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1 **Penicillin Binding Protein Substitutions Co-occur with Fluoroquinolone Resistance in**
2 **'Epidemic' Lineages of Multi Drug-Resistant *Clostridioides difficile***
3

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19 Running Head: Penicillin Binding Proteins of Epidemic *C. difficile*
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22 **ABSTRACT**

23 *Clostridioides difficile* remains a key cause of healthcare-associated infection, with multi-
24 drug-resistant (MDR) lineages causing high mortality (≥20%) outbreaks. Cephalosporin
25 treatment is a long-established risk factor, and antimicrobial stewardship a key control. A
26 mechanism underlying raised cephalosporin MICs has not been identified in *C. difficile*, but
27 among other species resistance is often acquired *via* amino acid substitutions in cell wall
28 transpeptidases (penicillin binding proteins, PBPs). Here, we investigated five *C. difficile*
29 transpeptidases (PBP1-5) for recent substitutions. Previously published genome assemblies
30 (n=7096) were obtained, representing sixteen geographically widespread lineages, including
31 healthcare-associated MDR ST1(027), ST3(001) and ST17(018). Recent amino acid
32 substitutions were found within PBP1 (n=50) and PBP3 (n=48), ranging from 1-10
33 substitutions per genome. β-lactam MICs were measured for closely related pairs of wild-
34 type and PBP substituted isolates separated by 20-273 SNPs. Recombination-corrected, dated
35 phylogenies were constructed to date substitution acquisition. Key substitutions such as PBP3
36 V497L and PBP1 T674I/N/V emerged independently across multiple lineages. They were
37 associated with extremely high cephalosporin MICs; 1-4 doubling dilutions >wild-type up to
38 ≤1506μg/ml. Substitution patterns varied by lineage and clade, showed geographic structure,
39 and notably occurred post-1990, coincident with the acquisition of *gyrA*/*B* substitutions
40 conferring fluoroquinolone resistance. In conclusion, recent PBP1 and PBP3 substitutions are
41 associated with raised cephalosporin MICs in *C. difficile*. The co-occurrence of resistance to
42 cephalosporins and fluoroquinolones hinders attempts to understand their relative importance
43 in the dissemination of epidemic lineages. Further controlled studies of cephalosporin and
44 fluoroquinolone stewardship are needed to determine their relative effectiveness in outbreak
45 control.

46

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47 **IMPORTANCE**

48 Fluoroquinolone and cephalosporin prescribing in healthcare settings have triggered
49 outbreaks of high-mortality, multi-drug resistant *C. difficile* infection. Here, we identify a
50 mechanism of acquired cephalosporin resistance in *C. difficile*, comprising amino acid
51 substitutions in two cell-wall transpeptidase enzymes (penicillin binding proteins). The
52 higher the number of substitutions, the greater the impact on phenotype. Dated phylogenies
53 revealed that resistance to both cephalosporins and fluoroquinolones was co-acquired
54 immediately before clinically important, outbreak strains emerged. PBP substitutions were
55 geographically structured within genetic lineages, suggesting adaptation to local
56 antimicrobial prescribing. Antimicrobial stewardship of cephalosporins and fluoroquinolones
57 is an effective means of *C. difficile* outbreak control. Genetic changes conferring resistance
58 likely impart a 'fitness-cost' after antibiotic withdrawal. Our study identifies a mechanism
59 that may explain the contribution of cephalosporin stewardship to resolving outbreak
60 conditions. However, due to the co-occurrence of cephalosporin and fluoroquinolone
61 resistance, further work is needed to determine the relative importance of each.

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62 **INTRODUCTION**

63 *Clostridioides difficile* is among the leading causes of healthcare-associated infection,
64 symptoms ranging from diarrhoea to potentially fatal pseudomembranous colitis (1). Over the
65 last 30 years, unrestricted antimicrobial use has selected multidrug resistant (MDR) *C.*
66 *difficile* lineages, which can be identified by multilocus sequence type (ST) and/or PCR-
67 ribotype (2-9). Uncontrolled prescribing of antimicrobials such as fluoroquinolones and
68 cephalosporins, which are associated with a high risk of *C. difficile* infection (CDI), creates
69 conditions under which MDR lineages can cause persistent, high-mortality (≥20%) outbreaks
70 (9-18). Such healthcare-associated transmission may be geographically widespread as in the
71 'hypervirulent' ST1(ribotype 027) lineage FQ-R1 (19), and/or prolonged, as in ST17(018),
72 predominating in Japanese and Italian healthcare settings since the 1990s (17, 20). Cases
73 associated with the rapid transmission of MDR lineages are typically superimposed over a
74 background of sporadic, unlinked cases caused by *C. difficile* strains, which lack acquired
75 antimicrobial resistance (21, 22).

76 Antimicrobial stewardship is an extremely effective means of preventing or resolving CDI
77 outbreaks in healthcare settings (23-29). This approach contributed to the marked decline in
78 fluoroquinolone resistant lineages in the UK a decade ago, with resistant ST1(027),
79 ST3(001), ST42(106) and ST37(017) falling from 67% to ~3% of cases (30, 31).
80 Fluoroquinolone resistance can be predicted from whole genome sequences by characteristic
81 single nucleotide polymorphisms (SNPs) in the chromosomal *gyrA* and/or *gyrB* genes (30,
82 32). Equivalent analysis for cephalosporins is lacking, because the genetic mechanism(s)
83 influencing cephalosporin susceptibility in *C. difficile* are unknown. Wild-type MICs are
84 already moderate to high for this species (up to, and >256µg/ml), (33-36), leading to the
85 concept of 'intrinsic' rather than acquired cephalosporin resistance (10). For this reason, and

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86 the practical difficulties of determining extremely high MICs near the limit of drug solubility,
87 cephalosporin MICs are rarely measured for *C. difficile*.

88 Studies aiming to understand the mechanism of *C. difficile* cephalosporin resistance have
89 focused on the endogenous *C. difficile* class D β-lactamase, but findings were inconclusive
90 (37, 38). In many bacterial species, reduced susceptibility to cephalosporins and other β-
91 lactams is conferred by amino acid substitutions in penicillin binding proteins (PBPs). These
92 are enzymes catalysing cell wall peptidoglycan biosynthesis, which are classified according
93 to molecular weight (high or low, H/LMW) and enzymatic activity (transpeptidase or
94 carboxypeptidase) (39). β-lactam antibiotics target PBPs by acting as inhibitory substrate
95 analogues (39), binding covalently to the active site serine (40) in the first of three conserved
96 motifs; SXXK, (S/Y)XN and (K/H)(S/T)G (41). β-lactam exposure selects substitutions
97 which reduce the affinity of the drug for the PBP, increasing the MIC (42, 43). However, the
98 nature and frequency of PBP substitutions among clinically important *C. difficile* lineages,
99 and their impact on cephalosporin MIC has not been investigated systematically. Here, our
100 aim was to determine whether PBP substitutions have contributed to the emergence and
101 spread of epidemic MDR *C. difficile*.

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102 **RESULTS**

103 The study was designed as follows. A globally distributed collection of published *C. difficile*
104 genomes was assembled (n=7094) representing sixteen genetic lineages; fourteen commonly
105 associated with CDI in healthcare settings, and two carried asymptomatically (non-toxigenic).
106 The occurrence of recent, within-lineage PBP substitutions was investigated. Cephalosporin
107 (and other β-lactam) MICs were measured for representative strains containing PBP
108 substitutions, and closely related 'wild-type' ancestors. Finally, the timing and sequence of
109 PBP substitution and fluoroquinolone resistance acquisition events was investigated
110 phylogenetically.

111 **Lineages Studied**

112 The 7094 genomes represented lineages ST1(ribotypes 027/198/176/181) (n=1918),
113 ST17(018) (n=279), ST42(106) (n=563), ST3(001) (n=411), ST37(017) (n=424) and its
114 recent descendant ST81(369) (n=39), ST63(053) (n=37), ST54(012) (n=148), ST8(002)
115 (n=593) and its recent descendant ST183 (n=14), ST11(078) (n=628), ST2(014/020) (n=790),
116 ST6(005) (n=404), ST10(015) (n=263), ST7(026) (n=190), ST56(058) (n=16) and two
117 prevalent non-toxigenic genotypes ST26(039) (n=175) and ST15(010) (n=202). Four
118 genetically distinct *C. difficile* clades; 1, 2, 4 and 5 were represented (44). Genomes,
119 accession numbers, AMR predictions from genotype and references are listed per lineage
120 (Table S1).

121 ***C. difficile* PBPs**

122 To date, nine PBPs have been described within the *C. difficile* genome (Table 1) five of
123 which are transpeptidases (PBP1-5) (45). HMW PBP1 and PBP3 are essential for growth *in*
124 *vitro* (46), while LMW PBP2 and PBP4 are not essential for growth, but are required for
125 sporulation (46). Only LMW PBP5 is variably present (45). PBP1-4 were present in all
126 genomes studied, while PBP5 occurred in lineages ST3(001), ST11(078), and

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127 ST37(017)/ST81(369). No additional PBPs were identified using known *C. difficile* PBP
128 sequences in low stringency BLAST searches.

129 **Recent PBP Substitutions**

130 PBP gene sequences were compared within each lineage to identify recent single nucleotide
131 polymorphisms (SNPs). These were absent or rare in LMW PBP2, PBP4 and PBP5. In
132 contrast, multiple SNPs occurred in HMW PBP1 and PBP3, almost all of which were non-
133 synonymous. The resultant amino acid substitutions affected a total of 48/993 (4.8%)
134 positions in PBP3 and 50/856-926 (5.8-5.4%) positions in PBP1 (variable PBP1 size due to
135 C-terminal repeats). Substitution data are shown per isolate (Table S1).

136 The frequency of each amino acid substitution was recorded per lineage (Table 2). This
137 identified the most common substitutions within and among lineages. In PBP3, V497L was
138 most frequent, occurring in 2897 genomes and 10 lineages, followed by A778V in 664
139 genomes and 10 lineages. In PBP1, T674I/N/V was most frequent, occurring in 1379
140 genomes of 10 lineages, followed by A555T in 442 genomes of 7 lineages. These data were
141 then plotted to visualise the relative positions and frequencies of substitutions within PBP1
142 and PBP3 (Figure 1). Virtually all substitutions occurred within the conserved transpeptidase
143 domains, flanking the active site motifs.

144 **Co-occurrence of PBP substitutions with fluoroquinolone resistance**

145 In addition to PBP substitutions, the presence of fluoroquinolone resistance was investigated
146 in all sixteen lineages (Figure 2, Table S1). Their co-occurrence was striking in lineages
147 ST1(027/198/176/181), ST17(018), ST42(106), ST3(001), ST37(017)/ST81(369),
148 ST63(053), ST54(012), ST8(002)/ST183 (Figure 2), suggesting the possibility of almost
149 simultaneous acquisition. The presence of further AMR determinants, *ermB* (clindamycin

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150 resistance) and, or *rpoB* substitutions (rifampicin resistance), was also recorded for each
151 genome (Table S1).
152 Among the fourteen clinically important lineages, PBP substitutions occurred relatively
153 rarely in the absence fluoroquinolone resistance (Figure 2). In this respect ST2(014/020)
154 (178/790 genomes, 22.5%), ST54(012) (46/148 genomes, 31.1%) and recent ST42(106) USA
155 genomes (44/180, 24.4%) were noteworthy. Interestingly, PBP substitutions occurred without
156 fluoroquinolone resistance in the majority of genomes belonging to the non-toxigenic
157 lineages ST26(039) (167/175, 95.4%) and ST15(010) (182/202 (90.1%), (Figure 2, Table
158 S1).

159 **Impact of PBP substitutions on β-lactam MIC**

160 β-lactam MICs were measured for isolates representing eight of the clinically important
161 lineages studied. Four of these lineages contained both 'wild-type' and PBP-substituted
162 strains; ST1(027), (both FQ-R1 and FQ-R2 (19)), ST17(018), ST3(001) and ST42(106). The
163 remaining four lineages, included for comparison, were 'wild-type' only, lacking PBP
164 substitutions; ST10(015), ST6(005), ST56(058) and ST7(026).
165 The choice of isolates from ST1(027), ST17(018), ST3(001) and ST42(106), was based on
166 low numbers of SNP differences between wild type and PBP substituted genomes (Figure
167 3A). In total, ten different PBP substitutions were represented, three in PBP3 and seven in
168 PBP1 (Figure 3B), including the four most frequently identified substitutions.
169 Isolates containing PBP substitutions showed increased cephalosporin and carbapenem MICs
170 relative to wild-type, but their penicillin MICs were unchanged (Figure 3A). The greatest
171 increases in cephalosporin MICs were associated with the highest numbers of substitutions,
172 for example cefuroxime MIC increased from 376 to 1506µg/ml in ST3(001) (four
173 substitutions) and ST17(018) (five substitutions). Cephradine MIC increased from 36 to
174 239µg/ml in the latter. Intriguingly, the wild-type ancestors of these four PBP substituted

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175 lineages had cefotaxime MICs which were still higher than the four lineages which have not
176 yielded PBP substituted strains; cefuroxime 128µg/ml vs 376µg/ml and cefotaxime 128µg/ml
177 vs 256µg/ml.

178 **Phylogenetic analyses**

179 Recombination-corrected phylogenies were constructed to date and identify the sequence in
180 which PBP substitution and fluoroquinolone resistance were acquired by seven genetic
181 lineages. These included the four PBP substituted lineages which had been phenotyped
182 (ST1(027), ST17(018), ST3(001) and ST42 (106)), and a further three lineages containing
183 notable MDR PBP substituted fluoroquinolone resistant strains. These were ST8(002)/ST183
184 and ST37(017)/ST81 - both important in South East Asia, and ST54(012) - notable in Costa
185 Rica (47-51) (Figures 4-7).

186 Irrespective of lineage, the sequence of PBP substitution acquisitions in epidemic strains
187 typically started with PBP3 V497L and/or A778V (ie. the most frequent PBP3 substitutions,
188 Table 2). Then further PBP substitutions followed, yielding a variety of patterns. Among
189 lineages well known for epidemic spread, the initial PBP3 V497L substitution occurred
190 simultaneously with fluoroquinolone resistance (Figures 4-7). One notable exception was the
191 ST1(027) FQ-R1 lineage in which PBP3 A726V substitution occurred first, while PBP3
192 V497L was absent (Figure 4B).

193 PBP substituted, fluoroquinolone resistant clades evolved more than once in ST1(027),
194 ST3(001), ST37(017)/ST81(369), ST42(106), and ST54(012) (Figures 4A,B, 5B, 6B, 7A,B).
195 Their PBP substitution patterns each showed geographic structure (Figures 4-8). MDR clades
196 with the highest numbers of PBP substitutions were identified within ST17(018) in Italy and
197 South East Asia (Figure 5A), ST3(001) in UK/Germany (Figure 5B), ST8(002)/ST183 in
198 Japan (Figure 6A), and ST37(017)/ST81(369) in South East Asia (Figure 6B).

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199 The occurrence of PBP substitutions in the absence of fluoroquinolone resistance was
200 investigated phylogenetically in ST2(014/020) (Figure 8), and to a lesser extent in ST42(106)
201 and ST54(012) (Figure 7). For ST2(014/020), a dated phylogeny was constructed using PBP
202 substituted genomes from six independent locations, together with wild type genomes from
203 the same locations and dates (Figure 8). The PBP substituted genomes clustered by location,
204 while the wild type strains did not. Interestingly, both ST42(106) and ST54(012) phylogenies
205 (Figure 7) contained clades where PBP substitution acquisition preceded fluoroquinolone
206 resistance. ST42(106) recently replaced ST1(027) as the most prevalent lineage in North
207 America (Carlson et al., 2020), but a single MDR clade was not apparent; PBP3 V497L
208 occurred on multiple independent occasions within the ST42(106) phylogeny.

209 **Evolutionary mechanisms of PBP substitution acquisition**

210 The almost total absence of non-synonymous SNPs within each lineage suggested that PBP
211 substitutions accumulate by the step-wise fixation of *de novo* point mutations in response to
212 β-lactam selection, rather than the import of novel variants by horizontal genetic exchange.
213 However, two important recombination events were identified by the co-import of
214 synonymous and non-synonymous SNPs, which changed PBP variants within toxin A-B+
215 ST81(369) (Figure S1) and in ST1(181), relative to their ancestral wild type A-B+ ST37(017)
216 and ST1(027) wild type respectively. The ST81(369) PBP3 gene was acquired as part of a
217 very long (~150kb) recombination event, the donor being closely related to ST8(002) (Figure
218 S1). In ST1(181), described in Greece and Romania (52, 53) recombination events affected
219 both PBP1 and PBP3, but their ~400kb separation around the chromosome suggests the
220 events were independent; (i) the PBP3 allele (100) was identical to clade 1 ST17(018),
221 indicating inter clade 1/2 recombination, and (ii) the PBP1 allele (360) contained 14 SNPs
222 and 6 amino acid differences relative to wild type ST1.

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223 **DISCUSSION**

224 To date, studies aiming to determine β-lactam resistance mechanisms in *C. difficile* have
225 focused on the endogenous *C. difficile* class D β-lactamase (37, 38). PBP substitutions have
226 been reported only occasionally, associated with raised carbapenem MICs in a single lineage
227 (45, 54). PBPs with reduced β-lactam affinity are clinically important in other Gram-positive
228 pathogens, for example *S. pneumoniae* (43), and methicillin resistant *Staphylococcus aureus*
229 (55). The present study was therefore performed to investigate systematically, and
230 phenotypically, recent PBP substitutions among clinically important lineages of *C. difficile*.

231 We identified multiple, recent PBP substitutions, which are focused in the conserved
232 functional domains of the two HMW *C. difficile* transpeptidases, PBP1 and PBP3 (Table 2,
233 Figure 1). Substitutions were associated with raised cephalosporin MICs, relative to closely
234 related wild type strains, and the higher the number of substitutions, the higher the MIC
235 (Figure 3A). The mechanism underlying substitution acquisition was not recombination, but
236 rather the accumulation of *de novo* chromosomal mutations in response to selective pressure.
237 This was indicated because virtually all SNPs were non-synonymous, flanked the catalytic
238 domains (Figure 1), and arose multiple times (Table 2). Only two major recombination events
239 were found involving ST81(369) (Figure S1), and ST1(181). Although PBP5 transpeptidase
240 is variably present, it was not recently acquired by MDR *C. difficile* lineages. Its constant
241 chromosomal location suggests that gradual loss, rather than recent acquisition may explain
242 its variable presence.

243 The co-occurrence of PBP substitutions with fluoroquinolone resistance in the clinically
244 important epidemic lineages was striking (Figure 2). This suggests that cephalosporin
245 stewardship may be equal to fluoroquinolone stewardship (30) in its effectiveness for
246 outbreak control. Furthermore, simultaneous stewardship of both drugs may have a greater
247 impact on the control of MDR lineages, than either drug alone. Studies performed over 20

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248 years ago, before widespread fluoroquinolone resistance emerged in *C. difficile*, reported
249 cephalosporin stewardship alone to be successful (24, 56, 57). Approximately 20 years
250 elapsed between the introduction of first generation cephalosporins (mid 1960s) and
251 fluoroquinolones (late 1980s) (58). Selection of the wild-type precursors of the current
252 epidemic lineages (Figure 3A), with cephalosporin MICs that exceed the wild type of other
253 lineages, (WT cefuroxime 128µg/ml vs 376µg/ml and WT cefotaxime 128µg/ml vs
254 256µg/ml) may have occurred during this time. However the mechanism underlying this
255 difference in wild type MICs remains unknown. At present, the co-occurrence of resistance to
256 both cephalosporins and fluoroquinolones hinders attempts to understand their relative
257 importance in epidemic spread. Further controlled studies of cephalosporin and
258 fluoroquinolone stewardship are needed.

259 Given the large number of PBP substituted, clinically important MDR clades which have
260 emerged in the last 30 years, in different geographic regions (Figures 4-8), it is surprising that
261 their elevated cephalosporin MICs have not been highlighted previously. This likely reflects
262 the 'intrinsic cephalosporin resistance' concept (10), established in the early 1980s, when
263 measured *C. difficile* cephalosporin MICs rarely exceeded 256µg/ml (33-36). These baseline
264 MICs, which predate the emergence of the MDR clades, are already high relative to other
265 pathogens, and were thought to exceed clinically relevant concentrations. The lack of a
266 known mechanism of acquired β-lactam resistance compounded the situation.

267 Our phylogenies revealed the sequence and timing of MDR acquisition by clinically
268 important lineages. The co-occurrence of PBP substitutions and fluoroquinolone resistance
269 predated epidemic spread, which was reflected in short-branched, geographically structured
270 clades (Figures 4-7). The first PBP substitution was typically PBP3 V497L, followed by
271 others, yielding a variety of final combinations. The presence of *ermB*, (clindamycin
272 resistance) and *rpoB* substitutions (rifampin resistance) was variable overall, but greater

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273 numbers of PBP substitutions frequently occurred with these in addition to fluoroquinolone
274 resistance (Table S1, ST17(018, ST37(017)/ST81(369), ST8(002)/ST81).

275 We dated the emergence of the two MDR ST1(027) clades (FQ-R1 and FQ-R2) to the
276 mid/late-1990s, as previously described (19) (Figure 4A,B). The emergence date of the MDR
277 ST17(018) clade was compatible with first reports of outbreaks in 1996-1999 (59), the
278 phylogeny root having a 95% credible interval dating of December 1998-July 2002 (Figure
279 5A). European and Asian ST17(018) clades then diverged, acquiring further region-specific
280 PBP substitutions (Figure 5A), arguing against recent their recent intercontinental spread.

281 Greater numbers of PBP substitutions were associated with the highest cephalosporin MICs
282 (Figure 3A). Consistent with this, highly substituted clades of multiple lineages (ST17(018),
283 ST81(369)/ST37(018) and ST183/ST8(002, Figures 5A, 6A,B) predominated in South East
284 Asia, where cephalosporin use is high (60-62). Adaptation to local prescribing conditions
285 through PBP substitution acquisition offers a possible explanation for the temporal and
286 geographic variation in prevalent *C. difficile* lineages (6, 8, 19, 47, 58, 63). This may extend
287 to competitive exclusion of lesser PBP substituted strains by more highly substituted ones, a
288 scenario requiring greater numbers of PBP substitutions to carry a fitness cost. This appears
289 possible as in *C. perfringens in vitro*, PBP substitutions are associated with slower growth
290 (64). We hypothesise that local levels of cephalosporin prescribing determine the prevalent *C.*
291 *difficile* strain(s) in a given region. For example, the unusually low levels of ST1(027) seen in
292 Asia, (65) may reflect competitive exclusion, under local prescribing conditions, by the more
293 highly PBP substituted clades which predominate here. The relative geographic restriction of
294 ST1(027)FQ-R1 (to USA, South Korea, Germany), compared to more globally distributed
295 FQ-R2 may also reflect the different PBP substitutions of the two clades (Figure 4), and
296 variations in MIC (Figure 3A).

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297 MDR strains exhibit high transmissibility in clinical settings when prescribing is uncontrolled
298 (17). The PBP1 and PBP3 transpeptidases function in cell wall biosynthesis, therefore
299 substitutions impacting their catalytic domain could affect transmissibility via sporulation. A
300 high sporulation phenotype has been reported in at least two epidemic lineages; ST3(001)
301 (UK), and ST81(369) (Asia), (66, 67). Sporulation phenotype is reportedly variable in
302 ST1(027), (68, 69), and we have observed variation in its PBP substitutions (Figure 4A,B,
303 Table S1). However, the possibility of a link remains to be investigated. It is relevant to
304 future experimental design that the MDR laboratory reference strains CD630 (ST54(012) and
305 R20291 (ST1(027)) both contain PBP substitutions (Table 1).

306 PBP substitutions occurred *without* fluoroquinolone resistance in reasonable numbers of
307 genomes of only three toxigenic lineages studied; ST2(020/014), ST54(012), and ST42(106)
308 (USA) (Table S1). The latter was of interest since it recently exceeded the prevalence of
309 ST1(027) in North America (8, 71). Visual inspection of phylogenies was used to assess
310 whether PBP substitutions might enhance transmissibility in the absence of fluoroquinolone
311 resistance. An ST2(020/014) phylogeny showed some possibility of locally enhanced
312 transmission (Figure 8), as did ST54(012), and ST42(106) (USA) (Figure 7A,B). However,
313 these events were small scale and this question remains to be answered. PBP substitutions
314 (without fluoroquinolone resistance) were, however, widespread within the two non-
315 toxigenic lineages ST15(010) and ST26(039), (Table S2) potentially explaining their high
316 prevalence over other non-toxigenic strains.

317 The cephalosporin MICs for PBP substituted strains were extremely high for certain
318 antibiotics (for example >512µg/ml for cefotaxime, and up to 1506µg/ml for cefuroxime,
319 Figure 3A). This raises questions about the *in vivo* conditions required for PBP substitution
320 selection. Intravenous β-lactams are eliminated in active form by biliary excretion, resulting
321 in highly variable intestinal concentrations (71). Intestinal concentrations ranging from 1.01

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322 to 1,345μg/ml have been reported (72) and so the potential exists for *C. difficile* to be
323 exposed *in vivo* to cephalosporin concentrations reaching the MICs measured here. Resistant
324 bacteria can also be selected experimentally at antimicrobial concentrations up to several
325 hundred-fold below lethal levels (73-77). However, the overall contribution made by such
326 'sub-MIC selection' to resistance in clinically important bacteria is unknown (78).

327 To date, only ~1% of known *C. difficile* lineages (949 STs identified as at 13 May 2022,
328 https://pubmlst.org/organisms/clostridioides-difficile) have evolved MDR clade(s).
329 Furthermore, members of this minority have tended to evolve >1 such clade (Figure 4-7).
330 This suggests a wild-type phenotype which favours the acquisition of chromosomal SNPs
331 which raise cephalosporin and fluoroquinolone MICs. As discussed above, the baseline
332 cephalosporin MICs for MDR-yielding lineages were higher than those lacking MDR strains
333 (Figure 3A). This potentially favours survival of MDR-yielding lineages in low
334 cephalosporin concentrations, allowing selection of PBP substitutions. An alternative,
335 mechanism might be a hypermutator phenotype, as in *S. pneumoniae* (73).

336 In summary, our findings identify a role for cephalosporin selection in the evolution of
337 epidemic CDI lineages. Specific regional prescribing practises may determine the locally
338 predominant epidemic strains, potentially explaining the marked international variation in *C.*
339 *difficile* molecular epidemiology. Since antimicrobial stewardship typically targets multiple
340 drug classes (29, 79) and epidemic strains have raised MICs for fluoroquinolones,
341 cephalosporins, (Figure 2) and more variably clindamycin (*ermB*) (Table S1), is difficult to
342 determine the relative contributions made by stewardship of each drug to CDI control. The
343 timing of cephalosporin and fluoroquinolone resistance acquisition, immediately before the
344 emergence of multiple epidemic strains from divergent *C. difficile* genetic backgrounds,
345 suggests AMR may be equally important as, or even exceed, strain-specific virulence
346 determinants in driving epidemic CDI.

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347 **MATERIALS AND METHODS**

348 WGS from 7094 *C. difficile* isolates, predominantly cultured from humans with CDI were
349 obtained. Clinical isolates from hospital and community patients from Europe, North and
350 South America, South East Asia, and Australia were included. Fourteen CDI lineages were
351 represented, and two non-toxigenic lineages. A complete list of genomes, their identifiers in
352 public databases, and references is provided (Table S1). Raw sequence reads were assembled
353 *de novo* as required using Velvet (version 1.0.7 – 1.0.18) (80) and Velvet Optimiser with
354 default settings (2.1.7) (81). A minority of genomes were obtained assembled, either from the
355 NCBI database (82) or EnteroBase (83, 84). Assemblies were imported to a BIGSdb database
356 (85) which was used to identify the seven loci used in multi-locus sequence typing (44).
357 Sequence types (STs) were assigned using the *C. difficile* PubMLST database
358 (https://pubmlst.org/organisms/clostridioides-difficile/). ST and PCR-ribotype were used to
359 indicate genetic lineages, identified by the notation ST1(027) (sequence type-1 (PCR-
360 ribotype-027)).
361 BLAST searches performed within BIGSdb (85) were used to identify and extract
362 chromosomal gene sequences for PBP transpeptidases (PBP1-5, (45)), together with *gyrA*,
363 *gyrB*, and *rpoB*, specific mutations in which confer AMR. Established amino acid
364 substitutions scored as conferring resistance to fluoroquinolones were GyrA T81I and GyrB
365 D426N, and to rifampin RpoB R505K, H502N, S498T. Acquisition of *ermB*, conferring
366 clindamycin resistance was also noted (86-89). Each unique allele sequence identified at
367 these loci (PBP1-5, *gyrA*, *gyrB*, *rpoB* and *ermB*) was assigned a number (Table S1) and can
368 be downloaded at https://pubmlst.org/organisms/clostridioides-difficile/ (44, 85). Newly
369 extracted gene sequences were queried against this database and the allele numbers were
370 recorded for each genome, together with the substitutions relevant to AMR (Table S1).

371

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372 **Identification of recent PBP substitutions**

373 This was achieved using MEGA (https://www.megasoftware.net/) (90), which facilitated
374 within-lineage comparisons of the nucleotide and amino acid sequences of PBP1-5 alleles.
375 Comparisons were made relative to the wild type PBP sequence for each lineage, wild-type
376 alleles being taken from non-MDR genomes within the lineage.

377 **Phenotyping**

378 Isolates were chosen for phenotyping from four lineages, each of which contained both PBP
379 substituted and wild type strains; ST1(027)FQ-R1 and FQ-R2, ST3(001), ST17(017) and
380 ST42(106). Representatives for each lineage were chosen on the basis of minimal SNP
381 distances (Figure 3A). Genomes of four lineages lacking MDR strains (wild type only), also
382 underwent phenotyping; ST10(015), ST6(005), ST56(058) and ST7(026).
383 Minimum inhibitory concentrations of cefotaxime, cefuroxime, cephradine, amoxicillin,
384 amoxicillin-clavulante, meropenem, imipenem and piperacillin-tazobatam were determined
385 by Wilkins Chalgren agar dilution methods (91, 92). Briefly, *C. difficile* isolates and controls
386 (*C. difficile* ATCC700057, E4 (PCR ribotype 010) and *B. fragilis* 25285) were cultured in
387 pre-reduced Schaedlers anaerobic broths at 37˚C for 24h, anaerobically. Isolates and controls
388 were diluted in pre-reduced saline to McFarland standard 1 equivalence and multipoint
389 inoculated onto prepared antibiotic-containing agar plates and controls. Agar plates were
390 incubated at 37˚C for 24h, anaerobically prior to MIC determination. MIC was defined as the
391 lowest concentration of antimicrobial that completely inhibited growth, showed only 1 or 2
392 colonies, or left a faint haze of growth on the plate.

393 Antimicrobial concentrations were prepared using solvents and diluents recommended in the
394 CLSI guidelines (93, 94). For amoxicillin clavulanate and piperacillin-tazobactam, clavulanic
395 acid and tazobactam were was added to agar at fixed concentrations of 2mg/L and 4 mg/L
396 respectively. In order to test susceptibility within normal doubling dilutions, further antibiotic

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397 concentrations were prepared for the antibiotic plate range. All antibiotics were tested at the
398 following dilutions: 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 36, 40, 46, 53, 64, 71, 80, 91, 107,
399 128, 142, 160, 182, 213, 256, 284, 320, 366, 427, 512 mg/L. Additionally, cefuroxime and
400 cephradine were prepared up to their limit of solubility and the following ranges of dilutions
401 were prepared. Cefuroxime: 102, 120, 143, 160, 205, 213, 240, 287, 319, 409, 478, 572
402 mg/L. Cephradine: 105, 118, 134, 157, 188, 209, 235, 269, 314, 376, 418, 471, 538, 627, 753,
403 837, 941, 1076, 1255, 1506 mg/L.

404 **Construction of dated phylogenies**

405 Dated phylogenies were constructed using genomes chosen to maximise geographic and
406 temporal spread of wild type and AMR strains, as well as to represent the diversity of PBP
407 substitutions detected in each lineage. Each set of genomes was first aligned to a reference
408 using MuMMER version 3.1 (95) to produce a genome-wide alignment. Initial phylogenies
409 were built using PhyML version 3.3 (96) which were then corrected for recombination using
410 ClonalFrameML version 1.12 (97). Finally these phylogenies were dated using BactDating
411 version 1.1 (98) assuming a mean evolutionary rate of 1.4 mutations per year per genome as
412 in previous similar studies (99).

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431 **DECLARATIONS**

432 DWE declares lecture fees from Gilead, outside the submitted work. No other author has a
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434 **FIGURE LEGENDS**

435 **Figure 1. Positions and relative frequency of amino acid substitutions within PBP3 (993**
436 **amino acids long) and PBP1 (856-926 amino acids).**
437 (A) Substitutions within PBP3 (n=48) are represented by purple circles, plotted according to
438 locations within the PBP (x-axis). Circles are scaled according to substitution frequency
439 within the entire dataset. Light grey shading indicates the position of conserved
440 transpeptidase domains identified by BLASTP. Red circles indicate transpeptidase catalytic
441 motifs.
442 (B) As (A), but PBP1 (n substitutions = 50), and dark grey indicates N-terminal glycosyl
443 transferase domain.
444 (C) As (A), except relative sizes of blue circle indicates the number of lineages in which each
445 substitution was identified.
446 (D) As (B), blue circles again indicating the number of lineages in which each substitution
447 was identified.
448 Raw data, including the identity of each substitution are shown in Table 2.

449 **Figure 2. Occurrence of PBP substitutions and fluoroquinolone resistance in the 16**
450 **lineages studied.**

451 **Figure 3. β-lactam MICs of wild type and PBP substituted *C. difficile* isolates.**
452 (A) MICs measured for the lineages, strains and β-lactams shown. PBP substitutions are
453 highlighted by colour. Intensity of grey shading indicates fold increase in MIC from wild
454 type. Numbers in superscript indicate the numbers of isolates tested, if >1. WT: wild-type,
455 NT: not tested.

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456 (B) Positions of the PBP substitutions (coloured as in (A)) contained in the isolates that
457 underwent phenotyping (A), relative to the conserved transpeptidase domains (grey) and the
458 active site motifs (red circles).

459 **Figure 4. Phylogenetic analysis of lineage ST1(027)**

460 (A) Dated phylogeny to show the emergence of lineage ST1(027) FQ-R1 (n=27) (19) (red
461 branches) from wild type (n=27).
462 (B) Dated phylogeny to show the emergence of lineage ST1(027) FQ-R2 (n=67) (19) (red
463 branches) from wild type (n=27).
464 Genomes were chosen to maximise temporal and geographic spread of wild type and AMR
465 strains, and to represent the diversity of PBP substitutions detected. AMR determinants and
466 PBP substitutions are as indicated in the key. Co-occurrence of fluoroquinolone resistance
467 and PBP substitutions is highlighted by red branches. PBP substitutions are highlighted by
468 coloured squares.

469 **Figure 5. Phylogenetic analysis of lineages ST17(018) and ST3(001)**

470 (A) Dated phylogeny to show the emergence of MDR lineage ST17(018) (n=66) (red
471 branches) from wild type (n=53). MDR strains from Europe, South East Asia and North
472 America were chosen to maximise geographic spread and PBP substitutions. These and other
473 AMR determinants and PBP substitutions are as indicated in the key. Co-occurrence of
474 fluoroquinolone resistance and PBP substitutions is highlighted by red branches.
475 (B) Dated phylogeny showing the evolutionary relationship of wild type (n=40) and PBP
476 substituted ST3(001) genomes (n=77).

477 **Figure 6. Phylogenetic analysis of lineages ST37(017)/ST81(369) and ST8(002)/ST183**

478 (A) Dated phylogeny showing the evolutionary relationship of wild type (n=43) and PBP
479 substituted ST8(002)/ST183 strains (n=47). Wild type genomes were chosen to maximise

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480 genetic diversity (inferred using a previously constructed phylogeny (Dingle et al., 2017)).
481 The MDR ST183 clade was identified here as emergent from MDR ST8(002). Co-occurrence
482 of fluoroquinolone resistance and PBP substitutions is indicated by red branches in ST8(002)
483 and light blue branches in ST183.
484 (B) Dated phylogeny showing the evolutionary relationship of wild type (n=25) and PBP
485 substituted ST37(017) (n=59) and ST81(369) (n=16) genomes. The MDR ST37(017)
486 genomes were chosen to include representatives of strains associated with well documented
487 outbreaks and other potential clusters identified by location and PBP substitution patterns.
488 The MDR ST81(369) clade was identified here as emergent from MDR ST37(017). The
489 majority of available wild type ST37(017) genomes represented healthcare-associated and
490 asymptomatically carried (infant) strains from a single location (Oxfordshire, UK (30; 104)).
491 Co-occurrence of fluoroquinolone resistance and PBP substitutions is indicated by red
492 branches in ST37(017) and light blue branches in ST81.

493 **Figure 7. Phylogenetic analysis of lineages ST42(106) and ST54(012)**
494 (A) Dated phylogeny for the ST42(106) lineage (n=111 genomes) showing the evolutionary
495 relationship of wild type and fluoroquinolone and/or PBP substituted strains, chosen to
496 represent overall diversity in terms of locations and PBP substitution patterns.
497 (B) As above, but for the ST54(012) lineage (n=107 genomes).
498 In both (A) and (B) the co-occurrence of fluoroquinolone resistance and PBP substitutions
499 indicated by red branches, and PBP substitutions alone by blue branches.

500 **Figure 8. Phylogenetic analysis of ST2(014/020) genomes**
501 Dated phylogeny showing the evolutionary relationship between wild type (n=63) and PBP
502 substituted ST2(014/020) strains (n=60) lacking fluoroquinolone resistance. Branch colour
503 indicates one of six locations. Clustering of PBP substituted genomes is compared with wild
504 type for six independent geographic locations. Wild type genomes from each location were

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505 collected concurrently with the PBP substituted strains. Occurrence of AMR determinants is
506 indicated as shown in the key.
507

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850

TABLE 1. PBPs and β-Lactamases of *C. difficile* Reference Genomes

R20291 loci NC_013316.1	**CD630** loci (former) NC_009089.1	**Alternate designation**	**PBP Classification**	**Size (aa)**	**Blastp predicted function/family**	**Blastp e value**[g]
0712[a]	RS04495 (07810)[d]	**PBP1**[f]	HMW class A	897	Bifunctional transglycosylase/transpeptidase pbp_1A_fam	2.14e-166
0985[a]	RS06420 (11480)[d]	**PBP3**[f]	HMW class B	992	Transpeptidase Pbp2_mrdA for cell elongation	1.53e-127
1067[b]	RS06830 (12290)	**PBP2**[f]	LMW class B	554	FtsI/Pbp2	2.85e-101
					Transpeptidase Pbp2_mrdA for cell elongation	1.81e-93
2544[b]	RS14215 (26560)[d]	**PBP4**[f] *spoVD*	LMW class B	659	spoVD_pbp transpeptidase	0e+00
					FtsI/Pbp2	3.23e-165
-	-	**PBP5**[e,f] strain M68	LMW class B	696	FtsI/Pbp2	1.00e-110
					Pbp2_mrdA transpeptidase	2.67e-104
1131[b]	RS07160 (12910)[d]	*dacF*	LMW class C	387	D-alanyl-D-alanine carboxypeptidase	1.68e-116
2048[b]	RS11615 (21410)		LMW class C	397	D-alanyl-D-alanine carboxypeptidase	2.31e-88
0441[c]	RS03150 (05150)		LMW class C	414	D-alanyl-D-alanine carboxypeptidase	8.55e-94
2390[c]	RS13415 (24980)	*dacF1*	LMW class C	429	D-alanyl-D-alanine carboxypeptidase	7.59e-97
3056[b]	RS17015 (31960)		PBP or β-lactamase?	340	Blastp: CubicO group peptidase, β-lactamase class C (R20291 'put. PBP'; CD630 'serine hydrolase')	3.06e-40
1318[c]	RS08060 (14690)	*cwp20*	PBP or β-lactamase?	1013	Blastp: β-lactamase, cell wall binding protein repeats (R20291 'put. PBP', 'cell surface protein')	2.40e-55
2283	RS12870 (23930)			338	Transglycosylase domain containing protein	1.37e-74
0399	RS02840 (04580)	blaCDD, CDD1/2	β-lactamase	312	YbaI Class D Beta-lactamase (37)	5.94e-51

Grey shading: proteins containing transpeptidase domains
[a]Essential for growth *in vitro* only PBP1 and PBP3 (46).
[b]Not essential for growth *in vitro*, but required for sporulation (46).
[c]Not essential for growth in vitro (46).
[d]Existence shown experimentally by mass spec (100, 101).
[e]PBP5 absent in R20291 and CD630; present in M68 (NC_017175.1) chromosomal locus RS02615, co-ordinates 501965-504052.
[f]PBP1 - 5 (45).

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860 [g]Blastp e-values for R20291 sequences; closer to zero, the more significant the match.

861

862 **MDR Reference strains contain the following PBP Substitutions relative to wild type of the identical genotype:**

863 R20291, ST1(027) UK 2006 (102) contains PBP3 V497L

864 CD630, ST54(012), Switzerland 1982 (103) contains PBP1 T674I and PBP3 N537K.

865 M68, ST37(017), Ireland 2006 (102) contains PBP3 Y721C.

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Table 2 PBP3 and PBP1 substitutions identified within each of the sixteen lineages studied.

Upper panel PBP3, lower panel PBP1, with lineages in the left hand column. 'Amino acid position' across the top of each panel indicates position within PBP3 or PBP1, together with the wild type amino acid. Amino acid substitutions are indicated within each panel; their frequency within-lineage shown in subscript. The overall frequency of each substitution within the entire data set is indicated in the bottom row. Coloured boxes indicate the substitutions occurring over 50 times within the dataset.

PBP3 Substitutions

Amino acid position:	45	92	125	260	300	303	316	339	378	403	416	424	450	460	484	496	497	498	501	518	530	537
Wild type aa:	N	H	K	S	P	S	P	V	T	S	P	A	P	E	T	M	V	T	A	V	V	N
ST1(027/198/176/181)																	L_{1740}					K_{8}
ST17(018)smz																	L_{188}					K_{10}
ST42(106)		N_{1}		Y_{1}													L_{360}					K_{3}
ST3(001)													Q_{1}	K_{1}			L_{234}					K_{1}
ST37(017)/ST81(369)trf	D_{2}						L_{5}		I_{1}		T_{2}					V_{4}	L_{74}				I_{1}	K_{2}
ST63(053)																	L_{23}					
ST54(012)															I_{2}							K_{83}
ST8(002)/ST183																V_{1}	L_{42}	I_{3}		G_{1}		K_{2}
ST11(078)			R_{2}																			
ST2(014/020)				Y_{17}	S_{1}			G_{1}		F_{2}							L_{65}		V_{7}	G_{17}	I_{2}	K_{1}
ST6(005)						A_{1}		G_{1}														
ST10(015)																						
ST7(026)																						
ST56(058)																						
ST26(039) non-toxigenic																	L_{166}					
ST15(010) non-toxigenic												V_{2}					L_{5}					K_{10}
Total occurrences (n)	2	1	2	18	1	1	5	2	1	2	2	2	1	1	2	5	2897	3	7	18	3	120

Amino acid position:	608	660	684	694	699	701	711	721	723	726	746	777	778	790	794	795	821	920	924	926	930	931	937	945	957	964
Wild type aa:	T	P	D	A	A	T	N	Y	A	A	S	V	A	F	V	A	S	T	A	A	D	P	C	S	G	P
ST1(027/198/176/181)										V_{98}			V_{35}													S_{1}
ST17(018)smz				V_{53}									V_{186}												E_{57}	
ST42(106)				V_{1}				S_{1}		V_{1}			V_{16}			V_{1}		S_{1}					F_{1}	I_{6}		
ST3(001)	A_{2}			T_{2}									V_{133}							V_{2}		L_{13}				
ST37(017)/ST81(369)trf				V_{2}				$S_{87}C_{30}H_{12}$	V_{5}	V_{37}	L_{13}	A_{1}	V_{61}	S_{13}	A_{3}	$V_{4}E_{28}$			T_{1}	V_{1}		L_{6}			R_{1}	
ST63(053)										V_{1}			V_{2}			E_{2}										
ST54(012)								$S_{1}C_{2}$		V_{1}						V_{1}										
ST8(002)/ST183						A_{1}	H_{3}			V_{1}			V_{40}				L_{13}									
ST11(078)		H_{1}								V_{1}						E_{3}								I_{1}		
ST2(014/020)			E_{3}	V_{22}	G_{19}	A_{4}		S_{22}	V_{1}	V_{1}			V_{23}			$V_{82}E_{1}L_{17}$										
ST6(005)																					E_{1}		F_{1}	I_{1}		
ST10(015)																										
ST7(026)																										
ST56(058)																										
ST26(039) non-toxigenic					G_{2}								V_{166}													
ST15(010) non-toxigenic								$S_{9}C_{2}$					V_{2}			V_{48}										
Total occurrences (n)	2	1	3	80	21	5	3	166	6	141	13	1	664	13	3	187	13	1	1	3	1	19	1	8	58	1

PBP1 Substitutions

Amino acid position:	48	216	237	439	467	491	493	507	519	522	532	533	539	540	543	550	551	555	556	557	577	595	596	600
Wild type aa:	D	F	E	V	G	P	A	A	F	N	G	G	L	P	L	I	A	A	E	M	G	K	F	T
ST1(027/198/176/181)					V_{1}	L_{7}								L_{1}	F_{18}			T_{335}			C_{10}	N_{21}	L_{2}	
ST17(018)smz							T_{1}					S_{61}				L_{121}		T_{5}				N_{187}		
ST42(106)		V_{1}										S_{2}	S_{2}		F_{1}									
ST3(001)								T_{130}							H_{1}		T_{1}	T_{15}	G_{3}			I_{2}		I_{93}
ST37(017)/ST81(369)trf									V_{3}	S_{10}		S_{1}						T_{40}						
ST63(053)																		T_{6}						
ST54(012)															F_{15}									
ST8(002)/ST183	N_{1}										V_{14}		S_{2}					T_{39}						
ST11(078)				A_{6}																				
ST2(014/020)															$F_{1}H_{17}$			T_{2}		I_{21}				
ST6(005)			D_{2}																					
ST10(015)																								
ST7(026)																								
ST56(058)																								
ST26(039) non-toxigenic																								
ST15(010) non-toxigenic																								
Total occurrences (n)	1	1	2	6	1	7	1	130	3	10	14	64	4	1	53	121	1	442	3	21	10	210	2	93

Amino acid position:	623	625	655	663	668	673	674	678	679	688	695	696	706	718	737	738	740	786	799	808	823	877	884	889	891	892
Wild type aa:	Q	V	D	T	S	G	T	A	T	G	S	A	P	A	S	T	T	A	G	P	T	G	G	P	G	S
ST1(027/198/176/181)	H	I_{21}	E_{21}		N_{1}		I_{796}		A_{21}	$S_{1}C_{2}$		V_{1}				I_{8}	N_{21}			T_{1}						
ST17(018)smz							I_{1}										N_{188}	V_{1}								
ST42(106)				A_{1}		D_{1}	$I_{5}N_{25}$												S_{2}							G_{2}
ST3(001)						D_{1}	N_{8}				I_{2}															
ST37(017)/ST81(369)trf							I_{1}	T_{1}		C_{39}					G_{14}	N_{3}		T_{1}							N_{4}	
ST63(053)																										
ST54(012)							$I_{82}V_{1}$																			
ST8(002)/ST183							$I_{1}N_{2}$																			
ST11(078)													T_{1}	S_{2}												
ST2(014/020)							I_{111}								G_{1}											
ST6(005)			E_{1}						A_{1}												I_{7}	N_{3}	V_{8}	T_{1}		
ST10(015)																										
ST7(026)																										
ST56(058)																										
ST26(039) non-toxigenic							I_{175}																			
ST15(010) non-toxigenic							I_{171}																			
Total occurrences (n)	21	21	22	1	1	2	1379	1	22	42	2	1	1	2	15	11	209	1	2	1	7	3	8	1	4	2

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875 **SUPPLEMENTAL MATERIAL**

876 **Figure S1.** ST81(369) PBP3 was acquired via a long recombination event.

877 Distance plots showing pairwise comparisons of the donor ST8(002), recipient ST37(017)
878 and recombinant ST81(369), with the aim of identifying the size and location of the
879 recombination event. The genomes used were ST8(002) isolate W0003a NZ_CP025047.1
880 (Yin et al., 2018), ST37(017) isolate M68 NC_017175.1 (He et al., 2010), and ST81(369)
881 isolate 28 WGS:QNWI01, (Bioproject PRJNA479396, Assembly GCA_003326885.1) (Wu
882 et al., 2019).

883 The plots extend from 0.9 to 1.4 Mbp relative to M68 (x axis) so the locations of both PBP1
884 and PBP3 are covered; PBP1 at 907,056-904,363 (gene CDM68_RS04280) and PBP3 at
885 1,219,021-1,221,999, (gene CDM68_RS05670), indicated by vertical red dashed lines.
886 Missing lines indicate alignment gaps.

887 The large recombination event involves ~150kbp (~3.5% of the 4,308,325bp genome). A
888 much smaller region occurred near the middle of the large event (position ~1.22Mbp) where
889 ST81(369) no longer resembles ST8(002), but resembles ST37(017) instead. It appears likely
890 that this short region, adjacent to PBP3, has recombined back with ST37(017).

Figure 1

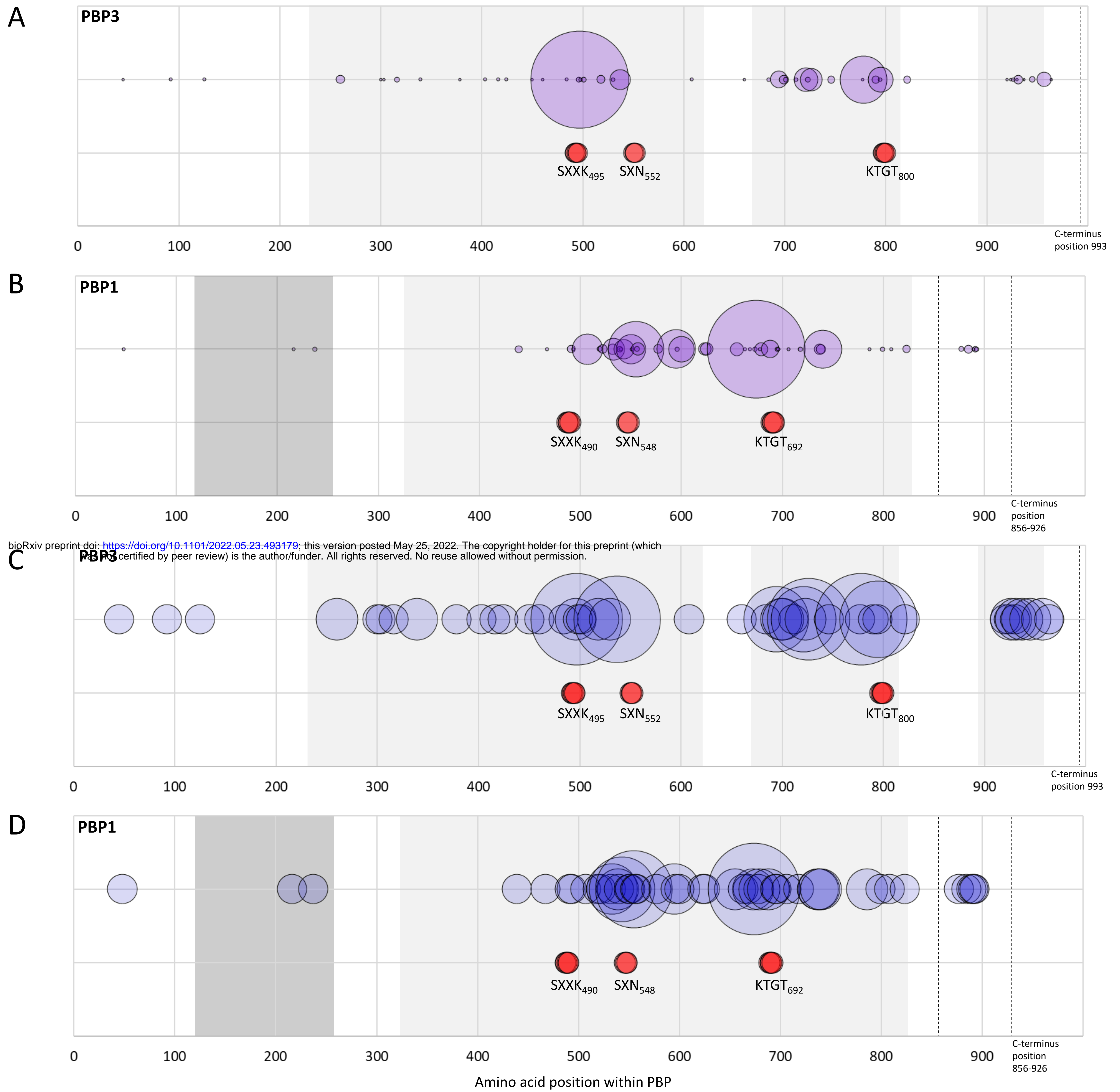

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Figure 2

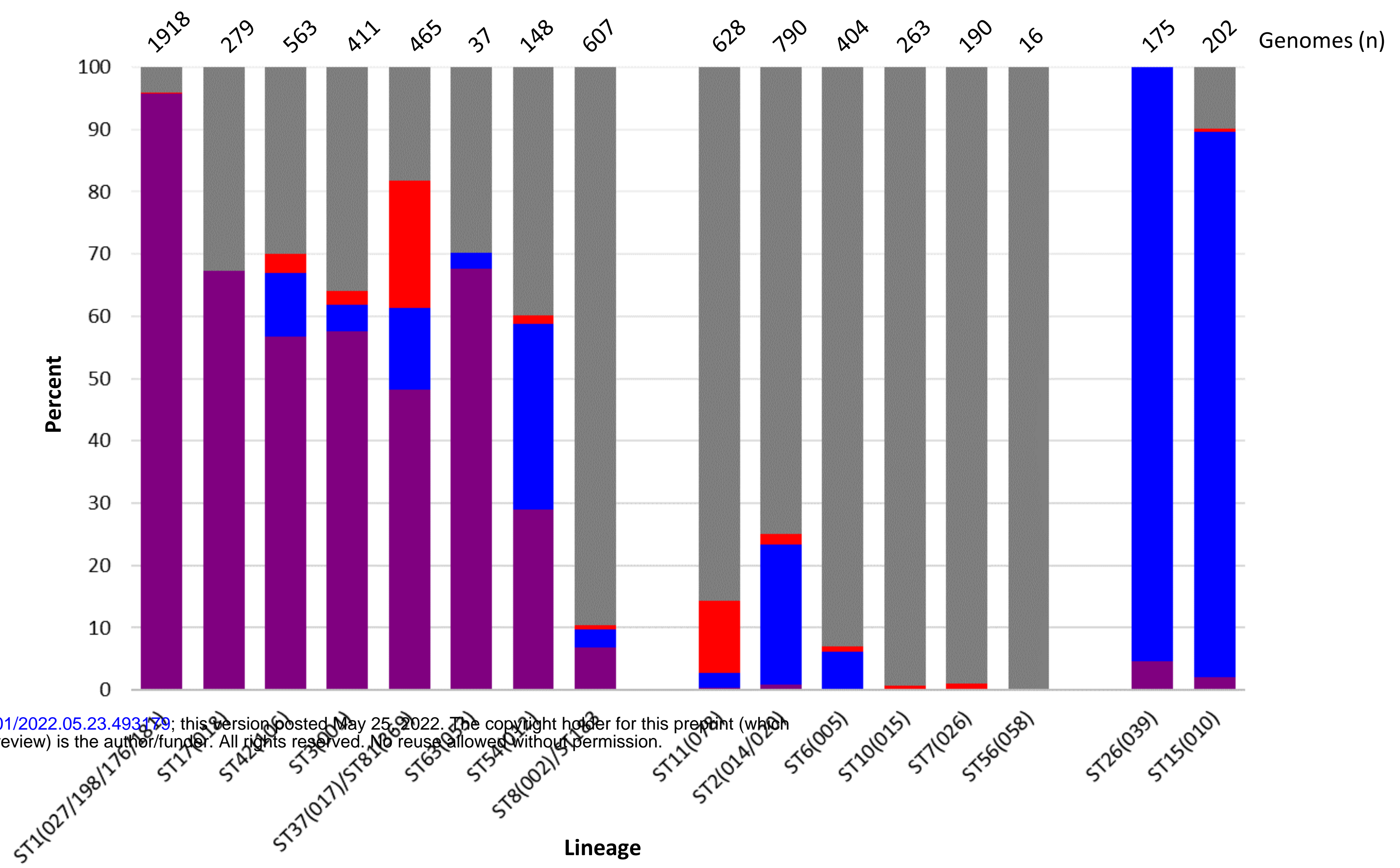

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Figure 3

A

Lineage	Isolate	Location	SNPs	PBP Substitutions: PBP3	PBP Substitutions: PBP1	Cephradine (first)	Cefuroxime (second)	Cefotaxime (third*)	Ampicillin	Co-amoxiclav	Meropenem	Imipenem	Piperacillin-tazobactam
ST1(027)	TN145	Oxfordshire, UK		WT	WT	72	376	256	2	2	4	8	16
ST1(027)-FQ-R2	1421	Oxfordshire, UK	30	V497L	WT	239	627	284	2	2	8	8	16
ST1(027)-FQ-R2	1582	Oxfordshire, UK	28	V497L	T674I	239	537	284	2	2	16	8	32
ST1(027)-FQ-R2	1503a	Oxfordshire, UK	29	V497L	T674I, A555T	239	627	284	2	2	16	8	32
ST1(027)	OPT_1687	Calgary, Canada		WT	WT	72	376	256	2	2	4	8	16
ST1(027) FQ-R1	OPT_1905	Columbus, USA	20	A726V	WT	143	752	365	2	2	8	8	32
ST1(027) FQ-R1	OPT_2787	Detroit, USA	27	A726V	A555T	143	752	512	2	2	8	8	16
ST17(018)	Oxf79	Oxfordshire, UK		WT	WT	36	376	256	1	1	4	8	8
ST17(018)	OPT_2644	Italy	118	V497L, A778V	I550L, K595N, T740N	573	1506	>512	2	2	8	16	32
ST3(001)	2915	Oxfordshire, UK		WT	WT	36	376	256	1	1	4	8	8
ST3(001)	Oxf746b	Oxfordshire, UK	162	A778V	WT	72	470	284	1	1	4	4	8
ST3(001)	1172-p1	Oxfordshire, UK	273	V497L	WT	143	752	284	1	1	4	8	8
ST3(001)	OPT_2456	Germany	134	V497L, A778V	T600I, A507T	239	1506	512	1	1	4	16	8
ST42(106)	Oxf1499	Oxfordshire, UK		WT	WT	72	376	256	2	1	4	8	16
ST42(106)	L,15.7921787	Leeds, UK	20	V497L	T674I	143	376	256	2	2	8	8	16
	Isolates (n)			**PBP3**	**PBP1**								
ST10(015)	11	UK	N/A	WT	WT	NT	128	128	0.5^{1}, 1^{10}	1	8	4	8
ST6(005)	10	UK	N/A	WT	WT	NT	128	128	1	1	8	4	8
ST56(058)	4	UK	N/A	WT	WT	NT	128	128	1	1	8	4	8
ST7(026)	5	UK	N/A	WT	WT	NT	128	64^{3}, 128^{2}	0.5^{1}, 1^{10}	1	8	2^{2}, 4^{3}	8

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*An additional third generation cephalosporin was tested, ceftazidime, all isolates MIC 128μg/ml.

B

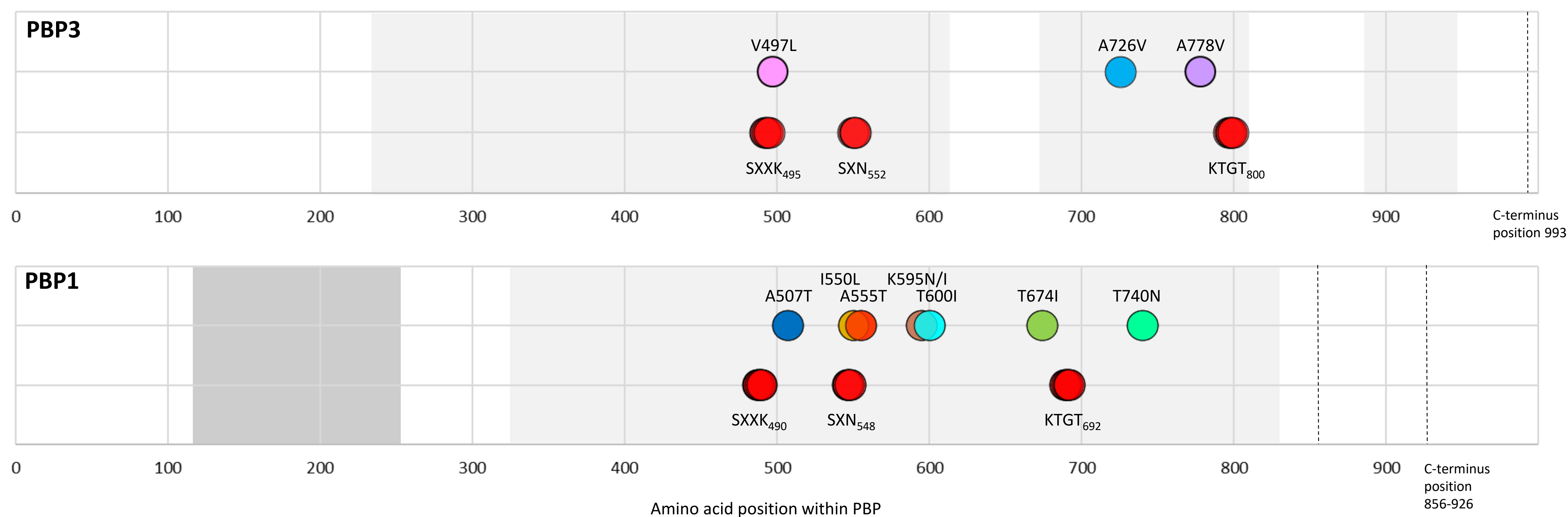

Figure 4

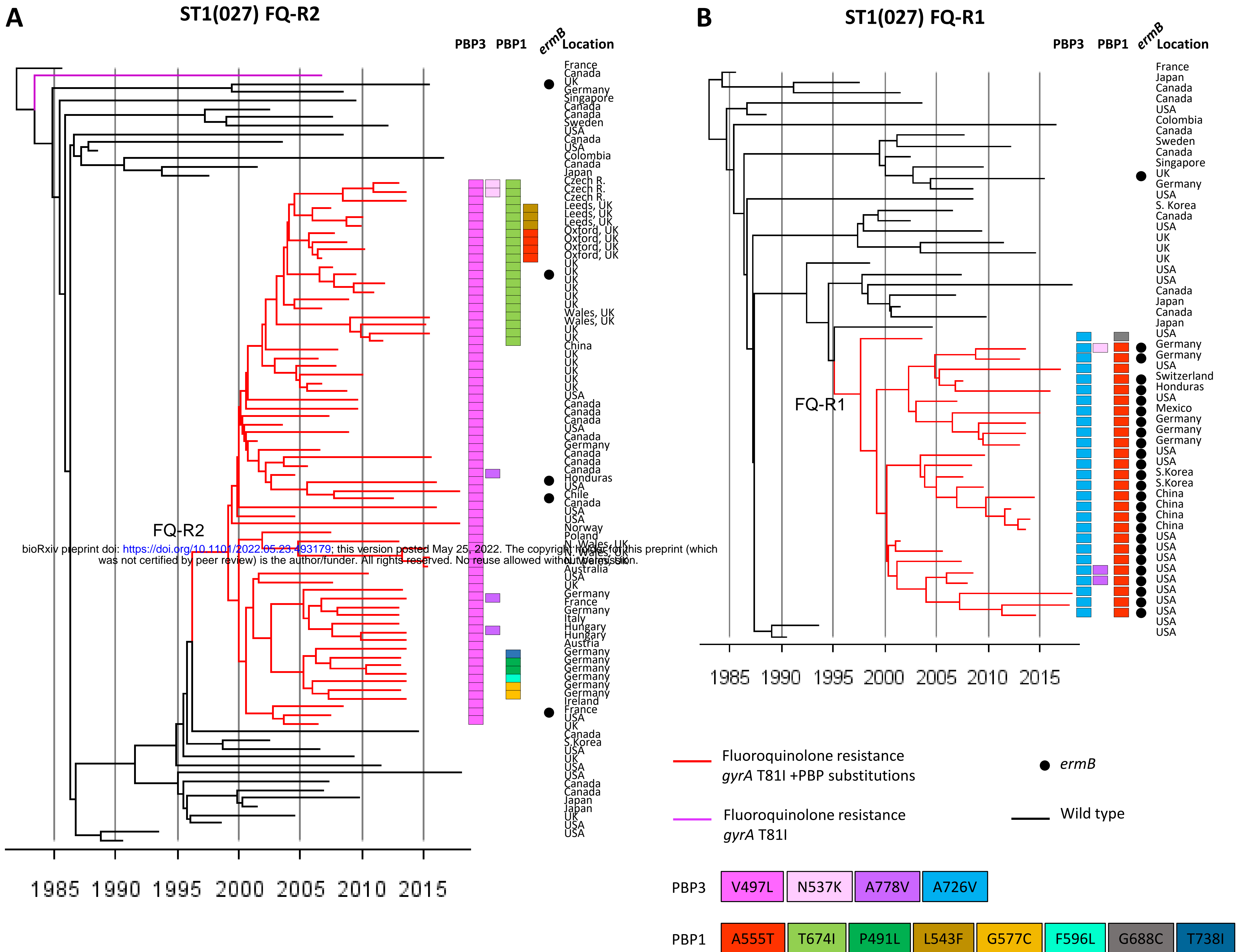

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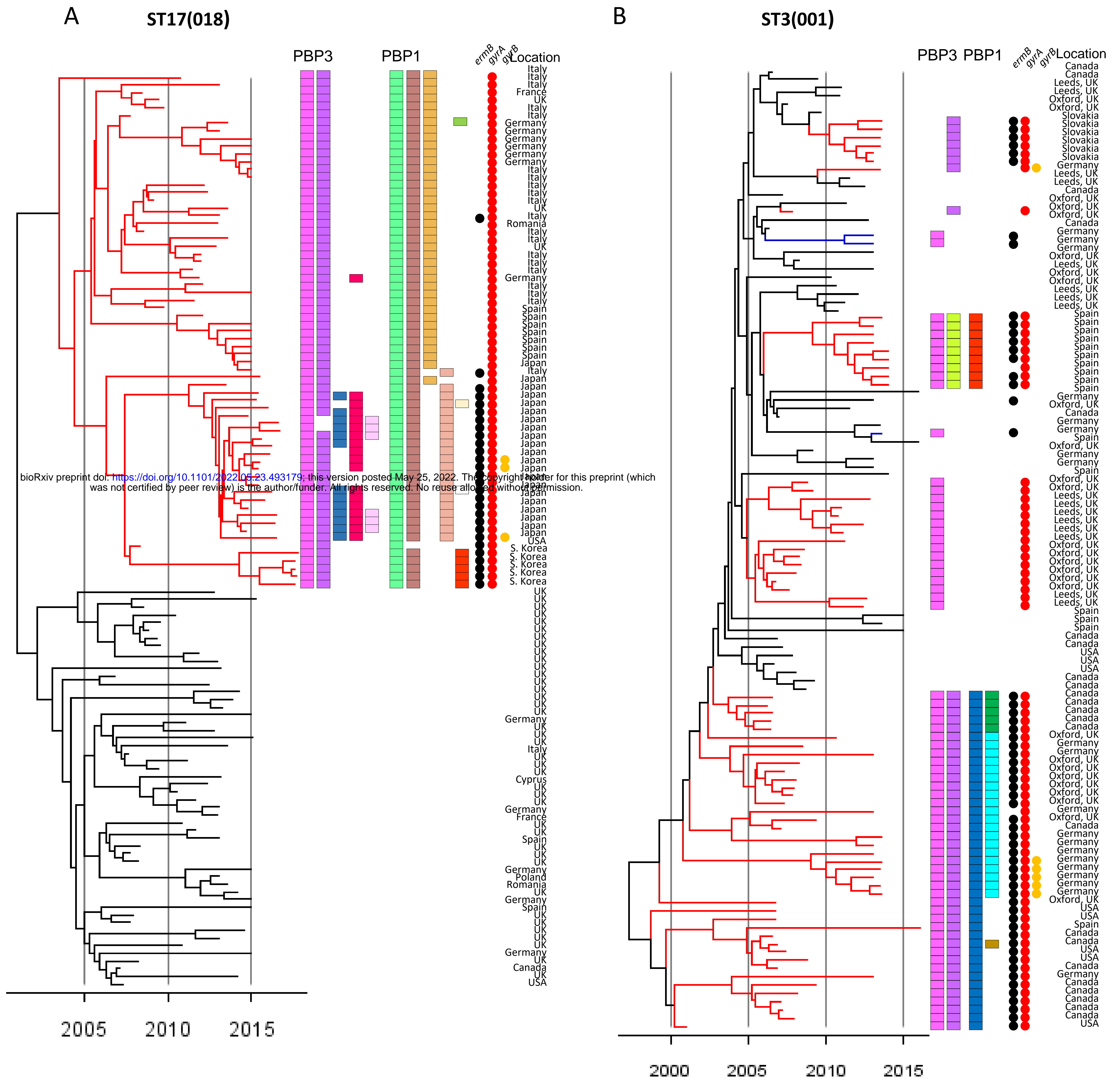
Figure 5



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Fluoroquinolone resistance *gyrA* T81I/V and/or *gyrB* D426N +PBP substitutions

Fluoroquinolone susceptible +PBP substitutions

Wild type

ermB

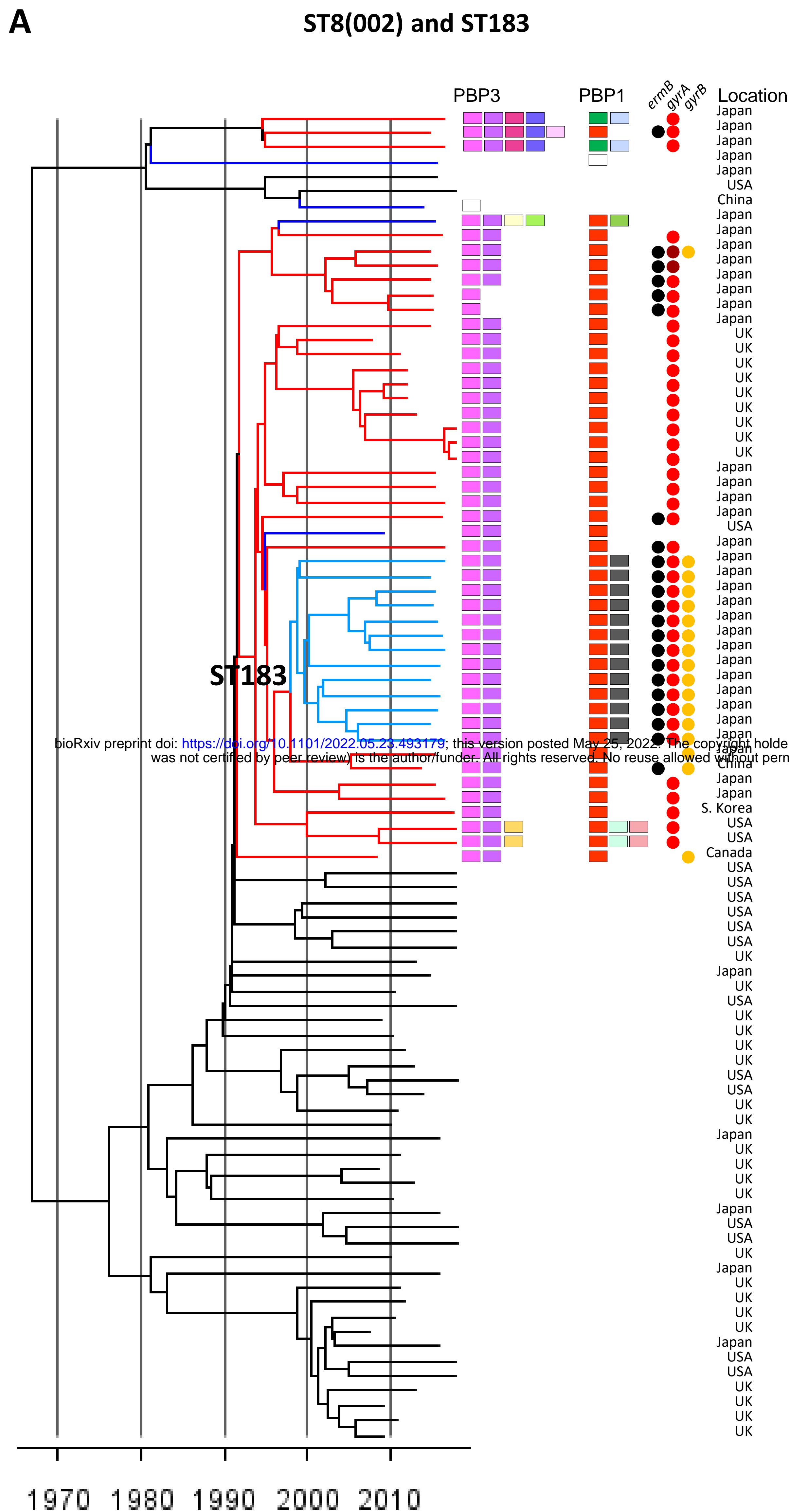
gyrA T81I

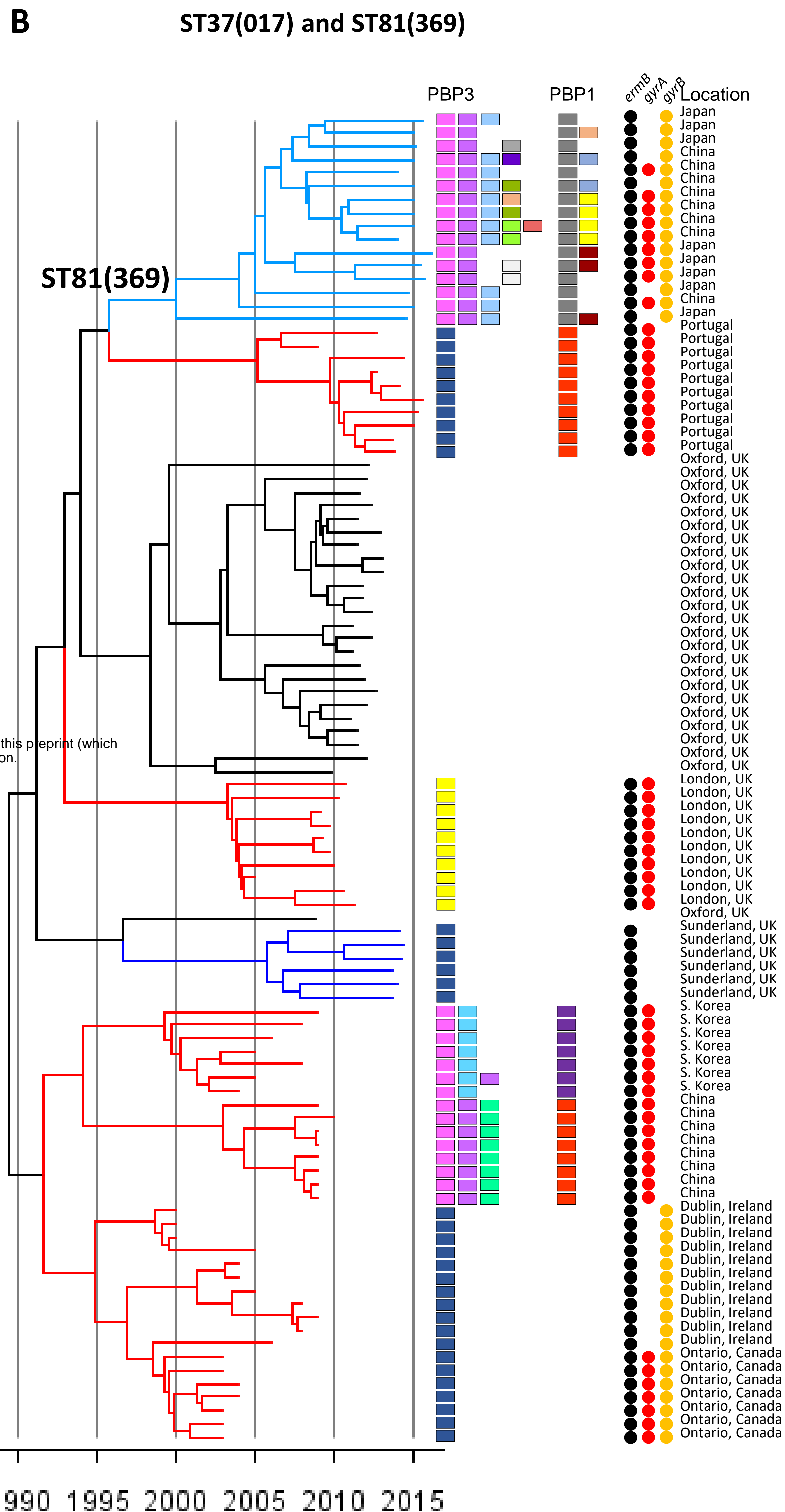
gyrB D426N

PBP3: V497L, N537K, A778V, A694V, G957E, P931L/I

PBP1: A555T, T674I, L543F, K595N, A507T, T600I, T740N, A786V, G533S, A493T, T674N

Figure 6





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Wild type

Fluoroquinolone susceptible +PBP substitutions

ST8(002) or ST37(017) Fluoroquinolone resistant *gyrA* T81I/V and/or *gyrB* D426N plus PBP substitutions

ST183 or ST81; fluoroquinolone resistance plus PBP substitutions

gyrA T81I

gyrA T81V

gyrB D426N

ermB

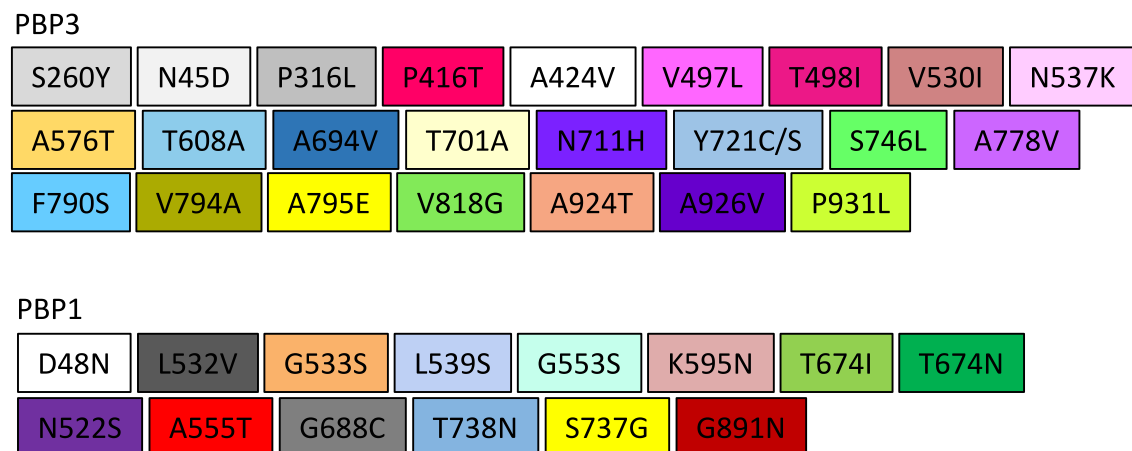

Figure 7

A **ST42(106)**

B **ST54(012)**

CD630 Reference, 1982

reference

1960 1980 2000

1990 2000 2010

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Fluoroquinolone resistance *gyrA* T81I/V +PBP substitutions

Fluoroquinolone susceptible +PBP substitutions

Wild type

● *ermB*

● *gyrA* T81I

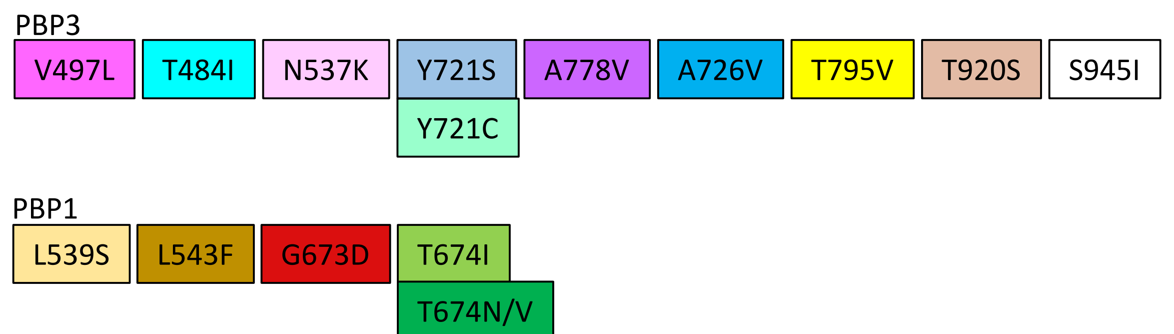
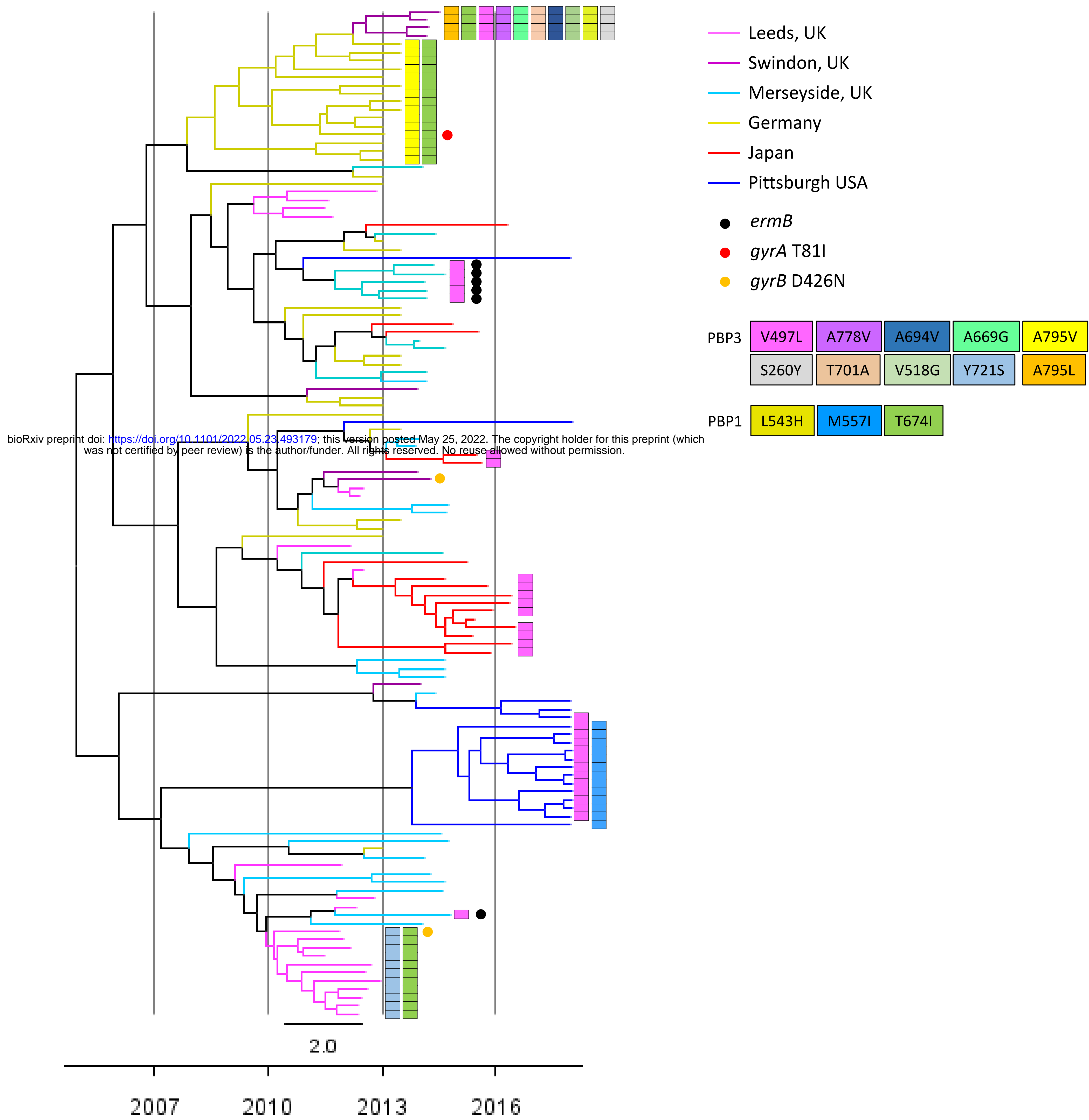


Figure 8



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