1 **Mechanisms of β-lactam resistance of *Streptococcus uberis* isolated from**

2 **bovine mastitis cases**

3 **Scott McDougall[a*], Laura Clausen[a], Hye-Jeong Ha[b], Isobel Gibson[c], Mark Bryan[d], Nazreen**

4 **Hadjirin[e], Elizabeth Lay[e], Claire Raisen[e], Xiaoliang Ba[e], Olivier Restif[e], Julian Parkhill[f] and**

5 **Mark A. Holmes[e]**

6 [a] *Cognosco, Anexa FVC, Morrinsville, New Zealand*

7 [b] *Animal Health laboratory, Ministry for Primary Industry, Upper Hutt, New Zealand*

8 [c] *New Zealand Veterinary Pathology, Hamilton, New Zealand*

9 [d] *VetSouth, Winton, New Zealand*

10 [e] *Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, UK*

11 [f] *Wellcome Sanger Institute, Hinxton, UK*

12 *Corresponding author at: PO Box 21, Morrinsville, New Zealand, 3300: Tel: +64 7 889 5159, Fax:

13 +64 7 889 3681, *Email address*: smcdougall@anexafvc.co.nzPage Break

Abstract

15 A number of veterinary clinical pathology laboratories in New Zealand have been reporting

16 emergence of increased minimum in inhibitory concentrations for β-lactams in the common

17 clinical bovine mastitis pathogen *Streptococcus uberis*. The objective of this study was to

18 determine the genetic basis of this increase in MIC for β-lactam amongst *S. uberis.* Illumina

19 sequencing and determination of oxacillin MIC was performed on 265 clinical isolates.

20 Published sequences of the five penicillin binding proteins *pbp1a*, *pbp1b*, *pbp2a*, *pbp2b,* and

21 *pbp2x* were used to identify, extract and align these sequences from the study isolates.

22 Amino acid substitutions resulting from single nucleotide polymorphisms (SNP) within these

23 genes were analysed for associations with elevated ($\geq$ 0.5 mg/L) oxacillin MIC together with

24 a genome wide association study. The population structure of the study isolates was

25 approximated using a phylogenetic tree generated from an alignment of the core genome. A

26 total of 53% of isolates had MIC $\geq$ 0.5 mg/L for oxacillin. A total of 101 substitutions within

27 the five *pbp* were identified, of which 11 were statistically associated with an MIC $\geq$ 0.5

28 mg/L. All 140 isolates which exhibited an increased β-lactam MIC had SNPs leading to

29 *pbp2x* $E_{381}K$ and $Q_{554}E$ substitutions. The phylogenetic tree indicated that the genotype and

30 phenotype associated with the increased MIC for oxacillin were present in several different

31 lineages suggesting that acquisition of this increased β-lactam MIC had occurred in multiple

32 geographically distinct regions. Reanalysis of the data from the intervention studies from

33 which the isolates were originally drawn found a tendency for the *pbp2x* $E_{381}K$ substitution to

34 be associated with lower cure rates. It is concluded that there is geographically and

35 genetically widespread presence of *pbp* substitutions associated with reduced susceptibility to

36 β-lactam antimicrobials. Additionally, presence of *pbp* substitutions tended to be associated

37 with poorer cure rate outcomes following antimicrobial therapy for clinical mastitis.

38 Key words: *Streptococcus uberis*; β-lactam resistance; penicillin binding proteins

1. Introduction

40 *Streptococcus uberis* is a common bovine mastitis pathogen (Bradley et al., 2007;

41 McDougall et al., 2007b). Its core genome consists of 1,530 genes and it is a highly

42 recombinant species (Lang et al., 2009). *Streptococcus uberis* is highly heterogeneous as

43 indicated by strain typing including random amplified polymorphic DNA (RAPD)

44 fingerprinting, multilocus sequencing typing (MLST), and pulsed field gel electrophoresis

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45 (PFGE) (Zadoks et al., 2011). Isolates from the UK and New Zealand have been shown to

46 have distinct MLST patterns, with a preponderance of ST-143 in New Zealand isolates, while

47 ST-5 was predominant among the UK isolates (Pullinger et al., 2006). *S. uberis* may behave

48 either as an environmental or contagious pathogen. Contagious transmission has been

49 demonstrated through longitudinal studies and inferred from cross sectional studies in which

50 the same sequence type is observed among cows or herds (Phuektes et al., 2001; Zadoks et

51 al., 2003).

52 Bacteriological cure rates following therapy of clinical *S. uberis* are reported to range

53 between 64 and 91% (McDougall, 1998; McDougall et al., 2007a; Owens et al., 1997). Many

54 factors influence the cure rate following mastitis therapy, including antimicrobial resistance

55 (Sandholm et al., 1990). Because they do not produce β-lactamase (Zapun et al., 2008),

56 Streptococci have historically been regarded as highly susceptible to β-lactams which are still

57 the therapy of choice for infections with these bacteria. However, phenotypically β-lactam

58 resistant bovine *S. uberis* isolates have been reported with bimodal distributions of minimum

59 inhibitory concentrations (MIC) for cloxacillin in French, German and New Zealand studies

60 (Guérin-Faublée et al., 2003; McDougall et al., 2014; Tenhagen et al., 2006).

61 Penicillin-binding proteins (PBPs) are cell wall transpeptidases that catalyse assembly

62 of cell wall peptidoglycan. Six *pbp* are found in *S. pneumoniae*: five high-molecular-mass

63 *pbp* (*pbp1a*, *pbp1b*, *pbp2x*, *pbp2a*, and *pbp2b*) and one low-molecular-mass PBP (*pbp3*).

64 *pbp2a* and *2b* are essential, at least in *S. pneumoniae*, as double deletion results in non-

65 viability (Peters et al., 2016). The active site of transpeptidase activity is formed by three

66 conserved amino acid motifs, SXXK, SXN, and KT(S)G. β-lactam resistance is generally

67 associated with changes within, or flanking, these motifs. Mutations that confer resistance

68 have only been described in the penicillin binding domains, that is, the transpeptidase

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69 domains (Hakenbeck et al., 2012a). Low affinity variants of all the *pbp* have been described

70 (Hakenbeck et al., 1999), but only mutations of *pbp1a*, *-2x*, and *-2b* appear to be associated

71 with clinical β-lactam resistance (Grebe and Hakenbeck, 1996; Nagai et al., 2002). Low level

72 resistance occurs with amino acid substitutions in *pbp2b* or *2x*, while high level resistance

73 requires additional amino acid substitutions in *pbp1b* or *pbp3* (Du Plessis et al., 2002; Smith

74 and Klugman, 1998). Alterations in the conserved motifs in *pbp2b* tend to be associated with

75 resistance to penicillin, and alterations in *pbp2x* appear to contribute to low-level resistance to

76 cephalosporins (Nagai et al., 2002). There is evidence of horizontal gene transfer amongst the

77 streptococci, with a common resistance gene pool for *S. pneumoniae*, *S. oralis* and *S. mitis*

78 (Dowson et al., 1994; Hakenbeck et al., 2012a). There is variation in the affinity of different

79 β-lactams to *pbp* variants, which may reduce the clinical efficacy of different β-lactams in the

80 face of emerging resistance (Garau, 2002; Grebe and Hakenbeck, 1996) in streptococci. It has

81 been shown that resistant *S. uberis* isolates generated in the laboratory arise from alterations

82 in *pbp1a, pbp2b* and/or *pbp2x* (Haenni et al., 2010b). β-lactam resistance mechanisms

83 independent of substitutions amongst the *pbp* have been reported including the presence of a

84 *murMN/fibAB* operon, a mutation in the gene coding for GlcNAc deacetylase, and mutations

85 in the gene *mraY* (Chewapreecha et al., 2014; Hakenbeck et al., 2012b).

86   This study was undertaken to test the hypothesis that increases in MIC in clinical *S.*

87 *uberis* isolates are associated with amino acid substitutions in one or more of the *pbp*.

2. Materials and Methods

89   *Streptococcus uberis* isolates collected during the conduct of two clinical mastitis

90 antimicrobial therapy intervention studies were used for this study (Bryan et al., 2016;

91 McDougall et al., 2019). The isolates were obtained from mastitic milk of cows prior to

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92 treatment from a total of 35 dairy farms in New Zealand (North Island and South Island) and

93 were collected with permission of Animal Ethics Committees.

94   Isolates that were Gram positive cocci, catalase negative, cleaved esculin, sorbitol and

95 inulin and which did not grow in SF broth were initially defined as *S. uberis*. All isolates

96 were subcultured onto an entire 5% blood agar plate containing 1% aesculin (Fort Richard,

97 Auckland, New Zealand). From a pure culture, a single colony was picked and inoculated

98 onto a Dorset egg slope (Fort Richard, Auckland, New Zealand), incubated overnight at 37°C

99 and checked for growth before storage at 4 °C for further testing. The species of isolates was

100 confirmed by MALDI-TOF (Pathology Associates LTD, Pathlab Bay of Plenty Division,

101 Tauranga, New Zealand).

102 *2.1 Susceptibility testing*

103   The MIC of oxacillin was determined using a broth microdilution method according to

104 CLSI standards (CLSI, 2013) using cation-adjusted Mueller-Hinton broth supplemented with

105 2.5% lysed horse blood (CAMHB-LHB). Oxacillin solutions were prepared in CAMHB-LHB

106 to a concentration range double of that the desired final concentrations of 0.0325 to 16 mg/L

107 and dispensed into 96-well plates at 50 µl per well. Inoculum was prepared using colony

108 suspension method to a turbidity equivalent to that of a 0.5 McFarland standard and was

109 diluted 1:100 in CAMHB-LHB. For each test isolate, 50 µl of diluted isolate inoculum was

110 added to the wells. Each isolate was tested in triplicate. The 96-well plates were then placed

111 in a plastic bag to minimize evaporation and incubated at 35 °C for 20 h. Oxacillin was

112 selected as we wished to determine the MIC for the penicillinase-stable penicillins including

113 cloxacillin, which is commonly used for treatment of both clinical (Bryan et al., 2016, and

114 subclinical mastitis at the end of lactation. The oxacillin MIC for the 265 isolates were used

115 to determine the epidemiological cut off (ECOFF) values by fitting a series of mixture

116 models (Everitt, 1996) to the log-transformed MIC by maximum likelihood.

117 Additional antibiotic susceptibility data was generated during the original clinical

118 studies using either eTest (Biomerieux, France) or a custom-designed broth microdilution

119 (Sensititre, Trek Diagnostics, Thermo Fisher, OH, USA) for penicillin, cefalexin, cefuroxime,

120 ceftiofur, and cefquinome for subsets of the isolates. *Streptococcus pneumoniae* (ATCC

121 49619) was run as a quality-control organism in parallel with the unknown isolates and the

122 results were within the CLSI defined quality assurance standards.

123 *2.2 Molecular biology*

124 Genomic DNA was extracted from overnight cultures using the MasterPure Gram

125 Positive DNA Purification Kit (Cambio, UK). Illumina library preparation was carried out as

126 previously described (Quail et al., 2008), and sequencing performed on an Illumina HiSeq

127 2000 following the manufacturer's standard protocols (Illumina, Inc, USA) at the Welcome

128 Sanger Institute, Hinxton, UK (WSI).

129 **2.3** *Bioinformatics*

130 Assemblies and annotations were generated using pipelines at the WSI (Page et al.,

131 2016). Assemblies were imported into Geneious (version 10.2.2, Geneious Inc, NZ) for

132 analysis. The sequences for *pbp1a*, *1b*, *2a*, *2b* and *2x* were obtained from O140J *S. uberis*

133 genome (NCBI accession number AM946015) and used to identify these genes in the study

134 isolates. The *pbp* genes were extracted, aligned and single nucleotide polymorphisms (SNP)

135 associated with amino acid substitution were identified. Amino acid sequences were aligned

136 (and numbered) with those previously reported (Haenni et al., 2010b) for *pbp1a*, *2b* and *2x*,

137 while amino acid numbering relative to the start of the open reading frame were used for

138 *pbp1b* and *2a*.

139   For comparison with the sequence data from the study isolates, the sequences of 13 UK

140   *S. uberis* (Hossain et al., 2015) were downloaded from European nucleotide archive

141   (http://www.ebi.ac.uk/ena) and 63 Canadian *S. uberis* sequences were downloaded from

142   PATRIC (www.patricbrc.org) (Vélez et al., 2017).

143   Multilocus sequence types were determined from the genome sequenced data (Coffey et

144   al., 2006) (http://pubmlst.org/suberis).

145   A core genome alignment was obtained using Roary (Page et al., 2015) and a maximum

146   likelihood phylogenetic tree was constructed using Randomized Accelerated Maximum

147   Likelihood (RAxML)(Stamatakis, 2014). This tree was annotated using iTOL (Letunic and

148   Bork, 2016).

149   Initial associations between non-synonymous SNPs and the presence of an MIC above

150   the ECOFF were examined using bivariate (χ^2) statistics and binary logistic regression

151   analyses. Subsequently forward and reverse multivariate logistic regression models were

152   constructed to which all SNPS that were significant ($P < 0.05$) at the bivariate level were

153   offered. This analysis was undertaken in STATA v10.2 (Stata Corp., College Station, TX,

154   USA). Comparisons of MIC amongst other β-lactams for the specific SNPs were undertaken

155   using Kruskal-Wallis non-parametric analyses, and regression analyses were used to compare

156   the MIC of oxacillin with the MICs for other β-lactams.

157   Subsequently a genome wide association study (GWAS) was undertaken using the

158   oxacillin resistance MIC as the outcome using sequence element enrichment analysis (SEER;

159   https://github.com/johnlees/seer) (Lees et al., 2016) and visualised using Phandango

160   (Hadfield et al., 2017). K-mers (10-593bp) were generated from the isolate assemblies using

161   FSM-lite. For the population structure an initial distance matrix was prepared using Mash

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162 (Ondov et al., 2016), which was projected onto a final distance matrix into 6 dimensions

163 (selected on the basis of a Scree plot, data not shown) using R (script available at MRC

164 Climb; climb.ac.uk) (Connor et al., 2016). SEER was then used to determine if any k-mers

165 were associated with the cloxacillin resistance phenotype using a threshold adjusted for

166 multiple testing of $P < 5 \times 10^{-8}$ (Barsh et al., 2012).

167   Frequency of recombination events within the *S. uberis* genomes was estimated by

168 performing a Genealogies Unbiased By recombinations In Nucleotide Sequences (Gubbins)

169 analysis, (https://github.com/sanger-pathogens/Gubbins; Croucher et al 2015). A Roary

170 alignment tree was used as the starting tree, the alignment was undertaken using SMALT,

171 with O140J as the reference, and visualised using Phandango (Hadfield et al., 2017).

172 *2.4 Cure following intramammary therapy*

173   The association between the presence of the $E_{381}K$ substitution in *pbp2x* and cure rate

174 (defined as absence of signs of clinical mastitis and/or non-isolation of the bacteria associated

175 with clinical mastitis pre-treatment at 2 or 3 time points post treatment) following

176 intramammary therapy was examined independently for the isolates from the two

177 intervention studies. In the first study quarters with clinical mastitis were infused on three

178 occasions at 24 hour intervals with either 1 g penicillin and 200 mg cloxacillin (PenClox

179 1200 High Potency Milking Cow, Virbac, (NZ) Ltd, Hamilton, NZ), or with a combination of

180 200 mg oxytetracycline, 100 mg oleandomycin, 100 mg neomycin and 5 mg prednisolone

181 (Mastalone, Pfizer NZ Ltd., Auckland, NZ) (Bryan et al., 2016). In the second intervention

182 study affected quarters were treated by intramammary infusion at 12 hourly intervals with

183 200 mg amoxycillin (as amoxycillin trihydrate), 50 mg clavulanic acid (as potassium

184 clavulanate), and 10 mg prednisolone (Clavulox LC, Zoetis New Zealand Limited, Auckland,

185 New Zealand) on three or five occasions (McDougall et al., 2019).

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186   Generalised linear mixed models were used to assess the effect of treatment, $E_{381}K$

187   *pbp2x* genotype, and the treatment by genotype interaction on cure. For the first intervention

188   study, herd was included as a random effect, and lactation number (i.e. categorised as first

189   and second versus greater than second lactation) was also included as fixed effect. For the

190   second intervention study, the model also included days in milk at clinical mastitis diagnosis

191   (categorised as ≤ 4 versus > 4 days), and age (categorised as 2, 3, 4-6, > 6-years-old).

3. Results

3.1 Distribution of minimum inhibitory concentrations and cut-off value

194   The frequency distribution of MICs of oxacillin are shown in Fig. 1. The MIC_{50} and

195   MIC_{90} were 1.0 and 2.0 mg/L. The ECOFF was defined as ≥ 0.5 mg/L based on visual

196   assessment and the mixture modelling, and 141/265 (53.2%) of the isolates had an ECOFF

197   greater or equal to this cutpoint.

3.2 Penicillin binding proteins

199   Penicillin binding proteins *1a*, *1b*, *2a*, *2b* and *2x* were identified in all 265 isolates. The

200   three conserved active site motifs were identified in *pbp1a* ($S_{214}TMK$, $S_{272}SN$, and $K_{401}TG$),

201   *pbp1b* ($S_{441}SIK$, $S_{497}WN$ and $K_{632}TG$), *pbp2a* ($S_{454}TIK$, $Y_{491}GN$ and $K_{632}TG$), PBP2b ($S_{353}VVK$,

202    $S_{408}SN$, and $K_{579}TG$), and in *pbp2x* ($S_{339}TMK$, $S_{398}SN$, and $K_{549}TG$: Fig. 2). There was perfect

203   alignment of the conserved active site motifs between the *S. uberis pbp2x* and *S. pneumoniae*

204   (NCBI gene ID 934744).

205   A total of 101 non-synonymous SNPs were identified across the 5 *pbp*. There were 19,

206   17, 19, 26 and 20 SNPs in *pbp1a*, *1b*, *2a*, *2b* and *2x*, respectively.

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207 *3.3 Associations between SNPs and oxacillin resistance*

208   At a bivariate level, the 11 most common SNPs (i.e. present in ≥ 26 (10%) of the

209 isolates) were associated with an oxacillin MIC greater or equal to the ECOFF within the

210 transpeptidase domains of *pbp1a, 2b* and *2x*, and *pbp1b* and *2a* (Table 1).

211   Of the 141 isolates having an oxacillin MIC greater or equal to the ECOFF, 140 of

212 these had the *pbp2x* $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions. There was only one isolate without

213 the $E_{381}K$ substitution that had an oxacillin MIC greater or equal to the ECOFF as it had an

214 oxacillin MIC of 1 mg/L. A total of 133 of the isolates with an oxacillin MIC greater or equal

215 to the ECOFF also had a *pbp2x* $V_{590}A$ substitution, and all of the $V_{590}A$ also had the $E_{381}K$,

216 $Q_{554}E$, and $G_{600}E$ substitutions. There was no difference in the oxacillin MIC for isolates that

217 did or did not have the $V_{590}A$ substitution within isolates with the $E_{381}K$ substitution ($1.79 \pm$

218 0.57 mg/L vs 1.66 ± 0.48 mg/L for isolates with and without the $V_{590}A$ substitution within the

219 $E_{381}K$ substitution; $P = 0.05$).

220   The $E_{381}K$ substitution was also associated with increases in MIC, relative to isolates

221 without the substitution, for penicillin, cefuroxime, ceftiofur, and cefquinome, but with a

222 decrease in MIC for cefalexin (Table 2; Fig. 3). There was a positive association between

223 oxacillin MIC and the MICs for penicillin ($R^2 = 0.58$; $P < 0.001$), cefuroxime ($R^2 = 0.60$; $P <$

224 0.001), ceftiofur ($R^2 = 0.29$; $P < 0.001$), ampicillin ($R^2 = 0.63$; $P < 0.001$), cefquinome ($R^2 =$

225 0.43; $P < 0.001$) and ampicillin/clavulanic acid ($R^2 = 0.19$; $P < 0.001$), but a negative

226 association between oxacillin and cefalexin ($R^2 = 0.07$; $P < 0.001$).

227   There were 5 non-synonymous SNPs in the transpeptidase domain of *pbp2x* gene of the

228 13 UK and 63 Canadian isolates (Table 1). All these variants, except the $A_{492}E$ substitution,

229 were also found in the New Zealand isolates.

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230   Multivariable models for oxacillin resistance did not converge if any one of the $E_{381}K$,

231    $Q_{554}E$, and $G_{600}E$ substitutions and any other SNPs were included.

232   *3.4 Multilocus sequence typing and Phylogenetic analysis*

233   A total of 146 sequence type were identified by MLST in the New Zealand isolates, a

234   proportion of which had not previously been described (listed in Supplementary Table 1).

235   While there was an association between MLST type and presence of the *pbp2x* $E_{381}K$

236   substitution (P < 0.001), the $E_{381}K$ substitution was widely distributed throughout the

237   phylogenetic tree and not found exclusively within one lineage (Supplementary Fig. 1).

238   *3.5 GWAS*

239   In the genome wide association study, the Manhattan plot (Supplementary Fig. 2)

240   reveals a high level of association (peaking at $P < 10^{-12}$) of k-mers within *pbp2x* and three

241   other genes positioned next to this gene. Statistically significant candidate loci were detected

242   in the *pbp2x* and *mraY* peptidoglycan biosynthesis pathway, and in two other genes *yxeM* and

243   *yxeN*. There were a total of 27, 42 and 11 SNPs in the *mraY*, *yxeM*, and *yxeN* genes,

244   respectively of which 10, 18 and 4 were non-synonymous. There were 3, 2, and 1 non-

245   synonymous SNPs with a prevalence of > 20% on *mraY*, *yxeM*, and *yxeN* genes, respectively,

246   all of which were associated (P < 0.001) with the *bpb2x* $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions.

247   No statistically significant SNPs were found in the *cshB* gene located between *mraY* and

248   *yxeM*.

249   *3.6 Gubbins analysis*

250   A high level of genomic recombination was observed amongst the *S. uberis* genomes

251   (Supplementary Fig. 3).

252 *3.7 Cure rate*

253   In the first intervention study (Bryan et al., 2016), there was no significant effect of

254 Treatment (P = 0.99), *pbp2x* $E_{381}K$ substitution (P = 0.13), or the treatment x *pbp2x* $E_{381}K$

255 substitution interaction (P = 0.41) on cure rate. However, the cure rate was numerically lower

256 in quarters affected with *S. uberis* with the $E_{381}K$ substitution where treatment occurred with

257 the β-lactam treatment (approximately 15% lower cure rate), while the cure rate of the $E_{381}K$

258 substitution was 4% lower following treatment with the non- β-lactam (Fig. 4a).

259   In the second study (McDougall et al., 2019), presence of the $E_{381}K$ substitution tended

260 (P = 0.07) to reduce bacteriological cure rate (Fig. 4b). While not significant (P = 0.11),

261 numerically there appeared to be an interaction with the duration of treatment, whereby

262 isolates with the $E_{381}K$ substitution treated for a longer duration (5 x 12 hourly) had higher

263 bacteriological cure rates than isolates treated for a shorter period (3 x 12 hourly), whereas

264 duration of treatment did not affect cure rate amongst the isolates without the substitution.

265 **4. Discussion**

266   A bimodal distribution of MICs for oxacillin was observed for *S. uberis* isolated from

267 cases of bovine mastitis. A total of 53% of isolates had an MIC greater than the ECOFF of ≥

268 0.5 mg/L. isolates with MICs greater than the ECOFF were present on 28 of 30 farms

269 located both in the North and South islands of New Zealand, indicating wide geographic

270 distribution and a low probability of direct cow to cow transmission of isolates with an MIC

271 greater than the ECOFF.

272   SNPs with possible association with the oxacillin MICs greater than the ECOFF were

273 found on all 5 *pbp* (*pbp1a*, *1b*, *2a*, *2b* and *2x*). Following multivariable modelling, those on

274 *pbp2x* resulting in a $E_{381}K$ substitution were found to account for the observed increased MIC

275 for oxacillin. This finding was confirmed by the results from a GWAS, which identified only

276 *pbp2x* and 3 genes flanking this region.

277 Substitutions in *pbp1b* and *pbp2a* have not been previously reported in *S. uberis*.

278 However, substitutions in all *pbp* associated with phenotypic resistance have been reported to

279 occur in *S. pneumoniae* (Hakenbeck et al., 2012a). The *pbp1b* $G_{768}S$ and the *pbp2a* $T_{397}A$

280 substitutions were also found in the Canadian and UK isolates, demonstrating wide

281 geographic distribution of these substitutions.

282 On *pbp2b*, 4 of the substitutions previously induced (Haenni et al., 2010b) were also

283 observed in the current study in New Zealand, Canada and the UK; $N_{366}I$, $T_{402}I$, $V_{570}A$ and

284 $P_{575}S$. Three other substitutions were located in *pbp2b* in the New Zealand isolates, but none

285 of these were associated with an increased MIC for oxacillin. As previously reported (Haenni

286 et al., 2010b), the $N_{366}I$ substitution was located 13 residues downstream of the SVVK motif,

287 the $T_{402}I$ SNP was located six residues before the SSN motif, and the $V_{570}A$ and $P_{575}S$ SNPs

288 were located nine and four residues upstream of the KTG motif, respectively.

289 For *pbp2x*, five substitutions associated with an increased MIC for oxacillin were

290 located in the present study. All 5 were also located in the Canadian and UK isolates. The

291 $E_{381}K$ and $Q_{554}E$ substitutions were previously reported as occurring in naturally occurring and

292 induced resistant *S. uberis* (Haenni et al., 2010b), being 42 amino acids downstream of the

293 STMK motif and five amino acids downstream of the KTG motif, respectively. All isolates

294 with the *pbp2x* $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions had oxacillin MIC $\geq$ 0.5mg/L. Of the

295 eight isolates with the *pbp2x* $A_{590}V$ substitution, seven of these also had the *pbp2x* $E_{381}K$,

296 $Q_{554}E$, and $G_{600}E$ substitutions. This suggests that *pbp2x* $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions

297 are the functionally important ones, rather than the $A_{590}V$ substitution. The mechanism of

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298 resistance for the one isolate without the $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions that had an

299 elevated oxacillin MIC remains to be determined.

300 The *pbp2x* $Q_{554}E$ substitution has been described in other penicillin resistant

301 streptococci (Haenni et al., 2018). Restoring the wild type genotype at this position results in

302 8 to 16 fold reduction in MIC (Dahesh et al., 2008), demonstrating the importance of this

303 substitution. The *pbp2x* $E_{381}K$ substitution has not been described in Streptococci other than *S.*

304 *uberis* (Haenni et al., 2010b; Hakenbeck et al., 2012a). The biological effect of the newly

305 identified substitutions ($I_{295}V$, $V_{590}A$, $G_{600}E$, $G_{600}D$) are unclear. These substitutions were

306 identified in all 3 populations of isolates examined. These substitutions were highly

307 correlated with the $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions, hence in the current population the

308 increased MIC for oxacillin could be entirely explained by the presence of the $E_{381}K$ and $Q_{554}E$

309 substitutions. The $V_{590}A$, $G_{600}E$, $G_{600}D$ substitutions are located in the α-helix of the

310 transpeptidase domain. In *S. pneumoniae*, a $N_{605}T$ substitution is associated with decreased

311 acetylation and reduced sensitivity to β-lactam antimicrobials (Carapito et al., 2006) and site-

312 directed mutagenesis has demonstrated that the reversion of the resistant $Y_{595}F$ substitution

313 reduces the MIC (Smith and Klugman, 2005). Both of these substitutions are also within the

314 α-helix of the transpeptidase domain (Hakenbeck et al., 2012a). Taken together these data

315 suggest that the newly identified substitutions could contribute to reduced susceptibility of *S.*

316 *uberis* to β-lactam antimicrobials.

317 The $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions were also associated with increased MIC for

318 other β-lactams including penicillin, ceftiofur, cefquinome, amoxicillin/clavulanic acid and

319 cefuroxime. Only ceftiofur has a validated bovine mastitis clinical breakpoint (2 mg/L) and

320 only 2 of 265 isolates in the current study had an MIC > 2 mg/L. For the other β-lactams, the

321 maximum MIC were 0.5 mg/L for penicillin, 1 mg/L for cefquinome, and 2 mg/L for

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322   cefuroxime, cephalexin and 2/1 mg/L for amoxicillin/clavulanic acid. In the absence of

323   clinical breakpoints, while bimodal distributions for many of these antimicrobials were

324   present, the clinical significance of this remains unclear. However, following intramammary

325   infusion of 1 g of penicillin, which is a commonly used therapy in New Zealand, it is likely

326   that concentrations greater than the maximum MIC for penicillin of the current isolates would

327   be achieved. Following infusion of 1 million international units (i.e. approximately 606 mg)

328   of penicillin G on 3 occasions at 12 hourly intervals, the mean milk concentration of

329   penicillin was 175 mg/L (Moretain and Boisseau, 1989), approximately 700-fold higher than

330   the MIC_{50} for penicillin found amongst the $E_{381}K$ substitute isolates in the current study. The

331    $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions were associated with increased MIC for all β-lactams

332   tested, other than cephalexin. Similarly, there was a positive association between the MIC for

333   oxacillin and all other β-lactams, other than cefalexin. Different effects on sensitivity within

334   antimicrobials class within mutations to *pbp2x* have been previously described. Laboratory

335   *pbp2x* mutants which increased cefotaxime MIC by more than 10 fold had no effect, or

336   increased, sensitivity to oxacillin (Grebe and Hakenbeck, 1996).

4.1 Population structure

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338   The current study found multiple MLST types including many novel types. In common

339   with a number of previous studies, it is clear that bovine mammary *S. uberis* are a highly

340   diverse population (Davies et al., 2016; Zadoks et al., 2011). There was limited evidence of

341   clonal expansion within or between dairy herds in New Zealand, suggesting a predominantly

342   environmental source of *S. uberis* in the New Zealand context, as distinct from cow to cow

343   transmission inferred from some previous studies (Davies et al., 2016; Zadoks et al., 2011).

344   Streptococci are generally found to be recombinogenic as seen in *S. pneumoniae*

345   (Croucher et al., 2014). This leads to substantial genome modification likely via a

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346 combination of point mutations, homologous recombination and movement of mobile genetic

347 elements (Croucher et al., 2014). This suggests that the association between the increased

348 MIC for oxacillin and the *pbp2x* genotype is more likely to be causal and not just a result of

349 clonal expansion of lineages carrying the *pbp2x* substitutions contained in a resistant

350 background genome. The Gubbins analysis indicated that recombination events are

351 particularly common in *S. uberis*. This places constraints on the interpretation of any

352 phylogeny within this species not taking recombination into account although it should be

353 noted that the maximum likelihood tree from the core genome shared much of the same

354 structure as the tree from the Gubbins analysis (which accounted for recombination). As

355 might be expected, the *pbp2x* $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions were conserved amongst

356 closely related isolates using the core genome phylogeny. However, there were many

357 examples of isolates within the same clade having different *pbp2x* genotypes suggesting

358 multiple *pbp2x* mutation or acquisition events.

359 *4.2 GWAS*

360   The GWAS independently identified the *pbp2x* locus. Additionally, the *mraY* locus was

361 identified which codes for phospho-N-acetylmuramoyl-pentapeptide-transferase, an enzyme

362 responsible for the formation of the first lipid intermediate of the cell wall peptidoglycan

363 synthesis. These two loci were also identified in a GWAS study of *S. pneumoniae*

364 (Chewapreecha et al., 2014). Two loci not previously reported as being associated with β-

365 lactam resistance in Streptococci were identified including *yxeM* which codes for an

366 extracellular solute-binding protein and *yxeN* which codes for an ABC transporter permease.

367 These later 2 genes flank *pbp2x*, and it is likely that they are associated due to linkage

368 disequilibrium. Interestingly *cshB*, which codes for a surface associated protein, and is

369 located between *mraY* and *yxeN*, contained a number of SNPs, none of which were associated

370 with oxacillin resistance. The reason for this is unclear; all isolates possessed this gene, and

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371 in the same location. A number of loci identified in the GWAS of *S. pneumoniae*

372 (Chewapreecha et al., 2014) including *clpL*, *ciaH*, *ftsL* and *gpsB* were not associated with β-

373 lactam resistance in the current study.

4.3 Cure rate

375 As the original intervention studies were not powered to specifically test the effect of

376 $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions on bacteriological cure rate, care should be taken when

377 interpreting the cure rate data given the relatively small sample size.

378 However, numerically the cure rate was lower amongst *S. uberis* isolates with the $E_{381}K$,

379 $Q_{554}E$, and $G_{600}E$ substitutions than those without these substitutions. The cure rate was

380 numerically lower for isolates with $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions following treatment

381 with a β-lactam compared with a non-β-lactam. This is biologically plausible as the SNP in

382 the *pbp2x* is only likely to affect β-lactams, and not the efficacy of antimicrobials operating

383 via different mechanisms. Increasing the duration of therapy with a β-lactam to 5 x 12 hourly

384 tended to overcome the depression in cure rate seen with a shorter duration of therapy (i.e. the

385 3 x 12 hourly treatment). Again, this is biologically plausible given that β-lactams are time-

386 dependent antimicrobials, hence with increasing duration it is feasible that concentrations

387 above MIC were of sufficiently long duration to result in bacteriological cure even amongst

388 isolates with increasing MIC. Failure to detect difference in cure rate between *pbp2x*

389 genotypes could also have been due to presence of other resistance mechanisms being present

390 masking any effect of the *pbp2x* genotype. For example, in the first study the control group

391 was treated with a combination of an aminoglycoside, a macrolide and a tetracycline. While

392 streptococci are considered to constitutively resistant to aminoglycosides (Jayarao and Oliver,

393 1992), there was no evidence of tetracycline resistance genes in the current study, and only 7

394 isolates had presence of the *ermB* gene conferring increased MIC to macrolides (unpublished

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395   data). Thus, it is considered unlikely that the failure to differentiate cure rate was due to a

396   reduced cure rate in the non-β-lactam treatment group.

397   The maximum MIC for cloxacillin of any isolates in the current study was 2 mg/L.

398   Ongoing monitoring of the MIC of *S. uberis* isolates, and the association between MIC and

399   clinical and bacteriological cure rates amongst clinical mastitis cases associated with *S.*

400   *uberis* is required. Additionally, as cloxacillin-based antimicrobials are the most commonly

401   used at the end of lactation (i.e. for dry-cow therapy), it is plausible that ongoing use of

402   antimicrobials at the end of lactation may result in selection of *S. uberis* with higher

403   cloxacillin MIC. However, this hypothesis remains to be tested.

5. Conclusions

405   This study has found widespread evidence for increased MIC of oxacillin amongst the

406   common bovine mastitis pathogen *S. uberis*. The great majority of isolates with an increased

407   MIC for oxacillin were found to have substitutions in *pbp2x*, and the $E_{381}K$, $Q_{554}E$, and $G_{600}E$

408   substitutions were predictive of the increased MIC for oxacillin phenotype and did not

409   require inclusion of substitutions on other *pbp* in the final predictive models. Isolates with the

410     $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions were also found in Canadian, European and UK isolates,

411   suggesting wide geographic distribution of this genotype. Phylogenetic analysis found the

412     $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions were widely distributed amongst New Zealand clades,

413   but there was variation in *pbp2x* genotype within closely related isolates. This suggests that

414   horizontal gene transfer may be occurring, as has been reported in other Streptococci, or that

415   multiple independent SNPs have occurred over time.

416   Presence of the $E_{381}K$, $Q_{554}E$, and $G_{600}E$ substitutions was numerically associated with

417   lower bacteriological cure rates following treatment with a β-lactam compared with a non-β-

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418 lactam intramammary therapy. Additionally, lower bacteriological cure rates occurred where

419 this genotype was treated with a shorter compared with the longer duration of therapy.

6. Acknowledgements

7. Funding

8. Transparency declarations

429 The authors declare that they have no competing interests.

430 LC, IG, and HJH undertook the original microbiology to isolate and confirm the

431 phenotypic identity of the *S. uberis* and initial MIC determination. NH, EL, XR and XB

432 undertook DNA sequence preparation and final MIC determinations. OR developed the

433 mixture models. JP contributed to manuscript reparation. MB undertook one of the

434 intervention studies. MH managed the sequencing processing, and contributed to the study

435 design, analysis and interpretation. SM undertook the design, analysis, interpretation and

436 manuscript preparation. All authors read and approved the final manuscript.

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9. Availability of data and materials statement

438 The assemblies of the isolates are in the European Nucleotide Archive

439 (https://www.ebi.ac.uk/ena).

440 Additional phenotypic data (New Zealand Island location, oxacillin MIC) and genotype

441 data (*pbp2x* $E_{381}K$ as 0/1) is included in Supplementary Table 1.

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602   Table **1**

603   Penicillin binding protein (PBP) number, residue number (based on previously reported

604   (Haenni et al., 2010b) numbering for *pbp1a*, *2b* and *2x*, otherwise from the start of the open

605   reading frame), most common residue at that position (core residue), the number of isolates

606   with the core residue at that position, the amino acid of the variant and the number of isolates

607   with this variant, the P-value from chi squared analysis of the variant against the isolate being

608   resistant (that is, an oxacillin MIC of > 0.5 mg/L), and the number and percentage of isolates

609   in core and variant amino acids with oxacillin resistance. Note only those substitutions with

610   >10% prevalence and within the transpeptidase domain (for *pbp1a*, *2b* and *2x*) are listed.

611   Where the same substitutions were identified in Canadian (Vélez et al., 2017) and UK

612   (Hossain et al., 2015) isolates these are listed.

613   **Table 2**

614   The mean, standard error of the mean (SEM) and median minimum inhibitory concentration

615   (MIC_{50}; mg/mL) for β-lactam antimicrobials for *Streptococcus uberis* isolates with and

616   without the $E_{381}K$ substitution in *pbp2x*.

617 **Fig. 1.** Frequency histogram of minimum inhibitory concentrations (mg/L) of oxacillin for

618 *Streptococcus uberis* from bovine clinical mastitis cases.

619 **Fig. 2 a, b, c**. Sites in (a) *pbp1a*, (b) *pbp2b*, and (c) *pbp2x* implicated in altered affinity to β-

620 lactams. The transpeptidase domain is represented as the horizontal black bar; the active site

621 motifs are indicated in blue and marked by blue triangles. Numbers in brackets indicate the

622 position of the first and last amino acids of the transpeptidase domain. The substitutions in

623 green are from the current study, and the red substitutions are from those induced in *S. uberis*

624 (Haenni et al., 2010a). Note only substitutions present in >10% of isolates in the current

625 study are represented.

626 **Fig. 3.** Box plots of the MIC of β-lactams for *S. uberis* isolates from bovine clinical mastitis

627 cases defined as oxacillin resistant (i.e. MIC $\geq$ 0.5 mg/L) or susceptible (< 0.5 mg/L).

628 **Fig. 4 a,b.** Estimated marginal mean (95% confidence intervals) for cure proportion for (a)

629 study 1 for quarters infected with *S. uberis* that had the $E_{381}K$ substitution (open bar) or not

630 (solid bar) by treatment type. The non-β-lactam treatment was daily intramammary infusion

631 for 3 days of a combination of 200 mg oxytetracycline, 100 mg oleandomycin, 100 mg

632 neomycin and 5 mg prednisolone, and β-lactam treatment was daily infusion 3 days of a

633 combination of 1 g penicillin and 200 mg cloxacillin, and (b) cure proportion for quarters

634 treated by intramammary infusion at 12 hourly intervals with 200 mg amoxycillin, 50 mg

635 clavulanic acid, and 10 mg prednisolone on three (hatched bar) or five (open bar) occasions.
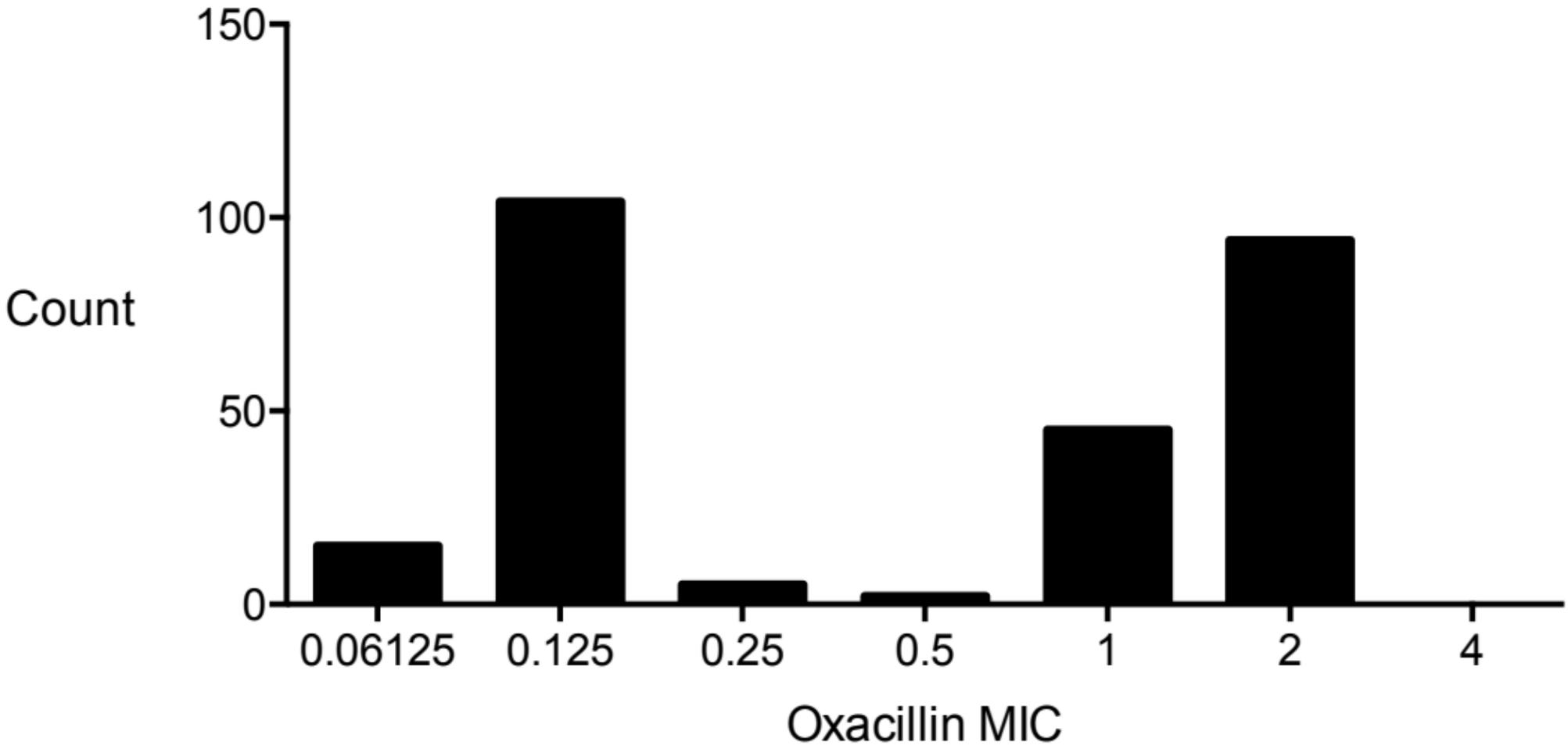
636 **Supplementary Table 1**. Isolate identity, multilocus sequence type (Sequence type),

637 oxacillin minimum inhibitory concentration (Ox MIC (mg/L)), resistance phenotype

638 (resistant (1) = $\geq$ 0.5 mg/L), resistance genotype (1 = *pbp2x* $E_{381}K$ substitution), location in

639 New Zealand (North or South Island) and the unique farm identity (Farm_ID), ENA sample

640 accession number, and ENA lane accession number.

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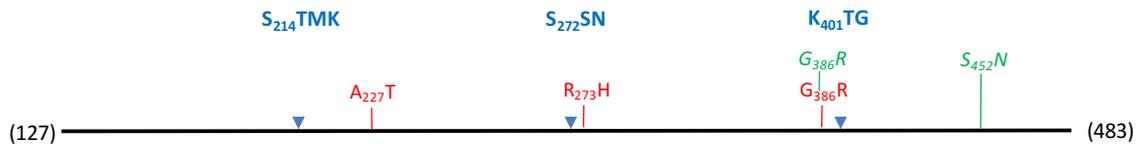1823
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641 **Supplementary Fig. 1.** Phylogenetic tree of 265 *S. uberis* isolates from bovine clinical

642 mastitis cases in New Zealand (classified as from the North or South Island) and *S. uberis*

643 isolates from Canada and the UK. The tree is created from the core genome (~1,500 genes) of

644 *S. uberis*. The meta data includes (from inner to outer) isolate number, multilocus sequence

645 type, island of New Zealand, farm identity, *pbp2x* $E_{381}K$ genotype (open green Square = wild

646 (sensitive) genotype; closed green square = resistant genotype), and oxacillin MIC phenotype

647 (red open square < 0.5 mg/L, closed red square ≥ 0.5 mg/L).

648 **Supplementary Fig. 2.** Manhattan plot showing the results of a k-mer based genome wide

649 association study using SEER visualised using Phandango. The reference genome used was

650 17652_8#12.gff. The annotation file was generated by Prokka as part of the Welcome Sanger

651 Institute Pathogen Informatics pipeline. The size of the dots indicates the length of positively

652 associated k-mer. A threshold of $P < 5 \times 10^{-8}$ was applied. The vertical axis is the negative of

653 the log10 value of P. The horizontal axis represents the base pair distance along the genome.

654 **Supplementary Fig. 3.** Gubbins plot of 265 *S. uberis* isolates from bovine clinical mastitis

655 cases in New Zealand. The phylogenetic tree (left panel) represents the maximum likelihood

656 tree. The *S. uberis* 0140J genome is represented as the blue vertical bars across the top of the

657 figure, while the vertical red bars in the centre of the figure represent the density estimates of

658 recombination events. The line graph at the bottom of the figure is the cumulative frequency

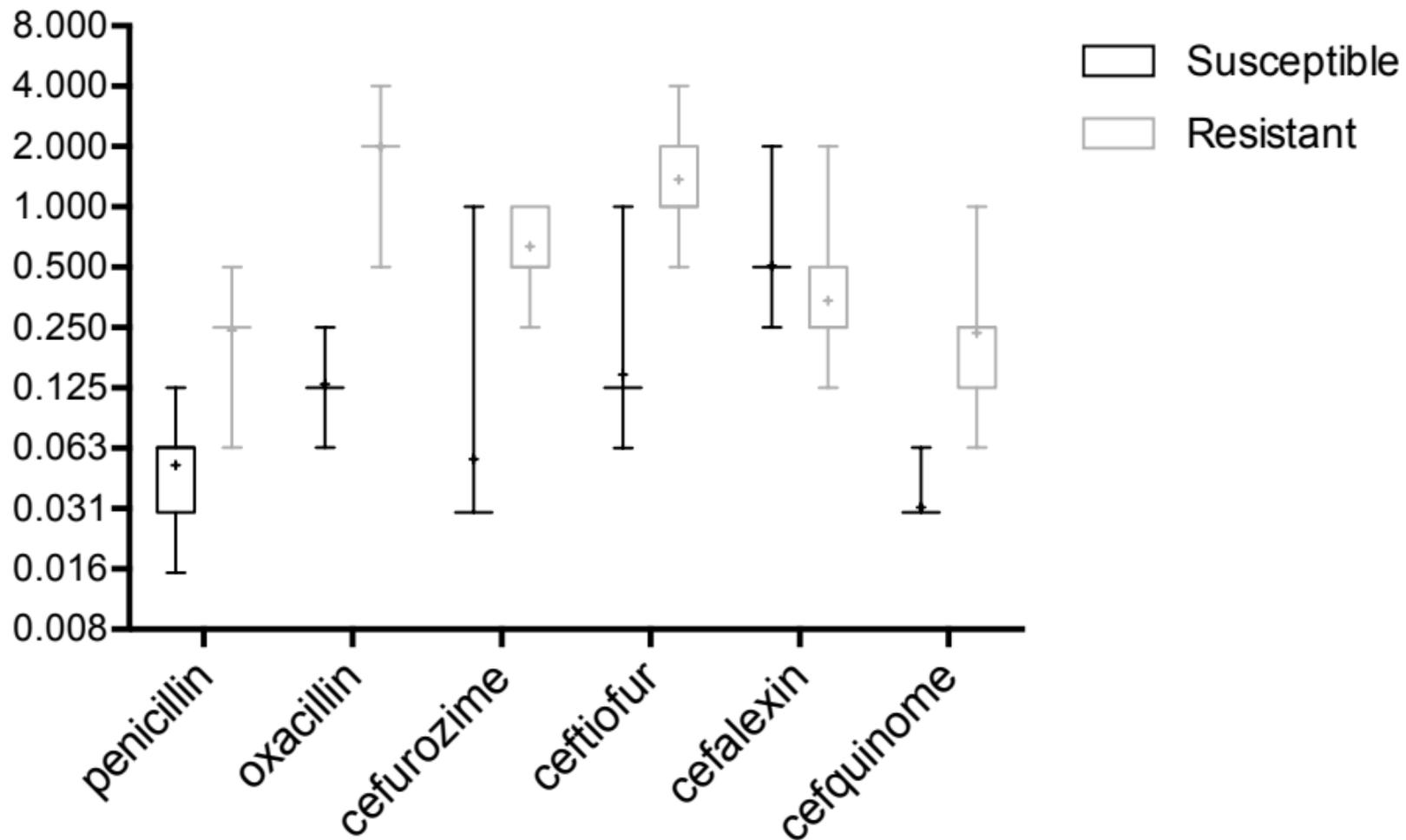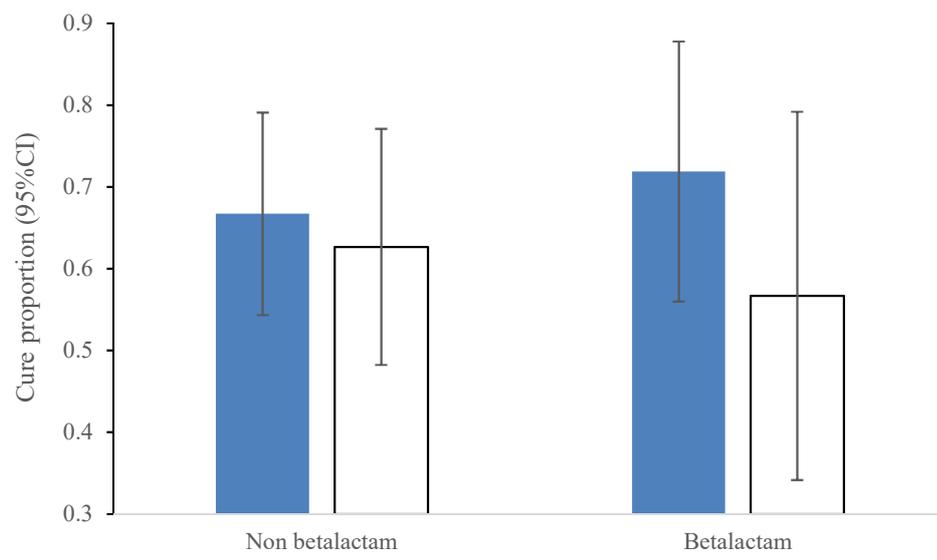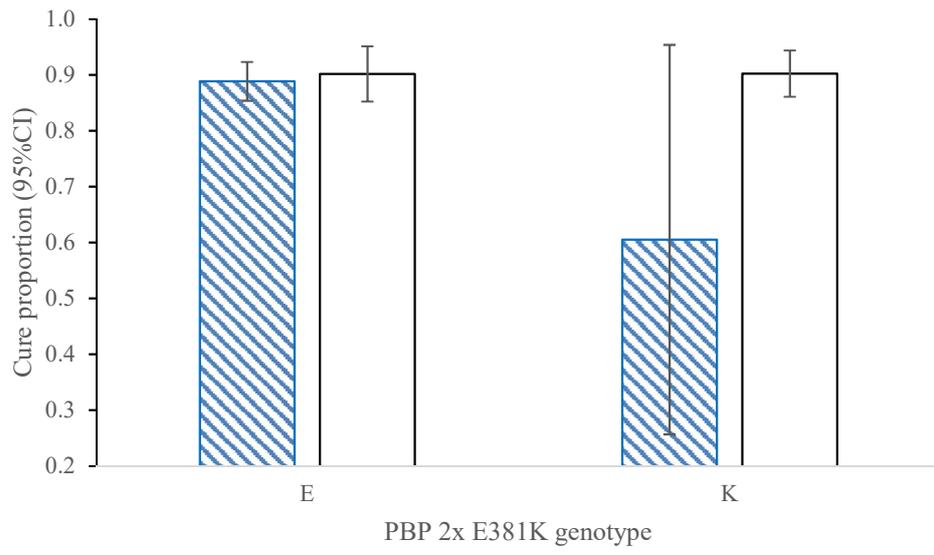659 of recombination events at that locus.

(a)

$S_{214}TMK$ $S_{272}SN$ $K_{401}TG$

$G_{386}R$ $S_{452}N$

$A_{227}T$ $R_{273}H$ $G_{386}R$

(127)   (483)

(b)

$S_{353}VVK$ $S_{408}SN$ $K_{579}TG$

$G_{371}R$

$N_{366}I$ $T_{402}I$ $V_{570}A$ $P_{575}S$

$N_{366}I$ $T_{402}I$ $V_{570}A$ $P_{575}E$

(280)   (648)

(c)

$S_{339}TMK$ $S_{398}SN$ $K_{549}SG$

$V_{295}I$ $E_{381}K$ $Q_{554}E$ $V_{590}A$ $G_{600}E$

$E_{381}K$ $A_{482}E$ $D_{524}A$ $Q_{554}E$

(264)   (619)

Tree scale: 0.001

Location
North Island
South Island

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mraW | pbpX | mraY | cshB | yxeM | yxeN | tcyN

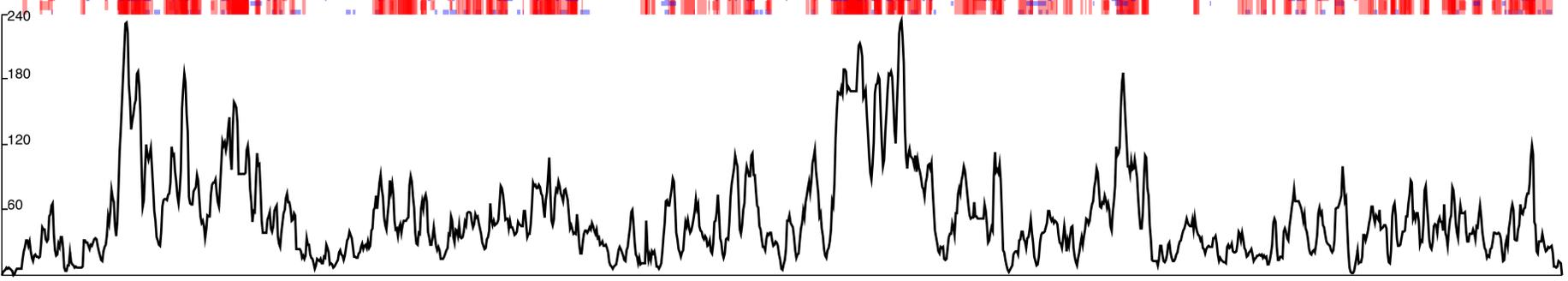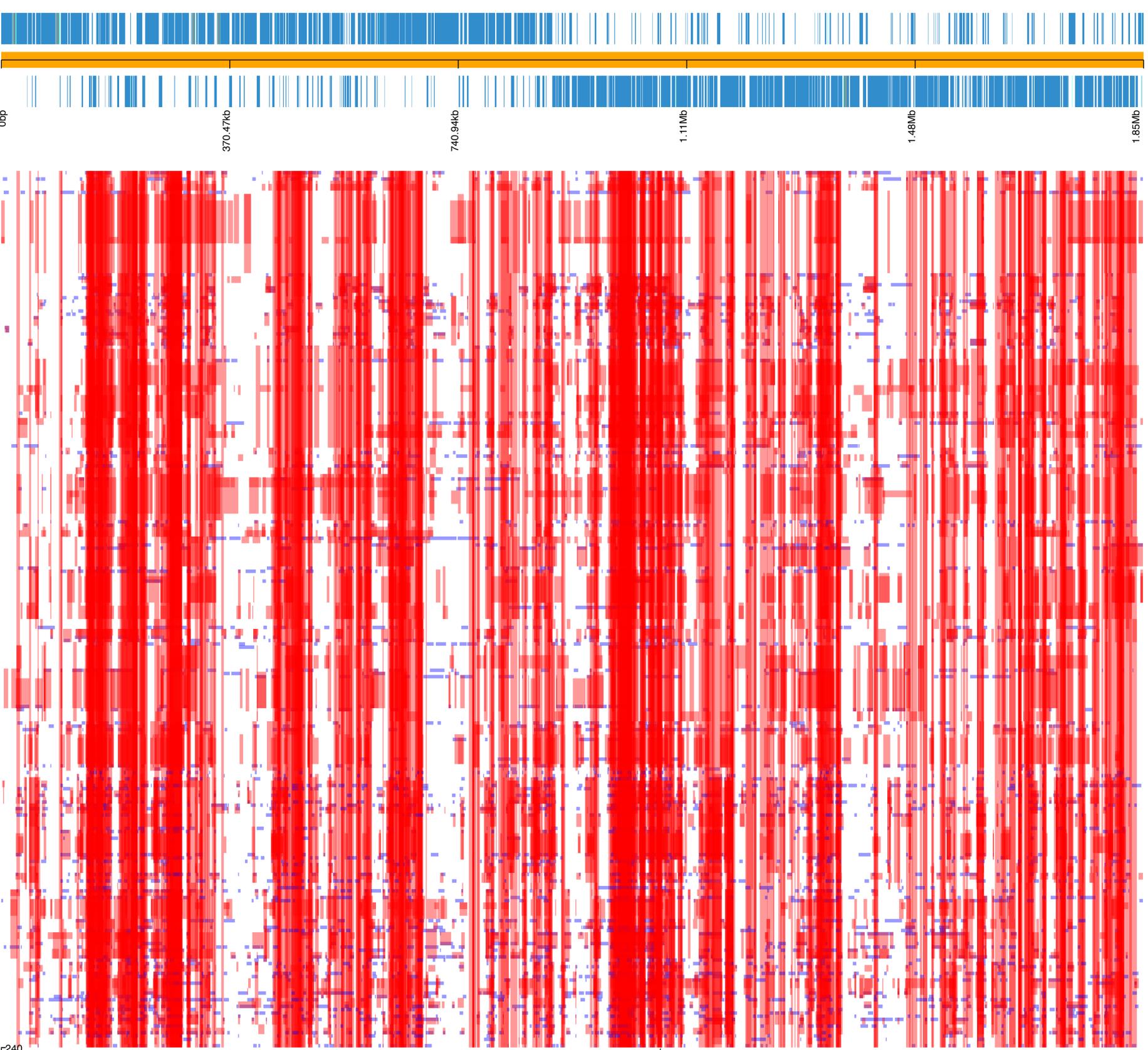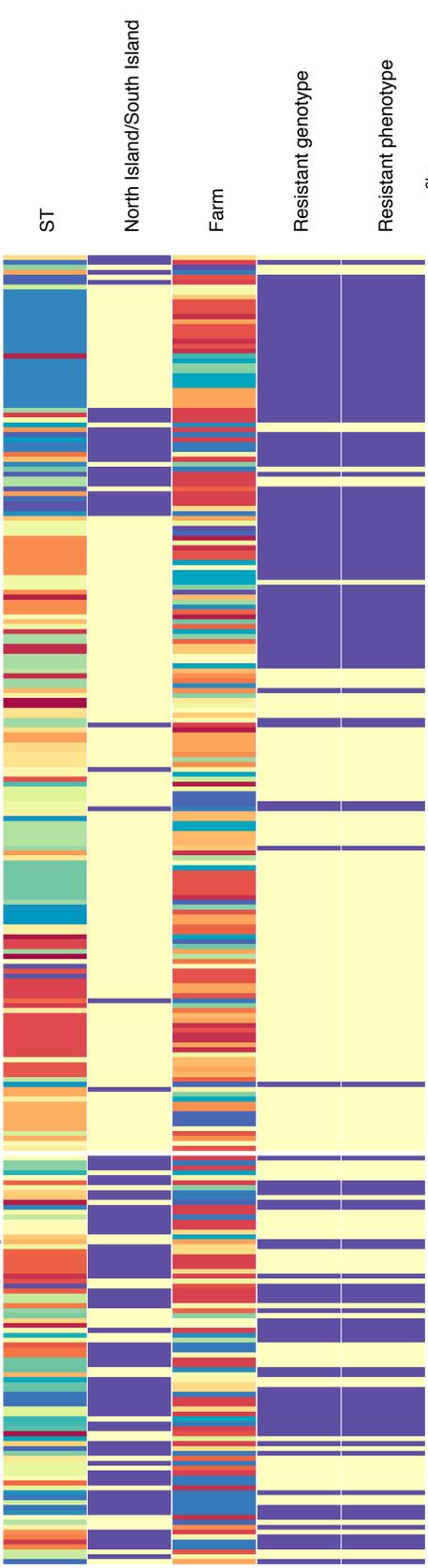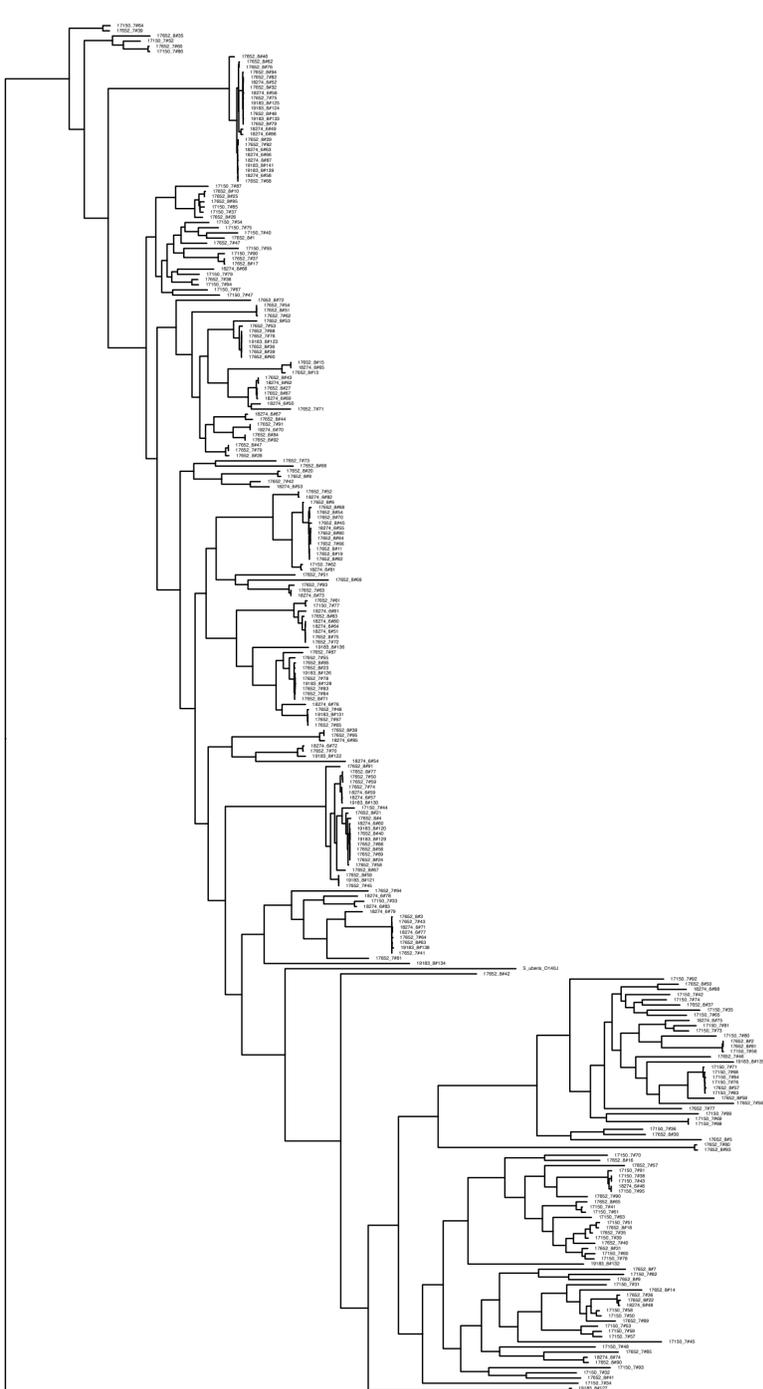2252 bp   1007 bp   1343 bp   818 bp   803 bp
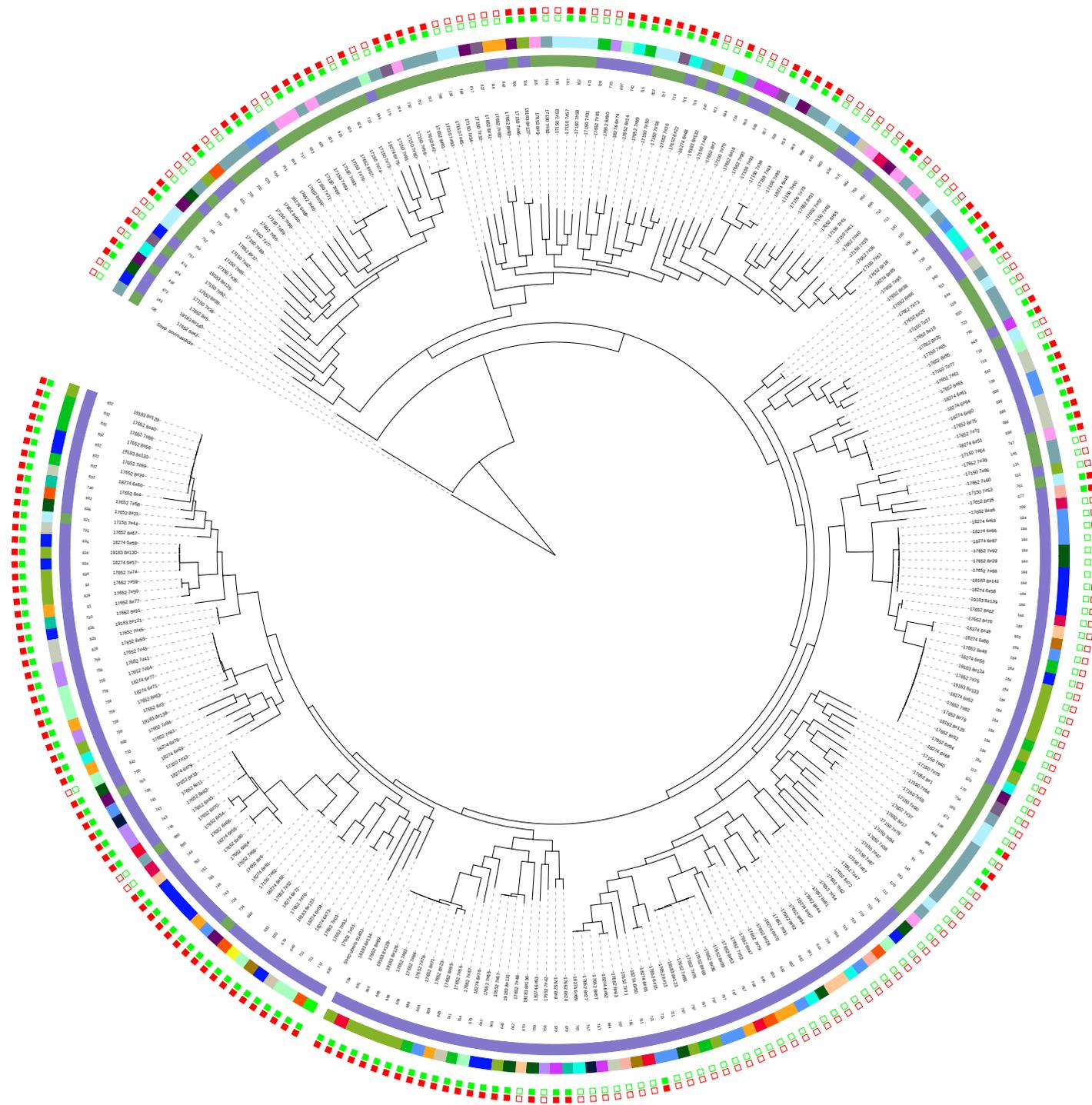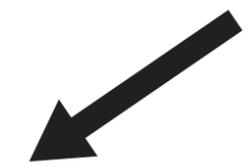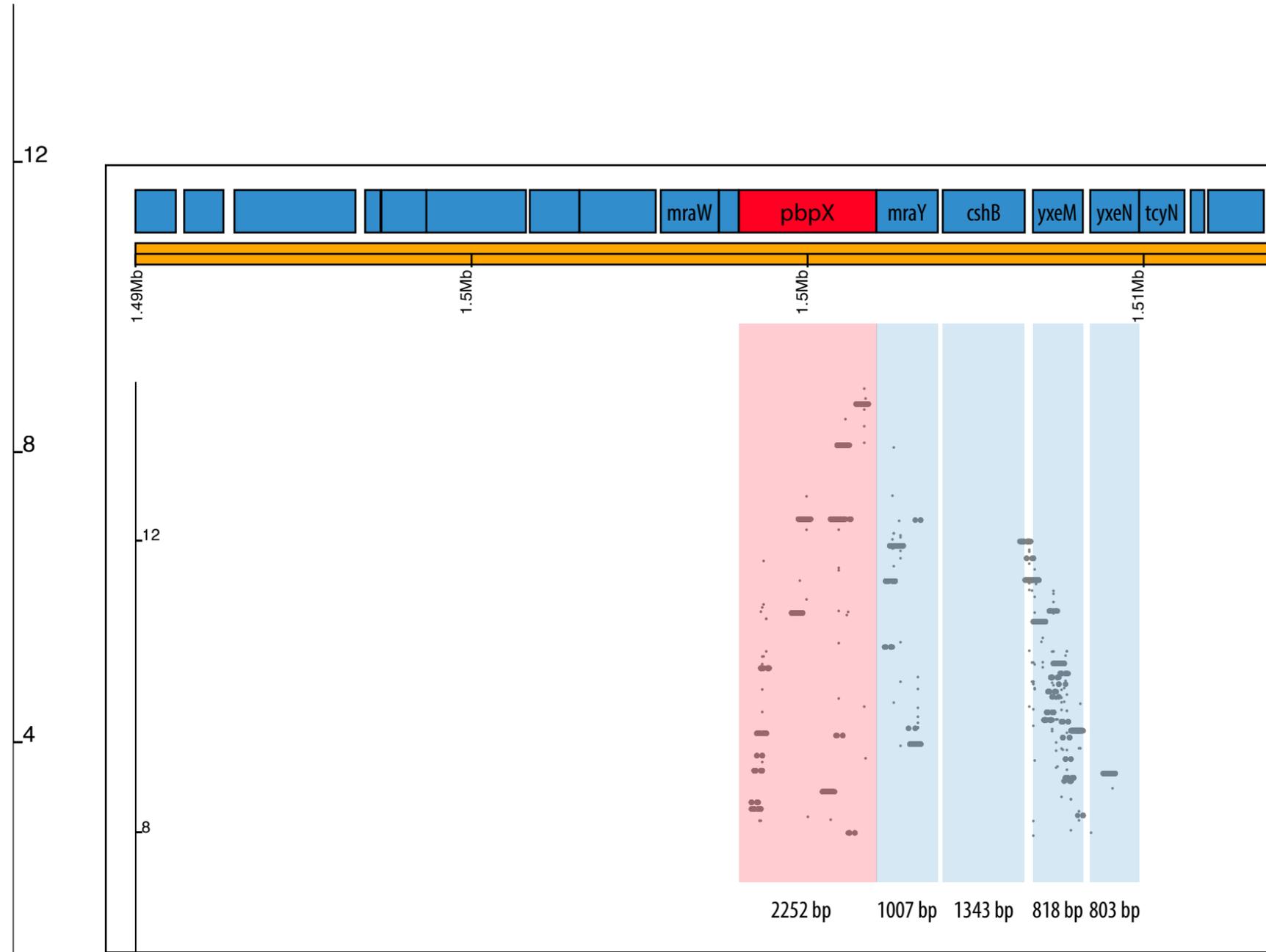
Table 1

Penicillin binding protein number (PBP), residue number (based on previously reported (Haenni et al., 2010b) numbering for *pbp1a*, *2b* and *2x*, otherwise from the start of the open reading frame), most common residue at that position (core residue), the number of isolates with the core residue at that position, the amino acid of the variant and the number of isolates with this variant, the P-value from chi squared analysis of the variant against the isolate being resistant (that is oxacillin MIC of >0.5 mg/L), and the number and percentage of isolates in core and variant amino acids with oxacillin resistance. Note only those substitutions with >10% prevalence and within the transpeptidase domain (for PBP1a, 2b and 2x) are listed. Where the same substitutions were identified in Canadian (Vélez et al., 2017) and UK (Hossain et al., 2015) isolates these are listed.

		New Zealand isolates									Canadian isolates						UK isolates					
		Core		Resistant		Variant		Resistant			Core		Variant		Variant		Core		Variant		Variant	
Gene	Position	AA	no.	no.	%	AA	no.	no.	%	P-value	AA	no.	AA	no.	AA	no.	AA	no.	AA	no.	AA	no.
pbp1a	452	S	175	73	41.7	N	90	68	75.6	0.000	N	50	S	13			N	11	S	2		
Pbp1b	768	G	164	83	50.6	S	101	58	57.4	0.280	G	34	S	28			G	9	S	4		
Pbp2a	44	E	179	107	59.8	G	86	34	39.5	0.002												
Pbp2a	397	T	235	125	53.2	A	30	16	53.3	0.843	T	45	A	18			T	12	A	1		
pbp2b	366	N	162	67	41.4	I	103	74	71.8	0.000	N	55	I	8			N	1	I	2		
pbp2b	370	S	237	129	54.4	T	28	12	42.9	0.246												
pbp2b	394	A	238	125	52.5	S	27	12	44.4	0.336												
pbp2b	402	T	161	67	41.6	I	104	74	71.2	0.000	T	55	I	8			T	11	I	2		
pbp2b	570	V	174	78	44.8	A	91	63	69.2	0.000	V	55	A	8			V	12	A	1		
pbp2b	575	P	174	78	44.8	S	91	63	69.2	0.000	P	55	S	8			P	12	S	1		
pbp2x	295	I	172	139	80.8	V	93	2	2.2	0.000	I	57	V	6			I	12	V	1		
pbp2x	381	K	140	140	100.0	E	125	1	0.8	0.000	K	53	E	10			E	10	K	3		
pbp2x	554	E	140	140	100.0	Q	125	1	0.8	0.000	E	52	Q	11			Q	11	E	2		
pbp2x	590	A	133	133	100.0	V	132	8	6.1	0.000	A	45	V	18			V	11	A	2		
pbp2x	600	E	140	140	100.0	G	125	1	0.8	0.000	E	49	G	11	D	3	G	10	E	2	D	1

	E$_{381}$K substitution			No substitution			
	Mean	SEM	MIC$_{50}$	Mean	SEM	MIC$_{50}$	P-value
Penicillin	0.24	0.09	0.25	0.05	0.02	0.0625	0.05
Cefuroxime	0.63	0.27	0.5	0.06	0.11	0.025	0.05
Ceftiofur	1.52	1.47	1.0	0.15	0.13	0.025	0.05
Cefquinome	0.23	0.15	0.25	0.03	0.01	0.025	0.05
Cefalexin	0.34	0.22	0.25	0.51	0.29	0.5	0.05