Reduced *in vitro* susceptibility of *Streptococcus pyogenes* to beta-lactam antibiotics associated with mutations in the *pbp2x* gene is geographically widespread

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Running head: *pbp2x* mutations and altered beta-lactam susceptibility

ABSTRACT

Recently two related *Streptococcus pyogenes* strains with reduced susceptibility to ampicillin, amoxicillin and cefotaxime, antibiotics commonly used to treat *S. pyogenes* infections were reported. The two strains had the same nonsynonymous (amino acid-substituting) mutation in the *pbp2x* gene encoding penicillin-binding protein 2X (PBP2X). This concerning report led us to investigate our library of 7,025 genome sequences of type *emm1*, *emm28*, and *emm89 S. pyogenes* clinical strains recovered from intercontinental sources for mutations in *pbp2x*. We identified 137 strains that combined had 37 nonsynonymous mutations in 36 codons in *pbp2x*. Although to a lesser magnitude than the two previously published isolates, many of our strains had decreased susceptibility *in vitro* to multiple beta-lactam antibiotics. Many *pbp2x* mutations were found only in single strains, but 16 groups of two or more isolates of the same *emm* type had an identical

amino acid replacement. Phylogenetic analysis showed that with one exception, strains of the same *emm* type with the same amino acid replacement were clonally related by descent. This finding indicates that strains with some amino acid changes in PBP2X can successfully spread to new human hosts and cause invasive infections. Mapping of the amino acid changes onto a three-dimensional structure of the related *Streptococcus pneumoniae* PBP2X suggests that some substitutions are located in regions functionally important in related pathogenic bacterial species. Decreased beta-lactam susceptibility is geographically widespread in strains of numerically common *emm* gene subtypes. Enhanced surveillance and further epidemiological and molecular genetic study of this potential emergent antimicrobial problem are warranted.

KEYWORDS: population genomics, bioinformatics, antibiotic resistance, whole genome sequencing, public health

INTRODUCTION

Generations of microbiologists, physicians and others with strong interests in infectious diseases have been taught that *Streptococcus pyogenes* (group A streptococcus, GAS) is universally susceptible to beta-lactam antimicrobial agents (1). Although the molecular basis for this resilient phenotype is unknown, given the global disease burden of greater than 700 million cases annually (2), universal susceptibility to these agents has been fortunate. Recently, Vannice et al. (3) identified two clonally related and epidemiologically linked strains of rare type

93 *emm43.4 S. pyogenes* that had eight-fold higher MICs for ampicillin and amoxicillin,
94 and three-fold higher MICs for cefotaxime, indicating decreased susceptibility to
95 these antibiotics. The two strains had an identical single nonsynonymous (amino
96 acid- altering) mutation in the *pbp2x* gene encoding penicillin-binding protein 2X
97 (PBP2X). This mutation confers a threonine to lysine replacement at amino acid 553
98 (Thr553Lys), a polymorphism that was not found in susceptible strains of type
99 *emm43.4*. The authors suggested that the Thr553Lys replacement may be a first step
100 toward *S. pyogenes* evolving resistance to beta-lactam antibiotics. The genomes of
101 these two strains were sequenced as part of an outbreak investigation being done
102 by Public Health-Seattle & King County in collaboration with the Centers for Disease
103 Control and Prevention (CDC). Standard analysis conducted by the CDC GAS genome
104 sequencing includes identification of features potentially contributing to antibiotic
105 resistance including PBP2X variants (4). Through this process the *pbp2x* missense
106 mutations associated with decreased antibiotic susceptibility reported by Vannice
107 et al. were detected. The identification of these two strains is concerning and may
108 signal a substantial public health problem because beta-lactams remain the frontline
109 treatment globally for the majority of GAS infections.
110 To assess the potentially unrecognized broader extent of this inauspicious
111 discovery, we felt compelled to interrogate our library of 7,025 *S. pyogenes* genome
112 sequences of type *emm1*, *emm28*, and *emm89* clinical isolates from intercontinental
113 sources for nonsynonymous mutations in *pbp2x*. Bioinformatic analysis identified
114 137 strains with 37 amino acid changes at 36 sites in *pbp2x* that could alter MIC
115 values for beta-lactam antibiotics. A subset of strains with *pbp2x* mutations was

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analyzed for beta-lactam MIC values using the gradient method (Etest strips). Our results indicate that clinical isolates with *pbp2x* mutations associated with small decreases in beta-lactam susceptibility in this common human-specific pathogen are more widespread than appreciated. Enhanced surveillance and fuller epidemiologic-al and molecular genetic study of this potentially emergent antimicrobial problem are warranted.

MATERIALS AND METHODS

***S. pyogenes* strains and whole-genome sequence data.** The *emm1* (*n* = 3,615), *emm28* (*n* = 2,095), and *emm89* (*n* = 1,315) *S. pyogenes* strains we studied have been described in our previous publications (5-8). The genome sequence data generated with Illumina instruments have been deposited previously in publicly available databases in the National Center for Biotechnology Information Sequence Read Archive (Bioprojects: PRJNA236767, PRJNA434389, PRJNA287922, PRJNA387243). Nucleotide polymorphisms in the *pbp2x* gene in these strains were identified by bioinformatics methods that have been extensively described previously (5).

Antibiotic susceptibility determinations. Forty-two strains with nonsynonymous mutations in *pbp2x* were tested for potential decreased susceptibility to penicillin by plating on tryptic soy agar supplemented with 6 ng/ml penicillin G (benzylpenicillin) or 15 ng/ml ampicillin (9, 10). These strains represent a diverse array of organisms with distinct *pbp2x* mutations from *emm1*,

emm28, and *emm89* organisms. Six reference strains lacking *pbp2x* mutations (wild-type strains; one strain of *emm1*, three strains of *emm28* and two strains of *emm89*) were included as PBP2X consensus wild-type comparators. The reference strains are *emm* type- and genetic clade-matched, have the most common allele representative of their genetic background for global transcriptional regulators of known virulence factors and several have been extensively studied both *in vitro* and in animal virulence experiments. The plates were incubated overnight at 37° C in 5% CO_2, and growth was assessed as present or absent. Minimal inhibitory concentration (MIC) values for six beta-lactam antibiotics (ampicillin, penicillin G, cefotaxime, cefoxitin, ceftazidime, and meropenem) were determined by the gradient method (Etest strips, BioMerieux) using standard clinical laboratory procedures. MIC values were scored independently by three investigators. Some strains were also tested for penicillin G and ampicillin susceptibility by broth microdilution in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). Liquid cultures were incubated overnight at 37° C in 5% CO_2, and growth was determined by optical density at 600 nm.

Phylogenetic analysis of whole genome sequence data. Phylogeny among the strains was inferred by Neighbor-Joining based on concatenated sequential core chromosomal SNPs by methods described previously (5). To constrain inferences to predominantly vertically acquired SNPs, regions of recombination were predicted based on entire core genome sequences using Gubbins, and putatively horizontally acquired SNPs were excluded. Clades of related strains were defined using

hierarchical Bayesian Analysis of Population Structure (hierBAPS), also as previously described (5).

Construction of isogenic strain MGAS27213-PBP2X-WT. Isogenic strain 27213-PBP2X-WT was constructed by replacing the naturally-occurring mutant *pbp2x* gene (Pro601Leu) of clinical isolate MGAS27213 with the wild-type allele encoding Pro601 using procedures previously described (11). Briefly, wild-type *pbp2x* of strain MGAS27566 was amplified by PCR using primers pbp2x-1 (5'-GTGAATACATGCGATAGGAGAACTCCAG-3') and pbp2x-2 (5'-CAATTGTACATTGATTCGCCAACTAAGTC-3'). The PCR amplicon was cloned into suicide vector pBBL740 and then transformed into parental strain MGAS27213, encoding the mutant *pbp2x* allele (Pro601Leu). Whole genome sequencing of isogenic strain MGAS27213-PBP2X-WT confirmed that the mutant *pbp2x* allele was replaced by the wild-type *pbp2x* allele, and the constructed strain lacked spurious mutations.

PBP2X structure modeling. The crystal structure of PBP2X from *Streptococcus pneumoniae* (PDB: 1RP5, chain A) was used to map the location of the amino acid substitutions relative to the active site of the transpeptidase domain. This structure was used because the structure of *S. pyogenes* PBP2X has not been determined. The two PBP2X proteins are well conserved in both amino acid sequence (54.1% identical, 82.1% similar) and structural-fold, and PBP2X from *S. pneumoniae* has been well studied by several investigators (12-16). The *S. pyogenes*

amino acid substitutions were mapped onto the *S. pneumoniae* PBP2X structure using Chimera (17). Chimera was also used to align PBP2X with PBP3 from *Pseudomonas aeruginosa* (PDB: 6UN3) and PBP2a from *Staphylococcus aureus* (PDB: 1VQQ, chain A) to assign the role of each residue in relation to PBP2X within *S. pyogenes*.

RESULTS

Identification of PBP2X amino acid replacements. To test the hypothesis that *S. pyogenes* strains in our international collection of human clinical isolates contained polymorphisms in the *pbp2x* gene, we interrogated the population genomic data generated in our previous studies of *emm1*, *emm28*, and *emm89* organisms (5-8). The vast majority of these strains were recovered from a normally-sterile site of patients with invasive infections such as bacteremia and necrotizing fasciitis. Among the 7,025 whole genome sequences examined, we identified 137 strains that in the aggregate had 37 nonsynonymous mutations altering 36 codons of the 2,259-nucleotide *pbp2x* gene. We also identified 161 strains with a synonymous single nucleotide polymorphism (that is, a silent mutation that would not alter the amino acid sequence of PBP2X) at 10 positions, each in a separate codon of *pbp2x*. Thus, 79% of SNP sites resulted in an amino acid replacement, a significantly greater percentage than expected by chance alone (for the 48 *pbp2x* alleles in the cohort by the Nei-Gojobori method the ratio of rates of nonsynonymous/synonymous site substitutions, Ka/Ks = 1.49 with Fisher P-value = 5.27e-41). This elevated percentage of nonsynonymous mutations is consistent with

the effect of positive selection acting on *pbp2x*. Among the strains with nonsynonymous mutations, with a single exception, each of the 137 strains had only one amino acid replacement relative to the consensus wild-type PBP2X sequence. The exception was an *emm28* strain (MGAS28532) recovered in the United States that had a unique combination of two contiguous amino acid replacements (Phe599Tyr and Gly600Asp) in PBP2X (**Table 1**). Of note, none of the 7,025 isolate sequences interrogated had an insertion or deletion mutation in *pbp2x* indicating that the peptidoglycan transpeptidase function of PBP2X is essential. This finding is consistent with the results of saturating transposon mutagenesis screens that during library generation also failed to recover strains with integrations in *pbp2x* (18, 19).

The analysis identified four sites that had the same amino acid replacement (Gly288Ser, Met342Ile, Gly600Asp, and Pro601Leu) present in multiple *emm* types (**Fig. 1**). In each case, these amino acid replacements were represented among strains of type *emm28* and *emm89* (**Fig. 1**). Additionally, these four replacements were present among multiple isolates within a single *emm* type. The finding of the same replacements in both multiple *emm* types and in multiple isolates of the same *emm* type strongly suggests that these changes have been selected by exposure to beta-lactam antibiotics. In contrast, there was no example of sharing of amino acid replacements between type *emm1* strains and either *emm28* or *emm89* strains. Despite the *emm1* cohort comprising the greatest number of isolates (*n* = 3,615) it had a lower frequency of nonsynonymous SNP sites (*n* = 8) than either the *emm28* (2,095 isolates and 21 nonsynonymous sites) or *emm89* (1,315 isolates and 12 sites)

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cohorts. Moreover, the nonsynonymous SNP sites among the *emm1* isolates differ in distribution compared to the *emm28* and *emm89* isolates, being among the *emm1* isolates somewhat less prevalent in the middle (i.e. the transpeptidase domain) and more prevalent toward the 3' end of *pbp2x* (i.e. the PASTA domains).

An alignment of PBP2X of *S. pyogenes*, *S. pneumoniae*, and *S. agalactiae* shows that the Gly residue at position 288 and Met residue at position 342 are conserved among the three species (**Fig. 2**). Of note Met342 is located in the conserved SxxK motif containing the transpeptidase activity catalytic Ser residue.

Phylogenetic analysis of strains of the same *emm* type with the identical PBP2X amino acid replacement using whole genome sequence data. We identified 16 instances in which two or more strains of the same *emm* type had the identical amino acid replacement (**Table 1** and **Fig. 1**). In general, strains of the same *emm* type with the identical *pbp2x* nonsynonymous mutation were identified in only one country, although a few exceptions to this were identified (**Table 1** and see Discussion). We tested the hypothesis that the strains with the same amino acid change were clonally related. This matter is important to address for public health and basic science reasons because if these organisms are clonally related it is unambiguous evidence that they can disseminate successfully to new human hosts and cause infections. Phylogenetic analysis of whole genome sequence data showed that with one exception, strains of the same *emm* type with the identical amino acid replacement are closely related, likely as a consequence of descent from a common progenitor (**Fig. 3**). The one exception is the 22 *emm28* strains with a Gly600Asp

replacement (**Fig. 3**). These findings indicate multiple independent evolutionary origins of the Gly600Asp polymorphism, that is, multiple episodes of evolutionary convergence. We note that the single strain with the combined Phe599Tyr and Gly600Asp replacement is very closely related to two strains having only the single Gly600Asp change. This phylogenetic relationship suggests that the Phe599Tyr amino acid change was acquired (likely by selection) after the Gly600Asp change occurred in a progenitor. Consistent with this idea, the dual amino acid replacement strain was isolated in 2006, years after the genetically related *emm28* strains with only the Gly600Asp replacement were initially found.

Association of PBP2X amino acid replacements with decreased susceptibility to beta-lactam antibiotics. We next tested the hypothesis that the PBP2X amino acid replacements are associated with decreased susceptibility to beta-lactam antibiotics. Strains were streaked onto tryptic soy agar plates supplemented with 6 ng/ml of penicillin G or 15 ng/ml ampicillin, and the plates were incubated overnight. These concentrations were previously determined to be minimally inhibitory for *S. pyogenes* (9, 10). Wild-type strains that lacked *pbp2x* mutations did not grow after overnight incubation in the presence of these beta-lactam antibiotics. In contrast, many strains with *pbp2x* nonsynonymous mutations grew well on both antibiotic-containing media, including organisms with Gly288Ser, Met342Ile, Phe599Tyr plus Gly600Asp, Gly600Asp, and Pro601Leu amino acid replacements (**Table 1, Fig. 1 and Fig. 3).** Of note, in contrast to the five strains with the Pro601Leu replacement, the single *emm89* strain (MGAS27308) with the

277 Pro601Ser change did not grow in the presence of either antibiotic under the plating
278 conditions tested. Similarly, none of the 10 *emm1* strains representing 8 different
279 amino acid replacements grew under the antibiotic conditions tested. The data are
280 consistent with the hypothesis of an association between some naturally occurring
281 *pbp2x* mutations and decreased susceptibility to these beta-lactams in some genetic
282 backgrounds. We next used Etest strips to determine the MICs for penicillin G and
283 found that many strains with *pbp2x* mutations had decreased susceptibility to this
284 agent as tested in this fashion, whereas all 18 wild-type comparator strains lacking
285 *pbp2x* mutations were fully susceptible (**Table 1**).

286 It is well known that the same PBP2X amino acid replacement can confer
287 divergent susceptibility phenotypes to different beta-lactam antibiotics. Thus, we
288 next performed MIC susceptibility testing with five additional beta-lactam
289 antibiotics (ampicillin, cefotaxime, cefoxitin, ceftazidime and meropenem) using the
290 Etest gradient method. We found that compared to the wild-type control strains,
291 many PBP2X mutant strains had reduced susceptibility to one or more beta-lactam
292 antibiotics (**Table 1**). The Etest MIC results for penicillin G and ampicillin were
293 confirmed for some strains using broth microdilution, and penicillin G and
294 ampicillin agar (**Table 1, Fig 4 and supplemental Fig. S1**). Of note, strains with the
295 Pro601Leu amino acid change, which occurs in both *emm28* and *emm89* strains, had
296 the highest MIC measurements against all beta-lactam antibiotics tested except
297 cefoxitin (**Table 1**). Specifically, the penicillin G MICs of strains with the Pro601Leu
298 change ranged between 23 ng/ml to 32 ng/ml, approximately 4- to 5-fold higher
299 than those of strains with the wild-type PBP2X (**Table 1**). To unambiguously

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demonstrate that the Pro601Leu PBP2X amino acid replacement was responsible for the altered MICs, we created an isogenic strain containing the wild-type *pbp2x* gene in place of the naturally occurring mutant *pbp2x* allele (encoding Pro601Leu). As expected, the isogenic Pro601 strain (i.e. PBP2X consensus wild-type engineered derivative strain) was more susceptible to beta-lactam antibiotics than the naturally occurring parental Pro601Leu substitution strain (**Fig. 4**). Also, the strain with both Phe599Tyr and Gly600Asp amino acid replacements had MIC measurements that were equal to or greater than strains with only the Gly600Asp change, suggesting that this dual amino acid replacement may have an additive effect on MICs

Relative location of amino acid changes in the PBP2X three-dimensional structure. To assess the potential consequence of the identified amino acid replacements on PBP2X, we mapped the location of the changes on a crystal structure available for *S. pneumoniae* PBP2X (**Fig. 5).** The structure of this protein has been well studied by several investigators because of its importance in beta-lactam resistance in this common human pathogen (12-14, 16). The variant amino acids at positions 342, 599, 600, and 601 mapped to regions known to influence structure-function relationships (20, 21). This determination of influence was derived from overlaying PBP2X from *S. pneumoniae* with the clinically relevant and structurally similar *P. aeruginosa* PBP3 (PDB: 6UN3) and *S. aureus* PBP2a (PDB: 1VQQ, chain A). Recently, it was discovered that residues on the bottom of the α-8 helix of PBP3 are essential in forming an aromatic pocket (20) comprised of Tyr532 and Phe533. This aromatic pocket is key in binding and stabilizing the side-chains of

beta-lactam antibiotics. In PBP2X, a similar, conserved aromatic pocket is formed with the neighboring His594 and Tyr595 residues. The amino acid substitutions at residues 599, 600, and 601 we observed in the clinical *S. pyogenes* isolates studied here are located directly above this aromatic pocket (**Fig. 5**). Substitutions at these positions may perturb binding interactions between the beta-lactam antibiotic and PBP2X and thereby decrease the acylation efficiency of the antibiotics leading to reduced susceptibility. It is noteworthy that the Pro601Leu substitution was associated with decreased penicillin G susceptibility, but the Pro601Ser substitution was not. The serine side chain is relatively small and hydrophilic, whereas that of leucine is larger and hydrophobic, and both differ from proline, a secondary (i.e. an imino acid) amino acid. Thus, both serine and leucine could potentially perturb the PBP2X structure and beta-lactam binding interactions but to a different extent, possibly leading to the differences in susceptibility observed.

Similarly, the Met342Ile substitution is noteworthy because residue 342 is located directly within the active site pocket of PBP2X near the catalytic Ser337. In PBP2X, a methionine cluster is conserved within the active site (15). Thus, the Met342Ile substitution present in the clinical *S. pyogenes* isolate may disrupt the conserved methionine cluster and thereby perturb binding and acylation of the enzyme by beta-lactam antibiotics. Decreased acylation efficiency, in turn, would explain the reduced beta-lactam susceptibility associated with clinical *S. pyogenes* isolates containing amino acid variants at position 342. In contrast, the Gly288Ser substitution is not located in a position to directly impact substrate binding or acylation (**Fig. 5**). Indicative of this, the Cα of residue 288 is 17.5 Å from the Oγ of

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the catalytic Ser337 in the *S. pneumoniae* PBP2X structure. Regardless, the substitution could detrimentally impact enzyme dynamics or stability to alter function. A more definitive conclusion on how the Gly288Ser substitution alters enzyme structure and function awaits further work.

DISCUSSION

Here we report on 137 strains of *S. pyogenes* from intercontinental sources that have 37 amino acid replacements at 36 sites in the PBP2X protein, some of which correlate with decreased susceptibility to beta-lactam antibiotics under the conditions tested. Importantly, none of the mutations we identified resulted in resistance *in vitro* to any of the six beta-lactams studied (as defined by CLSI) and none approached the level for ampicillin or cefotaxime MICs described for the PBP2X substitution Thr553Lys. This substitution evidently conferred an MIC at the CLSI-determined breakpoint for nonsusceptibility to ampicillin. However, isogenic mutant strains were not constructed to prove the mutant allele of *pbp2x* was solely responsible for the altered MIC value. This is an important point, because the two strains described by Vannice et al. also contain a Ser79Phe amino acid replacement in the topoisomerase ParC. Substituions in ParC can confer resistance to fluoroquinolone antibiotics. In principle, substitutions in ParC could produce a slowed growth phenotype potentially contributing to the altered beta-lactam MICs observed. As described by Vannice et al., all five *emm43.4* strains they analyzed also have a Thr236Ala amino acid replacement in a gene annotated as "glycoside hydrolase family 25." The only known enzymatic activity of the family 25 glycoside

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hydrolases is that of a lysozyme muramidase. Members of this protein family participate in peptidoglycan remodeling, and thus, in principle the Thr236Ala substitution might also contribute to the altered beta-lactam MIC. Clearly, much more work using isogenic mutant strains is required to deconvolute the role of specific amino acid replacements in these proteins to the observed altered MICs.

Our research was stimulated by the recent description of two clonally related type *emm43.4 S. pyogenes* strains with the same Thr553Lys amino acid replacement in PBP2X associated with altered MICs to beta-lactam antibiotics (3). We note that the Thr553Lys change likely reflects very recent antimicrobial selection in view of the described course of treatment of the two host patients. Our work was made possible in part by the availability of 7,025 genome sequences from geographically dispersed strains of types *emm1*, *emm28*, and *emm89* that we previously generated for molecular pathogenesis, population genomic and epidemiologic purposes. This unique resource permitted us to rapidly identify strains with mutations in the *pbp2x* gene by bioinformatic methods and subsequently assess the beta-lactam susceptibility phenotypes by standard clinical microbiology methods. Our findings indicate that decreased beta-lactam susceptibility associated with some PBP2X amino acid polymorphisms in this pathogen is geographically widespread and has arisen multiple times independently over many years in contemporary epidemic clones of serotype *emm1*, *emm28* and *emm89* GAS (Fig 2). The data support the interpretation that the nonsynonymous mutations have been selected by exposure to beta-lactam antibiotics used during treatment of infections caused by *S. pyogenes*. However, inasmuch as *S. pyogenes* can be carried asymptomatically in the upper

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392 respiratory tract or other anatomic site, it is also possible that selection occurred
393 during antibiotic treatment of an asymptomatic carrier for an infection caused by
394 another organism. The potential for reduced beta-lactam susceptibility to confer an
395 advantage during human infections (either invasive or non-systemic infection such
396 as pharyngitis or localized skin and soft tissue infections), or asymptomatic carriage
397 remains untested. More study is required to address these important issues.
398
399 **Examples of convergent evolution.** Several examples of convergent
400 evolution to decreased beta-lactam susceptibility were identified in this analysis.
401 Four instances of the presence of the same otherwise rare single amino acid
402 polymorphism in strains of different *emm* types were found. We identified strains of
403 *emm28* and *emm89* with each of the following amino acid replacements: Gly288Ser,
404 Met342Ile, Gly600Asp, or Pro601Leu. Given that strains of *emm28* and *emm89* are
405 very distantly related genetically, the only reasonable interpretation is that these
406 polymorphisms arose independently as a consequence of convergent evolution,
407 presumably due to selection following exposure to a beta-lactam antibiotic.
408 Similarly, the occurrence of the Gly600Asp replacement in some *emm28* strains that
409 have not shared a recent common ancestor serves as another clear example of
410 convergent evolution in *pbp2x*. As further evidence of convergent evolution,
411 Chochua et al. also found the Pro601Leu amino acid change in multiple unrelated
412 GAS lineages, including *emm4* and *emm75* isolates containing it as a single amino
413 acid replacement and *emm87* and *emm89* isolates containing it in combination with
414 a second substitution (4).

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Why is there an apparent difference between *emm1* strains and *emm28* and *emm89* strains? The majority of strains we identified with *pbp2x* nonsynonymous mutations are either type *emm28* or type *emm89* subclade 3 organisms. In addition, the linear location of PBP2X amino acid replacements differ in *emm1* compared to the *emm28* and *emm89* strains. We believe there are several factors that may contribute to these differences. First, it is important to note that essentially all *emm28* organisms do not produce hyaluronic acid capsule as a consequence of having an insertion of an adenine nucleotide after nucleotide 219 in an A-T rich region (7). This single nucleotide insertion severely truncates *hasA*, whose gene product is required for capsule biosynthesis. Similarly, *emm89* organisms of subclade 3 fail to make hyaluronic acid capsule because they lack the *hasABC* operon required for capsule biosynthesis (5, 22). The hyaluronic capsule among other interactions with the host, contributes to the capacity of *S. pyogenes* to resist phagocytosis. It is possible that a relationship exists between inability of a *S. pyogenes* strain to produce hyaluronic acid capsule and likelihood of generating a strain that is extant and has PBP2X amino acid changes that result in decreased beta-lactam susceptibility. Second, it is possible that the *in vivo* regulation and/or expression of *pbp2x* differ between strains of distinct clonal backgrounds. A third possibility is that for unknown reasons *emm1* strains with PBP2X amino acid replacements are simply less fit *in vivo* compared to *emm28* and *emm89* strains. A fourth possibility is that the *in vivo* topology of PBP2X differs between *emm1* and *emm28* and *emm89* strains, perhaps due to interaction with other currently

unknown proteins. However, all of these ideas are speculative and require more study to address these important observations. Finally, we note that the possibilities described above are not mutually exclusive.

Relationship of our findings to those reported for other pathogenic beta-hemolytic streptococci. Strains of *Streptococcus agalactiae* and *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) with decreased susceptibility to beta-lactam antibiotics have been reported (23-39). In the case of SDSE, four isolates cultured from the blood of three epidemiologically-associated patients were reported to be resistant to penicillin and oxacillin (25). Whole genome sequencing identified nonsynonymous mutations in PBP2X that were thought to be causally involved in the resistance phenotype. In particular, the investigators identified the occurrence of Thr341Pro and Gln555Glu amino acid replacements and noted that these two changes are located close to positions 337, 547 and 557 that are among the more prevalently found variant sites reported for penicillin-resistant *S. pneumoniae*. As described above, we identified decreased susceptibility in *S. pyogenes* strains with an amino acid change at position 342 (Met342Ile), and the altered amino acid reported by Vannice et al. was Thr553Lys (3).

Public health implications. Our population genomic analysis indicates that S. *pyogenes* strains with nonsynonymous mutations in *pbp2x* are not exceedingly rare in the *emm1*, *emm28*, and *emm89* organisms we studied, being present in approximately 2% of the collection of 7,025 isolates studied. Although this relatively

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low frequency is fortunate, two facts give us pause. First, the great majority of the isolates we previously characterized by whole-genome sequencing were cultured from patients with invasive episodes. Given the relative lack of strains causing pharyngitis in this sample, coupled with the well-documented treatment failures occurring among individuals with culture-positive *S. pyogenes* pharyngitis (40-48), it is possible that analysis of large samples of strains from pharyngitis patients will identify a different percentage of organisms with *pbp2x* mutations associated with altered susceptibility to beta-lactam antibiotics. Second, we identified strains with PBP2X amino acid changes that are clearly clonally related based on phylogenetic analysis of whole genome data. For example, we recovered clonally related type *emm28* organisms with the Gly600Asp replacement from patients in Canada and five different states in the US, indicating that they can successfully disseminate over geographic distances and cause infections. The same is true for the 27 clonally-related strains of type *emm1* with the Asp734Gly change, organisms causing invasive infections in Denmark, Sweden, and Iceland between 2002 and 2007 (**Table 1**). Taken together, our findings stress the importance of renewed efforts to monitor antimicrobial susceptibility rates and values in this pathogen on an ongoing basis, formulating an efficacious human vaccine against *S. pyogenes*, and the need for expanded vaccine efforts, as noted by many (49-51). Importantly, these needs also were highlighted in the report of a symposium held more than two decades ago dedicated to the topic of lack of penicillin resistance in *S. pyogenes* (1).

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What may the future hold? Although some favor the idea that we are not at the beginning of the end of universal susceptibility of *S. pyogenes* to beta-lactam antibiotics (52), we believe there are multiple reasons to be less sanguine in the long term. First, our data show that several distinct *pbp2x* mutations associated with decreased susceptibility occur in *S. pyogenes* strains of multiple *emm* types. Second, in contrast to the otherwise rare *emm43.4* strains reported by Vannice et al.(3), we identified PBP2X amino acid replacements in strains of types *emm1*, *emm2*8, and *emm89*, which in the aggregate are common causes of *S. pyogenes* pharyngitis and invasive episodes in many countries. Third, some of the organisms with amino acid changes are clonally related and have been recovered in multiple geographic locations, in some cases many years apart. Thus, if strains with PBP2X amino acid replacements have decreased fitness, at least in some cases the deficit is not sufficient to prohibit successful dissemination of some clonal progeny to new hosts and capacity to cause serious human invasive infections. Fourth, exchange of genetic material between *S. pyogenes* strains can produce progeny with altered phenotypes such as enhanced virulence and increased antimicrobial agent resistance. Thus, in principle, there is a risk of gene flow of a mutant *pbp2x* gene to a susceptible strain, a process that could accelerate the spread of decreased susceptibility to beta-lactams or frank resistance in this global human pathogen.

Concluding comment. To summarize, we used our library of 7,025 *S. pyogenes* genome sequences from strains of type *emm1*, *emm28*, and *emm89* to identify amino acid-altering mutations in *pbp2x*. Some of the strains with amino acid

replacements in PBP2X had decreased susceptibility under the laboratory conditions tested to some beta-lactam antibiotics, including the commonly used penicillin G. Although many *pbp2x* mutations occurred in only one or two strains, we found that some PBP2X amino acid replacements were present in multiple clonally-related strains causing infections many years apart. Decreased susceptibility to beta-lactams in *S. pyogenes* is geographically widespread and exists in strains of numerically common *emm* gene subtypes. We recommend increased basic science and translational research attention be applied to this potentially severe public health threat. For example, the availability of an efficacious human vaccine against *S. pyogenes* pharyngitis would significantly decrease use of beta-lactam antibiotic agents globally. We believe that for diagnostic laboratories not currently routinely performing beta-lactam susceptibility testing, it is reasonable to consider doing so, perhaps by measuring penicillin MIC.

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728

729 **[Table and Figures]**

730 **TABLE 1** Summary of available data for 137 *emm1*, *emm28*, and *emm89 S. pyogenes*
731 strains with amino acid replacements in PBP2X.

732

733 **Fig. 1.** Location of PBP2X amino acid replacements identified among the 7,025
734 genomes of *emm1*, *emm28*, and *emm89* clinical isolates. Amino acid replacements
735 identified in multiple strains are highlighted in yellow, with superscripts denoting
736 the number of strains. Replacements identified in both *emm28* and *emm89* strains
737 are in bold and enclosed in brackets. Replacements associated with reduced
738 susceptibility to one or more of the beta-lactam antibiotics tested under the in vitro
739 conditions analyzed are shown in red. The dimerization, transpeptidase and PASTA
740 domains are indicated.

741

742

743 **Fig. 2.** Aligned streptococcal PBP2X sequences. PBP2X of *S. pyogenes* strain
744 MGAS5005 (AAZ51984.1), *S. pneumoniae* (PBP reference sequence WP_050265832)
745 and *S. agalactiae* (PBP reference sequence WP_134808185) were aligned with
746 ClustalW. To facilitate comparisons, for each species, every tenth amino acid is in
747 red. Amino acids of the consensus sequence are highlighted to indicate conserved
748 domains as indicated in the inset, lower right. The three key conserved motifs (SxxK,
749 SxN and KSGT) of the transpeptidase are shown in red and bold below the aligned
750 sequences.

751

752 **Fig. 3.** Genetic relationships among *S. pyogenes emm1*, *emm28*, and *emm89* clinical
753 isolates. Phylogenies were inferred by Neighbor-Joining based on core chromosomal
754 SNPs. Isolates with nonsynonymous SNPs in *pbp2x* are colored according to the

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amino acid replacements in PBP2X as shown in the insets. Clades of more closely related strains are shown by shapes (e.g. circles and squares) as indicated. A) Relationships among 3,615 *emm1* strains. B) Relationships among 2,095 *emm28* strains. C) Relationships among 1,315 *emm89* strains.

Fig. 4. Beta-lactam antibiotic susceptibility assays. A-B) Shown is growth of *emm89* PBP2X wild-type strain MGAS27556 (on plate left) in comparison with PBP2X Pro601Leu amino acid replacement strain MGAS27316 (on plate right) on medium supplemented with 6 ng/ml penicillin G (panel A) or 15 ng/ml ampicillin (panel B). C-D) Graphed is MIC dilution growth of *emm1*, *emm28* and *emm89* PBP2X wild-type and amino acid replacement variant strains in THY broth supplemented with penicillin G (panel C) or ampicillin (panel D). E-F) Graphed is growth of *emm89* PBP2X Pro601Leu replacement strain MGAS27213 and its isogenic PBP2X wild-type engineered derivative in THY broth supplemented with penicillin G (panel E) or ampicillin (panel F). All THY broth growth experiments were done in quadruplicate and the results are given as mean ± SD. Significant differences in growth as determined by Student's t-test at $P < 0.05$ are indicated with *.

Fig. 5. Location of *S. pyogenes* PBP2X substitutions relative to the X-ray crystallography structure of PBP2X from *S. pneumoniae* (PDB: 1RP5, chain A). A) Variant sites influencing structure-function. Illustrated as spheres on the *S. pneumoniae* PBP2X ribbon diagram are the key amino acid replacement sites associated with reduced beta-lactam susceptibility from the *S. pyogenes* clinical

778 isolates with the relative amino acid positions labeled. Shown in red is the
779 transpeptidase catalytic serine residue (Spn residue 337 = Spy residue 340). The
780 amino acids depicted are those of the *S. pneumoniae* PBP2X. B) All variant sites. The
781 relative position of all 36 amino acid replacement sites observed among the 7,025
782 sequenced strains are shown as blue spheres with the catalytic serine shown in red.
783 C) Aromatic pocket. Illustrated is a surface representation showing the aromatic
784 pocket proposed to be involved in binding and stabilizing beta-lactam side chains.
785 The His594 and Tyr595 residues lining the pocket are shown in blue.
786
787

TABLE 1. PBP2X amino acid replacement strains (*n* = 137) and PBP2X wild-type control strains (*n* = 18)

	Strain (MGAS)	*emm type*	Amino acid change	Country	Year	Capsule	Penicillin G agar (a)	Ampicillin agar(b)	Penicillin G MIC (c)	Ampicillin MIC	Cefotaxime MIC	Cefoxitin MIC	Ceftazidime MIC	Meropenem MIC
1	2221	1	Wild-type	Australia	1998	positive	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006
2	23877	1	Wild-type	Canada	2002	positive			0.012*	0.016				
3	24791	1	Wild-type	Finland	2006	positive			0.012*	0.016				
4	7867	28	Wild-type	Canada	1991	negative			0.012*	0.016				
5	8357	28	Wild-type	Finland	1996	negative			0.012*	0.016				
6	10778	28	Wild-type	Canada	1998	negative			0.012*	0.016				
7	10783	28	Wild-type	Canada	1998	negative			0.012*	0.016				
8	11052	28	Wild-type	Finland	2000	negative			0.012*	0.016				
9	27961	28	Wild-type	United States	2005	negative	negative	negative	<0.016	0.016	0.023	1	0.19	0.006
10	28426	28	Wild-type	United States	1999	negative	negative	negative	<0.016	0.016	0.032	1.5	0.19	0.004
11	28737	28	Wild-type	United States	2012	negative	negative	negative	<0.016	0.016	0.032	1.5	0.19	0.003
12	28905	28	Wild-type	United States	2004	negative			0.012*	0.016				
13	23530	89	Wild-type	Italy	1997	weak			0.012*	0.016				
14	26568	89	Wild-type	United States	1996	positive			0.012*	0.016				
15	26645	89	Wild-type	United States	2009	positive			0.012*	0.016				
16	26844	89	Wild-type	United States	2008	negative	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006
17	27545	89	Wild-type	Finland	2010	weak			0.012*	0.016				
18	27566	89	Wild-type	Finland	2011	negative	negative	negative	<0.016	0.016	0.032	1.5	0.19	0.006
19	29554	28	Ile47Val	Finland	2004	negative	negative	negative	<0.016	<0.016	0.016	0.75	0.125	0.004
20	29632	28	Ile47Val	Finland	2002	negative								
21	28315	28	Asp52Gly	United States	1998	negative	negative	negative	<0.016	0.016	0.023	1	0.19	0.004
22	28329	28	Asp52Gly	United States	1998	negative								
23	28433	28	Asp52Gly	United States	2003	negative								
24	28894	28	Asp52Gly	United States	2004	negative								
25	31966	28	Thr70Asn	Norway	2014	negative								
26	5534	1	Gly85Ser	Finland	1995	positive	negative	negative	<0.016	<0.016	0.016	1	0.19	0.006
27	5546	1	Gly85Ser	Finland	1995	positive								
28	5555	1	Gly85Ser	Finland	1995	positive								
29	5556	1	Gly85Ser	Finland	1995	positive								
30	28692	28	Ser92Phe	United States	2008	negative								
31	26637	89	Met171Ile	United States	2009	negative								
32	26639	89	Met171Ile	United States	2009	negative								
33	26667	89	Met171Ile	United States	2009	positive	negative	negative	<0.016	0.016	0.023	1.5	0.25	0.006
34	29128	28	Ala174Val	Finland	2014	negative	negative	negative	<0.016	<0.016	0.012	0.75	0.125	0.003
35	29176	28	Ala174Val	Finland	2013	negative								
36	29178	28	Ala174Val	Finland	2013	negative								
37	29316	28	Ala174Val	Finland	2010	negative								
38	27413	89	Asp233Asn	United States	2011	negative								
39	29408	28	Thr245Ile	Finland	2008	negative	negative	negative	<0.016	<0.016	0.012	0.75	0.125	0.003
40	29254	28	Phe274Leu	Finland	2012	negative	positive	negative	0.016	0.023	0.023	2	0.19	0.006
41	28415	28	Val281Ile	United States	2003	negative								
42	28772	28	Gly288Ser	United States	2012	negative								
43	28773	28	Gly288Ser	United States	2012	negative	positive	positive	0.016	0.023	0.032	1.5	0.25	0.006
44	26860	89	Gly288Ser	United States	2003	positive	positive	positive	0.016	0.023	0.047	2	0.38	0.008
45	26929	89	Gly288Ser	United States	2003	positive	positive	positive	0.016	0.023	0.016	1.5	0.19	0.006
46	26932	89	Gly288Ser	United States	2003	positive	positive	positive	0.016	0.023	0.032	1.5	0.25	0.006
47	27438	89	Gly288Ser	United States	2006	positive	positive	positive	0.016	0.023	0.047	1.5	0.25	0.008
48	28711	28	Thr323Met	United States	2011	negative								
49	10786	28	Met342Ile	Canada	1998	negative	positive	positive	0.016	0.016	0.023	2	0.19	0.004
50	28367	28	Met342Ile	United States	2003	negative								
51	27033	89	Met342Ile	United States	2008	positive	positive	positive	0.023	0.023	0.032	3	0.25	0.006
52	7898	28	Lys422Arg	Canada	1995	negative	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.004
53	7922	28	Lys422Arg	Canada	1996	negative								
54	7973	28	Lys422Arg	Canada	1997	negative								
55	10752	28	Lys422Arg	Canada	1992	negative								

	Strain (MGAS)	*emm type*	Amino acid change	Country	Year	Capsule	Penicillin G agar (a)	Ampicillin agar(b)	Penicillin G MIC (c)	Ampicillin MIC	Cefotaxime MIC	Cefoxitin MIC	Ceftazidime MIC	Meropenem MIC
56	29061	28	Phe425Leu	Finland	2015	negative	positive	positive	0.016	0.016	0.023	2	0.19	0.006
57	29068	28	Phe425Leu	Finland	2015	negative								
58	29093	28	Phe425Leu	Finland	2014	negative								
59	29125	28	Phe425Leu	Finland	2014	negative								
60	29141	28	Phe425Leu	Finland	2014	negative								
61	30068	28	Phe425Leu	Finland	2015	negative								
62	7976	28	Ala438Val	Canada	1997	negative	positive	negative	<0.016	0.016	0.023	1.5	0.19	0.006
63	28782	28	Thr461Pro	United States	2012	negative	positive	negative	0.016	0.016	0.016	1.5	0.125	0.006
64	26899	89	Gln462His	United States	2003	negative	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006
65	25070	1	Thr515Aal	Norway	2004	positive	negative	negative	<0.016	<0.016	0.016	1	0.125	0.003
66	28088	28	Gly521Ser	United States	2010	negative	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.004
67	27590	89	Pro526Leu	Finland	2011	negative	positive	negative	<0.016	0.016	0.023	2	0.25	0.006
68	27612	89	Pro526Leu	Finland	2012	negative	positive	negative	0.016	0.016	0.032	2	0.25	0.008
69	23930	1	Thr535Ile	Canada	2003	positive	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006
70	28532	28	Phe599Tyr and Gly600Asp	United States	2006	negative	positive	positive	0.023	0.032	0.023	1.5	0.19	0.012
71	7873	28	Gly600Asp	Canada	1992	negative								
72	7935	28	Gly600Asp	Canada	1996	negative								
73	7936	28	Gly600Asp	Canada	1996	negative								
74	7937	28	Gly600Asp	Canada	1996	negative								
75	7938	28	Gly600Asp	Canada	1996	negative								
76	7947	28	Gly600Asp	Canada	1996	negative								
77	7948	28	Gly600Asp	Canada	1996	negative								
78	7953	28	Gly600Asp	Canada	1996	negative								
79	7987	28	Gly600Asp	Canada	1997	negative								
80	7989	28	Gly600Asp	Canada	1997	negative								
81	7994	28	Gly600Asp	Canada	1997	negative								
82	8014	28	Gly600Asp	Canada	1999	negative								
83	8015	28	Gly600Asp	Canada	1999	negative								
84	8016	28	Gly600Asp	Canada	1999	negative								
85	10813	28	Gly600Asp	Canada	2000	negative								
86	27982	28	Gly600Asp	United States	2005	negative								
87	28165	28	Gly600Asp	United States	1998	negative								
88	28336	28	Gly600Asp	United States	2000	negative								
89	28380	28	Gly600Asp	United States	2003	negative								
90	28425	28	Gly600Asp	United States	1999	negative	positive	positive	0.016	0.023	0.032	1.5	0.19	0.006
91	28792	28	Gly600Asp	United States	2000	negative	positive	positive	0.016	0.023	0.032	1.5	0.19	0.006
92	27143	89	Gly600Asp	United States	2012	negative	positive	positive	0.016	0.023	0.032	1.5	0.125	0.006
93	27326	89	Gly600Asp	United States	2013	negative	positive	positive	<0.016	0.016	0.023	1	0.094	0.004
94	29393	28	Pro601Leu	Finland	2008	negative	positive	positive	0.023	0.047	0.047	1.5	0.25	0.012
95	26837	89	Pro601Leu	United States	2011	negative								
96	27213	89	Pro601Leu	United States	2012	negative	positive	positive	0.032	0.047	0.064	1.5	0.38	0.012
97	27308	89	Pro601Ser	United States	2013	negative	negative	negative	<0.016	0.016	0.032	1.5	0.25	0.003
98	27316	89	Pro601Leu	United States	2013	negative	positive	positive	0.032	0.047	0.064	1.5	0.38	0.016
99	27453	89	Pro601Leu	United States	2010	negative	positive	positive	0.032	0.047	0.064	1.5	0.38	0.012
100	31677	89	Asp620Tyr	Scotland	2016	negative	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006
101	23875	1	Arg632His	Canada	2002	positive	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006
102	23888	1	Arg632His	Canada	2002	positive								
103	23904	1	Arg632His	Canada	2002	positive								
104	23905	1	Arg632His	Canada	2002	positive								
105	23906	1	Arg632His	Canada	2002	positive								
106	23950	1	Arg632His	Canada	2003	positive								
107	23980	1	Arg632His	Canada	2003	positive								
108	24056	1	Arg632His	Canada	2004	positive								
109	24141	1	Arg632His	Canada	2002	positive								
110	24177	1	Arg632His	Canada	2002	positive								
111	25364	1	Arg632His	Sweden	1998	positive	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006

	Strain (MGAS)	*emm type*	Amino acid change	Country	Year	Capsule	Penicillin G agar (a)	Ampicillin agar(b)	Penicillin G MIC (c)	Ampicillin MIC	Cefotaxime MIC	Cefoxitin MIC	Ceftazidime MIC	Meropenem MIC
112	25387	1	Arg632His	Sweden	2000	positive								
113	25388	1	Arg632His	Sweden	2000	positive								
114	28323	28	Gly647Asp	United States	2001	negative								
115	10792	28	Ser651Gly	Canada	1998	negative								
116	26743	89	Val662Ile	United States	1999	negative								
117	24008	1	Arg692Cys	Canada	2003	positive	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006
118	24019	1	Arg692Cys	Canada	2003	positive								
119	24202	1	Arg692Cys	Canada	1999	positive								
120	24203	1	Arg692Cys	Canada	1999	positive								
121	24212	1	Arg692Cys	Canada	1999	positive								
122	24645	1	Arg692Cys	Sweden	2009	positive								
123	24692	1	Arg692Cys	Sweden	2009	positive								
124	24709	1	Arg692Cys	Sweden	2009	positive								
125	25599	1	Glu695Asp	United States	2010	positive	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006
126	25603	1	Glu695Asp	United States	2010	positive								
127	1350	1	Lys708Arg	Germany (d)	1984	positive	negative	negative	<0.016	0.016	0.023	1.5	0.19	0.006
128	27009	89	Lys730Arg	United States	2007	negative								
129	23858	1	Asp734Gly	Iceland	2006	positive	negative	negative	0.016	0.016	0.023	1.5	0.19	0.004
130	25205	1	Asp734Gly	Sweden	2005	positive								
131	25221	1	Asp734Gly	Sweden	2007	positive	negative	negative	0.016	0.023	0.023	1.5	0.25	0.006
132	26024	1	Asp734Gly	Denmark	2002	positive								
133	26052	1	Asp734Gly	Denmark	2002	positive								
134	26055	1	Asp734Gly	Denmark	2002	positive								
135	26056	1	Asp734Gly	Denmark	2002	positive								
136	26057	1	Asp734Gly	Denmark	2002	positive								
137	26059	1	Asp734Gly	Denmark	2002	positive								
138	26060	1	Asp734Gly	Denmark	2002	positive								
139	26064	1	Asp734Gly	Denmark	2003	positive								
140	26071	1	Asp734Gly	Denmark	2003	positive								
141	26082	1	Asp734Gly	Denmark	2003	positive								
142	26083	1	Asp734Gly	Denmark	2003	positive								
143	26085	1	Asp734Gly	Denmark	2003	positive								
144	26096	1	Asp734Gly	Denmark	2003	positive								
145	26101	1	Asp734Gly	Denmark	2003	positive								
146	26109	1	Asp734Gly	Denmark	2003	positive								
147	26113	1	Asp734Gly	Denmark	2004	positive								
148	26159	1	Asp734Gly	Denmark	2009	positive								
149	26197	1	Asp734Gly	Denmark	2005	positive								
150	26213	1	Asp734Gly	Denmark	2006	positive								
151	26231	1	Asp734Gly	Denmark	2006	positive								
152	26267	1	Asp734Gly	Denmark	2007	positive								
153	26271	1	Asp734Gly	Denmark	2008	positive								
154	26279	1	Asp734Gly	Denmark	2008	positive								
155	26290	1	Asp734Gly	Denmark	2008	positive								

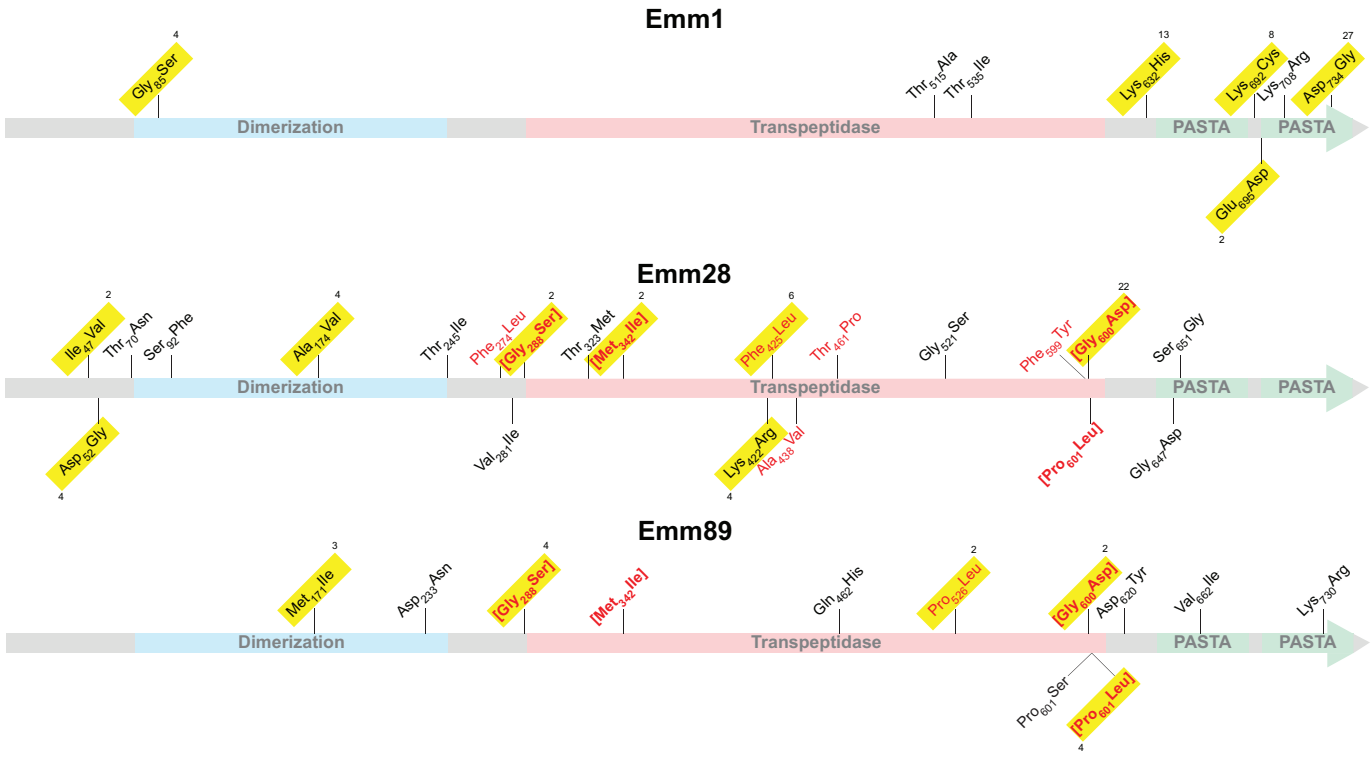
Note:

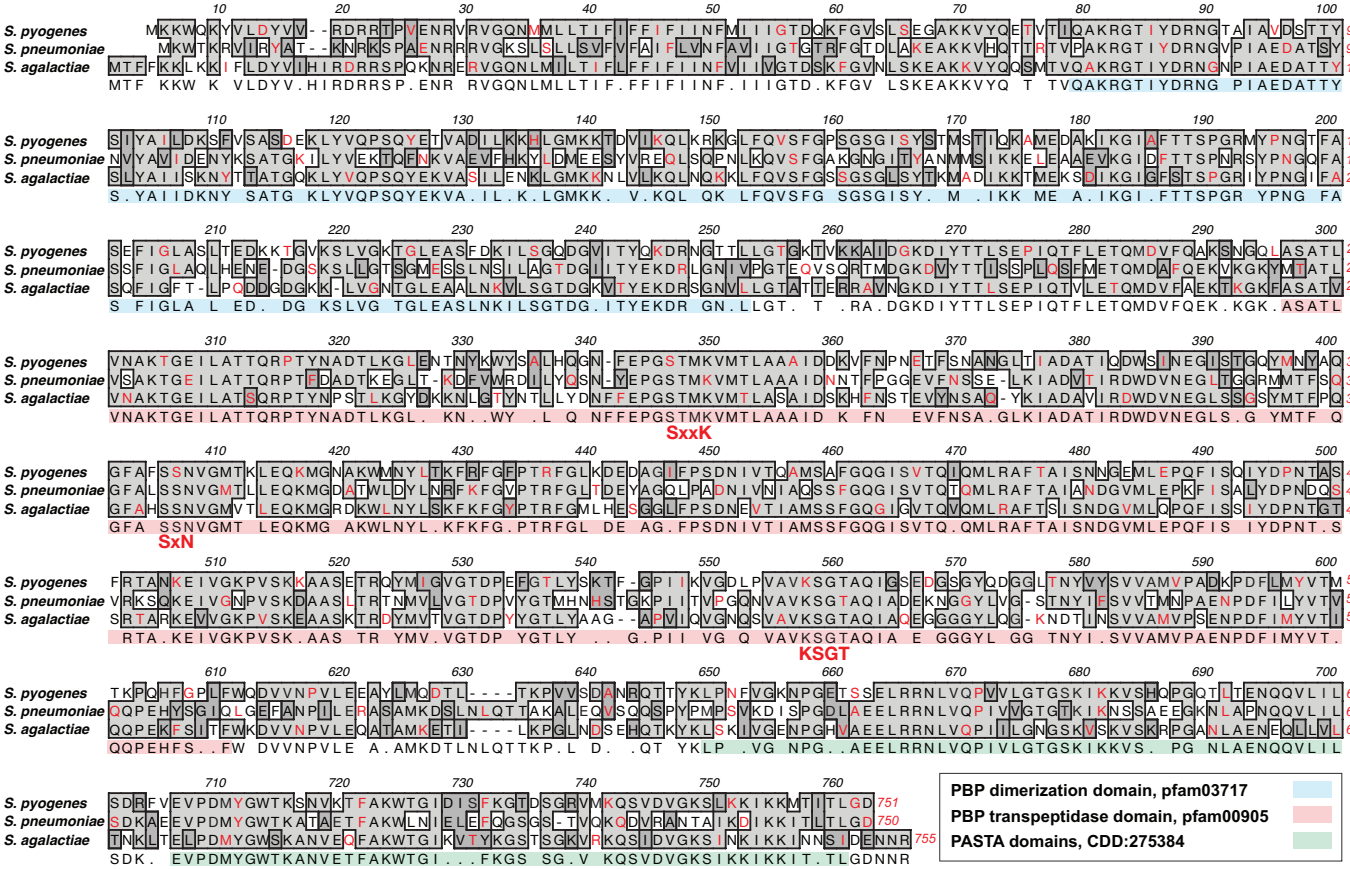
(a) Penicillin G agar is supplemented with 6 ng/mL of benzylpenicillin.

(b) Ampicillin agar is supplemented with 15 ng/mL of ampicillin.

(c) All anitibiotic MICs are given in ug/ml. Penicillin G ssays were done using strips with 2 dose ranges, standard = 0.016-to-256 ug/ml or low = 0.002-to-32 ug/ml, assays done with the low dose range strips are marked with *.

(d) From the German Democratic Republic (former East Germany).





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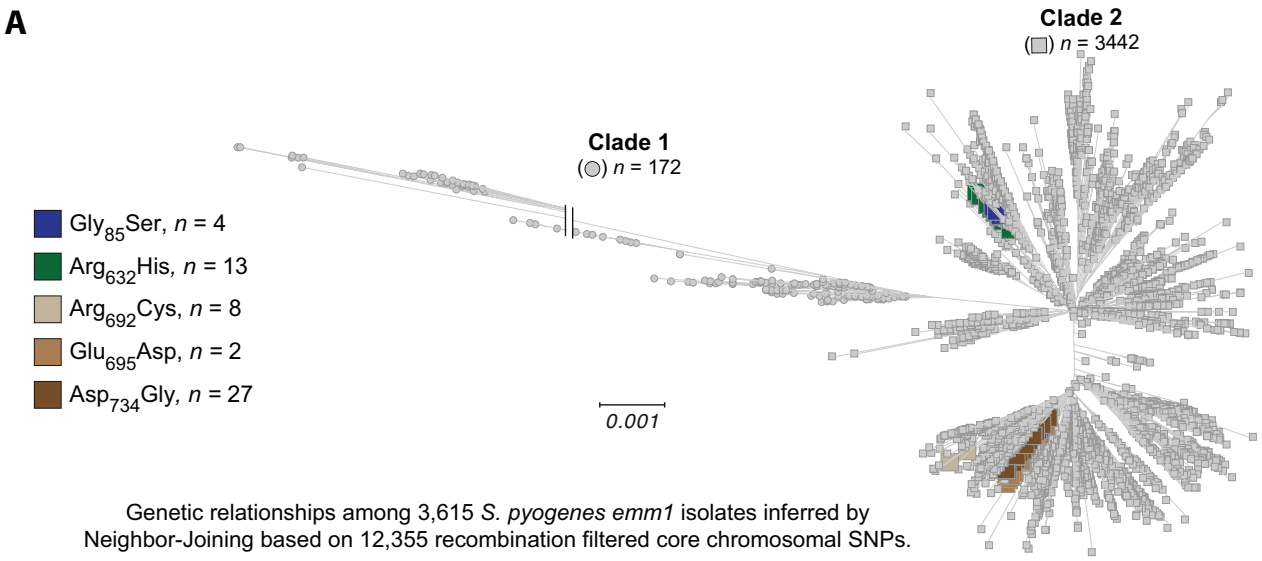
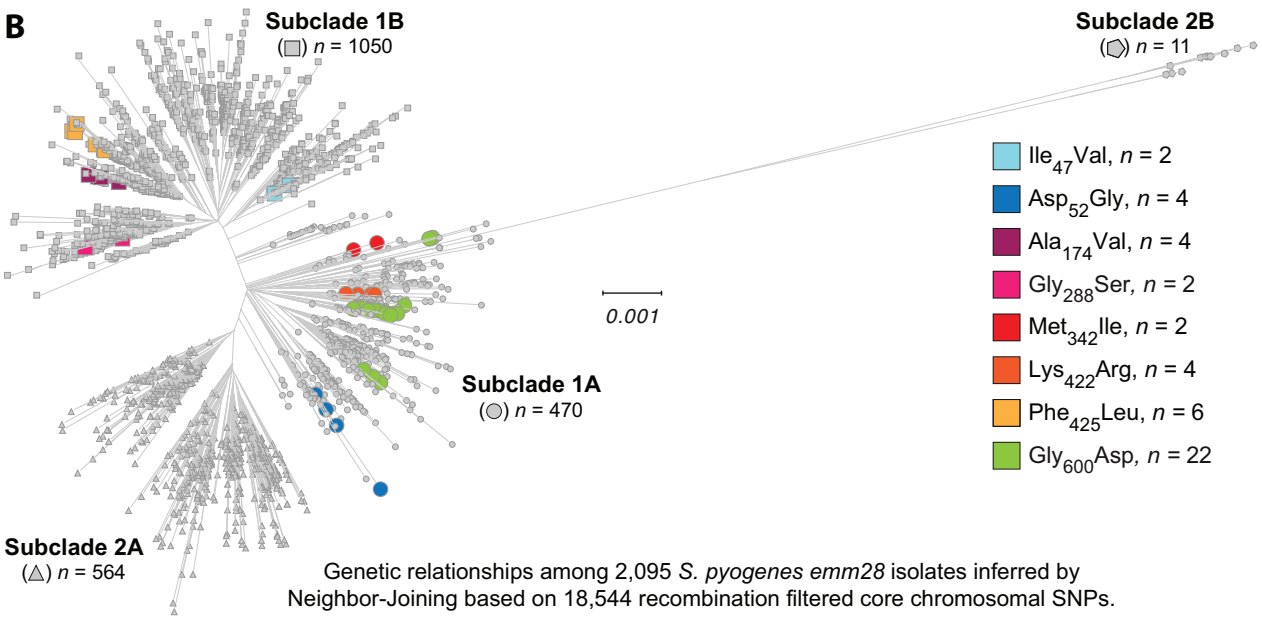
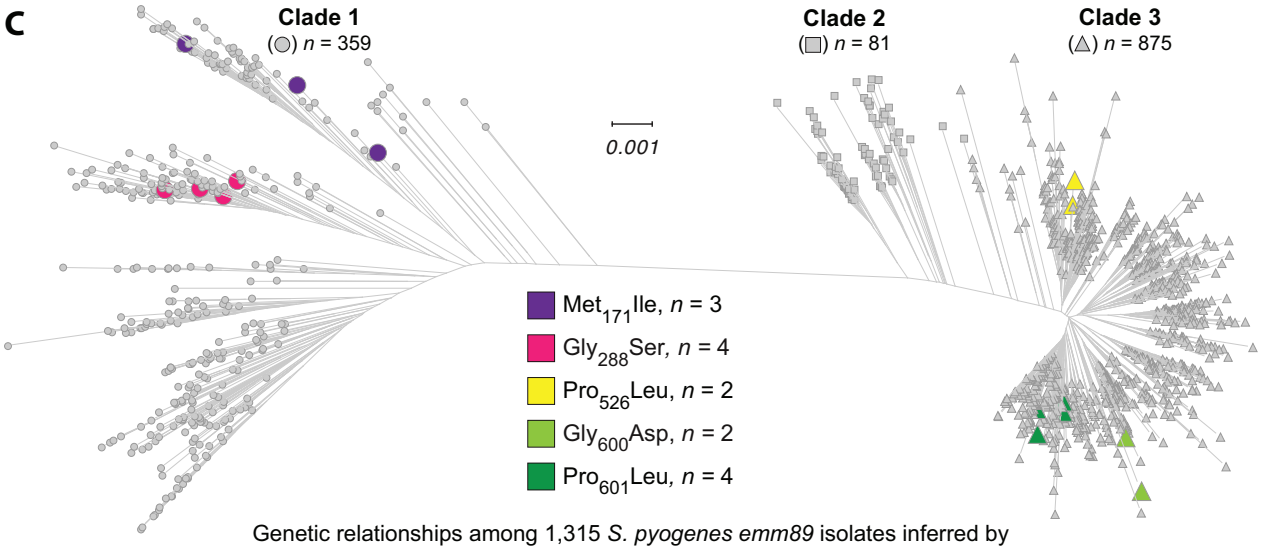


Genetic relationships among 3,615 *S. pyogenes emm1* isolates inferred by Neighbor-Joining based on 12,355 recombination filtered core chromosomal SNPs.



Genetic relationships among 2,095 *S. pyogenes emm28* isolates inferred by Neighbor-Joining based on 18,544 recombination filtered core chromosomal SNPs.



Genetic relationships among 1,315 *S. pyogenes emm89* isolates inferred by Neighbor-Joining based on 9,225 recombination filtered core chromosomal SNPs.