The role of the L421P mutation in Penicillin-Binding Protein 1 (PBP1) in the evolution of chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*

Running Title: Evolution of Penicillin Resistance in *N. gonorrhoeae*

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Abstract:

ponA^{L421P} encodes a mutated variant of penicillin-binding protein 1 (PBP1) and is a key resistance determinant that increases the penicillin MIC (MIC_{PEN}) above the clinical breakpoint in *Neisseria gonorrhoeae*. Despite the removal of penicillin from treatment guidelines for gonococcal infections in the 1980s, *ponA*^{L421P} is present in nearly 50% of current *N. gonorrhoeae* isolates in the PubMLST database. Bioinformatic analysis indicates that *ponA*^{L421P} is exclusive to *N. gonorrhoeae* isolates, whereas Leu-421 is 100% conserved in other *Neisseria* species. To understand the involvement of *ponA*^{L421P} in antibiotic resistance, we introduced *ponA* variants encoding 16 different amino acids at position-421 into FA6140, a penicillin-resistant gonococcal isolate that naturally harbors *ponA*^{L421P}. Proline-421 was the only mutation that increased the MIC_{PEN} to the same level as FA6140. We also assessed the fitness of strains with the 16 mutant *ponA* alleles over multiple serial passages, both with and without sub-MIC levels of penicillin. There was no fitness defect attributed to *ponA*^{L421P} under these experimental conditions; instead, our analyses suggest that the widespread occurrence of *ponA*^{L421P} is driven by its capacity to increase the MIC_{pen} above the clinical breakpoint. In FA6140 transformed with the mosaic *penA* allele from strain H041, a ceftriaxone-resistant isolate, *ponA*^{L421P} increased the MIC of ceftriaxone, suggesting that ceftriaxone targets PBP1 in this strain. We conclude that the *ponA*^{L421P} allele emerged in gonococcal isolates, increasing the MIC_{PEN} above the clinical breakpoint, and has remained in the population even after the removal of penicillin from treatment guidelines.

52 **Importance:**

53 The emergence of antibiotic-resistant *Neisseria gonorrhoeae* threatens effective
54 treatment of gonorrhea, one of the most common sexually transmitted infections
55 worldwide. Understanding the genetic changes that drive and maintain resistance is
56 crucial for anticipating future resistance trends. Here, we investigated the impact of a key
57 resistance mutation in PBP1 (encoded by *ponA*^{L421P}). Although penicillin has not been
58 used to treat gonorrhea for decades, this mutation remains widespread even in recent *N.*
59 *gonorrhoeae* isolates. *ponA*^{L421P} confers clinically relevant penicillin resistance without
60 imposing an *in vitro* fitness cost. *ponA*^{L421P} also increases resistance to ceftriaxone in
61 strains with *penA* alleles that are associated with ceftriaxone resistance. This work
62 highlights the role of the *ponA*^{L421P} allele in shaping the current antibiotic resistance
63 landscape and supports the need for ongoing surveillance and evolutionary studies of
64 such mutations in the gonococcal population.

65 **Introduction:**

66 *Neisseria gonorrhoeae* is the etiologic agent of the sexually transmitted infection
67 gonorrhea, which causes ~80 million infections annually (1, 2). The β-lactam antibiotic
68 penicillin was first used to treat gonorrhea in the early 1940s. For the following 40 years,
69 penicillin remained effective, but the minimum inhibitory concentrations of penicillin
70 (MIC_{PEN}) steadily increased, requiring higher doses to cure infections (3). By 1985, over
71 5% of circulating *N. gonorrhoeae* strains had MIC_{PEN} values above the breakpoint (≥2
72 μg/ml), prompting a switch in treatment guidelines to other antibiotics. The rise in penicillin
73 resistance was driven by two mechanisms: plasmid-mediated production of penicillinases
74 (TEM-1 and TEM-135 β-lactamases) and expression of resistance determinants that are
75 variants of chromosomal genes (4–11). In the US, the percentage of penicillinase-
76 producing *N. gonorrhoeae* isolates (PPNG) decreased by the 1990s, while the
77 percentage of chromosomally mediated penicillin-resistant *N. gonorrhoeae* isolates
78 (CMRNG) increased (2, 8).

79 β-lactam antibiotics target penicillin-binding proteins (PBPs), which are involved in
80 the cross-linking of the peptide chains of peptidoglycan. *N. gonorrhoeae* has four PBPs,
81 two of which (PBP1 and PBP2) are essential. PBP1 is a bifunctional PBP that catalyzes
82 both glycan polymerization (transglycosylation) and peptide cross-linking
83 (transpeptidation), and PBP2 is a monofunctional PBP, catalyzing transpeptidation
84 reactions. Because PBP2 has a much higher rate of acylation than PBP1 for penicillin
85 and other β-lactam antibiotics used to treat *N. gonorrhoeae* in susceptible strains, PBP2
86 is considered the lethal target for these antibiotics. CMRNG is facilitated by the step-wise
87 transfer (12–14) of at least four resistance determinants from a penicillin-resistant strain

88 to an antibiotic-susceptible strain: 1) mutant *penA* alleles, which encode variants of PBP2
89 that reduce the acylation rate of penicillin by 16-fold (15); 2) *mtr* mutations, which increase
90 *mtrCDE* operon expression and result in increased penicillin efflux through the MtrCDE
91 efflux pump (9, 16); 3) mutations in *porB*, which encodes the major outer membrane porin,
92 that reduce the influx of penicillin into the periplasm (7, 17); and 4) *ponA*^{L421P}, encoding a
93 PBP1 variant with an L421P missense mutation. Mutations in *penC* were thought to
94 contribute to high-level penicillin resistance (8), but *penC* was later shown to encode the
95 pore-forming secretion protein, PilQ (18). These *pilQ* mutations compromise its secretin
96 function and increase the MIC of multiple antibiotics (18, 19), but to date, *pilQ* mutations
97 have not been observed in clinical isolates, as they disrupt the type IV pili that play a key
98 role in gonococcal pathogenesis (20, 21). While the first three resistance determinants
99 are well characterized in the literature, the contribution of *ponA*^{L421P} to penicillin resistance
100 remains less well understood.

101 Our laboratory has shown that the L421P mutation in PBP1 lowers the acylation
102 rate by 2- to 3-fold for penicillin G and other β-lactam antibiotics (8), but it is not clear
103 whether this lower acylation rate explains the role of *ponA*^{L421P} in facilitating penicillin
104 resistance. Transformation of the *penA, mtr,* and *porB* determinants from strain FA6140,
105 a high-level-penicillin-resistant clinical isolate (MIC_{PEN} = 4 μg/ml), into the antibiotic-
106 susceptible strain FA19 (MIC_{PEN} = 0.01 μg/ml) results in MIC_{PEN} between 0.5 to 1.0 μg/ml
107 (12, 13), but further transformation with FA6140 DNA or *ponA*^{L421P} does not increase the
108 MIC_{PEN} (22). All attempts to reach donor levels of resistance in gonococcal isolates by
109 natural transformation have been unsuccessful (8, 10, 12). By contrast, we showed that
110 reversion of *ponA*^{L421P} in two penicillin-resistant clinical isolates, including FA6140, back

111 to the wild-type *ponA* allele decreases the MIC_{PEN} 2-fold (22). These data imply that
112 *ponA*^{L421P} requires a yet to be determined genetic background that cannot be transferred
113 between strains by transformation to exert its phenotypic effect on resistance.

114 We hypothesized that *ponA*^{L421P} arose during the years of penicillin use and has
115 continued to remain in the population because *ponA*^{L421P} continues to confer a fitness
116 benefit, even with the current β-lactam antibiotics recommended for use. In the present
117 study, we sought to test this prediction by investigating the origins of *ponA*^{L421P} to
118 elucidate the relationship between *ponA*^{L421P} and the evolution of β-lactam resistance in
119 *N. gonorrhoeae*.

120 **Results:**

121 ***ponA*^{L421P} is exclusive to *N. gonorrhoeae* among *Neisseria* species.**

122 *N. gonorrhoeae* isolates can acquire resistance-conferring mutations from commensal
123 *Neisseria* species and from *N. meningitidis* (23–25). We examined whether *ponA*^{L421P}
124 arose via transfer of DNA from other *Neisseria* species, or if this mutation is specific to
125 gonococcal isolates. We accessed the sequences of all unique *ponA* alleles across
126 *Neisseria* species from the PubMLST Database, and divided them into three groups: (1)
127 *N. gonorrhoeae* (n = 20,513 isolates, 172 *ponA* alleles), (2) *N. meningitidis* (n = 75,612
128 isolates, 897 *ponA* alleles), and (3) all other commensal *Neisseria* species (n = 1,604
129 isolates, 369 *ponA* alleles).

130 The PBP1 protein sequences across all three groups of *Neisseria* isolates are highly
131 conserved (Fig. S1), particularly the region surrounding residue 421 (Fig. 1). Leu-421 is
132 100% conserved across all *N. meningitidis* and commensal *Neisseria ponA* alleles (Fig.
133 1B-C), but for *N. gonorrhoeae* isolates, residue 421 is either a leucine or a proline (Fig.
134 1A). Moreover, *ponA*^{L421P} was observed in 49.5% of all gonococcal isolates in the
135 PubMLST database and was not found in other *Neisseria* species (Table S5). These data
136 strongly suggest that *ponA*^{L421P} arose in *N. gonorrhoeae* and not through horizontal gene
137 transfer from other *Neisseria* species.

138 ***ponA*^{L421P} is prevalent amongst gonococcal isolates with chromosomally-mediated**
139 **penicillin resistance.**

140 To investigate the emergence and distribution of *ponA*^{L421P} in the gonococcal
141 population, we assembled a dataset of sequenced *N. gonorrhoeae* isolates and the
142 associated MIC_{PEN} values (n=16,891). The first appearance of *ponA*^{L421P} in a sequenced

143 isolate was in 1961, and it rapidly expanded in the population in the 1980s (Fig. 2).
144 Interestingly, we first observed the insertion of aspartate after position 345 in *penA*
145 (Asp345a), which is a hallmark of early penicillin-resistant isolates (26, 27), in our dataset
146 of sequenced isolates in 1956, only a few years prior to the L421P mutation in *ponA* (Fig.
147 2). We examined the phylogenetic distribution of *ponA* alleles in a representative sample
148 of *N. gonorrhoeae* isolates without plasmid-mediated resistance (n=5,192) and found
149 $ponA^{L421P}$ emerged across multiple lineages in the gonococcal population (Fig. 3).

150 We next examined the relationship between $ponA^{L421P}$ and penicillin-resistant
151 CMRNG strains (Fig. 4, n=8,999). Less than 1% of isolates harboring the wild-type *ponA*
152 allele included in Fig. 4 have resistant MIC_{PEN} values (≥2 μg/mL), while the majority (90%)
153 fall within the intermediate MIC_{PEN} range (0.12–1 μg/mL). In contrast, 25% of isolates
154 harboring $ponA^{L421P}$ have resistant MIC_{PEN} (≥2 μg/mL), and 75% of isolates have
155 intermediate MIC_{PEN}. Few isolates, regardless of the *ponA* or $ponA^{L421P}$ allele, had
156 susceptible MIC_{PEN}. Additionally, 91.61% of isolates that harbor mosaic *penA* alleles, which
157 encode highly remodeled PBP2 variants that confer increased resistance to cephalosporins,
158 also harbor $ponA^{L421P}$ (Fig. 4B).

159 **Identification of other polymorphisms in gonococcal *ponA.***

160 Amongst *N. gonorrhoeae* isolates in PubMLST, there were only two other
161 mutations observed in gonococcal PBP1 at frequencies above 1%: A375T (7.07%) and
162 Y537C (2.35%) (Fig. 5). In keeping with these frequencies, a phylogeny of 6,082
163 representative gonococcal genomes reveals these mutations are present in fewer
164 lineages, compared to the phylogenetic distribution of the L421P mutation (Fig. S2). We
165 next examined the relationship between each mutation and penicillin-resistant CMRNG

166 strains (n=8,999). The majority (95%) of isolates harboring the A375T mutation in *ponA*
167 have intermediate (0.12–1 μg/mL) to resistant (≥ 2 μg/mL) $MIC_{PEN.}$ This proportion does
168 not change in isolates with alanine at residue 375 of *ponA*, as 95% of these isolates also
169 have intermediate to resistant MIC_{PEN}. The same trend is observed with the Y537C
170 mutation. We do not observe A375T and Y537C in sequenced isolates collected during
171 the penicillin treatment era, as they first appear in 1998 and 2001, respectively, well after
172 penicillin was removed as a treatment for gonorrhea infections in the US in 1985. It
173 remains unclear whether A375T and Y537C are directly involved in facilitating resistance
174 to penicillin, but, given the fact that isolates with those mutations are not enriched for
175 penicillin resistance and they are present in fewer gonococcal lineages than L421P, we
176 did not interrogate them further.

177 **Only proline and serine at PBP1 position 421 increase the MIC_{PEN}.**

178 To understand why only a proline mutation at residue 421 is observed in PBP1,
179 we created an isogenic library of 16 mutant strains in FA6140, each with a different amino
180 acid at position 421 (Table 1). FA6140 (MIC_{PEN}= 4 μg/mL) naturally harbors *ponA*^{L421P},
181 and reversion to *ponA*^{421L} reduces the MIC_{PEN} to 2 μg/mL (8, 22) (Table 1). In the strain
182 library, FA6140 *ponA*^{L421P} was the only strain with an MIC_{PEN} of 4 μg/mL. FA6140
183 *ponA*^{L421S}, which had a median MIC_{PEN} of 2.5 μg/mL, was the only other mutation that
184 increased the MIC_{PEN} > 2 μg/mL, with all the other strains having the same MIC_{PEN} as
185 FA6140 *ponA* (Table 1).

186 ***In vitro* serial passaging of the FA6140 *ponA*^{L421X} mutant library in the absence or**
187 **presence of sub-MIC concentrations of penicillin.**

188 The 2-fold higher MIC_{PEN} conferred by *ponA*^{L421P} and the timing of its first appearance
189 in sequenced isolates suggest that *ponA*^{L421P} emerged in response to selection by
190 penicillin. To test this, we performed *in vitro* serial passaging on our set of FA6140
191 *ponA*^{L421X} mutants in increasing sub-MIC_{PEN} concentrations (0.25, 0.5, and 1.0 µg/ml).
192 Illumina sequencing revealed that equal amounts of each FA6140 *ponA*^{L421X} mutant were
193 used to inoculate the cultures at hour 0 across all 3 trials (Fig. 6A, Fig. S3). In the absence
194 of penicillin, the amino acids proline, cystine, asparagine, and glycine at position 421
195 appear to be more fit in this growth environment, as they have the largest fold increase in
196 read counts at hour 36 relative to the inoculum (Fig. 6C). The *ponA*^{L421P} reads consistently
197 doubled by hour 36 in all three trials, while the *ponA*^{421L} reads remained similar to, or
198 decreased from, the number of reads in the inoculum. The proline and serine mutants
199 were the only strains with an MIC_{PEN} above 2 µg/mL (Table 1), but the read counts for the
200 serine mutant at hour 36 did not surpass its read counts at hour 0 (Fig. 6C). None of the
201 strains differed significantly in growth rate from the parental strain (Table S4).

202 We grew the collection of strains in growth medium with increasing concentrations of
203 penicillin to examine which strains would grow in the presence of selective pressure.
204 Optical Density at 600 nm (OD_{600}) values during growth in medium containing 0.25 mg/ml
205 penicillin were very similar to no antibiotic (Fig. S4), while there was more variability in
206 the OD_{600} values across trials for the cultures supplemented with 0.5 µg/mL of penicillin
207 (Fig. 6B). Of the 16 mutants, the two with the highest MIC_{PEN}, FA6140 *ponA*^{L421P} and
208 FA6140 *ponA*^{L421S}, increased in read counts when grown in 0.5 µg/mL penicillin, while all
209 the others decreased in read counts. FA6140 *ponA*^{L421P} consistently increased in read
210 counts across all three trials, while read counts for FA6140 *ponA*^{L421S} increased in trials

211 1 and 2, but not in trial 3. In trial 1, FA6140 *ponA*^{L421P} had more read counts at 36 h than
212 FA6140 *ponA*^{L421S}, and both strains had similar reads counts at 36 h in trial 2 (Fig. 6C).
213 FA6140 *ponA*^{L421P} is the only mutant that consistently increased in read counts over 36 h
214 in cultures either with or without sub-MIC_{PEN} concentrations.

215 At the highest sub-MIC_{PEN} concentration tested, we observed more variable results.
216 After 6 h of growth and passaging into fresh medium with 1 μg/ml penicillin, the OD_{600}
217 increased very little over the next 12 h (Fig. 7A). However, in two of the three trials (1 &
218 3), there were still live cells present, as later passages showed measurable growth during
219 the 6 h period. These data suggest that gonococcal cells remained viable but were not
220 dividing during the prior 6 h growth periods and eventually acquired either phenotypic or
221 genetic changes that allowed for measurable growth at the later growth periods. In trial 1,
222 we observed increasing growth during consecutive passages, beginning at 18 h. Illumina
223 sequencing of the *ponA* region revealed that serine was the predominant mutation at
224 position 421 (Fig. 7B).

225 **Whole genome sequencing of cells from *in vitro* serial passaging FA6140 *ponA*^{L421X}**
226 **mutant library reveals emergence of a mutation in *pilQ*.**

227 To determine if the FA6140 *ponA*^{L421X} mutants acquired additional mutations during
228 the 36 hours of *in vitro* growth, we sequenced the genomes of cells collected at 0 h and
229 36 h. We looked for mutations that were distinct from the inoculum and present in greater
230 than 20% of the reads, as anything detected below this would likely not be responsible
231 for any changes in the overall codon counts from the Illumina reads (Figs. 6D, 7B). Across
232 all 3 trials, most, but not all, mutations above the 20% threshold were either errors in the

233 reference genome sequence used for alignment (not true genetic changes), also present
234 in the inoculum, or in genes that would most likely be inconsequential to resistance.

235 In trial 1, we detected a mutation in *pilQ* at 36 h at 85% frequency in the culture
236 supplemented with 1.0 µg/mL of penicillin. The mutation was an insertion of two bases
237 (+CG) at position 459, which would result in a frameshift and early termination. Previous
238 work from our lab showed that missense and frameshift mutations in *pilQ* increase MIC_{PEN}
239 by inactivating PilQ and decreasing permeation of drug into the gonococcal cell (8, 18,
240 19, 28), which likely explains the marked increase in codon counts for the serine mutation
241 at 24 h seen in trial 1 (Fig. 7C). Our data suggest that during trial 1, FA6140 *ponA*^{L421S}
242 acquired a mutation in *pilQ* between hours 18 and 24, which allowed it to expand in the
243 culture compared to FA6140 *ponA*^{L421P}.

244 ***ponA*^{L421P} increases the ceftriaxone MICs of gonococcal isolates that also harbor a**
245 **mosaic *penA* allele.**

246 Our data indicate that *ponA*^{L421P} is involved in penicillin resistance, but penicillin
247 was removed as a recommended treatment in 1985, and ceftriaxone has been the only
248 recommended β-lactam antibiotic since 2012 for treating gonococcal infections. Given
249 that *ponA*^{L421P} appears to confer a modest fitness deficit *in vivo* (29), it might be expected
250 that *ponA*^{L421P} would decrease in prevalence without selection by penicillin use. However,
251 *ponA*^{L421P} is observed in a high percentage of isolates with a mosaic *penA* allele (91.61%,
252 Fig. 5B), which is the predominant determinant for decreased susceptibility to ceftriaxone
253 (30, 31). One possibility to explain the prevalence of *ponA*^{L421P} is that *ponA*^{L421P} influences
254 the MIC of ceftriaxone in the presence of a mosaic *penA* allele.

255 To test this hypothesis, we replaced the *penA4* allele in FA6140 and FA6140 *ponA*
256 strains with *penA41*, the mosaic allele from the multi-drug resistant gonococcal isolate,
257 H041 (32). This allele encodes a PBP2 with a markedly reduced acylation rate for
258 penicillin, cefixime, and ceftriaxone (31, 33, 34). Reversion of $ponA^{L421P}$ to wild-type *ponA*
259 reduced the MIC_{PEN} in both strain backgrounds, regardless of the *penA* allele (Fig. 8A),
260 suggesting that PBP1 is the lethal target of penicillin in these strains. While reversion of
261 $ponA^{L421P}$ to *ponA* in strain FA6140 had no effect on the MIC of ceftriaxone (Fig. 8B),
262 reversion of $ponA^{L421P}$ to *ponA* in strain FA6140 *penA41* caused a 3-fold decrease in the
263 MIC of ceftriaxone, suggesting again that PBP1 is the lethal target of ceftriaxone in this
264 strain.

265 **Discussion:**

266 In this study, we sought to understand the origins and role of the PBP1-L421P
267 mutation (encoded by the *ponA*^{L421P} allele) in β-lactam antibiotic resistance in *N.*
268 *gonorrhoeae.* Our data revealed that *ponA*^{L421P} is found exclusively in *N. gonorrhoeae*
269 and its emergence in gonococcal isolates is likely due to it increasing the MIC_{PEN} above
270 the clinical breakpoint of 2 µg/ml in CMRNG. While *ponA*^{L421P} has a modest fitness cost
271 *in vivo* (29), there is no apparent fitness cost during growth *in vitro*. Our data suggest that
272 this cost turns into a fitness benefit during growth in sub-MIC_{PEN} concentrations. Moreover,
273 while penicillin G is no longer used to treat gonococcal infections, *ponA*^{L421P} also
274 increases the MIC of ceftriaxone in strains harboring mosaic *penA41* alleles that confer
275 ceftriaxone resistance. Thus, the increases in MIC_{PEN} (in FA6140) and MIC_{CEF} (in FA6140
276 *penA41*) that result from harboring *ponA*^{L421P} appear to have provided enough of an
277 evolutionary advantage to remain in the population despite its modest fitness cost.

278 Mosaic *penA* alleles are defined by a sub-set of mutations that surround the active
279 site of PBP2 and decrease the conformational dynamics of the PBP, which reduces
280 acylation rates and increases MICs for various β-lactam antibiotics (15, 30, 31). These
281 mutations emerged in *N. gonorrhoeae* through interspecies recombination events with
282 *Neisseria* commensals. In contrast, *ponA*^{L421P} appears to have arisen spontaneously in
283 *N. gonorrhoeae,* and, unlike resistance-conferring mutations in PBP2, L421P is located
284 on a hinge that links the oligosaccharide-binding domain to the start of the transpeptidase
285 domain in PBP1 and is over 20 Å from the active-site serine residue (Fig. 5). How the
286 L421P mutation decreases acylation is unknown, and compared to mosaic PBP2 variants,

287 the effects on k_2/K_S are modest (8). The small decrease in acylation, however, could
288 explain the 2-fold increase in the MIC_{PEN}.

289 While *ponA*^{L421P} was the only mutation in FA6140 to increase the MIC_{PEN} to 4 μg/ml,
290 another mutation, *ponA*^{L421S}, increased the MIC_{PEN} to 2.5 μg/mL. However, to date
291 *ponA*^{L421S} has not been documented in clinical isolates. This could be due to three
292 reasons: 1) the L421P mutation requires only a single base change in the Leu-421 codon
293 in *ponA* (C<u>T</u>G → C<u>C</u>G), whereas the L421S mutation would require at least two base
294 changes (CTG → <u>TCT</u>/<u>TCC</u>/<u>TCA</u>/<u>TC</u>G/<u>AGT</u>/<u>AGC</u>); 2) strain FA6140 *ponA*^{L421S} is less fit
295 *in vitro* compared to other strains; and 3) the increase in resistance is insufficient to
296 overcome its fitness cost.

297 Following the six serial passages of the 16 mutants, we sequenced the genomes of
298 the 36-hour cultures from all three biological replicates to be sure that the results were
299 not skewed by the acquisition of random mutations, particularly under antibiotic selective
300 pressure. No consistent or biologically relevant mutations were detected, with the
301 exception of a single frameshift mutation in *pilQ* observed in the culture supplemented
302 with 1.0 μg/mL of penicillin during trial 1. PilQ increases the MICs of a range of
303 antimicrobials in *N. gonorrhoeae* strains that also have the *mtr* and *porB* resistance
304 determinants (18, 19), which decrease the concentration of antibiotics in the periplasm by
305 increasing efflux and decreasing influx of antibiotics, respectively. This implies that
306 permeation through the PilQ secretin is low and does not impact the MIC unless both *mtr*
307 and *porB* determinants are present, as they are in FA6140 (3, 8, 18, 22, 35). Based on
308 the increase in read counts for *ponA*^{L421S} at 24 hours, we surmise that the PilQ mutation
309 arose in FA6140 *ponA*^{L421S} sometime between 18-24 hours of growth, which increased

310 its MIC_{PEN} above that of FA6140 *ponA*^{L421P} and allowed it to survive in higher penicillin
311 concentrations. However, this is an outcome that would likely occur only during *in vitro*
312 growth, as expression of type IV pili (of which PilQ is an essential component) is important
313 for *N. gonorrhoeae* survival during pathogenesis (36). This is supported by the absence
314 of inactive PilQ mutants identified in clinical isolates (37, 38).

315 We recently reported that *N. gonorrhoeae* lineages harboring *ponA*^{L421P} between
316 1993 and 2013 were associated with a modest fitness disadvantage, compared to the
317 baseline type (lineages that do not carry any resistance determinants), suggesting that
318 *ponA*^{L421P} imposes a fitness cost (29). These data were consistent with results from
319 competitive co-infections between *ponA*- and *ponA*^{L421P}-containing strains of FA19 and
320 FA6140 (29). Despite this observed fitness cost, we showed here that *ponA*^{L421P} remains
321 prevalent in the gonococcal population and is present in over 90% of isolates that also
322 harbor a mosaic *penA* allele (as classified by the NG-STAR database). Why is *ponA*^{L421P}
323 found so frequently in strains with a mosaic *penA* allele? There are several possible
324 explanations for this: 1) mosaic *penA* alleles emerged by transformation almost
325 exclusively into penicillin-resistant strains that already had *ponA*^{L421P}, as these strains
326 also contain other resistance determinants and presumably compensatory mutations that
327 lessen their fitness cost; 2) with penicillin resistance at or near 100% in Asia (39), where
328 ceftriaxone resistance has been emerging, the link between *ponA*^{L421P} and ceftriaxone
329 resistance may reflect the gonococcal diversity/population structure in the places
330 experiencing the most selection for ceftriaxone resistance; and 3) *ponA*^{L421P} confers
331 additional resistance to ceftriaxone and thus provides a benefit to these strains, given the
332 widespread use of ceftriaxone to treat current infections. We tested the latter possibility

333 by determining the MIC_{CEF} values of FA6140 and FA6140 *ponA* also harboring the mosaic
334 *penA41* allele from H041, which confers ceftriaxone resistance to these strains.
335 Importantly, the MIC_{CEF} drops ~3-fold when *ponA*^{L421P} is replaced with *ponA*, indicating
336 that *ponA*^{L421P} confers additional resistance to ceftriaxone and potentially explaining, at
337 least in part, why *ponA*^{L421P} is so prevalent in strains also harboring mosaic *penA* alleles,
338 particularly with mosaic *penA* alleles that confer the largest increases in MIC_{CEF}.

339 The MIC data we present in this study can be best interpreted by knowing which PBP
340 is being targeted by penicillin and ceftriaxone (Fig. 9). The acylation constant k_2/K_S for
341 each antibiotic determines which PBP first becomes acylated as the antibiotic
342 concentration increases (the higher the k_2/K_S, the lower the concentration of antibiotic
343 needed to inhibit the PBP). Thus, k_2/K_S and the MIC have an inverse relationship, and the
344 essential PBP that first becomes acylated as the antibiotic concentration increases is the
345 killing target for that antibiotic and determines the MIC. For penicillin, the killing target for
346 FA6140 is likely a combination of PBP2^{FA6140} and PBP1^{L421P}, but when PBP1^{L421P} is
347 replaced by PBP1, the MIC decreases because PBP1 becomes the exclusive killing
348 target of penicillin. We showed previously that in 35/02, a ceftriaxone-intermediate
349 resistant (and penicillin-resistant) gonococcal isolate, reversion of *ponA*^{L421P} to *ponA* did
350 not alter MIC_{CEF} values (40). In contrast, expression of PBP2^{H041} in FA6140, which has a
351 much lower k_2/K_s for ceftriaxone than PBP2$^{35/02}$, shifts the killing target to PBP1^{L421P}. Thus,
352 exchanging PBP1^{L421P} for PBP1 in the FA6140 genetic background decreases MIC_{CEF} by
353 3-fold, suggesting that when strains acquire a "strong" mosaic PBP2 variant, e.g.
354 PBP2^{H041}, that has a sufficiently low acylation rate constant for ceftriaxone, the target
355 shifts from PBP2 to PBP1 and *ponA*^{L421P} confers a selective advantage over *ponA.*

356 In conclusion, this is the first study to investigate the origins of *ponA*^{L421P} in the context
357 of penicillin resistance and address why it is maintained in the *N. gonorrhoeae* population.
358 During the 40+ years of penicillin use, *N. gonorrhoeae* strains acquired β-lactamases, as
359 well as mutations in PBP1, PBP2, *mtr*, and *porB*, which eventually increased the MIC_{PEN}
360 enough to result in its removal as a treatment option. When expanded-spectrum
361 cephalosporins (ESCs) were introduced to treat gonococcal infections, mosaic PBP2
362 variants emerged (because PBP2 was the exclusive killing target of ESCs), in existing
363 penicillin-resistant strains, resulting in the proliferation of mosaic *penA* alleles that
364 increased resistance to ESCs. Over time, the MICs of ESCs for gonococcal isolates
365 increased as the mosaic *penA* alleles picked up additional mutations, and the killing target
366 shifted to PBP1, allowing the L421P mutation to further increase the MIC. These findings
367 reinforce the need to identify antibiotics that are PBP1-selective and thus can avoid the
368 highly mutated mosaic PBP2 variants that are compromising the use of ESCs. β-lactam
369 antibiotics that target both PBPs or a combination of two β-lactam antibiotics that target
370 PBP2 and PBP1 may provide a new strategy for treating strains with mosaic *penA* alleles.

371 **Methods:**

372 **Strains and plasmids.**

373 Strains, plasmids, and primers used in this study are listed in Tables S1 and S2. To
374 aid in subsequent screening for generating random mutations at codon-421, we
375 transformed FA6140 with the *ponA* allele from FA19, which naturally has a PstI site
376 encompassing codons 421 and 422. A portion of the *ponA* gene (bp 956 – 2400) from
377 FA19 genomic DNA was amplified via PCR and transformed into FA6140 via spot
378 transformation, performed as described (41). Colonies were screened for *ponA* by PCR
379 amplification of *ponA* and digestion with PstI. To generate FA6140 and FA6140 *ponA*
380 harboring the mosaic *penA* allele (*penA41*) from the ceftriaxone-resistant strain H041,
381 piliated cells were transformed with pUC18us-*penA41* plasmid containing *penA41* starting
382 at bp 133 and ending 200 bp downstream *penA41* to facilitate recombination.
383 Transformants that acquired *penA41* were selected on GCB agar plates containing 1.0
384 μg/mL cefixime. All transformants were verified by PCR and Sanger sequencing.

385 Descriptions of the cloning plasmids used to create the library of isogenic FA6140
386 *ponA*^{L421X} mutants can be found in Table S3. pUC18us-*ponA*^{421X}-Ω contains a portion of
387 the *ponA* gene (from bp 831 to 54 bp downstream of the stop codon) harboring
388 randomized codons to introduce amino acid substitutions at codon-421, the *aad1*
389 resistance cassette (Ω) conferring spectinomycin/streptomycin resistance (42), and 531
390 bp of sequence downstream of *ponA* to facilitate recombination. We employed overlap-
391 extension (43) PCR mutagenesis to introduce amino acid substitutions at codon-421 in
392 *ponA* fragments, using the primers listed in Table S2. pUC18us-*ponA*^{421X}-Ω plasmids
393 were created using the NEBuilder HiFi DNA Assembly Master Mix and transformed into

394 *Escherichia coli* XL-10 Gold cells, according to manufacturer's protocol. Clones were
395 screened by PCR amplification of the *ponA* gene followed by Sanger sequencing.
396 Plasmids containing unique mutations were isolated using a QIAPrep miniprep kit
397 (Qiagen). Plasmids (Table S3) were transformed into FA6140 *ponA* by allelic exchange,
398 and transformants were selected on GCB agar plates containing 25 μg/mL spectinomycin.
399 Individual colonies from each transformation were screened by PCR by amplifying the
400 *ponA* gene and digesting with PstI (loss of PstI indicated the presence of the mutation).
401 All clones were verified by Sanger sequencing.

402 PCR reaction conditions were as follows; 94°C for 30s (denaturation), 60°C for 30s
403 (annealing), and 72°C for 60s (elongation) for 35 cycles, and fragments were purified
404 using the QIAquick PCR Purification kit (Qiagen) as per manufacturers guidelines. All
405 gonococcal strains were propagated on solid GCB agar containing Kellogg's supplement
406 I (38) and 12 mM $Fe(NO_3)_3$ for 18 to 20 h at 37°C in a 5% CO_2-enriched humidified
407 atmosphere. Serial passaging was minimized to reduce the risk of acquiring secondary
408 mutations.

409 **Bacterial growth.**

410 FA6140 *ponA*^{L421X} mutants were grown in liquid culture to verify individual growth
411 phenotypes (Table S4). Nonpiliated bacterial colonies were swabbed from GCB agar
412 grown for 16 h and inoculated into GCB (per liter: 15 g protease peptone 3, 4 g K_2HPO_4,
413 dibasic, 1 g KH_2PO_4, 5 g NaCl) supplemented with Kellogg's supplement I (38) and 12
414 mM $Fe(NO_3)_3$ at a starting optical density at 600 nm (OD_{600}) of 0.08. Cultures were shaken
415 at 180 rpm at 37°C in a 5% CO_2-enriched atmosphere, and bacterial growth was
416 assessed by measuring the OD_{600} at hourly intervals for a total of 8 h. Experiments were

417 repeated in biological triplicate, and statistical significance between individual strains was
418 determined by using a repeated-measures 2-way analysis of variance (ANOVA) with
419 Tukey's multiple comparisons. Differences in growth rate were measured by comparing
420 the average time to reach an OD_{600} of 0.8 for each strain as previously described (44).
421 Results were compared using a one-way analysis of variance to determine overall
422 significance followed by Dunnett's multiple comparison test to determine significance
423 between individual strains.

424 ***Neisseria* sequence diversity analysis.**

425 The datasets used to identify polymorphisms and assess overall sequence diversity
426 of the *ponA* gene across all *Neisseria* species were all exported from the *Neisseria* page
427 of the PubMLST data base (last accessed September 30, 2024) (45). Allele mining
428 analysis of the *ponA* gene was performed as described (46). An individual search of the
429 profiles was made via the locus/sequence definitions tool to identify the locus assigned
430 to *ponA* (NEIS0414). Data was exported from the two-field breakdown section of the
431 *Neisseria* isolate database as a Microsoft Excel file, which provided a list of unique *ponA*
432 allelic profiles and a count for how many isolates possess that unique allele within each
433 *Neisseria* spp. Microsoft Excel was used to calculate the number of isolates that possess
434 each allele. Mutations identified in the *ponA* alleles from *N. gonorrhoeae* isolates with
435 frequency greater than 5% were mapped onto the predicted PBP1 structure, which was
436 generated using Alphafold (47). The PDB file for PBP1 from *N. gonorrhoeae* (UniProt:
437 O05131) was downloaded from the AlphaFold Protein Structure Database (AFDB) (48,
438 49) and mutations detected frequencies greater than 5% were mapped to the structure
439 using Pymol (2.0.7) (50). *ponA* sequences from PubMLST were exported using the script

440 available in the allele/sequence definition database. Molecular Evolutionary Genetics
441 Analysis software, version 7 (MEGA7) (51) was used to translate the sequences and
442 perform a multiple sequence comparison by log-expectation (MUSCLE) (52). Sequence
443 logo images were created using WebLogo (53). MUSCLE alignments of unique *ponA*
444 alleles from PubMLST were uploaded to WebLogo, background composition of proteome
445 was set to equiprobable, and y-axis units were set to bits.

446 **Genomic Analyses**

447 *Genome assembly and typing.* Publicly available *N. gonorrhoeae* genomic data with
448 available MICs of penicillin were downloaded from the European Nucleotide Archive (54–
449 72). Resistance-associated SNPs were determined from variant calls produced by Pilon
450 v 1.23 (73) after mapping to the NCCP11945 reference genome (NC_011035.1) using
451 BWA-MEM v 0.7.17 (74). Mosaic alleles and the presence or absence of resistance-
452 associated accessory genes (e.g. bla_{TEM}) were identified using blastn v 2.14.0 (75)
453 searches of *de novo* assemblies generated with SPAdes v 3.12.0 (76). Mosaic *penA*
454 alleles were typed according to the NG-STAR database (77).

455 Genomic data was included if the total *de novo* assembly length was within the range
456 of expected lengths for *N. gonorrhoeae* (1.89 Mb – 2.31 Mb) and that the number of
457 contigs was less than 250. We also required more than 40X coverage of the reference
458 genome, that at least 80% of reads mapped to the reference genome, and less than 12%
459 of positions in the reference genome had ambiguous calls defined as at least 90% of
460 reads supporting either the reference or alternate nucleotide. Lastly, we removed isolates
461 with ambiguous calls in genes that contribute to penicillin resistance, including *ponA,*
462 *penA*, and the *mtr* operon.

463 *Phylogenetic analysis.* An initial alignment and core genome phylogeny was estimated
464 using ParSNP v 2.0.5 (78) from 10,282 gonococcal *de novo* assemblies. To reduce the
465 computational resources needed for a recombination-corrected phylogenetic analysis, we
466 used Treemmer v 0.3 (79) to prune the ParSNP tree to 99.9% of the original tree length,
467 removing the most closely related pairs of isolates from our dataset. For this
468 representative subset of 6,082 isolates, pseudogenomes were generated from variant
469 calls and concatenated to produce an alignment for recombination detection and
470 phylogenetic analysis with Gubbins v 3.3.3 (80). Phylogenetic trees were visualized using
471 iTOL (81). We additionally pruned the dataset to remove isolates encoding *blaTEM*,
472 leaving a representative dataset of 5,192 isolates to investigate the distribution of *ponA*
473 among CMRNG.

474 **Antimicrobial susceptibility testing.**

475 All MIC values were determined by the agar dilution method exactly as described
476 previously (34). Briefly, FA6140 transformants were grown overnight on GCB plates,
477 resuspended in GCB broth at a final concentration of ~1 × 10^7 colony forming units
478 (CFU)/ml. Aliquots (5 µl) of each strain were spotted onto GCB plates containing
479 antibiotics at <2-fold dilutions. The MIC was defined as the lowest concentration of
480 antibiotic on which fewer than 5 colonies appeared after 24 hrs of incubation.

481 ***In vitro* serial passaging.**

482 Non-piliated colonies from each individual FA6140 *ponA*^{L421X} strain (Table 1) were
483 harvested and used to prepare a starting inoculum, which consisted of an equal ratio
484 (verified through Illumina sequencing) of all 16 strains. An equal volume of the starting
485 inoculum was used to inoculate liquid cultures at 0 h at an OD_{600} of 0.08. Three cultures

486 were supplemented with a different sub-MIC concentration of penicillin (0.25, 0.5, and 1.0
487 µg/mL), and one culture remained penicillin-free. Penicillin was added at hour 0 at the
488 specified concentration and was maintained throughout each of the six 6-hr growth
489 periods. After inoculation, cultures grew for 6 hours, at which time the OD_{600} of each
490 culture was measured to calculate the volume of cells needed to inoculate the next culture
491 at an OD_{600} of 0.08, and an aliquot was removed for analysis. This passaging procedure
492 was performed every 6 h for a total of 36 h. At every 6-hour timepoint, the cultures were
493 checked for contamination by dipping a sterile swab in the media and streaking it out on
494 a GCB agar plate. This experiment was repeated 3 separate times. All liquid cultures
495 consisted of 40 mL of GCB + supplements and were shaken at 180 rpm at 37°C in a 5%
496 CO_2-enriched atmosphere.

497 **Genomic DNA extraction.**

498 DNA was extracted from cells collected every 6 h during the *in vitro* serial passaging
499 experiments using the Promega Wizard genomic DNAs purification kit (Promega,
500 Madison, WI) according to the manufacturer's protocol. Extracted gDNA is from a mixture
501 of cells that represent the ratio of all sixteen FA6140 *ponA*^{L421X} mutants growing in the
502 culture at that time point. DNA concentrations were measured on the Invitrogen Qubit 2.0
503 Fluorometer. Samples were prepped using the Qubit™ dsDNA Quantification Assay Kits
504 according to the manufacturer' guidelines (Invitrogen).

505 **PCR and DNA sequencing of FA6140 *ponA*^{L421X} mutants during *in vitro* serial**
506 **passaging.**

507 *Library preparation.* Fragments for the library were prepped in a series of two PCR
508 reactions. The first PCR reaction (PCR 1; 20 cycles of 94°C for 30 s, 60°C for 30 s, and

509 72°C for 15 s) used genomic DNA extracted from cells collected during the serial *in vitro*
510 passaging experiments as a template. Primers were designed to target a region of the
511 *ponA* gene (bp 1212-1309), which encompasses codon-421, and to attach the
512 appropriate adaptor sequences to the 5' and 3' ends of each amplified fragment. The
513 amplicons obtained were then purified and used as templates in the second PCR (PCR
514 2; 12 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 15 s). Primers used in PCR 2
515 target the 5' and 3' Illumina adaptor sequences from PCR 1. Barcoding was achieved by
516 adding a unique, 5 bp sequence to the 5' end of fragments to signify the time point it refers
517 to. A unique index sequence was attached to the 3' ends of the fragments to mark the
518 penicillin concentration of the culture from which the fragment was obtained. All PCR
519 fragments were extracted from 3% agarose gels and purified using the QIAquick Gel
520 Extraction Kit (QIAGEN) according to manufacturers' guidelines. Concentrations were
521 measured on the Invitrogen Qubit 2.0 Fluorometer.

522 *Illumina sequencing.* Purified fragments from PCR 2 were pooled and sequencing was
523 performed on an Illumina Next-seq 1000. This was done three separate times, once per
524 *in vitro* serial passaging trial.

525 *Data analysis.* Read files generated from our libraries contained between 10 and 70
526 million reads. The reads in this library are essentially all genetically identical copies of the
527 amplified region of *ponA*, except for codon-421, meaning each read correlates to a strain
528 from the FA6140 $ponA^{L421X}$ library, based on the amino acid at codon-421. Data were
529 parsed using a UNIX script to count codons and condition specific barcodes. This process
530 yielded a table for each timepoint, listing all of the codons identified at position-421, and
531 a count of how many reads have that codon. Read counts across time points were

532 normalized to the total number of reads in the file. Only informative reads, containing the
533 expected codons at the expected positions, were analyzed to limit normalization and
534 counting based on uncertain read calls. After filtering, we retained ~99% of reads that
535 were informative. Bar graphs were generated by normalizing codon counts at 36 hr to the
536 strain's inoculum value (codon count at 0 h). Since this is a zero-sum competitive
537 experiment, any gain in codon counts by one isolate directly corresponds to a loss in
538 codon counts by the others.

539 **Whole genome sequencing and assembly.**

540 Whole genome sequencing was performed by SeqCenter (Pittsburgh, PA) on genomic
541 DNA from the inoculum and the 36 h timepoint. Illumina sequencing libraries were
542 prepared using the tagmentation-based and PCR-based Illumina DNA Prep kit and
543 custom IDT 10 bp unique dual indices (UDI) with a target insert size of 280 bp. No
544 additional DNA fragmentation or size selection steps were performed. Illumina
545 sequencing was performed on an Illumina NovaSeq X Plus sequencer in one or more
546 multiplexed shared-flow-cell runs, producing 2 x 151 bp paired-end reads. Demultiplexing,
547 quality control and adapter trimming was performed with bcl-convert1 (v4.2.4). Variant
548 calling analysis on the reads was also performed by SeqCenter, using their standard
549 methods. Illumina-generated 2 x 151 bp paired-end read data was used as the input for
550 variant calling against the reference genome of gonococcal clinical isolate, FA6140 (82),
551 the parental strain used to create the library of mutants (GCF_001047255.1). Variant
552 calling was carried out by SeqCenter using BreSeq1 under default settings.

553 **Data availability**

554 This publication made use of the PubMLST website (http://pubmlst.org/) developed by
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556 was funded by the Welcome Trust. Data was retrieved and is available from
557 https://pubmlst.org/organisms/neisseria-spp. Data supporting the findings are included in
558 the manuscript. All metadata and quality metrics for isolates included in the datasets for
559 our genomic and phylogenetic analysis are available at https://github.com/mortimer-
560 lab/ponA421. Experimental data, raw FASTQ files, and tables with codons and counts for
561 each Illumina sequencing trial, as well as the code used to generate these tables are
562 available upon request.

563 **Supplemental Material**
564
565 TABLE S1
566 TABLE S2
567 TABLE S3
568 TABLE S5
569 FIG S1
570 FIG S2
571 FIG S3
572 FIG S4
573 FIG S5
574
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Table 1 FA6140 *ponA*^{L421X} Isogenic Mutant Library and penicillin MICs.

Strains Name[a]	Amino Acid at Position 421 in *ponA*	Codon	Penicillin MIC (μg/mL)[b]
FA6140[c]	Proline (P)	CCG	**4**
FA6140 *ponA*[d]	Leucine (L)	CTG	**2**
FA6140 *ponA*^{L421P}	Proline (P)	CCG	**4**
FA6140 *ponA*^{L421L}	Leucine (L)	CTG	**2**
FA6140 *ponA*^{L421A}	Alanine (A)	GCC	**2**
FA6140 *ponA*^{L421R}	Arginine (R)	AGA	**2**
FA6140 *ponA*^{L421C}	Cysteine (C)	TGC	**2**
FA6140 *ponA*^{L421N}	Asparagine (N)	AAC	**2**
FA6140 *ponA*^{L421E}	Glutamic Acid (E)	GAA	**2**
FA6140 *ponA*^{L421G}	Glycine (G)	GGT	**2**
FA6140 *ponA*^{L421H}	Histidine (H)	CAT	**2**
FA6140 *ponA*^{L421Q}	Glutamine (Q)	CAG	**2**
FA6140 *ponA*^{L421W}	Tryptophan (W)	TGG	**2**
FA6140 *ponA*^{L421Y}	Tyrosine (Y)	TAT	**2**
FA6140 *ponA*^{L421S}	Serine (S)	AGT	**2.5**
FA6140 *ponA*^{L421F}	Phenylalanine (F)	TTC	**2**
FA6140 *ponA*^{L421I}	Isoleucine (I)	ATC	**2**
FA6140 *ponA*^{L421T}	Threonine (T)	ACT	**2**

[a]All strains are created in the FA6140 background, a penicillin-resistant gonococcal isolate, as described in Methods and Materials. Each strain possesses a different amino acid at position 421 in *ponA.*

[b]The MICs reported are the median values determined from a minimum of three independent experiments.

[c]Penicillin-resistant gonococcal isolate, FA6140, which harbors *ponA*^{L421P}.

[d]FA6140 with *ponA*^{L421P} replaced with WT *ponA*.

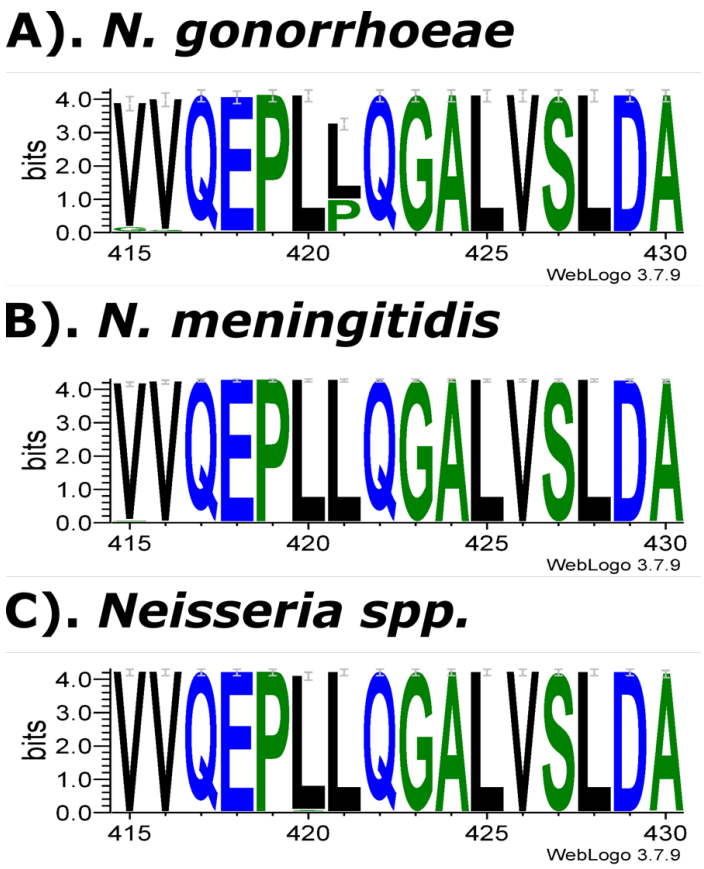

FIG 1 Sequence logo diagrams comparing the conservation of positions 415-430 in PBP1 from various *Neisseria* isolates. Diagram generated on WebLogo with MUSCLE-aligned *ponA* sequences. Sequences were downloaded from PubMLST and are a representative sub-set of all of the *ponA* alleles documented for each *Neisseria* species group: *N. gonorrhoeae* (n=172), *N. meningitidis* (n=897), and *Neisseria spp.* (n=369). The X-axis indicates the position of the amino acid. The height of the logo element (letter) represents its log-transformed frequency displayed in bits of information, and the overall height of the stack indicates the sequence conservation at that position.

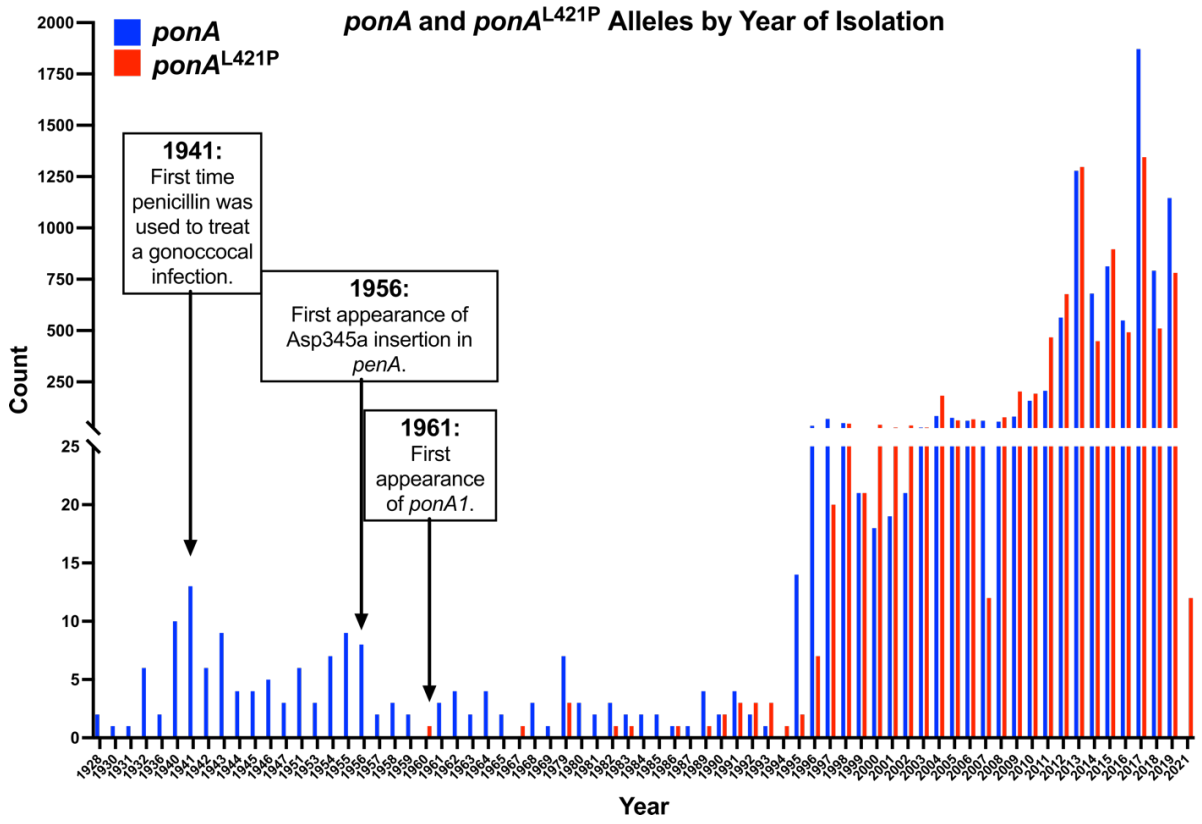

FIG 2 Historical analysis of *N. gonorrhoeae* isolates (n=16,891). Isolates are represented by the year the isolate was collected. Asp345a refers to the insertion of asparagine at position 345 in *penA* (encodes PBP2), a known penicillin-resistance-conferring mutation. Count represents the number of isolates that harbor *ponA* (blue) or *ponA*^{L421P} (red).

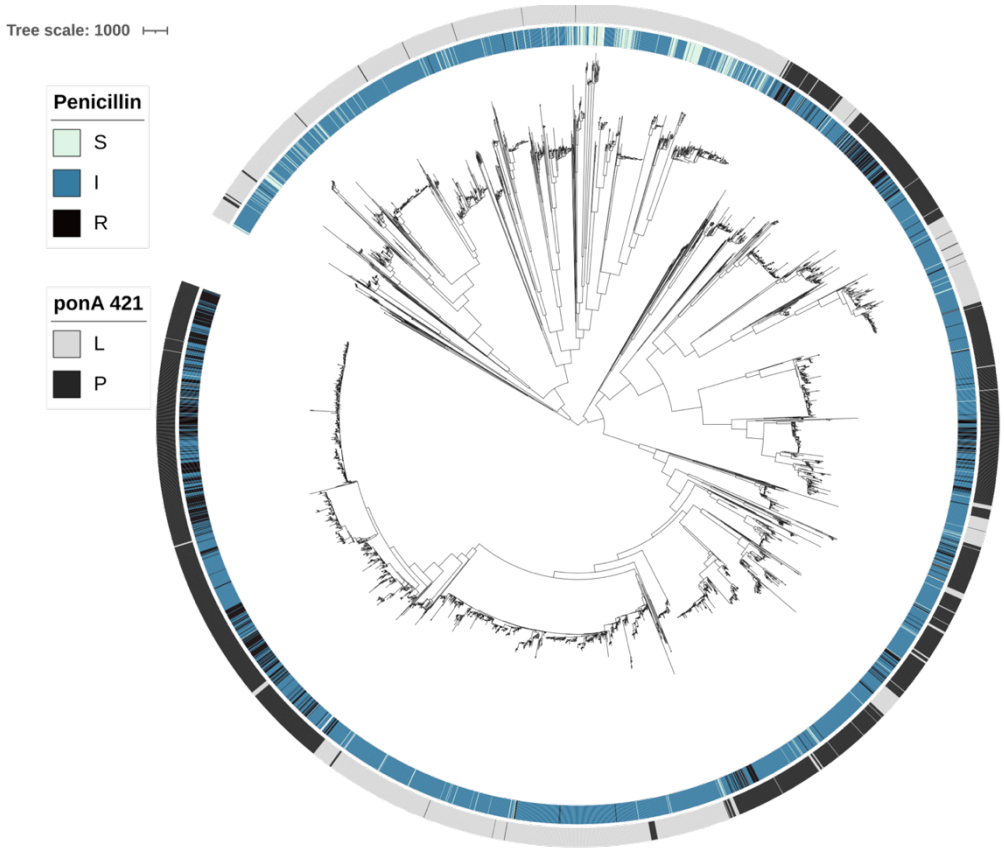

FIG 3 Phylogenetic distribution of *ponA* alleles in a representative sample of *N. gonorrhoeae* isolates without plasmid-mediated resistance (n=5,192). The outer annotation ring indicates the presence of *ponA* (L421, grey) and *ponA*^{L421P} (L421P, black). The inner ring indicates the level of penicillin resistance. S is susceptible (≤0.06 µg/mL), I is intermediate (0.25 - 1 µg/mL), and R is resistant (≥2 µg/mL) breakpoints of PenG as defined by the Clinical and Laboratory Standards Institute (CLSI) (83).

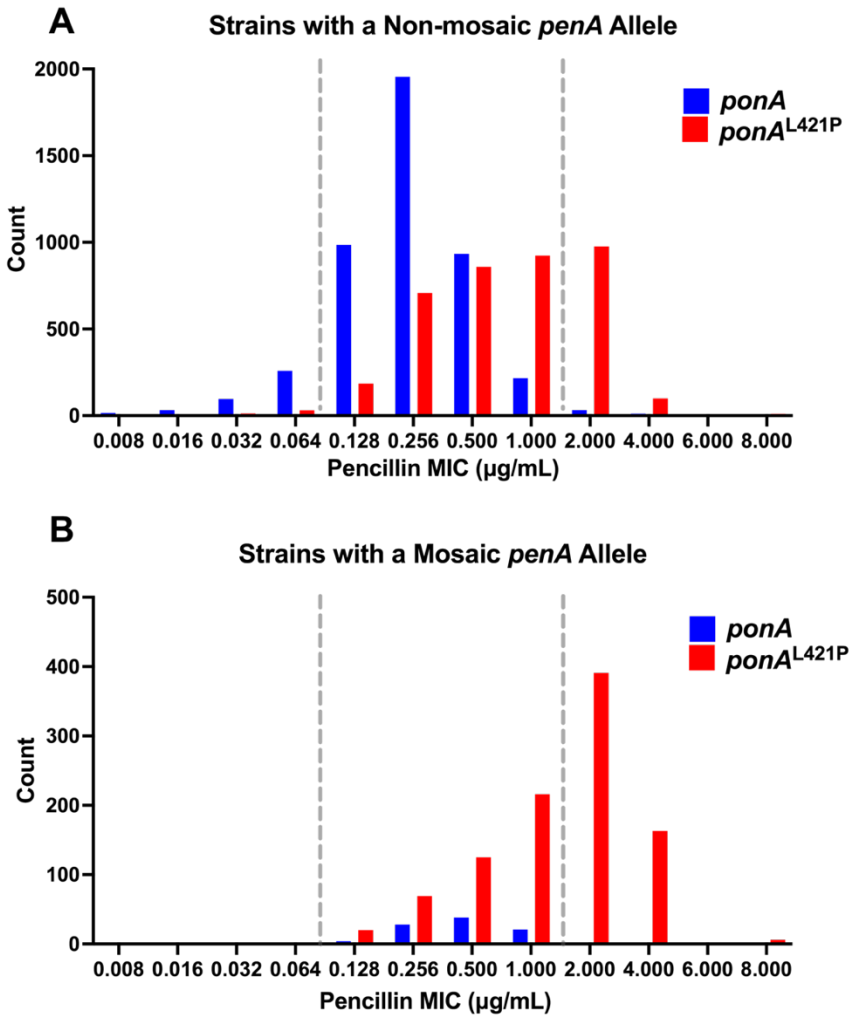

FIG 4 Distribution of MIC values for penicillin G (PenG) among *N. gonorrhoeae* isolates (n = 8,999). Strains are separated by harboring either (A) a non-mosaic *penA* allele, or (B) a mosaic *penA* allele. The X-axis shows the PenG MIC (MIC_{PEN}), and dotted lines indicate the susceptible (≤0.06 µg/mL), intermediate (0.25 - 1 µg/mL), and resistant (≥2 µg/mL) breakpoints of PenG as defined by CLSI (83). The Y-axis shows the number of isolated *N. gonorrhoeae* strains for each MIC_{PEN}, value. Red bars show distribution of strains with the $ponA^{L421P}$ allele and blue bars shows the number of strains with the wild-type *ponA* (L421) allele. The mosaic *penA* alleles in isolates were defined and typed according to the NG-STAR database (56).

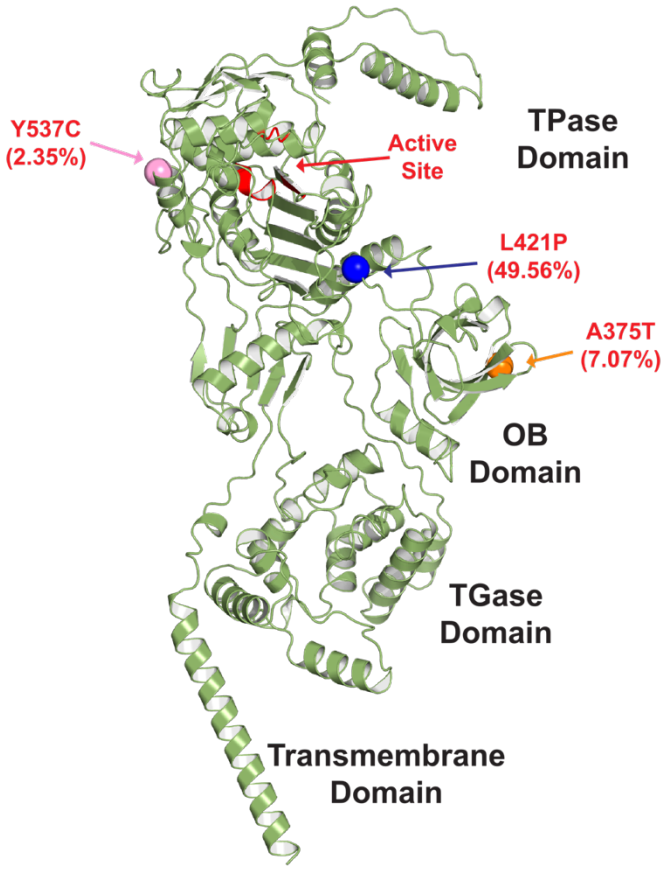

FIG 5 Polymorphisms of *N. gonorrhoeae* PBP1 mapped onto the predicted protein structure. Polymorphisms are colored according to their prevalence in the gonococcal population. Only polymorphisms detected at frequencies greater than 1% are shown: L421P (47.15%), A375T (7.07%), and Y537C (2.35%). The Transpeptidase (TPase), Transglycosylase (TGase), and the Oligosaccharide-Binding (OB) domains of PBP1 are labeled, with the TPase active site residues marked in red. MUSCLE-aligned sequences of gonococcal PBP1 were taken from PubMLST isolate database in order to identify polymorphisms and calculate population frequencies, see Materials and Methods. Predicted structure of gonococcal PBP1 is from the AlphaFold Protein Structure Database (AFDB).

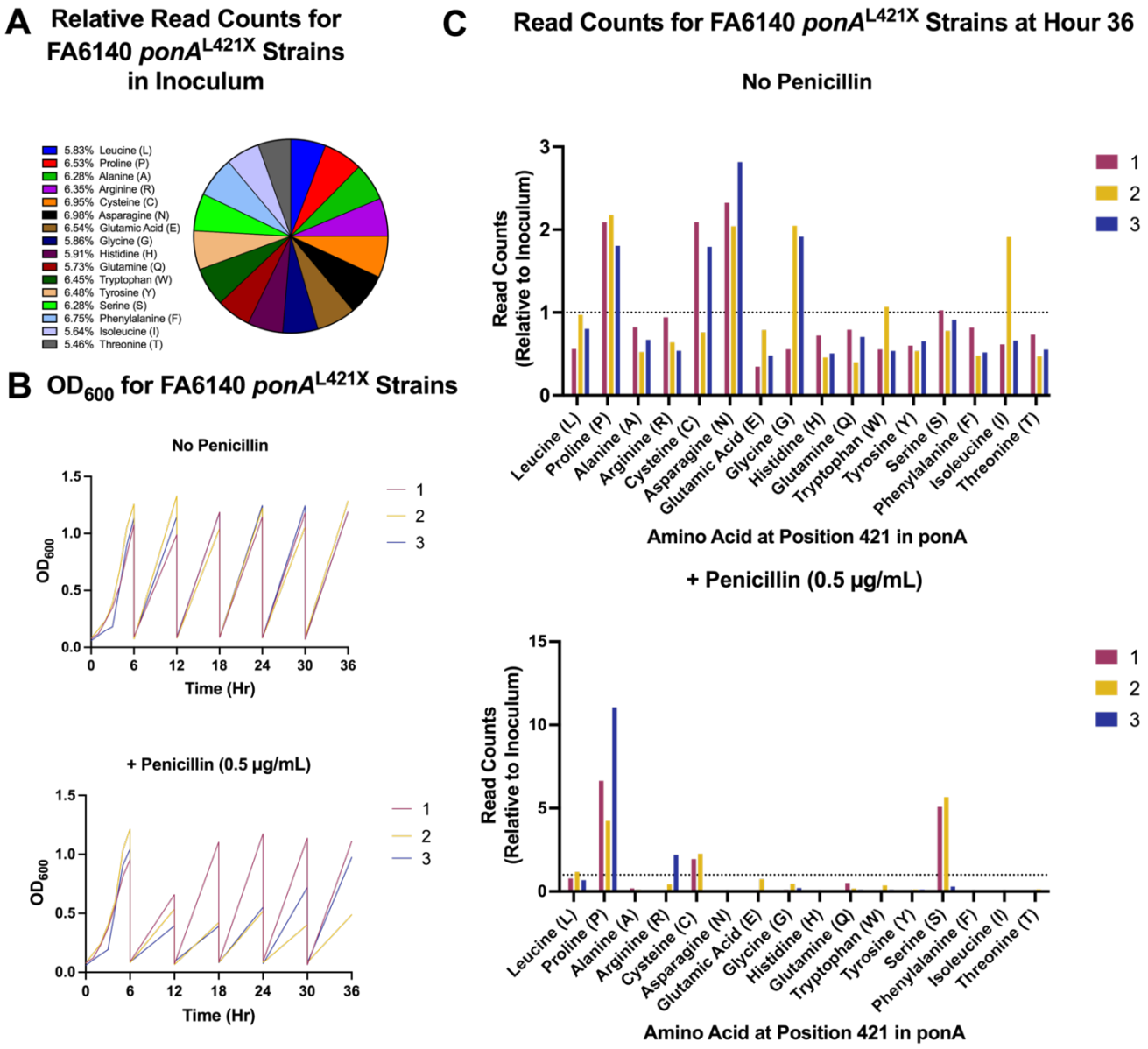

FIG 6 Optical densities (OD_{600}) and Illumina read counts from *in vitro* serial passaging of the FA6140 *ponA*^{L421X} mutant library in the absence or presence of sub-MIC concentrations of penicillin. (A) Percentage of reads, relative to the total number of reads, for each mutant at hour 0 (inoculum). The inoculum was used to inoculate each culture at 0.08 OD_{600}. (B) The serial passaging scheme included a total of 4 cultures, 3 of which were supplemented with different sub-MIC concentrations of penicillin (0.25, 0.5, and 1.0 µg/mL), and one with no penicillin added. Cultures were passaged every 6 hours, for a total of 36 hours, always with a starting OD_{600} of 0.08. (C) OD_{600} and (D) Illumina read counts of the cultures with no penicillin, compared to cultures supplemented with 0.5 µg/mL penicillin. (D) Reads for each mutant in the inoculum were normalized to 1, as indicated by the dotted line at y=1. Bars represent read counts for each mutant at hour 36, relative to their inoculum value. This experiment was performed 3 separate times

(trials) and each trial represents a separate Illumina sequencing run; (C-D) Trial 1 is shown in red, trial 2 in yellow, and trial 3 in blue. The pie chart in (A) is a representative figure from trial 1, but all 3 trials had similar results. See supplemental for read counts from the cultures supplemented with 0.25 μg/mL of penicillin (Fig. S4) and for the inoculum used in trials 2 and 3 (Fig. S3).

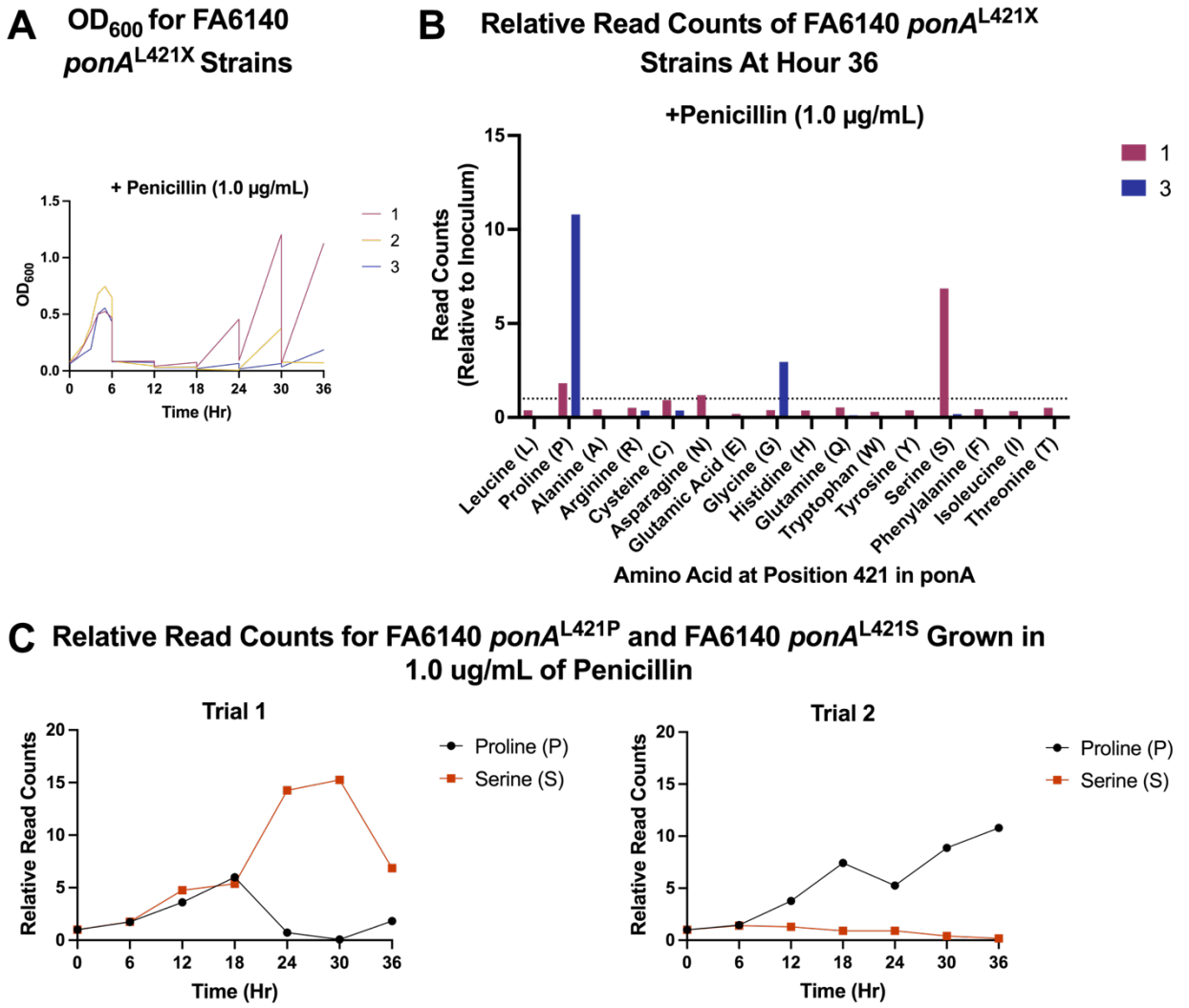

FIG 7 A *pilQ* mutation arose in the culture supplemented with 1.0 ug/ml of penicillin. Optical densities (A) and Illumina read counts (B) for the FA6140 *ponA*^{L421X} strains grown in cultures supplemented with 1.0 µg/mL of penicillin. (B) Read counts for each mutant at hour 36, relative to their inoculum (Hr 0) value. Inoculum reads for each mutant were normalized to 1, as indicated by the dotted line at y=1. (C) Read counts over time for FA6140 *ponA*^{L421S} (orange) FA6140 *ponA*^{L421P} (black), relative to their inoculum value (y=1), during trials 1 and 3. Each trial represents a separate Illumina sequencing run (A-B, trial 1 = red, trial 2 = yellow, trial 3 = blue). Cells growing in the culture from trial 2 appeared to die at hour 18 (A; yellow line), resulting in poor sequencing reads. See supplemental Fig. S5 for trial 2 data.

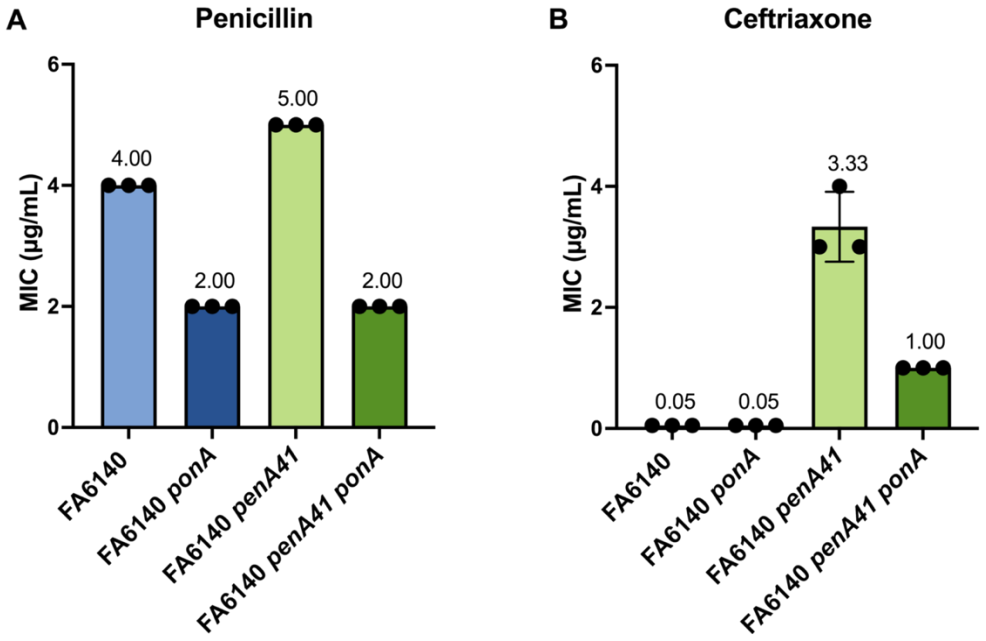

FIG 8 MICs of penicillin G and ceftriaxone for FA6140 transformants. The MICs reported above each bar are the median values (± the standard deviation) determined from three independent experiments. Data points within each bar represent the MIC values measured during each experiment.

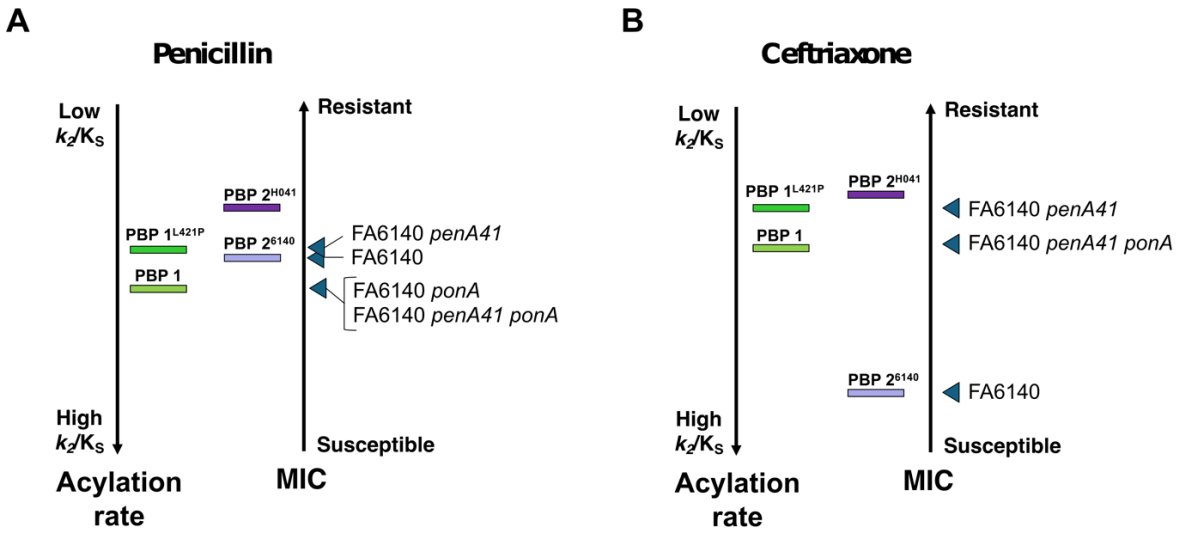

FIG 9. Diagram outlining the drug targets for penicillin and ceftriaxone in gonococcal strains with different *penA* and *ponA* alleles. PBP1 variants are marked by green rectangles and PBP2 variants are marked as purple rectangles. The left axis indicates the acylation rate, and the right axis indicates the MIC of the indicated strain. Arrows point to the killing target for each of the indicated strains. A). Penicillin has a low k_2/K_S for all PBP2 variants, so it kills gonococcal cells by inhibiting PBP1. Replacing PBP1^{L421P} with PBP1^{WT} decreases the MIC. B). Ceftriaxone normally kills by inhibiting PBP2, but PBP2^{H041} has a much lower k_2/K_S and this shifts the killing target to PBP1 (and maybe some PBP2^{H041}). Replacing PBP1^{L421P} with PBP1^{WT} decreases the MIC.