SHORT COMMUNICATION

A Single Amino Acid Replacement in Penicillin-Binding Protein 2X in *Streptococcus pyogenes* Significantly Increases Fitness on Subtherapeutic Benzylpenicillin Treatment in a Mouse Model of Necrotizing Myositis

Randall J. Olsen,^{*,†‡} Luchang Zhu,^{*} and James M. Musser^{*,†‡}

From the Center for Molecular and Translational Human Infectious Diseases Research,^{*} Houston Methodist Research Institute, Houston, Texas; the Clinical Microbiology Laboratory,[†] Department of Pathology and Genomic Medicine, Houston Methodist Hospital, Houston, Texas; and the Department of Pathology and Laboratory Medicine,[‡] Weill Cornell Medical College, New York, New York

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Address correspondence to
James M. Musser, M.D., Ph.D.,
6565 Fannin St., Ste. B490,
Houston, TX 77030. E-mail:
jmmusser@houstonmethodist.org.

Invasive strains of *Streptococcus pyogenes* with significantly reduced susceptibility to β -lactam antibiotics have been recently described. These reports have caused considerable concern in the international infectious disease, medical microbiology, and public health communities because *S. pyogenes* has remained universally susceptible to β -lactam antibiotics for 70 years. Virtually all analyzed strains had single amino acid replacements in penicillin-binding protein 2X (PBP2X), a major target of β -lactam antibiotics in pathogenic bacteria. We used isogenic strains to test the hypothesis that a single amino acid replacement in PBP2X conferred a fitness advantage in a mouse model of necrotizing myositis. We determined that when mice were administered intermittent subtherapeutic dosing of benzylpenicillin, the strain with a Pro601Leu amino acid replacement in PBP2X that confers reduced β -lactam susceptibility *in vitro* was more fit, as assessed by the magnitude of colony-forming units recovered from disease tissue. These data provide important pathogenesis information that bears on this emerging global infectious disease problem. (*Am J Pathol* 2020, 190: 1625–1631; <https://doi.org/10.1016/j.ajpath.2020.04.014>)

Streptococcus pyogenes, alias group A *Streptococcus* (GAS), causes >700 million human infections each year worldwide.^{1,2} This organism is a strict human pathogen that is responsible for a remarkable array of diverse health problems, ranging from relatively mild pharyngitis to life-threatening infections, such as septicemia and severe soft tissue infections.^{3–7} GAS is also responsible for acute rheumatic fever, a disease that remains the most common cause of preventable pediatric heart disease globally.⁶ No licensed vaccine against GAS is available despite 100 years of effort, and no promising vaccine is on the immediate horizon.^{8–11}

Firstline treatment of GAS infections typically involves a β -lactam antibiotic, such as penicillin or ampicillin, either alone or in conjunction with other classes of antibiotics (eg, macrolides or fluoroquinolones).^{3,7,12–14} Pediatric GAS

pharyngitis and attendant oral prescriptions account for enormous antibiotic use in the United States and many other countries.^{12–14} For reasons that remain poorly understood and contrast with many bacterial pathogens, GAS has remained universally susceptible to β -lactam antibiotics.¹⁵ However, GAS strains with significantly decreased susceptibility to β -lactams *in vitro* were described in two recent publications.^{16,17} The levels of decreased susceptibility did not exceed the threshold of frank *in vitro* resistance, but nevertheless, identification of these strains has resulted in

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widespread concern in the international infectious disease, microbiology, and public health communities.

Decreased susceptibility to β -lactams in GAS and other pathogenic streptococci is primarily associated with non-synonymous (amino acid–altering) mutations in the *pbp2x* gene encoding penicillin-binding protein 2X (PBP2X), a major target of these antibiotics in many pathogens.^{16–37} In the case of GAS, virtually all strains with altered susceptibility have only one amino acid replacement in PBP2X.¹⁶ Molecular genetic techniques were used to construct an isogenic mutant strain and showed that one of these changes (Pro601Leu in PBP2X) was the molecular cause of decreased *in vitro* susceptibility to β -lactams.¹⁶ Many questions remain unanswered about GAS strains with PBP2X amino acid replacements linked to decreased β -lactam susceptibility. One important area that has not been addressed is the effect, if any, of these amino acid changes on strain virulence or fitness. To begin to explore this association, isogenic strains were used to test the hypothesis that a single amino acid replacement in PBP2X alters fitness and/or virulence in mouse necrotizing myositis, a widely used model of deep tissue infection.^{38–42} We found that in infected mice given intermittent subtherapeutic dosing of benzylpenicillin, a naturally occurring strain with the Pro601Leu amino acid replacement in PBP2X was more fit than a strain with the wild-type (WT) PBP2X variant, as assessed by the number of colony-forming units (CFUs). The pilot studies described herein will form the basis for a more in-depth analysis of the relationship between β -lactam susceptibility and virulence *in vivo*, an area of GAS research that is greatly lagging compared with other pathogenic bacteria.

Materials and Methods

Construction of Isogenic Strain 27213-PBP2X-WT

MGAS27213 is a serotype M89 GAS strain recovered in 2012 in the United States from a patient with invasive infection.⁴³ This strain has a single amino acid replacement (Pro601Leu) that results in significantly decreased susceptibility to penicillin G, ampicillin, and other β -lactam antibiotics.¹⁶ We refer to this strain as 27213-PBP2X-mutant (MT). The isogenic derivative strain was constructed to restore a proline residue at position 601 in the PBP2X protein, which represents the WT variant of PBP2X that is fully susceptible to β -lactam antibiotics. Molecular construction and phenotypic characterization of isogenic strain 27213-PBP2X-WT has been described recently.¹⁶ Whole genome sequencing of 27213-PBP2X-WT confirmed that the constructed strain lacked spurious mutations.

Mouse Model of Necrotizing Myositis with Subtherapeutic Antibiotic Dosing

The mouse model of necrotizing myositis used in these experiments has been previously described.^{40–42} The model was modified to include intermittent subtherapeutic

benzylpenicillin treatment. Mice were treated on the day of infection with 7.3 IU/g benzylpenicillin (MilliporeSigma, Burlington, MA), and day 10 after infection with 11 IU/g, both of which were subtherapeutic doses of benzylpenicillin injected intramuscularly. The antibiotic was given intramuscularly as a 100- μ L injection in an identical manner as the GAS inoculation. The first dose was given at/near the GAS injection site in the right rear lower limb, as outlined in Figure 1; the second dose was given in the contralateral limb.

Near Mortality, CFU Determination, and Histopathology

For mouse survival experiments, infected mice were monitored for near mortality at least once daily for up to 17 days. CFU determination from infected muscle was assessed by culturing tissue homogenates, as previously described.^{41,44} Briefly, diseased muscle recovered from the infected limb was weighed and homogenized (Omni International, Kenosha, GA) in 1 mL phosphate-buffered saline, serially diluted, plated on sheep blood agar, cultured overnight, and counted. For histologic evaluation, tissue taken from the inoculation site was fixed, embedded, and processed using standard automated instruments. Statistical differences between strain groups were determined with the *U*-test. Mouse experiments were approved by the Institutional Animal Care and Use Committee of Houston Methodist Research Institute (protocol AUP-0318-0016).

Growth of GAS Strains in THY Broth

Growth of GAS strains in Todd Hewitt broth supplemented with 0.5% yeast extract (THY) with or without a subinhibitory concentration of benzylpenicillin (6 ng/mL) was performed, as described previously.¹⁶ Liquid GAS cultures were incubated for 12 hours at 37°C in 5% CO₂. Growth was determined by measuring the OD at 600 nm.

Results

To test the hypothesis that a single amino acid replacement in PBP2X alters virulence *in vivo*, a mouse model of necrotizing myositis that has been used extensively was employed.^{38–42} The naturally occurring strain MGAS27213 that has reduced susceptibility to β -lactams *in vitro* was compared with its isogenic derivative that differs in a single amino acid in PBP2X. The isogenic strain has a proline at amino acid position 601 of PBP2X, converting it to the WT PBP2X variant and a fully susceptible strain.¹⁶ We refer to these two strains as 27213-PBP2X-WT and 27213-PBP2X-MT.

Ten CD-1 outbred mice were inoculated with 1.5×10^8 CFUs of either 27213-PBP2X-MT or the isogenic derivative 27213-PBP2X-WT in the right hind limb, and near mortality was assessed at day 7 after infection. The

Figure 1 Group A *Streptococcus* inoculation and benzylpenicillin dosing schedule. Mice were inoculated with strain 27213—penicillin-binding protein 2X (PBP2X)—wild type (WT) or 27213-PBP2X-mutant (MT) followed by a subtherapeutic dose of benzylpenicillin via i.m. injection at the site of bacterial inoculation. A second dose was administered on day 10 in the contralateral limb. The mice were sacrificed on day 17, and infected tissue was harvested for colony-forming unit (CFU) counts. $n = 40$.

Kaplan-Meier survival curves for mice infected with these two strains were essentially superimposable (Figure 2A). Mice were sacrificed on day 7 after inoculation, and homogenates of the infected limbs were plated on sheep red blood cell agar and incubated overnight, and colonies were counted. The same numbers of CFUs were recovered from diseased tissue

from the wild-type and 27213-PBP2X-MT strains (Figure 2B). Sections of diseased tissue were fixed, stained, and analyzed for histopathology; both strains caused nearly identical lesions (Figure 2C). Taken together, the results show that in the absence of β -lactam treatment, the two isogenic strains had identical fitness and virulence, as assessed by the ability to

Figure 2 Comparison of 27213—penicillin-binding protein 2X (PBP2X)—wild type (WT) and 27213-PBP2X-mutant (MT) strains of Group A *Streptococcus* in a mouse model of necrotizing myositis in the absence and presence of benzylpenicillin (Bpen). **A:** Kaplan-Meier survival curves for mice inoculated with strain 27213-PBP2X-WT (WT) and the reduced susceptibility to β -lactam strain 27213-PBP2X-MT (MT). No significant differences are observed in near mortality. **B:** Colony-forming units (CFUs) recovered from the hind limbs of infected animals are similar. **C:** Histologic examination of hind limb sections from mice show no differences in bacterial dissemination or tissue destruction (hematoxylin and eosin stain). **A–C:** All analyses were performed with 10 mice in the absence of benzylpenicillin and at day 7 after infection. **D:** CFUs recovered from mice infected with strains 27213-PBP2X-WT and 27213-PBP2X-MT at day 17 after infection. Mice were injected following the schedule depicted in Figure 1. **E:** Kaplan-Meier survival curves for mice inoculated with WT and MT strains in the presence of benzylpenicillin. No significant difference in near mortality is observed. **F:** Growth of group A *Streptococcus* strains in Todd Hewitt broth supplemented with 0.5% yeast extract (THY) with or without a subinhibitory concentration of Bpen. $n = 40$ (**D**). * $P < 0.05$, **** $P < 0.0001$ versus WT (*U*-test). Original magnification, $\times 4$ (**C**).

replicate and persist (CFUs), induce tissue necrosis (histopathology), and cause near mortality.

We next tested the hypothesis that strain 27213-PBP2X-MT, which shows reduced *in vitro* sensitivity to β -lactams, would have a fitness advantage in the presence of subtherapeutic treatment with benzylpenicillin (penicillin G). This hypothesis is based on the clinical observation that patients with prolonged intermittent treatment with β -lactams can be infected by bacterial strains with significantly decreased susceptibility to this class of antibiotics.^{17,20,27,45,46} A pilot experiment was performed to test this hypothesis. Mice ($n = 10$ animals per strain) were inoculated in the right hind limb with bacteria, and a subtherapeutic dose of benzylpenicillin (7.3 IU/g) was administered by i.m. injection. The mice were followed up for 10 days, and then a second subtherapeutic dose of benzylpenicillin (11 IU/g) was administered (Figure 1). These doses were chosen on the basis of the recommended benzylpenicillin therapeutic dose of 22 IU/g for mice⁴⁷; the timing of the benzylpenicillin doses was somewhat arbitrary. Mice were sacrificed on day 17 after inoculation, and homogenates of the infected limbs were plated for CFU determination. Compared with mice infected with isogenic strain 27213-PBP2X-WT, more CFUs/g of mouse tissue were recovered from limbs infected with the 27213-PBP2X-MT strain. That is, mice infected with the strain with significantly reduced susceptibility to β -lactam antibiotics *in vitro* yielded more CFUs (data not shown). Thus, in the context of the intermittent subtherapeutic antibiotic dosing, the PBP2X mutant strain trended toward increased fitness, but the data did not reach statistical significance.

Using the previous infection and dosing strategy (Figure 1) and applying a power calculation, the study was repeated with 40 mice per GAS strain. Consistent with the data described in the previous paragraph, mice infected with strain 27213-PBP2X-MT yielded significantly higher CFUs than mice infected with the 27213-PBP2X-WT susceptible strain (Figure 2D). When benzylpenicillin was present, mortality (Figure 2E) and extent of tissue damage (data not shown) in mice infected with the 27213-PBP2X-WT and 27213-PBP2X-MT strains did not significantly differ. Consistent with the *in vivo* data, strain 27213-PBP2X-MT grew to a significantly higher OD than strain 27213-PBP2X-WT in THY broth supplemented with benzylpenicillin (Figure 2F). These data show that in the presence of subtherapeutic levels of benzylpenicillin, the PBP2X mutant strain is significantly more fit than the wild-type, susceptible strain.

Discussion

The goal of this study was to test the hypothesis that a single amino acid replacement in PBP2X significantly alters fitness in a mouse model of necrotizing myositis. The study data show that in the absence of intermittent subtherapeutic benzylpenicillin, there was no difference in fitness or

virulence between the isogenic PBP2X wild-type and mutant strains. In contrast, the introduction of intermittent dosing with subtherapeutic levels of benzylpenicillin showed that strain 27213-PBP2X-MT, with significantly decreased *in vitro* susceptibility to this β -lactam, was more fit in this mouse model of necrotizing myositis. These data provide important new fitness information about GAS strains that differ by a single amino acid replacement in PBP2X that confers altered β -lactam susceptibility. Many naturally occurring strains with single PBP2X polymorphisms have recently been identified, which has generated significant concern globally.^{16,17}

The relationship between resistance to antimicrobial agents and bacterial fitness and virulence is complicated and has not been assessed in pathogenic streptococci using isogenic strains. Work in other organisms, such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*, has not yielded a simple definitive theme: in some bacteria, investigators have reported no change in fitness in animal infection models, whereas other investigators have reported altered fitness.^{48–56} Although our study is the first analysis of altered antimicrobial susceptibility and fitness in GAS using isogenic strains, clearly more work is warranted given the spectrum of PBP2X amino acid polymorphisms and different diseases caused by GAS.

A large number of single amino acid replacements in PBP2X that are associated with decreased *in vitro* susceptibility to β -lactams were recently identified.¹⁶ Thus far, our studies have been confined to single amino acid changes in PBP2X identified by analysis of GAS strains cultured from patients with serious invasive infections.¹⁶ Currently, there are no data about the frequency of occurrence of such changes in strains causing pharyngitis or recovered from asymptomatic carriers. In this regard, *pbp2x* mutations in strains from these two clinical sources were recently identified, information that will likely add to the complexity of altered susceptibility to β -lactam antibiotics in GAS.

In addition, the extent of variation in *pbp1a* that may contribute to β -lactam resistance in GAS is unknown. Amino acid substitutions in PBP1A have been shown to contribute to β -lactam resistance in group B *Streptococcus* (*Streptococcus agalactiae*) and other pathogenic streptococci.^{23,25,27,36,57–59} The extent of amino acid substitutions in PBP1A that contribute to β -lactam resistance in GAS clinical isolates will need to be assessed to determine the full complement of changes contributing to reduced susceptibility in GAS to this important class of antibiotics.

The experiments described herein are important to assess potential changes in fitness and virulence associated with reduced susceptibility to β -lactams, an emerging problem that has raised concern in the microbiology, infectious disease, and public health communities. Future analyses will require construction, confirmation, and phenotypic characterization (eg, *in vitro* growth and β -lactam minimum inhibitory concentration) of additional isogenic strains,

followed by *in vivo* pathogenesis and fitness studies in various mouse models of infection. Finally, it will be important to assess the relative fitness of PBP2X and PBP1A isogenic strains in the upper respiratory tract of nonhuman primates, the gold standard infection model for GAS pharyngitis.

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Author Contributions

R.J.O. planned and conducted the mouse infection experiments and analyzed the resulting data. L.Z. generated and characterized the isogenic mutant strain. J.M.M. designed the studies, analyzed experiments, and oversaw the project. All authors wrote the manuscript and contributed to the intellectual atmosphere that resulted in this study. J.M.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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