Genomic insights into the rapid emergence and evolution of multidrug-resistance in *Staphylococcus pseudintermedius*

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Abstract

Objectives: Multidrug-resistant (MDR), methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) strains have emerged rapidly as major canine pathogens and present serious treatment issues, and concerns to public health due to their, albeit low, zoonotic potential. A further understanding of the genetics of resistance arising from a broadly susceptible backgound of *S. pseudintermedius* is needed.

Methods: We sequenced the genomes of 12 *S. pseudintermedius* isolates of varied sequence types (ST) and resistance phenotypes.

Results: Nine distinct clonal lineages had acquired either staphylococcal cassette chromosomes (SCC)*mec* elements and/or Tn*5405*-like elements carrying up to five resistance genes (*aphA3, sat, aadE, ermB, dfrG*) to generate MRSP, MDR-methicillin sensitive *S. pseudintermedius* (MSSP), and MDR-MRSP populations. The most successful and clinically problematic MDR-MRSP clones, ST68 SCC*mec*V(T) and ST71 SCC*mec*II-III, have further accumulated mutations in *gyrA* and *grlA* conferring resistance to fluoroquinolones. Carriage of additional mobile genetic elements (MGEs) was highly variable suggesting horizontal gene transfer is frequent in *S. pseudintermedius* populations.

Conclusions: Importantly, the data suggest that MDR-MRSP evolved rapidly by acquisition of a very limited number of MGEs and mutations, and that use of many classes of antimicrobials may co-select for the spread and emergence of MDR and extensively drug resistant strains.

37 Antimicrobial stewardship will need to be comprehensive encompassing human medicine and
38 veterinary disciplines to successfully preserve antimicrobial efficacy.

Introduction

Antimicrobial resistance has emerged as one of the most important challenges facing human and veterinary medicine in the 21st century. Animals are major reservoirs for multidrug-resistant (MDR) pathogens including zoonotic bacteria.[1, 2] Pet animals, in particular, are frequently treated with the same antimicrobial classes that are critical in human medicine, including first and third generation cephalosporins and fluoroquinolones.[3] Additionally, pets often have close contact with humans providing opportunities for evolution of resistance on either host with subsequent transmission of bacteria between hosts.[4-7]

S. pseudintermedius is a major opportunistic pathogen in dogs and cats that typically causes skin and wound infections.[8] Methicillin-susceptible *S. pseudintermedius* (MSSP) isolates typically have a widely susceptible phenotype, and infections respond well to treatment with penicillinase-stable β-lactams, lincosamides, fluoroquinolones and potentiated sulphonamides.[9-11] Methicillin-resistant *S. pseudintermedius* (MRSP) isolates carrying *mecA* were first described in 1999,[12] and have since spread clonally across North America, Europe and Asia.[13-15] MRSP now account for 20%-47% of all clinical *S. pseudintermedius* submissions from dogs and cats in many areas.[16-20] More recently, multidrug-resistant (MDR) phenotypes (resistant to four or more antimicrobial classes) have emerged, dramatically limiting treatment options, as often all clinically relevant antimicrobial agents are ineffective. Reports of MDR-MRSP infections in humans have highlighted a threat to human health from zoonotic transmission.[21-23]

The genome-wide diversity and the genetic basis underpinning the rapid evolution and accumulation of antibiotic resistance in *S. pseudintermedius* is relatively unknown, despite the sequencing of the genomes of one MDR-MRSP (E140, ST71 SCC*mec*II-III),[26] one MDR-MSSP (ED99, ST25)[27] and one MSSP (HKU10-03, ST308).[28] Broad β-lactam resistance in MRSP is encoded by *mecA* carried on staphylococcal cassette chromosome (SCC)*mec* elements that have been acquired by multiple *S. pseudintermedius* clonal lineages on multiple independent occasions.[29-31] MDR-MRSP ST68 SCC*mec*V and MDR-MRSP ST71 SCC*mec*II-III are the major clones that have spread in North America since 2003-2004 and Europe since 2005-2006, respectively and that continue to disseminate globally.[14, 15, 32, 32] In this study, we sequenced the genomes of 12 *S. pseudintermedius* isolates in order to characterise the population structure of resistant *S. pseudintermedius*, compare the gene content between major clonal lineages, identify the genetic basis of MDR and explain the evolutionary steps leading to the dominant MDR-MRSP clones.

Methods

Bacterial isolates

Genomes of 12 *S. pseudintermedius* isolates from canine infections were sequenced (Table 1). Isolates had been confirmed as S. pseudintermedius through demonstration of the *S. intermedius* group-specific *nuc* by PCR and as MSSP or MRSP by the absence or presence of *mecA*.[20] Antimicrobial susceptibility was assessed by disk diffusion tests for penicillin, ampicillin, amoxicillin/clavulanate, cefalexin, oxacillin, fusidic acid, gentamicin, kanamycin, erythromycin, clindamycin, tetracycline, trimethoprim, ciprofloxacin and rifampicin according to BSAC guidelines,[33] resistance to oxacillin according to CLSI guidelines.[34] Where breakpoints for a particular antimicrobial agent were not included in these documents, recommendations by the disk manufacturer were used.

An isolate was classed as MDR if it was resistant to antimicrobials in ≥4 antimicrobial classes.[35] Amongst MRSPs, the most extensively drug-resistant isolates from our collection of clinical European isolates were selected and for further variation within MRSP ST71, we selected isolates from the UK and from Germany and those with tetracycline resistance and those without. A MDR ST68 isolates was included to allow comparison of the current two most successful lineages and two usual clinical MRSPs were included for their lack of MDR phenotype as they were thought to provide information into the evolutionary events of MDR-MRSP emergence (Table 1). Three MSSPs were selected on varying resistance phenotypes including two MDR-MSSP isolates with uncharacterised STs, and one MSSP with an uncharacterised ST. For PCR reactions, genomic DNA was extracted from overnight cultures grown in brain heart

infusion broth (Sigma, UK) using the PureElute Bacterial Genomic DNA preparation kit (EdgeBiosystems, UK) at quarter scale following the standard protocol with the addition of 3µl lysostaphin (5mg/mL, Sigma, UK) to the spheroblast buffer.

Genome sequencing

Overnight cultures were grown in tryptic soy broth (TSB) at 37°C with 200 rpm shaking. Genomic DNA was extracted from 1 mL of cultures using the MasterPure Gram Positive DNA Purification Kit (Cambio, UK). This kit is recommended for extraction of total DNA for whole genome sequencing and has been used in a large number of studies including those assessing low molecular weight molecules such as plasmids.[36-38] Illumina library preparation was carried out as described.[39] Hi-seq sequencing was carried out following the manufacturer's standard protocols (Illumina, Inc, USA). Genomes were assembled *de novo* with Velvet,[40] which yielded on average 135 contigs/genome (Table 1) and contigs were realigned using Mauve[41] against genome ED99 (Accession: CP002478). Sequencing of the twelve isolates yielded an average total of 401,011 Kb per run, corresponding to genome coverage of between 118x and 213x (mean = 151x; Table 1). Nucleotide sequences of the isolates have been deposited in the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena/) and accession numbers are shown in Table 1.

Comparative genomics and phylogenetics

Draft genomes were then compared to the three *S. pseudintermedius* high quality genome sequences published before June 2013, ED99, HKU10-03 and E140 (26-28), using the Artemis and ACT genome visualisation tools.[42,43] MGEs were characterised by BLAST analysis of unique genome regions. Comparative genome figures were created using EasyFig.[44] Fastq files for the

115 isolates were mapped against the *S. pseudintermedius* genome ED99 using SMALT
116 (www.sanger.ac.uk/smalt) in order to identify single nucleotide polymorphisms (SNPs), as
117 previously described.[45] SNPs located in MGEs were identified by comparative genomics and
118 removed from the alignment. Regions of potential recombination were also identified and
119 removed from the alignment as previous described.[46] A maximum likelihood tree was
120 generated from core genome SNPs (the core genome being defined as regions of the
121 chromosome not excluded when MGEs and regions of potential recombination were removed)
122 using RAxML.[47] Trees were visualized and annotated using Figtree. Insertion sequence finder
123 was used to detect IS elements (www-is.biotoul.fr/). Restriction-enzymes were named
124 according to rebase.neb.com.[48] CRISPRfinder was used to detect and characterise CRISPR
125 elements (http://crispr.u-psud.fr/Server/).[49]

126 ## Population structure analysis

127 The sequence type (ST) of each of the isolates was determined using the recently developed 7-
128 gene multi-locus sequence typing method (MLST)(54). The clonal distribution and relatedness
129 of *S. pseudintermedius* isolates was assessed using all STs in the MLST database at the time of
130 analysis (http://pubmlst.org/spseudintermedius/) and eBURST clustering
131 (http://eburst.mlst.net/).[50]

132 ## Association of MDR phenotype and presence of Tn*5405*-like elements

133 The association of the MDR phenotype with the Tn*5405*-like elements was tested in a further
134 60 MSSP and 52 MRSP isolates from a collection of clinical canine isolates.[51] All strains were
135 grown on brain heart infusion agar (BHIA) overnight, prior to testing antibiotic
136 resistance/susceptibility by growth on BHIA plates containing 0.02mg/mL erythromycin,

0.02mg/mL kanamycin, 0.02mg/mL oxacillin, 0.02mg/mL streptothricin or 0.02mg/mL trimethoprim. Genomic DNA was extracted as previously described. The presence of *Tn*5405-like elements was detected by PCR that targeted the transposase gene using forward ($^{5'}$AAGCGATTCGATGATTTTGC$^{3'}$) and reverse primers ($^{5'}$CCTGGTTTTAGTTGGCCATT$^{3'}$). PCR reactions were performed using Bioline MyTaq kit (Bioline, UK) in 50 µL volumes containing 1 pmol forward and reverse primer, 1 x buffer, 0.1 units Bioline MyTaq polymerase, 1uL of DNA and dH_2O. Reactions were heated to 95 º C for an initial 2 minutes, followed by 35 cycles of 95 ºC for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 30 seconds, followed by of 1 cycle of 72 ºC for 10 minutes.

Results

Population structure of *S. pseudintermedius*

The 12 study genomes, respresenting the phenotypic groups MSSP (*n*=1), MRSP (*n*=2), MDR-MSSP (*n*=2) and MDR-MRSP (*n*=7), were compared to previously sequenced *S. pseudintermedius* genomes (*n*=3)(Table 1). All genomes were highly similar to each other, and sequence divergence was mainly due to SNPs and presence/absence of short genomic regions, and variation in the presence/absence of MGEs. Reconstruction of the phylogeny using 34,997 high-quality SNPs revealed nine clonal lineages (defined as a group of MLST genotypes in which each genotype shares at least five loci in common with another member of the group) amongst the 15 genomes, six of them sequenced for the first time (CC1, CC68, CC258, CC260, CC262 and CC263). (Figure 1). MRSP isolates were distributed over four clonal lineages (CC71, CC68, CC260

and CC258), whilst the MDR phenotype (3 MDR-MSSP and 7 MDR-MRSP) was distributed in six (CC25, CC68, CC71, CC258, CC262 and CC263). The six ST71 isolates only differed by 162 SNPs.

Clustering of 309 STs in the MLST-7 database using the eBURST program confirmed that MRSP emerged independently in 6/9 major *S. pseudintermedius* clonal lineages (CC1, CC45, CC68, CC71, CC84 and CC258) and in numerous doublet or unlinked STs (Figure 2). MRSP phenotypes existed in the majority of isolates belonging to STs CC45, CC71 and CC258 suggesting that SCC*mec* acquisition contributes to clonal lineage expansion.

Multidrug resistance was acquired by successful MDR-MRSP clones through a three-step accumulation of genomic changes

To understand the evolution of multidrug-resistance in *S. pseudintermedius*, we investigated the presence and absence of resistance genes in the core and accessory genome, characterised their presence on each identified MGE by BLAST analysis and compared their association with resistance phenotype. All phenotypic resistances could be putatively assigned to a genetic determinant in all isolates. Pairwise alignments of *S. pseudintermedius* genomes revealed they are co-linear to each other, and confirmed a stable component of genes found in all strains (Figure S3). MGEs accounted for up to 8% of the genome, providing evidence of extensive MGE acquisition.

All methicillin-resistant study strains carried *mecA* on a SCC*mec* element (Figures 1 and 4). All MDR-MRSP ST71 isolates had a homologous copy of the SCC*mec*II-III element (29), while the MDR-MRSP ST68 isolate (23929) had a SCC*mec*V(T) element.[14] The MDR-MRSP ST261 isolate, 1726, carried a novel SCC*mec*IV element that was homologous to an SCC*mec*IVg element in *S. aureus*,[52] but possessed an additional nine genes. MRSP ST260 isolates carried a novel SCC*mec*

element that shared blocks of homology to both SCC*mec*X and SCC*mec*V (containing a *ccrC* gene), however, it was not possible to obtain its full structure from the sequencing data (data not shown). Pairwise comparison of SCC*mec* element structures showed element divergence giving further support to an independent evolution of MRSP on multiple occasions (Figure 4).

Many other resistance genes were located on transposable elements, and all genomes carried at least two transposons or insertion sequence (IS) elements while *tra* genes for conjugative transfer were absent in all (Figure 1).

All eleven MDR isolates carried a large transposable element resembling Tn*5405*-like elements.[53,54] This element carried up to five antimicrobial resistance genes *aphA3-sat-aadE-dfrG-erm*(B) encoding resistance to aminoglycosides (including kanamycin, neomycin and amikacin), streptothricin, trimethoprim and erythromycin and was present in 8/8 MDR-MRSP and 3/3 MDR-MSSP isolates (Figures 1 and 5). Pairwise comparison revealed all Tn*5405*-like elements were integrated at the same core genome loci. Variation was seen in carriage of *dfrG* and *cat* (Figure 5), truncated versions were found in MDR-MSSP isolates GL118B and ED99, and one was located on a plasmid (GL117B).

Screening the larger collection of 60 MSSP and 52 MRSP, Tn*5405*-like elements were present in 8/11 (73%) MDR-MSSP and in all 46 (100%) MDR-MRSP and the MDR phenotype was found in 11/60 (18.3%) MSSP isolates and 46/52 (88.5%) MRSP isolates. Carriage of Tn*5405*-like elements was associated with the MDR phenotype ($p<0.001$, χ^2 test).

Additional resistance genes were present on transposons or IS elements integrated in the core *S. pseudintermedius* genome (Figure 1). The tetracycline resistance gene *tet*(M) was present on

Tn*5801* and Tn*916* elements in three and five genomes, respectively. All but one genome carried at least one *blaZ* gene, encoding resistance to penicillin, either on Tn*552* and/or on Tn*554*-like elements. Finally, the *aac-aph* gene, encoding resistance to gentamicin, was located on IS*256* or IS*1272* elements in seven and two genomes, respectively. The *cadA* gene, encoding resistance to cadmium, was located on IS*431* elements in four genomes.

Plasmids do not appear to play a role in the emergence of MRSP. In this study, 12/15 genomes carried the integrated plasmid first described in ED99,[27] but this plasmid did not carry any resistance genes. GL117B possessed an additional plasmid with a replication (*rep*) gene with 97% homology to plasmid pUB*112* from *S. aureus*, and resistance genes *aphA-sat-aadE-ermB* similar to the truncated version of the *Tn*5405-like element (Figure 5). There was no further evidence for the carriage of plasmid replication (*rep*) genes in any of the *S. pseudintermedius* genomes.

Of core genome mutations described to confer antimicrobial resistance in *S. pseudintermedius*,[80] individual mutations were present in all strains irrespective of their phenotype. However, only the six isolates phenotypically resistant to fluoroquinolones (all MDR-MRSP ST71 and the MDR-MRSP ST68 isolate), representing the most successful MDR-MRSP clones, had mutations *gyrA* S84L and *grlA* S80I simultaneously (Figure 1). Mutations in *folP* causing sulphonamide resistance are described in *S. aureus*.[57] However, of the eight strains (including ED99) showing phenotypic resistance to trimethoprim/sulfamethoxazole, the clinically relevant combination product available for veterinary use, only one strain (23923) showed *folP* mutations. Instead, *in vitro* resistance was more often correlated with the carriage

of *dfrG* on Tn*5405*-like elements (5/8 strains). Only the successful MDR-MRSP ST68, 23929, was phenotypically resistant to fusidic acid using BSAC breakpoints.[33] In *S. aureus*, *fusA* mutations cause low-level resistance to fusidic acid.[58, 59] Isolate 23929 had three non-synonymous mutations in *fusA* (A376V, I461V and V90I), while only 1 other strain had a *fusA* mutation (V90I). No strains carried the MGE-encoded *fusB*, *fusC* or *fusD*. This data indicates that mutations at sites 376 and 461 are implicated in phenotypic resistance to fusidic acid.

Comparison of fitness factors in the genomes

The successful survival and spread of MDR-MRSP ST71 and MDR-MRSP ST68 are likely depend on factors that influence colonisation or disease and other fitness factors that might influence survival and evolutionary success were widely distributed (Figure 1). Prophages were present in all *S. pseudintermedius* genomes except ED99, and were integrated into eight chromosomal locations (Figure 1). All MDR-MRSP ST71 isolates possessed three homologous prophages without evidence for loss/acquisition of additional prophages. The MDR-MRSP ST68 isolate possessed four prophages, whilst all other strains possessed only one prophage in their genome. Prophage genomes were diverse, with few shared genes, and chromosome integration sites differed (Figure S1). Genes encoding virulence associated protein E (VirE) and a surface protein with LPxTG motif were located on some prophages, but genes homologous to Panton-Valentine leukocidin (PVL) toxin were not found.

S. pseudintermedius pathogenicity island (SpPI)1, described in ED99, was present in all genomes except 1726 (Figure 1). SpPI1 variants existed (Figure 1 and Figure S2), but all shared the same integrase gene and the same core genome integration site. Hypothetical proteins were identified but none related to known virulence factors.

Potential virulence genes described in *Staphylococcus intermedius* group (SIG) isolates such as surface proteins, exoenzymes and toxins including novel toxins Se-int, the β-hemolysin and exfoliative toxins[53] were found in some but not all genomes. Four of 18 genes encoding surface proteins that adhere to extracellular matrix factors (*spsF, spsO, spsP* and *spsQ*)[60-63] were absent in some genomes (Figure 1). All surface protein genes were variable (non-synonymous and Indel variation) between lineages, with the most variation present in *spsD*, *spsF*, *spsJ*, *spsK*, *spsL*, *spsO* and *spsP*. There was no variation in the surface protein genes within ST71 isolates. Six of the seven exoenzyme genes (*coa, lip, geh, htrA, nuc* and *clpX*) were present in all genomes, whereas *nanB* encoding sialidase was present in only 5/15 genomes (Figure 1). Three of the four toxin genes (*hlb, se-int* and *speta*) were present in all genomes; in contrast *lukF-I* was present in only 9/15 *S. pseudintermedius* genomes (Figure 1). Genes encoding all previously identified two-component regulatory systems (*agrA*, *agrB*, *agrC*, *agrD*, *saeS*, *saeR*, *srrA*, *srrB*, *arlS*, *arlR*, *lytS* and *lytR*) and SarA protein families (*sarA*, *sarR*, *sarZ* and *rot*) were present in all genomes (Figure 1). Collectively, this data indicates that variation in virulence factors in the core genome is common in *S. pseudintermedius*, and possibly lineage-associated as in *S. aureus*.

Barriers to horizontal gene transfer in *S. pseudintermedius*

The success of particular lineages and/or clones, such as the major MDR-MRSP clones, may also depend on their ability to acquire novel MGEs through horizontal gene transfer (HGT). Restriction-modification (R-M) systems and competence genes *comG* and *comE* were present in all genomes irrespective of antimicrobial resistance. All isolates showed evidence of a type I R-M system that we therefore call Sps1, and is also known as SpsE140ORFAP (rebase.neb.com). A

full intact type I Sps1 R-M system, containing restriction (*hsdR*), modification (*hsdM*) and specificity (*hsdS*) genes, was present in the core genome of 11/15 *S. pseudintermedius* genomes (Figure 1). In contrast, incomplete systems that are predicted to be non-functional were present in the remaining genomes. HKU10-03 (ST308) possessed *hsdR* and *hsdM*, but no *hsdS*, 4639949 (ST309) possessed *hsdM* only, whilst ED99 (ST25) and GL118B (ST262) possessed truncated *hsdR* genes. Two types of *hsdS* variant in the type I R-M systems existed, whilst *hsdR* sequences and *hsdM* sequences were highly homologous across all genomes (Figure 1). These data suggest that the two clusters of lineages modify their DNA at different target sites, restricting horizontal transfer of DNA and divergent evolution of *S. pseudintermedius* into at least two populations.[64] Additionally, a second type I R-M system was carried on all SCC*mec*II-III elements of MDR-MRSP ST71 genomes and on the SCC*mec*IV element of the MDR-MRSP ST261 genome (Figure 1, Figure 4). The *hsdS*, *hsdM* and *hsdR* sequences from the SCC*mec* elements were highly homologous, but different from the sequences of respective core genome genes, and has been called SpsE140ORFGP. Predicted intact type II R-M systems, containing *hsdM* and *hsdR* genes were present in 6/15 genomes (Figure 1) at four different locations: (i) adjacent to *orfX* in MDR-MSSP ST25 (ED99)(Sps99ORF30P with a predicted cutsite of CTRYAG) and MSSP ST309 (4639949) genomes, (ii) adjacent to the *phoB* gene in the MSSP ST308 (HKU10-03) (SpsORF2234P with a predicted cutsite of CCNGG) and MRSP ST260 genomes (BNG1 and BNG3), (iii) at the type I R-M system locus in MDR-MRSP ST68 (23929) (called Sps68ORF1551), and (iv) on the SCC*mec*IV and novel SCC*mec* elements of MDR-MRSP ST68 (23929) and MRSP ST260 (BNG1 and BNG2) genomes (called SpsORF2234P), respectively , and was homologous to the type II system present at the *phoB* site.

Clustered regularly interspaced short palindromic repeats (CRISPR) systems were detected in 3/15 genomes (Figure 1). Six of the 22 spacers in CRISPR from ED99 were homologous to bacteriophage and plasmids,[27] suggesting this isolate is immune to uptake of specific elements encoding these sequences. Two of the 24 spacers in CRISPR from GL118B were homologous to bacteriophage, and 2/13 spacers in the CRISPR system found on SCC*mec*V(T) in 23929 were homologous to bacteriophage and plasmids. There was no evidence that CRISPR has recognised important SCC*mec* or transposons and MDR-MRSP ST71 and ST68 did not have greater or fewer R-M or CRISPR systems than other strains.

Discussion

Identifying the whole genome characteristics of MDR-MRSP is critical to explain the evolution and rapid emergence of successful clones. We have shown that only a three-step accumulation of an SCC*mec* element, Tn*5405*-like elements and SNPs in *gyrA*/*grlA* was required for the emergence of the two globally successful MDR-MRSP clones. At the same time, these MDR-MRSP have successfully spread worldwide, indicating that selection for survival of these resistant isolates is common.

Multiple SCC*mec* elements have now been sequenced in *S. pseudintermedius*.[26, 29, 68-71] They have given rise to multiple MRSP clones and we now report MRSP phenotypes in six of nine major lineages. The wide distribution of MRSP phenotypes in STs from CC45, CC71 and CC258 suggests that SCC*mec* elements were acquired ancestrally or that these lineages can acquire and spread SCC*mec* more effectively. SCC*mec* elements of *S. pseudintermedius* most closely matched SCC*mec* elements from *S. aureus* (SCC*mec*II-III, SCC*mec*IV and SCC*mec*V(T)) and/or

from coagulase-negative staphylococci (CNS) such as *S. epidermidis* (SCC*mec*II-III) and *S. haemolyticus* (ΨSCC*mec*$_{57395}$), supporting a role of HGT from CNS in MRSP emergence.

The acquisition of the Tn*5405*-like elements, encoding multiple antimicrobial resistances simultaneously was a consistent feature of the MDR phenotype seen in both MSSP and MRSP. Tn*5405*-like elements have been acquired by at least four *S. pseudintermedius* lineages, with variants also found in an additional two lineages. Driving pressures for the selection of Tn*5405*-like elements remain unclear as antimicrobial prescribing data for pets are available for only a few countries and the antimicrobial agents encoded on this element seem of little veterinary clinical relevance. Nevertheless, the use of only one antimicrobial could be sufficient to select for the survival of clones with Tn*5405*-like transposons and resistance to four classes of antimicrobial. Acquisition of specific *gyrA/grlA* SNPs associated with phenotypic resistance to fluoroquinolones is strongly correlated with the globally successful MDR-MRSP lineages. [14,69] Several fluoroquinolones were authorised for use in pets and have been widely used since the late 1980s suggesting a key role of fluoroquinolones in promoting survival and spread of MRSP. A similar association has been reported between fluoroquinolone resistance and the pandemic spread of major hospital-associated MRSA clones.[25, 65, 74]

The variation in distribution of other resistance genes and potential fitness factors suggests a genomic plasticity provided by transposons and suggests that HGT of transposons is frequent in *S. pseudintermedius*.[14, 75-77] Though prophages were present in all genomes (except ED99), MDR-MRSP ST71 and ST68 strains contained most prophages suggesting a potential role of prophages in the fitness of MRSP.

Although HGT mechanisms have been poorly studied in *S. pseudintermedius,* the frequent carriage of prophages makes it likely that transfer is predominantly by bacteriophage transduction rather than plasmid conjugation, in contrast to *S. aureus.*[24, 78] Experimental studies are required to test whether the prophage are capable of performing generalised transduction, and whether transformation occurs in *S. pseudintermedius* strains.

The distribution of type I and/or type II R-M systems, known barriers to HGT in *S. aureus*, suggested that MDR-MRSP genomes are no more efficient or inefficient in acquiring MGE-encoded resistance. R-M systems were present on all SCC*mec* elements in this study, and also on ΨSCC*mec*57395 from a previously described MDR-MRSP ST45.[69] To determine if populations are evolving independently, future studies could assess the extent of recombination occurring within and between *S. pseudintermedius* populations. As CRISPR systems were rare in genomes, and spacer sequences did not match any known SCC*mec* elements or transposons, it is unlikely that CRISPRs have a major role in governing HGT of resistance genes in *S. pseudintermedius*. Overall, ED99 carried the fewest R-M systems and no prophage, and is potentially an appropriate laboratory strain for genetic manipulation.

We conclude that MDR-MRSP evolved rapidly through step-wise accumulation of SCC*mec*, Tn*5405*-like elements and SNPs conferring fluoroquinolone resistance. Thus, this study indicates that the use of only a small number of antimicrobials may select for the pandemic spread of MDR strains. In particular, a unifying feature of the fittest clones, MDR-MRSP ST68 and MDR-MRSP ST71, was their fluoroquinolone resistance through core genome mutations, mirroring the success of hospital-associated MRSA clones, such as MRSA CC22. These findings

have implications for public health as they raise concern over additive selection pressure on zoonotic bacteria since antimicrobial classes used in pet animals are the same as those critical in human medicine. Therefore stewardship needs to be comprehensive and include medical and veterinary healthcare in order to successfully preserve antimicrobial efficacy.

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Transparency declaration

None to declare.

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587 Figure Legends

588 **Figure 1. Phylogenetic relationships and resistance and genomic features.** The phylogenetic

589 relationship of the *S. pseudintermedius* genomes based on SNP clustering is shown at the top of

590 each figure. Strain names are coloured according to their phenotypic group, MSSP = light blue,

591 MDR-MSSP = dark blue, MRSP = pink, MDR-MRSP = red. Grey or coloured bars indicate

592 phenotypic resistance or presence of genetic marker, whilst white indicates susceptibility or

593 absence. A) Phenotypic antibiotic resistance profiles and genetic resistance markersMGEs of

594 the same type integrated at the same chromosomal loci unless stated. SCC*mecA* elements are

595 SCC*mec*II-III (grey), SCC*mec*V(T) (blue), SCC*mec*IV (yellow) and a unique SCC*mec* (green), with

596 homology to SCC*mec*X and SCC*mec*V; structural differences are shown in Figure 4. Tn*5405*-like

597 elements carried *aphA3-sat-aadE-ermB-dfrG* (grey), *aphA3-sat-aadE-ermB* (blue), *aphA3-sat-*

598 *aadE* (yellow) or *aphA3-sat-aadE-cat* (green); structural differences are shown in Figure 5. All

599 Tn*5801* carried *tetM*. All Tn*916* carried *tetM* but were integrated into three different sites on

600 the genome (1, 2 or 3). All Tn*552* and Tn*554*-like carried *blaZ*. All IS256 and IS1272 carried *aac-*

601 *aph*. All IS431 carried *cadA*. SNPs associated with antimicrobial resistance to fluoroquinolones

602 (*gyrA* and *grlA)* and fusidic acid (*fusA)*. B) Content of other factors influencing fitness, survival

603 and horizontal gene transfer (HGT). MGEs of the same type integrated at the same

604 chromosomal loci unless stated. Three SpPI1 variants (grey, blue, yellow), two bacteriophage

605 phi1 variants (grey, blue) and three phi3 variants (grey, blue, yellow) were detected. Genes

606 encoding surface proteins, exoenzymes, toxins and regulatory systems are indicated. Barriers to
607 horizontal gene transfer (HGT) were restriction-modification (R-M) or clustered regularly
608 interspaced short palindromic repeats (CRISPR) systems. Two *hsdS* variants of type I R-M core
609 were detected (grey, blue). This figure appears in colour in the online version of JAC and in
610 black and white in the print version of JAC.

611 **Figure 2. Population structure of *Staphylococcus pseudintermedius*.** Clusters of related STs and
612 individual unlinked STs within the entire *S. pseudintermedius* MLST database are shown as an
613 eBURST diagram. Clusters of linked STs (black lines) correspond to clonal complexes (CCs). STs
614 for which MRSP phenotypes are reported in the MLST database are highlighted in black boxes.
615 STs for which genomes have been sequenced are highlighted in boxes as follows, light blue =
616 MSSP, dark blue = MDR-MSSP, pink = MRSP, red = MDR-MRSP. This figure appears in colour in
617 the online version of JAC and in black and white in the print version of JAC.

618 **Figure 3. Pairwise comparison of representative *Staphylococcus pseudintermedius* genomes.**
619 The alignment of genome sequences from ED99, 69687, 23929, 1726 and HKU10-03 are
620 displayed in Artemis Comparison Tool. Red bars present orthologue matches identified by
621 FASTA analysis. Mobile genetic elements (MGEs) including Staphylococcal cassette
622 chromosome (SCC)*mec* elements, transposons, plasmids, prophages and *S. pseudintermedius*
623 pathogenicity islands (SpPIs) are shown as coloured boxes. This figure appears in colour in the
624 online version of JAC and in black and white in the print version of JAC.
625
626 **Figure 4. Comparison of SCC*mec* elements from *Staphylococcus pseudintermedius* genomes**.
627 The SCC*mec*II-III element from ST71 (69687) is compared to SCC*mec*IV from ST261 (1726), and

SCC*mec*V(T) from ST68 (23929). Homology is indicated by a colour scale of blue (100% homology) to grey (78% homology). Genes are coloured by function *mecA* in red, *mecRI* in pink, *ccr* in yellow, type I restriction-modification (R-M) system genes in blue, type II R-M system genes in light blue, and clustered regularly interspaced short planidromic repeat (CRISPR) system genes in green. Figure produced using EasyFig and usingSCC*mec*II-III element sequence from 69876*. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

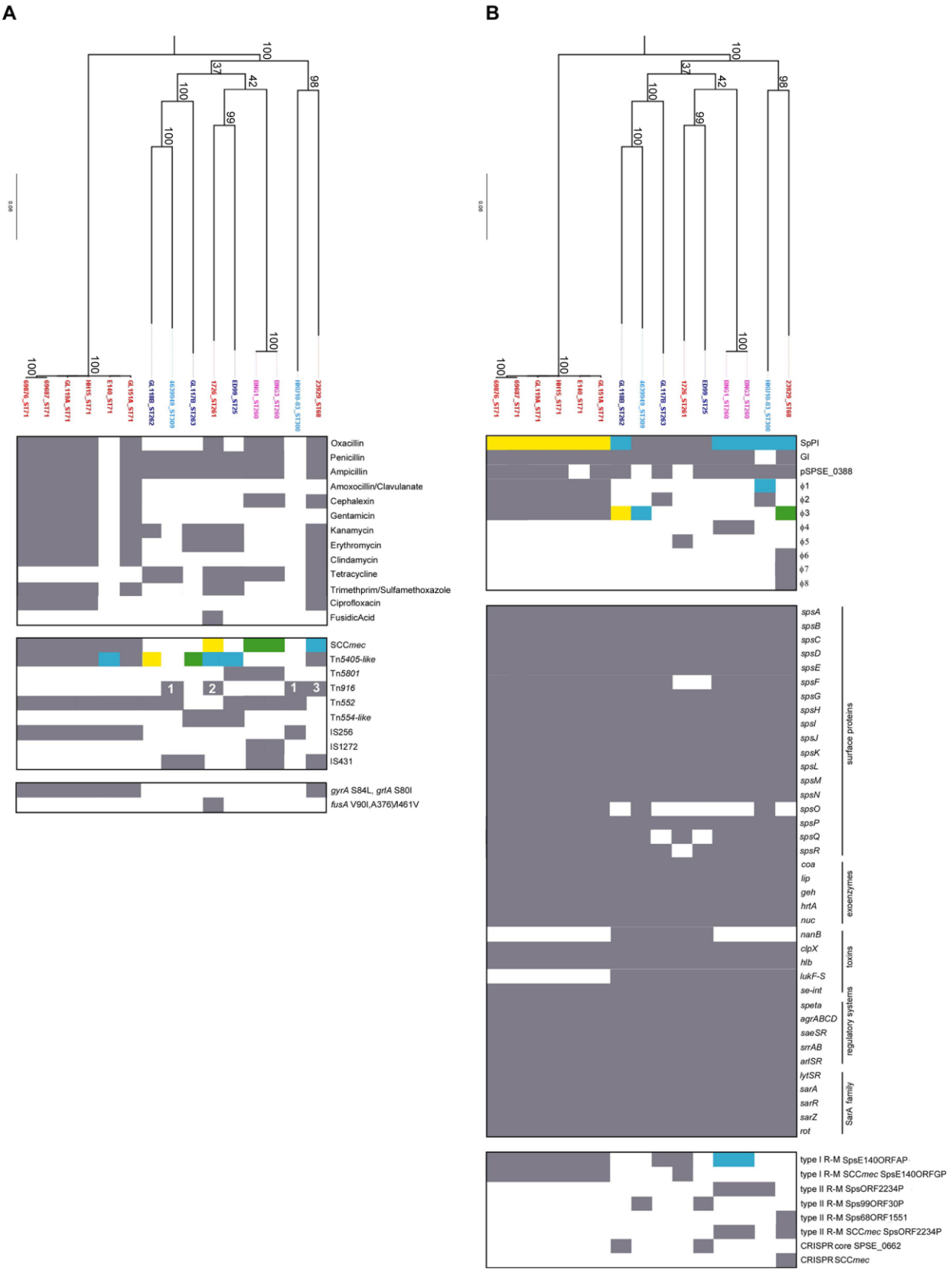
Figure 5. Comparison of *Tn*5405-like elements from *Staphylococcus pseudintermedius* genomes. The *Tn*5405-like element from ED99 is compared to *Tn*5405-like elements from 1726, E140, 69687, 23929, GL118B and GL117B. Homology is indicated by a colour scale of blue (100% homology) to grey (89% homology). Genes are coloured by function transposase in red, antibiotic resistance genes in yellow, topoisomerase genes in green, resolvase genes in light green, plasmid replication genes in bright blue and plasmid recombination genes in dull blue. Genes in grey were not homologous to any genes using BLAST. *aphA, aadE* encode resistance to aminoglycosides, *sat* to streptothricin, *dfrG* to trimethoprim and *cat* to chloramphenicol. Figure produced using EasyFig and *Tn*5405-like element sequence from 69876*. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

Figure S1. Comparison of representative prophage from *Staphylococcus pseudintermedius* strains. A representative prophage from each of the 8 insertion sites is shown. Homology is indicated by a colour scale of blue (100% homology) to grey (84% homology). The prophages are

named by insertion site (ϕX) and then the genome from which the sequence originates. Figure produced using EasyFig.

Figure S2. Comparison of SpPI1 from *Staphylococcus pseudintermedius* strains. The pathogenicity island SpPI1 from ED99 is compared to SpPI1 elements from other sequenced *S. pseudintermedius* strains. Homology is indicated by a colour scale of blue (100% homology) to grey (84% homology). SpPI1 genes are coloured pink, and integrase genes in red. Gene names are shown for genes that were homologous to annotated genes by pBLAST. Figure produced using EasyFig. *Note that *Tn*5405-like elements sequence from BNG1 and 69876 was used to create the figure.

Figure 1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HH15_ST71
E140_ST71
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GL117B_ST263
1726_ST261
ED99_ST25
BN03_ST260
BN01_ST260
HK0110-03_ST308
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Penicillin
Ampicillin
Amoxocillin/Clavulanate
Cephalexin
Gentamicin
Kanamycin
Erythromycin
Clindamycin
Tetracycline
Trimethprim/Sulfamethoxazole
Ciprofloxacin
FusidicAcid
SCCmec
Tn5405-like
Tn5801
Tn916
Tn552
Tn554-like
IS256
IS1272
IS431
gyrA S84L, grlA S80I
fusA V90I,A376V,I461V
SpPI
GI
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spsO
spsP
spsQ
spsR
surface proteins
coa
lip
geh
hrtA
nuc
exoenzymes
nanB
clpX
hlb
lukF-S
se-int
toxins
speta
agrABCD
saeSR
srrAB
arlSR
regulatory systems
lytSR
sarA
sarR
sarZ
rot
SarA family
type I R-M SpsE140ORFAP
type I R-M SCCmec SpsE140ORFGP
type II R-M SpsORF2234P
type II R-M Sps99ORF30P
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CRISPR core SPSE_0662
CRISPR SCCmec

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Figure 2

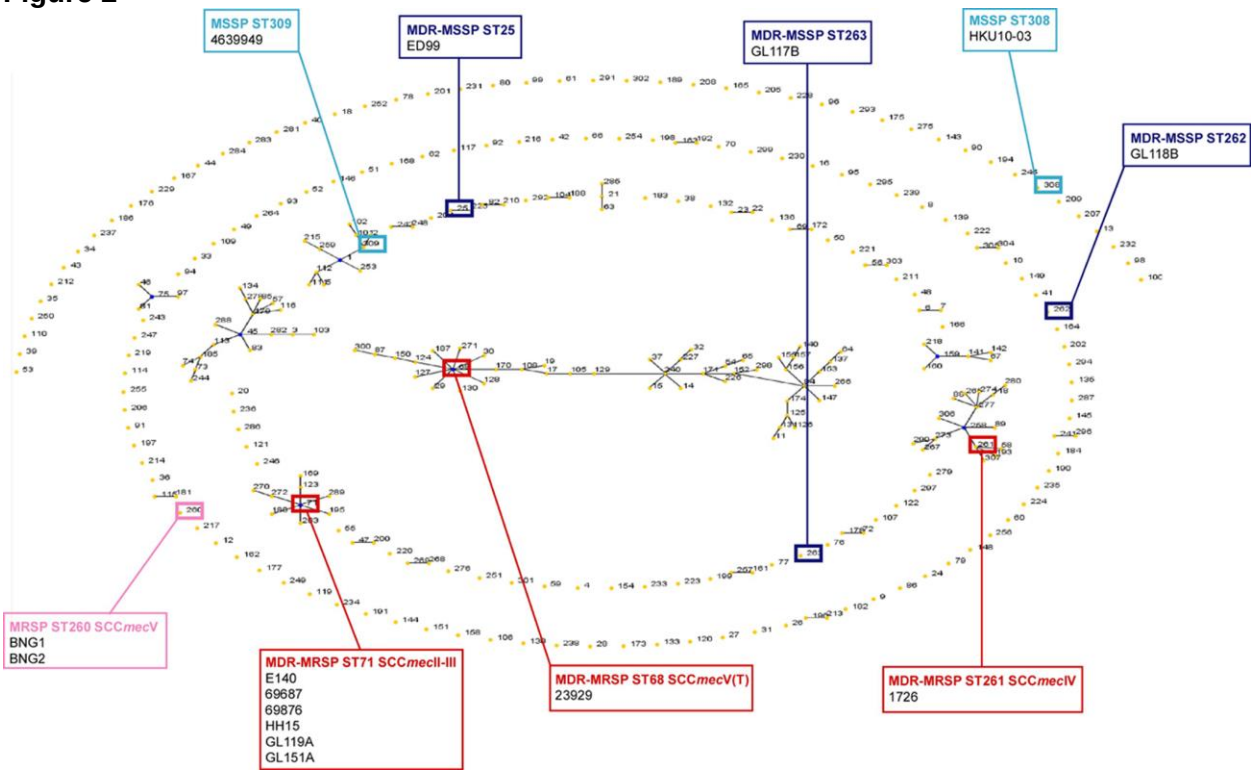

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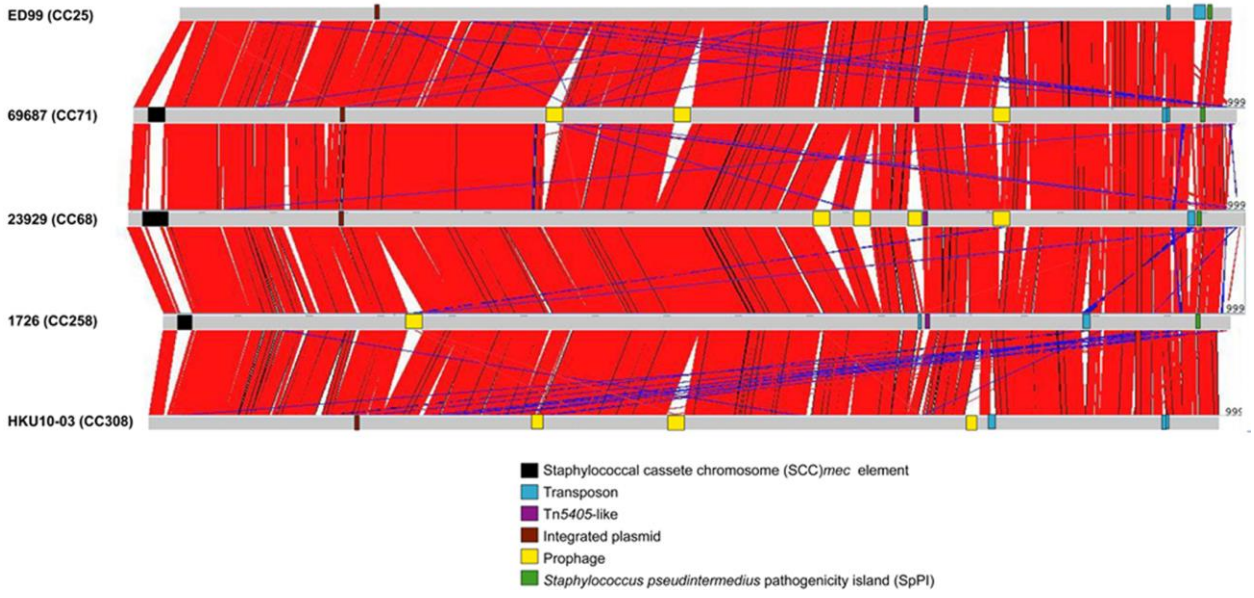
Figure 3



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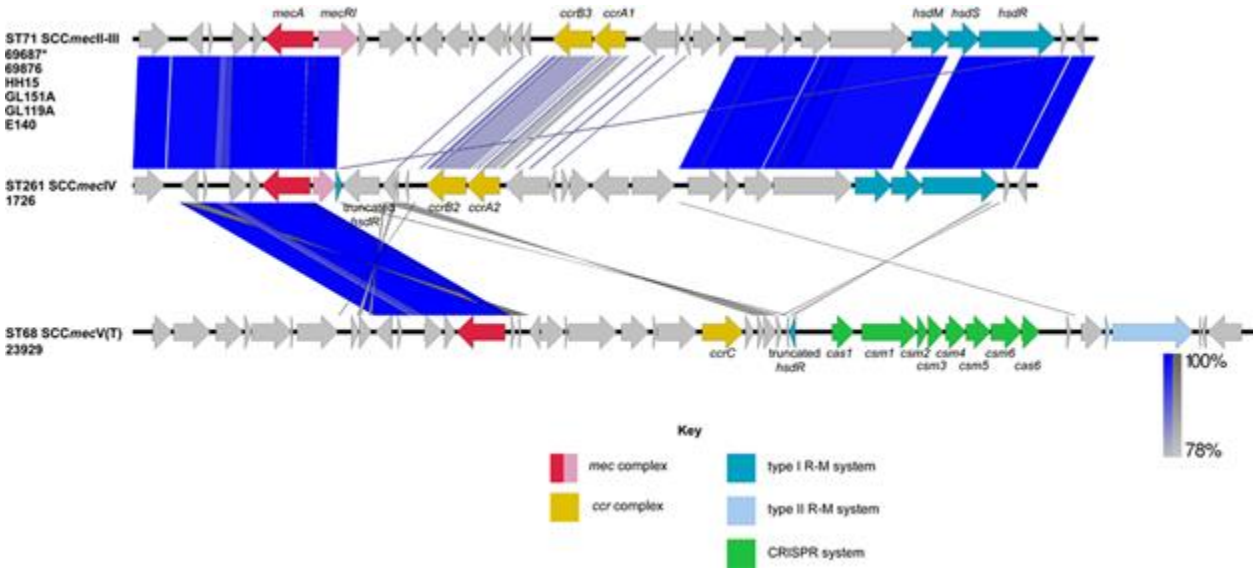
663 **Figure 4**



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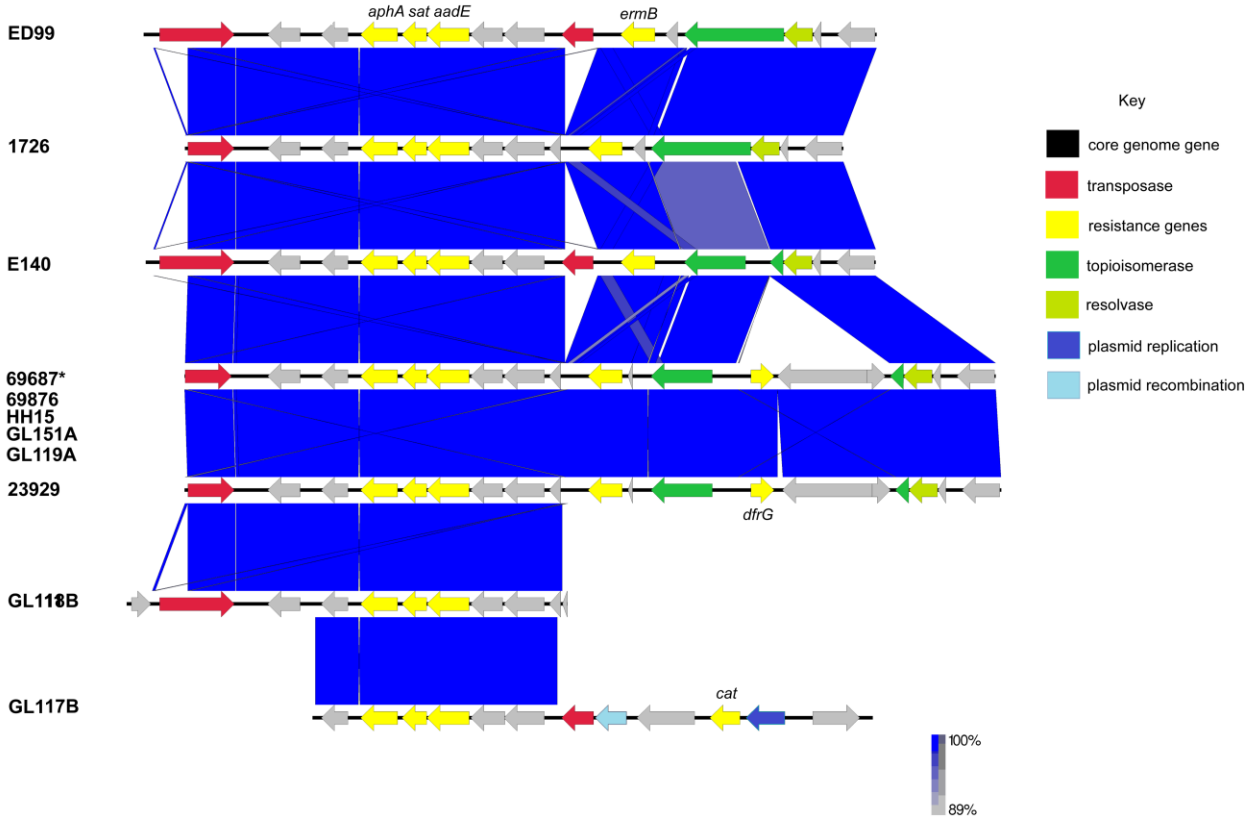
666 **Figure 5**



667

Strain	Phenotypic group	Country/ year/ isolation site	Phenotypic Resistance profile	№ Ab classes resistance	MLST	Total Yield Kb (~ fold coverage)	Number of contigs	Genome size (bp)	GC content	CDS number	GenBank Accession
69687	MDR-MRSP	UK/ 2012/skin	OXA-PEN-AMP-AMC-CL-GEN-KAN-ERY-CLI-SXT-CIP	7	71	346678 (128x)	130	2,712,000	37.4%	2,651	ERR163420
69876	MDR-MRSP	UK/ 2012/ear	OXA-PEN-AMP-AMC-CL-GEN-KAN-ERY-CLI-SXT-CIP	7	71	407664 (150x)	135	2,711,724	37.4%	2,695	ERR144842
HH15	MDR-MRSP	Germany/ 2012/skin	OXA-PEN-AMP-AMC-CL-GEN-KAN-ERY-CLI-TET-SXT-CIP	7	71	592337 (213x)	160	2,780,805	37.3%	2,747	ERR144844
GL119A	MDR-MRSP	Germany/ 2012/skin	OXA-PEN-AMP-AMC-CL-GEN-KAN-ERY-CLI-SXT-CIP	7	71	395970 (144x)	147	2,749,309	37.4%	2,784	ERR294366
GL151A	MDR-MRSP	Germany/ 2012/wound	OXA-PEN-AMP-AMC-CL-GEN-KAN-ERY-CLI-TET-SXT-CIP	7	71	359894 (131x)	156	2,753,286	37.3%	2,741	ERR294367
23929	MDR-MRSP	Ireland/ 2008/skin	OXA-PEN-AMP-CL-KAN-ERY-CLI-TET-SXT-CIP	7	68	322943 (118x)	157	2,742,394	37.3%	2,739	ERR175868
1726	MDR-MRSP	UK/ 2007/ear	OXA-PEN-AMP-KAN-ERY-TET-SXT	5	261	312702 (120x)	120	2,612,150	37.5%	2,568	ERR144810
BNG1	MRSP	UK/ Apr2011/skin	OXA-PEN-AMP-CL-TET	2	260	378309 (142x)	113	2,659,338	37.3%	2,608	ERR144839
BNG3	MRSP	UK/ Sep2011/skin	OXA-PEN-AMP-CL-TET	2	260	395712 (150x)	160	2,646,594	37.4%	2,597	ERR144767
GL117B	MDR-MSSP	Germany/ 2012/ear	PEN-AMP-KAN-ERY	3	263	419305 (166x)	148	2,532,911	37.6%	2,488	ERR310922
GL118B	MDR-MSSP	Germany/ 2012/skin	PEN-AMP-KAN-TET	3	262	454726 (180x)	117	2,527,818	37.6%	2,467	ERR310921
463949	MSSP	USA/ 2012/skin	PEN-AMP-TET	2	309	425893 (169x)	76	2,523,489	37.6%	2,463	ERR294364
ED99	MDR-MSSP	UK/ 2005	PEN-AMP-KAN-ERY-TET-SXT	5	25	-	-	2,572,216	37.6%	2,401	NC_017568
HKU10-03	MSSP	China/ unknown	unknown	-	308	-	-	2,617,381	37.5%	2,451	NC_014925
E140	MDR-MRSP*	Denmark/ 2009	unknown	-	71	-	-	2,769,458	38.0%	2,678	ANOI01000001
MEAN	**-**	**-**	**-**	**-**	**-**	**401011 (151x)**	**135**	**2,660,725**	**37.48%**	**2,605**	

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669 **Table 1:** Antimicrobial resistance and general genome characteristics of sequenced *Staphylococcus pseudintermedius*. All *S.*
670 *pseudintermedius* strains originate from clinical infections. For each isolate and genome, the phenotypic group, country origin and
671 year of isolation, resistance phenotype, number of antimicrobial classes the isolate is resistant to, 7-gene multilocus sequence type
672 (MLST)(54), total yield, fold coverage, number of contigs assembled, genome size, GC contents and number of coding domain
673 sequences (CDS) are shown. MSSP: phenotypic susceptibility to oxacillin and *mecA*-negative, and resistant to antimicrobials in <3
674 antimicrobial classes. MRSP: phenotypic resistance to oxacillin and *mecA*-positive, and resistant to antimicrobials in <3 antimicrobial
675 classes. MDR-MSSP: phenotypic susceptibility to oxacillin and *mecA*-negative, and resistant to antimicrobials in ≥3 antimicrobial
676 classes. MDR-MRSP: phenotypic resistance to oxacillin and *mecA*-positive, and resistant to antimicrobials in ≥3 antimicrobial classes.
677 OXA: oxacillin. PEN: penicillin. AMP: ampicillin. AMC: amoxicillin/clavulanate. CL: cefalexin. GEN: gentamicin. KAN: kanamycin. ERY:
678 erythromycin. CLI: clindamycin. TET: tetracycline. SXT: Trimethoprim/sulfamethoxazole. CIP: ciprofloxacin. RIF: rifampicin. All
679 phenotypic resistances could be putatively assigned to a genetic determinant in all isolates. *Phenotype not published but assumed
680 based on ST71.[26]
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