Figure 18: The *jep* deletion mutant and the complemented strain had lower coagulative effect on murine plasma than JSNZ WT.
Overnight cultures of the three strains in TSB were prepared. 65 µL of each culture was inoculated into 500 µL murine plasma and incubated at 37°C for 24 hours. The degree of plasma coagulation was determined visually according to a pre-set scale after 30 min, 1, 2, 4, 20 and 24 h. The investigator was blinded to experimental setup. JSNZ WT caused more rapid murine plasma coagulation than the deletion mutant and the complemented strains, where both showed an almost identical coagulation pattern. Mean +/- SD of three replicates are depicted.

4.3.6. Growth phase-dependent Jep expression was restored in the complemented strains

To investigate whether the genetic deletion and/or complementation influenced *jep* gene expression, we roughly assessed Jep production during the different bacterial growth phases by precipitating the extracellular proteins at defined time points and comparing the band sizes of Jep for the different strains. In this test, the JSNZ WT, the *jep* deletion mutant strain and two complemented strains were compared (here both the complemented strains were used; the one complemented with jep_A and the *jep* gene where the stop codon was not modified). The putative Jep band (25 kDa) was first detected at 2.5 h and steadily increased in intensity until 7.5 h. Patterns of the Jep band and the overall secretome were comparable for the WT strain and the two complemented strains. In contrast, the Jep band was completely absent in the extracellular protein supernatant of the JSNZΔ*jep* strain (Figure 19). Due to time constrains, the identity of Jep was not confirmed by Western Blot or MS analyses.

The result of this assay confirmed that the amount of Jep produced by the complemented strains and the pattern of its production was roughly the same as that of the wild type. This excluded the presence of a *jep* promoter gene mutation that might have influenced Jep production by the complemented strains and resulted in the detected phenotype. Also, the similar production pattern displayed by the complemented strain having mutation of the stop codon and the one that was complemented by a non-genetically modified *jep* gene provided more ground to the hypothesis that the accidental mutation occurred during the creation of the *jep* deletion mutant strain and not during its complementation.

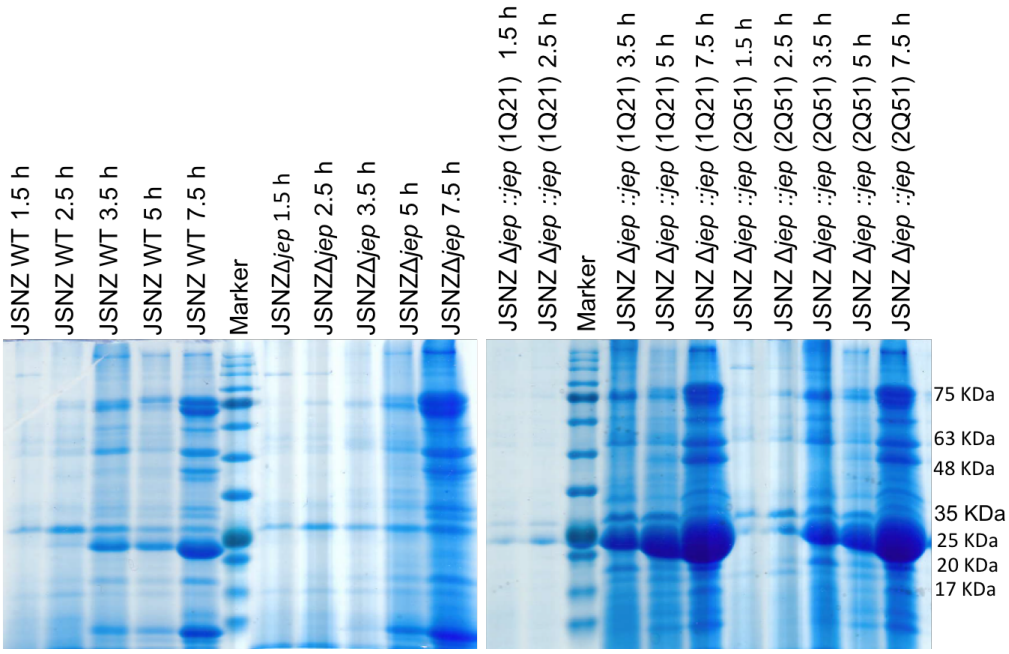


Figure 19: Protein electrophoresis gel images showing equal amount and timing of Jep production by JSNZ WT and the complemented strains and absence of the Jep band in the *jep* deletion mutant strain.

Comparing Jep production was done by precipitating the extracellular proteins from synchronized liquid cultures at 1.5, 2.5, 3.5, 5 and 7.5 h. The protein precipitate from 1 ml culture was loaded onto a 12 % SDS gel after the protein concentration was adjusted to the obtained OD. The compared strains were the JSNZ WT, the JSNZΔ*jep*, JSNZΔ*jep::jep* (complemented with un-mutated *jep* gene) and JSNZΔ*jep::jep*$_A$, Jep band at 25 KDa can be seen starting from the 2.5 h time point with increasing production up to the 7.5 h detection point. The Jep band was uniformly absent for the JSNZΔ*jep.*

To sum up, we have successfully complemented JSNZΔ*jep* with the *jep* gene using a chromosomal integration approach (4.1., 4.2.). Unexpectedly, the complemented

strains did not behave like the WT strain but rather like the mutant in a series of *in vitro* assays. Firstly, the growth of both the deletion mutant and the complemented strains was slightly retarded in TSB (4.3.1). Secondly, the JSNZ WT strain exhibited a strong growth advantage in murine whole blood compared to the mutant, but also the complemented strain (4.3.3). Finally, both the *jep* deletion mutant and the complemented strains had lower pro-coagulative effect on murine plasma than JSNZ WT (4.3.5). To exclude an unwanted defect in *jep* gene expression, we compared Jep expression during growth in TSB medium. The complemented strain produced Jep in a growth-phase dependent manner and in amounts similar to the WT strain, suggesting that Jep expression was not affected by the genetic engineering (4.3.6).

4.4. Genetic profiling of murine and matched human isolates

The Alere StaphArray allows the detection of target sequences of 336 distinct *S. aureus* genes and their allelic variants including species markers, resistance genes, exotoxins, exoenzymes, immune evasion factors, adhesins and *agr* group typing markers. Here, we compared the virulence gene profile of 25 *S. aureus* isolates from wild rodents and shrews with 18 *spa* type-matched human isolates to identify *S. aureus* factors involved in host adaptation.

The 25 animal strains belong to a cohort of 48 *S. aureus* strains isolated from 45 animals (wild rodents and shrews) collected during a period from 2012 to 2015 from remote locations in Mecklenburg-Western Pomerania, Thuringia, Baden-Wuerttemberg, Germany and South Moravian Region, Czech Republic.

Our research group previously *spa*-typed all 48 isolates and tested them by PCR for the *agr* type, methicillin resistance (*mecA* and *mecC*), and bacteriophage integrases (Table S1). Based on these characteristics, we chose 3 representative strains per genotype for the Staph Array analyses.

Whenever possible, *spa* type- matched human strains were included. The selected 25 murine isolates belonged to the *spa* types: t208 (CC49; n= 4), t4189 (CC49; n=3), t1736 (ST890; n=1), t1773 (ST890; n=3), t843 (CC130; n=2), t9909 (CC3033; n=3), t15027 (CC1956; n=2), t3058 (CC1956; n=3), t3830 (CC1956; n=3) (Table 10).

Table 10: Overview on representative murine S. aureus isolates that were genotyped. Whenever possible, spa type - matched human strains were included.

Strain ID	Host species	Host species	Place of isolation (region, Country)	clinical origin	date of isolation	*spa* type	MLST	deduced MLST	MLST CC	Staph array-derived CC	*mecA*	*mecC*
dmn150401-647	*Apodemus flavicollis*	yellow-necked mouse	MV, Germany	nasopharynx	13.07.14	t208	ST49	ST49	CC49	CC49-MSSA [lukF-P83/lukM+]	-	-
dmn150401-652	*Myodes glareolus*	Bank vole	MV, Germany	nasopharynx	24.09.14	t208	ND	ST49	CC49	CC49-MSSA [lukF-P83/lukM+]	-	-
dmn160622-849	*Myodes glareolus*	Bank vole	MV, Germany	nasopharynx	06.10.15	t208	ND	ST49	CC49	CC49-MSSA [lukF-P83/lukM+]	-	-
06-01225	*Homo sapiens*	Human	NI, Germany	wound infection	01.01.06	t208	ST49	ND	CC49	CC49-MSSA [lukF-P83/lukM+]	-	-
dmn150414-700	*Apodemus flavicollis*	yellow-necked mouse	BW, Germany	nasopharynx	28.07.14	t4189	ST49	ST49	CC49	CC49-MSSA [lukF-P83/lukM+]	-	-
dmn150521-800	*Myodes glareolus*	Bank vole	BW, Germany	nasopharynx	27.07.14	t4189	ND	ST49	CC49	CC49-MSSA [lukF-P83/lukM+]	-	-
09-03510	*Homo sapiens*	Human	SN, Germany	MRSA Screening	01.01.09	t4189	ND	ST49	CC49	CC49-MRSA-V	ND	ND
dsz140916-186-01(15)	*Microtus agrestis*	Field vole	TH, Germany	nasopharynx	24.09.13	t1736	ST890	ST890	ST890	ST890-MSSA	-	-
10-00991	*Homo sapiens*	Human	SH, Germany	wound infection	01.01.10	t1736	ST130	ND	CC130	CC130-MRSA-XI	-	+
08-02742	*Homo sapiens*	Human	BE, Germany	MRSA Screening	01.01.08	t1736	ST130	ND	CC130	CC130-MRSA-XI	-	+
11-01497	*Homo sapiens*	Human	BY, Germany	pneumonia	01.01.11	t1736	ND	ND	CC130	CC130-MRSA-XI	-	+
dmn150401-676	*Apodemus flavicollis*	yellow-necked mouse	MV, Germany	nasopharynx	22.09.14	t1773	ND	ST890	ST890	ST890-MSSA	-	-
dmn150421-721	*Apodemus flavicollis*	yellow-necked mouse	TH, Germany	nasopharynx	04.08.14	t1773	ND	ST890	ST890	ST890-MSSA	-	-
dmn160622-859	*Apodemus flavicollis*	yellow-necked mouse	MV, Germany	nasopharynx	08.10.15	t1773	ND	ST890	ST890	ST890-MSSA	-	-
09-01300	*Homo sapiens*	Human	TH, Germany	dermatitis	01.01.09	t1773	ST130	ND	CC130	CC130-MRSA-XI	-	+
13-01673	*Homo sapiens*	Human	NI, Germany	MRSA Screening	01.01.13	t1773	ND	ND	CC130	CC130-MRSA-XI	-	+
dsz140916-186-08 (77)	*Microtus agrestis*	Field vole	TH, Germany	nasopharynx	25.09.12	t2311	ST88	ST88	CC88	CC88-MSSA	-	-
JSNZ	*Mus musculus*	*House mouse (lab mouse)*	New Zealand	abscess	17.12.08	t729	ST88	ST88	CC88	JSNZ-ST88	-	-
M3	*Homo sapiens*	Human	New Zealand	SSTI	02.05.07	t186	ST88	ST88	CC88	CC88-MSSA	-	-
M25	*Homo sapiens*	Human	New Zealand	endocarditis	22.08.07	t186	ST88	ST88	CC88	CC88-MSSA	-	-
F25	*Homo sapiens*	Human	New Zealand	SSTI	01.04.08	t11192	ST88	ST88	CC88	CC88-MSSA	-	-

Table 10 cont.: Overview on representative murine S. aureus isolates that were genotyped. Whenever possible, spa type - matched human strains were included

A50	*Homo sapiens*	Human	New Zealand	intravenous device infection	13.04.07	t692	ST78	ST78	CC88	CC88-MSSA	-	-
A7	*Homo sapiens*	Human	New Zealand	febrile neutropenia	15.08.07	t186	ST78	ST78	CC88	CC88-MSSA	-	-
dsz140926-190-42(427)	*Mus musculus*	*House mouse (lab mouse)*	MV, Germany	nasopharynx	10.12.13	t843	ND	ST130	CC130	CC130-MSSA [lukF-P83/lukM+]	-	-
dsz140916-186-05(70)	*Apodemus flavicollis*	yellow-necked mouse	TH, Germany	nasopharynx	26.09.13	t843	ST130	ST130	CC130	CC130-MRSA-XI	-	+
15-01986	*Homo sapiens*	Human	ST, Germany	bacteremia	01.01.15	t843	ST130	ST130	CC130	CC130-MRSA-XI	-	+
12-03171	*Homo sapiens*	Human	ST, Germany	abscess	01.01.12	t843	ND	ST130	CC130	CC130-MRSA-XI	-	+
15-01861	*Homo sapiens*	Human	NI, Germany	MRSA screening	01.01.15	t843	ND	ST130	CC130	CC130-MRSA-XI	-	+
dmn150430-749	*Sorex araneus*	Common shrew	TH, Germany	nasopharynx	06.08.14	t9909	ST3033	ST3033	ST3033	ST3033-MSSA	-	-
dmn150430-754	*Sorex araneus*	Common shrew	TH, Germany	nasopharynx	05.08.14	t9909	ND	ST3033	ST3033	ST3033-MSSA	-	-
dmn150430-756	*Sorex araneus*	Common shrew	TH, Germany	nasopharynx	03.10.14	t9909	ND	ST3033	ST3033	ST3033-MSSA	-	-
dmn150421-724	*Microtus arvalis*	Common vole	BW, Germany	nasopharynx	22.10.14	t15027	ST3252	ST3252	CC1956	ST1959-MSSA	-	-
dmn150421-725	*Microtus arvalis*	Common vole	BW, Germany	nasopharynx	21.10.14	t15027	ST3252	ST3252	CC1956	ST1959-MSSA	-	-
dmn150421-726	*Microtus arvalis*	Common vole	BW, Germany	nasopharynx	21.10.14	t3058	ST3252	ST3252	CC1956	ST1959-MSSA	-	-
dmn160628-865	*Microtus arvalis*	Common vole	Brno, Czech Republic	nasopharynx	29.11.14	t3058	ND	ST3252	CC1956	ST1959-MSSA	-	-
dmn160628-867	*Microtus arvalis*	Common vole	Brno, Czech Republic	nasopharynx	28.11.14	t3058	ND	ST3252	CC1956	ST1959-MSSA	-	-
dmn150527-824	*Microtus arvalis*	Common vole	MV, Germany	nasopharynx	22.09.14	t3830	ST1956	ST1956	CC1956	ST1959-MSSA	-	-
dmn150527-829	*Microtus spp.*	Common vole	MV, Germany	nasopharynx	23.09.14	t3830	ND	ST1956	CC1956	ST1959-MSSA	-	-
dmn150527-830	*Microtus agrestis*	Field vole	MV, Germany	nasopharynx	23.09.14	t3830	ND	ST1956	CC1956	ST1959-MSSA	-	-

Since many virulence factors are lineage-associated, we compared the murine strains to *spa* type-matched human isolates. However, all detected murine lineages were extremely rare or completely absent in the human *S. aureus* population [7],[19]. For example, we did not detect lineages CC49, CC890, CC130, ST3033, and ST3252 in a large population-based study comprising more than 1000 *S. aureus* isolates [19]. If available, matched human isolates were kindly provided by the Robert Koch Institute, Wernigerode (Birgit Strommenger) and the Technical University Dresden (Stephan Monecke). Eventually, we analysed human strains of the following *spa* types: t1736/t1773 (CC130; n=8), t208/t4189 (CC49; n=2), and t186/t690/t692/t693/t730/t786/t1598/t2526/t3205/t3341/t4015/t11192/t14389 (CC88; n=18).

4.4.1 *S. aureus* sequence type 88 (CC88)

CC88 is the predominant lineage in laboratory mice (Schulz et al., 2017) and was also detected in a single field vole in Thuringia. This isolate belonged to the *agr* type III and tested negative for *mecA/mecC*, enterotoxin and exfoliative toxin genes and all resistance genes markers. This isolate was the only one in this study carrying the immune evasion cluster (IEC)-encoded genes *scn*, *chp*, and *sak*.

Microarray analyses revealed that JSNZ (prototypical laboratory mouse strain) and the field vole isolate are very similar. While JSNZ lacked Sa1int, Sa2int and the Sa3int phages [42] and the phage-encoded IEC genes, they were present in the field vole isolate (Table S2.). JSNZ and the field vole isolate responded to different probes of the staphylococcal superantigen-like protein 1 (*ssl1*), *clfB, lukS,* and *sdrC* suggesting the presence of allelic variants (Table S1). Moreover, the *sdrD* locus was present in the field mouse isolate and JSNZ but was variably present in the human CC88 isolates.

The human strains differed in virulence and resistance gene patterns from the two murine isolates. All human strains harboured the IEC-encoding phage (18/18). 7/18 reacted with the *setl01/set6 (RF 122)* probe and 11/18 encoded the leucocidins *lukF-PV* and *lukS-PV*, all of which were absent from the murine strains.

The methicillin resistance gene *mecA* that was totally absent in the murine isolates was found in some of the human CC88 isolates. This was also the case for the penicillin resistance gene complex of *blaZ, blaI* and *blaR*. (Table S2).

4.4.2 *S. aureus* murine sequence type 49 (CC49)

CC49 (ST49) is the predominant lineage in wild rodents and shrews. A comparison of the murine isolates (n=6) and matched human isolates (n=3) showed that all strains belonged to the *agr* type II, lacked SAg genes.
While all murine strains lacked the methicillin resistance genes *mecA* and *mecC,* the *mecA* gene was found in one human isolate. All murine strains were positive for the pore-forming toxins *LukF-PV(P83)/LukM* and *lukF/lukS,* while only one out of two of the human strains tested positive. All the murine strains also lacked the immune evasion cluster genes *sak*, *chp* and *scn* found in the human strains.

4.4.3 *S. aureus* sequence type 130 (CC130)

The murine CC130 isolates (n=2) belonged to the *agr* type III, lacked the enterotoxin and the exfoliative toxin genes as well as the IEC. One isolate (Thuringia yellow-necked mouse) was *mecC*-positive and *lukF-PV/lukM*-negative. The other isolate showed the opposite genetic profile, lacking *mecC* and harboring the leucocidin toxin. On the other hand, 100% of the human CC130 isolates were MRSA (*mecC*) positive. Around 90% of the human strains were positive for *ccrA-2,* the murine isolates were 100% negative. The penicillin resistance gene *blaZ-SCCmec XI* was positive in only one of the two murine isolates, while it was positive in all eight human isolates. The leucocidin genes *LukF-PV (P83)* and *LukM* were absent in all human strains and present in one murine isolate. Also, the *spIE* gene was missing in around 60% of the human strains.

4.4.4 *S. aureus* sequence type 890

Interestingly the ST890 isolates all lacked the resistance genes *mecA*/ *mecC*, enterotoxin and exfoliative toxin genes, the IEC genes as well as any resistance gene markers, except for the *blaZ* gene, which was detected in all tested murine

ST890 isolates. Remarkably, three out of the four isolates were negative for the coagulase gene *coA* and the beta-haemolysin gene *hlb.* The absence of an *hlb* hybridisation signal could be caused by a novel allelic variant, the true absence/destruction of the *hlb* gene locus or the disruption of the *hlb* gene by integration of Sa3int phages. Since all murine ST890 isolates lack Sa3int phages, we could exclude the last option. To differentiate between the first and second option, we tested the strains for Hlb production. Hlb disrupts the cell membrane of erythrocytes and Hlb-producing *S. aureus* colonies create a zone of ß haemolysis on red blood agar. Notably, all murine ST890 isolates did not cause ß haemolysis on sheep and also murine blood agar (personal communication D. Mrochen, Institute of Immunology). This implies that the murine strains do not produce functional Hlb.

The closely related *spa* types t1736 and t1773 have been previously identified in animals and humans and were always assigned to MLST 130. Our isolates, however, showed a different genetic profile than the human CC130 human isolates. The human t1736 and t1773 isolates (CC130) belonged to *agr*III, while the murine t1736/t1773 isolates belonged to *agr*IV. Moreover, murine t1736/t1773 isolates showed other differences like the lack of a hybridisation signal for *coA*, some leucocidin genes (*lukD, lukX, lukY*), *hlb*, and *splA/B/E* (table 2). Moreover, the murine strains carry different allelic variants of known core-variable genes, i.e. surface proteins and *ssls*. All these discrepancies suggest that the murine isolates belong to a different lineage. Indeed, the array software assigned the murine t1736/t1773 to ST890 which is not part of CC130, and in fact does not share a single allele of the 7 house-keeping genes used for MLST typing with it. Taken together, these findings point to a recombination event that involved only the *spa*-encoding region and not the MLST alleles. Consequently, the human t1736 and t1773 isolates could not be used for a comparison with the murine t1736 and t1773 isolates.

4.4.5 *S. aureus* sequence type 1956

Similarly, murine isolates belonging to CC1956 (ST1956, ST3252) lacked the *mecA/mecC*, IEC genes, SAg genes and any resistance genes markers. Human isolates of CC1956 have not been reported.

Table 10: Human and murine strains having spa type t1773/ t1736 show a different genetic profile.

	Human t1736/ t1773	Murine t1736/ t1773
MLST CC	**CC130**	**ST890**
MRSA (*mecC*)	+	-
SPECIES MARKER*:*		
coA (coagulase)	+	- (75%)
REGULATORY GENE:		
agr (Accessory gene regulator))	agrIII	agrIV
PENICILLIN RESISTANCE:		
Blaz (Beta-lactamase gene)	-	+
blaZ-SCCmec XI (beta-lactamase gene associated with SCCmec XI elements)	+	-
blaI (beta lactamase repressor (inhibitor)	-	+
blaR (beta-lactamase regulatory protein)	-	+
HLG AND LEUKOCIDINS:		
LukD (leucocidin D component)	+	-
LukX (leucocidin/haemolysin toxin family protein (= *lukB* or *lukG*))	+	-
LukY (leucocidin/haemolysin toxin family protein (= *lukA* or *lukH*))	+	-
HAEMOLYSINS:		
*hlb*_probe 1 (haemolysin beta)	+	-
*hlb*_probe 2	+	-
*hlb*_probe 3	+	-
un-disrupted *hlb* (haemolysin beta without phage insertion)	+	-
PROTEASES:		
SplA (Serine protease A)	+	-
SplB (Serine protease B)	+	-
SplE (Serine protease E)	+	-
STAPHYLOCOCCAL SUPERANTIGEN/ENTEROTOXIN-LIKE GENES (SET/SSL):		
ssl01/set6_probe1_11	+	-
ssl01/set6_probe2_11	-	+
ssl01/set6_probe1_12	+	-
ssl01/set6_probe2_12	-	+
ssl01/set6_probe4_11	+	-
ssl01/set6 (Mu50+N315)	+	-
ssl01/set6 (MW2+MSSA476)	-	+
ADHAESION FACTORS / GENES ENCODING MICROBIAL SURFACE COMPONENTS RECOGNIZING ADHESIVE MATRIX MOLECULES (MSCRAMM GENES):		
clfA (COL+RF122) (Clumping factor A)	+	-
can (Collagen binding Adhesin)	-	+
fnbB (total) (Fibronectin binding protein B)	+	-
fnbB (COL+Mu50+MW2)	+	-
sdrC (COL) (Ser-Asp rich fibrinogen-/bone sialoprotein-binding protein C)	+	-
TYPE I RESTRICTION-MODIFICATION SYSTEM, SINGLE SEQUENCE SPECIFICITY PROTEIN:		
hsdS2 (RF122) (type I site-specific deoxy ribonuclease subunit, 2nd locus)	+	-

4.4.6 *S. aureus* sequence type 3033

The ST3033 isolates belong to the *agr* type II and like other murine species lacked *mecA*/*mecC*, enterotoxin genes, exfoliative toxins genes, the *IEC* and all resistance genes markers. It was revealed to be positive for the disrupted hlb gene and positive PCR results for phage integrase, causing a lack of haemolysis on blood agar. ST3033 represents a novel CC and hence, no matched human isolates were available.

To conclude, the murine and matched human isolates showed differences in some cases like the lack of methicillin resistance genes in the murine isolates of the CC49 strain which were present in 50% of the human isolates. Also, the lack of the IEC genes in the murine isolates and their detection in 50% of the *spa*-type matched human isolates. All the murine isolates lacked the IEC genes (*sak*, *chp* and *scn*), excluding one wild mouse isolate of the CC88 strain that still retained the Sa3int phage. One CC130 murine isolate tested positive for the *mecC* resistance gene pointing to a possible reservoir of MRSA in wild rodents and voles.

Chapter 5: Discussion

JSNZ is a mouse-adapted *S. aureus* strain that colonizes laboratory mice at higher CFUs and for a longer time than human-adapted strains, suggesting that it is a better candidate for the murine *S. aureus* colonization model (Holtfreter et al., 2013). The putative extracellular protease Jep is an interesting candidate to study host adaptation, because our group detected it in murine isolates but not in human strains. Jep accounts for one third of the total extracellular protein production of JSNZ. The *jep* gene occupies the virulence module on the Sa1int phage, and it is related to other *S. aureus* proteases. All these factors point to a role for Jep in bacterial virulence and host adaptation.

To explore the role of Jep, our collaborators from the University of Auckland, New Zealand previously created a *jep* deletion mutant, which intriguingly showed a reduced survival and growth fitness in murine serum and whole blood.

To validate these findings and confine them to the action of Jep, a *jep*-complemented strain was created by chromosomal complementation. Unexpectedly, the complemented strain did not regain the phenotype of the JSNZ WT strain, but behaved in all assays (growth and survival in TSB, IMDM, murine whole blood, and human whole blood as well as coagulation of murine plasma) exactly like the JSNZ deletion mutant.

5.1. Was the chromosomal complementation of JSNZ∆*jep* successful?

The observation that the complemented strain did not show the phenotype of the WT strain prompted the question of whether the chromosomal complementation of JSNZ∆*jep* was successful. To confirm this, we first sequenced the *jep* region in the complemented strain using *jep*-flanking primers and observed that the *jep* ORF was intact, showed the marked stop codon, and had inserted in the correct genomic location. Sequencing results also showed the presence of two other point mutations up- and downstream of the *jep* gene. Those two mutations were outside of the *jep*

ORF. However, to exclude that a mutation maybe has affected a *jep* promotor region we analyzed the exo-proteome of synchronized cultures of the complemented strains and compared it to JSNZ WT. Notably, the complemented strains showed a strong band at 25 kDa, likely corresponding to Jep, with an expression kinetic and protein amount roughly equal for the two complemented strains and the WT strain.
However, the identity of the detected protein was not confirmed by MS or by Western blot using polyclonal anti-Jep mouse serum.
Since the *jep* ORF itself is intact, we speculate that at least one additional mutation was induced in the JSNZ chromosome accidentally during the creation of the *jep* deletion mutant and was maintained in the complemented strain. This hypothesis can be tested by whole genome sequencing of JSNZ WT and the complemented strain. Indeed, sequencing was performed by cooperation partners at the Robert-Koch-Institute, Wernigerode (Torsten Semmler), but unfortunately sequencing data have not been analyzed due to time restriction. To ease the following discussion, I will name this putative mutation 'mutation X'. Whatever the nature of the mutation X is, it seems to mediate a very interesting phenotype, as discussed below and deserves further studies.

5.2. Was the unidentified mutation X affecting the acquisition of nutrients by JSNZ?

S. aureus extracellular proteases are believed to play a role in the digestion of proteins providing the bacteria with a carbon source. Initially, we aimed to test whether Jep has a role in nutrient acquisition and compared the growth of JSNZ WT, the *jep* gene deletion mutant and the complemented strain in TSB. The JSNZ WT strain showed a growth advantage over the deletion mutant during the logarithmic growth phase as previously shown by our research group (Bachelor thesis, Evelyn Rüdiger). However, the complemented strain showed the same growth dynamics as the deletion mutant strain rather than the wild type. This suggests that mutation X mediates a slight growth defect in TSB.
S. aureus first uses glucose (carbon source) and amino acids (nitrogen sources) in TSB cultures and during the stationary growth phase turns to amino acids and acetate as alternative carbon sources [1]. Hence, the delayed growth of the

genetically modified strains could be due to the affection of a gene important for the uptake and utilization of glucose or amino acids.

5.3. Is the mutation X important for the survival in murine whole blood?

We initially aimed to test whether Jep is important for bacterial survival and growth in murine and human blood, because preliminary data using JSNZ WT and JSNZ∆*jep* showed striking defect of the mutant to survive in murine blood. Unexpectedly, upon comparing the growth of and survival of the WT, deletion mutant and complemented strains, the striking defect the deletion mutant showed was paralleled in the complemented strain. At this point we could say that a factor was giving the WT strain a growth advantage in murine whole blood over the deletion mutant, but it was highly doubtful that this factor could be Jep as it was clear from extracellular protein electrophoresis that the complemented strain regained the ability to produce Jep showing a band similar to that of the JSNZ WT. In consequence, the observed growth defect in murine whole blood was not mediated by Jep but rather the unknown mutation X.

Since fresh murine blood is highly bactericidal for *S. aureus* due to several immune mechanisms, the mutation X might reverse an immune evasive mechanism of JSNZ. Possible mechanisms would be that gene X renders JSNZ resistant to killing by phagocytosis by inhibiting opsonisation through the inactivation of the complement factors C3 and C5 by proteolytic cleavage in an effect similar to that of aureolysin [29]. Factor X may be achieving similar results by the proteolytic cleavage of antibodies. It could be that factor X acts as an inhibitor of host proteases (as a staphylococcal extracellular protein). Factor X could be producing toxins that cause lysis of the immune cells. It could be responsible for the production of phenol soluble modulins that interfere with neutrophil extravasation and chemotaxis, could be modulating the killing by neutrophilic granules through the production of catalases or superoxide dismutases. It could also be a factor responsible for the bacterial acquisition of cell-bound iron like a leucocidin or a haemolysin, or be responsible for the intracellular transportation of iron.

The difference in growth detected between the WT and the mutant strains in the murine whole blood was significant and the next question was if this growth discrepancy was related to JSNZ adaptation to its murine natural host or a general growth disadvantage in the mutant strain. To shed light on that the same experiment was repeated in human whole blood.

The same conditions of the murine blood survival assay were repeated but this time with human whole blood. All strains showed an inhibition of growth and a decline in CFU at all time points reaching almost 50% of the initial concentration. The difference in growth between the JSNZ WT and the mutant and complemented strains was not detected in human blood. This fortified the assumption that the gene X, which was mediating a growth advantage to the JSNZ WT, was a factor related to murine host adaptation. This could be explained by the fact that the composition and hence the antibacterial effects are different in murine and human blood. Murine blood cellular components differ from that of the human blood, where in human blood 50 – 70% of granulocytes are composed of neutrophils, while murine granulocytes are composed mainly of Lymphocytes and neutrophils occupy a smaller percentage of 10-25% [39]. Also, the human neutrophils express defensins that are not detected for the murine neutrophils [47].

5.4. The effect of the mutation X on murine plasma coagulation

One of the virulence mechanisms of *S. aureus* is its ability to hide itself from the host immune cells in a dense fibrin network. This is achieved by exploiting the host coagulation system, where the bacteria produce several factors that cause a shift towards coagulation or fibrinolysis. Previous work was done to assess the murine *S. aureus* strains, namely the CC88, effect on murine plasma coagulation compared to the human strains. Results have shown that plasma coagulation was more advanced when incubated with murine CC88 than with the human CC88 strains, also suggesting host specific coagulation system modulatory factors. To test the influence the mutation X had on the pro-coagulatory activity of the murine CC88, and to confirm that the deletion mutant and the complemented strains were acting similarly, the three strains were incubated with murine plasma for 24 h. Murine plasma

incubated with JSNZ WT showed more rapid coagulation than with the genetically modified strains during the first 5 hours.
This test showed that once more the complemented strain showed the same phenotype as the *jep* deletion mutant of slower coagulation of the murine plasma.
The obtained results reinforced the notion that Jep was most probably not the factor causing the growth advantage JSNZ had in murine blood, or the superior coagulation and thus not the element lying behind the murine host adaptation.

5.5. The importance of complementing knock-out strains

Countless studies have attempted exploring functions of certain genes by comparing the performance of a WT strain and its deletion mutant. To solidify conclusions a complemented strain should also be part of every study investigating a deletion mutant. This complementation can either be achieved through complementation with a plasmid containing the gene in question or, as in the case of this study, through chromosomal complementation. Chromosomal complementation with the introduction of the gene at its original position in the bacterial chromosome has undeniable advantages over plasmid complementation. Plasmid complementation has the benefit of easy manipulation and regulated gene expression, but when complementation is to be tested, aberrant phenotypes can result from the high plasmid copy number and the gene dosage that exceeds the chromosomal number. Another fundamental issue for plasmid complementation is the genetic instability, where plasmids can be easily lost in the absence of the appropriate selection conditions [15], [6].
Surprisingly, a considerable number of published studies fail to provide a complemented strain. In fact, the Kolar et al. study on which our study is based upon failed to provide a complemented strain of the protease null mutants. A literature search using the key words “Bacteria and mutant” in three microbiological journals revealed striking deficiencies: In The Journal of Infectious Diseases 25% of the papers testing a deletion mutant did not present data for a complemented strain (12 articles over the period from 01.01.2015 to 01.01.2017). Similarly, in PLoS Pathogens 27% of the papers did not provide a complemented strain (44 articles for the same period). Finally, 38% of the articles in MicrobiologyOpen lacked a

complemented strain.

Complementation is particularly important to exclude the presence of unexpected mutations that can lie behind all observations of the study. It is also important to prove that the expression of the gene in question in the complemented strain is accompanied by reversion to the wildtype phenotype.

The most solid finding of this work is that it is rather unlikely to be able to associate a certain function to any given gene without providing a complemented strain. Only the complemented strain can provide the proof that the gene in question is the only variable in the comparison of a WT strain and its deletion mutant. Once a complemented strain is created, it is important to show that (1) the gene sequence is correct, (2) that the protein expression resembles the WT and (3) that the protein-mediated functions are regained. In our case, points 1 and 2 were fulfilled, rendering the assumption that the *jep* gene deletion was the underlying cause rather plausible. However, we were not able to confirm point 3. This experience was unfavorable for this work, but it also provides us with a more critical view of research work involving bacterial mutants without complementation.

5.6. Genetic profiling of murine and matched human isolates

In addition to generating and characterizing a *jep* complemented strain, we also compared the virulence gene profile of 25 *S. aureus* isolates from wild rodents and shrews with 18 *spa* type-matched human isolates to identify *S. aureus* factors involved in host adaptation.

For this kind of genetic comparisons, it is essential to use *spa* type- or CC- matched strains, because the presence of many *S. aureus* virulence factors, which are part of the variable genome, are linked to the underlying lineage. Similarly, allelic variants of numerous surface proteins and regulators, collectively called core variable genes, are linked to the clonal cluster.

We applied the Alere *S. aureus* Genotyping Kit 2.0 to obtain a genetic profile of the murine and matched human isolates and to obtain some hints about host-specificity determinants in *S. aureus* strains isolated from wild mice. The *S. aureus* Genotyping Kit 2.0 can detect target sequences of 336 distinct *S. aureus* genes and their allelic

variants. Genetic differences between *S. aureus* isolates from wild rodents and shrews as compared to their matched human counterparts are outlined below.

Among the 25 isolates of the wild rodents and shrews the lineage CC88 was detected in only one field vole in Thuringia. The lineage CC88 was shown in a previous study of our group to be the predominant lineage in laboratory mice (Schulz et al., 2017). Not only was the CC88 lineage found to be rarely occurring in wild rodents and shrews in contrast to lab mice, but also the one Thuringia vole isolate still harbored the IEC complex which was lacking in all the murine CC88 isolates. This maybe indicate a recent host jump in this strain.

The study of Holtfreter et al., 2013 showed that the murine CC88 strains differed from the human CC88 isolates in their genome including the loss of the Saint phages and the SAg-encoding mobile genetic elements in the murine strains and the frequent presence of ampicillin sensitivity in the same strains in addition to their superior ability to coagulate human plasma. The microarray results revealed several differences between the mouse-adapted strain JSNZ and all other CC88 strains including an allelic variant of the *ssl1* gene the ssl protein *setl01/set6 (RF 122)* that are lacking in only JSNZ. The above-mentioned study also showed that JSNZ was found to be a better candidate for the mouse colonization model than the human-adapted strain Newman.

The most frequently isolated lineage in wild rodents and shrews was the CC49, and compared to the human isolates the murine strains showed clear signs of host adaptation. The murine isolates were 100% negative for the methicillin resistance genes *mecA* and *mecC,* which were found in isolated human strains (1 out of 2 isolates). Also, all the murine strains lacked the immune evasion cluster genes *sak*, *chp* and *scn* found in the human strain. These results come in agreement with a previous report of CC49 strains in literature, where this genetic profile was considered as a possible indicator of adaptation to wilderness rodents [40].

Other isolates including ST3033, CC1956 all lacked the resistance genes *mecA*/ *mecC*, enterotoxin and exfoliative toxin genes, the IEC genes as well as any resistance gene markers. This suggests adaptation of the murine strains to their murine host by shedding genes of no further pressing urgency for their survival. Also, CC130 isolates lacked the enterotoxin and exfoliative toxin genes as well as the IEC. CC130 has been previously found in humans but it is a common lineage in small ruminants (Merz et al., 2016b). Notably, one of two isolates from Thuringia yellow

necked mice still harboured a *mecC* gene. In consequence rodents carrying the *mecC*-positive *S. aureus* represent an important reservoir of such strains endangering livestock and humans.

Despite the overview it allowed and amount of information we obtained from this assay, it is important to point out the limitations of this analysis. First the sample size was rather small with only 25 animal strains from a cohort of 48 *S. aureus* isolates tested. Whenever possible, *spa*-type matched human strains were included in the assay for comparison of the results, however, this was not possible for all the isolated strains because some *spa* types were either very rare or have never been reported form human isolates.

It is also to be noted that the Alere *S. aureus* Genotyping Kit 2.0 was designed for the detection of genes of human *S. aureus* isolates and thus human-adapted strains. This necessarily means that it cannot detect new genes that may be involved in the adaptation to the animal host, which also entails that it cannot detect mouse-specific variants of already known genes. This means that whole genome sequencing remains the best method of researching the genes involved in host adaptation. However, using the Array in the future with a larger sample size and matched human strains can provide valuable data through the mutual collaboration with the assay providers. This collaboration can lead to the implementation of new genes and new allelic variants and identification of the host adaptation factors.

5.7. Outlook

The genetic mutation X that was created during the deletion process of the *jep* gene has accidently affected a gene that is highly relevant to the murine host adaptation capacity displayed by JSNZ. The identification of this gene could provide a key to understanding the mechanisms of animal host adaptation. Identifying the mutated gene could be achieved through whole genome sequencing, comparing the WT JSNZ genome to the deletion mutant strain (JSNZ∆*jep)*. Genome sequencing was already done and the sequence analysis should follow shortly. Once the mutation in question is found, proper analysis of the effects it might be conferring to host adaptation should be tested. After proper localization of gene X a deletion mutant and a chromosomally complemented strain should be created. The generated complemented strain together with the deletion mutant and the WT strains can all be

used for studying the specific role of gene X. Should factor X prove to be another protein produced by JSNZ, it could be analyzed by Western Blot or MS. The growth pattern of the strains in different media will be analyzed, comparing the survival and proliferation of gene X deletion mutant and the WT JSNZ in both murine and human whole blood and serum.
On the other hand, Jep still stands as an intriguing protein with an unknown function. Its role in virulence murine host adaptation remains to be elucidated. This is currently work under progress, where a new *jep* deletion mutant and a complemented strain are being created by members of our team. The deletion mutant and complemented strains will be created using the same protocol applied here for generation of the JSNZ∆*jep::jep* using the pIMAY plasmid system. Sequencing of each created strain will be done to exclude any unexpected outcomes.

One of the plausible questions that still needs to be answered is, what are the possible substrates of the putative serine protease Jep. There are several possible approaches that could be employed in tackling this question, one method would be performing a CLiPS-Assay. This is an assay that identifies a protease cleavage site using quantitative kinetic screening of cellular libraries of peptide substrates (CLiPS). In this assay, *E coli* are used to express a membrane-bound peptide which encodes an identifiable marker in the extracellular region. In front of the peptide binding site to the marker, a random amino acid sequence is introduced, creating an *E.coli* library of 10^7 different peptide sequences. Some of these sequences could be cut by a protease in question. The hydrolysis of the substrate by the protease can be quantitatively measured through the changes in the cell fluorescence by flow cytometry [3].
Another possible method is the incubation of Jep with individual protein candidates, e.g. complement factors, or protein cocktails. The latter could be surface proteins or extracellular proteins of *S. aureus*, as it is known for other *S. aureus* proteases that they cleave *S. aureus* virulence factors and might thereby regulate bacterial pathogenesis [28]. Cleavage could be detected by SDS-PAGE for individual substrates, or by MS analyses.

Finally, studying the factors and conditions that affect Jep production and comparing the production of the different strains at the transcription level would be of interest.

The expression of Jep at different stages of growth was done and compared for the wild type, the deletion mutant and the complemented strains. This assay was performed crudely to exclude defective production of Jep by the complemented strains, and so exclude this as a cause for the phenotype discrepancies detected. However, further exploration of the patterns of Jep production, whether at different growth phases or different culture conditions by extracellular proteins precipitation and Western blot analysis can provide valuable information about the nature and function of Jep.

The mouse-adapted strain JSNZ possesses certain factors that provide it with mechanisms for better adaptation to and survival in its murine host. It has been proven to be a promising tool in the *S. aureus* colonization model, but understanding how JSNZ has achieved this successful host jump is integral to the role it can play in studying the host-pathogen interaction.

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Summary

Background: The high incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) strengthens the need for new effective antibiotics and a protective vaccine. Up till now, mainly human-adapted S*taphylococcus aureus* strains were used to study *S. aureus* pathogenicity in mouse models. However, it is known that *S. aureus* is highly host-specific. Recently, a mouse-adapted *S. aureus* strain, JSNZ, was identified. This strain could be a promising tool in developing more appropriate infection models.

JSNZ produces high amounts of a putative extracellular protease, named JSNZ extracellular protease (Jep). Since the jep gene was only detected in *S. aureus* isolates from laboratory mice and wild small rodents and shrews, we hypothesize that Jep is important for colonization and infection in mice. The *jep* deletion mutant previously created by our collaborators from the University of Auckland, New Zealand, intriguingly showed a reduced survival and growth fitness in murine serum and whole blood as compared to the JSNZ wild type (WT) strain.

Objective: To elucidate the role of Jep in the interaction between *S. aureus* and its host by comparing the impact of JSNZ WT with a mutant and a complement strain on the murine immune system. In addition, the elucidation of possible genetic factors behind host-adaptation of *S. aureus* strains isolated from wild rodents and shrews.

Methods: A *jep* complemented strain was generated by chromosomal replacement. JSNZ WT, the *jep* mutant and the complement strain were subjected to functional assays (whole blood survival assay, coagulation assay). In addition, the genetic background that might confer host specificity was tested by staph array genotyping.

Results: The mutant strain JSNZ∆*jep* was successfully complemented with the *jep* gene using a chromosomal integration approach. The WT strain and the complemented strain produced the Jep protein in comparable amounts. Unexpectedly, the complemented strains did not behave like the WT strain but rather like the mutant in a series of *in vitro* assays. Firstly, the growth of both the deletion mutant and the complemented strains was slightly reduced in TSB as compared to the WT strain. Secondly, the *jep* knockout strain showed a strongly reduced survival in murine whole blood compared to its wild type counterpart, but so did the complemented strain. Finally, the coagulation of murine plasma was less pronounced for the *jep* deletion mutant and the complemented strain as compared to the JSNZ WT. To exclude a defect in *jep* gene expression, we compared the amount of Jep expressed during growth in TSB medium for the three strains. The complemented strain produced Jep in a manner similar to the WT strain in a growth-phase dependent manner, suggesting that Jep expression was not affected during the creation of the complemented strain.

The array data showed some differences in the genetic makeup between animal isolated strains and matched human strains. For example, while all animal isolates of the CC88 lacked the resistance *mecA* gene it was found in some human isolates of the same strain.

Conclusion: In conclusion, our unidentified mutation created during the generation of the jep knock-out strain rather than the jep gene itself manipulated the murine immune response. The responsible gene and the underlying mechanisms remain to be clarified. Genetic profiling of *S. aureus* strains allowed us to obtain some valuable information including data about CC49, the most frequently isolated lineage in wild rodents and shrews where compared to the human isolates the murine strains showed clear signs of host adaptation. However, the analysis had several limitations including the small sample size.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Datum Unterschrift

Supplementary Data

Table S1: Origin and genetic characteristics of murine *S. aureus* strains isolated from wild small rodents and shrews.

No.	Strain ID[1]	*spa* type	MLST/ST[2]	MLST CC	*agr*	*mecA/C*	Phage integrase (type)	Species	Place of capture (region/country)	date of capture
1	dmn150401-647	t208	ST49*	CC49	II	-	5	yellow-necked mouse	MV, Germany	13.07.14
2	dmn150401-652	t208	ST49	CC49	II	-	5	bank vole	MV, Germany	24.09.14
3	dmn150401-653	t208	ST49	CC49	II	-	5	bank vole	MV, Germany	24.09.14
4	dmn150401-659	t208	ST49	CC49	II	-	5	bank vole	MV, Germany	24.09.14
5	dmn150401-660	t208	ST49	CC49	II	-	5	bank vole	MV, Germany	22.09.14
6	dmn150401-661	t208	ST49	CC49	II	-	5	bank vole	MV, Germany	26.09.14
7	dmn150401-666	t208	ST49	CC49	II	-	5	bank vole	MV, Germany	22.09.14
8	dmn150401-672	t208	ST49	CC49	II	-	5	yellow-necked mouse	MV, Germany	22.09.14
9	dmn150401-680	t208	ST49	CC49	II	-	5	yellow-necked mouse	MV, Germany	23.09.14
10	dmn160622-849	t208	ST49	CC49	II	-	5	bank vole	MV, Germany	06.10.15
11	dmn160622-853	t208	ST49	CC49	II	-	5	bank vole	MV, Germany	08.10.15
12	dmn150414-700	t4189	ST49*	CC49	II	-	5	yellow-necked mouse	BW, Germany	28.07.14
13	dmn150521-792	t4189	ST49	CC49	II	-	5	bank vole	BW, Germany	28.07.14
14	dmn150521-797	t4189	ST49	CC49	II	-	5	bank vole	BW, Germany	28.07.14
15	dmn150521-800	t4189	ST49	CC49	II	-	5	bank vole	BW, Germany	27.07.14
16	dmn150521-805	t4189	ST49	CC49	II	-	5	bank vole	BW, Germany	28.07.14
17	dmn150521-806	t4189	ST49	CC49	II	-	5	bank vole	BW, Germany	28.07.14
18	dsz140916-186-01	t1736	ST890*	Sg	IV	-	7	field vole	TH, Germany	24.09.13
19	dsz140916-186-02	t1773	ST890*	Sg	IV	-	7	common vole	TH, Germany	24.09.13
20	dmn150401-671	t1773	ST890	Sg	IV	-	1	yellow-necked mouse	MV, Germany	22.09.14
21	dmn150401-676	t1773	ST890	Sg	IV	-	1	yellow-necked mouse	MV, Germany	22.09.14

22	dmn150421-721	t1773	ST890	Sg	IV	-	7	yellow-necked mouse	TH, Germany	04.08.14
23	dmn150521-786	t1773	ST890	Sg	IV	-	7	yellow-necked mouse	BW, Germany	26.07.14
24	dmn150521-786	t1773	ST890	Sg	IV	-	7	yellow-necked mouse	BW, Germany	26.07.14
25	dmn160622-858	t1773	ST890	Sg	IV	-	1	yellow-necked mouse	MV, Germany	08.10.15
26	dmn160622-859	t1773	ST890	Sg	IV	-	1	yellow-necked mouse	MV, Germany	08.10.15
27	dsz140916-186-08	t2311	ST88*	CC88	III	-	1,2,3	field vole	TH, Germany	25.09.12
28	dsz140916-186-05	t843	ST130*	CC130	III	*mecC*	*5*	yellow-necked mouse	TH, Germany	26.09.13
29	dsz140926-190-42	t843	ST130	CC130	III	-	1	house mouse	MV, Germany	10.12.13
30	dmn150430-749	t9909	ST3033*	Sg	II	-	3,5,7	common shrew	TH, Germany	06.08.14
31	dmn150430-754	t9909	ST3033	Sg	II	-	3,5,7	common shrew	TH, Germany	05.08.14
32	dmn150430-756	t9909	ST3033	Sg	II	-	3,5,7	common shrew	TH, Germany	03.10.14
33	dmn150503-760	t9909	ST3033	Sg	II	-	3,5,7	common shrew	TH, Germany	05.08.14
34	dmn150507-765	t9909	ST3033	Sg	II	-	3,5,7	common shrew	TH, Germany	03.10.14
35	dmn150421-724	t15027	ST3252*	CC1956	IV	-	3,5	common vole	BW, Germany	22.10.14
36	dmn150421-725	t15027	ST3252*	CC1956	IV	-	3,5	common vole	BW, Germany	21.10.14
37	dmn150421-726	t3058	ST3252*	CC1956	IV	-	3,5	common vole	BW, Germany	21.10.14
38	dmn160708-880	t3058	ST3252	CC1956	IV	-	3,5	common vole	SMR, Czech Republic	29.11.14
39	dmn160628-865	t3058	ST3252	CC1956	IV	-	3,5	common vole	SMR, Czech Republic	29.11.14
40	dmn160628-867	t3058	ST3252	CC1956	IV	-	3,5	common vole	SMR, Czech Republic	28.11.14
41	dmn160630-870	t3058	ST3252	CC1956	IV	-	3,5	common vole	SMR, Czech Republic	29.11.14
42	dmn160706-871	t3058	ST3252	CC1956	IV	-	3,5	common vole	SMR, Czech Republic	29.11.14
43	dmn160706-874	t3058	ST3252	CC1956	IV	-	3,5	common vole	SMR, Czech Republic	29.11.14
44	dmn160707-878	t3058	ST3252	CC1956	IV	-	3,5	common vole	SMR, Czech Republic	29.11.14
45	dmn150527-824	t3830	ST1956*	CC1956	IV	-	5,7	common vole	MV, Germany	22.09.14
46	dmn150527-830	t3830	ST1956	CC1956	IV	-	7	field vole	MV, Germany	23.09.14
47	dmn150527-831	t3830	ST1956	CC1956	IV	-	5,7	field vole	MV, Germany	23.09.14
48	dmn150527-834	t3830	ST1956	CC1956	IV	-	5,7	common vole	MV, Germany	22.09.14

Abbreviations: MLST, multi-locus sequence typing; spa, staphylococcus specific protein A; ST, sequence type; CC, clonal complex; *agr*, accessory gene regulator; *mec*, methicillin

resistance; sg, singleton; MV, Mecklenburg-Western Pomerania; BW, Baden-Wuerttemberg; TH, Thuringia; SMR, South Moravian Region

1 Isolates in grey were tested with the *S. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany)

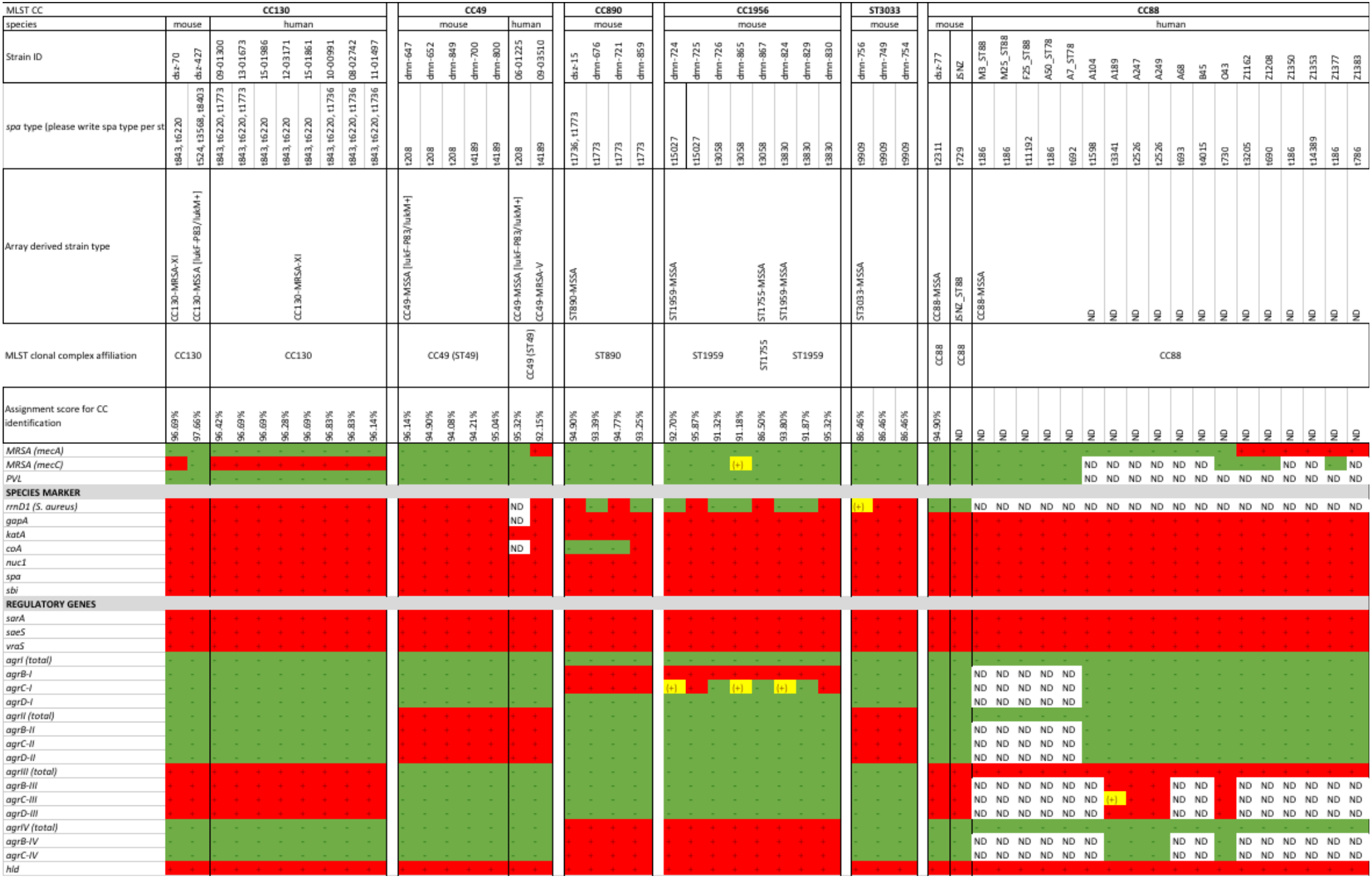
2 Isolates marked with an asterisk (*) were tested by MLST. ST and CC for unmarked isolates were derived from the *spa* type.

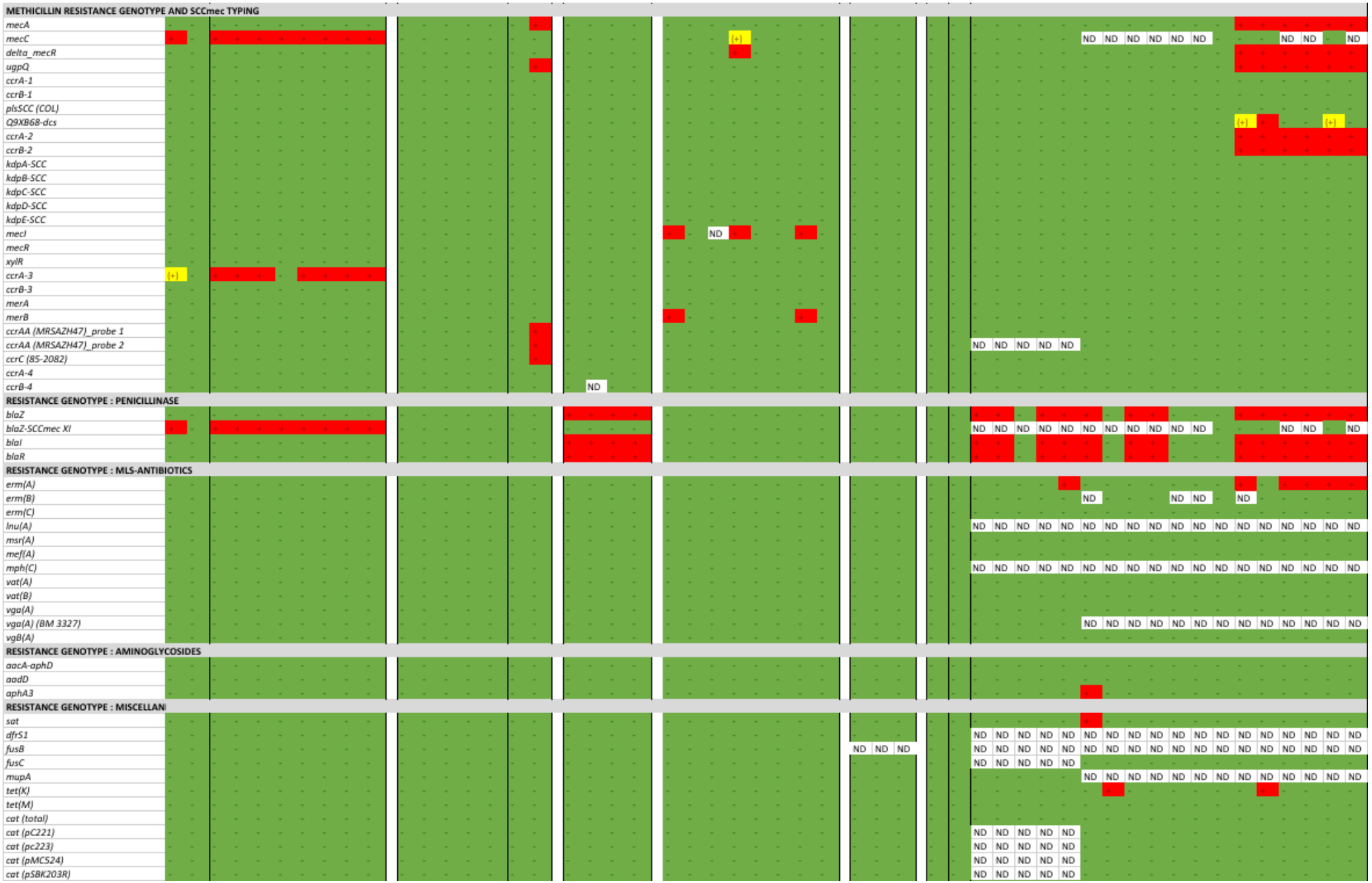
Table S2: Comparison of genetic traits of field vole CC88, murine CC88 (JSNZ) and human CC88

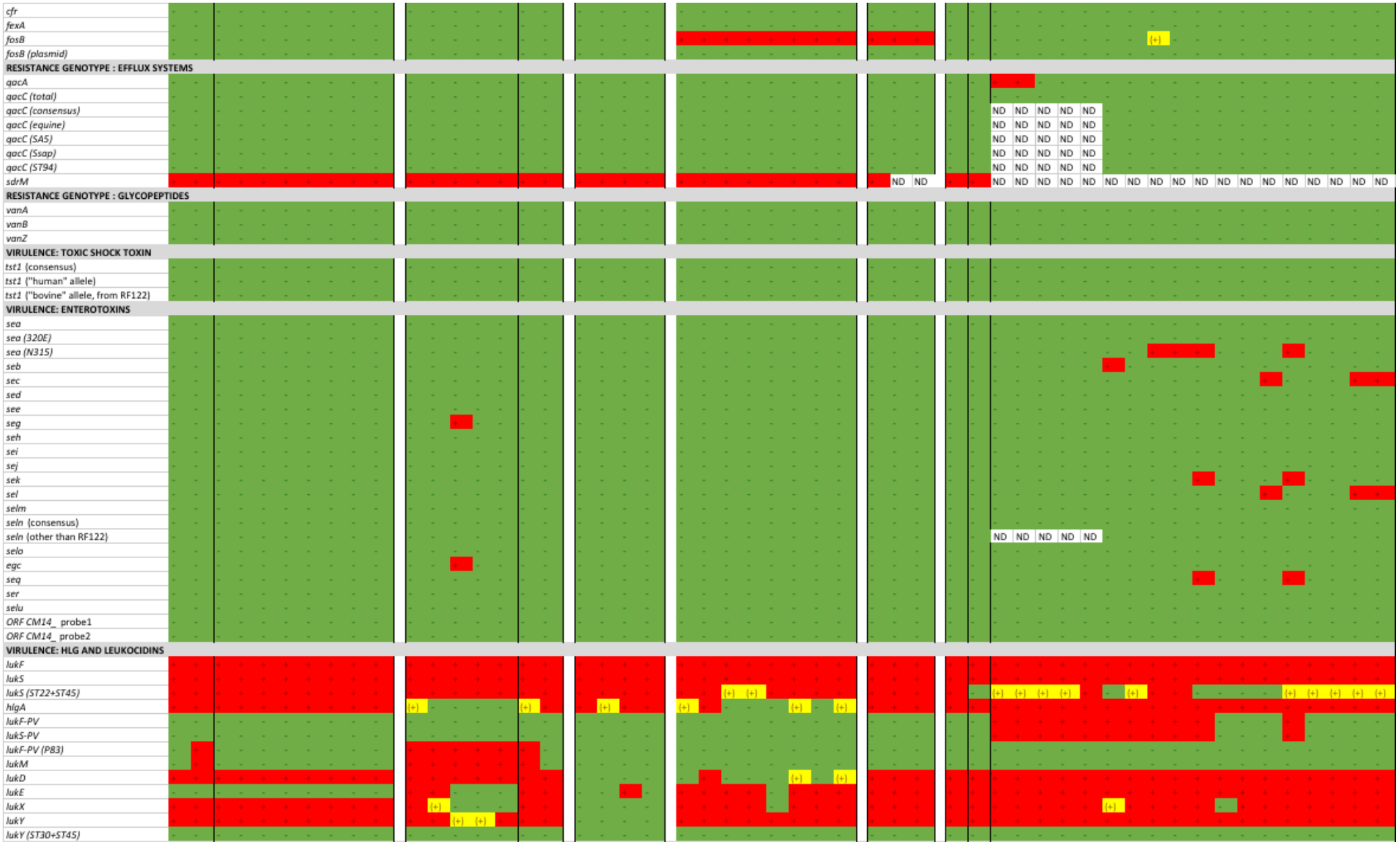
Reference number in Table 2/ name of *S. aureus* strain	**27**	**JSNZ**	**M3**	**M25**	**F25**	**A50**	**A7**	**A104**	**A189**	**A247**	**A249**	**A68**	**B45**	**O43**	**Z1162**	**Z1208**	**Z1350**	**Z1353**	**Z1377**	**Z1383**
***spa* type**	t2311	t729	t186	t186	t11192	t186	t692	t1598	t3341	t2526	t2526	t693	t4015	t730	t3205	t690	t186	t14389	t186	t786
Phage Integrase [2] (type)	1,2,3	none	1,3	1,3	3,7	2,3	3,4	2,3	2,3	2,3	2,3	1,2,3	3	3	3	1,2,3	3	3	3	3
MLST ST	ST88	ST88									ST88									
MLST CC	CC88	CC88									CC88									
origin	field vole	C57BL/6J (laboratory mouse)									human									
MRSA (*mecA)*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
METHICILLIN RESISTANCE GENOTYPE AND *SCCmec* TYPING																				
mecA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
delta_mecR																				
ugpQ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
ccrA-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
ccrB-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
RESISTANCE GENOTYPE : PENICILLINASE																				
blaZ	-	-	+	+	-	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+
blaI	-	-	+	+	-	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+
blaR	-	-	+	+	-	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+
RESISTANCE GENOTYPE: MLS-ANTIBIOTICS																				
erm(A)	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+	+	+	+
VIRULENCE: ENTEROTOXINS																				

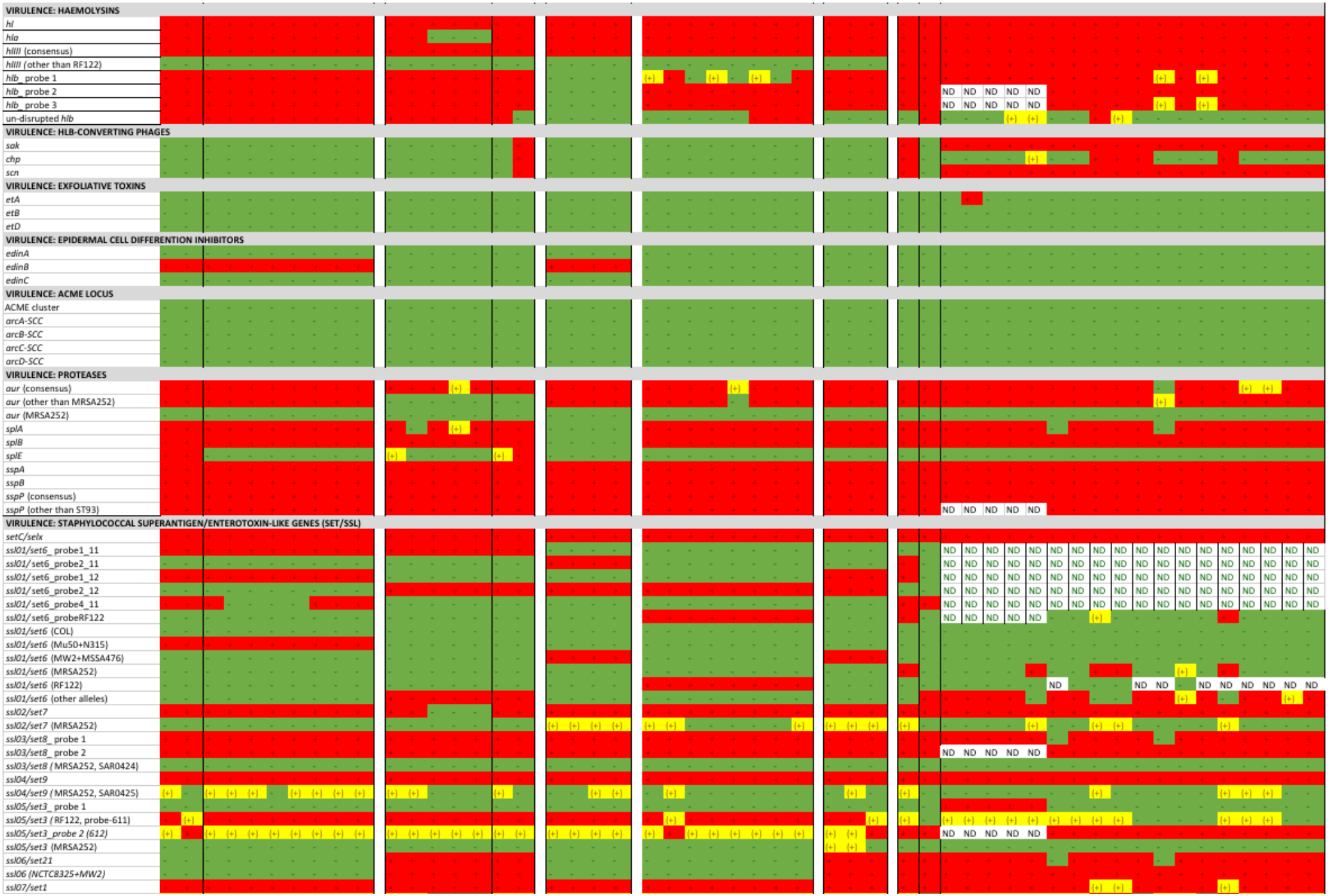
[2] Sa3int phage was detected by multiplex PCR by D. Mrochen

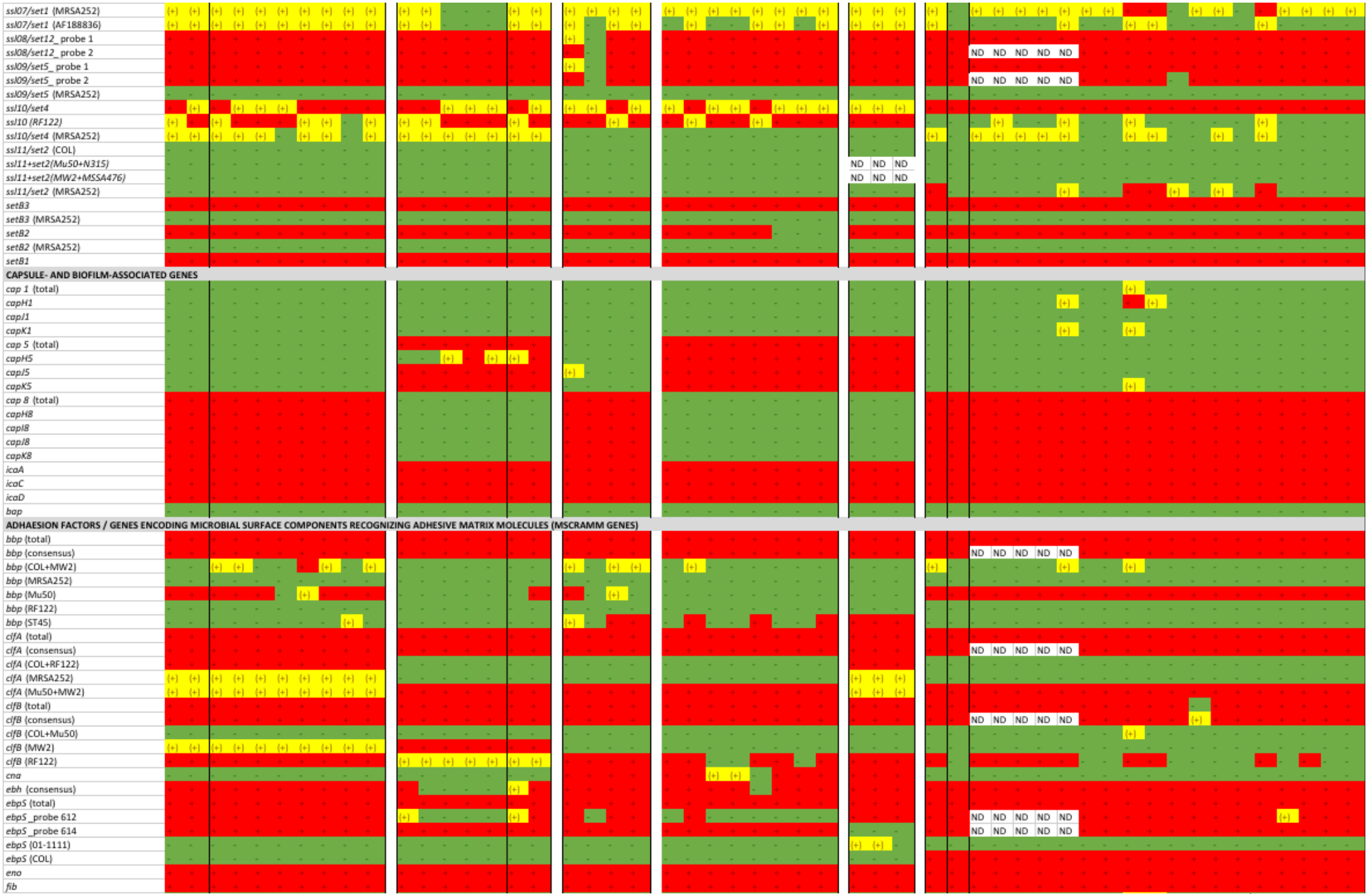
Table S311: Comparison of genetic traits of all the studied strains

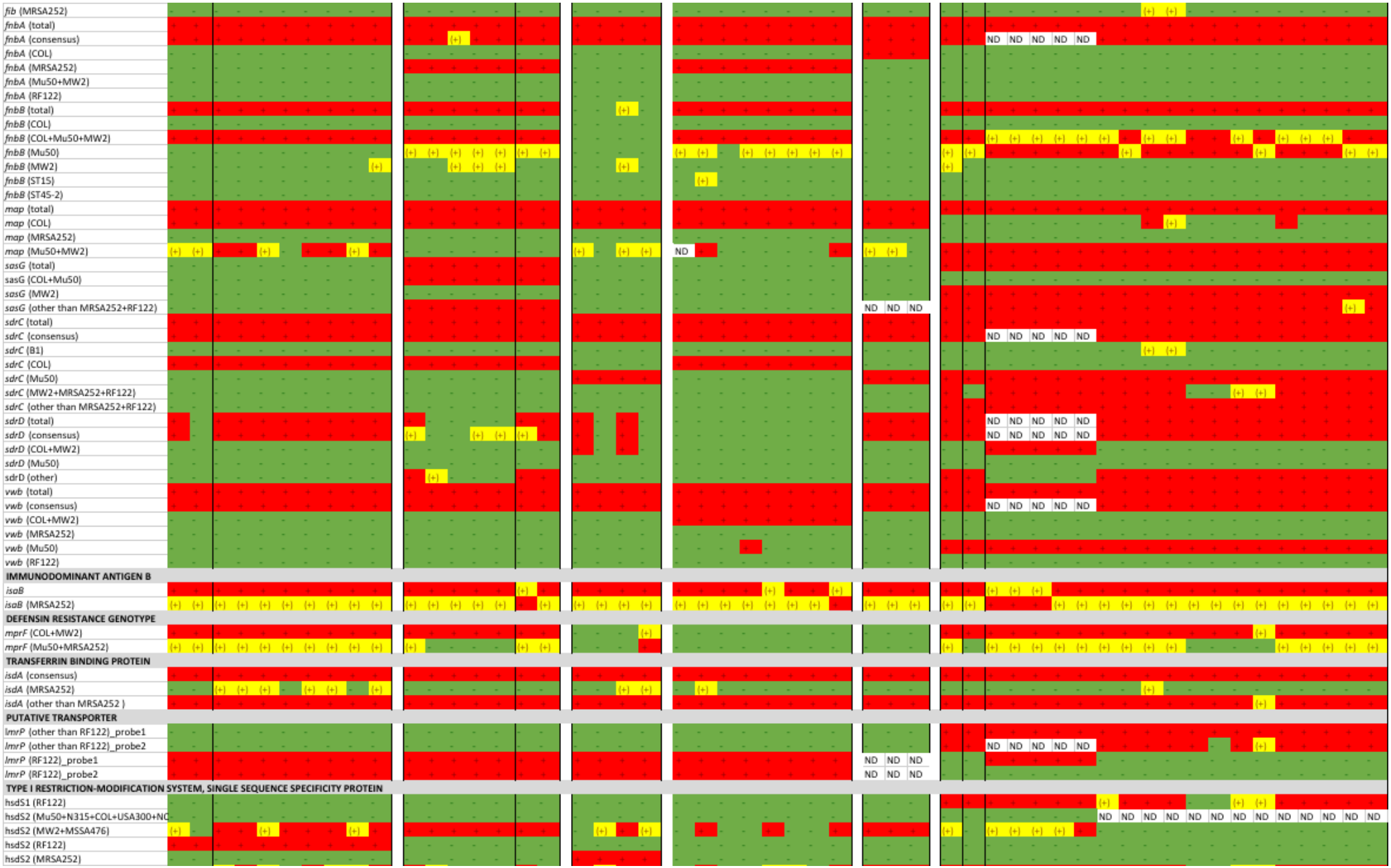

MLST CC
CC130
CC49
CC890
CC1956
ST3033
CC88
species
mouse
human
Strain ID
dsz-70
dsz-427
09-01300
13-01673
15-01986
12-03171
15-01861
10-00991
08-02742
11-01497
dmn-647
dmn-652
dmn-849
dmn-700
dmn-800
06-01225
09-03510
dsz-15
dmn-676
dmn-721
dmn-859
dmn-724
dmn-725
dmn-726
dmn-865
dmn-867
dmn-824
dmn-829
dmn-830
dmn-756
dmn-749
dmn-754
dsz-77
ISN2
M3_ST88
M25_ST88
F25_ST88
A50_ST78
A7_ST78
A104
A189
A247
A249
A68
B45
043
Z1162
Z1208
Z1350
Z1353
Z1377
Z1383
spa type (please write spa type per st
t843, t6220
t524, t3568, t8403
t843, t6220, t1773
t843, t6220, t1736
t208
t4189
t1736, t1773
t1773
t15027
t3058
t3830
t9909
t2311
t729
t186
t11192
t692
t1598
t3341
t2526
t2525
t693
t4015
t730
t3205
t690
t14389
t786
Array derived strain type
CC130-MRSA-XI
CC130-MSSA [lukF-PB3/lukM+]
CC49-MSSA [lukF-PB3/lukM+]
CC49-MRSA-V
ST890-MSSA
ST1959-MSSA
ST1755-MSSA
ST3033-MSSA
CC88-MSSA
ISNZ_ST88
CC88-MSSA
MLST clonal complex affiliation
CC130
CC49 (ST49)
ST890
ST1959
ST1755
CC88
Assignment score for CC identification
96.69%
97.66%
96.42%
96.69%
96.28%
96.83%
96.14%
96.90%
94.08%
94.21%
95.04%
95.32%
92.15%
94.77%
93.25%
92.70%
95.87%
91.32%
91.18%
86.50%
93.80%
91.87%
95.32%
86.46%
94.90%
MRSA (mecA)
MRSA (mecC)
PVL
SPECIES MARKER
rrnD1 (S. aureus)
gapA
katA
coA
nuc1
spa
sbi
REGULATORY GENES
sarA
saeS
vraS
agrI (total)
agrB-I
agrC-I
agrD-I
agrII (total)
agrB-II
agrC-II
agrD-II
agrIII (total)
agrB-III
agrC-III
agrD-III
agrIV (total)
agrB-IV
agrC-IV
hld
ND
(+)

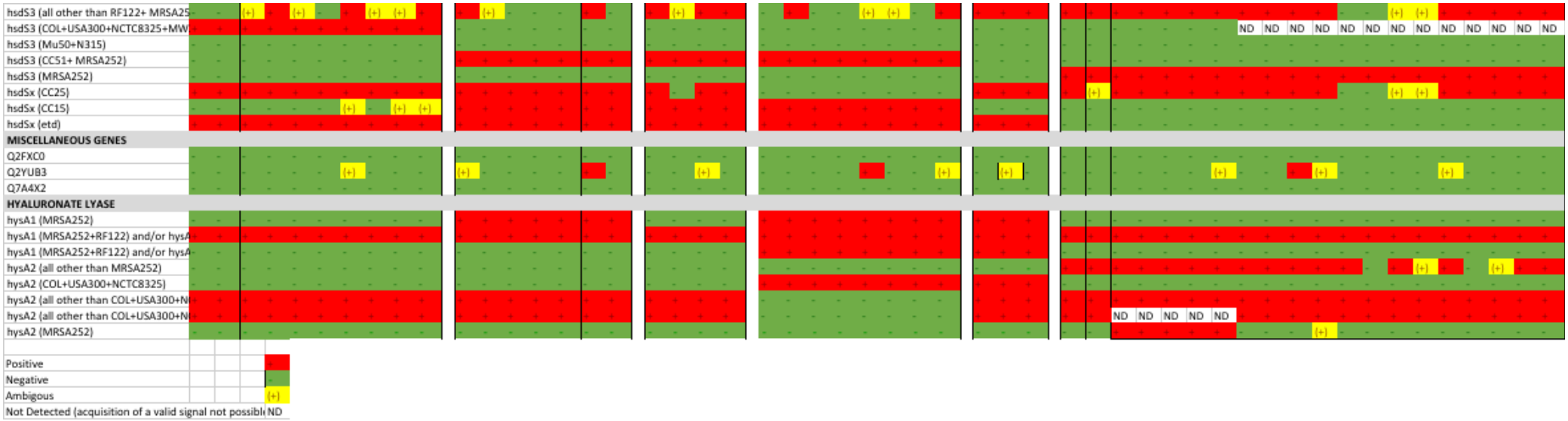

METHICILLIN RESISTANCE GENOTYPE AND SCCmec TYPING
mecA
mecC
delta_mecR
ugpQ
ccrA-1
ccrB-1
plsSCC (COL)
Q9XB68-dcs
ccrA-2
ccrB-2
kdpA-SCC
kdpB-SCC
kdpC-SCC
kdpD-SCC
kdpE-SCC
mecI
mecR
xylR
ccrA-3
ccrB-3
merA
merB
ccrAA (MRSAZH47)_probe 1
ccrAA (MRSAZH47)_probe 2
ccrC (85-2082)
ccrA-4
ccrB-4
RESISTANCE GENOTYPE : PENICILLINASE
blaZ
blaZ-SCCmec XI
blaI
blaR
RESISTANCE GENOTYPE : MLS-ANTIBIOTICS
erm(A)
erm(B)
erm(C)
lnu(A)
msr(A)
mef(A)
mph(C)
vat(A)
vat(B)
vga(A)
vga(A) (BM 3327)
vgB(A)
RESISTANCE GENOTYPE : AMINOGLYCOSIDES
aacA-aphD
aadD
aphA3
RESISTANCE GENOTYPE : MISCELLAN
sat
dfrS1
fusB
fusC
mupA
tet(K)
tet(M)
cat (total)
cat (pC221)
cat (pc223)
cat (pMC524)
cat (pSBK203R)


cfr
fexA
fosB
fosB (plasmid)
RESISTANCE GENOTYPE : EFFLUX SYSTEMS
qacA
qacC (total)
qacC (consensus)
qacC (equine)
qacC (SA5)
qacC (Ssap)
qacC (ST94)
sdrM
RESISTANCE GENOTYPE : GLYCOPEPTIDES
vanA
vanB
vanZ
VIRULENCE: TOXIC SHOCK TOXIN
tst1 (consensus)
tst1 ("human" allele)
tst1 ("bovine" allele, from RF122)
VIRULENCE: ENTEROTOXINS
sea
sea (320E)
sea (N315)
seb
sec
sed
see
seg
seh
sei
sej
sek
sel
selm
seln (consensus)
seln (other than RF122)
selo
egc
seq
ser
selu
ORF CM14_ probe1
ORF CM14_ probe2
VIRULENCE: HLG AND LEUKOCIDINS
lukF
lukS
lukS (ST22+ST45)
hlgA
lukF-PV
lukS-PV
lukF-PV (P83)
lukM
lukD
lukE
lukX
lukY
lukY (ST30+ST45)
ND
(+)


VIRULENCE: HAEMOLYSINS
hl
hla
hllll (consensus)
hllll (other than RF122)
hlb_ probe 1
hlb_ probe 2
hlb_ probe 3
un-disrupted hlb
VIRULENCE: HLB-CONVERTING PHAGES
sak
chp
scn
VIRULENCE: EXFOLIATIVE TOXINS
etA
etB
etD
VIRULENCE: EPIDERMAL CELL DIFFERENTION INHIBITORS
edinA
edinB
edinC
VIRULENCE: ACME LOCUS
ACME cluster
arcA-SCC
arcB-SCC
arcC-SCC
arcD-SCC
VIRULENCE: PROTEASES
aur (consensus)
aur (other than MRSA252)
aur (MRSA252)
splA
splB
splE
sspA
sspB
sspP (consensus)
sspP (other than ST93)
VIRULENCE: STAPHYLOCOCCAL SUPERANTIGEN/ENTEROTOXIN-LIKE GENES (SET/SSL)
setC/selx
ssl01/set6_ probe1_11
ssl01/set6_probe2_11
ssl01/set6_probe1_12
ssl01/set6_probe2_12
ssl01/set6_probe4_11
ssl01/set6_probeRF122
ssl01/set6 (COL)
ssl01/set6 (Mu50+N315)
ssl01/set6 (MW2+MSSA476)
ssl01/set6 (MRSA252)
ssl01/set6 (RF122)
ssl01/set6 (other alleles)
ssl02/set7
ssl02/set7 (MRSA252)
ssl03/set8_ probe 1
ssl03/set8_ probe 2
ssl03/set8 (MRSA252, SAR0424)
ssl04/set9
ssl04/set9 (MRSA252, SAR0425)
ssl05/set3_ probe 1
ssl05/set3 (RF122, probe-611)
ssl05/set3_probe 2 (612)
ssl05/set3 (MRSA252)
ssl06/set21
ssl06 (NCTC8325+MW2)
ssl07/set1
ND
(+)


sslO7/set1 (MRSA252)
sslO7/set1 (AF188836)
sslO8/set12_ probe 1
sslO8/set12_ probe 2
sslO9/set5_ probe 1
sslO9/set5_ probe 2
sslO9/set5 (MRSA252)
sslO10/set4
sslO10 (RF122)
sslO10/set4 (MRSA252)
sslO11/set2 (COL)
sslO11+set2(Mu50+N315)
sslO11+set2(MW2+MSSA476)
sslO11/set2 (MRSA252)
setB3
setB3 (MRSA252)
setB2
setB2 (MRSA252)
setB1
CAPSULE- AND BIOFILM-ASSOCIATED GENES
cap 1 (total)
capH1
capJ1
capK1
cap 5 (total)
capH5
capJ5
capK5
cap 8 (total)
capH8
capJ8
capJ8
capK8
icaA
icaC
icaD
bap
ADHAESION FACTORS / GENES ENCODING MICROBIAL SURFACE COMPONENTS RECOGNIZING ADHESIVE MATRIX MOLECULES (MSCRAMM GENES)
bbp (total)
bbp (consensus)
bbp (COL+MW2)
bbp (MRSA252)
bbp (Mu50)
bbp (RF122)
bbp (ST45)
clfA (total)
clfA (consensus)
clfA (COL+RF122)
clfA (MRSA252)
clfA (Mu50+MW2)
clfB (total)
clfB (consensus)
clfB (COL+Mu50)
clfB (MW2)
clfB (RF122)
cna
ebh (consensus)
ebpS (total)
ebpS _probe 612
ebpS _probe 614
ebpS (01-1111)
ebpS (COL)
eno
fib
ND


fib (MRSA252)
fnbA (total)
fnbA (consensus)
fnbA (COL)
fnbA (MRSA252)
fnbA (Mu50+MW2)
fnbA (RF122)
fnbB (total)
fnbB (COL)
fnbB (COL+Mu50+MW2)
fnbB (Mu50)
fnbB (MW2)
fnbB (ST15)
fnbB (ST45-2)
map (total)
map (COL)
map (MRSA252)
map (Mu50+MW2)
sasG (total)
sasG (COL+Mu50)
sasG (MW2)
sasG (other than MRSA252+RF122)
sdrC (total)
sdrC (consensus)
sdrC (B1)
sdrC (COL)
sdrC (Mu50)
sdrC (MW2+MRSA252+RF122)
sdrC (other than MRSA252+RF122)
sdrD (total)
sdrD (consensus)
sdrD (COL+MW2)
sdrD (Mu50)
sdrD (other)
vwb (total)
vwb (consensus)
vwb (COL+MW2)
vwb (MRSA252)
vwb (Mu50)
vwb (RF122)
IMMUNODOMINANT ANTIGEN B
isaB
isaB (MRSA252)
DEFENSIN RESISTANCE GENOTYPE
mprF (COL+MW2)
mprF (Mu50+MRSA252)
TRANSFERRIN BINDING PROTEIN
isdA (consensus)
isdA (MRSA252)
isdA (other than MRSA252)
PUTATIVE TRANSPORTER
lmrP (other than RF122)_probe1
lmrP (other than RF122)_probe2
lmrP (RF122)_probe1
lmrP (RF122)_probe2
TYPE I RESTRICTION-MODIFICATION SYSTEM, SINGLE SEQUENCE SPECIFICITY PROTEIN
hsdS1 (RF122)
hsdS2 (Mu50+N315+COL+USA300+NC
hsdS2 (MW2+MSSA476)
hsdS2 (RF122)
hsdS2 (MRSA252)


hsdS3 (all other than RF122+ MRSA25
hsdS3 (COL+USA300+NCTC8325+MW
hsdS3 (Mu50+N315)
hsdS3 (CC51+ MRSA252)
hsdS3 (MRSA252)
hsdSx (CC25)
hsdSx (CC15)
hsdSx (etd)
MISCELLANEOUS GENES
Q2FXC0
Q2YUB3
Q7A4X2
HYALURONATE LYASE
hysA1 (MRSA252)
hysA1 (MRSA252+RF122) and/or hysA
hysA1 (MRSA252+RF122) and/or hysA
hysA2 (all other than MRSA252)
hysA2 (COL+USA300+NCTC8325)
hysA2 (all other than COL+USA300+N
hysA2 (all other than COL+USA300+N
hysA2 (MRSA252)
Positive
Negative
Ambigous
Not Detected (acquisition of a valid signal not possibl
ND