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Ceftobiprole- and Ceftaroline-Resistant Methicillin-Resistant *Staphylococcus aureus*

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The role of *mecA* mutations in conferring resistance to ceftobiprole and ceftaroline, cephalosporins with anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity, was determined with MRSA strains COL and SF8300. The SF8300 ceftaroline-passaged mutant carried a single *mecA* mutation, E447K (E-to-K change at position 447), and expressed low-level resistance. This mutation in COL conferred high-level resistance to ceftobiprole but only low-level resistance to ceftaroline. The COL ceftaroline-passaged mutant, which expressed high-level resistance to ceftobiprole and ceftaroline, had mutations in *pbp2*, *pbp4*, and *gdpP* but not *mecA*.

Treatment for methicillin-resistant *Staphylococcus aureus* (MRSA) has been complicated by increasing rates of antibiotic resistance. MRSA strains express penicillin binding protein 2a (PBP2a), encoded by *mecA*, which provides resistance to β-lactams by protecting the reactive serine in a narrow, inaccessible cleft, allowing cell wall synthesis to continue in the presence of antibiotic (1, 2). Ceftobiprole and ceftaroline are anti-MRSA cephalosporins that inhibit PBP2a at therapeutically useful concentrations. The R2 group of ceftobiprole extends into the narrow cleft of PBP2a to access the active site, whereas ceftaroline binding causes an allosteric change in PBP2a, revealing the active site for binding by a second molecule (3, 4). Ceftobiprole has been evaluated in clinical trials, and ceftaroline is FDA approved for treatment of skin and skin structure infections, including those caused by MRSA (5–10).

TABLE 1 List of parental and mutant strains used in *mecA*-positive passage studies[a]

Strain	Description	Phenotype
COLn	Parental strain	Mc^r
COLnex	SCC*mec* excision strain derived from COLn	Mc^s
COLnex(pAW8)	COLnex with an empty plasmid	Mc^s
COLnex (pYK20)	COLnex with *mecA* on a plasmid	Mc^r
$COLnex_{pB}(pYK20_{COL}B^*)$	COLnex(pYK20) mutant passaged in ceftobiprole	BPR^r Mc^r
$COLnex_{pT}(pYK20_{COL}T^*)$	COLnex(pYK20) mutant passaged in ceftaroline	CPT^r Mc^r
$COLnex_{pB}$	$COLnex_{pB}(pYK20_{COL}B^*)$ cured of *mecA* plasmid	Mc^s
$COLnex_{pT}$	$COLnex_{pT}(pYK20_{COL}T^*)$ cured of *mecA* plasmid	Mc^s
SF8300	USA300 MRSA clinical isolate	Mc^r Em^r
SF8300ex	SCC*mec* excision strain derived from SF8300 ES	Mc^s Em^s
SF8300ex(pAW8)	SF8300ex with an empty plasmid	Mc^s Em^s
SF8300ex(pYK20)	SF8300ex with *mecA* on a plasmid	Mc^r
$SF8300ex_{pT}(pYK20_{8300}T^*)$	SF8300ex(pYK20) mutant passaged in ceftaroline	CPT^r Mc^r
$SF8300ex_{pT}$	$SF8300ex_{pT}(pYK20_{8300}T^*)$ cured of *mecA* plasmid	Mc^s

[a] An asterisk indicates a plasmid generated during ceftobiprole (B) or ceftaroline (T) selection in a COL or SF8300 background.

TABLE 2 Plasmids used in *mecA*-positive passage studies

Plasmid[a]	Origin
pAW8	Empty plasmid (see reference 14)
pYK20	pAW8 containing *mecA*
$pYK20_{COL}B^*$	pYK20 in COLnex passaged in ceftobiprole
$pYK20_{COL}T^*$	pYK20 in COLnex passaged in ceftaroline
$pYK20_{8300}T^*$	pYK20 in SF8300ex passaged in ceftaroline

[a] An asterisk indicates a passaged plasmid isolated during ceftobiprole (B) or ceftaroline (T) selection in a COL or SF8300 background.

Previous studies from our group have shown that passage of the COL strain in ceftobiprole selects for resistant mutants with mutations in PBP2a (11). This study examines whether ceftaroline passage also selects for similar mutations. Passage experiments were conducted with two different MRSA backgrounds: COL, an archaic homogeneously resistant isolate from 1961, and SF8300, a heterogeneously resistant contemporary USA300 MRSA strain. COLnex and SF8300ex, *mecA*-negative derivative strains of COLn and SF8300, were obtained by (12) excising chromosomal staphylococcal cassette chromosome *mec* (SCC*mec*) (13). Wild-type *mecA* was reintroduced on plasmid pYK20 (derived from plasmid pAW8 and containing wild-type *mecA* cloned from COL [14]) into COLnex and SF8300ex for passage studies. If *mecA* was mediating resistance, then either curing a resistant mutant of the plasmid or introducing it into a susceptible background should result in loss or gain of phenotype, respectively, allowing determi-

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TABLE 3 MICs of drugs for *mecA*-positive parent and passaged mutant strains

Strain	MIC (μg/ml) of[a]:						
	NAF	AMP	CFZ	CXT	CTX	CPT	BPR
COLn	256	16	>256	>256	>256	1	2
COLnex	1	0.5	1	4	4	<0.25	1
COLnex(pAW8)	0.5	<0.25	0.5	4	4	<0.25	1
COLnex(pYK20)	128	8	256	256	>265	1	0.5
$COLnex_{pT}$($pYK20_{COL}T^*$)	128	64	>256	8	>256	64	32
$COLnex_{pB}$($pYK20_{COL}B^*$)	>256	128	>256	>256	>256	64	64
SF8300	32	128	32	64	128	1	1
SF8300ex	0.5	<0.25	1	4	4	<0.25	0.5
SF8300ex(pAW8)	<0.25	<0.25	0.5	4	4	<0.25	0.5
SF8300ex(pYK20)	32	8	128	128	>256	0.5	1
$SF8300_{pT}$($pYK20_{8300}T^*$)	128	16	128	128	256	4	4

[a] NAF, nafcillin; AMP, ampicillin; CFZ, cefazolin; CXT, cefoxitin; CTX, ceftriaxone; CPT, ceftaroline; BRP, ceftobiprole.

nation of the contribution of *mecA* to resistance. Strains and plasmids used in this study are listed in Tables 1 and 2. COLnex (pYK20) and SF8300ex(pYK20) were serially passaged in tryptic soy broth containing increasing concentrations of ceftaroline (Forest Laboratories) as previously described (11).

After 28 days, COLnex(pYK20) and SF8300ex(pYK20) passaged in ceftaroline yielded two mutants, $COLnex_{pT}$ ($pYK20_{COL}T^*$) and $SF8300ex_{pT}$($pYK20_{8300}T^*$) (an asterisk indicates a plasmid generated during ceftobiprole [B] or ceftaroline [T] selection in a COL or SF8300 background) (Table 1). MICs to ceftaroline, ceftobiprole, and other β-lactams (Sigma-Aldrich) were determined by the broth dilution method according to CLSI standards (Table 3) (15). $COLnex_{pT}$($pYK20_{COL}T^*$) expressed high-level resistance to both ceftaroline and ceftobiprole, with MICs of 64 μg/ml and 32 μg/ml, respectively. $SF8300ex_{pT}$ ($pYK20_{8300}T^*$) expressed low-level resistance by passage day 4, with a MIC of 4 μg/ml, which remained unchanged despite 24 more passages (Table 3 and Fig. 1). The previously described ceftobiprole-passaged mutant of COLnex(pYK20) (11), $COLnex_{pB}$ ($pYK20_{COL}B^*$), showed high-level resistance to both ceftobiprole and ceftaroline, with a MIC of 64 μg/ml to each.

Plasmid *mecA* was sequenced for mutations in passaged strains. Since PBPs are the target of β-lactams, *pbp1*, *-2*, *-3*, and *-4* were also sequenced. Lastly, *gdpP* and *acrB* were sequenced, as mutations in these genes had been identified in a *mecA*-negative

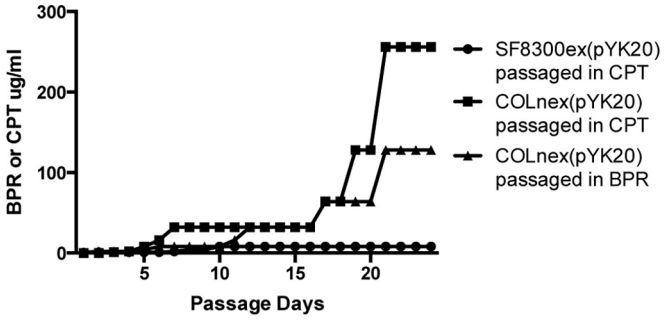

FIG 1 Emergence of ceftobiprole- and ceftaroline-passaged *mecA*-positive mutants. Resistance to ceftobiprole (BPR) and ceftaroline (CPT) was generated by passaging strains in subinhibitory concentrations of each antibiotic in broth. The highest concentration of drug in which strains grew each day is shown on the *y* axis.

TABLE 4 Mutations in *mecA*-positive mutant strains passaged with ceftobiprole and ceftaroline

Strain	Mutation(s) in[a]:						
	Plasmid-borne *mecA*	*pbp1*	*pbp2*	*pbp3*	*pbp4*	*gdpP*	*acrB*
$COLnex_{pB}$($pYK20_{COL}B^*$)	E150K, Y446L, E447K, F467Y, R589K, S649A	None	None	None	None	None	None
$COLnex_{pT}$($pYK20_{COL}T^*$)	None	None	D156N	None	T201A, F241L	H443Y	None
$SF8300ex_{pT}$($pYK20_{8300}T^*$)	E447K	None	None	None	None	None	None

[a] None, gene sequence was wild type.

TABLE 5 MICs of drugs for *mecA*-positive passaged mutant strains cured of plasmid and parental strains transduced with plasmids from passaged strains in the COL background

Strain	MIC (μg/ml) of[a]:						
	NAF	AMP	CFZ	CXT	CTX	CPT	BPR
COLn	256	16	>256	>256	>256	1	2
COLnex	1	0.5	1	4	4	<0.25	1
COLnex(pAW8)	0.5	<0.25	0.5	4	4	<0.25	1
COLnex(pYK20)	128	8	256	256	>265	1	0.5
COLnex(pYK20$_{8300}$T*)	>256	32	>256	64	32	4	64
COLnex(pYK20$_{COL}$B*)	>256	32	>256	128	>256	32	64
COLnex$_{pB}$(pYK20$_{COL}$B*)	>256	128	>256	>256	>256	64	64
COLnex$_{pB}$, plasmid cured	0.5	<0.25	0.5	4	4	<0.25	1
COLnex$_{pT}$(pYK20$_{COL}$T*)	128	64	>256	8	>256	64	32
COLnex$_{pT}$, plasmid cured	256	128	256	16	>256	64	32

[a] NAF, nafcillin, AMP, ampicillin; CFZ, cefazolin; CXT, cefoxitin; CTX, ceftriaxone; CPT, ceftaroline; BRP, ceftobiprole.

TABLE 6 MICs of drugs for *mecA*-positive passaged mutant strains cured of plasmid and parental strains transduced with plasmids from passaged strains in the USA300 strain SF8300 background

Strain	MIC (μg/ml) of[a]:						
	NAF	AMP	CFZ	CXT	CTX	CPT	BPR
SF8300	32	128	32	64	128	1	1
SF8300ex	0.5	<0.25	1	4	4	<0.25	0.5
SF8300ex(pAW8)	<0.25	<0.25	0.5	4	4	<0.25	0.5
SF8300ex(pYK20)	32	8	32	32	256	0.5	1
SF8300ex(pYK20$_{COL}$B*)	32	16	64	64	128	32	32
SF8300$_{pT}$(pYK20$_{8300}$T*)	128	16	128	128	256	4	4
SF8300$_{pT}$, plasmid cured	0.5	1	1	4	8	2	1

[a] NAF, nafcillin; AMP, ampicillin; CFZ, cefazolin; CXT, cefoxitin; CTX, ceftriaxone; CPT, ceftaroline; BRP, ceftobiprole.

ceftobiprole-resistant mutant of COL (16). Plasmid pYK20$_{8300}$T* (pYK20 from SF8300ex passaged in ceftaroline), had a single point mutation (G to A at coding sequence [CDS] position 1339) in *mecA*, resulting in a nonsynonymous amino acid change, E447K. No mutations were present in other PBP genes, *acrB*, or *gdpP* (Table 4).

Plasmid pYK20$_{COL}$T* (pYK20 from COLnex passaged in ceftaroline) had no mutations in *mecA*. A point mutation (C to T at CDS position 1327) introducing the H443Y mutation into GdpP, a point mutation (G to A at CDS position 466) introducing the D156N mutation into PBP2, and two point mutations (A to G at CDS position 601 and C to G at CDS position 723) introducing T201A and F241L mutations into PBP4 were found (Table 4). Of note, ceftaroline passage of COL containing *mecA* in its natural chromosomal location also resulted in a mutant with high-level resistance but lacking mutations in *mecA* (data not shown). Sequence analysis of that passaged mutant revealed a point mutation in *pbp2* (G to A at CDS position 1891), resulting in the amino acid change G631S, and one in *pbp4* (T to A at CDS position 414), introducing a N138K mutation. These data indicate that, even in the presence of *mecA*, ceftaroline can select for mutations in other genes, resulting in resistance.

Curing COLnex$_{pB}$(pYK20$_{COL}$B*) and SF8300ex$_{pT}$(pYK20$_{8300}$T*) of their plasmids, each of which had *mecA* mutations, decreased the MICs for all β-lactams tested (Tables 5 and 6). Curing COLnex$_{pT}$(pYK20$_{COL}$T*) of its plasmid, which lacked *mecA* mutations, had no effect on MICs (Table 5). Transforming pYK20$_{COL}$B*, which contains 6 substitution mutations in *mecA*, into the susceptible COLnex parent, yielding the transformant COLnex(pYK20$_{COL}$B*), resulted in high-level ceftaroline and ceftobiprole resistance. Transforming pYK20$_{8300}$T*, which contains the single mutation E447K in *mecA*, into COLnex, yielding the transformant COLnex(pYK20$_{8300}$T*), resulted in high-level resistance to ceftobiprole (MIC of 64 μg/ml) but only low-level resistance to ceftaroline (MIC of 4 μg/ml) (Table 5). Transforming pYK20$_{COL}$B* into SF8300ex, yielding the transformant SF8300ex(pYK20$_{COL}$B*), resulted in high-level resistance to ceftaroline and ceftobiprole (MIC of 32 μg/ml to each) (Table 6). Multiple attempts to transform SF8300ex with pYK20$_{8300}$T* were unsuccessful.

A single amino acid change at E447 in *mecA* appears to play a key role in resistance. It is present in ceftobiprole-passaged COL (among other mutations) (11), it is the only *mecA* mutation in the ceftaroline-passaged SF8300, and it has been reported in clinical isolates (17, 18). Structurally, E447 resides in the penicillin-binding domain of PBP2a and interacts with the R2 group of ceftobiprole and other β-lactams (3, 19). E447K conferred high-level ceftobiprole resistance and low-level ceftaroline resistance in the COLnex background, likely due to structural differences between the two compounds. Multiple mutations in *mecA* yield high-level resistance to both antibiotics (20). The genetic background also plays a role. Heterogeneous SF8300 passaged in ceftaroline developed low-level resistance to ceftobiprole and ceftaroline with E447K (MIC 4 μg/ml). This mutation has been associated with low-level resistance to ceftaroline in clinical isolates (17).

In conclusion, passage in either ceftaroline or ceftobiprole selects for an E447K mutation in PBP2a, a mutation found in ceftaroline-resistant clinical isolates, underscoring its importance in mediating the resistance phenotype (17, 18). Although whole-genome sequencing was not performed, which is a limitation of this study, genes other than *mecA* play a role because the level of resistance differed between COL and SF8300 backgrounds upon introduction of the E447K mutation and the highly resistant passaged COL mutant had no *mecA* mutations. Although there are likely others, mutations in genes encoding PBP4 and GdpP seem to be particularly important, as these have been repeatedly identified in ceftobiprole- and ceftaroline-passaged mutants. The role of these genes and others is under active investigation.

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