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SUSCEPTIBILITY

Activity of Imipenem-Relebactam against Multidrug- and Extensively Drug-Resistant *Burkholderia cepacia* Complex and *Burkholderia gladioli*

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ABSTRACT The *Burkholderia cepacia* complex (Bcc) and *Burkholderia gladioli* are opportunistic pathogens that most commonly infect persons with cystic fibrosis or compromised immune systems. Members of the *Burkholderia* genus are intrinsically multidrug resistant (MDR), possessing both a PenA carbapenemase and an AmpC β-lactamase, rendering treatment of infections due to these species problematic. Here, we tested the β-lactam–β-lactamase inhibitor combination imipenem-relebactam against a panel of MDR Bcc and *B. gladioli* strains. The addition of relebactam to imipenem dramatically lowered the MICs for Bcc and *B. gladioli*: only 16% of isolates tested susceptible to imipenem, while 71.3% were susceptible to the imipenem-relebactam combination. While ceftazidime-avibactam remained the most potent combination drug against this panel of Bcc and *B. gladioli* strains, imipenem-relebactam was active against 71.4% of the ceftazidime-avibactam-resistant isolates. Relebactam demonstrated potent inactivation of *Burkholderia multivorans* PenA1, with an apparent K_i ($K_{i\ app}$) value of 3.2 μM. Timed mass spectrometry revealed that PenA1 formed a very stable adduct with relebactam, without any detectable desulfation for as long as 24 h. Based on our results, imipenem-relebactam may represent an alternative salvage therapy for Bcc and *B. gladioli* infections, especially in cases where the isolates are resistant to ceftazidime-avibactam.

KEYWORDS β-lactamases, *Burkholderia*, β-lactam, PenA, carbapenemase, β-lactamase inhibitor, imipenem, relebactam, antibiotic resistance, avibactam, ceftazidime

Burkholderia spp. are Gram-negative bacteria that can infect humans, animals, and/or plants (1, 2). Major human pathogens include species within the *Burkholderia cepacia* complex (Bcc) and *Burkholderia gladioli*, which can cause chronic infections in persons with cystic fibrosis (CF), chronic granulomatous disease, or immunodeficiency. The Bcc includes >20 closely related species that are opportunistic and often of nosocomial origin (2). Infections caused by Bcc and *B. gladioli* are difficult to treat due to inherent antimicrobial drug resistance (e.g., polymyxin resistance). β-Lactam antibiotics, such as meropenem and ceftazidime, are often used to treat infections caused by Bcc and *B. gladioli*.

β-Lactams are the largest, most prescribed class of antibiotics and inhibit the transpeptidase domains of penicillin-binding proteins (PBPs), leading to altered cell wall metabolism and eventual bacterial cell death. The most common β-lactam resistance mechanism in Gram-negative bacteria is the production of β-lactamases, which hydrolyze the amide bonds of β-lactams, thus inactivating them and preventing them from inhibiting their PBP targets. β-Lactamases are structurally grouped into four classes: A, B, C, and D. Class A, C, and D enzymes possess a serine nucleophile, and class B

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β-lactamases require Zn^{2+} for catalysis. Within these classes, each β-lactamase possesses a unique spectrum of activity toward penicillins, cephalosporins, monobactams, and carbapenems (3). The conventional approach to circumventing β-lactamases is the addition of a β-lactamase inhibitor to a β-lactam; thus, the β-lactamase is inhibited, and the β-lactam is able to inactivate the PBP target (4, 5). Much as with β-lactams, the inhibition profiles of β-lactamase inhibitors differ across β-lactamase classes, and in some cases, β-lactamase inhibitors are substrates for β-lactamases (e.g., KPC-2 versus clavulanic acid) (3, 6).

Bcc and *B. gladioli* express two chromosomally encoded inducible β-lactamases: a class A and a class C β-lactamase (7–10). PenR, a LysR-type transcriptional regulator (LTTR), regulates the expression of these β-lactamase genes (*bla*) in response to β-lactam exposure (7, 10, 11). PenA1, the class A β-lactamase of *Burkholderia multivorans* ATCC 17616, possesses a very broad hydrolytic spectrum (e.g., ampicillin, ceftazidime, aztreonam, imipenem, clavulanic acid, sulbactam) (12). The class C β-lactamase of *B. multivorans* ATCC 17616, AmpC1, contributes only minimally to β-lactam resistance due to its poor hydrolytic activity (10). The PenA-like and AmpC-like β-lactamases of Bcc and *B. gladioli* have different β-lactam hydrolysis spectra, likely due to heterogeneity in their primary amino acid sequences (7, 10, 12, 13).

Clavulanic acid, sulbactam, and tazobactam were the first β-lactamase inhibitors approved for clinical use in the 1980s to 1990s and were partnered with various β-lactam antibiotics (e.g., amoxicillin, piperacillin) (5). *B. multivorans* PenA1 is able to hydrolyze clavulanic acid, sulbactam, and tazobactam, and cells producing bla_{penA1} test nonsusceptible to these historic β-lactam–β-lactamase inhibitor combinations (12). In the past decade, a resurgence has occurred in the β-lactamase inhibitor field with the advent of diazabicyclooctanes (DBOs) and boronates (4, 14). Ceftazidime-avibactam was the first contemporary β-lactam–β-lactamase inhibitor combination to be approved in the United States by the Food and Drug Administration (FDA), in 2015. Avibactam is a novel bridged DBO β-lactamase inhibitor with a non-β-lactam scaffold that possesses inhibitory activity toward class A, class C, and some class D β-lactamases (15). Avibactam has been shown to inactivate PenA1 (16), restore susceptibility to ceftazidime when tested *in vitro* in combination against multidrug-resistant (MDR) Bcc and *B. gladioli*, and successfully treat patients (16–25). However, several studies using the ceftazidime breakpoints established by the Clinical and Laboratory Standards Institute (CLSI) have revealed that some clinical isolates of Bcc and *B. gladioli* (10 to 46% of Bcc and 76% of *B. gladioli* isolates) are nonsusceptible to ceftazidime-avibactam (16, 19, 24, 26–30). Thus, combating Bcc and *B. gladioli* with alternative regimens remains a critical undertaking.

In 2019, the FDA approved another β-lactam–β-lactamase combination, imipenem/cilastatin-relebactam. Imipenem is a carbapenem β-lactam partner, as opposed to ceftazidime, which is the cephalosporin partner of avibactam. The cilastatin inhibits human renal dehydropeptidase, which would otherwise degrade imipenem. Relebactam possesses the same core DBO scaffold as avibactam with a piperidine side chain and demonstrates inhibitory activity toward class A and class C β-lactamases (31–33). Imipenem/cilastatin-relebactam is indicated for adult use in treating complicated intra-abdominal and urinary tract infections, including pyelonephritis, as well as hospital-acquired and ventilator-acquired bacterial pneumonia (34). Imipenem/cilastatin-relebactam is designated for the treatment of a variety of susceptible Gram-negative pathogens (e.g., *Enterobacterales* and *Pseudomonas aeruginosa*) (34). In this study, the *in vitro* antimicrobial activity of imipenem-relebactam was compared to those of imipenem, ceftazidime, and ceftazidime-avibactam against a challenge panel of Bcc and *B. gladioli* strains. Moreover, the inhibitory power of relebactam against PenA1 and AmpC1 was evaluated.

RESULTS

Imipenem-relebactam is more potent than ceftazidime alone and is effective against ceftazidime-avibactam-resistant strains. Clinical isolates of Bcc (i.e., *Burkholderia ambifaria*, *B. arboris*, *B. cenocepacia*, *B. cepacia*, *B. contaminans*, *B. diffusa*, *B. dolosa*, *B. multivorans*, *B. pseudomultivorans*, *B. pyrrocinia*, *B. seminalis*, *B. stabilis*, *B. ubonensis*, and *B. vietnamiensis*) and *B. gladioli* were subjected to antimicrobial

TABLE 1 *In vitro* activities of imipenem, imipenem-relebactam, ceftazidime, and ceftazidime-avibactam against150 Bcc and *B. gladioli* strains and controls[a]

Strains	Imipenem	Imipenem-relebactam*	Ceftazidime	Ceftazidime-avibactam*
MIC_{50}	16	1	8	4
MIC_{90}	64	8	128	8
% Susceptible	16%	71.3%	62.6%	90%
% Intermediate	9.3%	12%	9.3%	4.7%
% Resistant	74.7%	16.6%	28%	5.3%
B. multivorans ATCC 17616	32	1	1	1
E. coli DH10B pBC SK(+)	0.5	0.5	0.25	0.25
E. coli DH10B pBC SK(+) bla_{penA1}	1	0.5	8	0.25
E. coli DH10B pBC SK(+) bla_{ampC1}	0.5	0.5	ND	ND

[a]Imipenem breakpoints (susceptible, ≤2 μg/ml; intermediate, 4 μg/ml; resistant, ≥8 μg/ml) for *P. aeruginosa* were used to assign phenotypes for imipenem and the combination with relebactam. Ceftazidime breakpoints (susceptible, ≤8 μg/ml; intermediate, 16 μg/ml; resistant, ≥32 μg/ml) for Bcc were used to assign phenotypes for ceftazidime and the combination with avibactam. Categories are color coded as follows: green, susceptible; yellow, intermediate; orange, resistant. ND, not determined.
*Relebactam and avibactam were tested at a fixed concentration of 4 μg/ml.

susceptibility testing using agar dilution methodology against imipenem and imipenem-relebactam. *B. multivorans* ATCC 17616 was tested on every MIC panel to control for variability between experiments. A total of 15 independent experiments were conducted to test 150 strains (Table 1 and Fig. 1; see also Table S1 in the supplemental material).

The addition of relebactam to imipenem dramatically lowered the MICs for Bcc and *B. gladioli*: only 16% of isolates tested susceptible to imipenem, while 71.3% were susceptible to the imipenem-relebactam combination (Table 1 and Fig. 1). Moreover, imipenem-relebactam lowered MICs greatly from those of ceftazidime, a first-line treatment agent for *Burkholderia* sp. infections, since only 62.6% of isolates were susceptible to ceftazidime (Table 1). Adding avibactam to ceftazidime increased susceptibility to 90% (Table 1). Thus, ceftazidime-avibactam was comparatively more potent against this panel of isolates (Fig. 2). However, imipenem-relebactam was effective against 71.4% of 14 ceftazidime-avibactam-resistant Bcc and *B. gladioli* clinical isolates (Table 2 and Fig. 3). Only 4 of the 14 isolates (*B. cenocepacia* AU0756, *B. dolosa* AU12872, *B. dolosa* AU29985, and *B. multivorans* AU28442) were resistant to all of the agents tested (Table 2 and Fig. 3).

To determine if susceptibility patterns are influenced by species, a comparison was conducted. *B. gladioli* and *B. vietnamiensis* isolates were more susceptible to imipenem, at 100% and 70%, respectively, than the other *Burkholderia* species that were tested. The addition of relebactam increased susceptibility to 100% for the *B. vietnamiensis* strains (Table S2). However, the sample size of this subanalysis is small, so generalizations on potency versus species cannot be made at this time.

Relebactam inactivates the PenA1 carbapenemase, while imipenem inhibits AmpC1. Relebactam demonstrated potent inactivation of *B. multivorans* PenA1, with an apparent K_i ($K_{i\ app}$) value of 3.2 μM, which is comparable to that of avibactam ($K_{i\ app}$ = 0.5 μM) (Table 3) (16). The acylation rate (k_2/K) was nearly 300-fold lower for relebactam than for avibactam, while the k_{off} rate for relebactam was 7-fold higher than that for avibactam. The off-rate is evident even when one measures the on-rate as the final steady-state velocities for the progress curves with relebactam sloping upward instead of plateauing (data not shown).

Relebactam was a poor inhibitor of *B. multivorans* AmpC1, with a $K_{i\ app}$ value of

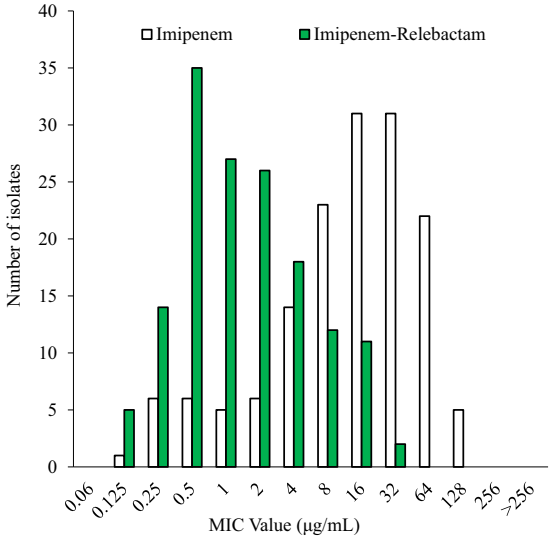

FIG 1 *In vitro* activity of imipenem alone (white bars) compared to that of imipenem combined with relebactam (green bars).

>800 μM; similarly, avibactam does not inhibit AmpC1, with a $K_{i\ app}$ value of >600 μM (10). Conversely, imipenem demonstrated measurable inhibition of AmpC1, with a $K_{i\ app}$ value of 13 $\pm$ 1 μM.

Relebactam forms a stable adduct with PenA1 and AmpC1. To discern any changes to relebactam during its reaction with PenA1 or AmpC1, timed mass spec-

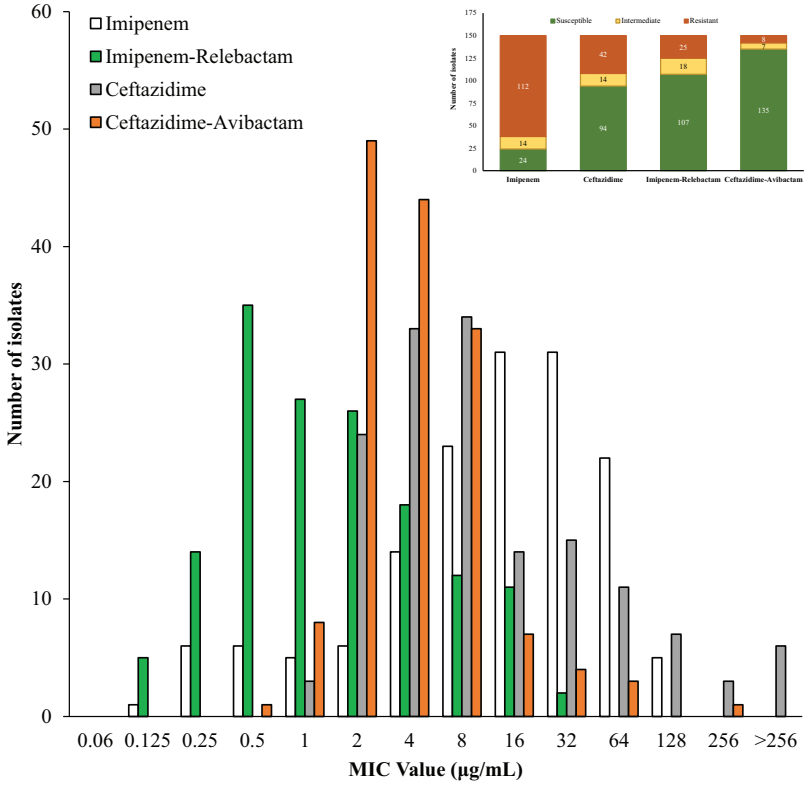

FIG 2 *In vitro* activity comparison for imipenem, imipenem-relebactam, ceftazidime, and ceftazidime-avibactam. (Inset) Numbers of isolates resistant (orange), intermediate (yellow), or susceptible (green) to imipenem, imipenem-relebactam, ceftazidime, and ceftazidime-avibactam.

TABLE 2 *In vitro* activities against *Burkholderia* sp. isolates with ceftazidime and ceftazidime-avibactam MICs of >8 μg/ml[a]

Strains	Imipenem	Imipenem-relebactam*	Ceftazidime	Ceftazidime-avibactam*
MIC_{50}	8	2	64	32
MIC_{90}	128	16	256	64
% Susceptible	35.7%	71.4%	0%	0%
% Intermediate	7.1%	0%	7.1%	42.9%
% Resistant	57.1%	28.6%	92.9%	57.1%
B. ambifaria AU11161	16	2	128	16
B. ambifaria AU20319	4	0.25	64	16
B. cenocepacia AU0756	128	32	128	32
B. cenocepacia AU19684	8	2	32	16
B. contaminans AU20979	8	2	128	32
B. dolosa AU12872	128	16	128	16
B. dolosa AU29985	32	16	512	64
B. gladioli AU16341	2	2	64	64
B. gladioli AU26456	0.25	0.25	16	16
B. gladioli AU29541	1	1	64	64
B. gladioli AU30473	1	0.5	32	32
B. multivorans AU28442	64	16	>128	>128
B. stabilis AU10235	16	2	256	32
B. vietnamiensis AU3578	0.5	0.5	64	16

[a]Imipenem breakpoints (susceptible, ≤2 μg/ml; intermediate, 4 μg/ml; resistant, ≥8 μg/ml) for *P. aeruginosa* were used to assign phenotypes for imipenem and the combination with relebactam. Ceftazidime breakpoints (susceptible, ≤8 μg/ml; intermediate, 16 μg/ml; resistant, ≥32 μg/ml) for *B. cepacia* were used to assign phenotypes for ceftazidime and the combination with avibactam. Categories are color coded as follows: green, susceptible; yellow, intermediate; orange, resistant.
*Relebactam and avibactam were tested at a fixed concentration of 4 μg/ml. The ceftazidime and ceftazidime-avibactam data presented here were obtained previously as part of another study (16).

trometry of PenA1 or AmpC1 incubated with relebactam was conducted. The molecular masses of PenA1 and AmpC1 alone were 29,412 ± 5 Da and 39,750 ± 5 Da, respectively (Fig. 4). By examining the reaction course of PenA1 and relebactam at a 1:1 ratio at 1 min, 15 min, 60 min, and 24 h, a single major peak of 29,761 ± 5 Da was observed, corresponding to the addition of 349 Da, essentially identical to relebactam's molecular mass of 348.38 Da (Fig. 4). Thus, PenA1 formed a very stable adduct with relebactam, without any detectable desulfation under the conditions tested (Fig. 4). Despite the high $K_{i\ app}$ values for AmpC1 and relebactam, by increasing the amount of relebactam relative to that of AmpC1 (1:100), an AmpC1–relebactam complex was obtained as early as 15 min and remained stable at 24 h (Fig. 4).

Imipenem-relebactam maintains induction of bla_{penA1}, while relebactam alone does not influence expression. Imipenem is a known inducer of bla_{penA1}. In order to determine the effects of imipenem-relebactam and relebactam on the expression of bla_{penA1} and bla_{ampC1}, *B. multivorans* ATCC 17616 cells were grown and exposed to sub-MICs of the test agents for 1 h. The cells were lysed and subjected to immunoblotting with an anti-PenA1 peptide antibody and an anti-AmpC1 antibody in order to detect the PenA1 and AmpC1 β-lactamases, respectively. An anti-RecA antibody was used as a loading control. Induction of bla_{penA1} was observed upon exposure to imipenem or imipenem-relebactam, but not upon exposure to relebactam alone (Fig. 5). Induction of bla_{ampC1} was not detected under any of the conditions (Fig. 5).

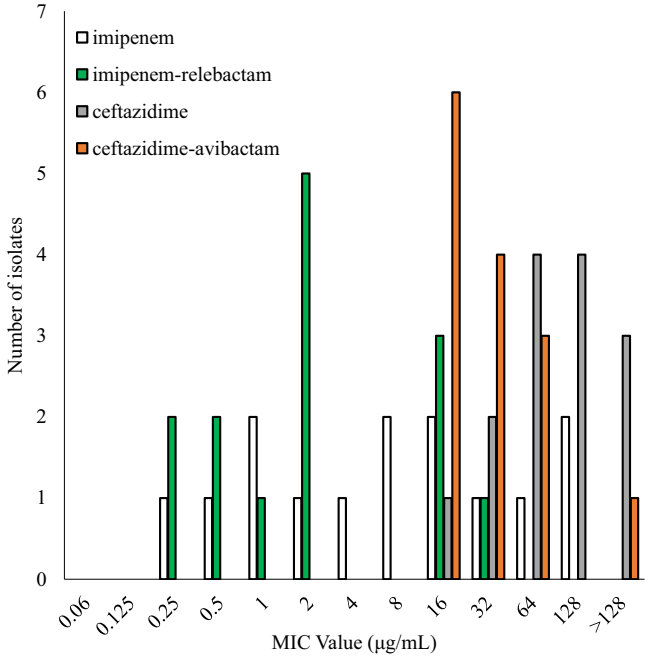

FIG 3 Comparison of *in vitro* activities of imipenem, imipenem-relebactam, ceftazidime, and ceftazidime-avibactam against 14 strains with ceftazidime and ceftazidime-avibactam MICs of >8 μg/ml.

DISCUSSION

By use of agar dilution susceptibility testing, the activity of imipenem-relebactam was evaluated against a challenge panel of Bcc and *B. gladioli* strains. The addition of relebactam lowered imipenem MICs overall, with only 16.6% of isolates remaining resistant. Imipenem-relebactam was superior to the first-line agent ceftazidime, which is commonly used to treat *Burkholderia* sp. infections. Ceftazidime-avibactam outperformed imipenem-relebactam overall. However, against ceftazidime-avibactam-resistant isolates, imipenem-relebactam demonstrated potent activity, with 71.4% testing susceptible. Unlike relebactam alone, imipenem and imipenem-relebactam are able to induce the expression of bla_{penA1}. Furthermore, the kinetics with PenA1 and relebactam revealed that despite inhibition of the enzyme, the on-rate was lower and the off-rate was higher, which, along with the induction of bla_{penA1}, may explain why imipenem-relebactam is not as potent against Bcc as ceftazidime-avibactam. Moreover, despite the lack of inhibitory activity against AmpC1, once relebactam formed a complex with AmpC1, the complex was stable for as long as 24 h. In summary, imipenem-relebactam represents a potential alternative therapy for infections caused by Bcc and *B. gladioli*, especially in cases where the isolates are resistant to ceftazidime-avibactam.

TABLE 3 Steady-state inhibitor kinetic values against *B. multivorans* PenA1[a]

Parameter (unit)	Value for:	
	Relebactam	Avibactam[b]
$K_{i\ app}$	3.2 ± 0.5 μM	500 ± 50 nM
k_2/K (M^{-1} s^{-1})	(7.2 ± 2.0) × 10^3	(2 ± 1) × 10^6
k_{off} (s^{-1})	(1.4 ± 0.5) × 10^{-2}	(2 ± 1) × 10^{-3}
$t_{1/2}$ (min)	0.83	5.8
t_n (15 min)[c]	30	1

[a]Individual data points were collected in triplicate, while all experiments were completed at a minimum in duplicate.
[b]Data from reference 16.
[c]t_n (15 min), turnover number at 15 min.

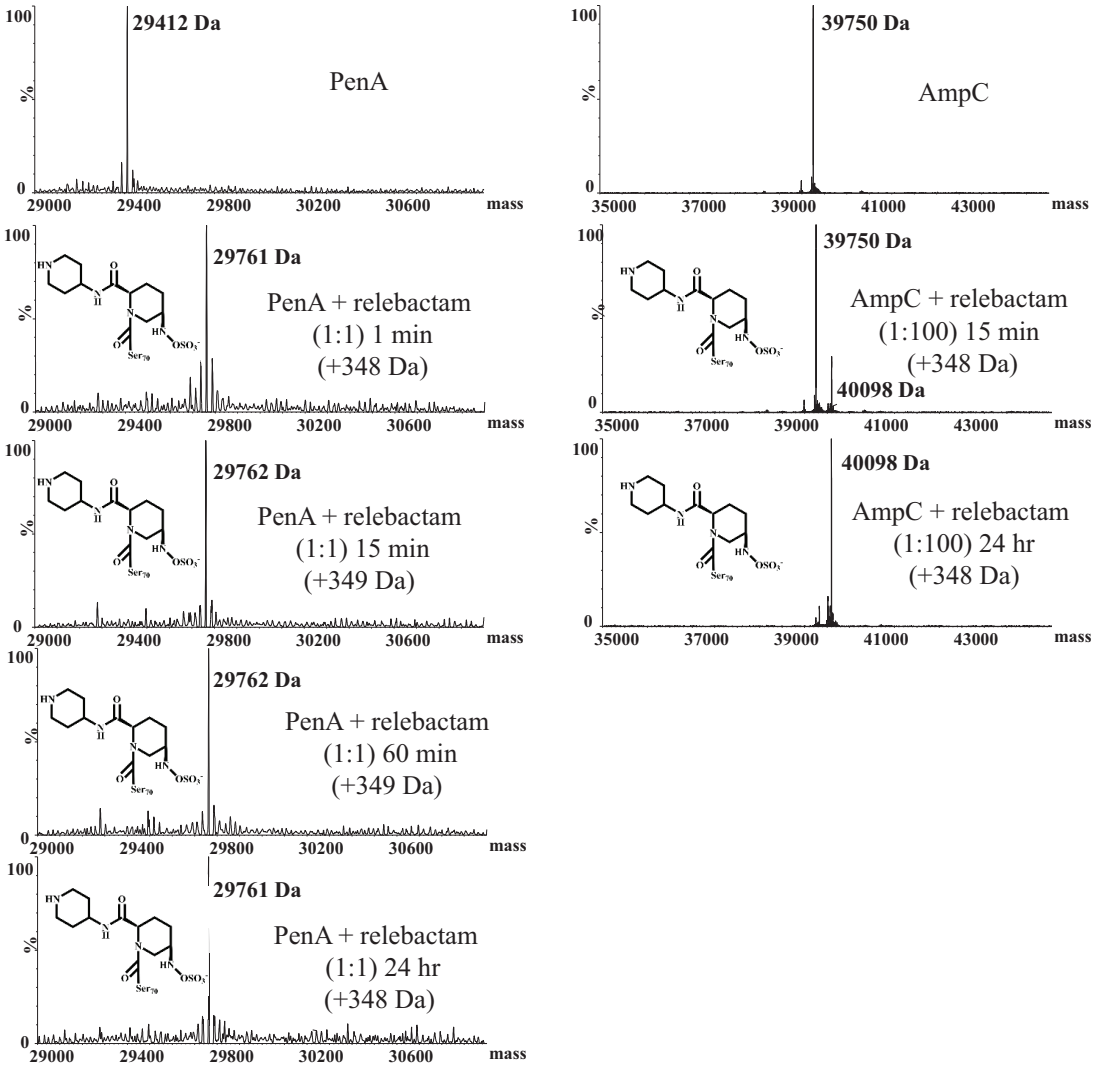

FIG 4 Timed mass spectrometry of PenA1 and AmpC1 with relebactam.

MATERIALS AND METHODS

Antibiotics and reagents. Imipenem and relebactam were provided by Merck. Nitrocefin was purchased from Thermo Scientific Oxoid. The data reported for ceftazidime and avibactam were from a previous study (16).

Bacterial strains. A total of 150 MDR clinical strains, including 140 Bcc (14 different species) and 10 *B. gladioli* strains, were obtained from the *Burkholderia cepacia* Research Laboratory and Repository (University of Michigan) (7, 16, 35). These 150 isolates were recovered from respiratory specimens from 150 individuals with CF receiving care in 68 cities in 36 states in the United States. Each Bcc isolate was identified to the species level by using species-specific PCR, *recA* restriction fragment length polymorphism (RFLP), and/or DNA sequencing of the *recA* gene (36, 37). If the species could not be determined, the isolate is listed as Bcc Indeterminate.

***In vitro* susceptibility test methods.** MICs for the bacterial isolates were determined by the cation-adjusted Mueller-Hinton (MH) agar dilution method. The MIC measurements were performed by using a Steers Replicator that delivered 10 μl at 10^4 CFU of overnight culture grown in MH broth at 37°C and diluting in fresh MH broth. Relebactam was tested at 4 μg/ml in combination with increasing concentrations of imipenem. MIC results were interpreted using CLSI breakpoints, where available (30).

Enzyme expression, purification, and steady-state kinetics. *E. coli* DE3 Origami 2 cells carrying pGEX-6p2 bla_{penA1} and pGEX-6p2-bla_{ampC1} were used for protein expression and purification, as described previously (10, 12). Briefly, cells were grown in Super Optimal Broth, and then isopropyl-β-D-1-thiogalactopyranoside was added to induce expression. Cells were pelleted and were frozen at −20°C. Subsequently, the cells were lysed, and the β-lactamase was purified and verified by electrospray ionization mass spectrometry, as described previously (10, 12). Kinetic parameters were obtained as described

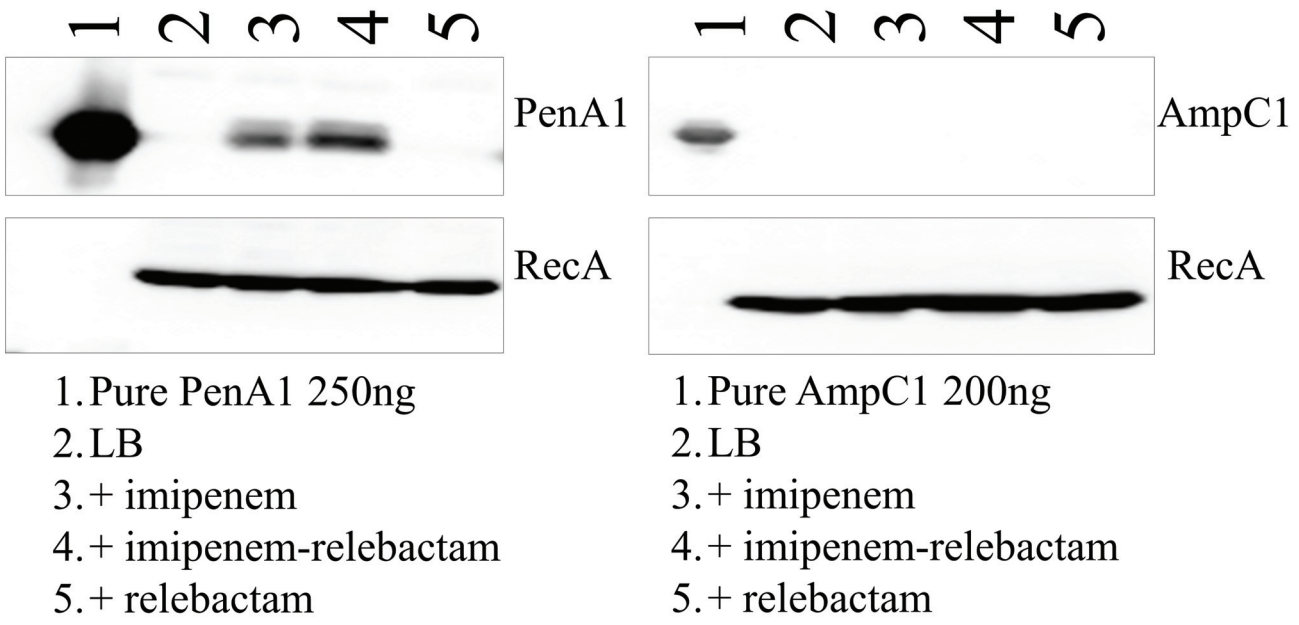

FIG 5 Immunoblotting for PenA1 and AmpC1 following induction by imipenem (1 μg/ml), imipenem-relebactam (1 μg/ml-4 μg/ml), or relebactam (4 μg/ml) using *B. multivorans* ATCC 17616. RecA was used as a control.

below; individual data points were obtained in triplicate, while each experiment was conducted at least in duplicate.

Kinetic parameters for PenA1 and AmpC1 were obtained using an Agilent 8453 Diode Array spectrophotometer in 10 mM phosphate-buffered saline, pH 7.4 (PBS), at room temperature using previously described methods (15, 38).

The apparent K_i ($K_{i\ app}$) value was determined for PenA1 and AmpC1 using a direct competition assay under steady-state conditions. PenA1 and AmpC1 were both maintained at 10 nM, while the relebactam concentration was varied. Nitrocefin was used as the reporter substrate at a fixed concentration of 100 μM. The β-lactamase, relebactam, and nitrocefin were mixed manually, and the initial reaction velocity was monitored. Data were linearized by plotting the inverse initial reaction velocities ($1/v_0$) versus inhibitor concentration (I). $K_{i\ app}$ was determined by dividing the value for the y-intercept by the slope of the line and accounting for the use of nitrocefin as a reporter.

To obtain the acylation rate (k_2/K), progress curves were obtained by mixing PenA1 at 5 nM with increasing concentrations of relebactam and using nitrocefin at 100 μM as a reporter substrate. Progress curves were fit to the following equation to obtain observed rate constant (k_{obs}) values:

$$y = v_f \cdot x + (v_0 - v_f) \cdot [1 - \exp(-k_{obs} \cdot x)]/k_{obs} + A_0$$

Here, v_f is final velocity, v_0 is initial velocity, and A_0 is initial absorbance at a λ of 482 nm. The data were plotted as k_{obs} versus [relebactam]. The k_2/K value was obtained by correcting the value obtained for the slope of the line for the use of nitrocefin as an indicator substrate.

The off-rate (k_{off}) of relebactam for PenA1 was determined by incubating 5 μM PenA1 with 25 μM relebactam for 5 min at room temperature, diluting the mixture to 1:10,000, and adding 100 μM nitrocefin. Progress curves measuring nitrocefin hydrolysis were collected for 1 h, and the data were fit to the equation given above to obtain k_{off}. PenA1 alone and nitrocefin alone were used as controls.

Timed mass spectrometry. The masses of intact PenA1 and AmpC1 with and without relebactam were measured by mass spectrometry on a Waters Synapt G2-Si quadrupole time-of-flight mass spectrometer. The Synapt G2-Si mass spectrometer was calibrated with a sodium iodide solution using a 50- to-2,000 m/z mass range. PenA1 at 10 μM was incubated with 10 μM relebactam at a 1:1 ratio for 15 min, 1 h, and 24 h in PBS. PenA1 and AmpC1 were also incubated alone as controls. AmpC1 at 10 μM was incubated with 1,000 μM relebactam at a 1:100 ratio for 15 min and 24 h in PBS. At the desired time points, the reactions were terminated by the addition of a final concentration of 0.1% formic acid and 1% acetonitrile. The samples were run using a Waters Acquity H class ultraperformance liquid chromatograph (UPLC) and an Acquity UPLC BEH C_{18} column (particle size, 1.7 μm; inside diameter, 2.1 mm; length, 100 mm). The aqueous phase consisted of 0.1% (vol/vol) formic acid in water (A), and the organic phase was made up of acetonitrile with 0.1% formic acid (B); the flow rate was maintained at 0.5 ml/min. Initial parameters consisted of 90% A and 10% B. At 1 min, a gradient of 19% A and 81% B was started, and it lasted until min 4. At 4 min, the gradient was adjusted to 15% A and 85% B and was maintained for 30 s. Thereafter, the gradient was adjusted to 10% A and 90% B for 30 s, and by min 5, the initial

conditions were reinitiated. The tune settings were as follows: capillary voltage at 3.5 kV, sampling cone at 35, source offset at 35, source temperature at 100°C, desolvation temperature at 500°C, cone gas at 100 liters/h, desolvation gas at 800 liters/h, and nebulizer bar at 6.0. The spectra were analyzed using MassLynx, v4.1, and were deconvoluted using the MaxEnt1 program.

Immunoblotting. *B. multivorans* ATCC 17616 was grown in lysogeny broth to log phase at an optical density at 600 nm (OD_{600}) between 0.6 and 0.7. The cells were treated with sub-MICs of imipenem (1 μg/ml) or imipenem-relebactam (0.5 μg/ml to 4 μg/ml) for 1 h. Subsequently, the cells were pelleted and lysed to prepare crude extracts, as described previously (39). These crude extracts, as well as purified full-length PenA protein, were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% (wt/vol) nonfat dry milk in 20 mM Tris-Cl with 150 mM NaCl (pH 7.4) (TBS) for 1 h and were probed in 5% nonfat dry milk in TBS with 1 μg/ml of a polyclonal anti-PenA peptide antibody or anti-AmpC antibody and 1 μg/ml of a polyclonal anti-RecA antibody. All antibodies were raised in rabbits by New England Peptide using a selected PenA 18-amino-acid peptide, the AmpC protein, and the RecA protein as the antigens, respectively (7, 10). The membranes were washed five times for 10 min with TBS with 0.05% Tween 20 (TBST). For protein detection, blots were incubated for 1 h with a 1:5,000 dilution of a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody in 5% nonfat dry milk in TBS. The blots were washed five times for 10 min with TBST and were developed using the ECL-Plus developing kit (GE Healthcare Life Sciences) according to the manufacturer's instructions. A Fotodyne Luminary/FX system was used to capture images.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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