



# Restricted Sequence Variation in *Streptococcus pyogenes* Penicillin Binding Proteins

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**ABSTRACT** A recent clinical report has linked *Streptococcus pyogenes*  $\beta$ -lactam antibiotic resistance to mutation in the penicillin binding protein (PBP) PBP2x. To determine whether this is an isolated case or reflects a broader prevalence of mutations that might confer reduced  $\beta$ -lactam susceptibility, we investigated the relative frequency of PBP sequence variation within a global database of 9,667 *S. pyogenes* isolates. We found that mutations in *S. pyogenes* PBPs (PBP2x, PBP1a, PBP1b, and PBP2a) occur infrequently across this global database, with fewer than 3 amino acid changes differing between >99% of the global population. Only 4 of the 9,667 strains contained mutations near transpeptidase active sites of PBP2x or PBP1a. The reported PBP2x T553K substitution was not identified. These findings are in contrast to those of 2,520 *S. pneumococcus* sequences where PBP mutations are relatively frequent and are often located in key  $\beta$ -lactam binding pockets. These data, combined with the general lack of penicillin resistance reported in *S. pyogenes* worldwide, suggests that extensive, unknown constraints restrict *S. pyogenes* PBP sequence plasticity. Our findings imply that while heavy antibiotic pressure may select for mutations in the PBPs, there is currently no evidence of such mutations becoming fixed in the *S. pyogenes* population or that mutations are being sequentially acquired in the PBPs.

**IMPORTANCE**  $\beta$ -Lactam antibiotics are the first-line therapeutic option for *Streptococcus pyogenes* infections. Despite the global high prevalence of *S. pyogenes* infections and widespread use of  $\beta$ -lactams worldwide, reports of resistance to  $\beta$ -lactam antibiotics, such as penicillin, have been incredibly rare. Recently,  $\beta$ -lactam resistance, as defined by clinical breakpoints, was detected in two clinical *S. pyogenes* isolates with accompanying mutations in the active site of the penicillin binding protein PBP2x, raising concerns that  $\beta$ -lactam resistance will become more widespread. We screened a global database of *S. pyogenes* genome sequences to investigate the frequency of PBP mutations, identifying that PBP mutations are uncommon relative to those of *Streptococcus pneumoniae*. These findings support clinical observations that  $\beta$ -lactam resistance is rare in *S. pyogenes* and suggest that there are considerable constraints on *S. pyogenes* PBP sequence variation.

**KEYWORDS** *Streptococcus pyogenes*, beta-lactams, penicillin resistance, penicillin binding proteins

*Streptococcus pyogenes* (group A *Streptococcus*, or GAS) has previously been understood to be uniformly susceptible to  $\beta$ -lactam antibiotics (1). Two *S. pyogenes* isolates with elevated MICs to  $\beta$ -lactam antibiotics have recently been reported (2). Both isolates were molecularly typed as *emm43.4* and had a penicillin binding protein

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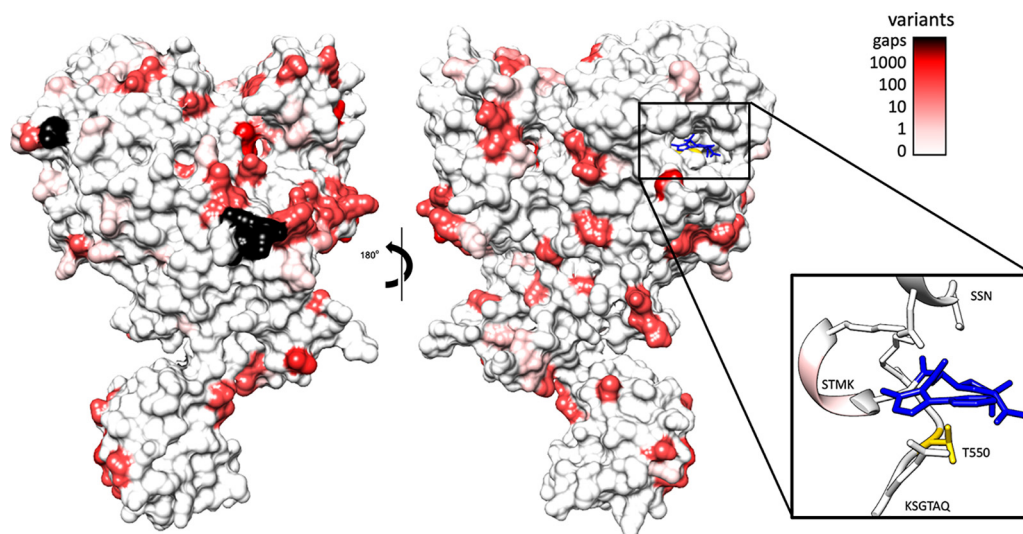
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**FIG 1** Global amino acid variation of *Streptococcus pyogenes* PBP2x mapped against the crystal structure of *Streptococcus pneumoniae* PBP2x. Crystal structure of PBP2x from *S. pneumoniae* (PDB entry 5OIZ) bound to oxacillin (blue), with the frequency of residue conservation from 9,667 *S. pyogenes* PBP2x sequences represented as a color gradient. Black residues represent regions absent from the alignment due to the absence of sequence relative to the *S. pneumoniae* crystal structure. Thresholds were chosen to represent differing orders of magnitude for conservation, with thresholds set at orders of magnitude (0, 1, 10, 100, and 1,000 sequences varying at the residue). (Inset) Ribbon diagram of binding pocket motifs SSN, STMK, and KSG with the position of the mutated residue (T553K) highlighted (yellow). Mutations were observed in the STMK motif in 4 of the 9,667 sequences.

(PBP) PBP2x missense mutation (T553K) at the transpeptidase active site, which was associated with an 8-fold and 3-fold increased MIC to ampicillin and cefotaxime, respectively, compared to levels for closely related isolates without the PBP2x mutation. In contrast to *S. pyogenes*, reduced susceptibility to  $\beta$ -lactams has been widely reported in *S. pneumoniae* and is strongly associated with sequence variation in PBPs (3, 4).

Using GAS genome sequences from global sources, we sought to determine the prevalence of substitutions across the transpeptidase domains of the GAS PBPs (PBP2x, PBP1a, PBP2a, and PBP1b) compared with domains of *S. pneumoniae* (which shares PBP2x and PBP1a).

## RESULTS

We examined sequence variation in PBP1a, PBP1b, PBP2a, and PBP2x among 9,667 *S. pyogenes* genome sequences, representing 115 different *emm* types and 321 multi-locus sequence types (see Table S1 and Text S1 to S4 in the supplemental material). These genome sequences were mostly from data sets from the United Kingdom and United States that focused on invasive disease (5–16). Mutations in the penicillin binding proteins (PBPs) have been associated with reduced clinical  $\beta$ -lactam susceptibility for *S. pneumoniae* (4), *S. agalactiae* (17), *S. dysgalactiae* (18), and now *S. pyogenes* (2). A comparison of PBP2x between  $\beta$ -lactam-susceptible reference genomes of *S. pyogenes*, *S. pneumoniae*, *S. agalactiae*, and *S. dysgalactiae* subspecies *equisimilis* demonstrated a high level of interspecies conservation (>72% similarity) (Fig. S1 and Table S2). In *S. pneumoniae*, substitutions at the PBP2x transpeptidase active site (SXXK, SXN, and KSTG) result in reduced  $\beta$ -lactam susceptibility. These three motifs were conserved across the four species (Fig. S1).

Given the similarity between PBP2x of *S. pneumoniae* and *S. pyogenes* (73.4% similarity) (Table S1), we mapped the conservation of residues from the alignment of 9,667 *S. pyogenes* PBP2x sequences onto the crystal structure of *S. pneumoniae* PBP2x (Fig. 1). The transpeptidase active-site motifs are SXXK at positions 340 to 343 in *S. pyogenes* (positions 337 to 340 in *S. pneumoniae*), SXN at positions 399 to 401 in *S. pyogenes* (positions 395 to 397 in *S. pneumoniae*), and KSGT at positions 550 to 553 in *S. pyogenes* (positions 547 to 550 in *S. pneumoniae*). There were 101 unique amino acid

**TABLE 1** Percentage of transpeptidase sequences with variation in the SXXK, SXN, or K(T/S)G motif of the transpeptidase active sites in PBP1a and PBP2x for *S. pneumoniae* and *S. pyogenes*

Motif	No. (%) of variants in:	
	<i>S. pneumoniae</i> (n = 2,520)	<i>S. pyogenes</i> (n = 9,667)
PBP1a		
STMK <sup>a</sup>	445 (17.7)	0 (0)
SRN	0 (0)	0 (0)
KTG(T)	0 (0)	0 (0)
PBP2x		
STMK <sup>a</sup>	639 (25.3)	4 (0.04)
SSN	0 (0)	0 (0)
KSG(T) <sup>b</sup>	0 (3) [0 (0.1)]	0 (0)

<sup>a</sup>The transpeptidase domain sequences as defined in Li et al. (4) were truncated between the S and T of S/TMK.

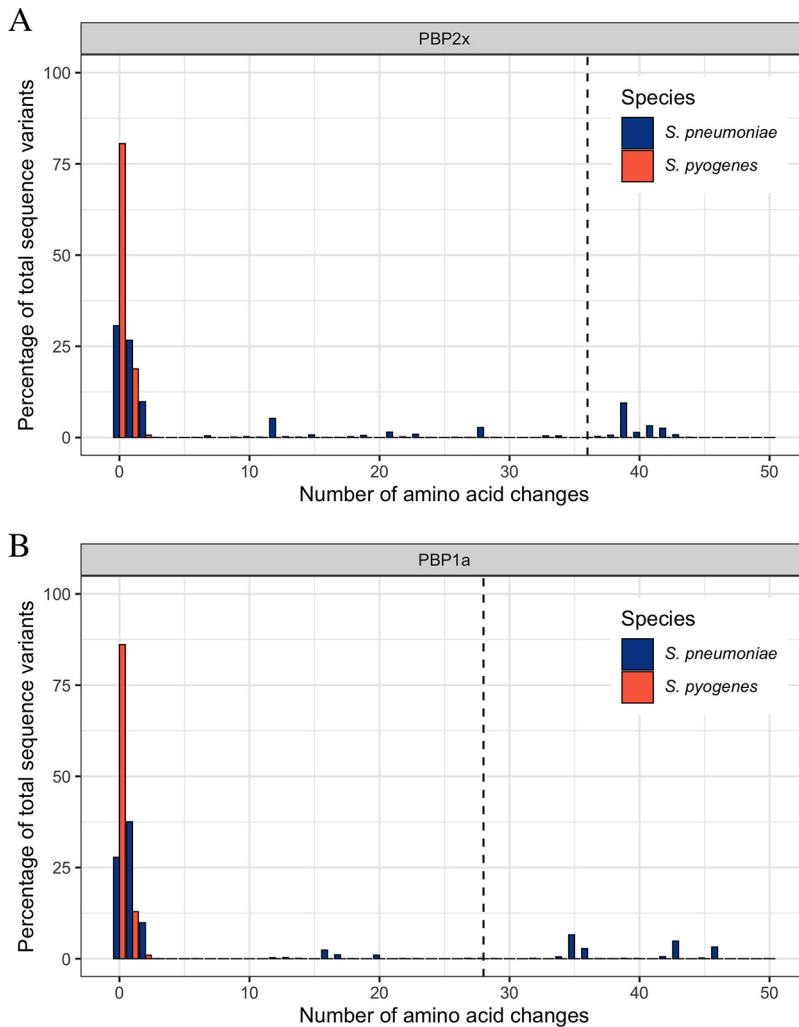
<sup>b</sup>The two T553K sequences reported to be associated with  $\beta$ -lactam resistance in *S. pyogenes* in Vannice et al. (2) are not included.

sequence variants of the *S. pyogenes* PBP2x sequence, with no frameshifts or premature stop codons (Text S1 and Table S3). We found no instances of the T553K substitution in the PBP2x KSGT motif, as reported in the recent *S. pyogenes*  $\beta$ -lactam-resistant isolates (2). Only four *S. pyogenes* isolate sequences (0.04%) had substitutions within the transpeptidase active-site motifs of PBP2x (Fig. 1 and Table 1), corresponding to STMK to SAMK and STMK to STIK. These changes may not have a phenotypic effect on penicillin susceptibility, as STIK recently has been reported in a penicillin-susceptible isolate (GASAR0057) (16). Another 84 (0.9%) of the 9,667 genomes contained mutations at one of four amino acid positions associated with increased tolerance to subclinical  $\beta$ -lactam MIC identified through a recent population genomics study of *emm1*, *emm28*, and *emm89* *S. pyogenes* (Table S3) (19). Furthermore, no amino acid substitutions were found in the active-site motifs of *S. pyogenes* PBP1a. In comparison, using population data from Li et al. (4), *S. pneumoniae* had active-site motif variants in 639/2,520 (25.3%) isolates for PBP2x and 445/2,520 (17.7%) for PBP1a (Table 1). A large proportion of *S. pneumoniae* substitutions mapped to areas near the active site (Fig. S2).

For *S. pneumoniae*, the number of substitutions across the whole transpeptidase domain of PBPs has been associated with penicillin resistance. Li et al. (4) found that penicillin MICs increased as the total number of divergent (defined as >10% amino acids different) transpeptidase domains of PBP2x, PBP1a, and PBP2b increased from 0 to 3. For *S. pyogenes* we used the most common amino acid sequences of PBP2x and PBP1a as our reference and, for *S. pneumoniae*, a previously defined wild type as the reference (4). There were considerably fewer PBP2x and PBP1a transpeptidase domains with multiple substitutions for *S. pyogenes* than for *S. pneumoniae* (Fig. 2). No *S. pyogenes* strains had sufficient mutations to reach the 10% threshold. For *S. pneumoniae*, 18.3% (462 of 2,520 strains) and 19.2% (485 of 2,520 strains) contained divergent PBP2x and PBP1a transpeptidase domains, respectively (Fig. 2). This pattern of greater conservation of *S. pyogenes* PBPs was also observed for PBP1b and PBP2a in *S. pyogenes* compared to PBP2b in *S. pneumoniae* (Fig. S3).

## DISCUSSION

Expanding on recent findings (19), we found no evidence that mutations are present in the  $\beta$ -lactam binding site KSGTAQ motif of PBP2x among 9,667 genetically and geographically diverse *S. pyogenes* genome sequences. While sporadic mutations were observed in PBP proteins, only four isolates contained mutations in the transpeptidase active sites of PBP2x and PBP1a. A further 84 strains (<1%) carried PBP2x amino acid variations recently associated with an increased tolerance to subclinical penicillin MIC (19). Although the report of two *S. pyogenes* isolates with clinical  $\beta$ -lactam resistance associated with *pbp2x* mutations is concerning (2), our findings provide reassurance that PBP mutations leading to clinical resistance are extremely limited, and perhaps



**FIG 2** Amino acid differences of the transpeptidase domains of PBP2x and PBP1a. (A and B) The percentage of isolates with changes in the transpeptidase domains of PBP2x (A) and PBP1a (B) relative to penicillin-susceptible references in *Streptococcus pneumoniae* (blue;  $n = 2,520$ ) and *S. pyogenes* (red;  $n = 9,667$ ). Sequences that are >10% divergent (indicated by dotted vertical lines) have been associated with increased penicillin MICs in *S. pneumoniae*.

unique, occurrences at this stage. Similar observations have been reported within closely related beta-hemolytic streptococci such as *Streptococcus agalactiae* and *Streptococcus dysgalactiae* subspecies *equisimilis*, where PBP mutations conferring reduced penicillin susceptibility or resistance were observed (18, 20), but without conclusive evidence of clonal expansion through current population-based surveillance investigations (6, 20).

We found a high degree of conservation of GAS PBP2x and PBP1a at transpeptidase active sites and across the broader transpeptidase domains. In comparison, PBP2x and PBP1a for *S. pneumoniae* were far less conserved, suggesting that there are strong evolutionary constraints in these domains for *S. pyogenes* that is not the case for *S. pneumoniae*. This may be due to several factors, including the lack of structural plasticity possible in PBP proteins of GAS (*S. pyogenes* lacks a PBP2b homolog), different  $\beta$ -lactam-resistant communities within the environmental niches occupied, lower natural transformation efficiency of GAS relative to that of *S. pneumoniae*, and a necessity for other chromosomal compensatory mutations to facilitate the maintenance of clinically relevant PBP mutations, as has been suggested for group B streptococci (2). Studies of penicillin-resistant *S. pyogenes* generated through mutagenesis (21) or serial

passage in penicillin-containing medium (22) demonstrated that mutants with raised penicillin MICs appeared to have alterations in PBPs with reduced penicillin affinity (21). Notably, mutants grow more slowly, have aberrant colony morphology compared to that of wild-type strains (21), and are avirulent, with a decrease in M protein production (22). These laboratory experiments, together with the absence of naturally occurring isolates with greater than five amino acid substitutions in PBP2x or PBP1a, strongly suggest that changes to the PBPs are associated with a significant fitness cost. However, as subclinical low-level  $\beta$ -lactam resistance theoretically could confer biological advantages to *S. pyogenes* carriage, maintaining vigilance through population-based *S. pyogenes* surveillance for PBP variants is encouraged (19).

## MATERIALS AND METHODS

We obtained publicly available genome sequence data for 9,667 *S. pyogenes* isolates from the short-read archive (see Table S1 in the supplemental material). We assembled genomes using shovill v.1.0.9 (<https://github.com/tseemann/shovill>) with an underlying SKESA v.2.3.0 assembler (23). Using the  $\beta$ -lactam-susceptible *S. pyogenes* serotype M3 strain ATCC BAA-595/MGAS315 as a reference, we determined the presence, amino acid sequence, and alignment (24) of each of PBP2x, PBP1a, PBP1b, and PBP2a in each genome with the screen\_assembly script (5) and BLASTP parameters of 100% coverage and 90% identity. Variant sites were identified from the multi-FASTA alignments using snp-sites (25).

To compare the conservation of the transpeptidase active-site motifs across streptococcal species, full-length PBP2x protein sequences of *S. pyogenes* serotype M3 strain ATCC BAA-595/MGAS315 (GenBank accession no. NC\_004070.1), *S. pneumoniae* strain ATCC BAA-255/R6 (NC\_003098.1), *S. agalactiae* strain 2603V/R (NC\_004116.1), and the *S. dysgalactiae* subspecies *equisimilis* strain RE378 (NC\_018712.1) reference genomes were aligned using Clustal Omega (26, 27). The percent sequence similarity was compared using Blosum62 with a threshold of 1 in Geneious Prime (28).

To investigate the inferred crystal structure location of *S. pyogenes* PBP2x mutations relative to that of the *S. pneumoniae* orthologue, *S. pyogenes* PBP2x sequence variations were plotted onto the *S. pneumoniae* PBP2x crystal structure bound to oxacillin (PDB entry 5OIZ) (29). Sequence conservation, as determined by the frequency (for *S. pyogenes*) and percentage (for *S. pneumoniae*) of variant amino acids compared to the consensus, was rendered onto the PBP2x crystal structure using UCSF Chimera (30).

We defined the PBP2x and PBP1a transpeptidase regions as those used in an assessment of 2,520 invasive *S. pneumoniae* isolates by Li et al. (4) and determined and plotted the number of pairwise amino acid differences within these regions using Distances Matrix in Geneious Prime (28) and ggplot2 in R version 3.6.1 (31). Similarly, we also assessed the conservation of PBP1b and PBP2a proteins for the 9,667 *S. pyogenes* genomes and the transpeptidase region of PBP2b for *S. pneumoniae*.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**TEXT S1**, TXT file, 0.2 MB.

**TEXT S2**, TXT file, 0.2 MB.

**TEXT S3**, TXT file, 0.2 MB.

**TEXT S4**, TXT file, 0.2 MB.

**FIG S1**, PDF file, 0.05 MB.

**FIG S2**, PDF file, 1 MB.

**FIG S3**, PDF file, 0.2 MB.

**TABLE S1**, XLSX file, 0.4 MB.

**TABLE S2**, DOCX file, 0.02 MB.

**TABLE S3**, DOCX file, 0.03 MB.

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