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A Chimeric Penicillin Binding Protein 2X Significantly Decreases in Vitro Beta-Lactam Susceptibility and Increases in Vivo Fitness of Streptococcus pyogenes

Randall J Olsen, Luchang Zhu, Regan E Mangham, Ahmad Faili, Samer Kayal, Stephen B Beres, Andrew J Musser

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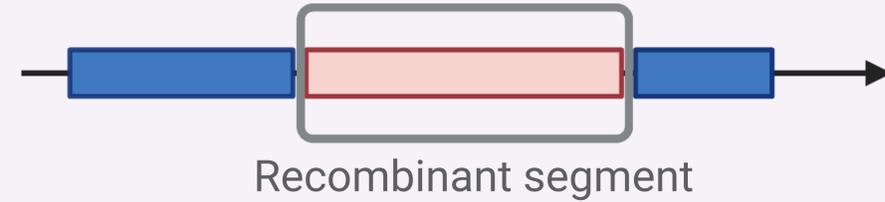
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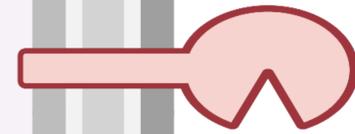
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Chimeric SDSE-like *pbp2x* gene

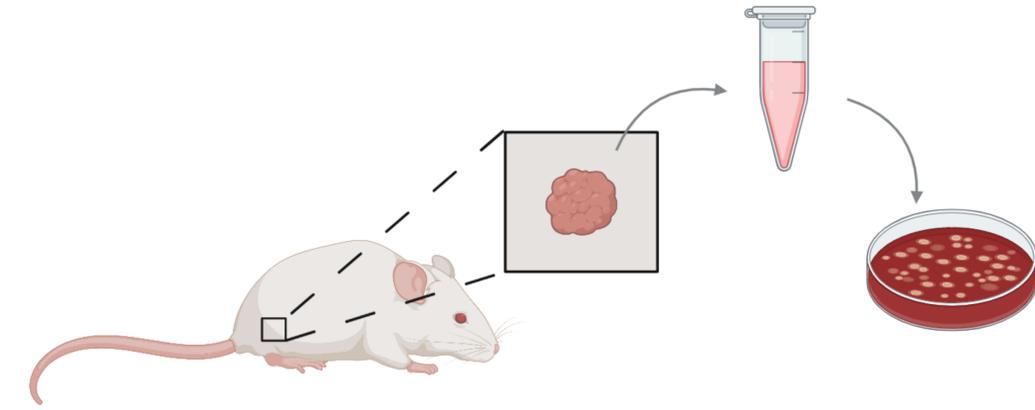


Chimeric SDSE-like PBP2X



Decreased
Beta-lactam antibiotic
susceptibility *in vitro*

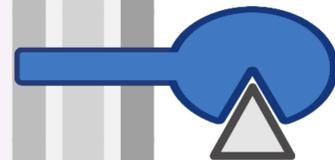
Increased fitness and virulence with intermittent
subtherapeutic penicillin therapy *in vivo*



WT GAS-like *pbp2x* gene

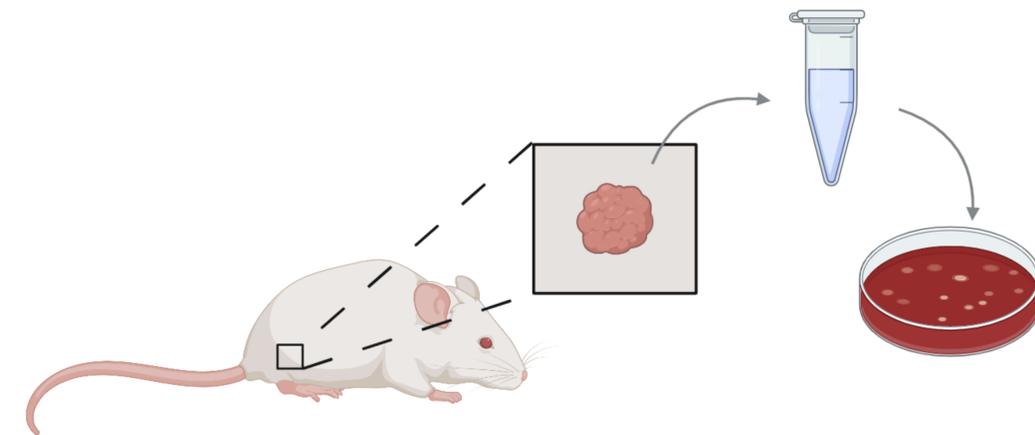


WT GAS-like PBP2X



WT
Beta-lactam antibiotic
susceptibility *in vitro*

WT fitness and virulence with intermittent
subtherapeutic penicillin therapy *in vivo*



Streptococcus pyogenes

1 **A Chimeric Penicillin Binding Protein 2X Significantly**
2 **Decreases *In Vitro* Beta-Lactam Susceptibility and Increases**
3 ***In Vivo* Fitness of *Streptococcus pyogenes***

4
5 Randall J. Olsen^{*†}, Luchang Zhu^{*†}, Regan E. Mangham^{*}, Ahmad Faili^{‡§¶}, Samer
6 Kayal^{‡¶#}, Stephen B. Beres^{*}, and James M. Musser^{*†}

7
8 ^{*}Laboratory of Molecular and Translational Human Infectious Disease Research, Center
9 for Infectious Diseases, Department of Pathology and Genomic Medicine, Houston
10 Methodist Research Institute and Houston Methodist Hospital, Houston, Texas, USA.

11 [†]Departments of Pathology and Laboratory Medicine and Microbiology and Immunology,
12 Weill Cornell Medical College, New York, New York, USA.

13 [‡]Inserm, CIC 1414, Rennes, France.

14 [§]Université Rennes 1, Faculté de Pharmacie, Rennes, France.

15 [¶]CHU de Rennes, Service de Bacteriologie-Hygiène Hospitalière, Rennes, France.

16 [#]Université Rennes 1, Faculté de Médecine, Rennes, France.

17

18 Address correspondence to:

19 Randall J. Olsen, M.D., Ph.D., 6565 Fannin Street, Ste B490, Houston, TX 77030

20 rjolsen@houstonmethodist.org

21

22 Running head: GAS strains with a chimeric PBP2X

23

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33

34 **ABSTRACT**

35 All tested strains of *Streptococcus pyogenes* (group A streptococcus, GAS) remain
36 susceptible to penicillin. However, GAS strains with amino acid substitutions in penicillin
37 binding proteins that confer decreased susceptibility to beta-lactam antibiotics have
38 been identified recently. This discovery raises concerns about emergence of beta-
39 lactam antibiotic resistance in GAS. Whole genome sequencing recently identified GAS
40 strains with a chimeric penicillin binding protein 2X (PBP2X) containing a recombinant
41 segment from *Streptococcus dysgalactiae subspecies equisimilis* (SDSE). To directly
42 test the hypothesis that the chimeric SDSE-like PBP2X alters beta-lactam susceptibility
43 *in vitro* and fitness *in vivo*, we generated an isogenic mutant strain and assessed
44 virulence in a mouse model of necrotizing myositis. Compared to naturally occurring
45 and isogenic strains with a wild-type GAS-like PBP2X, strains with the chimeric SDSE-
46 like PBP2X had reduced susceptibility *in vitro* to nine beta-lactam antibiotics. In a
47 mouse model of necrotizing myositis, the strains had identical fitness in the absence of
48 benzylpenicillin treatment. However, mice treated intermittently with a subtherapeutic
49 dose of benzylpenicillin had significantly more CFUs recovered from limbs infected with
50 strains with the chimeric SDSE-like PBP2X. These results show that mutations such as
51 the PBP2X chimera may result in significantly decreased beta-lactam susceptibility and
52 increased fitness and virulence. Expanded diagnostic laboratory surveillance, genome
53 sequencing, and molecular pathogenesis study of potentially emergent beta-lactam
54 antibiotic resistance among GAS is needed.

55 INTRODUCTION

56

57 *Streptococcus pyogenes* (group A *Streptococcus*, GAS) is a human-specific pathogen
58 that annually infects more than 700 million people worldwide^{1, 2}. GAS causes infections
59 that range in severity from pharyngitis (“strep throat”) and mild cellulitis to life-
60 threatening toxic shock syndrome and necrotizing myositis (“flesh-eating disease”)³⁻⁶.
61 GAS also causes acute rheumatic fever⁷. Despite decades of research, no licensed
62 vaccine against GAS is available⁸⁻¹¹.

63 Some streptococcal species such as *Streptococcus pneumoniae*, *Streptococcus*
64 *agalactiae* and *Streptococcus dysgalactiae subspecies equisimilis* (SDSE) are
65 increasingly becoming resistant to many different classes of antimicrobials¹²⁻¹⁶.
66 However, GAS remains universally susceptible *in vitro* to beta-lactam antibiotics that are
67 used for first line treatment^{3, 6}. The primary mechanism for beta-lactam antibiotic
68 resistance among some streptococcal species such as *S. pneumoniae* is an
69 accumulation of nonsynonymous (amino acid altering) mutations in genes encoding the
70 penicillin-binding proteins (PBPs) such as PBP2X¹⁷. Individual amino acid substitutions
71 can reduce the beta-lactam antibiotic binding activity of PBPs, and particularly under a
72 selective environment of suboptimal therapy, mutations may accumulate to result in
73 increasingly reduced susceptibility or outright resistance^{18, 19}. Of note, most penicillin
74 resistant *S. pneumoniae* strains have highly divergent alleles of the genes encoding
75 PBP2X, PBP2B or PBP1A that were created by recombination with other species such
76 as *Streptococcus oralis* or *Streptococcus mitis*^{20, 21}. The mosaic genes confer much
77 greater decreases in beta-lactam antibiotic susceptibility than single amino acid

78 substitutions. Importantly, the mosaic PBP2X genes in *S. pneumoniae* often decrease
79 susceptibility to multiple families of beta-lactam antibiotics^{20, 21}.

80 GAS infections are typically treated with a beta-lactam antibiotic such as
81 benzylpenicillin^{3, 6, 22-24}. In some cases, such as severe invasive infections or pharyngitis
82 with treatment failure, other antimicrobials may be used. Given their very high
83 incidence, GAS infections are a major cause of beta-lactam antibiotic use worldwide²⁵,
84 ²⁶. However, to date, no beta-lactam-resistant strains of GAS have been reported²⁷⁻²⁹.
85 For this reason, most clinical laboratories do not routinely perform beta-lactam
86 antimicrobial susceptibility testing on GAS isolates^{30, 31}. As a result, GAS strains with
87 decreased *in vitro* susceptibility to beta-lactam antibiotics may exist and unknowingly
88 disseminate. In support of this possibility, our laboratory and others have recently
89 identified GAS strains from geographically widespread locations with significantly
90 decreased *in vitro* susceptibility to benzylpenicillin and other beta-lactam antibiotics³²⁻³⁶.
91 The minimum inhibitory concentration (MIC) for these strains do not meet the Clinical
92 Laboratory Standards Institute (CLSI) threshold for resistance^{30, 31}. When studied by
93 whole genome sequencing, many of the GAS strains with a reduced penicillin
94 susceptibility phenotype have nonsynonymous mutations in PBP genes³²⁻³⁷. For the
95 most part, a single amino acid change is present in each strain, but in some instances,
96 multiple substitutions are found. This discovery has caused great concern among
97 clinical microbiologists and infectious disease physicians³⁸⁻⁴¹. GAS strains, particularly
98 under the selective pressure of intermittent or subtherapeutic beta-lactam therapy, could
99 continue evolving an increasingly reduced susceptibility phenotype and eventually
100 evolve to outright resistance.

101 A recent whole genome sequencing study investigating GAS collected in
102 Rennes, France identified five clonally related serotype *emm81* strains with a 4-kb
103 recombinant segment from *Streptococcus dysgalactiae subspecies equisimilis*
104 (SDSE)³⁷. The recombination created a chimeric gene encoding PBP2X (Fig. 1A). This
105 was the first discovery of an interspecies chimeric PBP2X in GAS^{39, 42}. Compared to the
106 most common (wild-type) PBP2X variant in GAS, the chimeric SDSE-like PBP2X differs
107 by 55 amino acids (Fig. 1B)³⁷. Importantly, several of the individual amino acid changes
108 in the chimeric SDSE-like PBP2X have been described in previous GAS studies and are
109 known to alter the MIC for penicillin and other beta-lactam antibiotics^{33, 36, 37, 43}.

110 Herein, we hypothesized that the chimeric SDSE-like PBP2X alters beta-lactam
111 susceptibility *in vitro* and strain fitness *in vivo*. To test this hypothesis, an isogenic
112 mutant strain was created. Compared to naturally occurring and isogenic strains with a
113 wild-type GAS-like PBP2X, strains with the chimeric SDSE-like PBP2X had markedly
114 reduced susceptibility *in vitro* to nine beta-lactam antibiotics. Using a mouse model of
115 necrotizing myositis (Fig. 1C)³⁶, the strains had no significant difference in virulence in
116 the absence of intermittent subtherapeutic benzylpenicillin treatment. However, in the
117 presence of intermittent subtherapeutic benzylpenicillin treatment, significantly more
118 CFUs were recovered from limbs infected with strains with the chimeric SDSE-like
119 PBP2X. In summary, the chimeric SDSE-like PBP2X variant in GAS significantly
120 decreases beta-lactam susceptibility *in vitro* and increases fitness *in vivo*, resulting in
121 increased virulence.

122

123 MATERIALS AND METHODS

124

125 Growth of GAS strains

126

127 GAS strains were grown in Todd Hewitt broth supplemented with 0.5% yeast extract
128 (THY) with or without a subinhibitory concentration of benzylpenicillin (MilliporeSigma,
129 Burlington, MA, USA) as described previously³⁶. Cultures were incubated at 37° C in 5%
130 CO₂ for 20 h, and growth was determined by measuring the optical density at 600 nm in
131 an automated plate reader (Synergy H1 Hybrid Reader, BioTek, Santa Clara, CA, USA).
132 Six replicates of each strain in each penicillin concentration (0, 6 or 12 ng/mL) were
133 performed. GAS strains were also grown on THY agar supplemented with 5% sheep
134 blood (Cardinal Health, Dublin, OH, USA) under the same conditions.

135

136 GAS strains and PBP2X isogenic mutant strain construction

137

138 Naturally occurring strain MGAS34668 has a wild-type GAS-like PBP2X. This strain is
139 referred to as MGAS34668 (GAS-like PBP2X). Naturally occurring strain MGAS34772
140 has a chimeric PBP2X from SDSE. This strain is referred to as MGAS34772 (SDSE-like
141 PBP2X). An isogenic mutant strain of MGAS34772 with the GAS-like PBP2X restored
142 from wildtype strain MGAS2221 was constructed as previously described⁴⁴. This strain
143 is referred to as MGAS34772 (GAS-like PBP2X). Briefly, primer sets PBP-1-FWD and
144 PBP-1-REV, PBP-2-FWD and REV, and PBP-3-FWD and PBP-3-REV (Table 1) were
145 used to amplify a 1533-bp, 1602-bp, and 1560-bp fragment using genomic DNA of
146 MGAS34772, MGAS2221, and MGAS34772, respectively. The three DNA fragments

147 were seamlessly ligated and cloned into suicide vector pBBL-740 using the NEBuilder
148 HiFi DNA Assembly kit (New England Biolabs, Ipswich, MA). The resulting construct
149 was transformed into MGAS34772 to restore the GAS-like allele encoding PBP2X.
150 Whole-genome sequence analysis was performed on the isogenic mutant strain to
151 confirm the desired mutation and an absence of spurious mutations.

152

153 **Minimum inhibitory concentration (MIC) determination**

154

155 MICs for beta-lactam antibiotics were determined by the Etest gradient method
156 (bioMerieux, Marcy-l'Etoile, France) or the MIC Test Strip method (Liofilchem, Waltham,
157 MA, USA) according to the manufacturer's instructions. The MIC of each strain was
158 measured three times for each antibiotic. All replicate measurements were within +/- 1
159 dilution, and mean MIC is reported in Table 2. Statistically significant differences were
160 determined by ANOVA (GraphPad Prism V9.3.1, San Diego, CA, USA). The MIC
161 determinations for penicillin and ampicillin were confirmed by broth dilution.

162

163 **Mouse model of necrotizing myositis in the absence or presence of** 164 **intermittent subtherapeutic benzylpenicillin treatment**

165

166 The mouse model of necrotizing myositis used in these experiments has been
167 previously described³⁶. The model includes the presence of intermittent subtherapeutic
168 benzylpenicillin treatment. Mice were infected intramuscularly in the right lower hindlimb
169 with 1×10^9 CFU of the indicated strain. This dose was selected by performing a dose

170 escalation study to identify the inoculum that results in approximately 50% survival on
171 day 7 after infection in the absence of benzylpenicillin therapy. Mice were then
172 intermittently treated with a subtherapeutic dose of benzylpenicillin (MilliporeSigma,
173 Burlington, MA, USA) on the day of infection (7.3 IU/g in the right lower hindlimb near
174 the infection site) and day 10 after infection (11 IU/g in the left lower hindlimb). Group
175 sizes were selected with a power calculation.

176

177 **Mouse survival and GAS CFU determination**

178

179 For survival experiments, mice were monitored for near mortality at least once daily
180 using standard criteria⁴⁵. Survival was graphed as a Kaplan-Meier curve, and statistical
181 differences were determined using a log-rank test (GraphPad Prism V9.3.1, San Diego,
182 CA, USA). CFUs from infected muscle were determined by culturing tissue
183 homogenates from limbs as previously described³⁶. Briefly, each infected limb was
184 amputated, weighed, homogenized (Omni International, Kennesaw, GA, USA) in 1 mL
185 sterile phosphate buffered saline, serially diluted, plated, and cultured overnight. CFU
186 data were graphed as mean +/- SEM, and statistical differences were determined with a
187 Mann-Whitney test. Mouse experiments were approved by the Institutional Animal Care
188 and Use Committee of Houston Methodist Research Institute (protocol IS00006169).

189

190 **RESULTS**

191

192 **Naturally occurring strain MGAS34772 (SDSE-like PBP2X) has**
193 **reduced susceptibility to beta-lactam antibiotics *in vitro***

194
195 Whole genome sequencing of GAS strains recovered from patients in Rennes, France
196 identified serotype *emm81* strain MGAS34772 with a chimeric PBP2X with a
197 recombinant segment from SDSE (Fig. 1A)³⁷. This strain is referred to as MGAS34772
198 (SDSE-like PBP2X). Compared to the wild-type GAS PBP2X, the chimeric SDSE-like
199 PBP2X differs by 55 amino acid substitutions (Fig. 1B). To test the hypothesis that the
200 chimeric SDSE-like PBP2X confers reduced susceptibility to beta-lactam antibiotics *in*
201 *vitro*, MGAS34772 (SDSE-like PBP2X) was compared to genetically closely related
202 strain MGAS34668 with a GAS-like PBP2X. This strain is referred to as MGAS34668
203 (GAS-like PBP2X). The minimum inhibitory concentration (MIC) was measured for nine
204 beta-lactam antibiotics, including four penicillins, four cephalosporins, and one
205 carbapenem. Vancomycin was also tested as a control. Compared to MGAS34668
206 (GAS-like PBP2X), MGAS34772 (SDSE-like PBP2X) had significantly higher MICs to all
207 beta-lactam antibiotics tested (Table 2). As expected, the strains had an identical MIC
208 for vancomycin (Table 2).

209
210 **Naturally occurring strain MGAS34772 (SDSE-like PBP2X) has**
211 **increased fitness resulting in increased virulence in the presence of**
212 **intermittent subtherapeutic penicillin treatment**

213

214 To test the hypothesis that the chimeric SDSE-like PBP2X alters GAS fitness *in vivo*, a
215 mouse model of necrotizing myositis was used³⁶. This mouse model, including an
216 intermittent subtherapeutic benzylpenicillin treatment strategy (Fig.1C), was previously
217 used to demonstrate that a single amino acid replacement in PBP2X significantly
218 increased GAS fitness *in vivo*³⁶.

219 First, the fitness of naturally occurring strains MGAS34772 (SDSE-like PBP2X)
220 and MGAS34668 (GAS-like PBP2X) were compared in the absence of intermittent
221 subtherapeutic benzylpenicillin treatment. Mice were infected with the indicated strain
222 and followed for survival and CFU recovery from infected limbs. Survival was assessed
223 at day 7 after infection, and the resulting Kaplan-Meier survival curves were essentially
224 superimposable (Fig. 2A). CFUs were recovered from limbs on day 3 after infection, and
225 similar numbers of colonies were counted from mice infected with either strain (Fig. 2B).
226 These data demonstrate that in the absence of intermittent subtherapeutic
227 benzylpenicillin therapy, naturally occurring strains MGAS34772 (SDSE-like PBP2X)
228 and MGAS34668 (GAS-like PBP2X) have indistinguishable fitness and virulence.

229 Next, the fitness of naturally occurring strains MGAS34772 (SDSE-like PBP2X)
230 and MGAS34668 (GAS-like PBP2X) was compared in the presence of intermittent
231 subtherapeutic benzylpenicillin treatment. Mice were infected with the indicated strain
232 and given a subtherapeutic dose of benzylpenicillin on the day of infection and day 10
233 after infection (Fig. 1C). Limbs were harvested on day 17 after infection. All mice
234 survived to day 17 (Fig. 2C). Consistent with the hypothesis that strain MGAS34772
235 (SDSE-like PBP2X), which has reduced susceptibility to beta-lactam antibiotics *in vitro*,
236 is more fit in the presence of intermittent subtherapeutic penicillin *in vivo*, significantly

237 more CFUs were recovered from limbs infected with this strain (Fig. 2D). These data
238 show that in the presence of subtherapeutic benzylpenicillin treatment, the SDSE-like
239 PBP2X increases GAS fitness and results in increased virulence.

240

241 **Creation of isogenic mutant strain MGAS34772 (GAS-like PBP2X)**

242 **from MGAS34772 (SDSE-like PBP2X)**

243

244 The naturally occurring strains differ in other parts of the genome besides the
245 recombinant segment encoding PBP2X³⁷. To unambiguously demonstrate that the
246 chimeric SDSE-like PBP2X is responsible for the increased fitness phenotype of
247 MGAS34772 (SDSE-like PBP2X), we created an isogenic mutant strain by restoring the
248 wild-type GAS *pbp2x* allele (that is, the most common allele found in GAS)³⁷. This
249 isogenic mutant strain is referred as MGAS34772 (GAS-like PBP2X).

250 First, the MIC of strains MGAS34772 (SDSE-like PBP2X) and MGAS34772
251 (GAS-like PBP2X) was measured for nine beta-lactam antibiotics. Consistent with the
252 hypothesis that the chimeric SDSE-like PBP2X confers a reduced beta-lactam
253 susceptibility phenotype *in vitro*, strain MGAS34772 (SDSE-like PBP2X) had
254 significantly increased MICs for all beta-lactam antibiotics tested (Table 2). As expected,
255 the strains had an identical MIC for vancomycin (Table 2).

256 Next, the growth of strains MGAS34772 (SDSE-like PBP2X) and MGAS34772
257 (GAS-like PBP2X) was compared *in vitro*. In the absence of benzylpenicillin, the
258 isogenic strains had nearly superimposable growth curves (Fig. 3A). However, in the

259 presence of two different subinhibitory concentrations of benzylpenicillin, strain
260 MGAS34772 (SDSE-like PBP2X) grew to a markedly higher optical density (Fig. 3B-C).

261
262 **Isogenic strain MGAS34772 (SDSE-like PBP2X) has increased fitness**
263 **resulting in increased virulence in the presence of intermittent**
264 **subtherapeutic penicillin treatment**

265
266 Using the previously described GAS infection model and intermittent subtherapeutic
267 benzylpenicillin dosing (Fig. 1C), mouse necrotizing myositis studies were performed
268 with isogenic strains MGAS34772 (SDSE-like PBP2X) and MGAS34772 (GAS-like
269 PBP2X). In the absence of benzylpenicillin treatment, mice infected with the isogenic
270 strains did not differ in survival or CFU recovery (Fig. 4A-B). In the presence of
271 intermittent subtherapeutic benzylpenicillin treatment, all mice survived to day 17 after
272 infection (Fig. 4C). However, significantly more CFUs were recovered from limbs
273 infected with MGAS34772 (SDSE-like PBP2X) (Fig. 4D).

274 Taken together, these data show that in the presence of intermittent
275 subtherapeutic benzylpenicillin treatment, the naturally occurring and isogenic strains
276 with the chimeric SDSE-like PBP2X are more fit, resulting in more virulence, than
277 strains with the wild-type GAS-like PBP2X.

278

279 **DISCUSSION**

280

281 In this study, our goal was to test the hypothesis that the chimeric PBP2X with a
282 recombinant segment from SDSE alters beta-lactam susceptibility *in vitro* and GAS
283 fitness *in vivo*. Naturally occurring and isogenic mutant strains were compared. Results
284 demonstrate that the chimeric SDSE-like PBP2X confers significantly decreased
285 susceptibility to all beta-lactam antibiotics tested. For mice with experimental necrotizing
286 myositis, the strains had indistinguishable virulence in the absence of benzylpenicillin
287 therapy. However, in the presence of intermittent subtherapeutic benzylpenicillin
288 treatment, strains with the chimeric SDSE-like PBP2X had increased fitness resulting in
289 significantly increased virulence. These data add important new information bearing on
290 PBP2X variants and GAS fitness and virulence.

291 Our laboratory recently demonstrated that a single amino acid substitution in
292 PBP2X (P601L) significantly decreases susceptibility to beta-lactam antibiotics *in vitro*
293 and increases GAS fitness *in vivo*³⁶. The P601L change was important to study because
294 it has convergently (independently) evolved in multiple GAS serotypes over
295 geographically distant locations³⁷. The effect of some other amino acid changes in
296 PBP2X has been investigated³²⁻³⁷, including use of isogenic mutant strains to test
297 penicillin susceptibility³⁷. However, more amino acid substitutions and beta-lactam
298 antibiotics need to be tested. Also, the lack of *in vivo* animal model data leaves many
299 important hypotheses untested.

300 The PBP2X variant studied herein is the first documented example of an
301 interspecies chimeric PBP2X in GAS³⁷. The chimeric PBP2X was identified in five
302 clonally related strains of serotype *emm81* GAS recovered from patients with invasive
303 infections in Rennes, France⁴². The epidemiology indicates that the GAS strains with a

304 chimeric PBP2X were successfully transmitted from human to human. Of note, two of
305 the isolates were recovered from patients with recurrent infection and treatment failure,
306 which could occur in a selective environment of suboptimal therapy⁴². The same PBP2X
307 variant, but with a different recombination segment containing additional SDSE gene
308 content, was also discovered in a type *emm238* GAS strain recovered in New
309 Zealand³⁷. That is, like the P601L single amino acid substitution mentioned above, the
310 same chimeric PBP2X variant has evolved in different GAS serotypes in geographically
311 distant locations. It is unknown if the second example of an SDSE-like recombinant
312 segment is the result of an intraspecies or interspecies recombination. However,
313 because of this recurring theme, we speculate that other instances of interspecies
314 PBP2X recombination have likely occurred, but since GAS strains are not routinely
315 tested for susceptibility, the variants have gone undiscovered.

316 The recent discovery of GAS strains with amino acid substitutions in PBP2X and
317 reduced susceptibility to beta-lactam antibiotics has generated significant concern
318 among clinical microbiologists and infectious disease physicians³⁸⁻⁴¹. Most PBP2X
319 variants in GAS have a single amino acid substitution compared to the wild-type
320 sequence, but some strains with two or more amino acid changes have been
321 discovered³⁷. Under selective conditions such as suboptimal therapy, these strains may
322 accumulate more mutations that further reduce susceptibility to beta-lactam antibiotics.
323 In support of this idea, among the 55 amino acid substitutions identified in the chimeric
324 PBP2X, 3 were not present in either the GAS or SDSE reference strain (Fig. 1B).
325 Notably, the G600D amino acid substitution present in the chimeric PBP2X but not
326 either reference PBP2X has independently arisen in multiple GAS serotypes. Additional

327 concern stems from the recent report of four clonally related penicillin resistant strains of
328 SDSE in Denmark¹⁴. These are the first known penicillin resistant SDSE. Compared to
329 susceptible strains, the penicillin resistant SDSE strains have two amino acid changes
330 in close proximity to active sites of the PBP2X transpeptidase domain. One of the amino
331 acid changes, Q555E (corresponding to Q552E in *S. pneumoniae* and Q557E in *S.*
332 *agalactiae*) has been reported in other penicillin resistant streptococcal species^{18, 46}, but
333 it has not been identified in GAS³⁷. Of note, the penicillin resistant SDSE strains had
334 additional amino acid substitutions in PBP1A and PBP1B, so the contribution of each
335 substitution to the overall beta-lactam antibiotic resistance phenotype is uncertain¹⁴.
336 Regardless, if a chimeric PBP2X were to arise in GAS by recombination with a penicillin
337 resistant SDSE, the resulting phenotype could be markedly decreased susceptibility or
338 outright resistance, both very concerning scenarios.

339 Despite more than 70 years of penicillin use as a first line therapy and the
340 immense global burden of GAS infections, GAS remains universally susceptible *in vitro*
341 to beta-lactam antibiotics^{3, 6}. The molecular basis for the enduring susceptibility
342 phenotype is uncertain⁴⁷. However, our recent discovery of GAS strains, in varied
343 genomic backgrounds and geographically widespread locations^{33, 36, 37}, may be an early
344 signal of emerging resistance. Furthermore, the lack of a commercially licensed vaccine
345 against GAS leaves the world's population unprotected against ongoing transmission
346 and evolution of these strains⁸⁻¹¹. Expanded diagnostic laboratory surveillance, genome
347 sequencing, and molecular pathogenesis study of potentially emergent beta-lactam
348 antibiotic resistance among GAS is needed.

349

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351
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357 **Author Contributions**

358
359 R.J.O. planned and conducted the mouse infection experiments and analyzed the
360 resulting data. L.Z. and R.M. generated and characterized the isogenic mutant strain.
361 A.F. and S.K. provided strains and epidemiology data. S.B.B. performed genomic
362 analyses. J.M.M. designed the studies, analyzed the experiments, and oversaw the
363 project. All authors wrote the manuscript and contributed to the intellectual atmosphere
364 that resulted in this study. R.J.O. is the guarantor of this work, and as such, had full
365 access to all the data in the study and takes responsibility for the integrity of the data
366 and the accuracy of the analysis.

367

368

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370

371

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525 **Figure Legends**

526

527 **FIG 1. A.** Comparison of the chimeric strain with SDSE and GAS reference strains. The
528 sequence of SDSE reference strain NCTC7136 is shown in red (upper panel), and the
529 sequence of GAS reference strain MGAS2221 is shown in blue (lower panel). The
530 chimeric GAS strain MGAS34772 (middle panel) contains a 4 kb recombinant segment
531 from SDSE (boxed region) that creates a SDSE-like PBP2X. **B.** Amino acid alignment of
532 PBP2X from SDSE reference strain NCTC7136 (upper sequence), chimeric strain
533 MGAS34772 (middle sequence), and GAS reference strain MGAS2221 (lower
534 sequence). In the sequence downstream of the 5' crossover (position 201, red arrow),
535 amino acid residues ($n=52$) that differ between the GAS reference strain and chimeric
536 SDSE-like strain are shown in red font. Amino acid changes ($n=3$) present in the
537 chimeric SDSE-like strain but not the GAS or SDSE reference strains are shown in
538 green font. The dimerization domain (brown box), transpeptidase domain (orange box),
539 and PASTA domains (yellow box) of PBP2X are shown. Naturally occurring strain
540 MGAS34668 (GAS-like PBP2X) and isogenic mutant strain MGAS34772 (GAS-like
541 PBP2X) have the GAS-like PBP2X sequence. Strain MGAS34772 (SDSE-like PBP2X)
542 has the chimeric PBP2X sequence. **C.** Mouse model of necrotizing myositis in the
543 presence of intermittent subtherapeutic benzylpenicillin treatment. Mice were inoculated
544 in the right lower hindlimb with a naturally occurring or isogenic GAS strain with the
545 SDSE-like PBP2X (upper panels) or GAS-like PBP2X (lower panels). Subtherapeutic
546 doses of benzylpenicillin were given in the same limb on the day of infection (left

547 panels) and the contralateral limb on Day 10 after infection (right panels). Virulence was
548 assessed on Day 17 by survival analysis and CFU recovery from infected limbs.

549

550 **FIG 2.** Comparison of naturally occurring strains MGAS34772 (SDSE-like PBP2X) and
551 MGAS34668 (GAS-like PBP2X) in a mouse model of necrotizing myositis. For GAS
552 infections occurring in the absence of penicillin therapy, Kaplan-Meier survival curves
553 **(A)** and CFU recovery **(B)** are shown. Survival was monitored for 7 days, and CFU
554 recovery was performed on day 3 after infection. Replicate data are expressed as mean
555 +/- SEM ($n=10$ mice per strain). No significant differences in survival (log rank test) or
556 CFU recovery (Mann-Whitney test) are observed in the absence of penicillin. For
557 infections occurring in the presence of intermittent subtherapeutic penicillin therapy as
558 described in Fig. 1, Kaplan-Meier survival curves **(C)** and CFU recovery **(D)** are shown.
559 Replicate data are expressed as mean +/- SEM ($n=40$ mice per strain). No significant
560 differences in survival (log-rank test) are observed. *** $P<0.001$ (Mann-Whitney test).

561

562 **Fig 3.** Comparison of isogenic strains MGAS34772 (SDSE-like PBP2X) and
563 MGAS34772 (GAS-like PBP2X). In the absence of penicillin **(A)**, the growth curves of
564 MGAS34772 (SDSE-like PBP2X) and MGAS34772 (GAS-like PBP2X) are nearly
565 superimposed. In the presence of 6 ng/mL **(B)** or 12 ng/mL **(C)** benzylpenicillin, strain
566 MGAS34772 (SDSE-like PBP2X) grew to a markedly higher optical density. Replicate
567 data are expressed as mean +/- SD ($n=6$ replicates per strain).

568

569 **FIG 4.** Comparison of isogenic strains MGAS34772 (SDSE-like PBP2X) and
570 MGAS34772 (GAS-like PBP2X) in a mouse model of necrotizing myositis. For GAS
571 infections occurring in the absence of penicillin therapy, Kaplan-Meier survival curves
572 **(A)** and CFU recovery **(B)** are shown. Survival was monitored for 7 days, and CFU
573 recovery was assessed on day 3 after infection. Replicate data are expressed as mean
574 +/- SEM ($n=10$ mice per strain). No significant differences in survival (log rank test) or
575 CFU recovery (Mann-Whitney test) are observed in the absence of penicillin. For
576 infections occurring in the presence of intermittent subtherapeutic penicillin therapy as
577 described in Fig. 1, Kaplan-Meier survival curves **(C)** and CFU recovery **(D)** are shown.
578 Replicate data are expressed as mean +/- SEM ($n=40$ mice per strain). No significant
579 differences in survival (log-rank test) are observed. *** $P<0.001$ (Mann-Whitney test).

580

581 **Tables**

582

583 **Table 1.** Primers used in this study. Lowercase bases are complimentary to the
 584 adjacent sequence for ligation. Uppercase bases are complimentary to the target
 585 sequence.

586

Primer	Oligonucleotide sequence	DNA template
PBP-1_fwd	5'-gtgaatacatAGTCGCTCTTAACAGCTTAC-3'	MGAS34772
PBP-1_rev	5'-ctagacctgtTTTTCCAATAACTCTTAACAC-3'	MGAS34772
PBP-2_fwd	5'-agttggaaaaACAGGTCTAGAAGCTTCTTTTG-3'	MGAS2221
PBP-2_rev	5'-taaaaacatgTTAATCTCCTAAAGTAATGGTCATTTTTTTTATTTTT-3'	MGAS2221
PBP-3_fwd	5'-aggagattaaCATGTTTTTAACCTTTATCGCAGG-3'	MGAS34772
PBP-3_rev	5'-caattgtacaCGACCCGGAGTTCCAATG-3'	MGAS34772

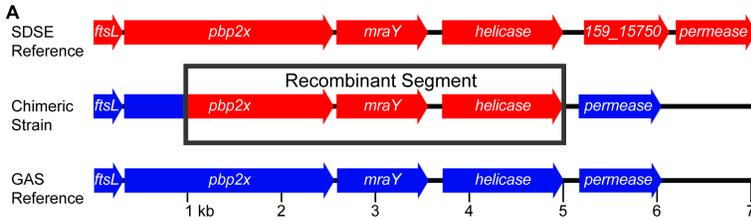
587

588 **Table 2.** Minimum inhibitory concentration (MIC) of strains MGAS34772 (SDSE-like
 589 PBP2X), MGAS34668 (GAS-like PBP2X), and MGAS34772 (GAS-like PBP2X) to beta-
 590 lactam antibiotics. * $P < 0.05$ and *** $P < 0.001$, ANOVA, compared to MGAS34772 (SDSE-
 591 like PBP2X). N.D., not defined.

592

Beta-lactam antibiotic	34772 (SDSE-like PBP2x)	34668 (GAS-like PBP2X)	34772 (GAS-like PBP2x)	CLSI breakpoint
amoxicillin	0.060	0.016***	0.016***	<0.25
ampicillin	0.047	0.016***	0.016***	<0.25
cefazolin	0.235	0.19*	0.125***	N.D.
cefotaxime	0.043	0.016***	0.016***	<0.5
cefoxitin	3	1***	1***	N.D.
ceftazidime	0.25	0.19***	0.19***	N.D.
meropenem	0.036	0.008***	0.011***	<0.5
oxacillin	0.470	0.094***	0.115***	N.D.
penicillin	0.036	0.012***	0.012***	<0.12
vancomycin	0.25	0.25	0.25	<1

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B

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SDSE      10      20      30      40      50      60      70      80      90     100
Chimeric  MRKQKRVLDYVVRDRRTFVQNRVRVQGNMMLLTFVFFPIINFMIIIGTDQKFGVLSSEGAKKVVQETVTVQAKRGTIYDRNGTALAVDSTTYSIYAI
GAS       MRKQKRVLDYVVRDRRTFVQNRVRVQGNMMLLTFVFFPIINFMIIIGTDQKFGVLSSEGAKKVVQETVTVQAKRGTIYDRNGTALAVDSTTYSIYAI
          110     120     130     140     150     160     170     180     190     200
          LDKSPVSADEKLYVQPSQYETVADILKHLGKMKKTDVILKQKRRGLFQVSPGSGSISYSTMTIQKAMEDAKIKGLAFTTSPGRMYPMGTFASEFIFG
          LDKSPVSADEKLYVQPSQYETVADILKHLGKMKKTDVILKQKRRGLFQVSPGSGSISYSTMTIQKAMEDAKIKGLAFTTSPGRMYPMGTFASEFIFG
          210     220     230     240     250     260     270     280     290     300
          LASLTDKKTGVKSLVKGSGLEASFDKILSGDGVITYQKDRNGWTLGKTKVKAADGDIYTLSEPIQTFLETQMDIFQAKSNGKLAATLVNAKT
          LASLTDKKTGVKSLVKGSGLEASFDKILSGDGVITYQKDRNGWTLGKTKVKAADGDIYTLSEPIQTFLETQMDIFQAKSNGKLAATLVNAKT
          LASLTDKKTGVKSLVKGSGLEASFDKILSGDGVITYQKDRNGWTLGKTKVKAADGDIYTLSEPIQTFLETQMDIFQAKSNGKLAATLVNAKT
          310     320     330     340     350     360     370     380     390     400
          GEILATTQRPYTNADTLKGLNKDYKYSALHQQNFEPGSTMKVMTLAAIDDKVFNPNETFSNANGLTIADATIQDWAINEGISTGYMVAQGFAPSS
          GEILATTQRPYTNADTLKGLNKDYKYSALHQQNFEPGSTMKVMTLAAIDDKVFNPNETFSNANGLTIADATIQDWAINEGISTGYMVAQGFAPSS
          GEILATTQRPYTNADTLKGLNKDYKYSALHQQNFEPGSTMKVMTLAAIDDKVFNPNETFSNANGLTIADATIQDWAINEGISTGYMVAQGFAPSS
          410     420     430     440     450     460     470     480     490     500
          NVGKTLKQKMGNAKMNWILKFRFGPFTRFGLQDEYAGHLPSDNIIVQAMSAFGQISVTOAQMLRAPTAISNDGEMLEPQFVSOIYDPMTASFRTAK
          NVGKTLKQKMGNAKMNWILKFRFGPFTRFGLQDEYAGHLPSDNIIVQAMSAFGQISVTOAQMLRAPTAISNDGEMLEPQFVSOIYDPMTASFRTAK
          NVGKTLKQKMGNAKMNWILKFRFGPFTRFGLQDEYAGHLPSDNIIVQAMSAFGQISVTOAQMLRAPTAISNDGEMLEPQFVSOIYDPMTASFRTAK
          510     520     530     540     550     560     570     580     590     600
          EVVGFVSKRAASFRYMIQVGTDFEFTLYSKTGFIIKVGDLPAVKSQTAQIGADSGSQDGGLTNYVSVVAVPADKDFVHYVMTQPEHFG
          EVVGFVSKRAASFRYMIQVGTDFEFTLYSKTGFIIKVGDLPAVKSQTAQIGADSGSQDGGLTNYVSVVAVPADKDFVHYVMTQPEHFG
          EVVGFVSKRAASFRYMIQVGTDFEFTLYSKTGFIIKVGDLPAVKSQTAQIGADSGSQDGGLTNYVSVVAVPADKDFVHYVMTQPEHFG
          610     620     630     640     650     660     670     680     690     700
          PLFWQDVNPNVLEAYLMDQTLTRPVAADAKHTTYQLPDPFGKMPGETSSELRNVLQPVVLGTGSKIKKISQKAKGKLSENQQLILSDRFTLEPDMY
          PLFWQDVNPNVLEAYLMDQTLTRPVAADAKHTTYQLPDPFGKMPGETSSELRNVLQPVVLGTGSKIKKISQKAKGKLSENQQLILSDRFTLEPDMY
          PLFWQDVNPNVLEAYLMDQTLTRPVAADAKHTTYQLPDPFGKMPGETSSELRNVLQPVVLGTGSKIKKISQKAKGKLSENQQLILSDRFTLEPDMY
          710     720     730     740     750
          GWTKSNVKTFAKWTGIDVIFKGSDSGRVTKQSLDVGKSLKIKKITTILGD
          GWTKSNVKTFAKWTGIDVIFKGSDSGRVTKQSLDVGKSLKIKKITTILGD
          GWTKSNVKTFAKWTGIDVIFKGSDSGRVTKQSLDVGKSLKIKKITTILGD
    
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