ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2010, p. 4900–4902 Vol. 54, No. 11
0066-4804/10/$12.00 doi:10.1128/AAC.00594-10

A *mecA*-Negative Strain of Methicillin-Resistant *Staphylococcus aureus* with High-Level β-Lactam Resistance Contains Mutations in Three Genes[▽]

Ritu Banerjee,[1]* Michael Gretes,[2] Christopher Harlem,[3] Li Basuino,[3] and Henry F. Chambers[3]

Division of Pediatric Infectious Diseases, Mayo Clinic, 200 First Street, Rochester, Minnesota 55905[1]; Department of Biochemistry and Molecular Biology, University of British Colombia, Vancouver, Canada[2]; and Division of Infectious Diseases, San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, California 94110[3]

Received 29 April 2010/Returned for modification 10 July 2010/Accepted 7 August 2010

We previously generated a ceftobiprole-resistant *Staphylococcus aureus* strain after high inoculum serial passage of a *mecA*-negative variant of strain COL (R. Banerjee, M. Gretes, L. Basuino, N. Strynadka, and H. F. Chambers, Antimicrob. Agents Chemother. 52:2089–2096, 2008). Genome resequencing of this strain, CRB, revealed that it differs from its parent by five single-nucleotide polymorphisms in three genes, specifically, those encoding PBP4, a low-molecular-weight penicillin-binding protein, GdpP, a predicted signaling protein, and AcrB, a cation multidrug efflux transporter. CRB displayed resistance to a variety of β-lactams but was hypersusceptible to cefoxitin.

The efficacy of antimicrobials currently used to treat methicillin-resistant *Staphylococcus aureus* (MRSA) is decreasing (2, 7, 12, 20, 26). Novel, investigational β-lactams with activity against MRSA, including ceftobiprole and ceftaroline, are in clinical development and bind with high affinity to staphylococcal PBP2a, the penicillin-binding-protein encoded by the gene *mecA* (5, 6, 18, 22). Widespread clinical use of these newer cephalosporin antibiotics will likely generate organisms resistant to them.

Our laboratory previously reported the emergence of ceftobiprole-resistant MRSA *in vitro* (1). We demonstrated that serial passage of MRSA strain COL in subinhibitory ceftobiprole concentrations selected for point mutations in *mecA* that conferred resistance to ceftobiprole and other β-lactams. This is not surprising, given that ceftobiprole's anti-MRSA activity is due to its ability to bind to the active site of all PBPs, including that of PBP2a. However, an unexpected result was that passage of a *mecA*-negative COL variant (COLnex) also selected for high level β-lactam resistance in a derivative, named CRB (1). We undertook genome resequencing of strain CRB to identify the molecular basis for *mecA*-independent resistance to β-lactams.

Strain COLnex (a tetracycline- and methicillin-susceptible, β-lactamase negative variant of COL) lacks chromosomal *mecA*, which had been eliminated by complete excision of the SCCmec cassette element and selection for loss of methicillin resistance (14). COLnex was transformed with a plasmid vector, pAW8, and serially passaged in increasing concentrations of ceftobiprole and 10 μg/ml of tetracycline as previously described (1). A resistant mutant, CRB, was selected after 21 days of serial passage in ceftobiprole; MICs increased from 1 for the COL parent to 256 μg/ml for mutant strain CRB.

CRB displayed reduced growth rate and colonies that are smaller, less hemolytic, and less pigmented than those of the parent strain. These multiple phenotypic abnormalities suggest that CRB has alterations of global gene expression and/or cell signaling pathways. Scanning electron microscopy of CRB and COLnex in the presence and absence of β-lactam did not reveal any gross ultrastructural differences between the strains. CRB also displayed high-level resistance to all β-lactams tested but demonstrated hypersensitivity to cefoxitin, with an MIC of 8 μg/ml. Combinations of cefoxitin (at 1 μg/ml) with nafcillin and with ceftobiprole were synergistic (Table 1).

To identify candidate genes that may mediate β-lactam resistance in CRB, we used a whole-genome shotgun sequencing strategy. In collaboration with 454 Life Sciences, we resequenced the genomes of two strains: COLnex, the ceftobiprole-sensitive parent strain, and CRB. Genomes were sequenced to a depth of 16×, with average read lengths in excess of 200 bp. Individual reads were aligned against the reference sequence of COL (NC_002951). Overlapping reads were joined into contigs using the 454 Newbler assembler, and for each strain, more than 80 contigs of at least 500 bases were assembled to provide a total length of 2.7 megabase pairs and over 99.9% coverage. For strain-to-strain comparison, reads from one strain were aligned against assembled large contigs of the other strain. In addition, the entire CRB genome was manually curated and compared to that of COLnex. Three gaps in the CRB genome were identified and filled in by Sanger sequencing, yielding 100% coverage of the genome. CRB and COL differed by only five point mutations in three loci (Table 2). The differences between the two genomes were verified by sequencing using locus-specific forward and reverse primers. Mutations in all three genes appeared in the earliest stored isolate, from passage day 13.

CRB has three point mutations in locus SACOL0699 in the *pbp4* gene (also known as *pbpD*), which encodes penicillin-

* Corresponding author. Mailing address: Division of Pediatric Infectious Diseases, Mayo Clinic, 200 First St., Rochester, MN 55905. Phone: (507) 255-8464. Fax: (507) 255-7767. E-mail: Banerjee.ritu@mayo.edu.

▽ Published ahead of print on 30 August 2010.

TABLE 1. β-lactam MICs

Strain[a]	*mecA*	MIC (μg/ml)[b]							
		Naf	Amp	Cefaz	Cefox	CTX	BPR	Naf + Cefox[c]	BPR + Cefox[c]
COLn	+	256	16	256	256	>256	1	256	1
COLnex	−	0.5	<0.25	0.5	4	4	<0.25		
CRB	−	128	256	>256	8	256	128	4	8

[a] COLnex is a *mecA*-negative variant of COL; CRB is a β-lactam-resistant derivative of COLnex; COLn is a tetracycline-sensitive derivative of COL.
[b] Naf, nafcillin; Amp, ampicillin; Cefaz, cefazolin; CTX, ceftriaxone; BPR, ceftobiprole; Cefox, cefoxitin.
[c] The cefoxitin concentration was 1 μg/ml.

binding-protein 4 (PBP4). The mutations result in amino acid substitutions E183A and F241R, which cause the removal of a negative charge and the introduction of a positive charge, respectively, as well as substantial change in polarity. The mutations occur on the surface of PBP4 and close to the active site (21) and so may affect catalytic function (Fig. 1). In the PBP4-cefotaxime cocrystal structure, F241 is adjacent to the R1 group of cefotaxime, which is closely comparable to the R1 group of ceftobiprole. A major change in polarity at this amino acid position may directly modulate interactions with ceftobiprole to confer resistance (Fig. 1).

PBP4 is a low-molecular-weight penicillin-binding protein which has transpeptidase and carboxypeptidase activities and participates in peptidoglycan cross-linking. It was recently crystallized and shown to have β-lactamase activity as well (21). In COL, a prototypical hospital-associated strain, PBP4 has little effect on β-lactam resistance and is not essential (15, 17). However, it was recently shown that PBP4 is necessary for β-lactam resistance in community MRSA strains (17). It is unclear why disruption of *pbp4* results in such different phenotypes among *S. aureus* strains. Also, drastically reduced *pbp4* levels have been found in vancomycin-intermediate *S. aureus* (VISA) strains (9, 25, 27), while overexpression of PBP4 is associated with low-level methicillin resistance in strains BB255R, CDC6, and PVI (3, 10, 11). Decreased deacylation of PBP4 in strain BB255R suggested that it harbored mutations that affected protein function (3).

Interestingly, CRB demonstrated hypersensitivity to cefoxitin, a β-lactam with high affinity for PBP4. We hypothesize that mutant PBP4 has a role in mediating resistance in CRB, perhaps because of a reduced affinity for β-lactams other than cefoxitin. Because PBP2, PBP2a, and PBP4 work together in *S. aureus* in cell wall biosynthesis (16), it is possible that PBP4 has a crucial role in mediating β-lactam resistance in strains that lack PBP2a, like CRB, but may be less important in PBP2a-expressing strains, like COL.

TABLE 2. Mutated loci in strain CRB

Mutated locus	Description	Nucleotide change	Amino acid change
SACOL0699	PBP4 (penicillin-binding protein 4)	A723504C A723505G T723678G	E183A F241R
SACOL0014	GdpP (signaling protein)	C18889A	N182K
SACOL2252	AcrB/D/F (cation efflux pump)	T2316786C	I960V

CRB also contains a mutation in the locus SACOL0014, which encodes a hypothetical signaling protein that was named GdpP by Holland et al. (13). This protein consists of two domains. The N-terminal domain consists of a modified GGDEF motif that is typically found in diguanylate cyclases. In GdpP, this domain may be catalytically inactive because key catalytic residues are altered. The C-terminal domain contains a Desert Hedgehog (DHH) superfamily motif and is predicted to have phosphodiesterase activity (4). CRB harbors a mutation (N182K) that introduces a positive charge in a highly conserved residue in the GGDEF domain. GGDEF domains are widely present in bacteria and are often linked to nonhomologous domains in a variety of signaling proteins (23). The only other GGDEF-containing protein in staphylococcal species, GdpS, has a catalytically active GGDEF motif and is thought to have roles in biofilm formation and expression of virulence factors (13, 24, 29). It is possible that the mutation in GdpP may account for CRB's growth and phenotypic abnormalities.

The third locus with a mutation in CRB is SACOL2252, encoding a homolog of the AcrB/AcrD/AcrF multidrug resistance (MDR) pump in the resistance nodulation division (RND) superfamily of transporters. In CRB, the mutation in AcrB, I960V, affects a residue that is conserved among homologs in several Gram-positive species. Homologs of this transporter are found in several Gram-positive bacteria but are best characterized in Gram-negative species. In *E. coli*, AcrB

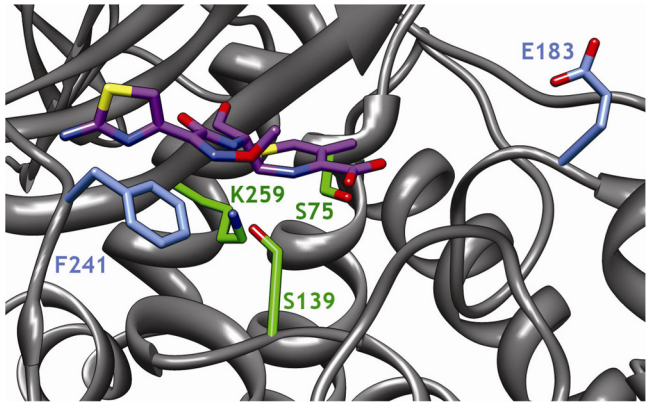

FIG. 1. PBP4 crystal structure (gray ribbon) in complex with cefotaxime (purple) as recently reported (21), shows that residues F241 and E183 (blue; mutated in ceftobiprole-resistant strain CRB) are adjacent to the active site. Active-site residues S75, S139, and K259 are shown in green. Noncarbon atoms are colored according to the CPK convention.

forms a homotrimer with AcrA and TolC, resides in the cytoplasmic (inner) membrane, and couples energy stored in a transmembrane proton gradient to export substrates (19, 28). The *S. aureus* genome does not contain homologs of AcrA and TolC. A role for transporters in β-lactam resistance is unexpected, since the targets of β-lactams are extracellular transpeptidases. Single-nucleotide polymorphisms in AcrB of *E. coli* did not alter drug resistance (8). It is possible that the conservative amino acid change in AcrB in CRB may also not affect resistance.

Strain CRB demonstrates *mecA*-independent β-lactam resistance, a mechanism that is likely to become more important as PBP2a-targeted β-lactams are developed and used clinically. Genetic studies are needed to determine if one or more of the mutations identified in CRB confer β-lactam resistance.

We thank Dale Webster and Joseph Derisi, University of California, San Francisco, for their assistance with genome sequencing analysis.

This study was supported by grants from Johnson and Johnson Pharmaceutical Research and Development (H.F.C.) and a University of California Department of Pediatrics T32 training grant (R.B.).

REFERENCES

1. **Banerjee, R., M. Gretes, L. Basuino, N. Strynadka, and H. F. Chambers.** 2008. *In vitro* selection and characterization of ceftobiprole-resistant methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **52:** 2089–2096.
2. **Boucher, H. W., G. H. Talbot, J. S. Bradley, J. J. E. Edwards, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg, and J. Bartlett.** 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin. Infect. Dis. **48:**1–12.
3. **Chambers, H. F., M. J. Sachdeva, and C. J. Hackbarth.** 1994. Kinetics of penicillin binding to penicillin-binding proteins of *Staphylococcus aureus*. Biochem. J. **301:**139–144.
4. **Chen, Y.-J., J. Sims-Mourtada, J. Izzo, and K. S. C. Chao.** 2007. Targeting the hedgehog pathway to mitigate treatment resistance. Cell Cycle **15:**1826–1830.
5. **Davies, T. A., M. G. P. Page, W. Shang, T. Andrew, M. Kania, and K. Bush.** 2007. Binding of ceftobiprole and comparators to the penicillin-binding proteins of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **51:**2621–2624.
6. **Davies, T. A., W. Shang, and K. Bush.** 2006. Activities of ceftobiprole and other β-lactams against *Streptococcus pneumoniae* clinical isolates from the United States with defined substitutions in penicillin-binding proteins PBP1a, PBP2b, and PBP2x. Antimicrob. Agents Chemother. **50:**2530–2532.
7. **Deresinski, S.** 2009. Vancomycin heteroresistance and methicillin-resistant *Staphylococcus aureus*. J. Infect. Dis. **199:**605–609.
8. **Elkins, C. A., L. B. Mullis, D. W. Lacher, and C. M. Jung.** 2010. Single nucleotide polymorphism analysis of the major tripartite multidrug efflux pump of *Escherichia coli*: functional conservation in disparate animal reservoirs despite exposure to antimicrobial chemotherapy. Antimicrob. Agents Chemother. **54:**1007–1015.
9. **Finan, J. E., G. L. Archer, M. J. Pucci, and M. W. Climo.** 2001. Role of penicillin-binding protein 4 in expression of vancomycin resistance among clinical isolates of oxacillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **45:**3070–3075.
10. **Henze, U. U., and B. Berger-Bachi.** 1996. Penicillin-binding protein 4 overproduction increases β-lactam resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**2121–2125.
11. **Henze, U. U., and B. Berger-Bachi.** 1995. *Staphylococcus aureus* penicillin-binding protein 4 and intrinsic β-lactam resistance. Antimicrob. Agents Chemother. **39:**2415–2422.
12. **Hiramatsu, K., N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi, and I. Kobayashi.** 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet **350:**1670–1673.
13. **Holland, L. M., S. T. O'Donnell, D. A. Ryjenkov, L. Gomelsky, S. R. Slater, P. D. Fey, M. Gomelsky, and J. P. O'Gara.** 2008. A Staphylococcal GGDEF domain protein regulates biofilm formation independently of cyclic dimeric GMP. J. Bacteriol. **190:**5178–5189.
14. **Katayama, Y., H. Z. Zhang, D. Hong, and H. F. Chambers.** 2003. Jumping the barrier to β-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **185:**5465–5472.
15. **Katayama, Y., H.-Z. Zhong, and H. F. Chambers.** 2003. Effect of disruption of *Staphylococcus aureus* PBP4 gene on resistance to β-lactam antibiotics. Microb. Drug Resist. **4:**329–336.
16. **Leski, T. A., and A. Tomasz.** 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.
17. **Memmi, G., S. R. Filipe, M. G. Pinho, Z. Fu, and A. Cheung.** 2008. *Staphylococcus aureus* PBP4 is essential for β-lactam resistance in community-acquired methicillin-resistant strains. Antimicrob. Agents Chemother. **52:** 3955–3966.
18. **Moisan, H., M. Pruneau, and F. Malouin.** 2010. Binding of ceftaroline to penicillin-binding proteins of *Staphylococcus aureus* and *Streptococcus pneumoniae.* J. Antimicrob. Chemother. doi:10.1093/jac/dkp503.
19. **Murakami, S.** 2008. Multidrug efflux transporter, AcrB—the pumping mechanism. Curr. Opin. Struct. Biol. **18:**459–465.
20. **Mwangi, M. M., S. W. Wu, Y. Zhou, K. Sieradzki, H. de Lencastre, P. Richardson, D. Bruce, E. Rubin, E. Myers, E. D. Siggia, and A. Tomasz.** 2007. Tracking the *in vivo* evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. Proc. Nat. Acad. Sci. U. S. A. **104:** 9451–9456.
21. **Navratna, V., S. Nadig, V. Sood, K. Prasad, G. Arakere, and B. Gopal.** 2010. Molecular basis for the role of *Staphylococcus aureus* penicillin binding protein 4 in antimicrobial resistance. J. Bacteriol. **192:**134–144.
22. **Noel, G. J.** 2007. Clinical profile of ceftobiprole, a novel β-lactam antibiotic. Clin. Microbiol. Infect. **13**(S2)**:**25–29.
23. **Ryjenkov, D. A., M. Tarutina, O. V. Moskvin, and M. Gomelsky.** 2005. Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. J. Bacteriol. **187:**1792–1798.
24. **Shang, F., T. Xue, H. Sun, L. Xing, S. Zhang, Z. Yang, L. Zhang, and B. Sun.** 2009. The *Staphylococcus aureus* GGDEF domain-containing protein, GdpS, influences protein A gene expression in a cyclic diguanylic acid-independent manner. Infect. Immun. **77:**2849–2856.
25. **Sieradzki, K., M. G. Pinho, and A. Tomasz.** 1999. Inactivated pbp4 in highly glycopeptide-resistant laboratory mutants of *Staphylococcus aureus*. J. Biol. Chem. **274:**18942–18946.
26. **Sieradzki, K., R. B. Roberts, S. W. Haber, and A. Tomasz.** 1999. The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection. N. Engl. J. Med. **340:**517–523.
27. **Sieradzki, K., and A. Tomasz.** 2003. Alterations of cell wall structure and metabolism accompany reduced susceptibility to vancomycin in an isogenic series of clinical isolates of *Staphylococcus aureus*. J. Bacteriol. **185:**7103–7110.
28. **Tornroth-Horsefield, S., P. Gourdon, R. Horsefield, L. Brive, N. Yamamoto, H. Mori, A. Snijer, and R. Neutze.** 2007. Crystal structure of AcrB in complex with a single transmembrane subunit reveals another twist. Structure **15:** 1663–1673.
29. **Tu Quoc, P. H. T., P. Genevaux, M. Pajunen, H. Savilahti, C. Georgopoulos, J. Schrenzel, and W. L. Kelley.** 2007. Isolation and characterization of biofilm formation-defective mutants of *Staphylococcus aureus*. Infect. Immun. **75:**1079–1088.