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PBP4 Mediates β-Lactam Resistance by Altered Function

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ABSTRACT Penicillin binding protein 4 (PBP4) can provide high-level β-lactam resistance in *Staphylococcus aureus*. A series of missense and promoter mutations associated with *pbp4* were detected in strains that displayed high-level resistance. We show here that the missense mutations facilitate the β-lactam resistance mediated by PBP4 and the promoter mutations lead to overexpression of *pbp4*. Our results also suggest a cooperative interplay among PBPs for β-lactam resistance.

KEYWORDS PBP4, *Staphylococcus aureus*, β-lactam resistance

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Antibiotic resistance in *Staphylococcus aureus* is an important cause of concern for the health care system worldwide. β-Lactam antibiotics are a prominent class of antibiotics used to treat infections caused by the bacteria. Resistance to traditional β-lactam antibiotics, such as penicillin, methicillin, or their derivatives, is widespread among *S. aureus* and is primarily mediated by penicillin binding protein 2a (PBP2a) (1), which is encoded by *mecA* or *mecC* (2, 3).

We previously reported that *S. aureus* strains lacking *mecA* can develop high-level β-lactam resistance on passage (4–6). Appearance of this mode of resistance in different strains of *S. aureus* (COLnex and SF8300ex, i.e., COLn and SF8300 strains lacking *mecA*) and in different β-lactam drugs (ceftaroline, ceftobiprole, and nafcillin) suggested that the underlying resistance mechanism is a general one. Genome sequences of the strains obtained from passage in ceftaroline and ceftobiprole indicated high frequencies of missense and promoter mutations in *pbp4* among these strains (6). Although the role of the *pbp4* mutations remains unclear, wild-type strains lacking *pbp4* and *mecA* were unable to develop high-level resistance (5), suggesting that PBP4 is essential for this mode of resistance. PBP4 is an uncanonical, low-molecular-weight penicillin binding protein of *S. aureus* whose mechanism of action is poorly characterized.

To determine whether the basis of high β-lactam resistance of the previously generated nafcillin-passaged strains (COLnex and SF8300ex resistant to nafcillin [CRN and SRN, respectively] [Table 1]) follows the same underlying principles of ceftaroline and ceftobiprole resistance, their genomes were sequenced. First, three colonies each from CRN- and SRN-passaged strains were chosen. All three strains displayed high-level resistance to nafcillin and ceftaroline (Table 1), suggesting a common mechanism of action. One clone each from CRN and SRN were randomly chosen, and their genomes were sequenced using the method described in Text S1 in the supplemental material.

Both CRN and SRN showed mutations targeting PBPs similar to those observed before in ceftaroline- and ceftobiprole-resistant strains. CRN had R200L and F241L PBP4

TABLE 1 Wild-type, passaged, and mutant strains used in this study

Strain	Driver for selection[a]	MIC (μg/ml) to[b]:			Mutations in:				
		CPT	NAF	VAN	PBP1	PBP2	PBP3	*pbp4* promoter	PBP4
COLnex[c]	NA	<0.25 S	0.5 S	2 S					
CRB	BPR	64 R	128 R	0.5 S				36-bp duplication at 290 bp upstream of *pbp4* start codon[e]	E183A; F241R
CRB Δ*pbp4*		0.25 S	0.25 S					36-bp duplication at 290 bp upstream of *pbp4* start codon[e]	
CmTc	CPT	>64 R	>256 R	0.5 S		D156N		A → C at −399 bp upstream of *pbp4* start codon[f]	T201A; F241L
CmTc Δ*pbp4*		0.25 S	0.25 S			D156N		A → C at −399 bp upstream of *pbp4* start codon[f]	
CRN	NAF	64 R	256 R	1 S				191-bp deletion at 135 bp upstream of *pbp4* start codon[f]	R200L; F241L
CRN Δ*pbp4*		0.25 S	0.5 S					191-bp deletion at 135 bp upstream of *pbp4* start codon[f]	
SF8300ex[d]	NA	0.25 S	0.5 S	1 S					
SRB	BPR	4 S	8 S	1 S	H499R; E567K	Y437C; V445L; Q453R; M559I	W228X[g]		E183V; F241R
SRB Δ*pbp4*		0.125 S	0.25 S		H499R; E567K	Y437C; V445L; Q453R; M559I	W228X[g]		
SRT	CPT	>64 R	64 R	1 S				"A" deletion at −378 bp upstream and 11-bp deletion at 300 bp upstream of *pbp4* start codon[f]	N138K; H270L
SRT Δ*pbp4*		0.125 S	0.25 S					"A" deletion at −378 bp upstream and 11-bp deletion at 300 bp upstream of *pbp4* start codon[f]	
SRN	NAF	32 R	128 R	1 S		Δ105 bp	T619R		
SRN Δ*pbp4*		0.25 S	0.5 S			Δ105 bp	T619R		

[a]BPR, ceftobiprole; CPT, ceftaroline; NAF, nafcillin.
[b]VAN, vancomycin; R, resistant; S, susceptible.
[c]COL strain with *mecA* excised, parent of CRB, CmTc, and CRN.
[d]SF8300 strain with *mecA* excised, parent of SRT, SRB, and SRN.
[e]See reference 13.
[f]See reference 6.
[g]X, truncation of protein.

missense mutations near the active site of PBP4 (5) and a 191-bp deletion starting at −135 bp upstream of the *pbp4* start codon. Notably, SRN lacked *pbp4* mutations and had a 105-bp deletion at the C-terminal end of the *pbp2* gene and a T619R missense mutation in PBP3 (Table 1). The 105-bp deletion in *pbp2* did not affect the transpeptidase (TPase) or glycosyltransferase (GTase) domains of PBP2 but affected a region that shares no similarity with any known domains through BLAST searches.

To identify accessory gene mutations that might be commonly present among the passaged strains, genome sequences of all six passaged strains were compared (Table 1). This revealed a total of six genes to be mutated at a frequency of at least twice among the six passaged strains (see Table S1 in the supplemental material). Five of these candidates, i.e., GdpP, FmtA, RpoB, Stp1, and ClpX, have already been implicated in β-lactam resistance (7–11), but their precise role in resistance is currently unknown. The sixth candidate, TcaA, was shown to be upregulated on treatment with cell wall-active antibiotics (12). Notably, all six passaged strains had mutations in *gdpP* (Table S1).

PBP4 drives β-lactam resistance among passaged strains. We previously showed that *pbp4* plays an important role in the resistance among passaged strains, as deletion of *pbp4* in CRB, SRB, and SRT rendered them completely susceptible to β-lactams (5, 13). All three strains had mutations in *pbp4* (Table 1). To determine *pbp4*'s role in CRN and SRN, in-frame deletion of *pbp4* was carried out as previously described (5). Deletion of *pbp4* in CRN made it completely susceptible to β-lactams (ceftaroline and nafcillin; (MICs, ≤0.5 μg/ml)). This result was expected, but strikingly, *pbp4* deletion in SRN, although it has no *pbp4* mutation, also made it completely susceptible to β-lactam drugs (ceftaroline and nafcillin; MICs, ≤0.5 μg/ml) (Table 1). These results indicated that *pbp4* played a central role in resistance not only in the CRN strain but also in the SRN strain. The roles of *pbp2* and *pbp3* mutations in SRN are unknown, but they may play a supportive role in the resistance process, underscoring the complex interplay among PBPs in *S. aureus*. Deletion of *pbp4* in CmTc, which also has *pbp4* mutations, likewise turned it into a completely susceptible strain (Table 1).

PBPs perform the penultimate steps of bacterial cell wall synthesis through their transpeptidase (TPase) and glycosyltransferase (GTase) domains (14). They are also the exquisite targets of the β-lactam class of antibiotics (1). *S. aureus* has five PBPs, of which PBP4 is considered uncanonical because it possesses only the TPase domain and is roughly half the size of the other PBPs. TPase activity mediates the cross-linking of bacterial peptidoglycan by the formation of a pentaglycine crossbridge between two adjacent PG molecules, whereas GTase activity mediates formation of a glycosidic bond between peptidoglycans (15–17). Thus, in principle, a monofunctional PBP (such as PBP4) has to work in concert with a bifunctional PBP (such as PBP2, the only known bifunctional PBP in *S. aureus*) for effective cell wall synthesis. PBP2 was previously implicated to function in concert with PBP4, although a direct interaction between them has not been shown experimentally (18). Thus, PBP2 missense mutations that were detected in CmTc and SRB apart from SRN (Table 1) probably also play a yet-to-be-determined role in resistance.

Missense mutations in PBP4 provide β-lactam resistance. A total of six missense mutations surrounding the active site of PBP4 (S75) were detected among passaged strains (5). Whether these mutations provide β-lactam resistance or have an indirect role in resistance, such as facilitating interactions with other PBPs or proteins that mediate cell wall synthesis, is currently unknown. To precisely determine the contribution of *pbp4*, we cloned the wild-type and mutated *pbp4* strains from the COLn and passaged strains (CRB, CmTc, CRN, SRB, and SRT) in a constitutive expression, high-copy-number vector (pTX_{Δ}) as described before (5). These clones were introduced to a surrogate recipient, COLnex Δ*pbp4*, a wild-type background that lacks *pbp4*, and the β-lactam resistance of these strains was evaluated. Population analysis of the resultant strains showed that the PBP4 missense mutations conferred significant nafcillin resistance to the recipient compared with that of wild-type PBP4 (Fig. 1).

Promoter mutations in *pbp4* lead to its overexpression. Four of our passaged strains

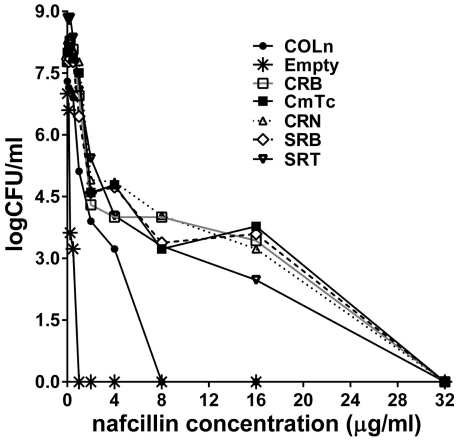

FIG 1 PBP4 missense mutations confer β-lactam resistance. *pbp4* from wild-type (COLn) and mutant passaged strains were cloned in constitutively expressed vector pTX_{Δ}. The resultant plasmids were transformed into the surrogate recipient COLnex Δpbp4 strain, and population analysis was carried out in nafcillin. Two-way analysis of variance of the data revealed a significant difference ($P < 0.0417$) between strains.

had mutations in the *pbp4* promoter region (Table 1). The promoter mutations varied widely from a small insertion and deletion to a large duplication and deletions. The 36-bp duplication that was detected in the *pbp4* promoter region of CRB resulted in its overexpression (13). To determine the role of the other *pbp4* promoter mutations in CRB expression, we cloned the *pbp4* promoters (*Ppbp4*) from wild-type (COLn) and mutant (CRB, CmTc, CRN, and SRT) passaged strains into a *lux* reporter plasmid (19). The resultant plasmids were introduced into the COLnex strain through transformation, and reporter activity was measured. All strains with a mutated *Ppbp4* had higher lux signals than the wild-type strain, suggesting enhanced *pbp4* expression due to the promoter mutations (Fig. 2). The results also suggest that the promoter mutations are responsible for *pbp4* overexpression and probably lead to considerable β-lactam resistance, as shown previously in the CRB strain (13).

PBP4 is generally expressed in very small amounts, as suggested by transcriptional analysis and bocillin assays performed using bacterial whole-cell lysates (13). Thus, *pbp4* expression is generally under tight regulatory control in bacterial cells. Enhanced *pbp4* expression due to promoter mutations suggests a lack of regulatory control that leads to enhanced *pbp4* expression. The regulators that control *pbp4* expression are currently unknown.

The resistant passaged strains displayed increased cell wall thickening and abnormal

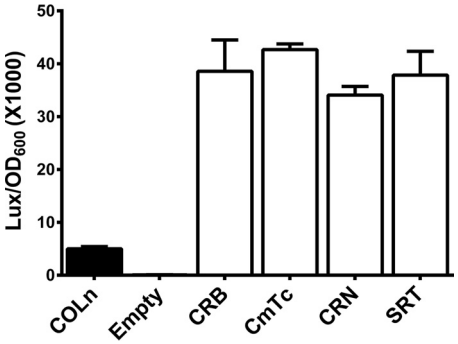

FIG 2 *pbp4* promoter mutations cause enhanced *pbp4* expression. *pbp4* promoters from wild-type (COLn) and mutant passaged strains were cloned into *lux* reporter plasmid pAmilux. Lux signals and bacterial optical density at 600 nm (OD_{600}) were measured at 4 h postculture, and data are represented by dividing the lux signal by OD_{600}. *P* values of <0.0001 were revealed by nonparametric *t* test between wild-type and other strains.

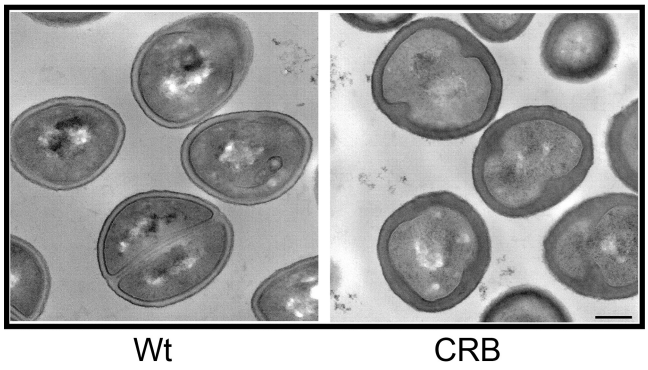

FIG 3 Transmission electron microscopy reveals cell wall thickening of CRB.

cell morphology. We previously reported that CRB had highly cross-linked peptidoglycan as a consequence of *pbp4* overexpression (13). Because peptidoglycans are the building blocks of the bacterial cell wall, enhanced cross-linking may affect bacterial cell wall morphology. To determine if peptidoglycan cross-linking affects bacterial cell wall structure, we performed transmission electron microscopic (TEM) analysis on the COLnex and CRB strains, as described in Text S1. This revealed cell wall thickening of CRB compared with its parental COLnex strain (Fig. 3). TEM analysis on other passaged strains also showed cell wall thickening and abnormal cell morphologies, such as uneven cell division, roughness of the cell surface, and compromised structural integrity (see Fig. S1 in the supplemental material). Thus, cell wall thickening was a common phenomenon among all of the passaged strains.

Since thickening of the bacterial cell wall has been attributed as one of the primary underlying factors of vancomycin intermediate resistance, we analyzed vancomycin resistance among the passaged strains. MIC to vancomycin was unaltered among passaged strains versus their parental strains (Table 1), suggesting that cell wall thickening *per se* does not impart vancomycin resistance among these strains.

In summary, our results suggest that PBP4 played a critical role in mediating high-level β-lactam resistance among all of the passaged strains. Resistance mediated through *pbp4* promoter and missense mutations contributed to the resistance phenotype. Further studies are required to determine its precise mechanism of action.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00932-17.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

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REFERENCES

1. Chambers HF, Deleo FR. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol 7:629–641. https://doi.org/10.1038/nrmicro2200.
2. Berger-Bachi B. 1999. Genetic basis of methicillin resistance in *Staphylococcus aureus*. Cell Mol Life Sci 56:764–770. https://doi.org/10.1007/s000180050023.
3. Laurent F, Chardon H, Haenni M, Bes M, Reverdy ME, Madec JY, Lagier E, Vandenesch F, Tristan A. 2012. MRSA harboring *mecA* variant gene *mecC*, France. Emerg Infect Dis 18:1465–1467. https://doi.org/10.3201/eid1809.111920.
4. Banerjee R, Gretes M, Basuino L, Strynadka N, Chambers HF. 2008. *In vitro* selection and characterization of ceftobiprole-resistant methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 52:2089–2096. https://doi.org/10.1128/AAC.01403-07.
5. Chan LC, Gilbert A, Basuino L, da Costa TM, Hamilton SM, Dos Santos KR, Chambers HF, Chatterjee SS. 2016. PBP4 Mediates high-level resistance to new-generation cephalosporins in *Staphylococcus aureus*. Antimicrob Agents Chemother 60:3934–3941. https://doi.org/10.1128/AAC.00358-16.
6. Greninger AL, Chatterjee SS, Chan LC, Hamilton SM, Chambers HF, Chiu CY. 2016. Whole-genome sequencing of methicillin-resistant *Staphylo-*

coccus aureus resistant to fifth-generation cephalosporins reveals potential non-*mecA* mechanisms of resistance. PLoS One 11:e0149541. https://doi.org/10.1371/journal.pone.0149541.
7. Aiba Y, Katayama Y, Hishinuma T, Murakami-Kuroda H, Cui L, Hiramatsu K. 2013. Mutation of RNA polymerase beta-subunit gene promotes heterogeneous-to-homogeneous conversion of beta-lactam resistance in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 57:4861–4871. https://doi.org/10.1128/AAC.00720-13.
8. Argudin MA, Dodemont M, Taguemount M, Roisin S, de Mendonca R, Deplano A, Nonhoff C, Denis O. 2017. In vitro activity of ceftaroline against clinical *Staphylococcus aureus* isolates collected during a national survey conducted in Belgian hospitals. J Antimicrob Chemother 72:56–59. https://doi.org/10.1093/jac/dkw380.
9. Baek KT, Grundling A, Mogensen RG, Thogersen L, Petersen A, Paulander W, Frees D. 2014. β-Lactam resistance in methicillin-resistant *Staphylococcus aureus* USA300 is increased by inactivation of the ClpXP protease. Antimicrob Agents Chemother 58:4593–4603. https://doi.org/10.1128/AAC.02802-14.
10. Rahman MM, Hunter HN, Prova S, Verma V, Qamar A, Golemi-Kotra D. 2016. The *Staphylococcus aureus* methicillin resistance factor FmtA is a D-amino esterase that acts on teichoic acids. mBio 7:e02070–02015. https://doi.org/10.1128/mBio.02070-15.
11. Tamber S, Schwartzman J, Cheung AL. 2010. Role of PknB kinase in antibiotic resistance and virulence in community-acquired methicillin-resistant *Staphylococcus aureus* strain USA300. Infect Immun 78:3637–3646. https://doi.org/10.1128/IAI.00296-10.
12. Utaida S, Dunman PM, Macapagal D, Murphy E, Projan SJ, Singh VK, Jayaswal RK, Wilkinson BJ. 2003. Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. Microbiology 149:2719–2732. https://doi.org/10.1099/mic.0.26426-0.
13. Hamilton SM, Alexander JA, Choo EJ, Basuino L, da Costa TM, Severin A, Chung M, Aedo S, Strynadka NC, Tomasz A, Chatterjee SS, Chambers HF. 2017. High-level resistance of *Staphylococcus aureus* to beta-lactam antibiotics mediated by penicillin-binding protein 4 (PBP4). Antimicrob Agents Chemother 61:pii=e02727-16.
14. Egan AJ, Biboy J, van't Veer I, Breukink E, Vollmer W. 2015. Activities and regulation of peptidoglycan synthases. Philos Trans R Soc Lond B Biol Sci 370:pii=20150031.
15. Bush K. 2012. Antimicrobial agents targeting bacterial cell walls and cell membranes. Rev Sci Tech 31:43–56. https://doi.org/10.20506/rst.31.1.2096.
16. Macheboeuf P, Contreras-Martel C, Job V, Dideberg O, Dessen A. 2006. Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. FEMS Microbiol Rev 30:673–691. https://doi.org/10.1111/j.1574-6976.2006.00024.x.
17. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. 2008. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev 32:234–258. https://doi.org/10.1111/j.1574-6976.2008.00105.x.
18. Leski TA, Tomasz A. 2005. Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *Staphylococcus aureus*: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. J Bacteriol 187:1815–1824. https://doi.org/10.1128/JB.187.5.1815-1824.2005.
19. Mesak LR, Yim G, Davies J. 2009. Improved *lux* reporters for use in *Staphylococcus aureus*. Plasmid 61:182–187. https://doi.org/10.1016/j.plasmid.2009.01.003.