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PBP4 activity and its overexpression are necessary for PBP4-mediated high-level β-lactam resistance

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Background: PBP4 is typically considered unimportant for conferring high-level β-lactam resistance in *Staphylococcus aureus*. Mutations in PBP4 have been associated with β-lactam non-susceptibility among natural strains of *S. aureus*. We have previously shown that PBP4 can mediate high-level β-lactam resistance in laboratory-generated strains passaged in β-lactam antibiotics. Mutations in the *pbp4* promoter that up-regulate its expression and missense mutations that surround PBP4's active site were detected in high frequencies among passaged strains, suggesting PBP4 plays a key role in resistance. How these mutations participate in PBP4's ability to provide high-level β-lactam resistance is unknown.

Objectives: To determine whether enzymatic activity of PBP4 is required for high-level β-lactam resistance and to investigate how the *pbp4*-associated mutations provide β-lactam resistance.

Methods: The catalytic activity of PBP4 was disabled through introduction of a serine to alanine point mutation in its active site (Ser-75→Ala) in a representative and well-studied passaged strain, CRB. *pbp4* promoter and missense mutations detected in CRB were reconstituted in a WT strain individually and in combination. β-Lactam resistance of the resultant strains was evaluated by population analysis. Bacterial peptidoglycan composition of the *pbp4* mutants was evaluated with and without antibiotic treatment using LC.

Results: PBP4 inactivation imparted complete β-lactam susceptibility of CRB. Reconstitution of PBP4 missense mutations alone did not impart β-lactam resistance, but did so in synergism with *pbp4* promoter mutation. A similar synergistic interaction of *pbp4* mutations was observed in enhanced peptidoglycan cross-linking upon antibiotic treatment.

Conclusions: PBP4's activity and overexpression both contribute to high-level β-lactam resistance.

Introduction

PBPs, the primary targets of β-lactam antibiotics, perform the final steps of bacterial cell wall biosynthesis.[1] High-level resistance to β-lactam drugs in *Staphylococcus aureus* is generally mediated by PBP2a.[2] The hallmark of PBP2a-mediated β-lactam resistance is its lower affinity to the drug, allowing cell wall biosynthesis even in the presence of high concentrations of the drug.[2] *S. aureus* possesses four other PBPs (PBP1–4), which are not considered as important as PBP2a for β-lactam resistance and consequently have received little attention.

We have recently shown that *S. aureus* when passaged in β-lactams can attain high-level, broad-spectrum β-lactam resistance in the absence of *mecA* (the gene that codes for PBP2a).[3,4] Mutations targeting *pbp4* were common among the resistant passaged strains.[5] Deletion of *pbp4* in these strains restored full susceptibility to β-lactams, and *mecA*-negative WT strains without *pbp4* were unable to attain the high-level resistance when passaged in a similar manner.[4,6] These results suggested that PBP4 has a vital role in the pathway leading to the observed high-level β-lactam resistance that is independent of PBP2a.

PBPs constitute the core of the bacterial cell wall biosynthesis machinery. How each component participates in cell wall biogenesis is not well understood. The mechanism by which PBP4 mediates resistance in our passaged strains is also unknown. PBP4 could either play an active role through its enzymatic activity or it could also play an accessory role to facilitate stability and/or

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interactions with the core components of the cell wall biosynthesis machinery or with the components of the cell wall itself.

One of the resistant passaged strains, CRB (COLnex strain resistant to ceftobiprole) has a 36 bp (ATTTATATGATAGAATATTTC TATTGCATTTTTTGT) duplication in the *pbp4* promoter region and two missense mutations, Glu-183→Ala and Phe-241→Arg, that surround the PBP4 active site.[4,6] Reconstitution of the 36 bp *pbp4* promoter mutation in the parental COLnex strain led to PBP4 up-regulation, increased peptidoglycan cross-linking and enhanced β-lactam resistance.[6] The role of the PBP4 missense mutations is currently unclear. Since affinity to β-lactams between recombinant PBP4s with or without the missense mutations appears to be similar,[6] the mechanism of PBP4-mediated resistance may not be due to low drug binding affinity, in contrast to PBP2a. The fact that PBP4 missense mutations are common among passaged strains,[5] and that similar mutations targeting PBP4 were recently reported among ceftaroline-non-susceptible clinical isolates,[7] suggested an as-yet unidentified role for these mutations in β-lactam resistance.

In this paper we show that the enzymatic activity of PBP4 is essential for the manifestation of high-level β-lactam resistance independent of PBP2a, and that the PBP4 missense mutations with their promoter mutations synergistically contribute to β-lactam resistance.

Materials and methods

Bacterial strains and plasmids

Bacterial strains were grown in tryptic soy broth (TSB) as previously described.[4,6] Allelic replacements were carried out with the plasmid *pJB38* as previously described.[8] The bacterial strains used in this study are listed in Table S1 (available as Supplementary data at *JAC* Online). The primers used in this study are listed in Table S2.

Population analysis

Population analyses were done by the agar method as previously described.[4] Briefly, a 10 μL volume of serially diluted bacterial culture was spotted onto agar plates containing increasing concentrations of nafcillin (Sigma). The plates were incubated at 37°C for 72 h and were read for cfu determination.

Additional methods used in this study are described in the Supplementary Methods available as Supplementary data at *JAC* Online.

Results

Activity of PBP4 is necessary for high-level β-lactam resistance of CRB

To determine if the activity of PBP4 is important for high-level β-lactam resistance in the *mecA*-negative CRB strain, the active site serine at amino acid position 75 of PBP4 was substituted with an alanine residue. Although the resultant mutant, CRB *pbp4*$^{(Ser\text{-}75\rightarrow Ala)}$, expressed similar amounts of *pbp4* compared with CRB, it was completely susceptible to β-lactam antibiotics, comparable to the CRB Δ*pbp4* strain (Figure 1, Figure S1 and Table S3), indicating that the enzymatic activity of PBP4 was indeed essential for the resistance of CRB.

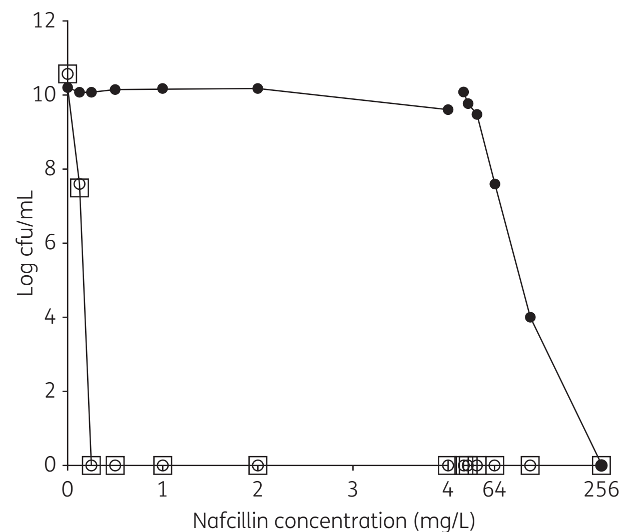

Figure 1. Activity of PBP4 is important to confer β-lactam resistance in strain CRB. Population analysis in nafcillin was carried out with highly resistant CRB (filled circles), its Δ*pbp4* deletion mutant (open circles) and its PBP4 (S-75→A) substitution mutant (open squares). Two-way ANOVA of the data revealed a significant difference ($P < 0.0001$) between CRB and the other strains.

pbp4 up-regulation is essential for PBP4-mediated high-level β-lactam resistance

As mentioned above, PBP4 missense mutations were not only common among our passaged strains, but a recent clinical surveillance study also reported similar missense mutations in PBP4.[7] Another surveillance study carried out in the Asia-Pacific region identified several ceftaroline-non-susceptible *S. aureus* strains.[9] Ceftaroline non-susceptibility was predominantly due to mutations in PBP2a. Notably, the same study also detected six ceftaroline-non-susceptible strains without any PBP2a mutations. Sequencing of *pbp4* revealed identical missense mutations (Thr-189→Ser; Thr-409→Ala) in three out of these six strains. This suggested that missense mutations in PBP4 are associated with β-lactam non-susceptibility or resistance and might play an unidentified role in resistance.

To determine the role of the missense mutations of PBP4 detected in CRB (Glu-183→Ala and Phe-241→Arg), these mutations were reconstituted in the parental COLnex strain (*mecA*-deleted COLn strain). The resultant mutant strain (COLnex *pbp4*$^{(Glu\text{-}183\rightarrow Ala\ and\ Phe\text{-}241\rightarrow Arg)}$) did not show enhanced β-lactam resistance (Figure 2 and Table S3). This result was consistent with our previous finding where the same set of mutations failed to confer any β-lactam resistance in the SF8300ex strain (a PBP2a-deleted USA300 background strain).[4]

Since PBP4 missense mutations were commonly identified along with *pbp4* promoter mutations among our passaged strains,[5] and the promoter mutation in CRB led to PBP4 up-regulation,[6] we hypothesized that PBP4 up-regulation might be a prerequisite for the missense mutations to manifest resistance. To this end, we reconstituted the Glu-183→Ala and Phe-241→Arg mutations in the COLnex strain bearing the *pbp4* promoter mutation (COLnex *Ppbp4*$^{(CRB)}$). The resultant strain COLnex *Ppbp4*$^{(CRB)}$ *pbp4*$^{(Glu\text{-}183\rightarrow Ala\ and\ Phe\text{-}241\rightarrow Arg)}$ showed enhanced β-lactam

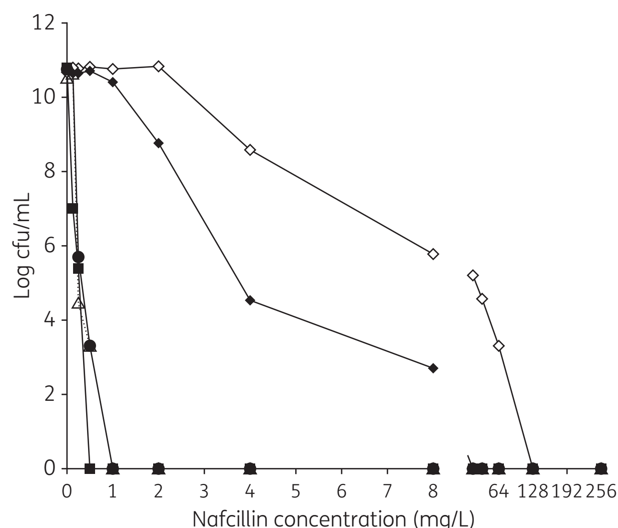

Figure 2. PBP4 missense mutations provide β-lactam resistance in synergism with its promoter mutation. Population analysis in nafcillin was carried out with COLnex (filled circles), COLnex *pbp4*$^{(E\text{-}183\rightarrow A \text{ and } F\text{-}241\rightarrow R)}$ (open triangles), COLnex *Ppbp4*$^{(CRB)}$ (filled diamonds), COLnex *Ppbp4*$^{(CRB)}$ *pbp4*$^{(E\text{-}183\rightarrow A \text{ and } F\text{-}241\rightarrow R)}$ (open diamonds) and COLnex *Ppbp4*$^{(CRB)}$ *pbp4*$^{(E\text{-}183\rightarrow A \text{ and } F\text{-}241\rightarrow R)\ (S\text{-}75\rightarrow A)}$ (filled squares). Two-way ANOVA of the data revealed a significant difference ($P < 0.0001$) between COLnex *Ppbp4*$^{(CRB)}$, COLnex *Ppbp4*$^{(CRB)}$ *pbp4*$^{(E\text{-}183\rightarrow A \text{ and } F\text{-}241\rightarrow R)}$ and the other strains.

resistance compared with the COLnex *Ppbp4*$^{(CRB)}$ strain both through population analysis and in MICs (Figure 2 and Table S3). Further, a Ser-75→Ala mutation in the PBP4 of COLnex *Ppbp4*$^{(CRB)}$ *pbp4*$^{(Glu\text{-}183\rightarrow Ala \text{ and } Phe\text{-}241\rightarrow Arg)}$ rendered it completely β-lactam susceptible, similar to the parental COLnex strain (Figure 2 and Table S3). Thus, although the Glu-183→Ala and Phe-241→Arg mutations can enhance β-lactam resistance, up-regulation of PBP4 is required for detectable levels of resistance and intact enzymatic activity of PBP4 is equally important for the resistance phenotype.

pbp4 mutations are synergistic in producing highly cross-linked cell wall upon β-lactam challenge

We previously observed that COLnex *Ppbp4*$^{(CRB)}$ has a highly cross-linked (represented by 18+ muropeptide fractions) cell wall pattern that is dramatically reduced by nafcillin treatment.[6] This led us to hypothesize that PBP4 overexpression in COLnex *Ppbp4*$^{(CRB)}$ allowed cells to resist the β-lactam challenge by catalysing the essential cross-linking reaction.[6] To evaluate if a synergism similar to the results mentioned above exists in COLnex *Ppbp4*$^{(CRB)}$ *pbp4*$^{(Glu\text{-}183\rightarrow Ala \text{ and } Phe\text{-}241\rightarrow Arg)}$ in producing highly cross-linked cell wall, bacterial peptidoglycans were analysed. The PBP4 missense mutations mediated a decrease in highly cross-linked cell wall when grown in TSB medium in the absence of nafcillin. However, in the presence of nafcillin the same mutations aided the formation of enhanced cell wall cross-linking (Figure S2). Growth curves of these strains showed identical growth in TSB without nafcillin, but nafcillin treatment inhibited COLnex *Ppbp4*$^{(CRB)}$ more efficiently compared with COLnex *Ppbp4*$^{(CRB)}$ *pbp4*$^{(Glu\text{-}183\rightarrow Ala \text{ and } Phe\text{-}241\rightarrow Arg)}$ (Figure S3). These results support our previous hypothesis that PBP4's ability to form highly cross-linked cell wall confers the resistance phenotype.[6]

Discussion

PBP4 is a low molecular weight, non-essential PBP in *S. aureus* that is usually expressed in minute amounts compared with other PBPs in bacterial membrane proteins labelled with a saturating concentration of bocillin.[6] PBP4 has transpeptidase (TPase) activity that is needed to cross-link the peptidyl moieties in newly synthesized bacterial peptidoglycan.[6] Our previous and current results suggest that up-regulation of PBP4 (mediated by *pbp4* promoter mutation) in *mecA*-negative strains and its activity are important for β-lactam resistance (Figures 1 and 2).[6] PBP4 up-regulation leads to increased cell wall cross-linking as a probable consequence of its increased TPase activity.[6] A similar association of PBP4 overproduction leading to increased cell wall cross-linking and β-lactam resistance has been described previously.[10]

Our current results suggest that PBP4 missense mutations also contribute to PBP4-mediated β-lactam resistance although the phenotype depends on overexpression of PBP4. Notably, the β-lactam-non-susceptible clinical strains described above that bear PBP4 missense mutations lack *pbp4* promoter mutations.[7,9] Perhaps PBP4 does not mediate the resistance phenotype in these strains or the phenotype varies with strain background. Further studies are needed to precisely decipher the role that PBP4 missense mutations play in resistance. The mechanisms for decreased cell wall cross-linking associated with PBP4 missense mutations in the absence of antibiotic are unknown (Figure S2) and are currently under investigation.

CRB displayed much enhanced β-lactam resistance compared with the strain in which we reconstituted CRB's *pbp4* promoter and missense mutations (COLnex *Ppbp4*$^{(CRB)}$ *pbp4*$^{(Glu\text{-}183\rightarrow Ala \text{ and } Phe\text{-}241\rightarrow Arg)}$) (Table S3). Thus, other factors in addition to PBP4 are probably involved in the resistance of CRB. Prior genome sequence analysis of CRB revealed a point mutation in *gdpP*, which encodes a newly identified signalling protein in bacteria that has recently been associated with β-lactam resistance.[11,12] In fact, a recent clinical surveillance study has also reported mutations in *gdpP* in ceftaroline-non-susceptible strains.[7] Thus, *gdpP* could play an important role in the hyper-resistant phenotype of CRB. Our results suggest that PBP4, and its overexpression in particular, can provide an alternative mechanism of resistance to β-lactam resistance in *S. aureus*.

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Transparency declarations

None to declare.

Supplementary data

Supplementary Methods, Tables S1 to S3 and Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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