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Microevolution of S. pseudintermedius isolated from one canine patient over a time span of seven years

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List of abbreviations

agr	accessory gene regulator
ack	housekeeping gene: acetate kinase
ATP	adenosine triphosphate
BHI	brain heart infusion
bla	beta-lactamase encoding gene
BLAST	basic local alignment tool
bp	base pairs
BRIG	blast ring image generator
BSA	bovine serum albumin
CHL	chloramphenicol
CLI	clindamycin
CoPS	coagulase-positive staphylococci
DNA	deoxyribonucleic acid
dNTP	desoxyribonucleotide triphosphate
DOX	doxycycline
Dsg	desmoglein
EDTA	ethylene diamine tetraacetic acid
EMBOSS	European molecular biology open software suite
ENR	enrofloxacin

List of abbreviations

Exp	exfoliative toxin of *S.pseudintermedius*
fdh	housekeeping gene: formate dehydrogenase
GEN	gentamicin
GOR	Garnier-Osguthorpe-Robson
HCL	hydrochloric acid
*hsp*60	heat shock protein 60 encoding gene
IMT	Institute of Microbiology and Epizootics
kb	kilobase
KCL	potassium chloride
KOH	potassium hydroxide
Luk	leukotoxin
MAR	marbofloxacin
MDR	multidrug resistance
MGE	mobile genetic elements
ml	milliliter
µl	microliter
MLST	multilocus sequence typing
MRS	methicillin-resistant staphylococci
MRSP	methicillin-resistant *S. pseudintermedius*
MSCRAMM	microbial surface components recognizing adhesive matrix molecule

MSSP	methicillin-susceptible *S. pseudintermedius*
NaCL	sodium chloride
nm	nanometer
NaOH	sodium hydroxide
NGS	next generation sequencing
nuc	nuclease encoding gene
ORF	open reading frame
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PEN	penicillin
PFGE	pulsed field gel electrophoresis
PHAST	phage search tool
PK	proteinase K
pta	phosphate acyltransferase encoding gene
*pur*A	housekeeping gene: adenylosuccinate synthetase
RAST	rapid annotation using subsystem technology
ref	reference
ResFinder	antimicrobial resistance gene finder
S.	*Staphylococcus*
sar	housekeeping gene: sodium sulfate symporter

SCC*mec*	staphylococcal cassette chromosome mec
SDS	sodium dodedyl sulfate
SE	staphylococcal enterotoxin
SIET	*Staphylococcus pseudintermedius* exfoliative toxin
SIG	*Staphylococcus intermedius*-group
sp.	species
sps	*S. pseudintermedius* surface protein encoding gene
ssp.	subspecies
ST	sequence type
SXT	sulfamethoxacole trimethoprim
syn	synonymus
tn	transposon
TSB	tryptic soy broth
tst	toxic shock syndrome toxin encoding gene
UPGMA	unweighted pair group method using arithmetic averages
WGS	whole genome sequence analysis

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1. Introduction

Opportunistic pathogens can cause infectious diseases when natural barriers of the host are lowered. Prominent examples of those bacteria that colonize the skin and mucous membranes of humans and animals are *Staphylococcus* (*S.*) species. *S. aureus* is a common asymptomatic colonizer of humans and it has been shown that carriage of this opportunistic pathogen is an important factor for developing various staphylococcal infections [1].

Although dogs can be colonized with *S. aureus* as well, the natural staphylococcal colonizer of the canine skin and mucous membranes is *S. pseudintermedius*. Dogs can be either colonized over a long time period or they can change the colonization status from carrier to non-carrier and vice versa. Additionally, some dogs are non-carriers for a long time span. It is also known that dogs are frequently colonized with different genetic lineages at different body sites [2-4].

S. pseudintermedius is responsible for a broad range of infections and recent studies revealed the skin as cause of pyoderma as one of the predominant infection sites [5,6]. The carrier status of the animal has been identified as an important risk factor for developing atopical dermatitis caused by *S. pseudintermedius* [2,7-9].

While different screening studies investigated the frequency and genetic variation of this opportunistic pathogen obtained from colonized and infected animals, there is no information available on the diversity of *S. pseudintermedius* in single patients that are frequently affected by infections with this pathogen.

Therefore the aims of this study were

i) To identify the diversity of *S. pseudintermedius* obtained from several infections of one canine patient during seven years
ii) To unravel microevolutionary changes of *S. pseudintermedius* sharing a similar genetic background by use of whole genome sequence analysis
iii) To analyze changes in the phenotypic behavior of the investigated *S. pseudintermedius* population.

2. Staphylococcus pseudintermedius

2.1 Taxonomy and characteristics

In general, staphylococci are coccoid shaped, Gram-positive, mainly facultative anaerobic, non-motile bacteria with a diameter of 0.5-1.5µm. The name *staphylococcus* (staphylé: Greek for grape) was chosen based on the appearance of the bacteria during microscopy, where they are frequently ordered in bunches of grapes or pairs. [10,11]. Up to date 81 different staphylococcal species and subspecies have been registered in the German collection of Microorganisms and Cell cultures (January 2016). Staphylococci are a member of the family *Staphylococcaceae*, class Bacillus and phylum Firmicutes.

Staphylococci are able to colonize the skin and mucous membranes of various animals and humans [12]. Furthermore, several staphylococcal species are opportunistic pathogens. Thus, hosts can be infected with staphylococci due to colonization by invasion of injured tissue or during immune suppression. Apart from *S. aureus*, an important opportunistic pathogen for humans and animals that was first isolated from humans in 1884 [13], *S. hyicus, S. schleiferi* ssp. *coagulans* and species of the *Staphylococcus intermedius*-group (SIG) are further relevant pathogenic species in veterinary medicine [14]. The SIG group includes the three closely related species *S. delphini*, *S. intermedius* and *S. pseudintermedius.* All these species express coagulase. Thus, they are categorized as coagulase positive staphylococci (CoPS) [15]. While *S. aureus* is the most important staphylococcal pathogen for humans, *S. pseudintermedius* accounts for the predominant CoPS in dogs. However, infections induced by other species like *S. aureus* and *S. schleiferi* ssp. *coagulans* can be observed during routine diagnostic of canine samples, as well [15].

Changes in the taxonomy of SIG species complicate the comparison of recent research with work performed before 2005, when *S. pseudintermedius* was defined as a new CoPS species [16].

Historically, *S. intermedius* was first described in 1976. The staphylococcal species was isolated from pigeons, dogs, minks and horses [17]. Different working groups compared isolates of this species and identified phenotypic [17,18] as well as genetic diversity [19]. *S. delphini* was defined as a new species in 1988 [20]. After description of *S. pseudintermedius* in 2005 it was noted that colonies of the three species mentioned above share similar phenotypic profiles, resulting in frequent misclassification. In 2007, detailed phylogenetic analyses of *S. intermedius* collections were conducted by two working groups, showing that these collections contained three distinct staphylococcal species, including *S. delphini* and *S. pseudintermedius* [21,22]. Furthermore, it was possible to point out an association between the host and the species. Isolates obtained from dogs, cats and humans were classified as *S. pseudintermedius* while most strains from horses and domesticated pigeons were categorized as *S. delphini*. *S. intermedius* were mainly isolated from feral pigeons. Even though the phylogenetic analysis allowed a clear distinction between each of these species, it became evident that they are closely related. Therefore, the term *Staphylococcus intermedius* group was introduced, including *S. delphini*, *S. intermedius* and *S. pseudintermedius*. Extensive investigation and reclassification of *S. intermedius* collections obtained before 2005 indicate that dogs are mainly infected with *S. pseudintermedius*. Thus, *S. intermedius* infections of dogs reported before introduction of this new SIG species should be considered as *S. pseudintermedius* [15].

The close relatedness of SIG species is not only displayed by phenotypic similarities according to colony morphology but also by difficulties to differentiate these species by use of biochemical tests. However, reliable distinction can be achieved by different molecular typing methods [22,23]. The comparison of sequences of the thermonuclease or heat shock protein encoding genes *nuc* or *hsp*60, for example, allows to differentiate SIG species [22]. However, this cost intensive and time consuming approach is not applicable as standard method in routine diagnostic. Therefore a sequencing-independent method was developed in 2009. Digestion of the housekeeping gene *pta* by use of the restriction enzyme *Mbo*I was reported to allow the identification of *S. pseudintermedius* based on a specific digestion pattern [23]. In 2010, another working group identified *S. pseudintermedius* with different *pta*

restriction pattern after digestion with *Mbo*I [24]. Thus, clear distinction cannot be achieved by use of this method in routine diagnostic.

2.2 Epidemiology of *S. pseudintermedius*

Although *S. pseudintermedius* has been isolated from different animals like cats and minks as well as humans in the past, the vast majority of studies identified dogs as the predominant host species of this opportunistic pathogen, including both, isolates from colonization and infection.

In order to determine the percentage of healthy colonized dogs, several point prevalence studies were conducted in the past, revealing a considerably high proportion of colonized animals, ranging between and 37 and 87% [8,9,25-27]. However, comparative analysis of these study results is not possible due to different study designs (e.g. enrichment of the sample, choice of the body site and inclusion of various sampling sites) as well as investigation of different study groups. Nonetheless, based on these studies it was possible to gain basic knowledge with respect to the epidemiology of *S. pseudintermedius*. Nowadays it is well known that *S. pseudintermedius* is part of the normal cutaneous microbiota of the dog and colonizes the skin and hair follicles as well as mucocutaneous membranes of mouth, nose and the anus. These findings provide strong evidence that dogs are the natural habitat of *S. pseudintermedius* [28].

Carriage rates at different body sites of dogs were compared in order to identify sampling sites that allow drawing a conclusion about the carrier status of a dog. Different studies recommend the simultaneous sampling of perineum and additionally one or two different sampling sites. These studies state that only the investigation of more than one sample site is sufficient to detect a high proportion of carriers [27,29-31]. Interestingly, genotypic characterization of isolates from different sampling sites of each animal revealed the capability of dogs to be infected with more than one genetic lineage [2-4]. Indeed, it was shown that the majority of dogs are colonized with isolates of different genetic lineages at different body sites.

Limitations of point-prevalence studies conducted to identify carriers are given by the lacking possibility to get information about the length of carriage. By consideration of the time it is possible to differentiate three colonization levels:

i) persistent carriage (permanent colonization during a long time period)
ii) intermittent carriage (alternating time points with and without colonization)
iii) non-carriage (lack of colonization at each investigated time point).

Based on the consideration of one sampling time point in point prevalence studies intermittent carriers can be misclassified as negative. Thus, longitudinal studies are essential to gain more knowledge about the role of intermittent and persistent carriage. So far, four longitudinal studies have been conducted, all of them describing a high proportion of persistently colonized dogs [29-32]. While most of the non-persistent carriers were intermittently tested positive for *S. pseudintermedius* only a limited number of dogs achieved the status of a non-carrier [30,31].

So far, little is known about the role and importance of transmission between dogs as well as between different mammalians. However, it was shown that vertical transmission between bitches and their puppies occurs frequently [33-35]. Furthermore, the study conducted by Paul and colleagues provides strong evidence that clones acquired by puppies due to vertical transmission may persist even after separation from the bitch [33]. Thus, vertical transmission seems to play an important role with respect to establishment of the individual microbiota of dogs.

Transmission between dogs living in the same household has been described as well in the past. Especially the investigation of animals in close contact with *S. pseudintermedius* infected dogs revealed high carriage rates for these contact dogs [29,36]. Even after successful treatment of the infection it was possible that both, former infected dogs and contact dogs, remained positive. Furthermore, one study described the existence of indistinguishable *S. pseudintermedius* in healthy dogs [33]. Based on current knowledge, it is evident that transmission between dogs and humans can take place even though humans are not typically colonized with this opportunistic pathogen [37-42]. However, these transmission events seem to be less frequent compared to transmission between dogs.

2.3 Pathogenicity of *S. pseudintermedius*

2.3.1 Infections in dogs

The opportunistic pathogen is an inhabitant of the normal canine cutaneous microbiota. Infections can be caused in dogs when the natural barrier of the colonized host is lowered. *S. pseudintermedius* can cause a variety of infections, including infections of the skin, ear, respiratory tract and urinary tract as well as wound infections [7-9,38,43-49]. Increasing reports on surgical site infections caused by *S. pseudintermedius* lead to the assumption that this species is an important nosocomial pathogen [6,50,51]. Current surveys identified the skin as cause of pyoderma as one predominant infection site [5,6]. In addition, colonization studies identified higher proportions of *S. pseudintermedius* carriers among dogs with clinical signs of atopical dermatitis in comparison to healthy dogs [2,7-9]. Various virulence factors have been identified in *S. pseudintermedius* but the virulence mechanisms are still not well understood. An overview of important factors is shown in figure 1. However, it is known that this opportunistic pathogen shows better adherence to corneocytes from diseased dogs (atopical dermatitis) in comparison to healthy animals [52,53].

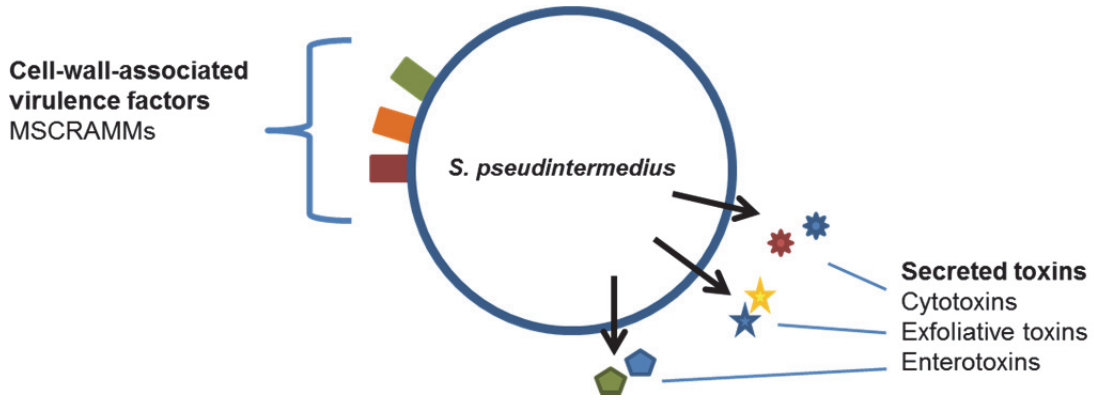

Figure 1: Schematic figure of selected cell-wall-associated as well as secreted virulence-associated factors of *Staphylococcus (S.) pseudintermedius*. Abbreviations: MSCRAMMs: microbial surface components recognizing adhesive matrix molecules. Figure modified from [28].

Only recently it was shown that the carrier status of the donor is an important factor with respect to adherence. Thus, independent of the genetic background from tested isolates, *S. pseudintermedius* adhered stronger to canine corneocytes obtained from persistent carriers compared to intermittent and non-carriers [54].

2.3.2 Virulence-associated factors

2.3.2.1 Cell-wall-associated virulence factors

Cell-wall-associated virulence factors are important for the initial adhesion of a pathogen to the host environment. In 2009, a study conducted by Geoghegan and colleagues identified several adhesins binding to the three important host extracellular matrix components fibrinogen, fibronectin and cytokeratin 10 that resemble well-characterized surface proteins of *S. aureus* [55]. Furthermore, whole genome analysis of *S. pseudintermedius* ED99 identified a variety of genes showing a typical cell-wall anchoring motif as well as additional characteristic motifs of cell-wall-associated adhesins (figure 2) [56].

Figure 2: Scheme of typical motifs for genes encoding cell-wall-anchored proteins. Red: signal sequence of MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), light blue: A-domain, green: repeat regions, dark blue: cell-wall-anchoring motif. Figure adapted from [56].

In total, the genome encodes 18 putative adhesins *sps*A to *sps*R (Abbreviation Sps: *S. pseudintermedius* surface protein). Different combinations of these 18 putative adhesins have been further identified in other *S. pseudintermedius* whereas all investigated isolates encoded at least 14 different *sps* genes [56]. Functional analyses were able to show that different adhesins mediate binding to canine corneocytes as well as to identical extracellular matrix components like fibrinogen [56,57].

2.3.2.2 Secreted toxins

Three different cytotoxins have been identified in *S. pseudintermedius* so far. The α- and ß-hemolysin cause hemolysis whereas the ß-hemolysin has a high affinity for sphingomyelin due to its sphingomyelinase activity [16,28]. In addition, *S. pseudintermedius* can produce a bicomponent leukotoxin Luk-I which is encoded on the two genes *luk*S and *luk*F. The toxin is responsible for cytotoxic activity on leukocytes [58]. However, the relevance of this virulence factor for pathogenesis remains unknown.

Exfoliative toxins are important virulence factors in CoPS such as *S. aureus* and *S. hyicus*. Until know, three exfoliative toxins are described for *S. pseudintermedius*. EXI (abbreviation: exfoliative toxin of *S. pseudintermedius*) was first characterized in 2009 [59]. The toxin causes intradermal splitting of desmoglein 1 (Dsg-1) in neonatal mice and dogs [59,60]. Another exfoliative toxin ExpB with the ability to split and degradade Dsg-1 was identified only one year later. Similar characteristics of the two toxins EXI and ExpB led to the suggestion to rename EXI to ExpA [61]. The *S. pseudintermedius* exfoliative toxin (SIET) has a rounding effect on epithelial cells. The relevance of SIET as virulence factor remains unclear since two independent study groups observed opposite effects in trials where dogs were confronted with SIET. While the first trial indicated that injection of SIET in healthy dogs leads to clinical signs that are similar to those of canine pyoderma [62,63] it was shown in vitro that SIET does not cause intradermal splitting [60].

The enterotoxins SEC_{canine} and SE-int have been identified in *S. pseudintermedius* [64,65]. Like other staphylococcal enterotoxins SEC_{canine} is able to induce vomiting and T-cell proliferation [64]. Only recently other enterotoxin encoding genes have been reported in *S. pseudintermedius*, including staphylococcal enterotoxin (*se*) encoding genes *sea*, *seb*, *sed*, *sei, sej sek* and *ser* as well as *tst* (toxic shock syndrome encoding gene) [7,66].

2.3.3 Beta-lactam antibiotics

Beta-lactam antimicrobials have a bactericidal effect based on the inhibition of the cell wall synthesis. The beta-lactam ring is the essential structure for these antibiotics and allows binding to staphylococcal penicillin-binding proteins (PBP). In case of interaction between the beta-lactam ring and PBP an essential transpeptidase is inactivated and therefore, not able to crosslink peptidoglycans during cell wall synthesis. Thus, structural integrity is lost during cell growth and the osmotic pressure results in disruption of the cell [67].

In staphylococci, resistance to beta-lactam antimicrobials can be achieved by two different mechanisms:

(i) Expression of a beta-lactamase
(ii) Expression of PBP 2A.

Beta-lactamases are able to destroy the essential beta-lactam ring structure due to hydrolytic splitting [68]. Four beta-lactamases A-D have been described so far. The four beta-lactamases are either constantly expressed (type D) or after induction due to treatment with beta-lactams (type A-C). The enzymes are encoded on mobile genetic elements (MGE), including a transposon (Tn552) as well as different plasmids [69].

Different studies were able to show that a high proportion of *S. pseudintermedius* harbor the ß-lactamase containing transposon Tn552, encoding the gene *blaZ* [26,70,71]

The second very successful strategy to avoid the bactericidal effect of beta-lactam antibiotics is the production of the alternative penicillin binding protein PBP 2A. Due to expression of PBP 2A resistance is conferred to all beta-lactam antibiotics including penicillinase-stable beta-lactams. Like PBP, PBP 2A functions as a transpeptidase. However, in contrast to PBP this enzyme has a low affinity to beta-lactam antimicrobials. Therefore, interaction with beta-lactams is very low and the function of the essential transpeptidase remains intact. Based on the name of the first semi-synthetic beta-lactamase-stable beta-lactam antimicrobial methicillin these

staphylococci were named as methicillin-resistant staphylococci (MRS). The methicillin resistance encoding gene *mec*A is located on a mobile genetic SCC*mec* (staphylococcal cassette chromosome *mec*) element [72]. In total, 11 different SCC*mec* types as well as SCC*mec* hybrids have been identified in staphylococci. Different SCC*mec* types have been identified in MRSP screening studies. However, SCC*mec* type II-III, a hybrid containing elements from SCC*mec* II and SCC*mec* III [73-76] as well as SCC*mec* type III [49,77,78] were described frequently in the past. Beside resistance to beta-lactams, *S. pseudintermedius* are frequently multidrug resistant (MDR) and thus, express resistance to three or more different antimicrobial classes [79,80].

2.3.4 Further antimicrobial classes

Antimicrobial resistance encoding genes are either chromosomally encoded or they are located on mobile genetic elements like transposons or plasmids. Mobile genetic elements carrying resistance genes can be transferred between different bacteria. Frequently, MGE are transferred between isolates of the same species (intra-species transmission) but transmission between different species occurs as well [84]. However, in *S. pseudintermedius* transposons seem to be the predominant MGE referring resistance to this staphylococcal species [79,80]. Table 1 provides an overview on identified mobile resistance genes in *S. pseudintermedius* isolates.

Besides the possibility to acquire resistance encoding genes that are located on mobile genetic elements, resistance can be also obtained by mutations of chromosomally encoded genes. Resistance to fluoroquinolones can be transferred by mutations of the genes *gyr*A, *gyr*B, *grl*A or *grl*B. Rifampicin resistance can be acquired due to point mutations of the gene *rpo*B [79].

Table 1: Overview on relevant resistance encoding genes modified from [79].

Antimicrobial class	**Resistance gene**	**encoding protein**
Aminoglycosides	*aac*A-*aph*D	acetyltransferase and phosphotransferase
	*aph*A3	phosphotransferase
	*sat*4	acetyltransferase
	*aad*E	adenyltransferase
	ant	aminoglycoside nucleotidyltransferase [85]
Chloramphenicol	cat_{pC221}	acetyltransferase
Fusidic acid	*fus*B	protective protein [75]
	*fus*C	protective protein
Macrolides and lincosamides	*erm*(A), (B), (C)	rRNA-methylase
	mph(C)	phosphotransferase [75]
	msr(A)	ABC-transporter
	lnu(A)	nucleotidyltransferase
Mupirocin	*ile*S2	isoleucyl-tRNA synthetase [86]
Tetracyclines	*tet*(M), (O)	ribosome protective protein
	tet(K), (L)	efflux system
Trimethoprim	*dfr*G	dihydrofolate reductase
	*dfr*K	dihydrofolate reductase [75]

2.4 Characterization of *S. pseudintermedius*

While subpopulations within each bacterial species can be differentiated by specific phenotypic as well as genotypic characteristics, the distinction of bacterial subpopulations based on genetic diversity forms the basis for investigation of epidemiological pathways. Various genetic characterization methods have been introduced in the past for each bacterial species and are essential in order to understand the genetic relationship of bacterial subpopulations.

In 2001, Achtmann distinguished the two characterization approaches classification and typing [87]. In this context, classification is defined as a top-down strategy. Thus, classification is used to differentiate subgroups of one bacterial species and therefore to subdivide species into clonal groups. Classification methods need to allow the detection of relationships at the species level. Therefore, they are used for long-term global epidemiology. In contrast, typing methods are defined as a bottom-up approach. Hence, they are used to distinguish the maximum number of isolates. Typing methods are useful to investigate short-term, local epidemiology within one bacterial species.

In general, molecular characterization methods need to be robust and reproducible so that comparison of results between different laboratories is possible. Classification methods usually include a limited number of information like the sequence analysis of seven housekeeping genes during multilocus sequence typing (MLST) or the investigation of restriction sites within one genome by use of pulsed field gel electrophoresis (PFGE). With the ability to consider information of the entire genome by use of whole genome sequence analysis (WGS) a new typing era was introduced. WGS data provide information about classical molecular typing methods like MLST. In addition, they can provide valuable information on the entire gene repertoire as well as on sequence variations. In recent years, high-throughput sequencing allows detailed phylogenetic analyses of various bacterial species and provides valuable information on the spread and distribution as well as the capacity to adapt to specific ecological niches or hosts [88].

While many different typing methods have been successfully introduced for *S. aureus*, only a limited number of techniques have been successfully implemented for the rather young species *S. pseudintermedius*. The two predominant typing methods used in order to differentiate *S. pseudintermedius* subpopulations are MLST and PFGE analysis.

2.4.1 Multilocus Sequence Typing (MLST)

Multilocus sequence typing provides information on the genetic relatedness of bacterial isolates within one species based on the investigation of six to eight housekeeping genes. MLST analysis is a very robust and reliable method to gain knowledge about the existence and distribution of distinct genetic lineages. In 1999, the first MLST scheme was developed for Neisseria meningitidis [89]. Since then, MLST databases were established for various microorganisms. In general, MLST typing is based on sequencing results of highly conserved regions (alleles) from well-characterized essential genes. These so-called housekeeping genes are present in all isolates of one species and therefore, they are reliable targets for typing. The sequence of each allele can be assigned to a specific allele number by use of the specific MLST database for the respective microorganism. The allele numbers from each investigated locus define a specific multilocus sequence type (ST). Due to a slow accumulation of point mutations in these stable alleles, MLST provides information about evolutionary changes over a long time-scale. The first *S. pseudintermedius* MLST scheme was introduced in 2007 only a short time after definition of this new species and the description of the SIG complex. This MLST was based on alleles of the four housekeeping genes *tuf*, *cpn*60, *pta* and 16S rRNA [21] and is therefore named MLST-4 in the literature [90]. By use of the MLST-4 scheme it was possible to differentiate each SIG species. In addition, MLST-4 allowed in combination with the investigation of the *agr*D locus a distinction between different *S. pseudintermedius* genotypes [74,78]. In order to obtain a higher resolution, Solyman and colleagues expanded the MLST-4 scheme by inclusion of alleles from the housekeeping genes *ack*, *fdh*, *pur*A and *sar* as well as exclusion of 16SrRNA [90]. The use of this new scheme based on information of seven loci shows a greater genetic diversity and a better distinction of genetic lineages in comparison to the MLST-4 scheme.

2.4.2 Pulsed Field Gel Electrophoresis (PFGE)

PFGE analysis investigates DNA fingerprints obtained after fragmentation of chromosomal DNA with a specific restriction enzyme. DNA fragment patterns from different isolates can be compared, providing information about the putative relatedness. For *S. pseudintermedius*, *Sma*I is the restriction enzyme of choice in the literature [2,3,30,66,74,75,78]. PFGE has been very useful to compare individual isolates of putative outbreaks and to investigate potential nosocomial infection routes, when linked to epidemiological data [29,31]. However, expansion of PFGE analysis between different laboratories as well as between different countries is very difficult due to the band-based typing approach.

2.4.3 Whole Genome Sequence Analysis (WGS)

WGS offers the opportunity to consider and compare information of the whole genome, including the gene content as well as structural differences of specific genes. So far, WGS datasets are available for four *S. pseudintermedius* in the NCBI database (March 2016) ranging in size from 2.6 to 2.8 Megabases (Mb) and encoding more than 2400 genes. The different genome size already indicates the variability of the genome content in *S. pseudintermedius*. In general, each bacterial genome can be divided in a very stable part that consists of a highly conserved gene set as well as in a variable part. The conserved part of the genome has been denominated as core genome in the past and consists of genes that can be found in each isolate within a distinct species. The accessory genome includes genes that can be found in subsets of strains and provide specific individual features for these isolates [88]. As one important part of the accessory genome, mobile genetic elements (MGE) can easily transmit important virulence as well as resistance encoding genes and thus, enhance the diversity of a genetic population. By use of WGS data it is possible to consider each part of the genome and investigate fine-scale evolution of bacteria.

2.4.3.1 Extended MLST analysis

Population structure analyses provide important information of bacterial evolution and spread of successful lineages. Like described above, WGS enables researcher to include information on each gene of the genome. Nevertheless, the method chosen to compare isolates within one species needs to be robust. Therefore, core genomes were defined for several bacterial species in the past. These defined core genomes contain all genes that can be identified in each isolate of the respective species [91]. However, in 2014 von Mentzer and colleagues used a different approach in order to analyze the phylogeny of 362 enterotoxigenic *E. coli* (ETEC). This approach assumes a variability of shared genes in each investigated subpopulation that depends on the diversity of isolates included in the analysis. In order to use the maximum number of genes that are present in all investigated isolates it is necessary to define these genes for each analyzed subpopulation. This subset of genes that is present in all investigated isolates has been defined as maximum common genome (MCG) by von Mentzer and colleagues [92]. Investigation of multiple whole genome data sets from isolates of one bacterial species by extended MLST analyses follow a similar approach. By comparison of all available genes in a defined subpopulation it is possible to identify all genes that are present in all investigated isolates, which resembles the MCG described above [93]. While investigation of the MCG was based on the analysis of the occurrence and frequency of single nucleotide polymorphisms (SNPs), extended MLST is performed by construction of allele profiles based on differences in the gene structure considering SNPs as well as deletions and insertions.

During SNP analysis all point mutations in genes as well as single deletions or insertions of nucleotides of the MCG gene content are considered in order to unravel the relatedness of isolates. SNPs are the most frequent form of variation in bacteria. Thus they represent very good targets to define specific subpopulations. It is possible to differentiate synonymous and non synonymous mutations. While synonymous mutations do not alter the amino acid sequence, non synonymous changes lead to an alternative amino acid structure which might have impact on the protein structure and the functionality of the protein. These non synonymous mutations might result in

a selective advantage for the bacterium. Therefore, SNPs are able to give insights into the evolution of bacteria [94].

However, allele-based attempts allow additionally consideration of insertions and deletions and thus, provide the opportunity to consider more genetic changes in the genes of the maximum common genome by use of a gene-by-gene comparison approach. Comparative analyses showed similar population structures for a subpopulation using SNP analysis and the allele based approach. Thus, both methods are capable to analyze the phylogeny of bacterial populations [95].

2.4.3.2 The accessory genome

The accessory genome contains all genes that are not defined as MCG. Therefore, all genes that cannot be identified in all genomes account for the accessory genome. Furthermore, genes that are present in one single isolate are a component of the accessory genome [96]. It is possible to differentiate the accessory genome, considering chromosomally encoded genome content and plasmids. The main part of the accessory genome is provided by various mobile genetic elements (MGE), including transposons, insertion elements, pathogenicity islands, bacteriophages and plasmids. These MGE can be transmitted between different isolates and strongly contribute to diversification of bacteria.

The investigation of the accessory genome can provide essential information on virulence and resistance determinants by detection of virulence-associated or resistance encoding genes [80,96]. These gene subsets that contain all virulence-associated or resistance encoding genes are defined as virulome resp. resistome. Relevant genes that define the resistome and virulome of *S. pseudintermedius* are defined in section 2.3.2 - 2.3.4. Although information on the resistome and virulome of a subset of isolates provides important information on the diversity and putative advantages of a specific strain, phenotypic characterization is necessary to investigate the impact of the identified genes on the bacterial behavior.

Corresponding to the resistome it is important to determine the phenotypic resistance profile. Like shown in section 2.3.4, the predominant mechanism in order to acquire

resistance is the uptake of mobile elements harboring resistance encoding genes. However, resistance can be also transferred by point mutations like it has been shown for resistance to fluoroquinolones for *S. pseudintermedius*. Furthermore, resistance genes are frequently encoded on plasmids. By use of the Illumina Sequencer (Illumina Inc. ©, San Diego, CA, USA) chromosomal DNA is sequenced sufficiently, while plasmids are only partly present in WGS data sets.

In addition, changes in the gene structure based on mutations, insertions or deletions can inhibit or enhance the effect of the gene. Therefore, only phenotypic tests can be used in order to determine the true resistance profile of the investigated isolate. However, genotypic characterization of the resistome provides basal information on the presence or absence of important resistance encoding genes.

3. Material and Methods

3.1 Material

3.1.1 Bacterial isolates, reference strains and oligonucleotide primers

In total, 37 colonies of 25 *S. pseudintermedius* positive samples were investigated in this study. All samples were obtained from wound swabs as well as one blood culture of one specific canine patient from the Small Animal Clinic of the Freie Universität Berlin. Further samples of the same animal patient were obtained from swabs taken by the owner of the animal or a different veterinary practice. From samples that were not provided by the Small Animal Clinic of the Freie Universität Berlin further information on treatment as well as sampling site are not available.

The samples arrived between 2008 and 2014 and species as well as phenotypic resistance profiles were determined during routine diagnostic of the Institute of Microbiology and Epizootics (IMT). Mainly, one *S. pseudintermedius* colony per sample was stored in stocks containing 15% glycerol at -80 °C in the strain collection of the IMT for further investigation. In three cases, multiple phenotypically identical colonies were stored in the strain collection.

Genotypic characterization was performed for all available isolates (table 2). Multiple colonies obtained from one swab are marked in purple (June 2011), orange (July 2011) and blue (August 2014).

Table 2: *S. pseudintermedius* of one canine patient obtained between 2008 and 2014 from the Small Animal Clinic

strain ID	isolation date	sample
16402	25.08.2008	wound swab
16403	25.08.2008	wound swab
16543	20.10.2008	blood culture
18149	13.02.2009	wound swab
19298	14.09.2009	wound swab
20293	15.12.2009	wound swab
21171	27.04.2010	wound swab
21356	10.05.2010	wound swab
21485	28.05.2010	wound swab
21759	20.07.2010	wound swab
23677	27.08.2010	wound swab
25558	05.05.2011	wound swab
25688	20.05.2011	wound swab
26148	10.06.2011	wound swab
26149	10.06.2011	wound swab
26150	10.06.2011	wound swab
26151	10.06.2011	wound swab
26154	10.06.2011	wound swab
26155	10.06.2011	wound swab
26152	10.06.2011	wound swab
26153	10.06.2011	wound swab
26542	05.07.2011	wound swab
26543	05.07.2011	wound swab
27972	19.09.2011	wound swab
28733	27.01.2012	wound swab
28796	22.02.2012	wound swab
29108	13.03.2012	wound swab
29328	13.04.2012	wound swab
30243	05.09.2012	wound swab
31163	16.05.2013	wound swab
31164	16.05.2013	wound swab
33113	04.04.2014	wound swab
33600	20.06.2014	wound swab
33875	17.07.2014	wound swab
33972	08.08.2014	wound swab
33974	08.08.2014	wound swab
33973	08.08.2014	wound swab

Table 3: Reference strains

strain	species	properties	reference
NCTC 8325	*S. aureus*	PFGE reference strain	
ED99	*S. pseudintermedius*	complete genome sequence available	
W3110	*E. coli*	Positive control for biofilm formation	
AAEC189	*E. coli*	negative control for biofilm formation	

Table 4: Oligonucleotide primers

target gene	primer sequence	Tm (°C)	product size	reference
*tet*K	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	54°C	360bp	[97]

The oligonucleotide primers were obtained from Eurofins MWG Operon, Ebersberg, Germany in lyophilized form, suspended in Millipore water and stored at -20°C.

3.1.2 Consumables, equipment and chemicals

Most consumables were obtained from companies based in Germany. Therefore the country is only mentioned for cities located outside of Germany.

Consumables

15 ml tubes, conical	Sarstedt, Nümbrecht
50 ml tubes, conical	Sarstedt, Nümbrecht
Cryotubes 1.5 ml	Greiner Bio-One, Frickenhausen
Culture tubes, glass 10 ml, 20 ml	Carl Roth, Karlsruhe
Deep well plate, 96 well	Sarstedt, Nümbrecht
Disposable pipette 10 ml, 25 ml	Sarstedt, Nümbrecht
Disposable pipette tips 10ml	Sarstedt, Nümbrecht
Disposal bags	Sarstedt, Nümbrecht
Gas permeable sealing membrane	Carl Roth, Karlsruhe
Glass ware	Schott, Mainz
Gloves	Carl Roth, Karlsruhe
Inoculation loops	Greiner Bio-One, Frickenhausen
Microtest plate, F-profile, polystyrene (biofilm formation)	Carl Roth, Karlsruhe
Microtest plate, sterile, F-profile (fibrinogen / fibronectin binding)	Brand, Wertheim

Parafilm	Carl Roth, Karlsruhe
PCR reaction tubes 0.2 ml	Sarstedt, Nümbrecht
Petridishes	Sarstedt, Nümbrecht
Plastic cuvettes	Brand, Wertheim
Reaction tubes 1.5 ml, 2 ml	Sarstedt, Nümbrecht
Spatula	Sarstedt, Nümbrecht

Equipment

Autoclave	Systec, Wettenberg
Centrifuge	Sigma, Osterode am Harz
Chef-DR III Pulsed Field	Bio-Rad, München
ELISA reader	Bio Tek, Bad Friedrichshall
Gel chambers for agarose electrophoresis	AGS, Heidelberg
Gel documentation system	herolab Laborgeräte, Wiesloch
Ice machine	Dr. Heinekamp, Karlsfeld
Incubator for bacteria plates	Binder, Tuttlingen
Incubator, shaker for liquid bacteria cultures	GFL, Burgwedel
Laboratory centrifuge	Eppendorf, Wesseling-Berzdorf
Laminar flow cabinet	Nuaire, Plymouth, USA
Magnetic stirrer	IKA Labortechnik, Staufen
Microwave	
MiSeq Desktop Sequencer	Illumina, San Diego, California
Multichannel pipet, 100 µl, 300 µl	Eppendorf, Wesseling-Berzdorf
pH/ ion meter	Knick, Berlin
Pipettes	Eppendorf, Wesseling-Berzdorf
Pipettor	Brand, Wertheim
Power Supply for Electrophoresis	Bio-Rad, München
Spectrophotometer for cultures	Eppendorf, Wesseling-Berzdorf
Spectrophotometer for DNA, Nanodrop	Thermo Scientific, Schwerte
Table centrifuge	Eppendorf, Wesseling-Berzdorf
Thermocycler, PCR	Biometra, Göttingen
Thermomixer	Eppendorf, Wesseling-Berzdorf
Vortex	IKA Labortechnik, Staufen
Water bath	Theodor Karow, Berlin
Water preparation	Merck Millipore, Darmstadt

Chemicals

Acetone	Carl Roth, Karlsruhe
Agarose	Biodeal, Markkleeberg
Ammoniumacetate	Ambion, Berlin
Ammonium sulphate	Sigma-Aldrich, Steinheim
Boric acid	Carl Roth, Karlsruhe
Bovine serum albumin V (BSA)	Carl Roth, Karlsruhe

Brain heart infusion	Oxoid, Basingstoke, UK
Bromphenolblue	Merck, Darmstadt
Crystal violet	Carl Roth, Karlsruhe
dNTP mix	Carl Roth, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe
Ethanol	Carl Roth, Karlsruhe
Fibrogen, human	Calbiochem, Nottingham, UK
Fibronectin, human	Biochrom, Berlin
Gel Red	Biotium, Köln
Glacial Acetic Acid	Carl Roth, Karlsruhe
Glucose, Monohydrat	Merck, Darmstadt
Glycerol	Carl Roth, Karlsruhe
Hydrochloric acid (HCl)	Carl Roth, Karlsruhe
Isopropanol	Carl Roth, Karlsruhe
Midori Green, DNA dye	Biozym Scientific, Hessisch Oldendorf
Peptone (Casein)	Oxoid, Basingstoke, UK
Phenol/Chloroform/Isoamylalcohol	Carl Roth, Karlsruhe
Potassium chloride (KCl)	Carl Roth, Karlsruhe
Potassium hydroxide (KOH)	Sigma-Aldrich, Steinheim
Potassium Phosphate	Merck, Darmstadt
Sodium Dodecyl Sulfate (SDS)	Serva, Heidelberg
Sodium acetate	Serva, Heidelberg
Sodium citrate	Carl Roth, Karlsruhe
Sodium chloride (NaCl)	Carl Roth, Karlsruhe
Sodium hydroxide (NaOH)	Carl Roth, Karlsruhe
Tris base	Carl Roth, Karlsruhe
Tri-sodium citrate	Carl Roth, Karlsruhe
Tryptic soy broth (TSB)	Carl Roth, Karlsruhe

Ladders

100 bp DNA ladder	Fermentas, St. Leon-Rot
1 kbp DNA ladder	Fermentas, St. Leon-Rot
GeneRuler High Range DNA Ladder	Fermentas, St. Leon-Rot
Lambda DNA ladder	New England Bio Labs Inc., Schwalbach/Taunus

Enzymes

DNA taq polymerase	Fermentas, St. Leon-Rot
Proteinase K	Carl Roth, Karlsruhe
Lysostaphin	Sigma-Aldrich, Steinheim
Restriction endonuclease *Sma*I	Fermentas, St. Leon-Rot
Tango buffer with BSA	Fermentas, St. Leon-Rot

Commercial kits

QIA amp DNA Mini Kit	Qiagen, Hilden
QIA quick PCR Purification Kit	Qiagen, Hilden
Nextera XT kit	Illumina Inc., San Diego, California, USA

3.1.3 Media, buffers and solutions

Media

Brain heart infusion (BHI)	Oxoid, Wesel
Columbia agar with sheep Blood Plus	Oxoid, Wesel
Tryptic soy broth (TSB)	Carl Roth, Karlsruhe

Buffers and solutions

0.9 NaCl			
	NaCl		9.0 g
	distilled water		ad 1 l
DNA extraction solutions			
TE buffer (pH 7)	EDTA		1mM
	Tris Ultra Quality		10mM
	distilled water		ad 10 ml
	HCl	to adjust the pH	
Solutions for Agarose Gel electrophoresis			
Tris Borate EDTA (TBE) 10x			
	Tris Ultra Quality	890 mM	107.82 g
	Boric acid	890 mM	55.03 g
	EDTA solution (pH 8.0)	500 mM	18.62 ml
	NaOH	to adjust the pH	
	distilled water		ad 100 ml
2. 1.5 % Agarose Gel			
	Agarose		6.0 g
	TBE 1x		400 ml
Midori Green, DNA dye			4 µl
Gel Red, DNA dye			
Solution for Polymerase chain reaction (PCR)			
	10x Green Buffer		
	PCR Nucleotide Mix (dNTPs: dATP, dCTP, dGTP, dTTP)		10mM each
	Primer-Mix		10 pmol each
	DreamTaqDNA Polymerase		5 U/µl
	DNA-Template		2 µl

Solutions and buffers for pulsed-field gel electrophoresis (PFGE)

Cell suspensions buffer (pH 7.2)			
	EDTA	50 mM	1.861g
	NaCl	20 mM	0.117g
	Tris Ultra Quality	10 mM	1.211g
	HCl	to adjust the pH	
	distilled water		ad 100 ml
Lysis buffer (pH 7)			
	EDTA	20 mM	1.861g
	NaCl	50 mM	0.292g
	Tris Ultra Quality	10 mM	1.211g
	Deoxycholat	0,20%	0.2g
	Sarcosyl	0,50%	0.5g
	HCl	to adjust the pH	
	distilled water		ad 100 ml
Wash buffer (pH 7)			
	EDTA	0.1 mM	0.004g
	Tris Ultra Quality	10 mM	1.211g
	HCl	to adjust the pH	
	distilled water		ad 100 ml
PK buffer (pH 9)			
	EDTA	250 mM	9.305g
	Sarcosyl	1%	1g
	NaOH	to adjust the pH	
	distilled water		ad 100 ml

Solutions for biofilm formation assay

Crystal violet staining solution	Crystal violet		0.5 g
	distilled water		ad 500 ml
Ethanol-acetone (80:20)	Ethanol		400 ml
	Acetone		100 ml

solutions for plasmid preparation

500 mM EDTA (pH 8)	EDTA		186.15 g
	distilled water		ad 1l
Loading buffer (pH 8)	Bromphenolblue		40 mg
	Glycerin		20 ml
	EDTA	0.5M (pH 8)	8 ml
	Tris	10 mM	400.00 µl
	Millipore water		11.60 ml

Lysis buffer	Millipore water		0.95 ml
	SDS	10%	0.60 ml
	Tris	250 mM	0.40 ml
	NaOH	5 N	30.00 µl
10% SDS	SDS		5,00 g
	Millipore water		50ml
1 M Tris-Cl (pH 8)	Tris		60.57 g
	distilled water		ad 500 ml
	HCl	-25%	to adjust the pH
250 mM Tris	Tris		15.14 g
	distilled water		ad 500 ml
50x TAE Buffer	Tris		242 g
	Glacial Acetic Acid		57.10 ml
	EDTA	0.5 M (pH 8)	100.00 ml
	distilled water		ad 1l

3.1.4 Software

Antimicrobial Resistence Gene Finder (ResFinder), version 2.1, (freeware)	Identification of resistance encoding genes
BioNumerics version 7.5, Applied Maths ©, Belgium	Analysis and dendrogram construction for *S. pseudintermedius* macrorestriction pattern
Blast Ring Image Generator (BRIG), version 0.95 (freeware)	Comparative analysis of the genome content, visualization tool
geneious, version 6.1.5, Biomatters Ltd. ©, New Zealand	Comparative analysis of the genome content, identification of single nucleotide polymorphisms
Microsoft Office 2010, Microsoft ©, USA	Graphs, Tables, Images, Text
NanoDrop 1000, version 3.6.0, Thermo Fisher, USA	DNA quantification
Phage Search Tool PHAST (freeware)	characterization of phage gene containing regions
Phyre2, version 2, (freeware)	protein modelling of the three dimensional structure
Ridom Seq Spere+, version 2.3.1, Ridom GmbH ©, Münster	Genome assembly, extended multilocus sequence typing (MLST) analysis, construction of the minimum spanning tree
SPSS Statistics, version 20, IBM ©, USA	Statistics, Graphs

3.2 Methods

3.2.1 DNA isolation, whole genome sequencing and polymerase chain reaction

DNA isolation

A suspension of one *S. pseudintermedius* colony in 25µl lysostaphin (0.1mg/ml) was incubated at 37°C for 10 minutes. After adding 25µl proteinase K (0.1mg/ml) and 75µl TE buffer a second incubation step followed at 37°C for 20 minutes. After boiling the suspension for 5 minutes, the DNA was purified by use of the DNA Mini Kit (Quiagen, Hilden).

Whole genome sequence analysis

In total, 38 *S. pseudintermedius* were sequenced. The DNA-library was prepared by use of the Nextera XT kit (Illumina Inc. ©, San Diego, CA, USA) and sequencing was performed on an Illumina MiSeq sequencer according to the manufacturer´s instructions aiming for minimum coverage of 100 fold. Quality trimming and *de novo* assembly were carried out using the Velvet assembler [98] integrated in Ridom SeqSphere+ software (version 2.3.1, Ridom GmbH ©, Münster, Germany) [93]. Technical assistance during sequencing was kindly provided by Dr. Inga Eichhorn and Petra Krienke.

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to determine the presence of the tetracycline resistance encoding gene *tet*K. PCR was performed for all *S. pseudintermedius* using the following pipette scheme:

10 x green buffer	2.5 µl
dNTPs (10mM each)	0.6 µl
Primer mix (10pM each)	5 µl
DreamTaq DNA-polymerase 5 U/µl	0.1µl
template DNA	2 µl
Millipore water	ad 14.8 µl

The PCR reaction was carried out with following temperatures:

step	temperature	time
pre-denaturation	95°C	5 minutes
denaturation	95°C	1 minute
annealing	55°C	30 seconds
elongation	72°C	45 seconds
end elongation	72°C	10 minutes

The steps denaturation, annealing and elongation were performed in 24 circles.

Gel electrophoresis was performed by use 1.5% agarose. The product size was determined by use of a 100 bp DNA ladder after staining of the products with Midori Green.

3.2.2 Pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

Pulsed field gel electrophoresis

Plug preparation

A single *S. pseudintermedius* colony was picked from a culture incubated overnight at 37°C on sheep blood agar. The colony was suspended in 5 ml BHI and incubated overnight at 37°C in an incubator which allows shaking at 200 rpm (rotations per minute). 150µl of the incubated bacterial solution were pipetted into a 1.5ml reaction tube and pelleted by centrifugation at 13.000g for 1min. Suspension of the pellet followed by use of 150µl cell suspension buffer. The pre-warmed suspension was kept at 37°C in a thermomixer. Liquid 1.4% peqGOLD agarose was stored in a water bath at 56°C.

2µl lysostaphin (1mg/ml) were added to the cell suspension. After short mixing of the tube, 150µl agarose were immediately added to the suspension and mixed by pipetting. The mixture was transferred into plug molds. Plugs dried at room temperature for 10-15 minutes. Afterwards plugs were moved into a 1.5ml tube and covered with 500µl lysis buffer. Incubation followed at 37°C for one hour. After removing the lysis buffer another incubation step followed in 500µl PK/PK buffer (PK buffer substituted with proteinase K at 500µg/ml) at 55°C for 30 minutes. Next, the buffer was removed and replaced by 1ml washing buffer. After incubation for one hour, the step was repeated two more times. The plugs were stored in the fridge at 4°C.

Pulsed field gel electrophoresis

One third of each plug was placed in a new 1.5ml tube and equilibrated in 300µl restriction buffer (30 minutes at 4°C). The buffer was replaced with fresh restriction

buffer. 2µl lysostaphin were added to the buffer, followed by incubation at 37°C for 5 hours.

1.2% peqGOLD agarose gel was prepared and used to prepare a PFGE gel. The plugs as well as the lambda ladder were placed into the gel and sealed with agarose. Electrophorese was performed in 0.5 TBE (cooled at 14°C) by use of following run conditions:

First block: switching time 8 hours, angle 120°, pulse time 5-15 seconds

Second block: switching time 10 hours, angle 120°, pulse time 15-60 seconds

The gel was stained with Gel Red for 15 minutes. The restriction pattern was stored by use of a gel documentation system (Herolab Lab Systems, Wiesloch).

PFGE analysis by use of Bionumerics (Applied Maths©, Belgium)

Bionumerics (Applied Maths©, Belgium) was used to analyze the macrorestriction patterns obtained for each *S. pseudintermedius*. Bands were marked in each pattern and similarity of all patterns was analyzed by calculation of dice coefficient with a tolerance set at 1.5% and optimization of 0.5%. The dendrogram was constructed by use of “unweighted pair group method using arithmetic averages” (UPGMA) clustering like it has been described before [78].

Multilocus sequence typing (MLST)

MLST was performed for all *S. pseudintermedius* isolates by investigation of the seven housekeeping genes *ack*, *cpn*60, *fdh*, *pta*, *pur*A, *sar* and *tuf* according to the recently developed scheme published by Solyman et al. [99]. Alleles were obtained from whole genome sequence data. Unknown sequence types were assigned to the MLST database (http://pubmlst.org/ spseudintermedius/) in order to obtain a sequence type.

3.2.3 Extended MLST analysis

Phylogenetic analysis was carried out for assembled genomes by extended MLST using SeqSphere+ v2.3.1 (Ridom GmbH ©, Münster). Genes present on the published genome ED99 (Accession number CP002478) [100] served as basis for the extended MLST scheme. Genes shared by all isolates of this study, excluding those with premature stop codons and ambiguous nucleotides, were included for typing. Altogether, 1845 targets were determined as shared genes. The minimum spanning tree was generated based on extended MLST typing results.

3.2.4 Comparative analysis of the genome content

Comparative analysis of the genome content was performed for isolates sharing the same sequence type. Therefore, the scaffold order of the contigs was determined for each genome by mapping to the reference genome ED99 using geneious (version 6.1.5) software (Biomatters Ltd. ©, New Zealand). Assembled contigs were submitted to the RAST server (http://rast.nmpdr.org/) [101] for genome annotation and manually inspected using geneious (version 6.1.5) software (Biomatters Ltd. ©, New Zealand). Variability of the genome content was investigated for isolates sharing distinct genotypes by use of progressive mauve alignment (geneious version 6.1.5; Biomatters Ltd. ©, New Zealand) and visualised by use of the freeware BRIG (Blast Ring Image Generator) [102]. Single nucleotide polymorphisms (SNPs) were determined in open reading frames (ORFs) with geneious (version 6.1.5) (Biomatters Ltd. ©, New Zealand). SNPs located in mobile genetic elements or low-quality regions (insertions and deletions (indels) / low coverage/repeat regions) were excluded from the analysis.

The online tool PHAST (phage search tool) was used to characterize regions harbouring phage encoding genes (http://phast.wishartlab.com/) [103].

Resistance encoding genes were determined by use of the freeware ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) [104].

3.2.5 Protein prediction

Two different tools were used in order to predict the secondary as well as the three dimensional structure. Prediction of the secondary structure was performed by use of geneious (version 6.1.5) software (Biomatters Ltd. ©, New Zealand). In this program, protein prediction is modelled with the EMBOSS (European Molecular Biology Open Software Suite) 6.5.7 tool garnier. The tool garnier allows the prediction of secondary protein structures by use of the GOR (Garnier-Osguthorpe-Robson) method. The prediction is based on mathematical modelling. The database available for the modelling includes 26 protein chains with a total number of 2,400 amino acids [105]. By use of this prediction tool it is possible to differentiate between alpha-helices, beta-strands, coils and turns based on the amino acid structure of a protein. Furthermore, geneious allows to comparatively analyze predicted secondary structures of two amino acid sequences. The method is very fast since it is computationally fast and utilizes less CPU memory compared to other prediction tools.

The freeware Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) was used to predict the three dimensional structure of the amino acid sequences of interest by use of template-based modelling. This approach is based on the comparative analysis of the amino acid sequence of interest with the protein database UniProtKB (http://www.uniprot.org/uniprot/) which contains more than 100,000 experimentally determined three dimensional protein structures.

3.2.6 Plasmid profile analysis

An overnight culture was prepared by inoculation of a single colony in 5ml BHI medium and incubation at 37°C on a shaker. The bacterial cells were pelleted by centrifugation of 1ml overnight culture for 5 minutes at 12,000g in a 1.5ml tube. After discarding the supernatant, the cells were resuspended in 20µl TE buffer by vortexing. Afterwards, 100µl lysis buffer were added to the suspension and mixed by inverting the tube four times. After incubating the suspension for 25 minutes at 58°C, 100µl phenol/chlorophorm/isoamylalcohol (lower phase) were added. The tube content was mixed by inverting the tube 100 times. After centrifugation at 16,000g for

15 minutes the supernatant was pipetted into a fresh 1.5ml tube and 20µl DNA loading dye were added. The tube was incubated for 30 minutes on ice. 50µl of the supernatant were then loaded into a freshly prepared 0.4% gel by use of TAE buffer. 1kb as well as Gene ruler high range DNA ladder served as size references. The electric field was applied to the gel for 16 hours at 25 volt. Afterwards the gel was stained in a 10% Gel Red solution for 15 minutes.

3.2.7 Fibrinogen and fibronectin binding assays

An overnight culture was prepared by inoculation of a single colony in 5ml BHI medium and incubation at 37°C on a shaker. In addition, a fibrinogen dilution (40ng/µl) was prepared with 1x PBS. 200µl of the solution were pipetted in the first lane of a 96 well plate. The remaining wells were filled with 100µl PBS by use of a multichannel pipette. Afterwards a dilution series was prepared along seven rows. The last row yielded only 100µl PBS and served as control. The plate was covered and stored over night at 4°C.

After incubation, the supernatant was discarded and washed with PBS by submerging the whole plate in a container filled with PBS and dried on a tissue paper. The wells were then blocked with 100µl of a 5% bovine serum albumin solution and the covered plate was incubated for 2 hours at 37°C.

After incubation, the BSA was removed and the plate was washed three times in PBS.

The bacterial cells obtained from the overnight culture were pelleted by centrifugation for 10 minutes at 1,500g. The pellet was resuspended in 1ml PBS and the optical density was adjusted to OD_{600}=1. 100µl of the cell suspension were added to each well of the 96 well plate that was coated with fibrinogen and incubated for two hours at 37°C. Non adherent cells were removed with the pipette after incubation. The wells were washed three times with 200µl PBS and afterwards fixated with 100µl 99% methanol for 20 minutes. After removing the methanol and washing the plate in PBS staining of the cells was performed by use of 1% crystal violet and incubation for 1 minute. The remaining stain solution was removed and the plate was washed three times with PBS. Cells were disrupted by adding 100µl acetic acid (5%) to each well and incubation for 30 minutes with gentle shaking. The absorbance was measured on an ELISA plate reader at 590nm.

Fibronectin binding assay was performed with the same protocol by coating the well with fibronectin instead of fibrinogen.

3.2.8 Biofilm formation assay

An overnight culture was prepared by inoculation of a single colony in 5ml BHI medium and incubation at 37°C on a shaker. All overnight cultures were diluted 1:10 in TSB medium and the optical density was adjusted to OD_{600}=0.05 in fresh TSB medium. 200µl were pipetted in triplicates into 96 well plates and covered with a permeable film. After incubation for 24 hours at 37°C without shaking, bacterial suspension was removed and each well was washed three times with 1x PBS. After drying the plate 100µl methanol were added to each well and incubated for 20 minutes to fixate the cells. After removing methanol, the plate was dried. Staining of the cells was performed by adding 200µl of 0.1% crystal violet solution to each well and incubation for one minute. After removing the solution, the plate was washed three times in PBS and air-dried. Afterwards cells were disrupted by use of 100µl acetic acid (5%) and incubation for 30 minutes with gentle shaking. The absorbance was measured on an ELISA plate reader at 590nm.

4. Results

4.1 Cultivation and isolation of bacteria and detection of methicillin-resistance

This study investigates the genetic relationship of *S. pseudintermedius* isolates obtained from one canine patient between 2008 and 2014 as cause of multiple wound and soft tissue infections. In total, 33 clinical samples were investigated during routine diagnostic at the Institute of Microbiology and Epizootics (IMT) identifying 25 *S. pseudintermedius* positive samples. While *S. pseudintermedius* was mainly identified in pure culture, five samples contained *S. pseudintermedius* in mixed culture. Among others, species identified in mixed culture included *Trueperella pyogenes*, *Streptococcus sp.*, *E. coli* and *Klebsiella pneumoniae*.

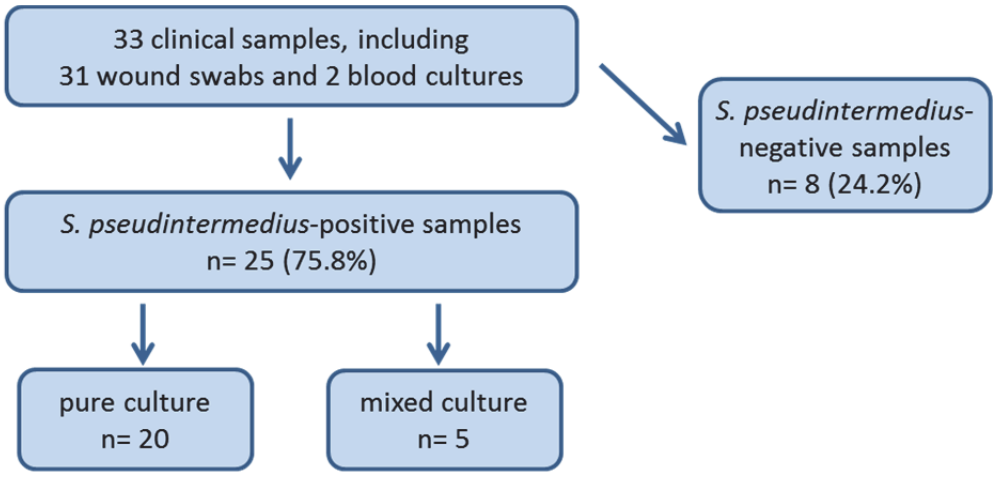

Figure 3: Overview on clinical samples obtained from one canine patient between 2008 and 2014

In addition to colonies expressing the classical *S. pseudintermedius* phenotype (grey-white opaque colonies with a diameter of 1-2mm and production of double zone hemolysis on sheep blood agar) smaller grey-white colonies with identical hemolysis were observed in similar number on blood agar plates on three occasions (Mai 2010, January 2012 and July 2014). However, sub-cultivation of these colonies resulted in

colonies showing similar colony sizes of 1-2mm diameter like usually reported for *S. pseudintermedius*.

Colonies of *S. pseudintermedius* isolated from each infection were stored in the strain collection of the IMT. On limited occasions more than one colony was stored and therefore available for further characterization. In general, each available isolate was included into the analysis. Thus, some isolates originate from identical clinical samples. Figure 4 provides an overview on the number of colonies per clinical sample that were included in this study.

In total, 37 isolates were available for investigation. MRSP as well as MSSP were identified at 18 respectively 10 time points, including three occasions were mixed infections with MRSP and MSSP where determined. Initial infections (in 2008) were caused by MSSP. In September 2009, the first MRSP infection was detected. Since then, MRSP as well as MSSP were identified as cause of multiple soft tissue infections until August 2014 (figure 4). Table 2 (page 31) provides additional information on the isolation site of each *S. pseudintermedius* positive swab.

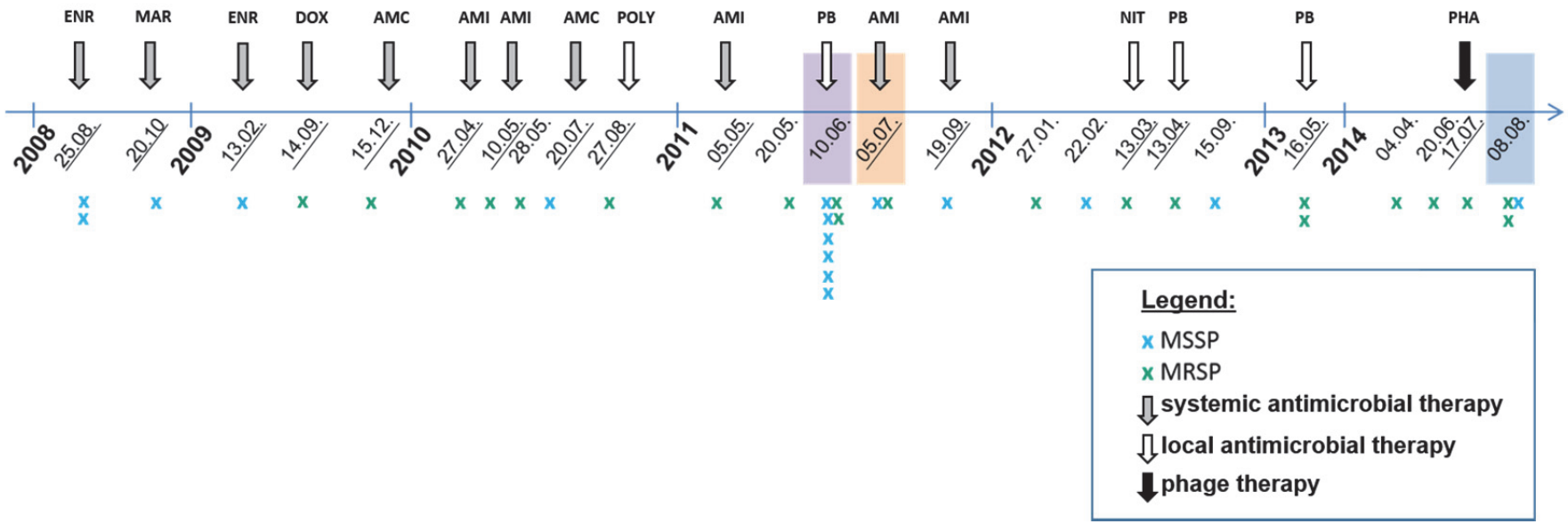

Figure 4: Isolation dates of *S. pseudintermedius* from clinical samples. Islolates cultivated from swabs obtained during investigation of the Small Animal Clinic are marked with underlined isolation dates. Further samples were obtained directly from the animal´s owner or another veterinarian. Information on treatment for isolates originating from these dates were not available in this study. Arrows above the timeline indicate treatment: systemic treatment = grey shaded arrows, local application = white filled arrows, phage therapy (PHA) = black arrow. Abbreviations of antibiotics used for treatment: AMI: amikacin; AMC: amoxicillin / clavulanic acid; DOX: doxycycline; ENR: enrofloxacin; MAR: marbofloxacin; NIT: nitrofurantoin; PB: polymyxin B. Crosses below the timeline indicate the detection of methicillin-resistant *S. pseudintermedius* (MRSP) (marked in green) and methicillin-susceptible *S. pseudintermedius* (marked in blue). The number of crosses visualizes the number of isolates included in the analysis for each distinct time point. Purple, orange and light blue shaded time points show clinical samples with mixed *S. pseudintermedius* infections.

4.2 Genotypic characterization

To obtain an overview on the genetic variability as well as relatedness of the bacterial study population, genotypic characterization was performed for all available *S. pseudintermedius* from the canine patient.

4.2.1 Species verification and detection of the methicillin-resistance encoding gene *mec*A

Species verification, that was carried out by investigation of conserved alleles of the housekeeping genes *cpn*60 and *pta* as has been described before [49], confirmed all isolates as *S. pseudintermedius.* Furthermore, all phenotypically methicillin-resistant isolates (n=21) harboured the resistance encoding gene *mec*A.

4.2.2 PFGE and MLST analysis

To unravel the genetic diversity PFGE was performed for all isolates. The analysis was carried out using the band based dice coefficient analysis (optimization: 0.5%, tolerance: 1.5%) as it has been described for *S. pseudintermedius* before [78]. Using a cut-off at 85%, three distinct PFGE-clusters were identified. While all MRSP (n=21) showed identical band patterns in one specific cluster, MSSP were grouped into two distinct clusters. The majority of MSSP isolates (n=14) formed one distinct cluster. Two MSSP isolated in September 2011 and February 2012 formed a second MSSP-cluster. Comparison of the 12 MSSP within the first cluster showed an identical pattern for eleven isolates. IMT 28796, isolated in February 2012, expressed a slightly different profile (92% similarity).

Further genotypic characterization was conducted for all isolates based on data of whole genome sequence analysis (WGS). Overall, MLST revealed three sequence types ST71, ST241 and ST529. All MRSP (n=21) belonged to ST71, while the MSSP belonged to two different sequence types ST529 (n=14) and ST241 (n=2). ST529-MSSP were detected at eight distinct time points between 2008 and February 2012. MSSP isolated in September 2012 and August 2014 belonged to sequence type ST241.

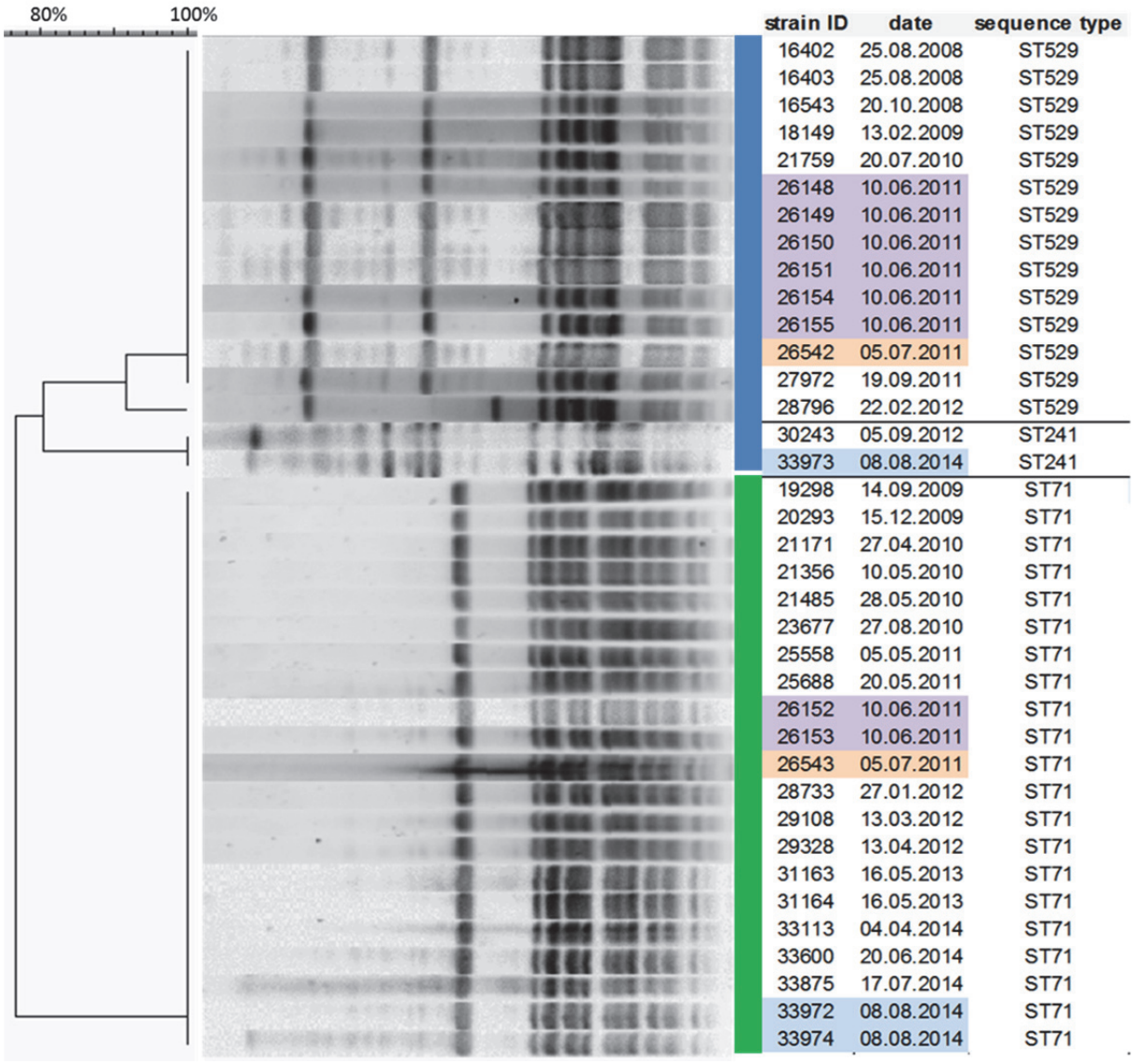

Figure 5: PFGE (Pulsed field gel electrophoresis) analysis of 37 *Staphylococcus (S.) pseudintermedius* using the restriction enzyme *Sma*I. The dendrogram provides information about the pattern-similarity of the investigated strains. Methicillin resistance is indicated by color (green=methicillin-resistant *S. pseudintermedius* (MRSP), blue=methicillin-susceptible *S. pseudintermedius* (MSSP)). Purple, orange and light blue shaded strain IDs have been isolated from clinical samples with mixed *S. pseudintermedius* infections. Sequence types (STs) are based on the results of multilocus sequence type (MLST) analysis.

4.2.1 Whole genome sequence analysis

Whole genome sequence analysis provides the opportunity to compare the set of shared genes within each of the isolates investigated in this study by use of extended MLST analysis as well as the accessory genome like it has been described in section 2.4.2.

4.2.1.1 Extended MLST analysis

Extended MLST was conducted by use of RidomSeqSphere+ (Ridom GmbH ©, Münster) in order to investigate the diversity of genes present in each of the isolates of the study population. In total, 1845 target genes were present in all isolates (n=37) and therefore included for identification of the allelic diversity. Extended MLST analysis revealed three distinct clusters (figure 6). The largest cluster contained all ST71-MRSP. MSSP belonged to two different clusters: all ST529-MSSP formed one large cluster and the two ST241-MSSP shaped the third cluster.

Within each of the two main clusters (ST71-MRSP and ST529-MSSP) minor variations were observed between isolates obtained at different time points. Identical extended MLST patterns were observed for some isolates of both predominant genotypes. Those isolates were either obtained at different or identical time points. For MSSP, identical patterns were observed for isolates IMT26148, IMT26149, IMT26150, IMT26154, IMT26155, IMT26543 and IMT26544. While most MSSP with identical extended MLST type originated from a wound swab taken in June 2011 (IMT26148-IMT26155), the two strains IMT26543 and IMT26544 were isolated in July 2011. Within the cluster of MRSP, identical patterns were observed for IMT33600 (June 2014), IMT33875 and IMT33876 (both July 2014) as well as for IMT31163 and IMT31164 (both May 2013).

Allelic variation between both predominant clusters was identified in 1431 of the 1845 alleles (77.6%).

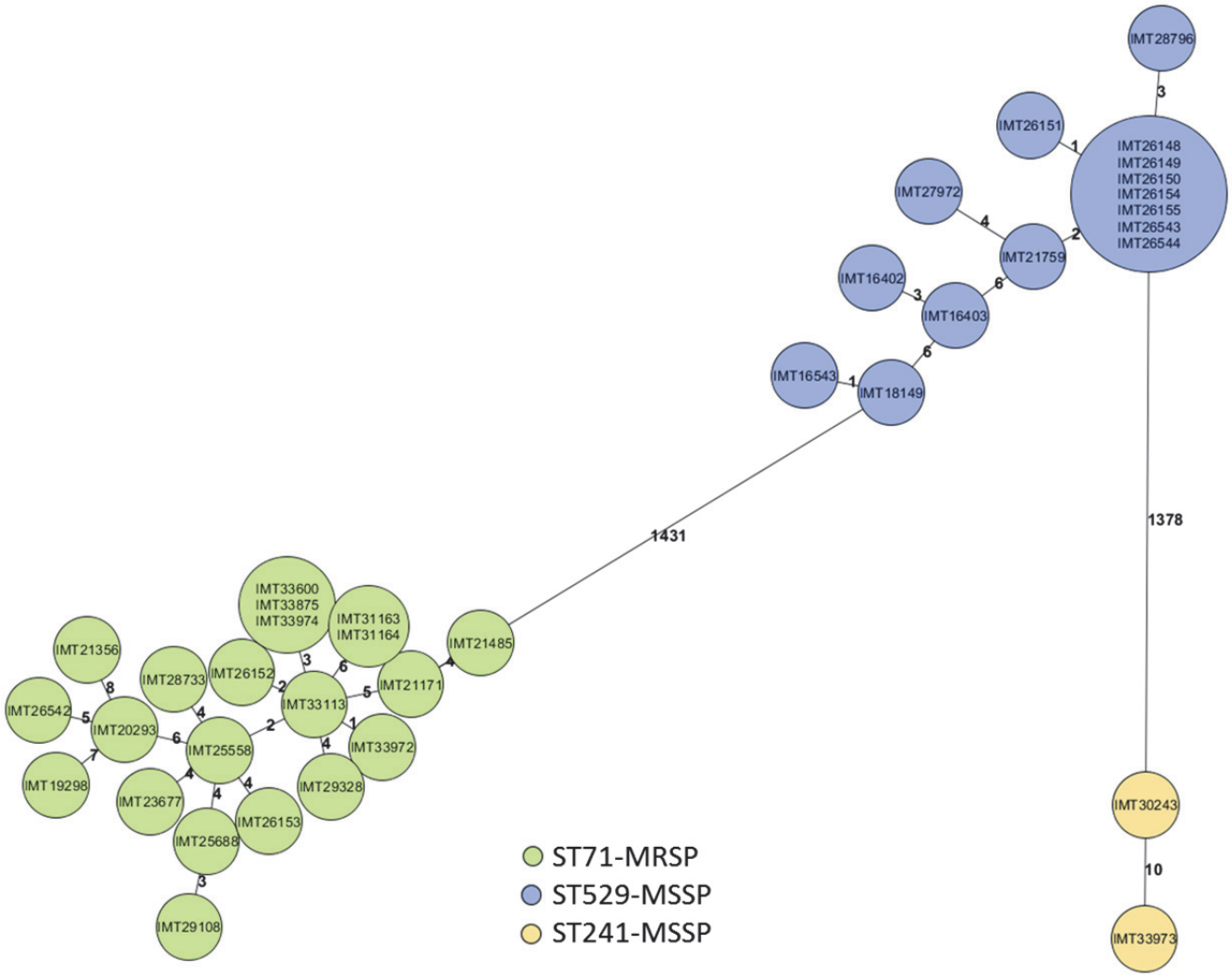

Figure 6: Minimum spanning tree (MSTree) based on results of extended multilocus sequence type (MLST) analysis of 37 *S. pseudintermedius* isolates by consideration of 1845 alleles. The circle size corresponds to the number of isolates. Each color represents a distinct sequence type based on MLST analysis of seven housekeeping genes according to [99]. Each number next to a connecting line defines the number of different alleles between two isolates. Abbreviations: MRSP: methicillin-resistant *S. pseudintermedius*; MSSP: methicillin-susceptible *. pseudintermedius,*ST: sequence type.

4.2.1.2 SNP analysis

To gain more knowledge about the genetic diversity of both main genetic lineages ST71-MRSP and ST529-MSSP, single nucleotide polymorphisms (SNPs) were analyzed separately for each group of isolates. SNPs were identified in coding regions for ST529-MSSP (n=17) as well as ST71-MRSP (n=19). For MRSP, an accumulation of SNPs over time was observed in ten different genes including six non synonymous as well as four synonymous point mutations. The remaining SNPs in nine genes (five synonymous and four non synonymous point mutations) were unique at specific isolation time points (table 5).

For MSSP, an accumulation of SNPs over time was observed for seven genes, including six non synonymous mutations. Six SNPs were identified at single time points (three synonymous and three non synonymous point mutations) (table 6). In addition, four SNPs were observed in isolates IMT16543 and IMT18149 originating from the second and third sampling but were missing in ST529-MSSP isolated at later time points.

Table 5: Overview of single nucleotide polymorphisms (SNPs) (point mutations) identified for methicillin-resistant *S. pseudintermedius* (MRSP). The MRSP isolates are ordered chronologically according to their isolation date. Dots indicate the presence of the reference base defined according to the base identified in the first MRSP isolate IMT19298. Abbreviations: Ref: reference, syn: synonymous, s: synonymous, ns: non synonymous. * Genes that are also present in ST529-MSSP.

open reading frame	protein	Ref. base	SNP base	syn. / non syn.	IMT 19298	IMT 20293	IMT 21171	IMT 21356	IMT 21485	IMT 23677	IMT 25558	IMT 25688	IMT 26152	IMT 26153	IMT 26542	IMT 28733	IMT 29108	IMT 29328	IMT 31163	IMT 31164	IMT 33113	IMT 33600	IMT 33875	IMT 33972	IMT 33974
EC 2.1.1.14*	homocysteine methyltransferase	T	G	ns	•	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
EC 4.3.1.17*	L-serine dehydratase	A	C	s	•	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
FIG00859	hypothetical protein	T	G	s	•	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
*maz*G*	tetrapyrrole methyltransferase	T	G	s	•	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
*sug*E*	quaternary ammonium compound-resistance protein	A	G	s	•	•	G	•	G	G	G	G	G	G	•	G	G	G	G	G	G	G	G	G	G
*gre*A*	transcription elongation factor	T	C	ns	•	•	C	•	C	C	C	C	C	C	•	C	C	C	C	C	C	C	C	C	C
*trk*A*	potassium uptake protein	G	A	ns	•	•	•	•	•	A	A	A	A	A	•	A	A	A	A	A	A	A	A	A	A
EC 3.6.1.11*	exopolyphosphatase	G	A	ns	•	•	•	•	•	•	A	A	A	A	•	A	A	A	A	A	A	A	A	A	A
*rps*M*	SSU ribosomal protein S13p	G	A	ns	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	A	•	A
EC 3.1.3.25*	inositol-monophosphatase	G	A	ns	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	A	•	A
EC 1.97.1.4*	ribonucleotide reductase	C	T	s	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
*sbn*F like gene*	siderophore staphylobactin biosynthesis protein	G	A	s	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
EC 1.1.1.25*	shikimate-5 dehydrogenase	A	G	ns	•	•	•	•	•	•	•	•	•	•	G	•	•	•	•	•	•	•	•	•	•
FIG 12669975	hypothetical protein	A	T	ns	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	•	•
groES*	heat shock protein	C	T	ns	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	•
SPSE2322*	rRNA-subunit methyltransferase	T	C	s	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•
EC 1.1.1.140*	sorbitol-6-phosphate 2-eehydrogenase	T	C	s	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	C	•	•	•	•	•
FIG01109162	hypothetical protein	T	C	ns	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	C	•	•	•	•	•
*pbu*G*	hypoxanthine permease	A	G	s	•	•	•	•	•	•	•	•	•	•	•	•	•	•	G	G	•	•	•	•	•

Table 6: Overview of single nucleotide polymorphisms (SNPs) (point mutations) identified for methicillin-susceptible *S. pseudintermedius* (MSSP). The MSSP isolates are ordered chronologically according to their isolation date. Dots indicate the presence of the reference base defined according to the base identified in the first MSSP isolate IMT16402. Abbreviations: Ref: reference, syn: synonymus, s: synonymus, ns: non synonymus. * Genes that are also present in ST71-MRSP.

genes with SNPs	protein	Ref. base	SNP base	syn. / non syn.	IMT 16402	IMT 16403	IMT 16543	IMT 18149	IMT 21759	IMT 26148	IMT 26149	IMT 26150	IMT 26151	IMT 26154	IMT 26155	IMT 26543	IMT 26544	IMT 27972	IMT 28796
*mal*F*	ABC transporter permease protein	T	C	ns	•	•	C	C	C	C	C	C	C	C	C	C	C	C	C
c*yd*C*	transport ATP-binding protein	A	C	ns	•	•	C	C	C	C	C	C	C	C	C	C	C	C	C
EC 4.2.1.70*	pseudouridine synthase D	C	A	ns	•	•	•	•	A	A	A	A	A	A	A	A	A	A	A
EC 1.1.99.1*	choline dehydrogenase	C	T	ns	•	•	•	•	T	T	T	T	T	T	T	T	T	T	T
FIG01239740*	hypothetical protein	A	G	ns	•	•	•	•	G	G	G	G	G	G	G	G	G	G	G
*ald*A* (EC 1.2.1.3)	aldehyde dehydrogenase	A	G	s	•	•	•	•	G	G	G	G	G	G	G	G	G	G	G
*mut*S-related gene*	*mut*S-related DNA mismatch repair protein	G	T	ns	•	•	•	•	•	T	T	T	T	T	T	T	T	•	T
SPSE_2475*	hypothetical protein	G	A	s	•	•	A	A	•	•	•	•	•	•	•	•	•	•	•
FIG01109163*	hypothetical protein	G	A	ns	•	•	A	A	•	•	•	•	•	•	•	•	•	•	•
EC 1.1.1.28*	D-lactate dehydrogenase	T	C	s	•	•	C	C	•	•	•	•	•	•	•	•	•	•	•
*rec*J*	exonuclease	T	G	ns	•	•	G	G	•	•	•	•	•	•	•	•	•	•	•
*obg**	GTP-binding protein	C	T	s	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•
*pur*H* (EC 3.5.4.10)	purine biosynthesis protein	A	G	s	•	•	•	•	•	•	•	•	G	•	•	•	•	•	•
EC 2.5.1.30*	heptaprenyl-diphosphate synthase component	G	A	ns	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•
*prf**	peptide chain release factor 2	C	T	s	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•
*yei*H*	membrane protein	T	A	ns	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•
*cam*S*	pheromone lipoprotein	G	A	ns	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•

4.2.1.3 Prediction of the protein structure

Prediction of protein structures is a powerful tool to identify putative beneficial changes for bacteria based on the evolvement of genetic modifications. However, as theoretical model protein prediction is clearly limited. While it is able to identify putative changes in the structure which might result in phenotypic changes only phenotypic analyses allow confirming these suggestions based on genome analysis.

Protein structures were predicted for genes with stable non-synonymous SNPs after initial detection in order to investigate putative modifications. First, predicted secondary structures were compared using BioNumerics version 7.5 (Applied Maths ©, Belgium). In this program secondary structures are displayed in following order: alpha-helix = pink structure, beta-strand = yellow arrow, turn = blue arrow, coil = grey structure. In order to predict the three-dimensional structure the freeware Phyre2 was used. In this prediction tool, beta-strands are displayed as arrows and alpha-helices appear as helix-structure. Different colors are used to determine the localization of each structure. By use of Phyre2 protein structures are predicted based on known structures. Therefore, each analysis provides additional information about the reference gene with known protein structure used for comparative analysis. Furthermore, it provides information on the coverage of the gene of interest and the reference gene as well as about the confidence of the model. The confidence is a measure of the likelihood of homology but is not a direct indicator of model quality.

Comparative analyses with Phyre2 were conducted on August 5th 2015.

For MRSP, stable non-synonymous mutations were observed in six genes. The gene *gre*A encodes a transcription elongation factor, which induces RNA-polymerase molecules to cleave RNA and thus, assures efficient elongation of the transcript. Comparison of the secondary structure showed differences at amino acid position 93. The point mutation results in shortening of a beta-strand (figure 7).

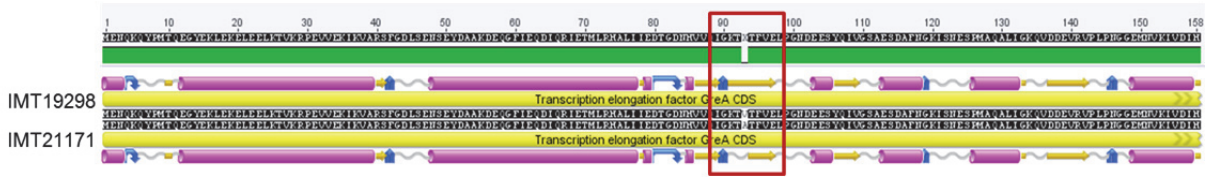

Figure 7: Predicted secondary structure alignment based on the *gre*A gene sequence for isolates IMT19298 and IMT21171.

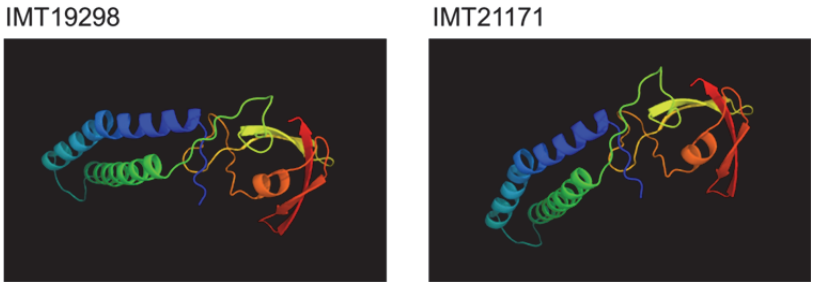

Figure 8: Predicted three-dimensional structure based on the *gre*A gene sequences for isolates IMT19298 and IMT21171.

However, the Phyre2 model showed identical predicted three-dimensional structures (figure 8). The model was based on the crystal structure of the transcript cleavage factor that is encoded on the gene *greb*2 and covers 96% of the gene *gre*A. 100% confidence was given for the prediction.

The gene *trk*A encodes a potassium transporter. Comparison of the predicted secondary structure showed differences at amino acid position 156. The point mutation results in shortening of an alpha-helix structure (figure 9). The predicted three-dimensional model did not reveal differences for the protein structure of IMT19298 and IMT23667. The model was based on the structure of a potassium transporter of *Bacillus subtilis* that is encoded on the gene *ktrap.* The reference gene covers 98% of the target gene *trk*A. 100% confidence was given for the prediction.

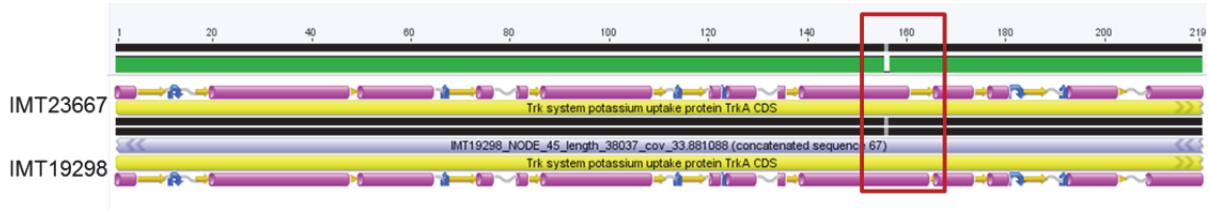

Figure 9: Predicted secondary structure alignment based on the *trk*A gene sequences for isolates IMT19298 and IMT23667.

The exopolyphosphatase that is encoded by gene EC 3.6.1.11 is involved in phosphorus metabolism. Prediction of the secondary and three-dimensional structures of this protein revealed differences of an alpha-helix structure. IMT25558 showed a longer alpha-helix compared to IMT19298 (Figure 10 / 11).

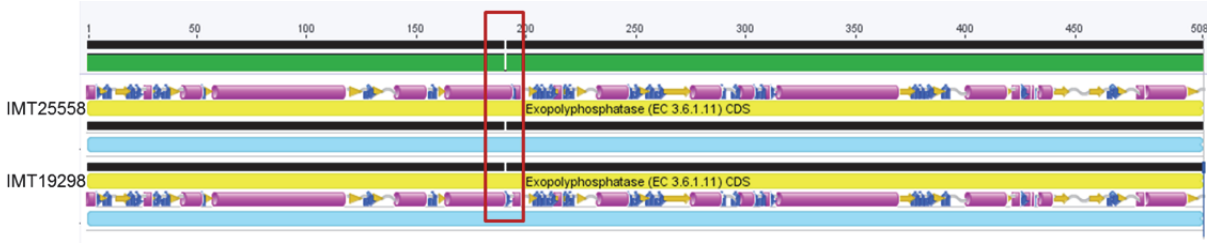

Figure 10: Predicted secondary structure alignment based on the exopolyphosphatase encoding gene sequences for isolates IMT19298 and IMT25558.

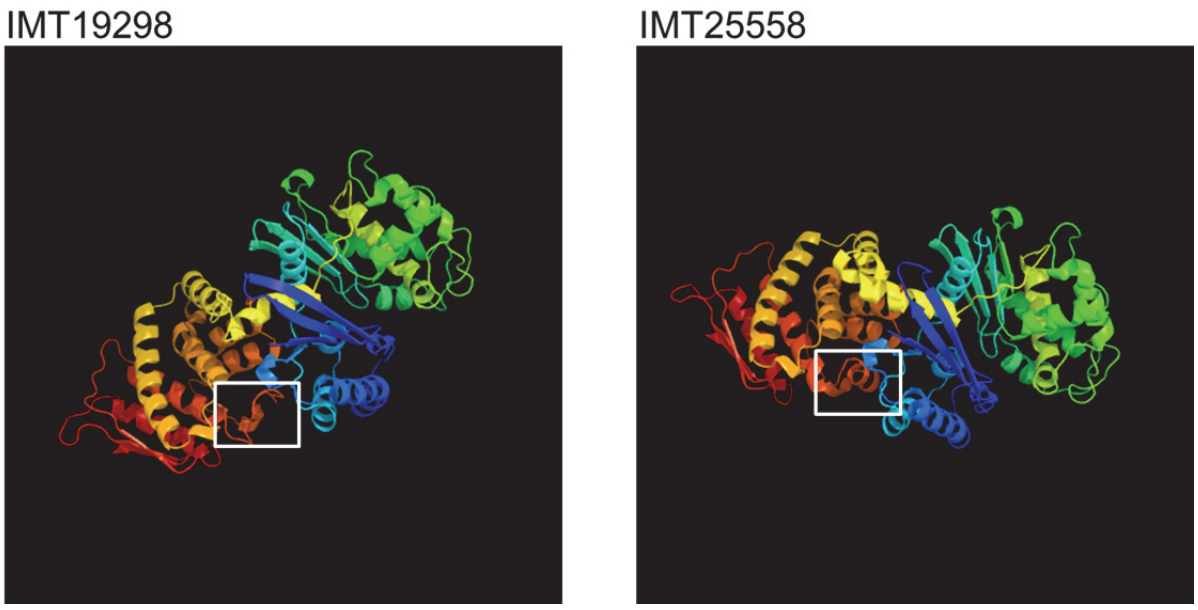

Figure 11: Predicted three-dimensional structure based on the exopolyphosphatase encoding gene sequences for isolates IMT19298 and IMT25558.

The model is based on the crystal structure of an exopolyphosphatase encoded by the gene *ppx* that is expressed in *Escherichia coli*. The reference gene covers 96% of the target gene EC 3.6.1.11. 100% confidence was given for the prediction.

The genes *rps*M that encodes a subunit of the ribosomal protein S13p and EC3.1.3.25 encoding an inositol-monophosphatase were identical in their predicted protein structures.

The point mutation in gene EC 2.1.1.14 resulted in an elimination of a stop codon (figure 12). The differences in gene number with two homocysteine transferase encoding genes in IMT19298 and one large homocysteine transferase encoding gene in all other MRSP (exemplarily shown for IMT20293) do not allow a comparative analysis of the secondary and three-dimensional protein structure.

Figure 12: EC 2.1.1.14 gene alignment of isolates IMT19298 and IMT20293.

For MSSP, stable non-synonymous mutations were observed in six genes including the hypothetical protein encoding gene FIG01239740 that was excluded from the analysis. The gene *mal*F encodes an ABC transporter permease protein which is involved in the export and import of substrates as well as in ATP (adenosine triphosphate) hydrolysis. Comparison of the secondary structure showed differences at amino acid position 93. The point mutation results in shortening of a beta-strand (figure 13).

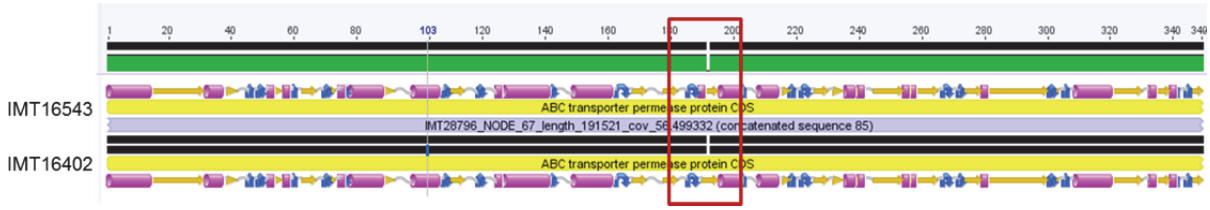

Figure 13: Predicted secondary structure alignment based on the *mal*F sequences for isolates IMT16402 and IMT16543.

Building a three-dimensional model was not possible by use of PHAST2.

*Cyd*C encodes a transport ATP-binding protein that is involved in the ATPase activity as well as transmembrane movement of substances. Prediction of the protein sequences revealed no differences of the secondary structure. However, a three-dimensional structure predicted with PHAST2 identified differences, whereas IMT16543 lacked a small alpha-helix structure (figure 14). The model that was based on the crystal structure of a heterodimeric abc transporter reached a high coverage (99%) and confidence (100%).

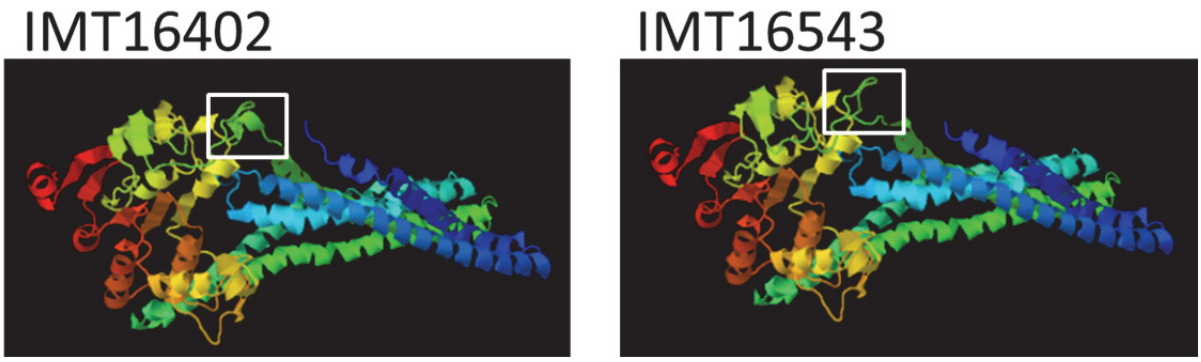

Figure 14: Predicted three-dimensional structure based on *cyd*C sequences for isolates IMT16402 and IMT16543.

The pseudouridine synthase encoding gene EC 4.2.1.70 showed identical patterns in the predicted secondary structure. However, the three-dimensional model constructed with Phyre2 that was based on the crystal structure of *rlud* from *E. coli* showed differences in the presence of a short beta strand (Figure 15). The reference gene *rlud* covered 95% of the gene EC 4.2.1.70. The confidence given for the model was 100%.

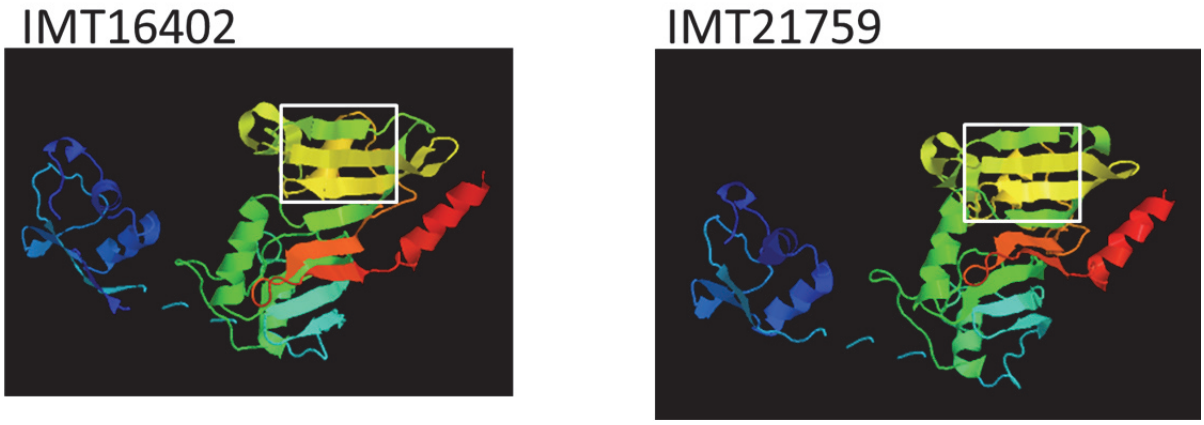

Figure 15: Predicted three-dimensional structure based on the pseudouridine synthase encoding gene sequences for isolates IMT16402 and IMT21759.

The choline dehydrogenase encoding gene EC 1.1.99.1 shows differences in the predicted secondary as well as three-dimensional structure. In comparison to IMT16402, isolate IMT21759 shows an additional beta strand structure (figure 16 / 17). The three-dimensional structure was predicted based on crystal structure of aryl-

alcohol-oxidase from pleurotus eryingii. The reference gene covered 94% of the gene EC 1.1.99.1. The confidence given for this prediction reached 100%.

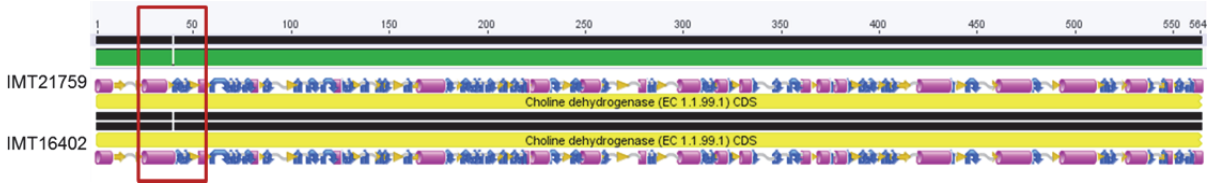

Figure 16: Predicted secondary structure alignment based on the choline dehydrogenase encoding gene sequences for isolates IMT16402 and IMT21759.

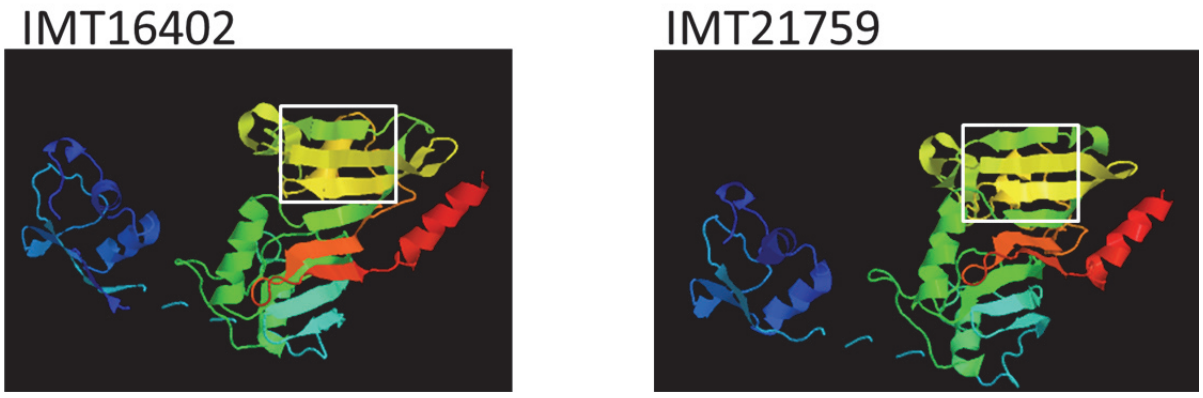

Figure 17: Predicted three-dimensional structure based on the choline dehydrogenase encoding gene sequences for isolates IMT16402 and IMT21759.

The predicted protein structures based on the sequences of the *mut*S-related protein encoding genes were identical. The three-dimensional model was predicted based on the crystal structure of *taq muts*, which covered 87% of the gene of interest. The confidence of this prediction reached 100%.

4.2.1.4 Mobile genetic elements

Comparative analysis of the genomes was performed separately for both main genotypes. Comparison of the sequence data for 21 ST71-MRSP identified a highly similar genome content for all isolates except for IMT19298 (figure 18).

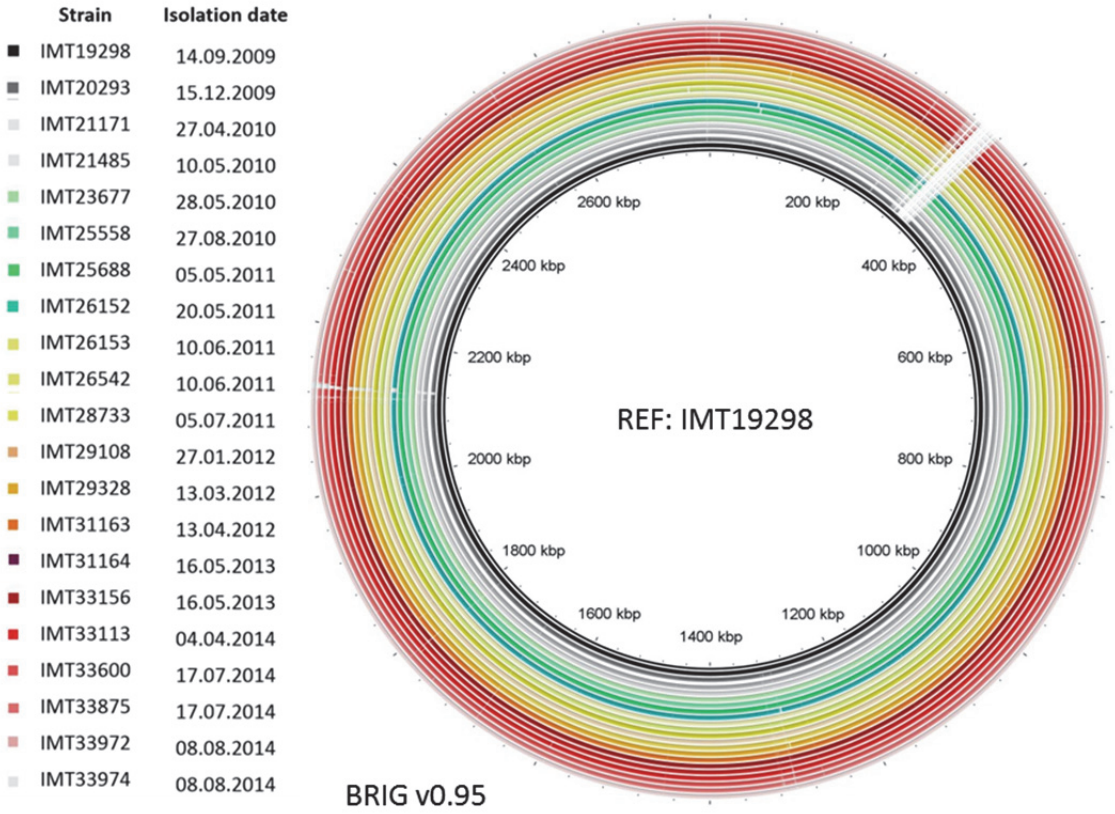

Figure 18: Comparison of the genome content of 21 methicillin-resistant *S. pseudintermedius* (MRSP) visualized by use of BRIG v.0.95 (Blast Ring Image Generator). The first identified MRSP IMT19298 serves as reference. All other MRSP appear in chronological order. Abbreviations: REF: reference, kbp: kilo basepairs

The initial MRSP isolate IMT19298 contains unique genomic regions of 11 kilobases (kb) (region A) and 25.9kb (region B) located on two separate contigs that harbor phage encoding genes (figure 19). Both regions A and B are missing in all MRSP obtained after the initial isolate IMT19298. Mapping of the phage integration sites (int-site 1 and 2) to MRSP obtained after initial infection revealed close proximity of both regions (<300b) (exemplarily shown for IMT20293 in figure 20).

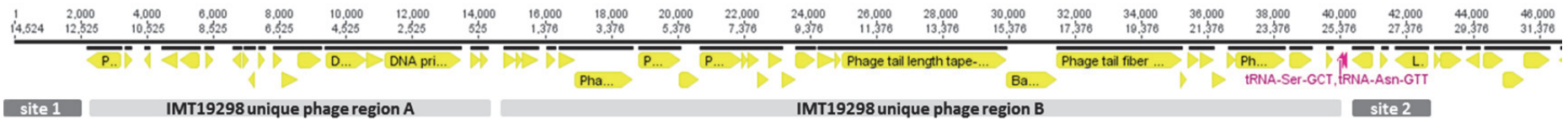

Figure 19: Unique phage regions A and B of the first isolated ST71-MRSP. The integration sites of the phages are marked in dark grey and named as site 1 and site 2.

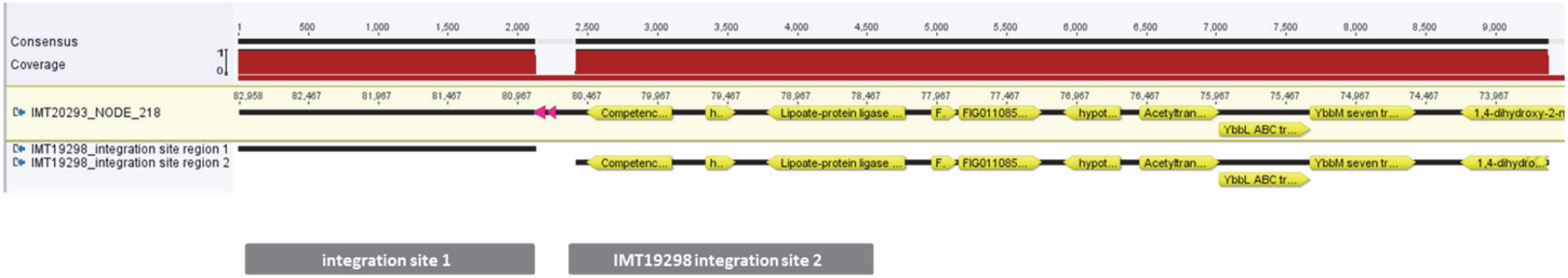

Figure 20: Mapping of the phage integration sites site 1 and site 2 (IMT19298) to the WGS data of IMT20293 by use of geneious (Biomatters Ltd ©, New Zealand). Both integration sites are located on one contig (node_218) and appear in close proximity to each other.

By use of the freeware PHAST (Zhou et al., 2011) the phage regions A and B were classified as intact phage containing an integrase and a terminase as well as genes encoding tail proteins, a portal protein, head proteins, other phage-like proteins and hypothetical proteins. It was not possible to identify an identical phage structure by use of BLAST (basic local alignment tool). However, the phage genome identified in isolate IMT19298 showed partial similarity with phage IME-SA4 that had been identified in *S. haemolyticus*. Analyses of the phage regions using PHAST and BLAST were conducted in August 2015.

Other small variations that are displayed in figure 18 (e.g. at 2120kbp) are caused by different lengths of contigs.

Therefore, closure of gaps would be needed for a sound investigation of these regions. Based on Illumina whole genome sequence data it is not possible to sufficiently display these regions.

Comparative analysis of the genome content of all ST529-MSSP revealed highly similar sequences for all investigated isolates. Small variations are induced by differences in the length of contigs (figure 21).

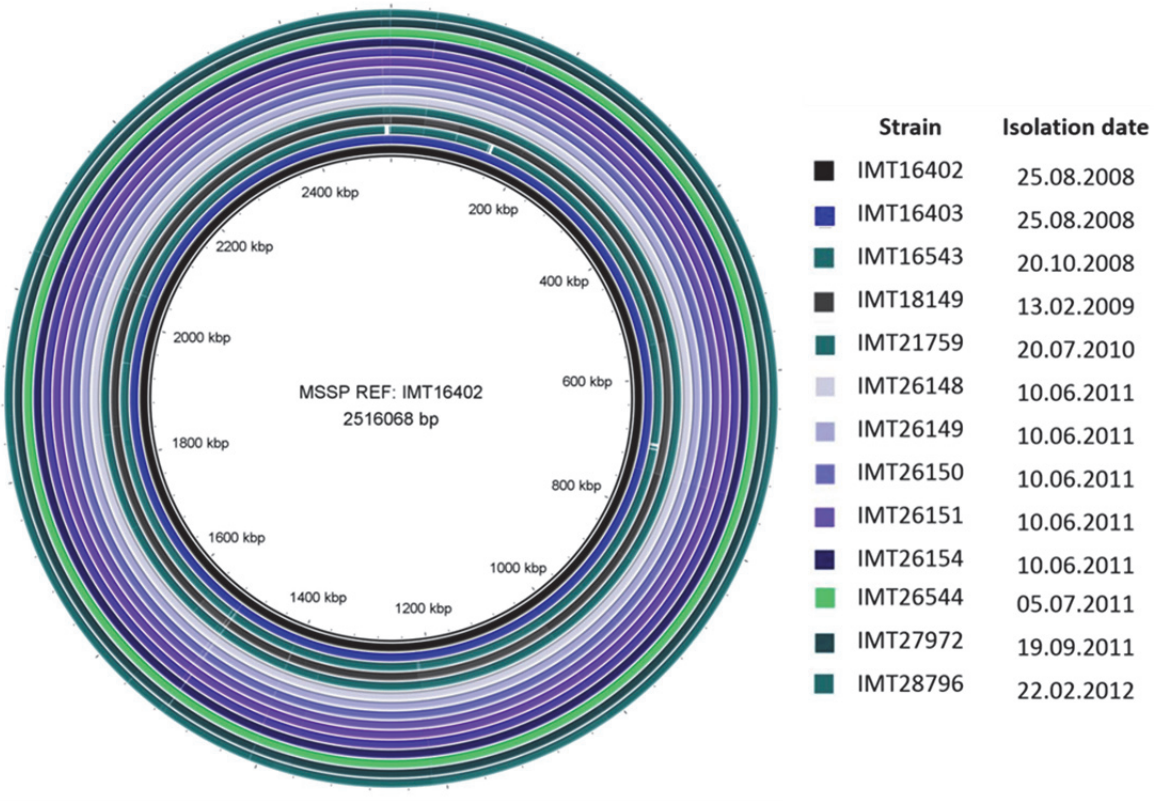

Figure 21: Comparison of the genome content of 15 methicillin-susceptible *S. pseudintermedius* (MSSP) belonging to ST529 visualized by use of BRIG v.0.95 (Blast Ring Image Generator). The first identified MSSP IMT16402 serves as reference. All other ST529-MSSP appear in chronological order. Abbreviations: REF: reference, kbp: kilo basepairs

Mobile genetic elements can be either located chromosomally (e.g. transposons, pathogenicity islands, phages) or extra chromosomally (plasmids) like it has been described in section 4.2.1.2. In order to gain an overview on putative changes of all mobile genetic elements plasmid profile analysis was conducted for all isolates. In summary, all MSSP carried one large plasmid (~48,000bp). For ST71-MRSP differences were observed during plasmid profile analysis. While the first MRSP (isolated in September 2009) carried only one large plasmid (~48,000bp), all other ST71-MRSP which were isolated after September 2009 carried in addition smaller plasmid structures (~3,000bp and ~1,000bp). Figure 22 exemplarily shows the plasmid profiles of the first and second MSSP and MRSP isolates.

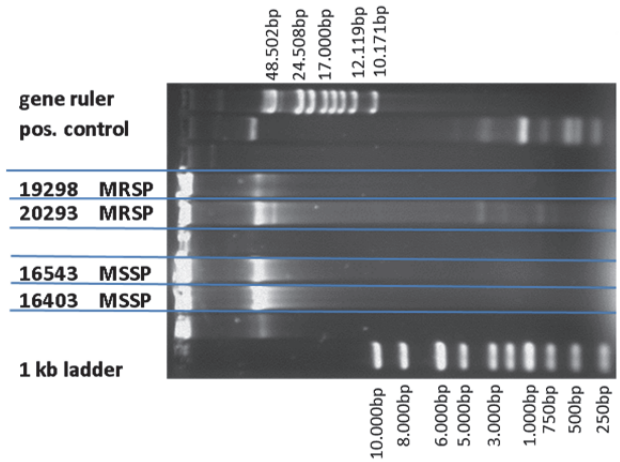

Figure 22: Plasmid profile analysis of the first and second methicillin-susceptible (MSSP) (IMT 16403 and IMT16543) as well as methicillin-resistant *S. pseudintermedius* (MRSP) isolates (IMT19298 and IMT20293).

4.2.1.5 Presence of resistance and virulence-associated genes

The resistome provides information about the presence of resistance encoding genes (Table 7 / 8 / 9). Information on the resistome of all isolates was obtained by use of the freeware ResFinder 2.1. Additionally, conventional PCR was conducted to determine the presence of the tetracycline resistance encoding gene *tet*(K) which is encoded on a plasmid.

Table 7: Identified resistance encoding genes for all ST71-MRSP. Resistance encoding genes were identified by use of the freeware ResFinder 2.1. as well as conventional PCR (polymerase chain reaction).

resistance encoding gene	resistance to	IMT 19298	IMT 20293	IMT 21171	IMT 21356	IMT 21485	IMT 23677	IMT 25558	IMT 25688	IMT 26152	IMT 26153	IMT 26542	IMT 28733	IMT 29108	IMT 29328	IMT 31163	IMT 31164	IMT 33113	IMT 33600	IMT 33875	IMT 33972	IMT 33974
aac(6´)-aph(2``)	aminoglycosides	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ant(6)-Ia	aminoglycosides	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
aph(3`)-III	aminoglycosides	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
blaZ	beta-lactam antibiotics	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
erm(B)	macrolides	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
dfrG	trimethoprim	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tet(K)	tetracycline	Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 8: Identified resistance encoding genes for all ST529-MSSP. Resistance encoding genes were identified by use of the freeware ResFinder 2.1. as well as conventional PCR (polymerase chain reaction).

resistance encoding gene	resistance to	IMT 16402	IMT 16403	IMT 16543	IMT 18149	IMT 21759	IMT 26148	IMT 26149	IMT 26150	IMT 26151	IMT 26154	IMT 26155	IMT 26543	IMT 26544	IMT 27972	IMT 28796
ant(6)-Ia	aminoglycosides	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
aph(3´´)-III	aminoglycosides	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
blaZ	beta-lactam antibiotics	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
erm(B)	macrolides	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tet(M)	tetracyclines	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 9: Identified resistance encoding genes for both ST241-MSSP. Resistance encoding genes were identified by use of the freeware ResFinder 2.1. as well as conventional PCR (polymerase chain reaction).

resistance encoding gene	**resistance to**	**IMT 30243**	**IMT 33973**
ant(6)-Ia	aminoglycosides	+	+
aph(3´´)-III	aminoglycosides	+	+
blaZ	beta-lactam antibiotics	+	+
erm(B)	macrolides	+	+
tet(M)	tetracyclines	Ø	Ø

The presence of important virulence-associated genes (which have been introduced in chapter 2.3.2) within the study population is summarized in table 10. No differences were observed for isolates with identical sequence types. Thus, the results shown summarize findings for all isolates that belong to the same genetic lineage.

Table 10: Presence of important virulence-associated factors for isolates of each identified sequence type (ST).

	ST71-MRSP	**ST529-MSSP**	**ST241-MSSP**
***exp*A**	Ø	Ø	Ø
***exp*B**	Ø	Ø	Ø
***luk*S**	+	+	+
***luk*F**	+	+	+
***hl* ß**	+	+	+
siet	+	+	+
sec	Ø	Ø	Ø
SE-*int*	+	+	+
***sps*A**	+	+	+
***sps*B**	+	+	+
***sps*C**	+	+	+
***sps*D**	+	+	+
***sps*E**	+	+	+
***sps*F**	+	Ø	Ø
***sps*G**	+	+	+
***sps*H**	+	+	+
***sps*I**	+	+	+
***sps*J**	+	+	+
***sps*K**	+	+	+
***sps*L**	+	+	+
***sps*M**	+	+	+
***sps*O**	Ø	Ø	Ø
***sps*P**	+	+	Ø
***sps*Q**	+	Ø	+
***sps*R**	+	+	+

4.3 Phenotypic characterization

Genotypic characterization is an important and powerful tool to investigate microevolutionary changes of a subset of isolates as well as to hypothesize putative advantages or disadvantages due to observed genotypic adaptation. However, only phenotypic experiments allow confirmation of these putative changes in the biology of a microorganism that has been predicted based on genetic information. While genotypic analysis was performed for all available *S. pseudintermedius* from the patient of interest, representative isolates were chosen for phenotypic analysis. The collection was selected based on following selection criteria: time point of isolation (early: 2008-2009, middle: 2010-2012, late phase: 2013-2014) methicillin resistance and sequence type. The aim was to include diverse isolates with respect to the mentioned selection criteria.

4.3.1 Resistance profile

Phenotypic resistance profiles were obtained for all isolates of this study and revealed resistance to three or more antimicrobial classes for MSSP as well as MRSP (table 11). In total, four different resistance profiles were obtained, including two different patterns for MSSP as well as for MRSP.

MSSP isolated at eight time points between 2008 and February 2012 were resistant against chloramphenicol, clindamycin, doxycycline and penicillin whereas MSSP isolated in September 2012 and 2014 (n=2) showed a similar resistance profile with one exception. The isolates were susceptible to doxycycline. The initial MRSP isolate IMT19298 conferred resistance to chloramphenicol, clindamycin, enrofloxacin, marbofloxacin, gentamicin, penicillin, trimethoprim-sulfamethoxazole and cefoxitin. All MRSP isolated after the initial infection with IMT19298 were in addition resistant against doxycycline.

Table 11: Phenotypic resistance profile and sequence types of representative isolates from each isolation date. Abbreviations: AMI: amikacin; AMC: amoxicillin / clavulanic acid; CHL: chloramphenicol; CLI: clindamycin; DOX: doxycycline; ENR: enrofloxacin; GEN: gentamicin; MAR: marbofloxacin; PEN: penicillin; PB: polymyxin B; SXT: sulfamethoxazole/trimethoprim, R: resistant, S: susceptible, ST: sequence type.

isolate	date	sequence type (ST)	AMC	AMI	CHL	CLI	DOX	ENR	GEN	MAR	PEN	PB	SXT
IMT16402	25.08.2008	ST529	S	S	R	R	R	S	S	S	R	S	S
IMT16543	20.10.2008	ST529	S	S	R	R	R	S	S	S	R	S	S
IMT18149	13.02.2009	ST529	S	S	R	R	R	S	S	S	R	S	S
IMT21759	20.07.2010	ST529	S	S	R	R	R	S	S	S	R	S	S
IMT26148	10.06.2011	ST529	S	S	R	R	R	S	S	S	R	S	S
IMT26542	05.07.2011	ST529	S	S	R	R	R	S	S	S	R	S	S
IMT27972	19.09.2011	ST529	S	S	R	R	R	S	S	S	R	S	S
IMT28796	22.02.2012	ST529	S	S	R	R	R	S	S	S	R	S	S
IMT30243	05.09.2012	ST241	S	S	R	R	S	S	S	S	R	S	S
IMT33973	08.08.2014	ST241	S	S	R	R	S	S	S	S	R	S	S
IMT19298	14.09.2009	ST71	R	S	R	R	S	R	R	R	R	S	R
IMT20293	15.12.2009	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT21171	27.04.2010	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT21356	10.05.2010	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT21485	28.05.2010	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT23677	27.08.2010	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT25558	05.05.2011	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT25688	20.05.2011	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT26153	10.06.2011	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT26543	05.07.2011	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT28733	27.01.2012	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT29108	13.03.2012	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT29328	13.04.2012	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT31163	16.05.2014	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT33113	04.04.2014	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT33600	20.06.2014	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT33875	17.07.2014	ST71	R	S	R	R	R	R	R	R	R	S	R
IMT33972	08.08.2014	ST71	R	S	R	R	R	R	R	R	R	S	R

4.3.1 Biofilm formation

The ability to form biofilms on a polystyrene surface after 24 hours was tested for eleven representative isolates, including ST71-MRSP (n=5) and ST529-MSSP (n=4) as well as for both ST421-MSSP. Representative ST71-MRSP and ST529-MSSP were chosen based on the time point of infection, including isolates from early (2008-2009), middle (2010-2012) as well as late isolation dates (2013-2014). In general, similar results were obtained for isolates with the same genetic background. One exception was observed for IMT16543 (figure 23). This isolate showed a significantly decreased biofilm formation ability compared to all other ST529-MSSP. P-values for all isolates compared to each other by use of Mann-Whitney-U test are provided in table 12. Based on this initial analysis, isolates with the same genetic background were grouped for the final statistical analysis since they did not reveal statistically significant differences with respect to their biofilm formation ability. ST529-MSSP as well as ST241-MSSP showed very weak biofilm formation ability. In contrast, ST71-MRSP were significantly higher biofilm producers compared to MSSP of this study (figure 24).

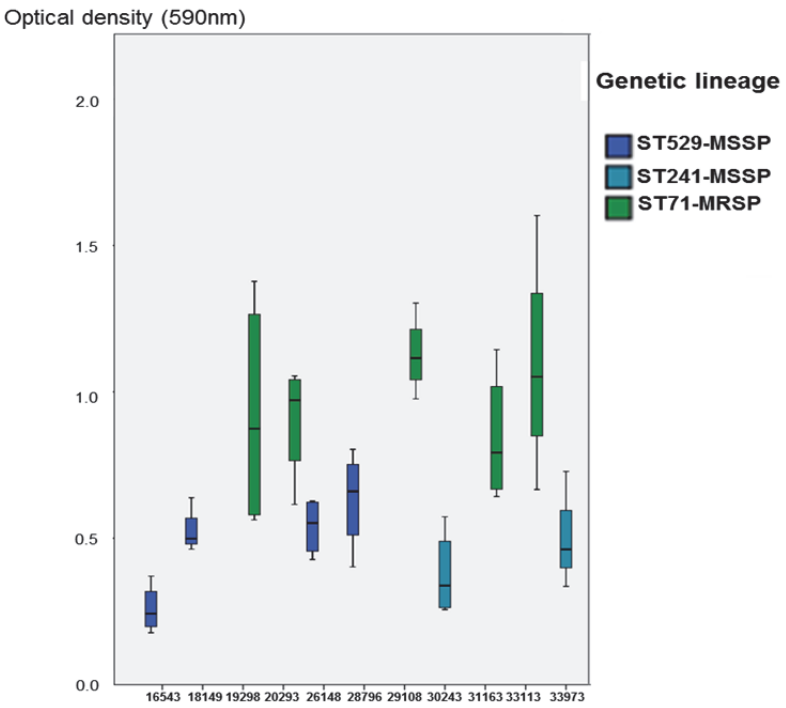

Figure 7: Biofilm formation ability on polystyrene surface after 24 hours incubation time at 37°C. The x-axis provides information on the tested isolates. Genetic lineages are marked in color: methicillin-susceptible *S. pseudintermedius* (MSSP) sequence type (ST)529: dark blue, MSSP ST241: light blue, methicillin-resistant *S. pseudintermedius* (MRSP) green. The y-axis shows the optical density measured at 590nm. A high optical density was achieved for strong biofilm producers.

Table 12: P-values obtained for eleven representative isolates based on the ability to form biofilms. Statistical analysis was performed by use of Mann-Whitney-U test. Statistically significant values ($p<0.05$) are marked (*).

IMT	16543	18149	19298	20293	26148	28796	29108	30243	31163	33113
16543										
18149	0.021*									
19298	0.034*	0.034*								
20293	0.021*	0.021*	0.480							
26148	0.021*	1.0	0.034*	0.021*						
28796	0.021*	0.149	0.157	0.043*	0.248					
29108	0.021*	0.021*	0.724	0.083	0.021*	0.021*				
30243	0.149	0.386	0.034*	0.021*	0.149	0.083	0.021*			
31163	0.021*	0.021*	0.289	0.386	0.021*	0.248	0.149	0.021*		
33113	0.021*	0.021*	0.289	0.773	0.021*	0.083	0.386	0.021*	0.564	
33973	0.077	1.0	0.127	0.034*	1.0	0.480	0.034*	0.289	0.157	0.077

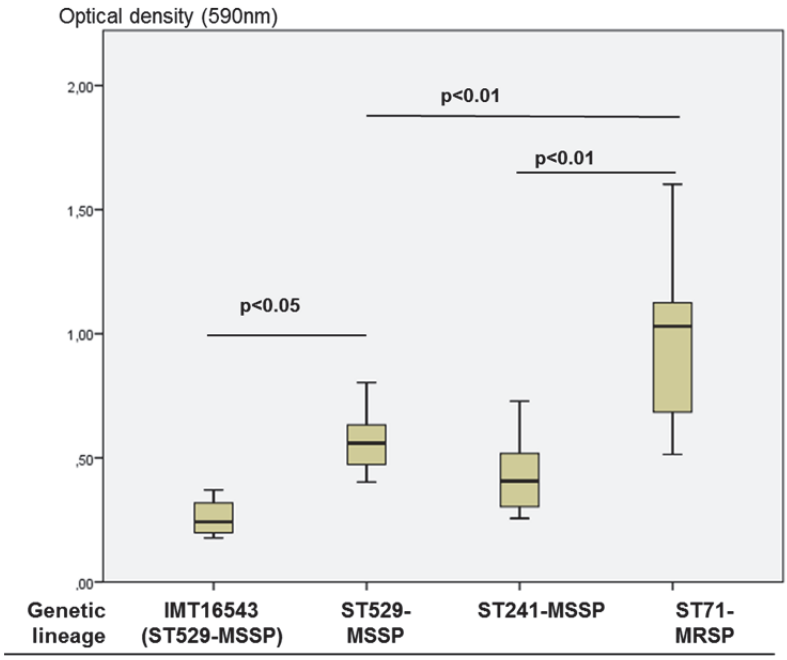

Figure 8: Biofilm formation ability of representative methicillin-resistant *S. pseudintermedius* (MRSP) sequence type (ST)71 (n=5), methicillin-susceptible *S. pseudintermedius* (MSSP) ST529 (n=4) and MSSP ST241 (n=2). The y-axis shows the optical density measured at 590nm. A high optical density was achieved for strong biofilm producers. Statistical analysis was conducted by use of the Mann-Whitney-U test.

4.3.2 Adhesion to extracellular matrix components

The initial step for successful colonization and infection of a host is the adherence of bacteria to host cells. Thus, adhesins that are able to initiate the attachment are recognized as important virulence factors. In general, staphylococci encode a variety of different adhesins [106]. In *S. pseudintermedius* 18 putative adhesins have been identified so far, mediating attachment to extracellular matrix components [56]. Two important components are fibrinogen and fibronectin.

The main aims of these experiments were:

i) To investigate putative changes in the binding capacity for isolates with the same genetic background, which have been isolated at different sampling time points
ii) To compare the ability of isolates representing different genetic lineages to adhere to fibrinogen and fibronectin.

4.3.2.1 Fibrinogen binding assay

In order to investigate the ability of *S. pseudintermedius* to adhere to fibrinogen, binding assays were performed for the same representative isolates that were chosen for biofilm formation assays.

In general, three different binding patterns were observed, whereas isolates within the same genetic lineage displayed similar binding capacity to fibrinogen (figure 25). ST529-MSSP revealed the highest binding. In contrast, ST71-MRSP displayed a very weak adherence to this extracellular ligand. Compared to these findings, ST241-MSSP showed a moderate binding ability. Statistical analysis was performed using the Mann-Whithey-U test and significance level was set at $p<0.05$. Statistical significant differences were obtained for isolates from different genetic lineages but missing between isolates with the same genetic background (p-values are given in table 13). Therefore, results for isolates were pooled according to each genotype and final statistical analysis was conducted (figure 26).

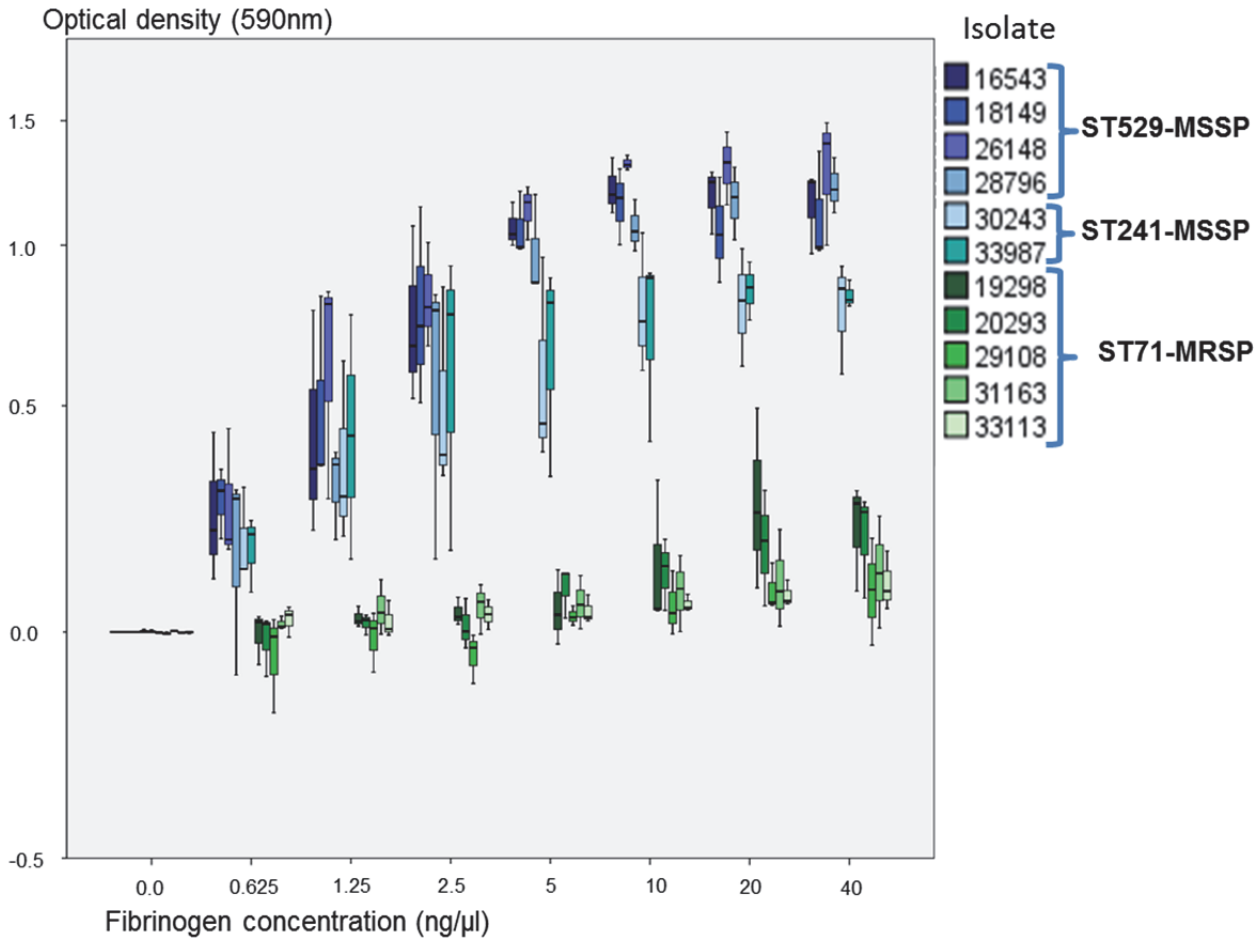

Figure 9: Adherence of eleven representative *S. pseudintermedius* to fibrinogen. The x-axis provides information on the fibrinogen concentration. The y-axis shows the optical density measured at 590nm. A high optical density was achieved for isolates with strong binding capacity. Methicillin-susceptible *S. pseudintermedius* (MSSP) are colored in blue. Methicillin-resistant *S. pseudintermedius* (MSRSP) are shown in green. The sequence type (ST) is given for each isolate.

Table 13: P-values obtained for 11 representative isolates based on adherence to fibrinogen (40ng/µl). Statistical analysis was performed by use of Mann-Whitney-U test. Statistically significant values ($p<0.05$) are marked (*).

IMT	16543	18149	19298	20293	26148	28796	29108	30243	31163	33113
16543										
18149	0.827									
19298	0.050*	0.050*								
20293	0.050*	0.050*	0.513							
26148	0.275	0.127	0.513	0.050*						
28796	0.827	0.513	0.050*	0.050*	0.513					
29108	0.050*	0.050*	0.275	0.275	0.050*	0.050*				
30243	0.050*	0.050*	0.050*	0.050*	0.050*	0.050*	0.050*			
31163	0.021	0.050*	0.275	0.275	0.050*	0.050*	0.513	0.050*		
33113	0.021	0.050*	0.184	0.275	0.050*	0.050*	0.827	0.050*	0.218	
33973	0.050*	0.050*	0.050*	0.050*	0.050*	0.050*	0.050*	0.827	0.050*	0.050*

Final analysis revealed significant higher binding for all ST529-MSSP compared to ST71-MRSP ($p<0.0001$) as well as to ST241-MSSP ($p=0.01$). Furthermore, ST241-MSSP adhered significantly better to fibrinogen in comparison to ST71-MRSP ($p<0.0001$).

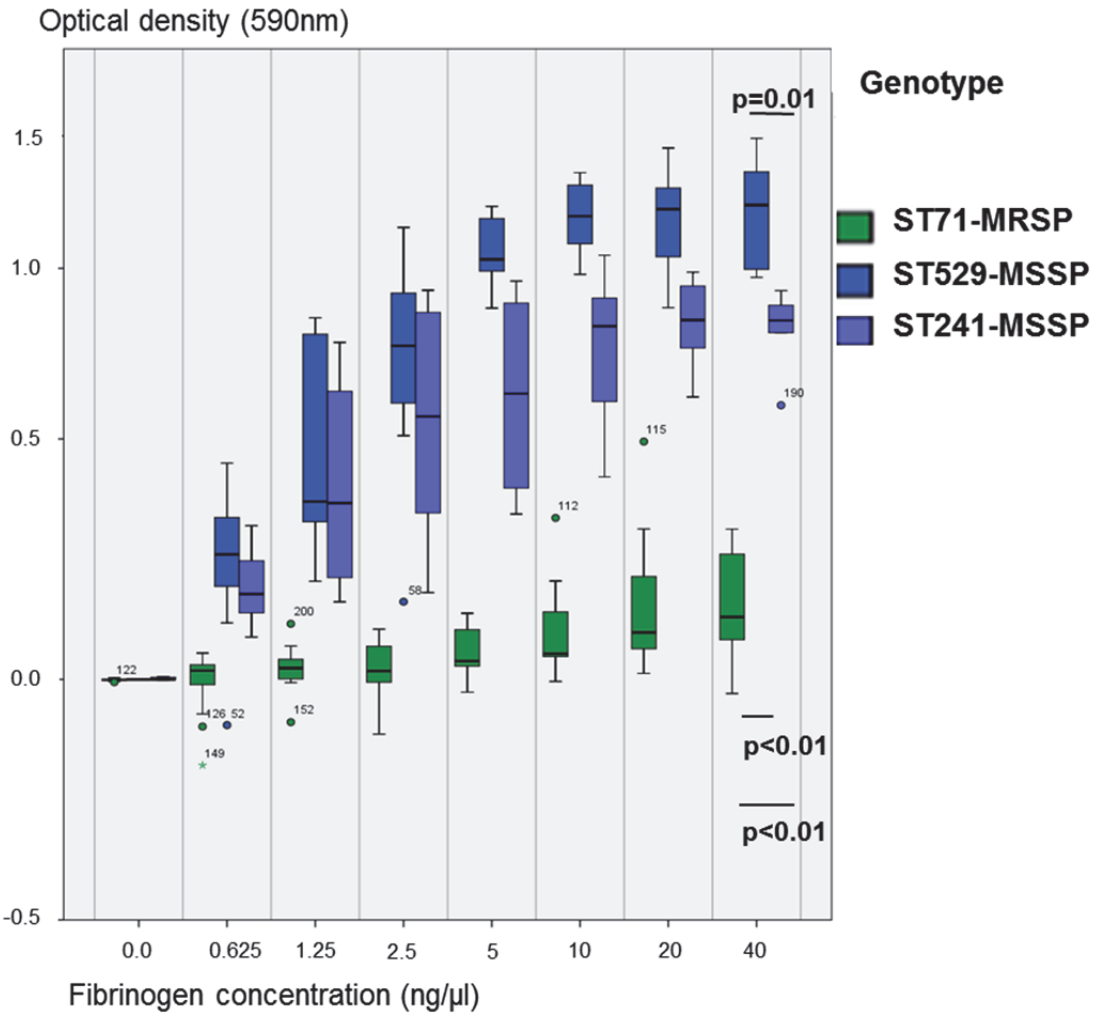

Figure 10: Adherence of representative isolates of methicillin-resistant *S. pseudintermedius* (MRSP) sequence type (ST)71 (n=5), methicillin-susceptible *S. pseudintermedius* (MSSP) ST529 (n=4) and MSSP ST241 (n=2) to fibrinogen. The y-axis shows the optical density measured at 590nm. A high optical density was achieved for isolates with strong binding capacity. Statistical analysis was conducted by use of the Mann-Whitney-U test.

4.3.2.2 Fibronectin binding assay

Adherence to the extracellular ligand fibronectin was tested for two representative isolates of each genotype. In total, six isolates displayed very similar binding capacity by use of a concentration of 40ng/µl. No statistical significant differences were observed between the tested isolates as well as between different genetic lineages (figure 27).

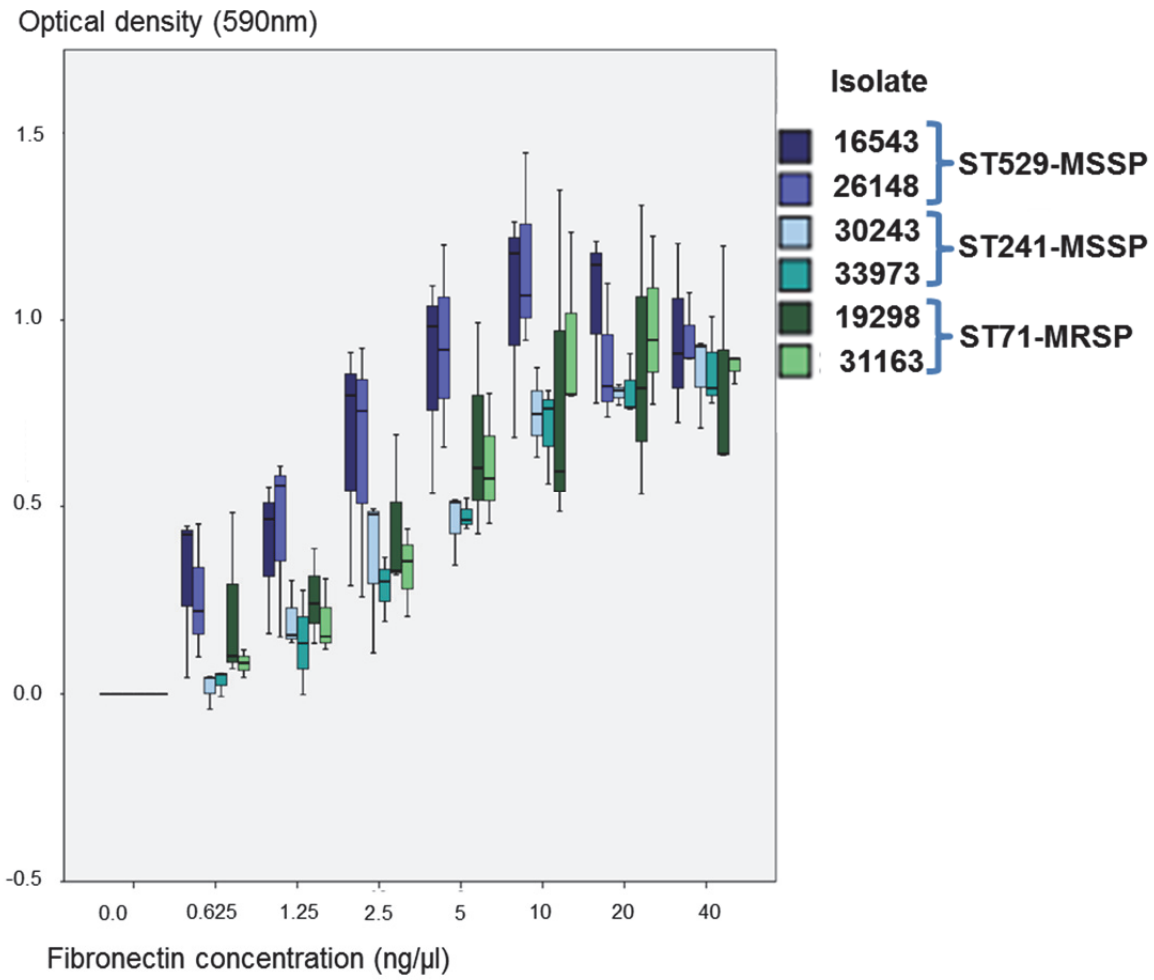

Figure 11: Adherence of representative isolates of methicillin-resistant *S. pseudintermedius* (MRSP) ST71 (n=2), methicillin-susceptible *S. pseudintermedius* (MSSP) ST529 (n=2) and MSSP ST241 (n=2) to fibronectin. The x-axis provides information on the tested fibronectin concentration. The y-axis shows the optical density measured at 590nm. A high optical density was achieved for isolates with strong binding capacity. Methicillin-susceptible *S. pseudintermedius* (MSSP) are colored in blue. Methicillin-resistant *S. pseudintermedius* (MRSP) are shown in green. The sequence type (ST) is given for each isolate.

5. Discussion

This study investigates the genetic diversity and microevolution as well as phenotypic appearances of a *S. pseudintermedius* subpopulation obtained from multiple infections of one individual patient. The study population consists of *S. pseudintermedius* isolated from one blood culture as well as 24 wound and soft tissue infections including several isolates from follow-up microbiological investigations due to wound healing disorder.

5.1 Sampling population

MRSP as well as MSSP were identified as cause of infection at 18 respectively 10 time points during a period of six resp. seven years. Infections in 2008 were caused by MSSP. In September 2009, MRSP infections was detected as well. Since then MRSP as well as MSSP were identified as cause of multiple soft tissue infections until August 2014. These findings lead to the suggestion of a highly susceptible patient regarding infections caused by *S. pseudintermedius*.

Due to investigation of multiple phenotypically identical *S. pseudintermedius* colonies obtained on a blood agar plate from one swab, mixed infections with MSSP and MRSP were detected on three occasions. These findings are of major concern for routine bacteriological diagnostic since it was shown during this work that even phenotypically similar appearing colonies can exhibit different resistance profiles. This indicates that the verification of susceptibility to cefoxitin for one colony per sample does not necessarily provide a full picture of all resistance phenotypes that might be present during infection. In staphylococci, the observation of phenotypically identical isolates showing different resistance patterns has been described only recently for colonized humans [107]. For other bacterial species like for *Pseudomonas aeruginosa* it is well known that isolates with identical colony morphologies obtained from individual patients can vary with regard to their resistance patterns [108]. However, further investigation is needed to identify the frequency of *S pseudintermedius* infections caused by both MSSP and MRSP with

undistinguishable colony morphology to understand the importance for routine diagnostic for this bacterial species.

5.2 Molecular epidemiology of the analyzed *S. pseudintermedius* subpopulation

Various different genotyping analyses were used in this study in order to obtain detailed information concerning the molecular epidemiology of the investigated *S. pseudintermedius* study population.

Pulsed field gel electrophoresis (PFGE) provided a first overview on the variety of genetic lineages within the study population. In total, three different clusters were identified (cut-off at 85% similarity). While band patterns of isolates within each cluster appeared very similar or identical, comparison of the three different clusters showed very different restriction patterns, indicating the presence of three distinct genetic lineages. Furthermore, the absent or very limited variation that was observed with respect to the PFGE patterns for isolates representing the same genetic lineage suggests a rather limited genetic variation of isolates within the same lineage. This is of special interest, since the samples were obtained over a seven year period (2008-2014) suggesting the presence of very stable clones within this patient. However, as macrorestriction analysis does only reveal differences when the restriction sites are changed through mutation or recombination, the discriminatory power of this method within single genetic lineages is scarce. Thus, further methods providing a higher resolution (extended MLST and investigation of single nucleotide polymorphisms) were applied to investigate the intra-lineage diversity.

In concordance with the results provided by PFGE analysis, MLST and extended MLST analysis identified three distinct genotypes showing a high allelic diversity between different genotypes. The two predominant lineages ST71-MRSP and ST529-MSSP were obtained from several infections over six respectively five years. ST71-MRSP has been described as a highly successful lineage in several European countries, including Germany, in the past [78]. In contrast, the MLST type of the predominant MSSP lineage, to the best of our knowledge, has not been described so

far. However, genotyping data on MSSP are scarce and therefore, evaluation of this finding will only be possible during future investigations.

Detailed investigation of the two predominant genetic lineages showed only minor variation with respect to the occurrence of SNPs in coding regions for both ST71-MRSP (n=19) and ST529-MSSP (n=17). The accumulation of SNPs over time in nine, resp. seven genes provides evidence of a common ancestor for each lineage that underwent microevolutionary changes over time. Interestingly, accumulating SNPs were observed for synonymous as well as non synonymous mutations. In total, a very low number of SNPs was detected, revealing a low mutation rate for both ST71-MRSP and ST529-MSSP during six respectively five years. SNPs that occurred only in one or two isolates might be explained by intra strain variability like it has been shown for MRSA only recently [109]. Furthermore, sequencing error needs to be considered at least as a partial cause of this observed result. Resequencing of these regions using Sanger sequencing would be needed to exclude putative technical errors.

In the study reported here, different genetic lineages (ST71-MRSP in combination with ST529- or ST241-MSSP) were isolated from three wound swabs by investigation of three to eight colonies isolated from a single swab. Especially worrisome is the identification of both phenotypically identical MSSP and MRSP, a result that has a huge impact on therapy options like it has been discussed in the previous section. While all MRSP belonged to ST71, characterization of MSSP revealed two distinct genotypes. However, the absence of ST529-MSSP in the last two MSSP-positive swabs has to be interpreted with caution due to the limited number of colonies investigated for both sampling time points. Results obtained in this study proof that it is possible to detect two lineages simultaneously from one swab by investigating more than one colony. Thus, it might be as well possible that both genetic lineages ST529-MSSP as well as ST241-MSSP were present in the last two MSSP infections. Another explanation for the observed change of the genetic lineage might as well be the successful treatment of ST529-MSSP infection and infection with a new MSSP lineage.

There is no study available discussing the diversity of *S. pseudintermedius* colonies isolated from one clinical sample so far, although it is known that dogs can be colonized with different genetic lineages [2-4]. A study published in 2015 investigated the diversity of MRSA lineages during carriage, infection and transmission. In this publication, Harrison and colleagues were able to demonstrate considerable within-host diversity for colonization samples highlighting the need to investigate multiple isolates obtained from colonization. However, comparison of multiple MRSA colonies from one infection site identified isolates from identical genetic lineages. These isolates showed only minor differences of two SNPs [109]. Genotypic heterogeneity has been described for similar appearing colonies in individual samples for other bacterial species like *Pseudomonas aeruginosa* and *Burkholderia dolosa* in patients with cystic fibrosis [110,111] or *Clostridium difficile* infected patients [112]. In a review Balmer and Tanner discuss implications of multiple strain infections indicating that the main benefits can be changes in pathogen dynamics, disease course and transmission [113]. However, knowledge on the impact of mixed infections with different genetic lineages is still scarce and future studies are needed to investigate the burden by these infections.

5.3 Adaptive capacity

5.3.1 Genetic adaptation

5.3.1.1 Genotypic variation

Analysis of changes in the accessory genome revealed different results for ST529-MSSP and ST71-MRSP. While comparison of the genomic content and plasmids showed no variation for ST529-MSSP, changes were observed for ST71-MRSP, indicating the flexibility as well as the need of ST71-MRSP to react to environmental changes by uptake or loss of mobile genetic elements. The initial ST71-MRSP IMT19298 (isolated in 2012), which was susceptible to doxycycline harboured one large plasmid. All following MRSP infections were caused by doxycycline resistant MRSP, which contained the large as well as smaller plasmid structures. Doxycycline resistance can be either encoded chromosomally or on plasmids. Genes referring

resistance to doxycycline and all other tetracyclines have been denominated as tetracycline resistance encoding genes (*tet*). So far, four different *tet* genes have been identified in *S. pseudintermedius* [79]. Verification of doxycycline resistance encoding genes in all MRSP proofed resistance due to the uptake of the *tet*K gene in all doxycycline resistant isolates. *Tet*K is usually encoded on plasmids [114]. This knowledge matches the observation of a plasmid uptake that took place after initial infection. In order to determine the possible reason for this uptake the case history was analysed showing that the plasmid uptake was presumably triggered by therapy of the first MRSP infection with a doxycycline susceptible isolate with this antimicrobial agent (figure 4, page 49). Treatment with doxycycline provided the selective pressure necessary to force the MRSP to include a doxycycline resistance encoding gene into the bacterial DNA content. It is well known that plasmids have a high impact on the emergence and spread of antimicrobial resistance encoding genes [115]. However, the source of the plasmid remains unclear. Another (commensal) bacterium that could have been additionally present in the environment might have served as source for the *tet*K encoding plasmid. Plasmids encoding the *tet*K gene have been identified in various staphylococcal species like *S. aureus*, *S. cohnii*, *S. haemolyticus, S. epidermidis, S. saprophyticus* and *S. sciuri* [116-120]. Small *tet*K gene encoding plasmids identified in various staphylococcal species are very similar indicating the transferability between different staphylococcal species. Furthermore, the *tet*K gene shares considerable homology to a tetracycline resistance encoding gene detected on plasmids of Bacillus species [121]. It has been shown that some staphylococcal plasmids (e.g. pEF418) originate from other bacterial species like Enterococci [122]. Furthermore, an extensive exchange of plasmids between isolates of the same staphylococcal species has been identified *in vivo* for S. aureus isolates [123] and *in vitro* for isolates belonging to different staphylococcal species (e.g. *S. haemolyticus* and *S. aureus*) [124]. For Enterobacteriaceae *in vivo* spread of a plasmid between *Citrobacter freundii* and *E. coli* resp. *Klebsiella* sp. has been investigated only recently [125].

Although the plasmid profile appears similar for all other MRSP and all ST529-MSSP further sequence analysis would be needed in order to identify putative differences of each plasmid containing the same size with respect to the gene content since it is

well known that plasmids can be very diverse [115,126]. However, highly variable plasmid regions hamper the analysis of DNA sequences obtained from a MySeq (Illumina Inc. ©, San Diego, CA, USA) that was used in this study. This sequencer belongs to the group of bench-top instruments and its main advantage compared to other instruments is cost-effective sequencing with minimal preparation time in the laboratory. The disadvantage is the short read-length that is produced during sequencing which complicates efficient assembly. However, plasmid fragments obtained by sequencing could be used to manually close the sequence gaps between contigs (DNA consensus sequence constructed from overlapping reads) by PCR amplification and subsequent Sanger sequencing [127,128]. Currently, the method of choice for plasmid sequencing is the use of the PacBio RS (Pacific Biosciences ©, CA, USA) sequencing platform that aims for very long read ends producing fragments as long as 40kb [127]. Thus, it is possible to obtain the information for smaller plasmids on one contig enabling a sound investigation of the complete plasmid. Disadvantages of sequencing by use of the PacBio RS system (Pacific Biosciences ©, CA, USA) are high costs and a high error rate [128,129].

One tool that can be used to investigate plasmid sequences from whole genome data sets is called plasmid constellation network (short: PLACNET). In addition, PLACNET allows to create a network of plasmid and chromosomal contig interaction [130]. Of course, comparison of plasmid sequences from different isolates could be conducted by other programs like geneious (Biomatters Ltd. ©, New Zealand) by comparative sequence alignment as well.

Investigation of the accessory genome variability of ST71-MRSP revealed in addition to the uptake of a plasmid the loss of a phage for the first isolate IMT19298. While it is possible to demonstrate a cause-and-effect relationship between usage of doxycycline and uptake of a plasmid that provides resistance against this antimicrobial agent, reasons for the loss of the phage after initial MRSP infection are unclear. The phage mainly encodes genes with unknown function so that it is impossible to understand the benefit for *S. pseudintermedius* carrying this phage. One possible reason for the phage loss after initial infection might be a benefit due to the loss of a fitness burden that might be induced by carriage of the phage like it has

been discussed for phages before [131]. To prove this hypothesis, functional assays would be needed to compare the fitness of the ST71-MRSP harbouring the phage and a mutant which lost the phage. The genome content of further isolated ST71-MRSP remained stable after the initial adaptation described above.

Small genome gaps that are visible during comparative analysis of the genome data using BRIG analysis are caused by different lengths of contigs. Therefore, closure of gaps would be needed for a sound investigation of these regions. Based on the MySeq (Illumina Inc. ©, San Diego, CA, USA) data it is not possible to sufficiently display these regions. In order to investigate the complete genome sequence closure of gaps could be performed by use of PCR and sequencing of the amplified regions like it has been described before [100]. Like already discussed for plasmid sequencing use of the PacBio RS system (Pacific Biosciences ©, CA, USA) would be the method of choice to generate long contigs. This would decrease the number of contigs and simplify manual closure of gaps. Comparison of complete genomes would allow to include all genomic regions into the comparative analyses of the genome structures and SNP analysis. Furthermore, it would be possible to include positional information into the analysis, resulting in investigation of structural rearrangements, deletions or insertions of large regions that might have taken place [129]. This is of special interest for bacteria with high homologous recombination rates like *Streptococcus* pyogenes [132]. Literature on recombination rates in staphylococci is inconsistent but based on whole genome sequencing it was possible to identify recombination as an important diversification mechanism in staphylococci as well [133,134]. Thus, investigation of recombination events due to PacBio sequencing (Pacific Biosciences ©, CA, USA) and gap closure would additionally contribute to better understand the microevolution of the investigated subpopulation. However, even without closure of gaps whole genome sequence data allow to determine microevolutionary changes by consideration of approximately 98% of the whole genome content and thus are applicable for phylogenetic investigation of population structures [135].

Overall, a low genetic diversity was observed for ST71-MRSP and ST529-MSSP during the whole time span. Furthermore, ST71-MRSP demonstrated an adaptive

potential very likely driven by antibiotic treatment in case of the plasmid-uptake. Only recently, Azarian and colleagues published a study that investigated the intrahost evolution of MRSA obtained from four individual patients with three to six cases of recurring skin and soft tissue infections during five years. In this study the authors identified development of antimicrobial resistance and intrahost evolution based on accumulation of mutations as well as uptake or loss of mobile genetic elements [136]. Antimicrobial resistance was acquired due to the uptake of resistance gene encoding plasmids. Overall, the number of SNPs observed by comparison of isolates with similar genetic background from individual patients was considerably low. These observations for MRSA infected patients suffering from recurrent skin and soft tissue infections are in concordance with the findings in this study. However, a remarkable difference of both studies is the detection of recurrent infections with isolates of the two stable lineages ST71-MRSP and ST529-MSSP during the whole time span. Instead, recurrent infections investigated in the study described above were either caused by MRSA belonging to the same genetic lineage for the whole time span (two patients) or MRSA of one lineage were completely replaced by MRSA showing another genetic background (two patients) [136]. Due to the limited number of patient investigated in both studies (four human patients by Azarian and colleagues and one dog patient in our study) it is not possible to state whether or not these different observations with respect to inter-lineage diversity within one patient are typical findings for the two investigated staphylococcal species. However, the remarkable similarities in both studies regarding the low mutation rate and the flexibility to integrate mobile genetic elements suggest that both, *S. aureus* and *S. pseudintermedius* have very successful survival strategies in the patients' environment and thus, recurrent infections caused by bacteria of the same lineages can occur over long time spans. As opportunistic pathogens, it is very likely that both species achieve this goal by colonization of the patients' skin and mucosa. Dogs can be colonized with *S. pseudintermedius* and persistent carriage is frequently detected in these animals. Interestingly, colonization studies identified higher proportions of *S. pseudintermedius* carriers among dogs with clinical signs of atopical dermatitis in comparison to healthy dogs [2,7-9]. Based on this observation it is also necessary to investigate host characteristics which might contribute to a persistent colonization status.

Our study was conducted retrospectively. Hence, it was not possible to determine the colonization status of the dog during as well as between infections. Furthermore, genotypic characterization was limited to the isolates stored in the strain collection of the Institute of Microbiology and Epizootics and could not be extended on more colonies per swab. By use of PFGE analysis it has been shown in the past for MRSP infected animals, that these patients can also be colonized with identical or very similar genotypes even after successful treatment of infection [29,31,38,44]. Thus, re-infection due to the animal microbiota could be another explanation for the infection with identical genetic lineages during the investigated time period. In addition, it has been shown in the past that humans can be colonized with *S. pseudintermedius* as well [39]. Therefore, multiple transmission events from the colonized owner to the dog might have led to re-infection as well like it has been already suggested in a previous study [38]. Further examples are given by multiple re-infection events of humans with *S. aureus* due to colonized animals [137,138]. Furthermore, the tenacity of *S. pseudintermedius* is high, enabling the opportunistic pathogen to survive in the environment like the feeding site or the sleeping place of the animal for considerable long time periods. Van Duijkeren and colleagues were able to demonstrate a high proportion of domestic contamination in households with *S. pseudintermedius*-infected dogs (70% of the investigated households) [37]. Thus, other contaminated sources in the surrounding area of the animal might have led to re-infection as well.

5.3.1.2 Protein prediction based on WGS data

Protein prediction of genes with coding SNPs that were stable over time after initial introduction showed different results:

i) No differences were observed in the predicted protein structures (three genes)
ii) A difference was predicted in the secondary structure but missing in the three-dimensional model (two genes)
iii) Both predictions revealed differences (two genes)
iv) Differences were only predicted for the three-dimensional structure but missing in the secondary structure model (two genes)

In general, it needs to be addressed that prediction of the secondary and three dimensional structures were obtained by use of different prediction tools. In order to understand the different outcomes (i-iv) it is necessary to understand the methodology used in both prediction tools.

Proteins are highly complex constructions. It is possible to differentiate three different structures [139].

i) The primary structure consists of the amino acid sequence. This structure can be easily obtained from WGS data.
ii) The secondary structure describes amino acids that arrange themselves based on hydrogen bonds between the backbone atoms.
iii) The tertiary structure considers the three-dimensional arrangement of the peptide, including information on the secondary structure. The tertiary structure is build based on ionic interactions, hydrogen bonds, van der Waals dispersion forces and sulphur bridges.

Prediction of the secondary structure by use of geneious (Biomatters Ltd. ©, New Zealand) is based on the EMBOSS (European Molecular Biology Open Software Suite) 6.5.7 tool garnier. The tool garnier allows the prediction of secondary protein structures by use of the GOR (Garnier-Osguthorpe-Robson) method. The prediction is based on mathematical modelling. To calculate the predictions, a database is used that includes 26 protein chains with a total number of 2,400 amino acids. This first version of the GOR method has been updated several times and the latest version GOR-IV is able to predict the secondary structure by use of a larger database including 267 protein chains and 63,556 amino acids. However, in geneiuos (Biomatters Ltd. ©, New Zealand) prediction of the secondary structure is only possible by use of the oldest version GOR-I [105]. This initial version allows to get very fast insight into the predicted secondary structure, since it is computationally fast and utilizes less CPU memory. However, it needs to be addressed, that this method is not as accurate in protein structure prediction as other prediction tools since the prediction is based on simulated folding [140].

Instead, the freeware Phyre2 predicts protein structures by use of a template-based modelling approach. This approach is based on the comparative analysis of the amino-acid sequence of interest with the protein database UniProtKB (http://www.uniprot.org/uniprot/) which contains more than 100,000 experimentally determined three dimensional protein structures. It is possible to build reliable models based on comparative analysis of the protein sequences since it is known that protein structures are more conserved than protein sequences and that a finite and relatively small (1,000–10,000) number of unique protein folds exist in nature [140]. Thus, it can be assumed that Phyre2 allows a more accurate prediction in comparison to the secondary structure obtained from geneious (Biomatters Ltd. ©, New Zealand). However, it needs to be kept in mind that both methods are not able to reveal the true structure of the protein based on information of the respective gene sequence. While Phyre2 predicts a more precise model compared to geneious (Biomatters Ltd. ©, New Zealand) it is computational more intensive and does not allow a direct comparative analysis of the secondary structures of two protein sequences of interest.

Interestingly, mutations were mainly observed in genes encoding transporter proteins (*malF*, *cydC*, *trkA*) as well as in genes encoding proteins that are involved in different metabolic pathways (exopolyphosphatase, choline dehydrogenase). In order to determine the relevance of predicted structural differences based on non synonymous mutations for the bacterium, it would be necessary to perform phenotypic assays which are able to allow a comparative analysis of the metabolic systems. Furthermore, construction of mutants would be needed in order to allow a clear connection between observed phenotypic differences and the mutation in the corresponding gene. Another serious problem is the ability of bacteria to use different proteins as well as different enzymes to maintain important metabolic pathways. While no data are available for *S. pseudintermedius*, *Staphylococcus aureus* has been described as one of the most complex organisms with respect to metabolic reactions and metabolites so far [141]. Thus, use of alternative metabolic pathways could further falsify the role of the gene of interest.

5.3.2 Phenotypic adaptation

All MRSP and MSSP isolated within this study were multidrug resistant, conferring resistance to three or more antimicrobial classes. It can be noted that phenotypic resistance profiles differed for isolates with different genetic background while they were highly stable for isolates of the same lineage. However, one remarkable difference was the lack of doxycycline resistance in the first MRSP isolated in September 2009. Medical history of the animal patient revealed treatment of this initial MRSP-infection with doxycycline. Thus, the acquisition of the doxycycline resistance encoding gene *tet*K can be linked to the selective pressure based on the use of the specific antimicrobial agent and demonstrates the adaptive capacity of *S. pseudintermedius* to react to environmental changes.

The detection of small colonies – so called small colony variants (SCVs) – during routine diagnostic supports the hypothesis of persistence in the host cell rather than re-infection. Small colony variants have been observed in various staphylococcal species [142] including *S. pseudintermedius* [143]. In contrast to SCVs detected for *S. aureus*, the *S. pseudintermedius* SCVs described by Savini and colleagues showed a very sticky phenotype and were stable even after sub-cultivation. In our study, SCVs observed in mixed culture with *S. pseudintermedius* expressing a classical phenotype (grey-white opaque colonies with a diameter of 1-2mm and production of double zone hemolysis on sheep blood agar) were unstable. After sub-cultivation of SCVs, colonies were indistinguishable from classical *S. pseudintermedius*. Thus, SCVs identified in this study resemble more closely the behaviour that has been described for SCVs in *S. aureus*. Multiple studies have been conducted on *S. aureus* SCVs in the recent past and allow drawing a connection between SCVs and successful persistence in the host cell [144]. Even though, persistence of *S. pseudintermedius* in the host cell has not been described so far, research on SCVs for *S. aureus* provides evidence of a coagulase positive staphylococcal species which is capable to persist in the host. Therefore, the detection of SCVs in *S. pseudintermedius* infections and the very low genomic diversity might indicate the possibility of *S. pseudintermedius* to persist in host cells as well. However, research is needed to investigate this hypothesis.

Biofilm formation assays revealed different results for MSSP and MRSP. MRSP tested in this study were significantly better biofilm producers compared to isolates of both MSSP lineages.

All MSSP isolates showed similar capacities to form biofilms, irrespective of the genetic lineage. Information on the relevance of biofilm formation as a virulence factor for *S. pseudintermedius* is scarce but it has been described as an important factor for other bacterial species. The ability to produce biofilms can be observed for many bacteria, whereas most of them are colonizers of humans or animals. Due to the ability to form an agglomeration with other microorganisms that attaches to surfaces, bacteria are able to survive hostile environmental changes, like for example limited access to nutrients, changes of the pH or temperature. In addition, biofilms protect bacteria from host defenses during colonization as well as during infection [145].

Several studies investigated the ability of *S. pseudintermedius* to form biofilms [50,146-150]. The results obtained from these studies suggest that the ability to form biofilms provides a pathogenic potential for *S. pseudintermedius* like it has been described for other staphylococci as well [145]. Furthermore, recent studies were able to show that both MSSP as well as MRSP isolates are able to form biofilms. These findings are in accordance with the results obtained in this study. However, it can be assumed that the intensity to perform biofilms is not necessarily an indicator for the virulence of *S. pseudintermedius*. Instead, both ST529-MSSP and ST71-MRSP were able to infect the patient of interest in this study multiple times during several years. Interestingly, all ST529-MSSP isolated after one of the initial infections in October 2008 showed a significantly increased biofilm formation intensity compared to one of the initial isolates (IMT16543). It is not possible to directly link this phenotypic difference to point mutations identified in genes for isolate IMT16543 and isolates obtained after the infection with IMT16543. In contrast, IMT18149 the isolate obtained directly after the infection with IMT16543 showed no differences with respect to non synonymous mutations by comparative analysis of the genome but significantly increased biofilm formation capacity. A possible explanation for this controversial finding might be that only single nucleotide polymorphisms with a high

coverage were included in the analysis. Furthermore, it needs to be considered, that changes in genes with repetitive regions were not investigated in this study due to frequent assembly errors in those regions. The ability of bacteria to form biofilms is very complex and besides the presence of genes that are involved in biofilm formation other factors like the regulation of gene expression are also very important in this process. Thus, further investigations to analyze the expression of genes of interest for example by use of real-time PCR could provide more information on the differing ability to form biofilms [151].

Three different adherence patterns to fibrinogen were observed for MSSP and MRSP tested in this study, whereas all isolates with the same genetic background were able to adhere to fibrinogen in the same intensity. ST529-MSSP were able to strongly adhere to fibrinogen, ST241 showed moderate binding capacity and ST71-MRSP revealed a low ability to bind to the tested ligand. In other studies, the ability of *S. pseudintermedius* to adhere to fibrinogen has been demonstrated as well [55,56]. Unfortunately, information about the presence of *mec*A is missing for isolates tested in these studies. Thus, it is not possible to compare the results. In our study MSSP express a higher binding capacity compared to ST71-MRSP. Due to the small sampling size it is not possible to generalize these findings for a large *S. pseudintermedius* population. In order to examine the putative impact of the genetic background for the binding capacity to fibrinogen a large sample size would be needed, including isolates of various different genetic lineages.

In staphylococci, multiple adhesins allow binding to fibrinogen. Extensive research on the proteins SpsD and SpsL (encoded on the adhesins *sps*D and *sps*L) revealed that both proteins mediated binding to fibrinogen. Further proteins encoded by the other known *S. pseudintermedius* adhesins could additionally contribute to fibrinogen. The adhesins *sps*D and *sps*L were present in all investigated isolates within this study. Furthermore, comparison of the adhesins present in isolates of the three genetic lineages (table 10, page 73) identified 17 of the 18 characterized adhesins of *S. pseudintermedius* while both MSSP-lineages contained 15 adhesins. Thus, it is not possible to link the observed phenotypic differences with respect to the fibrinogen binding capacity to genetic differences identified during whole genome analysis. The

analysis of whole genome sequence data does not provide information on the protein expression of these genes. Investigation of the gene expression would be necessary to provide more information on the presence of proteins involved in adherence to fibrinogen. In addition, it should be noted that there might be additional adhesins encoded on *S. pseudintermedius* genomes that have not been described so far. Nevertheless, the observed results indicate that (at least under *in vitro* conditions) binding to fibrinogen is not necessary to successfully infect a dog patient. In contrast to observed differences in the behavior of MRSP and MSSP to produce biofilms and to adhere to fibrinogen, fibronectin binding assays did not reveal significant differences between isolates of different genetic lineages as well as within each lineage. A limitation of the binding experiments to fibrinogen and fibronectin is the use of human ligands due to the lacking availability of canine fibrinogen and fibronectin. However, a study conducted in 2011 compared the binding capacity of *S. pseudintermedius* to fibrinogen obtained from different hosts, including humans and dogs [56]. In this experiment very similar results were obtained for adherence to human as well as to canine fibrinogen. Thus, it can be assumed that the binding behavior is similar for fibrinogen obtained from dogs and humans. In order to rule out a different binding pattern to canine fibrinogen and fibronectin experimental verification would be needed.

Adherence of bacteria to host cells and / or host cell components like extracellular matrix molecules such as fibrinogen and fibronectin is the necessary initial step to successfully colonize and / or infect the skin and mucous membranes of hosts. Binding to fibrinogen for example is useful for colonization of mucous membranes or for skin with micro lesions like from mammalians with atopic skin. Fibronectin is a component of the normal skin. While it is primarily detectable in basal layers it can be additionally detected in the epidermal stratum corneum of human patients with atopical dermatitis [152]. More complex methods – that take into account the complexity of colonization to the skin – would be needed to gain more knowledge about the ability of single isolates from the *S. pseudintermedius* subpopulation investigated in this study to adhere to skin of dogs. This is for example possible by *ex vivo* investigations. Corneocytes can be collected from donor dogs to investigate the ability of *S. pseudintermedius* to colonize these cells [53,54,57,153,154]. In addition,

it is possible to investigate the relevance of one specific adhesin after cloning and overexpression of the gene of interest into a bacterial species that does not adhere to canine corneocytes. By use of this method the role of the two proteins SpsD and SpsL (encoded by the adhesins *sps*D and *sps*L) in binding to corneocytes could be determined [57]. In order to additionally include responses of the host it is necessary to conduct *in vivo* animal experiments like for example mouse or rabbit skin infection experiments. In 2015, a rabbit skin infection model was conducted to determine the host-pathogen interaction. Therefore, rabbits were infected by subcutaneous injection of *S. aureus*. Abscess samples were collected on different days. RNA isolation and microarray analysis from each sample allowed to investigate changes in the gene expression pattern of the pathogen. In addition, real-time PCR was performed to determine the expression level of genes encoding host inflammatory cytokines and receptors. In this study, the authors unravelled some of the dynamics of both host and pathogen. Furthermore, they showed that *S. aureus* uses a network of virulence factors rather than one or two important virulence molecules [155].

6. Conclusion

This study enabled to unravel the microevolution of *S. pseudintermedius* obtained from several infections of one individual dog, who despite antibiotic treatment suffered from infections over a period of seven years. Independent of the genetic background as well as resistance to methicillin, two predominant successful lineages ST71-MRSP and ST529-MSSP were identified. Isolates belonging to the same sequence type showed only minor variations with consideration of the gene content as well as the occurrence of SNPs, indicating the isolation of the same identical lineages at least in this specific patient. However, ST71-MRSP showed the flexibility to adapt to environmental changes by uptake of a doxycycline resistance gene encoding plasmid as well as by the loss of a phage after initial infection. Phenotyping revealed opposite abilities for ST71-MRSP and ST529-MSSP regarding adherence to fibrinogen and biofilm formation. These findings suggest that MSSP and MRSP are able to use different pathways to successfully infect their hosts. No respectively minor phenotypical differences were observed for isolates sharing the same genetic background. This outcome is in accordance with the stable genomes of strains isolated over six (ST71-MRSP) respectively five years (ST529-MSSP). Reasonable explanations for the lack of variability within the identified lineages might be recurrent auto-infection, persistent infection as well as re-infection due to an external source.

Worryingly, mixed MSSP and MRSP infections were determined multiple times by investigation of phenotypically identical colonies. This finding has an impact on treatment decision and possible treatment failure due to underestimation of the resistance status. It needs to be addressed in future studies, if different pheno- and genotypes can be obtained regularly by investigation of more than one phenotypically identical colony. Testing multiple phenotypically identical colonies from basic media (e.g. sheep blood agar) or comparison of colony growth and number of colonies cultivated after streaking the swab onto basic media as well as on selective media (e.g. cefoxitin-substituted agar) could contribute to identify more mixed infections.

7. Summary

Staphylococcus (S.) pseudintermedius is part of the normal skin microbiota of dogs. As the most important canine coagulase-positive staphylococcal species, the opportunistic pathogen can cause a variety of different infections when the natural barrier of the host is lowered. The number of infections caused by multidrug resistant *S. pseudintermedius* increased in the last years – including infections with methicillin-resistant *S. pseudintermedius* (MRSP) – limiting treatment options for veterinarians. Different screening studies investigated the frequency and genetic variation of this opportunistic pathogen obtained from colonized and infected animals, revealing a high genotypic variability. However, there is no information available on the diversity of *S. pseudintermedius* in single patients that are frequently affected by infections with this pathogen although recurrent infections have been described as a serious problem for single dog patients in the past. Therefore, the aims of this study were to investigate the genetic and phenotypic variability of *S. pseudintermedius* isolated from multiple infections of a single patient.

In order to unravel the microevolution as well as the phenotypic diversity of this pathogen within one patient phenotypic and genotypic characterization was performed for methicillin-susceptible *S. pseudintermedius* (MSSP) and MRSP isolated from multiple wound infections of one dog between 2008 and 2014. Both, MRSP and MSSP were obtained from the patient at 18 respectively 10 time points, suggesting that the dog of interest was highly susceptible for infections caused by *S. pseudintermedius*. Furthermore, mixed infections with MRSP and MSSP were determined on three occasions. Worryingly, these isolates expressed the same colony morphology. This finding is of major concern for routine bacteriological diagnostic since it was shown during this work that even phenotypically similar appearing colonies can exhibit different resistance profiles.

Genotypic characterization identified three distinct genotypes, whereas the two lineages ST71-MRSP and ST529-MSSP were predominant. Analysis of single nucleotide polymorphisms and the variability of mobile genetic elements showed only minor variation, revealing the isolation of very stable lineages during the investigated

time period. Regarding the variability of the accessory genome content, ST71-MRSP showed the flexibility to adapt to environmental changes by the uptake of a doxycycline resistance gene encoding plasmid as well as by the loss of a phage after initial infection. The patient history revealed that the uptake of the plasmid was induced by treatment of the patient with doxycycline.

Phenotypic characterization showed opposing results for ST71-MRSP and ST529-MSSP with respect to biofilm formation as well as adherence to fibrinogen. These *in vitro* findings lead to the assumption that MSSP and MRSP are able to use different pathways to successfully infect their hosts. No respectively minor phenotypical differences were observed for isolates sharing the same genetic background. This outcome is in accordance with the stable genomes of strains isolated over six (ST71-MRSP) respectively five years (ST529-MSSP). A reasonable explanation for the lack of variability within the identified lineages might be recurrent auto-infection, persistent infection or re-infection due to an external source.

8. Zusammenfassung

Mikro-Evolution der *S. pseudintermedius*-Subpopulation eines Hundes über einen Zeitraum von sieben Jahren

Staphylococcus (S.) pseudintermedius ist ein Bestandteil der normalen Haut-Mikrobiota des Hundes. Als wichtigste Coagulase-positive Staphylokokken-Spezies bei Hunden kann der opportunistische Erreger eine Vielzahl unterschiedlicher Infektionskrankheiten hervorrufen wenn die natürlichen Abwehrmechanismen reduziert sind. Die Anzahl der Infektionen mit multiresistenten *S. pseudintermedius* (inklusive Methicillin-resistenten *S. pseudintermedius* (MRSP)) limitieren die Behandlungsoptionen der Tierärzte zunehmend. Verschiedene Screening-Studien haben das Vorkommen und die genetische Variabilität des opportunistischen Erregers bei kolonisierten und infizierten Tieren untersucht und dabei eine große genetische Vielfalt aufgezeigt. Allerdings gibt es bisher keine Untersuchungen zur genetischen Variabilität von *S. pseudintermedius*, die von multiplen Infektionen eines einizigen Patienten isoliert wurden, obwohl bekannt ist, dass rezidivierende *S. pseudintermedius*–Infektionen bei Patienten ein ernsthaftes Problem darstellen können. Ziel dieser Studie war es deshalb, die genetische und phänotypische Variabilität von multiplen *S. pseudintermedius*-Infektionen eines caninen Patienten zu untersuchen.

Um die Mikro-Evolution und die phänotypische Diversität des Erregers bei einem Patienten zu untersuchen, wurden Methicillin-sensitive *S. pseudintermedius* (MSSP) und MRSP aus multiplen Infektionsgeschehen zwischen 2008 und 2014 von einem Patienten charakterisiert. Sowohl MRSP als auch MSSP wurden zu 18 beziehungsweise 10 Zeitpunkten nachgewiesen. Die häufige Nachweisrate lässt darauf schließen, dass der Hund sehr empfänglich gegenüber *S. pseudintermedius*-Infektionen ist. Zusätzlich wurden zu drei Zeitpunkten gemischte Infektionen mit MSSP und MRSP nachgewiesen. Die Isolate ließen sich nicht anhand ihrer Koloniemorphologie voneinander unterscheiden. Dieses Ergebnis ist sehr beunruhigend in Hinblick auf die phänotypische Resistenztestung von nur einer Kolonie im Rahmen der Routine-Diagnostik, da in der Studie gezeigt werden konnte

dass auch Kolonien mit identischer Koloniemorphologie unterschiedliche Resistenzprofile aufweisen können.

Mittels genotypischer Charakterisierung wurden drei separate Genotypen identifiziert, wobei die beiden genetische Linien ST71-MRSP und ST529-MSSP dominierten. Innerhalb dieser beiden genetischen Linien zeigten die Isolate im Vergleich nur geringe Unterschiede in Hinblick auf Punktmutationen und die Variabilität mobiler genetischer Elemente. Dieses Ergebnis deutet darauf hin, dass die Isolate beider Genotypen zumindest in diesem caninen Patienten sehr erfolgreich sind.

Bei ST71-MRSP waren allerdings im akzessorischen Genom Unterschiede zu erkennen. Diese Variabilität zeigt die Flexibilität von ST71-MRSP sich durch die Aufnahme eines Tetrazyklin-Resistenzgen-kodierenden Plasmids an ändernde Umweltbedingungen anzupassen. Die Aufnahme des Plasmids wurde vermutlich durch die Behandlung des Patienten mit Doxyzyklin während seiner ersten MRSP-Infektion mit einem sensiblen Isolat induziert.

Die phänotypische Untersuchung zeigte bei der Biofilmbildung und der Bindung zu Fibrinogen entgegengesetzte Ergebnisse für ST71-MRSP und ST529-MSSP. Diese *in vitro* erzielten Ergebnisse deuten darauf hin, dass MSSP und MRSP einen Wirt über unterschiedliche Mechanismen erfolgreich infizieren können. Isolate mit dem gleichen genetischen Hintergrund zeigten nur geringe bzw. keine phänotypischen Unterschiede in den durchgeführten Untersuchungen. Dieses Ergebnis ist im Einklang mit den sehr stabilen Genomen von Isolaten der gleichen Linie obwohl sie über einen Zeitraum von sechs (ST71-MRSP) beziehungsweise fünf Jahren (ST529-MSSP) isoliert wurden. Wiederkehrende Autoinfektionen oder persistierende Infektionen könnten mögliche Erklärungen für die fehlende Variabilität sein.

9. Literature

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* Markierte Tagungen wurden durch Reisekostenstipendien teilfinanziert.

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12. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig, ohne unzulässige fremde Hilfsmittel und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Szilvia Vincze

Berlin, 24.05.2017