Ceftaroline Is Active against Heteroresistant Methicillin-Resistant *Staphylococcus aureus* Clinical Strains despite Associated Mutational Mechanisms and Intermediate Levels of Resistance

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important infectious human pathogen responsible for diseases ranging from skin and soft tissue infections to life-threatening endocarditis. β -Lactam resistance in MRSA involves acquisition of penicillin-binding protein 2a (PBP2a), a protein with low affinity for β -lactams that mediates cell wall assembly when the normal staphylococcal PBPs (PBP1 to -4) are blocked by these agents. Many MRSA strains display heterogeneous expression of resistance (HeR) against β -lactam antibiotics. The β -lactam-mediated homoresistant (HoR) phenotype is associated with both expression of the *mecA* gene and activation of the LexA-RecA-mediated SOS response, a regulatory network induced in response to DNA damage. Ceftaroline (CPT) is the only FDA-approved cephalosporin targeting PBP2a. We investigated the mechanistic basis of CPT activity against HeR-MRSA strains, including a set of strains displaying an intermediate level of resistance to CPT. Mechanistically, we found that 1 exposure of HeR-MRSA to subinhibitory concentrations of CPT selected for the HoR derivative activated the SOS response and increased mutagenesis. Importantly, CPT-selected HoR cells remained susceptible to CPT while still being resistant to most β -lactams, and 2-CPT activity in HeR-MRSA resided in an attenuated induction of *mecA* expression in comparison to other β -lactams. In addition, 3-CPT intermediate-resistant strains displayed a significant increase in CPT-induced *mecA* expression accompanied by mutations in PBP2, which together may interfere with the complete repression by CPT of both PBP2a and PBP2a-PBP2 interactions and thus be a determining factor in the low level of CPT resistance in the absence of *mecA* gene mutations. The present study provides mechanistic evidence that CPT represents an alternative therapeutic option for the treatment of heteroresistant MRSA strains.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important infectious human pathogen responsible for diseases ranging from skin and soft tissue infections to life-threatening endocarditis, both in hospital (HA) and community (CA) settings (1). β -Lactam resistance in MRSA involves the acquisition of *mecA*, a gene encoding penicillin-binding protein 2a (PBP2a), a protein that is refractory to inhibition by virtually all β -lactams, while the normal staphylococcal PBPs (PBP1 to -4) are blocked by these agents (2, 3). In MRSA strains, PBP2a catalyzes, in concert with the transglycosylase activity of PBP2, the biosynthesis of the bacterial cell wall (2, 3). Moreover, PBP2 localizes at the division septum, which is the main site of cell wall synthesis in *S. aureus* (4). In MRSA strains, in the presence of oxacillin, PBP2 is maintained in place by functional PBP2a (4). Clinical heteroresistant (HeR)-MRSA strains, isolated from either hospital (HA-MRSA) or community (CA-MRSA) settings and associated mainly with persistent infections, are composed of mixed cell populations where only a small portion ($\leq 0.1\%$) of the cells expresses resistance to oxacillin (OXA) levels of ≥ 10 $\mu\text{g/ml}$ (5, 6).

Ceftaroline fosamil is approved by the U.S. Food and Drug Administration (FDA) for the treatment of acute bacterial skin and skin structure infections (ABSSSI) and community-acquired bacterial pneumonia (CABP) (7) and by the European Medicines Agency (EMA) for the treatment of complicated skin and soft tissue infections (cSSTI) and community-acquired pneumonia (CAP) (7). The active metabolite, ceftaroline (CPT), is a cephalosporin with potent bactericidal activity against resistant Gram-positive organisms, including MRSA, and common Gram-negative organisms (8). The activity of

CPT on PBP2a resides in the fact that CPT binds at an allosteric region located some 60 Å away from the active site, causing the molecule to open, allowing CPT binding, which predisposes PBP2a to inactivation (9, 10). CPT maintains activity against MRSA with reduced susceptibility to vancomycin (VAN), including heteroresistant vancomycin-intermediate *S. aureus* (VISA) and daptomycin (DAP) (11, 12). In previous studies, we demonstrated that acquisition of the homoresistant (HoR) phenotype during exposure to subinhibitory concentrations of β -lactam antibiotics was associated with both increased expression of the *mecA* gene and activation of the *lexA/recA*-SOS response, a conserved regulatory network in bacteria that is induced in response to DNA damage, together leading to high levels of cross-resistance to many β -lactam antibiotics (13, 14). The present results demonstrate that HeR-MRSA, when exposed to subinhibitory concentrations of CPT, activates the SOS response and increases the mutation rate despite inhibition of PBP2a by CPT, a process that results in selection of CPT-selected HoR cells. Interestingly, CPT-selected HoR cells became highly resistant to most β -lactams while remaining susceptible to CPT. From a mechanis-

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TABLE 1 Primers used in real-time RT-PCR assays

Primer name	Primer sequence (5' to 3')
16S-F	TCCGGAATTATTGGGCGTAA
16S-R	CCACTTTCCTCTTCTGCACTCA
UmuC-F	TGCGAGTGTCTTCTTGATTG
UmuC-R	CCCTGTCTTGATGCCTAA
pbp1-GF	AGGTAGCGGTTTGTGTCC
pbp1-GR	TATCCTTGTCAGTTTACTGTC
pbp2-GF	TATTTAGCCGGTTTACCTCA
pbp2-GR	TTTTGACGTTCTTCAGGAGT
pbp3-GF	GTTGACCAACCTCATCTTA
pbp3-GR	CGGGAGACCCCTTATTATTCT
pbp4-GF	TGGTGCTAACTGCTTTGTAA
pbp4-GR	GCTAAAGCTATCGGAATGAA
PBP2a-GF	GTTAGATTGGGATCATAGCGTCATT
PBP2a-GR	TGCCTAATCTCATTGTGTTCTGTAT
PBP2a-FL	ACACATATCGTGAGCAATGAATG
PBP2a-RL	GACTCGTTACAGTGTCACTTTCAAC

tic standpoint, we found by *in vitro* and *in vivo* studies that CPT activity in HeR-MRSA also relies on attenuated induction of *mecA* expression at the level of both mRNA and protein compared to other β -lactams. In addition, we provide evidence that CPT intermediate-resistant (CPT-IR) strains displayed very significant increases in CPT-induced *mecA* expression accompanied by mutations in PBP2, which together may interfere with the ability of CPTs to completely repress both PBP2a and PBP2a-PBP2 interactions and thus be determining factors in the low level of CPT resistance in the absence of *mecA* gene mutations. Together, these observations provide evidence showing that CPT is highly active against both hetero- and homoresistant MRSA, including those clinical strains displaying intermediate levels of CPT resistance, and represents an alternative therapeutic option for the treatment of these pathogens.

MATERIALS AND METHODS

Materials and media. Trypticase soy agar with 5% sheep blood (BBL, Sparks, MD) and Mueller-Hinton (MH) agar (BBL Microbiology System, Cockeysville, MD) with and without additives (Sigma-Aldrich, St. Louis, MO, and United States Biochemicals, Cleveland, OH) were used for subculture and maintenance of *S. aureus* strains.

Antibiotics and susceptibility testing. Standard reference powders (oxacillin [OXA], nafcillin [NAF], cefotaxime [CTX], and imipenem [IPM]) were obtained from Sigma-Aldrich. Ceftaroline (CPT) was provided by Forest Research Institute (Jersey City, NJ). Antimicrobial susceptibility to OXA, NAF, CTX, cefoxitin (FOX), and CPT were determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) broth dilution methods (15) and by Etest (AB Biodisk, Solna, Sweden).

Strains and growth conditions used in this study. HeR-MRSA strains were collected from diverse sources of infections, including blood, bone, and soft tissue infections (Table 1). For this study, isogenic clinical MRSA heteroresistant *S. aureus* 13011 (HeR) (OXA MIC, 2 μ g/ml) and its highly homoresistant derivative (HoR) (OXA MIC, 256 μ g/ml) were used. The SA13011 strain is representative of a heteroresistant MRSA collection previously described (13, 16), which were determined to be OXA susceptible and *mecA* positive (13, 16). SA13011-HeR and USA100 (OXA MIC, 256 μ g/ml) were identified as ST5, SCC*mec* type II, *spa* type 2, or TJMBMDGMK. USA300 (SCC*mec* type IVa, HGFMBQBLO, and ST8) was included as a different heteroresistant SCC*mec* type strain.

Population analysis profile. Population analysis profiles (PAPs) were performed as described previously (16). Overnight cultures of SA13011-HeR and SA13011-(CPT)-HoR (SA13011-HeR selected with subinhibitory concentrations of CPT [0.125 μ g/ml]) were plated at various dilutions on Trypticase soy agar (TSA) plates containing a series of concentrations of CPT (0 to 128 μ g/ml); bacterial colonies were counted after incubation of the plates at 37°C for 48 h.

DNA manipulation and cloning. Chromosomal DNA was prepared by using the Qiagen genomic DNA preparation kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Sequence analysis of *mecA* was performed for strains SA135 and SA156 using chromosomal DNA at SeqWright (Houston, TX); primers used were consensus sequences assembled from both orientations with Vector NTI Advance 10 software for Windows (Life Technologies, Thermo Fisher Scientific, Waltham, MA). A ceftobiprole-resistant strain, namely *S. aureus* BAL16, was used as a positive-control strain containing *mecA* mutations, and *S. aureus* N315 (GenBank accession number BA000018) was used as a wild-type control.

HeR/HoR selection by sublethal concentrations of OXA or CPT. Selection of SA13011 from the HeR to the HoR phenotype was performed as we previously described (13). Briefly, bacteria were grown overnight in 5 ml LB broth without antibiotic, diluted to an optical density at 600 nm (OD₆₀₀) of ~0.025 in 300 ml LB broth, with or without subinhibitory concentrations of CPT (0.12 μ g/ml) or OXA (0.5 μ g/ml), and grown at 37°C with shaking (180 rpm). The OD₆₀₀ values were monitored every hour for up to 35 h. β -Lactam antibiotics and CPT activity MICs were determined by Etest (AB Biodisk, Solna, Sweden).

Determination of mutation frequency. Mutation frequencies for resistance to rifampin were determined as previously described (13). All of the variants were selected on TSA plates containing rifampin 200 μ g/ml and TSA plates used for serial dilutions to determine CFU/ml. Mutation frequencies were expressed as the number of rifampin-resistant mutants recovered as a fraction of the viable count. Three independent cultures were sampled in triplicate to minimize error caused by inter- and intrasample variation during the HeR/HoR selection process. Inoculated flasks were incubated at 37°C with shaking at 145 rpm; aliquots of 100 μ l were taken at different time intervals, including 6, 27, and 33 h, as we previously described (13, 14).

RNA extraction and RT-PCR assay. RNA extractions for real-time reverse transcription-PCR (RT-PCR) analysis were performed as previously described (17). Total RNA was extracted using an RNeasy isolation kit (Qiagen); all RNA samples were analyzed by A₂₆₀/A₂₈₀ spectrophotometry to assess concentration and integrity and cleaned of potential DNA contamination by treating them with DNase per manufacturer recommendations (Ambion, Inc., Life Technologies/Thermo Fisher, Austin, TX). Real-time reverse transcription-PCR analysis was done using a SensiMix SYBR one-step kit (Bioline, Taunton, MA) according to the manufacturer's protocol. Gene expression was compared according to the threshold cycle (C_T) values converted to fold change with respect to a sample considered the reference (value = 1, using $\log_2 - \Delta\Delta CT$). The change (n -fold) in the transcript level was calculated using the following equations: $\Delta CT = C_T(\text{test DNA}) - C_T(\text{reference cDNA})$, $\Delta\Delta CT = \Delta CT(\text{target gene}) - \Delta CT(16S \text{ rRNA})$, and ratio = $2 - \Delta\Delta CT$ (18). The quantity of cDNA for each experimental gene was normalized to the quantity of 16S cDNA in each sample determined in a separate reaction. Each RNA sample was run in triplicate; values represent the means from at least three separate RNA samples. Probes used are listed in Table 1.

Western blot analysis of PBP2a. Analysis of PBP2a protein expression was performed by Western blotting using protein cell lysates prepared from the SA352 strain grown to an OD₆₀₀ of 0.6 in the absence or presence of 1/2 MIC CPT, IPM, FOX, and OXA. Cell pellets were washed with 5 ml phosphate-buffered saline (PBS) and resuspended in the same buffer containing the Halt protease inhibitor cocktail as recommended by the manufacturer (Thermo Fisher, Rockford, IL). Cells were lysed with 0.25 ml of

zirconia beads (0.1 mm in diameter) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY, USA) twice for 20 s at a speed of 6,500 rpm. After centrifugation, the supernatant was transferred and diluted with loading buffer (Invitrogen, Thermo Fisher Scientific) also added to the Halt protease inhibitor. Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific). Equal protein concentrations (20 µg) were subjected to SDS-PAGE using precast gels (Invitrogen, Thermo Fisher Scientific) and electroblotted to a nitrocellulose membrane. After incubation with the corresponding primary and secondary antibodies, blots were developed by enhanced chemiluminescence (Pierce, Thermo Fisher Scientific). Primary rabbit antibody for PBP2a protein (RayBiotech, Norcross, GA) was used at a 1:10,000 dilution. Secondary horseradish peroxidase-conjugated anti-rabbit antibody was obtained from KPL (Gaithersburg, GA) and used at a 1:10,000 dilution.

Time-kill curves. Bactericidal time-kill assays (at 0, 2, 4, 6, 8, and 24 h) for CPT and β -lactams OXA, FOX, and IPM were performed using MH broth with an initial inoculum of 1×10^6 CFU/ml at $1 \times$ MICs (based on individual strain Etest data [see Table 3]), as previously described (19, 20). A minimum of two independent experiments were run for each β -lactam.

Treatment of infected larvae with HoR and CPT. Groups of *Galleria mellonella* larvae (10/group) were inoculated with the corresponding live *S. aureus* strains (1.5×10^6 CFU) as previously described (20). Repeat treatment doses of antibiotics or PBS (to control for multiple injections) were given at 24 and 48 h.

Statistical analyses. Statistical tests were performed using SPSS v. 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). The survival data were plotted using the Kaplan-Meier method.

RESULTS

In vitro activity of CPT against clinical MRSA and HeR-MRSA isolates. The MICs to CPT were determined in multiple clinical HeR-MRSA strains recovered from different sites of infection. As summarized in Table 2, CPT was active against all HeR-MRSA isolates under consideration with MICs in the range of 0.25 to 1 µg/ml and MICs to OXA between 0.5 and ≥ 32 µg/ml. CPT was also active against USA300 (HeR-MRSA) (CPT MIC, 1 µg/ml; OXA MIC, 64 µg/ml). Interestingly, two of the HeR-MRSA strains, SA135 and SA156 (OXA MIC, 256 µg/ml; IPM MIC, ≥ 32 µg/ml), displayed MIC to CPT of 2 µg/ml. These strains were classified as intermediate resistant according to CLSI breakpoints.

The activity of CPT was also tested against homoresistant (HoR)-MRSA derivatives obtained from HeR-MRSA strains SA13011, USA300, and SA352 exposed to subinhibitory concentrations of OXA (13, 16). Importantly, as shown in Table 3, CPT was very active (MIC, 0.5 µg/ml) against the highly resistant SA13011-HoR derivative, while still displaying high levels of resistance against most of the β -lactams tested (e.g., CTX, OXA, NAF, and FOX) (MICs of ≥ 16 µg/ml). Similar results were observed with HoR-MRSA USA100 (MICs for CPT, 1 µg/ml; OXA, 64 µg/ml; CTX, 256 µg/ml; NAF, 32 µg/ml; FOX, 128 µg/ml; IPM, 2 µg/ml).

In vitro time-kill analysis of CPT in comparison to other β -lactam class antibiotics in HeR- and HoR-MRSA. Isogenic strains SA352-HeR/-HoR and SA13011-HeR/-HoR, as well as control strains USA300 (HeR) and USA100 (HoR), were then tested to determine the *in vitro* efficacy of CPT by using time-kill analysis at time points 0, 2, 4, 6, 8, and 24 h. Cells were grown in MH broth with an initial inoculum of 1×10^6 CFU/ml in the absence or presence of $1 \times$ MICs of OXA, IPM, FOX, and CPT, as indicated in Fig. 1. In the case of HeR-MRSA SA352 and SA13011 strains, while no bactericidal effects were observed in cells incu-

TABLE 2 *In vitro* susceptibility to β -lactams

HeR-MRSA strain	Source	Molecular type ^a	MIC (µg/ml) ^b for:	
			OXA	CPT
SA20	Blood	ST8-IV	2	0.25
SA37	Blood	ST8-IV	4	0.5
SA44	Tissue	ST8-IV	>32	0.5
SA46	Tissue	ST8-IV	8	0.25
SA53	Bone	ST8-IV	32	0.5
SA67	Bone	ST8-IV	>32	0.5
SA352	Tissue	ST8-IV	2	0.5
SA148	Tissue	ST8-IV	2	0.5
SA73	Tissue	ST8-IV	4	0.5
SA103	Bone	ST5-II	32	1
SA106	Bone	ST5-II	>32	1
SA107	Tissue	ST5-II	32	1
SA123	Blood	ST5-II	>32	1
SA135	Tissue	ST5-II	>32	2
SA147	Blood	ST5-II	0.5	0.5
SA156	Blood	ST5-II	>32	2
SA157	Blood	ST5-II	>32	1
SA168	Blood	ST5-II	>32	1
SA187	Tissue	ST5-II	>32	1
SA221	Blood	ST5-II	>32	1
SA242	Blood	ST5-II	8	0.5
SA279	Blood	ST5-II	32	1
SA300	Blood	ST5-II	>32	1
SA387	Blood	ST5-II	>32	1
SA417	Blood	ST5-II	>32	1
SA13011	Blood	ST5-II	2	0.5

^a Combination of MLST (ST) type and SCCmec (I to IV).

^b MICs (µg/ml) of HeR *S. aureus* strains against oxacillin (OXA) and ceftaroline (CPT).

bated in the presence of OXA, IPM, or FOX, those exposed to CPT showed significant bactericidal effects (with cell killing of ≥ 5 log CFU at 24 h) compared with that achieved without or with other agents tested (Fig. 1B and C). Similarly, CPT showed dramatic bactericidal activity against the corresponding HoR-MRSA (i.e., USA100, SA352-HoR, and SA13011-HoR), demonstrating a ≥ 3 to 5 log CFU reduction compared to cells exposed to either OXA, IPM, or FOX.

We were interested in determining whether CPT was still active against CPT-IR strains. Time-kill analyses were performed for strains SA135 and SA156 (Fig. 1D) in the absence or presence of $1 \times$ MICs of OXA, IPM, FOX, and CPT, and no bactericidal effects were observed with OXA, IPM, and FOX. In the case of CPT at concentrations of $1 \times$ MIC, a bactericidal effect was observed until 8 h, which was followed by cell regrowth until 24 h. In contrast, concentrations of CPT at $2 \times$ MIC demonstrated sustained bactericidal activity, with cell killing of ≥ 5 log CFU at 24 h.

Thus, these results showed that CPT is highly active *in vitro* against multiple clinical MRSA and, more importantly, HeR-MRSA strains exhibiting variable levels of heteroresistance (HoR-MRSA), including those exhibiting intermediate CPT resistant levels.

In vivo activity of CPT against HoR-MRSA. We used a wax worm model to investigate whether CPT therapy regimens have enhanced *in vivo* efficacy against clinical HeR- and HoR-MRSA compared with the efficacies of other β -lactam regimens, including IPM, NAF, and FOX. Larvae of the greater wax moth (*Galleria mellonella*) have recently been used as an alternative to vertebrates

TABLE 3 MICs of HeR and HoR (HeR plus OXA) strains to CPT and different β -lactams

Antimicrobial agent ^a	MIC (μ g/ml) for:					
	SA13011-HeR	SA13011-HoR	USA300-HeR	USA300-HoR	SA352-HeR	SA352-HoR
CPT	0.5	2	1	1	0.38	0.5
OXA	2	256	64	≥ 256	2	≥ 256
FOX	16	256	64	192	2	8
AMX	1	8	48	≥ 256	6	24
CRO	64	256	256	256	256	256
CAZ	256	256	≥ 256	≥ 256	128	128
IPM	0.25	8	0.25	1	0.12	4

^a CRO, ceftriaxone; CAZ, ceftazidime.

as a model host for studying pathogenic microbes, virulence, and therapeutic regimens (21–23), including the assessment of the efficacy of antistaphylococcal agents (17, 20). *G. mellonella* larvae possess an immune system relatively homologous to the innate immune system of vertebrates, a digestive tract, a loosely organized muscular system, a biosynthetic fat body, and hemolymph that, analogous to blood, transports nutrients, hemocytes, and immune molecules. Numerous enzymatic cascades akin to complement fixation and blood coagulation occur in the hemolymph, resulting in hemolymph clotting and melanin production, key defense mechanisms against invading microbes (21, 24). These tissue types are similar to those encountered by *S. aureus* during invasive infections in humans. Groups of larvae (10/group) were inoculated with a bacterial suspension containing USA300-HeR, USA100-HoR, SA352-HoR, or SA13011-HoR strains and incubated for 2 h at 37°C, after which NAF (5 mg/kg) (OXA was replaced by NAF because of recommendations for its clinical use), IPM (10 mg/kg), FOX (10 mg/kg), or CPT (10 mg/kg) was administered (0 h), and then larvae were reincubated for an additional 24 h at 37°C. An uninfected group received PBS treatment to control for multiple injections. After the first 24 h of incubation, treatments were repeated. Worms were checked daily, and any deaths were recorded for a total of 10 days. While groups of worms injected with either USA300-HeR or USA100-HoR, SA352-HoR, SA13011-HoR, or PBS (untreated) or treated with either IPM, NAF, or FOX displayed very low survival rates by day 10, worms treated with CPT had survival rates between 70% and 100% (Fig. 2). Uninfected worms treated with PBS showed 90 to 100% survival. These results show that CPT is highly effective against both HeR- and HoR-MRSA strains and are consistent with the effects of CPT observed in *in vitro* time-kill studies.

Quantitation of *pbp2* and *mecA* expression levels. Ceftaroline activity against MRSA resides in its high affinity against PBP2a (9, 10). As we showed by *in vitro* and *in vivo* studies, CPT may constitute an alternative therapeutic option for the treatment of highly resistant HoR-MRSA strains. To explore more in detail the mechanistic effects of CPT in both HeR- and HoR-MRSA strains, *mecA* gene transcription levels and native *pbp1* to -4 were determined. We used RNAs extracted from HeR- and HoR-MRSA strains grown under similar conditions (uninduced/induced) to those used to perform time-kill experiments (Fig. 1). As depicted in Fig. 3A (left panel), the β -lactam antibiotics OXA, FOX, and IPM produced a strong induction in *mecA* transcription (10- to 13-fold); however, *mecA* induction by CPT occurred to a significantly lesser extent, achieving only a 3.8-fold increase. These changes in *mecA* transcription translated into smaller amounts of

PBP2a protein, as determined by Western blotting in protein lysates from cells exposed to CPT versus other β -lactams (Fig. 3A, left panel, inset). While no changes were found in the expression levels of *pbp1*, -3, or -4 (data not shown), CPT at $1\times$ MIC induced an increase in *pbp2* mRNA contents to levels slightly superior to those observed with IPM, which appeared as the only other β -lactam with that capacity (no changes in *pbp2* expression were observed with either FOX or OXA) (Fig. 3A, right panel). Taken together, these results indicate that improvement of CPT killing against HeR-MRSA may result from both its well-recognized effect on inhibiting PBP2a protein (9, 10) and, as we show herein, its ability to produce a more moderate response in terms of *mecA* induction.

Intermediate resistance to CPT in *S. aureus*. During the screening of CPT activity on HeR-MRSA, we found two strains, namely, SA135 and SA156, that displayed intermediate levels of resistance to CPT. To determine whether other β -lactam antibiotics may be associated with this effect, we screened the activity of other β -lactams and carbapenems in these two CPT intermediate-resistant strains. We found that they exhibited high levels of resistance to IPM (MICs to IPM of ≥ 32 mg/ml), especially compared to the susceptible CPT strains.

Intermediate-resistant strains have recently been characterized by Alm et al. in strains collected both in Taiwan and Spain in 2010, before CPT was introduced in Europe (25). CPT intermediate-resistant strains have also been described in Greece (26). In both studies, mutations in *mecA* have been demonstrated to be responsible for decreased CPT activity (25, 26). Two mutations, N₁₄₆K and E₁₅₀K, were found to be harbored in the non-penicillin-binding domain (nPBD) (25, 26). This region is far from the transpeptidase active site and within a domain not involved in β -lactam binding. However, alterations in this region, including E₁₅₀K, have been indirectly implicated in decreased susceptibility to β -lactam agents, mainly due to modified protein-protein interactions (26). Other mutations located at position G239L have also been described in strains displaying MICs of 2 μ g/ml, whereas the isolates with MICs of 8 μ g/ml carried additional changes in the penicillin-binding domain (G447L) (25). Taking into account these observations, we tested for the presence of mutations by sequencing the *mecA* gene and its promoter operator in both SA135 and SA156 strains expressing intermediate levels of resistance to CPT (2 μ g/ml). Analysis of *mecA* in both strains revealed no differences compared to wild-type *mecA* from *S. aureus* N315 (data not shown). As a positive control for sequencing, we used the strain BAL16, which displays resistance to ceftobiprole (BAL) and contains a mutation in *mecA* at position S643N that corre-

FIG 2 *In vivo* activity of CPT against HeR- and HoR-MRSA strains in *Galleria mellonella*. Groups of larvae (10/group) were inoculated into the last left proleg with 10 μ l of a bacterial suspension containing 1.5×10^6 CFU/ml of either HeR (USA300) or HoR (USA100, SA352-HoR, and SA13011-HoR) strains and incubated for 2 h at 37°C. After this, 10 μ l of PBS (control, no treatment), NAF (5 mg/kg), FOX (10 mg/kg), IPM (10 mg/kg), or CPT (10 mg/ml) was administered (time zero h) into the right hindmost proleg and reincubated for 24 h at 37°C. An uninfected control group received PBS treatment to control for multiple injections. The treatment was repeated after the first 24 h of incubation. Worms were checked daily and any deaths were recorded for a total of 10 days. A minimum of three independent experimental runs were performed for each antibiotic. The survival data were plotted using the Kaplan-Meier method.

sponds to the transpeptidase domain of PBP2a (A. E. Rosato, unpublished results).

We showed previously that *mecA* is among the genes for which transcription is induced directly by CPT, although to levels moderately inferior to those of other β -lactams (Fig. 3A). We investigated *mecA* expression levels by using real-time RT-PCR in both intermediate-resistant SA135 and SA156 strains under CPT-uninduced and -induced conditions. As shown in Fig. 3B, a very significant increase (between 8- and 12-fold) in *mecA* mRNA content was determined in SA135 and SA156 cells exposed to CPT. Similarly, IPM, OXA, and FOX also induced high levels of *mecA* in both strains (~6- to 12-fold) in comparison to strain SA13011 induced under similar conditions (2- to 4-fold). We then examined whether the high increases in *mecA* transcription observed in SA135 and SA156 may be due to alterations in the *mecI* repressor;

the *mecI* gene was sequenced in both SA125 and SA156 strains and no differences were found when we compared them to the N315 control strain (data not shown). These observations led us to speculate that increased *mecA* expression in CPT intermediate-resistant SA135 and SA156 was not due to an unrepressed *mecI*-mediated mechanism. It is known that in the presence of β -lactam antibiotics, PBP2a and PBP2 work in concert through cooperative effects between the PBP2 transglycosylase domain and PBP2a transpeptidase domain. PBP2 status was then explored in both intermediate-resistant strains. Sequence analysis of SA135 and SA156 *pbp2* gene revealed two nonsynonymous mutations at nucleotide positions 590 and 2120 that result in amino acid replacements C197Y and S707L, respectively, with C197Y located at the transglycosylase domain of the protein. In addition, as shown in Fig. 3C, while increased expression of *pbp2* (3.5-fold) was ob-

FIG 1 Comparative *in vitro* activity of CPT and β -lactams against heteroresistant (HeR) and homoresistant (HoR) MRSA strains. Analysis of CPT and β -lactam antibacterial efficacies against HeR/HoR-MRSA strains USA300/USA100 (A), SA352-HeR/HoR (B), SA13011-HeR/HoR (C), and CPT intermediate-resistant SA135 and SA156 (D), respectively, were determined by time-kill analysis performed using Mueller-Hinton (MH) broth; a 10^6 CFU/ml inoculum was used; samples were collected at 0, 2, 4, 6, 8, and 24 h. Concentrations of antibiotics used are indicated in the corresponding insets. A minimum of three independent experimental runs were performed for each analysis.

FIG 3 Quantitation of *mecA* and *pbp2* mRNA levels and PBP2a protein contents. Sample RNAs (SA352, SA13011, SA135, and SA156) and protein lysates (methicillin-susceptible *S. aureus* and SA352-HeR) were collected from strains after 6 h exposure to FOX, OXA, IPM, or CPT at the same concentrations used in time-kill experiments in the case of SA352 (A) or subinhibitory concentrations (1/4 MICs) for SA13011, SA135, and SA156 (B and C). Graphs show RT-PCR relative fold change values $\pm$ standard error of the mean (SEM) of specific mRNAs (reference value = 1) on the vertical axis; 16S rRNA was used as an internal control. (A) (left inset) Western blot analysis of PBP2a; 50 μ g of total lysates were run, blotted, and analyzed using a commercially available rabbit anti-PBP2a antibody. *, a $P < 0.01$ (statistically significant).

served in control strain SA13011 under CPT, FPX, and IPM induction, no changes were observed under any conditions in SA135 and SA156.

Together, these results show that CPT-intermediate resistance observed in strains SA135 and SA156 involves both a significant

increase in CPT-induced *mecA* expression and mutations in PBP2, indicating that together they may interfere with CPT's ability to repress completely both PBP2a and PBP2a/PBP2 interactions, determining a low level of CPT resistance in the absence of *mecA* gene mutations.

Exposure of HeR-MRSA strains to subinhibitory concentrations of CPT selects for β -lactam HoR populations while maintaining sensitivity to CPT. In previous studies, we have demonstrated that the highly resistant HoR-MRSA derivative strains are selected when HeR-MRSA undergoes exposure to subinhibitory concentrations of β -lactams (e.g., OXA, 0.5 μ g/ml) (13, 14). Furthermore, we have shown that this process required both *mecA* expression and SOS-mediated mutagenesis (13). The fact that CPT targets and specifically inhibits PBP2a led us to hypothesize that, under exposure to subinhibitory concentrations of CPT, selected HoR-MRSA cells may become impaired in their capacity to express high levels of resistance, despite increased mutation rates. To test this hypothesis, and to determine whether CPT may induce the SOS response, we exposed HeR-MRSA SA13011 to subinhibitory concentrations of CPT (0.12 μ g/ml) and monitored its growth up to 35 h following addition of the antibiotic. As shown in Fig. 4A, as cells growing in the absence of CPT reached the exponential phase after 20 to 24 h, when in the presence of the antibiotic, we observed an increase in the optical density (OD₆₀₀) during the first 6 h of growth, which was followed by the killing of the more susceptible cells and selection of the HoR derivative. Interestingly, and in agreement with our hypothesis, we observed that CPT-selected HoR cells [SA13011-(CPT)-HoR] did not express high levels of resistance to CPT (MIC, 0.75 μ g/ml) (Fig. 4BI); in contrast, it displayed the characteristically high level of resistance to other β -lactams, including OXA, CTX, FOX, and CEC (Fig. 4BII). Consistent with these observations, population analysis profiles (PAPs) performed in SA13011-HeR and CPT-selected homoresistant SA13011 [SA13011-(CPT)-HoR] plated on both OXA- and CPT-containing plates (Fig. 4C, upper and lower panels, respectively), reflected that while SA13011-(CPT)-HoR cells (10^2 to 10^3) grew at concentrations of OXA up to 64 μ g/ml on OXA agar plates (Fig. 4C, upper panel), same number of cells (i.e., 10^2 to 10^3) grew only up to 2 μ g/ml on CPT agar plates (Fig. 4C, lower panel). To determine whether CPT-mediated HeR/HoR selection involved the activation of the SOS response/increased mutagenesis as we previously showed with other β -lactams (e.g., OXA) (13), we analyzed the mutation frequency at 6-, 20-, and 24-h time intervals during the HeR/HoR selection. Exposure to subinhibitory concentrations of CPT determined an ~ 4 -log increase in mutation rates at both 21-h and 24-h time intervals (i.e., $2.20 \times 10^{-8}/1.04 \times 10^{-4}$ and $5.09 \times 10^{-9}/1.43 \times 10^{-4}$ with or without CPT at 21 and 24 h, respectively). As expected, cells growing in the absence of CPT did not display changes in the mutation frequency during this time (i.e., $8.1 \times 10^{-8}/6.7 \times 10^{-8}$ at 21 and 24 h, respectively). After activation, the SOS response initiates the highly dynamic expression of SOS genes, including the error-prone DNA pol V (product of the *umuC* and *umuD* genes) that results in increased mutagenesis (27). Consistent with the observations showing increased mutation rates, expression analysis of *umuC* levels as an indicator of mutagenesis showed marked increases in the amount of specific mRNA in CPT-selected cells [SA13011-(CPT)-HoR], similar to those observed in OXA-selected [SA13011-(OXA)-HoR] cells (Fig. 4D). These results indicate that although CPT induces SOS response and increases mutagenesis during the process of CPT-mediated HeR/HoR selection, effects of CPT on *mecA* (weaker induction of *mecA* and inactivation of the protein) may determine the inability of cells to express high levels of resistance to CPT, confirming our initial hypothesis in which both factors (i.e., increased *mecA* expression

and SOS-mediated mutagenesis) are required for selection of HoR-resistant cells.

DISCUSSION

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a major public health problem worldwide and a therapeutic challenge to treat (28). The changing epidemiology of MRSA infections, varying resistance to commonly used antibiotics, and involvement of MRSA in community- and hospital-associated infections are influencing the use and clinical outcomes of currently available anti-infective agents. CPT is a cephalosporin with demonstrated activity against MRSA clinical strains, including those exhibiting reduced susceptibility to vancomycin, linezolid, and daptomycin (11, 12, 29). However, less is known about the mechanistic basis of CPT activity against HeR-MRSA. The current study yields relevant findings.

First, we found that CPT was highly effective against HeR-MRSA, with the exception of two MRSA strains in which CPT demonstrated reduced susceptibility, with MIC values corresponding to an intermediate level of resistance. Previous studies performed with strains isolated in Greece (26) and Spain and Taiwan (25) identified mutations in *mecA* as responsible for CPT-intermediate resistance levels. Importantly, these CPT-IR strains were collected and analyzed prior to the launch of CPT in Europe (25, 26). In contrast, the two CPT-IR strains used in the present study were identified after CPT was introduced in the market. Moreover, they do not show mutations in the *mecA* gene, although a very significant increase in CPT-induced *mecA* expression was observed that was not linked to mutations in the *mecI* gene that could explain the high level of *mecA* expression in CPT-IR strains. Interestingly, we found that both CPT-IR strains harbored two identical nonsynonymous mutations in PBP2 that localize at the transglycosylase domain. We speculate that these features (i.e., strong CPT-induced *mecA* expression and mutations in PBP2) interfere with the efficacy of CPT to completely repress both PBP2a and PBP2a-PBP2 interactions, an effect which in turn determines the low level of CPT resistance in the absence of *mecA* gene mutations. Furthermore, since PBP2 is one of the targets of IPM (30), we hypothesized that alterations in PBP2 (e.g., mutations and changes in expression levels) may result from pre-exposure of these isolates to IMP. In support of our observations, a recent study performed in 14,902 organisms (2008 to 2010) sampled in the United States, including *S. aureus* clinical strains, determined that surrogate β -lactam markers predict CPT activity against *S. aureus*. In fact, IPM or meropenem MICs of ≤ 8 μ g/ml (susceptible and intermediate categories) were proposed as the best predictors of the outcome of CPT activity (31). Ongoing studies in our laboratory are addressing the role of PBP2/IPM in the *S. aureus* acquisition of CPT-intermediate resistance.

Second, in studies published by our group, we showed that β -lactam-mediated HeR/HoR selection required increases in both expression of *mecA* and mutagenesis in order to achieve the selection of the highly resistant HoR phenotype (13, 14). In the current study, and based on the mechanism of action of CPT, we determined that *mecA* plays a pivotal role during the selection process. Indeed, inhibition of PBP2a by CPT sensitizes HoR-MRSA to CPT while HoR-MRSA remains resistant to other β -lactams, although CPT-mediated selection was associated with increased mutagenesis through SOS induction.

FIG 4 Exposure of HeR-MRSA strains to subinhibitory concentrations of CPT selects for β -lactam HoR-populations while maintaining sensitivity to CPT. (A) Time course analysis of SA13011 selection from HeR to HoR as determined by OD₆₀₀ in cells exposed to subinhibitory concentrations of CPT (0.125 μ g/ml). (B) Susceptibility testing of SA13011-HoR was performed using SA13011-HoR selected as described for panel A; after overnight incubation, the strain was plated and CPT Etest strips (BI) or disk susceptibility testing (BII) were used; plates were incubated at 37°C for an additional 24 h. CEC, cefaclor; CTX, cefotaxime; OXA, oxacillin; FOX, cefoxitin; IMP, imipenem. (C) Population analysis profile (PAP) of SA13011-HeR and SA13011-(CPT)-HoR strains; SA13011-(CPT)-HoR was obtained after selection with subinhibitory concentrations of CPT (0.125 μ g/ml) as described in Materials and Methods. Aliquots from these cultures (10 μ l) were inoculated in agar plates containing increasing concentrations of either OXA (top) or CPT (bottom) to determine the mode of phenotypic expression of resistance. (D) Quantitation of *umuC* mRNA levels; RNA was extracted from SA13011-HeR, OXA-selected SA13011-HoR, or CPT-selected SA13011-HoR strains after 6 h exposure to either 0.5 μ g/ml OXA or 0.125 μ g/ml CPT. RT-PCR relative fold change values $\pm$ standard error of the mean (SEM) of specific *umuC* mRNAs (SA13011-HeR reference value = 1) are shown on the vertical axis; 16S rRNA was used as an internal control.

Third, the improvement in CPT killing against HeR-MRSA compared to other β -lactams was associated with a substantial reduction in CPT-induced *mecA* at the transcription and protein levels. In addition to *mecA*, we observed CPT-mediated increases in PBP2 mRNA expression. Other investigators have shown that *pbp2* and *pbp2a* are both induced in the presence of cell wall active

agents, including VAN and OXA (32). It is conceivable that inhibition of PBP2a by CPT may determine a compensatory effect targeting *pbp2* expression.

In conclusion, in the present study, we demonstrated that CPT activity is independent of the degree of heteroresistance and the clonal type expressed by different HeR-MRSA, includ-

ing USA300. Furthermore, our data suggest that CPT may be an excellent therapeutic alternative against HeR-MRSA, which is notable because these strains are composed of subpopulations of cells often associated with persistent infections that are difficult to eradicate.

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