

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

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15   Short Title: Multidrug resistant *S. pseudintermedius* emergence

16   Key works: Antimicrobial resistance, horizontal gene transfer, MRSP, whole genome  
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18    **Abstract**

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

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

26    **Results:** Nine distinct clonal lineages had acquired either staphylococcal cassette chromosomes  
27    (SCC)mec elements and/or Tn5405-like elements carrying up to five resistance genes (*aphA3*,  
28    *sat*, *aadE*, *ermB*, *dfrG*) to generate MRSP, MDR-methicillin sensitive *S. pseudintermedius*  
29    (MSSP), and MDR-MRSP populations. The most successful and clinically problematic MDR-MRSP  
30    clones, ST68 SCCmecV(T) and ST71 SCCmecII-III, have further accumulated mutations in *gyrA*  
31    and *gyrB* conferring resistance to fluoroquinolones. Carriage of additional mobile genetic  
32    elements (MGEs) was highly variable suggesting horizontal gene transfer is frequent in *S.*  
33    *pseudintermedius* populations.

34    **Conclusions:** Importantly, the data suggest that MDR-MRSP evolved rapidly by acquisition of a  
35    very limited number of MGEs and mutations, and that use of many classes of antimicrobials  
36    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.

39    **Introduction**

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

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

59 The genome-wide diversity and the genetic basis underpinning the rapid evolution and  
60 accumulation of antibiotic resistance in *S. pseudintermedius* is relatively unknown, despite the  
61 sequencing of the genomes of one MDR-MRSP (E140, ST71 SCCmecII-III),<sup>26</sup> one MDR-MSSP  
62 (ED99, ST25)<sup>27</sup> and one MSSP (HKU10-03, ST308).<sup>28</sup> Broad β-lactam resistance in MRSP is  
63 encoded by *mecA* carried on staphylococcal cassette chromosome (SCC)*mec* elements that  
64 have been acquired by multiple *S. pseudintermedius* clonal lineages on multiple independent  
65 occasions.<sup>29-31</sup> MDR-MRSP ST68 SCC*mecV* and MDR-MRSP ST71 SCC*mecII-III* are the major  
66 clones that have spread in North America since 2003-2004 and Europe since 2005-2006,  
67 respectively and that continue to disseminate globally.<sup>14, 15, 32, 33</sup> In this study, we sequenced the  
68 genomes of 12 *S. pseudintermedius* isolates in order to characterise the population structure of  
69 resistant *S. pseudintermedius*, compare the gene content between major clonal lineages,  
70 identify the genetic basis of MDR and explain the evolutionary steps leading to the dominant  
71 MDR-MRSP clones.

72 **Methods**

73 **Bacterial isolates**

74 Genomes of 12 *S. pseudintermedius* isolates from canine infections were sequenced (Table 1).

75 Isolates had been confirmed as *S. pseudintermedius* through demonstration of the *S.*

76 *intermedius* group-specific *nuc* by PCR and as MSSP or MRSP by the absence or presence of

77 *mecA*.<sup>20</sup> Antimicrobial susceptibility was assessed by disk diffusion tests for penicillin, ampicillin,

78 amoxicillin/clavulanate, cefalexin, oxacillin, fusidic acid, gentamicin, kanamycin, erythromycin,

79 clindamycin, tetracycline, trimethoprim, ciprofloxacin and rifampicin according to BSAC

80 guidelines,<sup>33</sup> resistance to oxacillin according to CLSI guidelines.<sup>34</sup> Where breakpoints for a

81 particular antimicrobial agent were not included in these documents, recommendations by the

82 disk manufacturer were used.

83 An isolate was classed as MDR if it was resistant to antimicrobials in ≥4 antimicrobial classes.<sup>35</sup>

84 Amongst MRSPs, the most extensively drug-resistant isolates from our collection of clinical

85 European isolates were selected and for further variation within MRSP ST71, we selected

86 isolates from the UK and from Germany and those with tetracycline resistance and those

87 without. A MDR ST68 isolates was included to allow comparison of the current two most

88 successful lineages and two usual clinical MRSPs were included for their lack of MDR phenotype

89 as they were thought to provide information into the evolutionary events of MDR-MRSP

90 emergence (Table 1). Three MSSPs were selected on varying resistance phenotypes including

91 two MDR-MSSP isolates with uncharacterised STs, and one MSSP with an uncharacterised ST.

92 For PCR reactions, genomic DNA was extracted from overnight cultures grown in brain heart

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

96 **Genome sequencing**

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

110 **Comparative genomics and phylogenetics**

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

115 isolates were mapped against the *S. pseudintermedius* genome ED99 using SMALT  
116 ([www.sanger.ac.uk/smalt](http://www.sanger.ac.uk/smalt)) in order to identify single nucleotide polymorphisms (SNPs), as  
117 previously described.<sup>45</sup> 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.<sup>46</sup> 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.<sup>47</sup> Trees were visualized and annotated using Figtree. Insertion sequence finder  
123 was used to detect IS elements ([www-is.biotoul.fr/](http://www-is.biotoul.fr/)). Restriction-enzymes were named  
124 according to rebase.neb.com.<sup>48</sup> CRISPRfinder was used to detect and characterise CRISPR  
125 elements (<http://crispr.u-psud.fr/Server/>).<sup>49</sup>

## 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/>).<sup>50</sup>

## 132 **Association of MDR phenotype and presence of Tn5405-like elements**

133 The association of the MDR phenotype with the Tn5405-like elements was tested in a further  
134 60 MSSP and 52 MRSP isolates from a collection of clinical canine isolates.<sup>51</sup> 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,

137 0.02mg/mL kanamycin, 0.02mg/mL oxacillin, 0.02mg/mL streptothricin or 0.02mg/mL  
138 trimethoprim. Genomic DNA was extracted as previously described. The presence of *Tn*5405-  
139 like elements was detected by PCR that targeted the transposase gene using forward  
140 (<sup>5'</sup>AAGCGATTGATGATTTCGC<sup>3'</sup>) and reverse primers (<sup>5'</sup>CCTGGTTTAGTTGCCATT<sup>3'</sup>). PCR  
141 reactions were performed using Bioline MyTaq kit (Bioline, UK) in 50 µL volumes containing 1  
142 pmol forward and reverse primer, 1 x buffer, 0.1 units Bioline MyTaq polymerase, 1uL of DNA  
143 and dH<sub>2</sub>O. Reactions were heated to 95 °C for an initial 2 minutes, followed by 35 cycles of 95  
144 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds, followed by 1 cycle of 72  
145 °C for 10 minutes.

## 146 Results

### 147 Population structure of *S. pseudintermedius*

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

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

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

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

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

174 All methicillin-resistant study strains carried *mecA* on a SCCmec element (Figures 1 and 4). All  
175 MDR-MRSP ST71 isolates had a homologous copy of the SCCmecII-III element (29), while the  
176 MDR-MRSP ST68 isolate (23929) had a SCCmecV(T) element.<sup>14</sup> The MDR-MRSP ST261 isolate,  
177 1726, carried a novel SCCmecIV element that was homologous to an SCCmecIVg element in *S.*  
178 *aureus*,<sup>52</sup> but possessed an additional nine genes. MRSP ST260 isolates carried a novel SCCmec

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

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

186 All eleven MDR isolates carried a large transposable element resembling Tn5405-like  
187 elements.<sup>53,54</sup> This element carried up to five antimicrobial resistance genes *aphA3-sat-aadE-*  
188 *dfrG-erm(B)* encoding resistance to aminoglycosides (including kanamycin, neomycin and  
189 amikacin), streptothrinicin, trimethoprim and erythromycin and was present in 8/8 MDR-MRSP  
190 and 3/3 MDR-MSSP isolates (Figures 1 and 5). Pairwise comparison revealed all Tn5405-like  
191 elements were integrated at the same core genome loci. Variation was seen in carriage of *dfrG*  
192 and *cat* (Figure 5), truncated versions were found in MDR-MSSP isolates GL118B and ED99, and  
193 one was located on a plasmid (GL117B).

194 Screening the larger collection of 60 MSSP and 52 MRSP, Tn5405-like elements were present in  
195 8/11 (73%) MDR-MSSP and in all 46 (100%) MDR-MRSP and the MDR phenotype was found in  
196 11/60 (18.3%) MSSP isolates and 46/52 (88.5%) MRSP isolates. Carriage of Tn5405-like  
197 elements was associated with the MDR phenotype ( $p<0.001$ ,  $\chi^2$  test).

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

200 Tn5801 and Tn916 elements in three and five genomes, respectively. All but one genome  
201 carried at least one *blaZ* gene, encoding resistance to penicillin, either on Tn552 and/or on  
202 Tn554-like elements. Finally, the *aac-aph* gene, encoding resistance to gentamicin, was located  
203 on IS256 or IS1272 elements in seven and two genomes, respectively. The *cadA* gene, encoding  
204 resistance to cadmium, was located on IS431 elements in four genomes.

205 Plasmids do not appear to play a role in the emergence of MRSP. In this study, 12/15 genomes  
206 carried the integrated plasmid first described in ED99,<sup>27</sup> but this plasmid did not carry any  
207 resistance genes. GL117B possessed an additional plasmid with a replication (*rep*) gene with  
208 97% homology to plasmid pUB112 from *S. aureus*, and resistance genes *aphA-sat-aadE-ermB*  
209 similar to the truncated version of the *Tn5405*-like element (Figure 5). There was no further  
210 evidence for the carriage of plasmid replication (*rep*) genes in any of the *S. pseudintermedius*  
211 genomes.

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

221 of *dfrG* on Tn5405-like elements (5/8 strains). Only the successful MDR-MRSP ST68, 23929, was  
222 phenotypically resistant to fusidic acid using BSAC breakpoints.<sup>33</sup> In *S. aureus*, *fusA* mutations  
223 cause low-level resistance to fusidic acid.<sup>58, 59</sup> Isolate 23929 had three non-synonymous  
224 mutations in *fusA* (A376V, I461V and V90I), while only 1 other strain had a *fusA* mutation  
225 (V90I). No strains carried the MGE-encoded *fusB*, *fusC* or *fusD*. This data indicates that  
226 mutations at sites 376 and 461 are implicated in phenotypic resistance to fusidic acid.

227 **Comparison of fitness factors in the genomes**

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

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

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

## 258 **Barriers to horizontal gene transfer in *S. pseudintermedius***

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

264 full intact type I Sps1 R-M system, containing restriction (*hsdR*), modification (*hsdM*) and  
265 specificity (*hsdS*) genes, was present in the core genome of 11/15 *S. pseudintermedius* genomes  
266 (Figure 1). In contrast, incomplete systems that are predicted to be non-functional were  
267 present in the remaining genomes. HKU10-03 (ST308) possessed *hsdR* and *hsdM*, but no *hsdS*,  
268 4639949 (ST309) possessed *hsdM* only, whilst ED99 (ST25) and GL118B (ST262) possessed  
269 truncated *hsdR* genes. Two types of *hsdS* variant in the type I R-M systems existed, whilst *hsdR*  
270 sequences and *hsdM* sequences were highly homologous across all genomes (Figure 1). These  
271 data suggest that the two clusters of lineages modify their DNA at different target sites,  
272 restricting horizontal transfer of DNA and divergent evolution of *S. pseudintermedius* into at  
273 least two populations.<sup>64</sup> Additionally, a second type I R-M system was carried on all SCCmecII-III  
274 elements of MDR-MRSP ST71 genomes and on the SCCmecIV element of the MDR-MRSP ST261  
275 genome (Figure 1, Figure 4). The *hsdS*, *hsdM* and *hsdR* sequences from the SCCmec elements  
276 were highly homologous, but different from the sequences of respective core genome genes,  
277 and has been called SpsE140ORFGP. Predicted intact type II R-M systems, containing *hsdM* and  
278 *hsdR* genes were present in 6/15 genomes (Figure 1) at four different locations: (i) adjacent to  
279 *orfX* in MDR-MSSP ST25 (ED99)(Sps99ORF30P with a predicted cutsite of CTRYAG) and MSSP  
280 ST309 (4639949) genomes, (ii) adjacent to the *phoB* gene in the MSSP ST308 (HKU10-03)  
281 (SpsORF2234P with a predicted cutsite of CCNGG) and MRSP ST260 genomes (BNG1 and BNG3),  
282 (iii) at the type I R-M system locus in MDR-MRSP ST68 (23929) (called Sps68ORF1551), and (iv)  
283 on the SCCmecIV and novel SCCmec elements of MDR-MRSP ST68 (23929) and MRSP ST260  
284 (BNG1 and BNG2) genomes (called SpsORF2234P), respectively , and was homologous to the  
285 type II system present at the *phoB* site.

286 Clustered regularly interspaced short palindromic repeats (CRISPR) systems were detected in  
287 3/15 genomes (Figure 1). Six of the 22 spacers in CRISPR from ED99 were homologous to  
288 bacteriophage and plasmids,<sup>27</sup> suggesting this isolate is immune to uptake of specific elements  
289 encoding these sequences. Two of the 24 spacers in CRISPR from GL118B were homologous to  
290 bacteriophage, and 2/13 spacers in the CRISPR system found on SCCmecV(T) in 23929 were  
291 homologous to bacteriophage and plasmids. There was no evidence that CRISPR has recognised  
292 important SCCmec or transposons and MDR-MRSP ST71 and ST68 did not have greater or fewer  
293 R-M or CRISPR systems than other strains.

294 **Discussion**

295 Identifying the whole genome characteristics of MDR-MRSP is critical to explain the evolution  
296 and rapid emergence of successful clones. We have shown that only a three-step accumulation  
297 of an SCCmec element, Tn5405-like elements and SNPs in *gyrA/grlA* was required for the  
298 emergence of the two globally successful MDR-MRSP clones. At the same time, these MDR-  
299 MRSP have successfully spread worldwide, indicating that selection for survival of these  
300 resistant isolates is common.

301 Multiple SCCmec elements have now been sequenced in *S. pseudintermedius*.<sup>26, 29, 68-71</sup> They  
302 have given rise to multiple MRSP clones and we now report MRSP phenotypes in six of nine  
303 major lineages. The wide distribution of MRSP phenotypes in STs from CC45, CC71 and CC258  
304 suggests that SCCmec elements were acquired ancestrally or that these lineages can acquire  
305 and spread SCCmec more effectively. SCCmec elements of *S. pseudintermedius* most closely  
306 matched SCCmec elements from *S. aureus* (SCCmecII-III, SCCmecIV and SCCmecV(T)) and/or

307 from coagulase-negative staphylococci (CNS) such as *S. epidermidis* (SCCmecII-III) and *S.*  
308 *haemolyticus* ( $\Psi$ SCCmec<sub>57395</sub>), supporting a role of HGT from CNS in MRSP emergence.

309 The acquisition of the Tn5405-like elements, encoding multiple antimicrobial resistances  
310 simultaneously was a consistent feature of the MDR phenotype seen in both MSSP and MRSP.  
311 Tn5405-like elements have been acquired by at least four *S. pseudintermedius* lineages, with  
312 variants also found in an additional two lineages. Driving pressures for the selection of Tn5405-  
313 like elements remain unclear as antimicrobial prescribing data for pets are available for only a  
314 few countries and the antimicrobial agents encoded on this element seem of little veterinary  
315 clinical relevance. Nevertheless, the use of only one antimicrobial could be sufficient to select  
316 for the survival of clones with Tn5405-like transposons and resistance to four classes of  
317 antimicrobial. Acquisition of specific *gyrA/grlA* SNPs associated with phenotypic resistance to  
318 fluoroquinolones is strongly correlated with the globally successful MDR-MRSP lineages.<sup>14,69</sup>  
319 Several fluoroquinolones were authorised for use in pets and have been widely used since the  
320 late 1980s suggesting a key role of fluoroquinolones in promoting survival and spread of MRSP.  
321 A similar association has been reported between fluoroquinolone resistance and the pandemic  
322 spread of major hospital-associated MRSA clones.<sup>25, 65, 74</sup>

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

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

333 The distribution of type I and/or type II R-M systems, known barriers to HGT in *S. aureus*,  
334 suggested that MDR-MRSP genomes are no more efficient or inefficient in acquiring MGE-  
335 encoded resistance. R-M systems were present on all SCCmec elements in this study, and also  
336 on  $\Psi$ SCCmec57395 from a previously described MDR-MRSP ST45.<sup>69</sup> To determine if populations  
337 are evolving independently, future studies could assess the extent of recombination occurring  
338 within and between *S. pseudintermedius* populations. As CRISPR systems were rare in genomes,  
339 and spacer sequences did not match any known SCCmec elements or transposons, it is unlikely  
340 that CRISPRs have a major role in governing HGT of resistance genes in *S. pseudintermedius*.  
341 Overall, ED99 carried the fewest R-M systems and no prophage, and is potentially an  
342 appropriate laboratory strain for genetic manipulation.

343 We conclude that MDR-MRSP evolved rapidly through step-wise accumulation of SCCmec,  
344 Tn5405-like elements and SNPs conferring fluoroquinolone resistance. Thus, this study  
345 indicates that the use of only a small number of antimicrobials may select for the pandemic  
346 spread of MDR strains. In particular, a unifying feature of the fittest clones, MDR-MRSP ST68  
347 and MDR-MRSP ST71, was their fluoroquinolone resistance through core genome mutations,  
348 mirroring the success of hospital-associated MRSA clones, such as MRSA CC22. These findings

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

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365 **Transparency declaration**

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367

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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. SCCmecA elements are  
595     SCCmecII-III (grey), SCCmecV(T) (blue), SCCmecIV (yellow) and a unique SCCmec (green), with  
596     homology to SCCmecX and SCCmecV; structural differences are shown in Figure 4. Tn5405-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     Tn5801 carried *tetM*. All Tn916 carried *tetM* but were integrated into three different sites on  
600     the genome (1, 2 or 3). All Tn552 and Tn554-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 SCCmec elements from *Staphylococcus pseudintermedius* genomes.**  
627 The SCCmecII-III element from ST71 (69687) is compared to SCCmecIV from ST261 (1726), and

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

635 **Figure 5. Comparison of *Tn5405*-like elements from *Staphylococcus pseudintermedius***  
636 **genomes.** The *Tn5405*-like element from ED99 is compared to *Tn5405*-like elements from 1726,  
637 E140, 69687, 23929, GL118B and GL117B. Homology is indicated by a colour scale of blue (100%  
638 homology) to grey (89% homology). Genes are coloured by function transposase in red,  
639 antibiotic resistance genes in yellow, topoisomerase genes in green, resolvase genes in light  
640 green, plasmid replication genes in bright blue and plasmid recombination genes in dull blue.  
641 Genes in grey were not homologous to any genes using BLAST. *aphA*, *aadE* encode resistance to  
642 aminoglycosides, *sat* to streptothricin, *dfrG* to trimethoprim and *cat* to chloramphenicol. Figure  
643 produced using EasyFig and *Tn5405*-like element sequence from 69876\*. This figure appears in  
644 colour in the online version of JAC and in black and white in the print version of JAC.

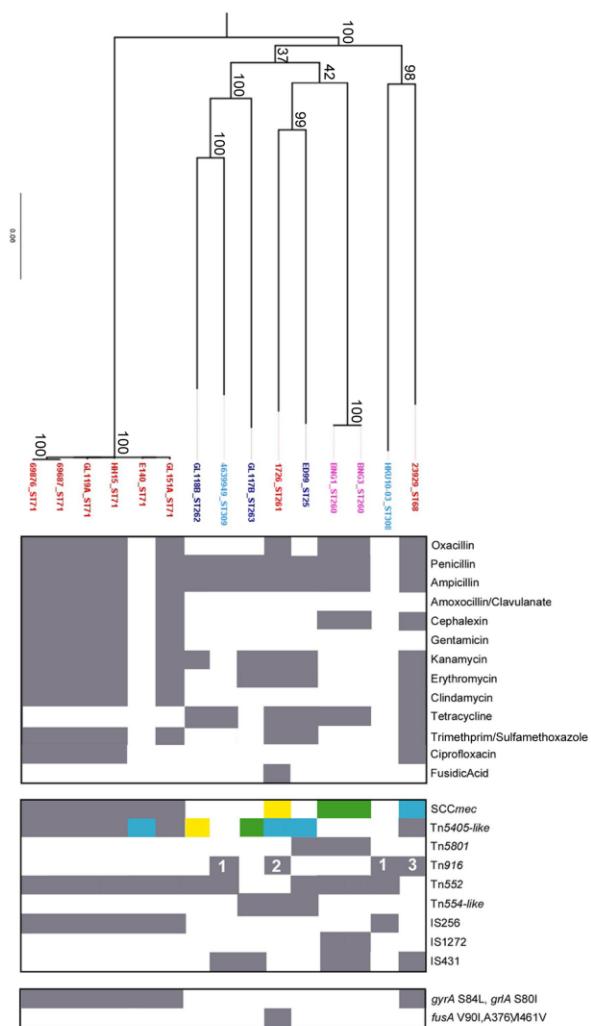
645 **Figure S1. Comparison of representative prophage from *Staphylococcus pseudintermedius***  
646 **strains.** A representative prophage from each of the 8 insertion sites is shown. Homology is  
647 indicated by a colour scale of blue (100% homology) to grey (84% homology). The prophages are

648 named by insertion site ( $\phi$ X) and then the genome from which the sequence originates. Figure  
649 produced using EasyFig.

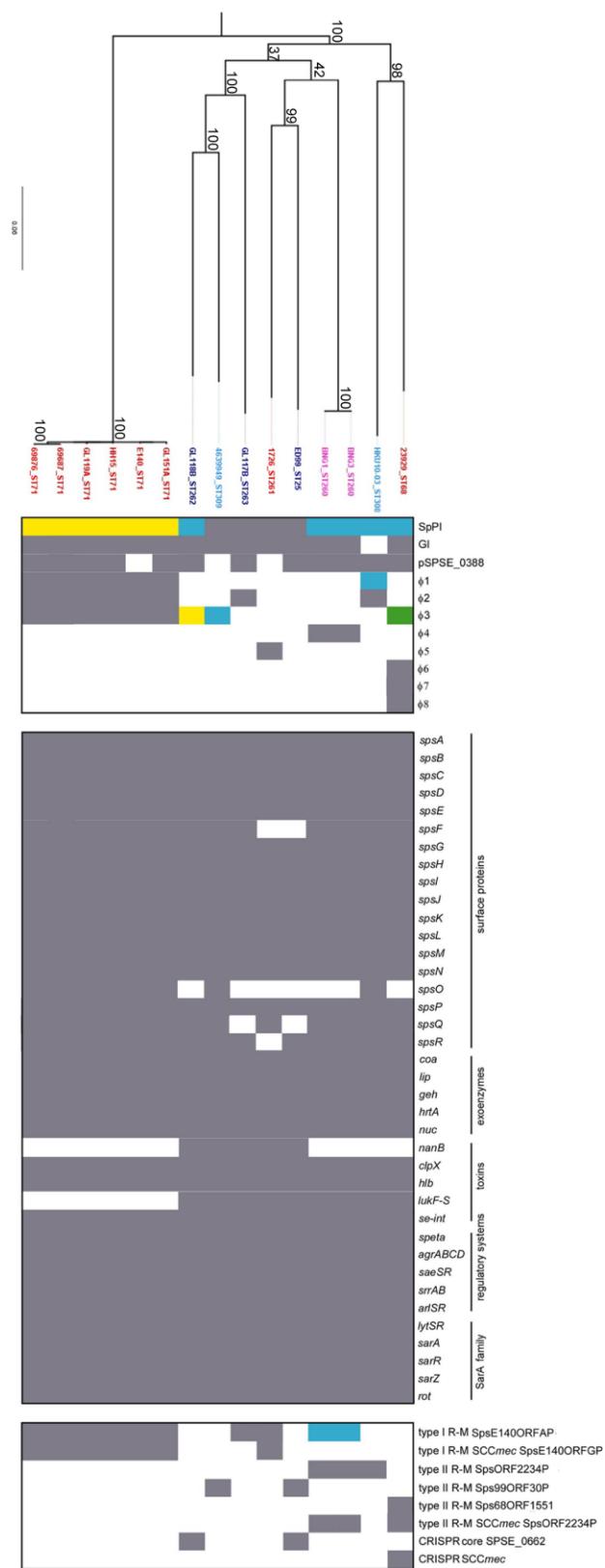
650 **Figure S2. Comparison of SpPI1 from *Staphylococcus pseudintermedius* strains.** The  
651 pathogenicity island SpPI1 from ED99 is compared to SpPI1 elements from other sequenced *S.*  
652 *pseudintermedius* strains. Homology is indicated by a colour scale of blue (100% homology) to  
653 grey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

**Figure 1**

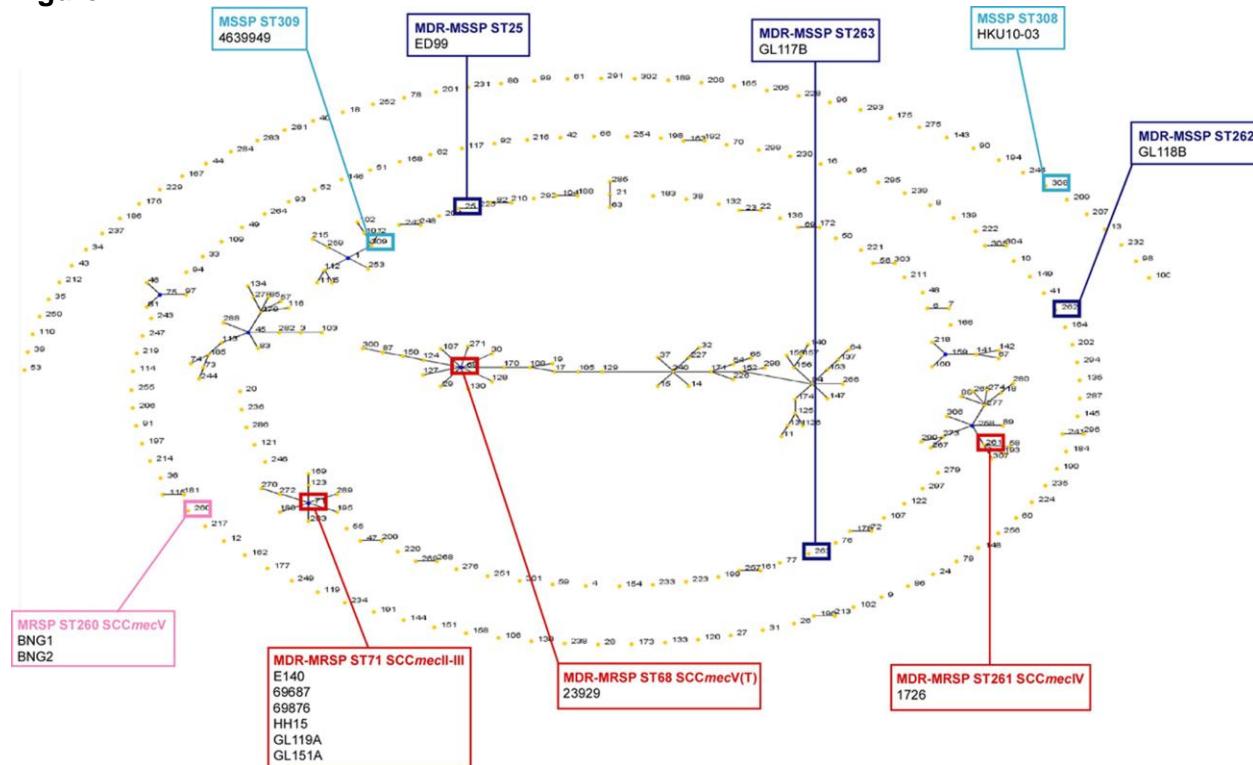
A



B



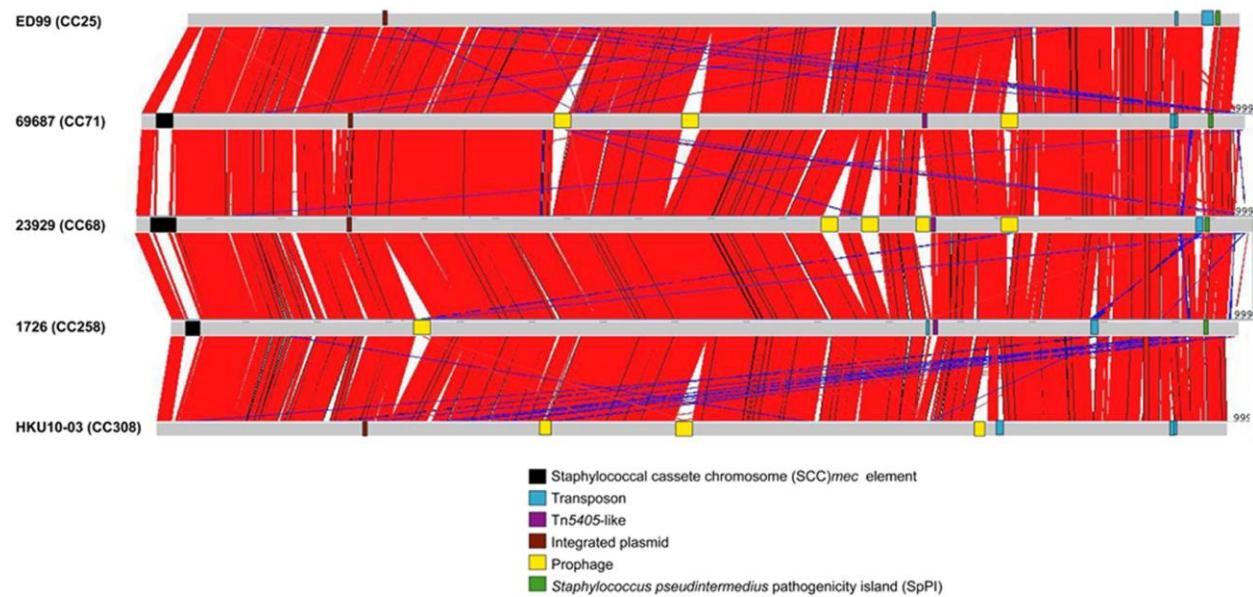
**Figure 2**



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**Figure 3**

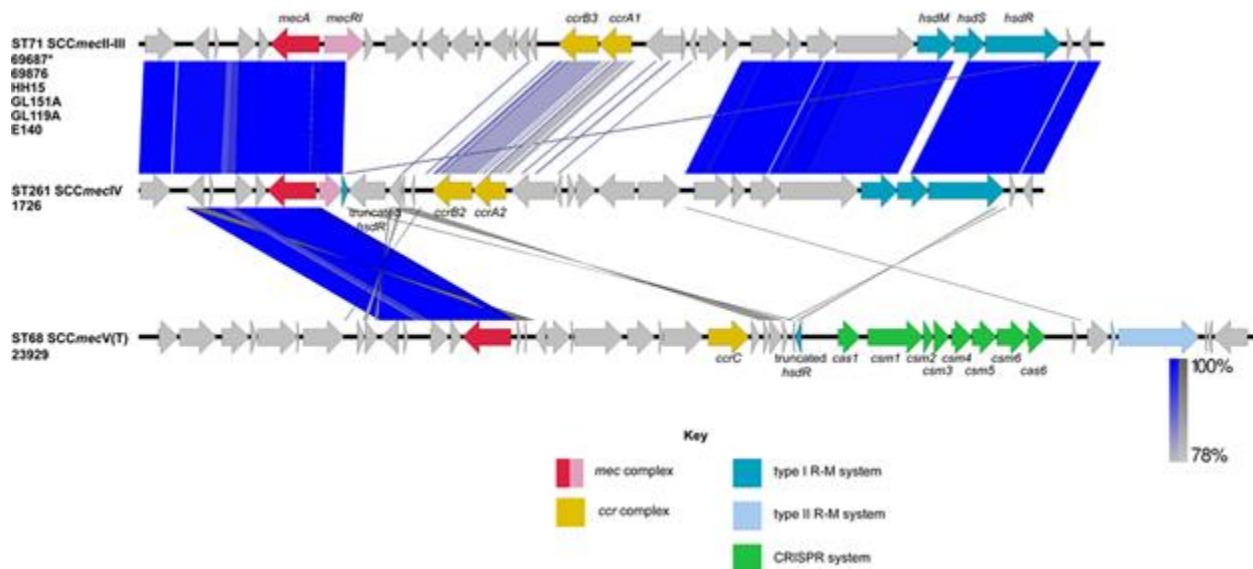


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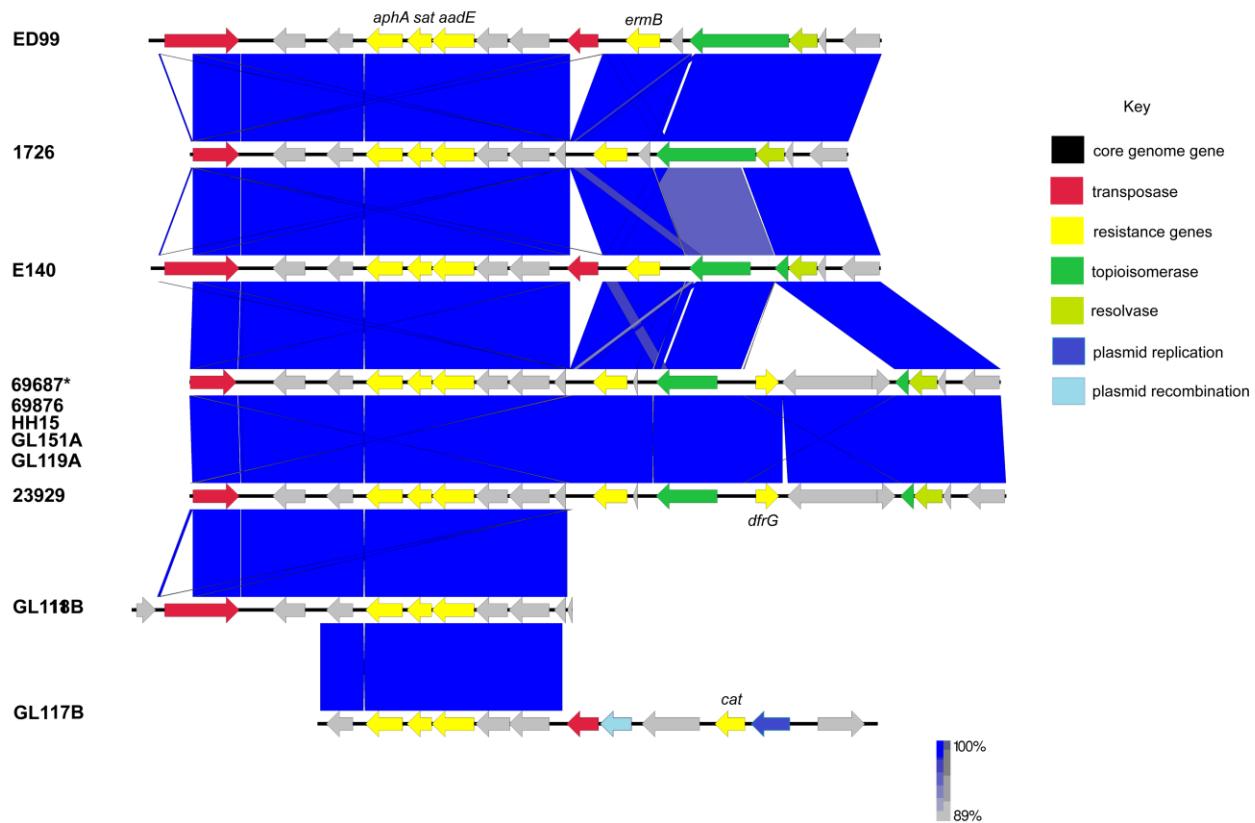
663 **Figure 4**



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666 **Figure 5**



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Strain	Phenotypic group	Country/ year/ isolation site	Phenotypic Resistance profile	Nº 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
<b>MEAN</b>	-	-	-	-	-	<b>401011 (151x)</b>	<b>135</b>	<b>2,660,725</b>	<b>37.48%</b>	<b>2,605</b>	

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.<sup>26</sup>

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