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Substrate Spectrum Extension of PenA in *Burkholderia thailandensis* with a Single Amino Acid Deletion, Glu168del

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We describe a deletion mutation in a class A β-lactamase, PenA, of *Burkholderia thailandensis* that extended the substrate spectrum of the enzyme to include ceftazidime. Glu168del was located in a functional domain called the omega loop causing expansion of the space in the loop, which in turn increased flexibility at the active site. This deletion mutation represents a rare but significant alternative mechanical path to substrate spectrum extension in PenA besides more common substitution mutations.

Burkholderia pseudomallei is the etiological agent of septicemic melioidosis, which is endemic in Southeast Asia and northeastern Australia (4). *Burkholderia mallei*, the cause of glanders, is a species derived from a clone of *B. pseudomallei* (20). Bcc, which is a complex composed of more than 10 *Burkholderia* species, including *Burkholderia cepacia*, *Burkholderia cenocepacia*, and *Burkholderia multivorans*, is a group of nosocomial pathogens that cause respiratory and systemic infections in patients with cystic fibrosis or chronic granulomatous disease and in other immunocompromised patients (10). The antibiotic regimen used to treat infections with these bacteria generally includes ceftazidime (10, 26). To date, only a few cases of ceftazidime resistance in *Burkholderia* spp. have been reported and resistance has pointed mostly to a single gene, *penA*. Two single amino acid substitutions, Pro167Ser and Cys69Tyr (amino acid residue numbering according to Ambler et al. [1]), have been described in PenA of ceftazidime-resistant *B. pseudomallei* isolates (15, 17, 18, 22). Similarly, two orthologs of PenA, PenB2 and PenB3, with seven and two amino acid alterations, respectively, were shown to be associated with ceftazidime resistance in clinical isolates of *B. cenocepacia* (13).

Here we report a new mutation that extends to ceftazidime the substrate spectrum of PenA of *B. thailandensis* (3) (BTH_II1450 of *B. thailandensis* strain E264), which is closely related to PenA enzymes of the pathogenic species *B. pseudomallei*, *B. mallei*, and Bcc (13, 17, 22). The resistant isolate arose against a high level of ceftazidime (5 μg/ml) in LB medium. The MIC of ceftazidime for this isolate was 48 μg/ml, which was significantly higher than the 1.75 μg/ml of the wild type (Fig. 1). By PCR amplifying *penA* from its genomic DNA using primers penA-F (5′-CGTCAATCCGATG CAGTACC-3′) and penA-R (5′-GCCGTTATCGCACCTTTAT C-3′) and sequencing the amplicon in both directions using a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA), we found a three-base deletion mutation in the coding region of *penA* (Fig. 1). To confirm that this *penA* gene with the mutation was responsible for the ceftazidime resistance that developed in the mutants, we inactivated the *penA* gene by replacing a region spanning 196 bp in the middle of the coding region with a Tet^r cassette, which was obtained from broad-host-range vector pRK415K (9). The *penA*-null mutant was verified by PCR using primers penA_LF (5′-AACAGATCGCCGAGATGG-3′) and penA_LR (5′-GCGAACGTTGCCCGATAC-3′), which hybridize to the genomic regions outside *penA*. The mutant strain with inactivated *penA*-Glu168del (that is, with Δ*penA*-Glu168del) lost resistance to ceftazidime, whose MIC was comparable to that for the wild-type strain with Δ*penA*-WT (Fig. 1). The MICs were measured by the Etest (8) in accordance with the manufacturer's instructions (AB Biodisk, Solna, Sweden). When a mutant strain with Δ*penA*-Glu168del was provided with an intact copy of *penA*-Glu168del in *trans*, carried by pRK415K, ceftazidime resistance was restored (Fig. 1), indicating that *penA* was the factor responsible for ceftazidime resistance.

The mutation resulted in the deletion of an amino acid residue located in the omega loop, a structural domain constituting part of the active-site pocket (7, 16). Specifically, this deleted residue Glu168, which is the first of the three residues constituting the helical structure in the middle of the loop and is also located in a conserved domain in class A β-lactamases ^{166}EXXLN^{170} (numbering according to Ambler et al. [1]) (11) (Fig. 1). Glu168del artificially constructed in TEM-1 resulted in ceftazidime resistance in *Escherichia coli* (19). However, this deletion mutation has never been found in natural variants of class A β-lactamases, including TEM and SHV enzymes (TEM and SHV tables summarizing variants in each group can be found at http://www.lahey.org/Studies/).

To investigate the structural change in PenA that correlates with the substrate spectrum extension to ceftazidime, we conducted modeling analyses using SED-1 (Protein Data Bank identification code 3BFE; 54% amino acid sequence identity to PenA) (12) as a template with SYBYL-X (Tripos Inc., St. Louis, MO). With the predicted structures, molecular dynamic simulations were conducted using OpenMM Zephyr 2.0.3 (6) for 500 ps (0.002 ps/step) at 303.15 K with the Amber03 force field to release any structural constraints originating from the template. Energy minimization was then performed using SYBYL-X (Tripos Inc., St. Louis, MO) with a Tripos force field until the energy gradient reached 0.001 kcal/(mol · Å). We chose SED-1 for the simulation

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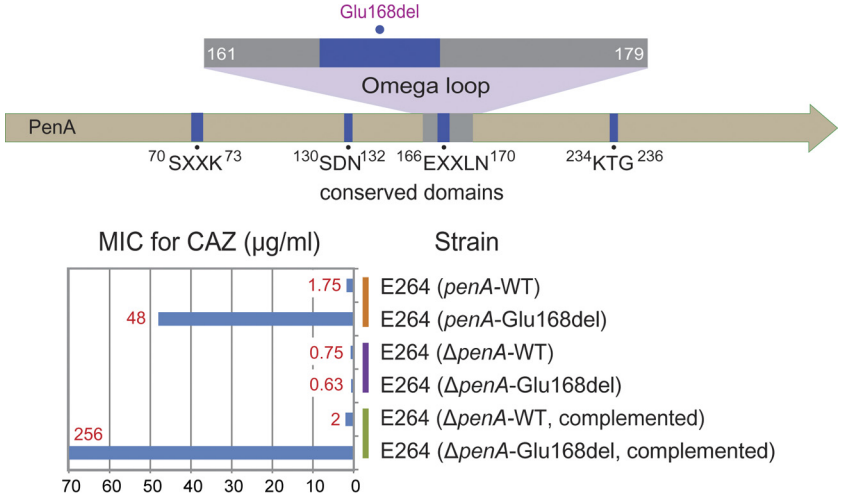

FIG 1 A single amino acid deletion mutation in PenA. The PenA protein is represented by an arrow in which four conserved domains of sequences and the omega loop common to class A β-lactamases (11) are indicated. The positions are numbered according to Ambler et al. (1). *B. thailandensis* strain E264 with the intact *penA* allele (*penA*-WT) and others with various *penA* alleles are listed, and the MICs of ceftazidime (CAZ) are shown in the bar graph. Complemented strains contain the *penA* alleles, corresponding to the disrupted genes, carried by plasmid pRK415K.

because, similar to wild-type PenA, it does not hydrolyze ceftazidime and also has high homology to PenA. In our simulation, the size and shape of the binding pocket did not show significant changes resulting from the amino acid deletion. Instead, we observed that Glu168del resulted in disruption of the α-helix structure in the omega loop and increased distance between positions 164 and 179, possibly affecting the stabilizing salt bridge between the two residues that clamp the omega loop structure at both ends (7) (Fig. 2). This, in turn, suggests that the possible disruption of the ionic bond between residues 164 and 179 may destabilize the omega loop (11, 14, 23). In addition, we noted that the space in the omega loop (calculated as the distances between amino acid resi-

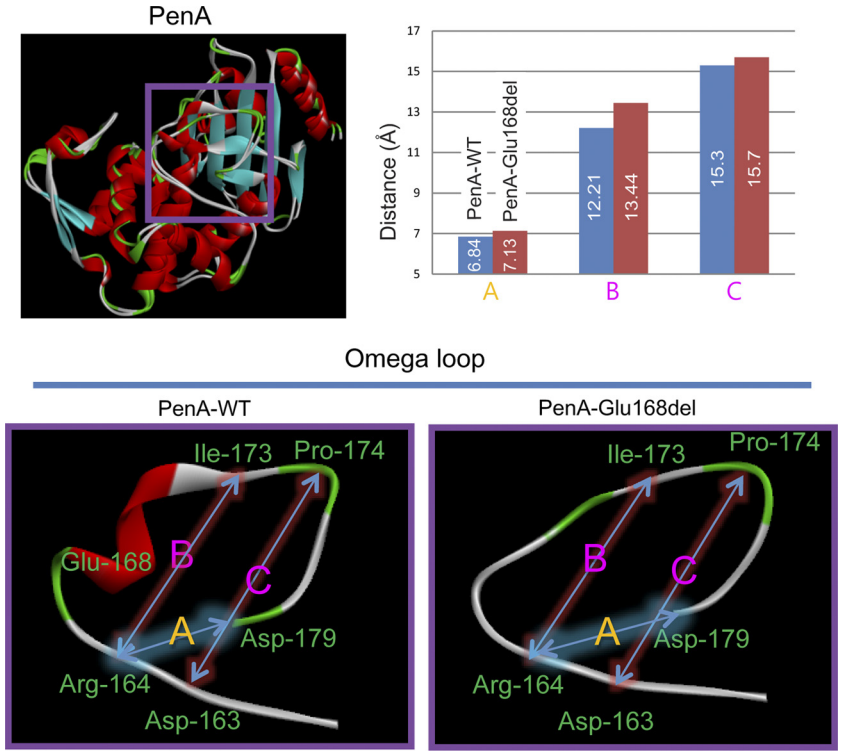

FIG 2 Altered α-helix structure and the internal space in the omega loop of PenA. Glu168del resulted in disruption of the α-helix structure and expansion of the internal space in the omega loop. The measured distances are designated A, B, and C in the simulated three-dimensional omega loop structure. The distance between positions 164 and 179, where the ionic bond is present (at least in the wild type), in PenA-Glu168del is compared to that in the wild type in the bar graph. The distances between positions 164 and 173 and positions 163 and 174 in the omega loop, representing the internal space of the omega loop, are also compared to those in the wild type in the bar graph.

TABLE 1 MICs of various β-lactams for *B. thailandensis* strains

Strain	MIC (μg/ml)[a]					
	AMX	AMX-CLA	CTXM	CRX	CEF	MER
Wild-type E264 (*penA*-WT)	36	6	6	16	18	0.75
Mutant E264 (*penA*-Glu168del)	4	2.25	4.5	2.5	9	0.63
penA-null mutants						
E264 (Δ*penA*-WT)	4	1.5	1	0.5	3	0.75
E264 (Δ*penA*-Glu168del)	3	2.25	0.75	0.22	3	0.38
Complemented *penA*-null mutants[b]						
E264 (Δ*penA*-WT, complemented)	>256	48	>32	>32	64	0.75
E264 (Δ*penA*-Glu168del, complemented)	10	2.25	12	12	48	1.13

[a] MICs were measured by the Etest. Abbreviations: AMX, amoxicillin; AMX-CLA, amoxicillin-clavulanic acid; CTXM, cefotaxime; CRX, ceftriaxone; CEF, cefepime; MER, meropenem.
[b] Complemented with *penA* alleles carried by pRK415K.

dues 163 and 174 and between 164 and 173) increased in the mutated PenA sequence compared to that in the wild-type PenA sequence, notably between 164 and 173 (Fig. 2). We postulate that the removal of the α-helix structure and increased space in the omega loop may accompany increased flexibility of the loop in the mutant enzymes. This flexibility would then, in turn, relieve steric hindrance between the omega loop and the bulky 7β side chain of ceftazidime, thereby increasing the accessibility of ceftazidime to the binding pocket (25).

To investigate the extent to which the structural changes in PenA that were adjusted to ceftazidime affect the enzyme's activity toward other β-lactam antibiotics, we measured the MICs of selected β-lactam antibiotics. These antibiotics included the four that the wild-type enzyme was able to hydrolyze, a carbapenem antibiotic (meropenem), and a β-lactamase inhibitor (clavulanic acid with amoxicillin). Mutated PenA exhibited decreased levels of resistance to the original substrates, as observed in many mutant forms of the class A β-lactamase that acquired activity against broad-spectrum cephalosporins (24) (Table 1). The hydrolytic activity of the mutated PenA enzyme against amoxicillin was effectively inhibited by clavulanic acid. In addition, resistance to meropenem was not observed in the wild type or the mutant (Table 1).

The levels of ceftazidime resistance in clinical *B. pseudomallei* isolates (5, 21) and in Bcc (2, 16) have been determined to be low. However, the continued use of ceftazidime in clinical settings suggests a high potential for the increased emergence of ceftazidime resistance in these bacterial groups. In this regard, predetermination of possible mutations in PenA capable of altering the substrate spectrum of the enzyme is significant. Furthermore, these data have substantial value, as they suggest that Glu168del, which was characterized only in an artificially constructed TEM-1 derivative (19), indeed is highly likely to occur in natural settings in PenA.

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REFERENCES

1. **Ambler RP, et al.** 1991. A standard numbering scheme for the class A beta-lactamases. Biochem. J. **276**(Pt 1):269–270.
2. **Bonacorsi S, Fitoussi F, Lhopital S, Bingen E.** 1999. Comparative in vitro activities of meropenem, imipenem, temocillin, piperacillin, and ceftazidime in combination with tobramycin, rifampin, or ciprofloxacin against *Burkholderia cepacia* isolates from patients with cystic fibrosis. Antimicrob. Agents Chemother. **43**:213–217.
3. **Brett PJ, DeShazer D, Woods DE.** 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int. J. Syst. Bacteriol. **48**(Pt 1):317–320.
4. **Cheng AC, Currie BJ.** 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. **18**:383–416.
5. **Dance DAB, Wuthiekanun V, Chaowagul W, White NJ.** 1989. The antimicrobial susceptibility of *Pseudomonas pseudomallei*. Emergence of resistance in vitro and during treatment. J. Antimicrob. Chemother. **24**: 295–309.
6. **Friedrichs MS, et al.** 2009. Accelerating molecular dynamic simulation on graphics processing units. J. Comput. Chem. **30**:864–872.
7. **Jelsch C, Mourey L, Masson JM, Samama JP.** 1993. Crystal structure of *Escherichia coli* TEM1 beta-lactamase at 1.8 A resolution. Proteins **16**:364–383.
8. **Joyce LF, Downes J, Stockman K, Andrew JH.** 1992. Comparison of five methods, including the PDM Epsilometer test (E test), for antimicrobial susceptibility testing of *Pseudomonas aeruginosa*. J. Clin. Microbiol. **30**: 2709–2713.
9. **Keen NT, Tamaki S, Kobayashi D, Trollinger D.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70**:191–197.
10. **Leitão JH, et al.** 2010. Pathogenicity, virulence factors, and strategies to fight against *Burkholderia cepacia* complex pathogens and related species. Appl. Microbiol. Biotechnol. **87**:31–40.
11. **Matagne A, Lamotte-Brasseur J, Frere JM.** 1998. Catalytic properties of class A beta-lactamases: efficiency and diversity. Biochem. J. **330**:581–598.
12. **Petrella S, Clermont D, Casin I, Jarlier V, Sougakoff W.** 2001. Novel class A β-lactamase Sed-1 from *Citrobacter sedlakii*: genetic diversity of β-lactamases within the *Citrobacter* genus. Antimicrob. Agents Chemother. **45**:2287–2298.
13. **Poirel L, Rodriguez-Martinez J-M, Plesiat P, Nordmann P.** 2009. Naturally occurring class A beta-lactamases from the *Burkholderia cepacia* complex. Antimicrob. Agents Chemother. **53**:876–882.
14. **Raquet X, et al.** 1994. TEM beta-lactamase mutants hydrolysing third-generation cephalosporins: a kinetic and molecular modelling analysis. J. Mol. Biol. **244**:625–639.
15. **Rholl DA, et al.** 2011. Molecular investigations of PenA-mediated β-lactam resistance in *Burkholderia pseudomallei*. Front. Microbiol. **2**:139. doi: 10.3389/fmicb.2011.00139.
16. **Saika T, Kobayashi I, Hasegawa M, Nishida M.** 2002. Antimicrobial resistance and DNA-fingerprinting pattern of *Burkholderia cepacia* blood isolates. J. Infect. Chemother. **8**:341–344.
17. **Sam IC, See KH, Puthucheary SD.** 2009. Variations in ceftazidime and amoxicillin-clavulanate susceptibilities within a clonal infection of *Burkholderia pseudomallei*. J. Clin. Microbiol. **47**:1556–1558.
18. **Sarovich DS, et al.** 2012. Characterization of ceftazidime resistance mechanisms in clinical isolates of *Burkholderia pseudomallei* from Australia. PLoS One **7**:e30789. doi:10.1371/journal.pone.0030789.
19. **Simm AM, Baldwin AJ, Busse K, Jones DD.** 2007. Investigating protein structural plasticity by surveying the consequence of an amino acid deletion from TEM-1 β-lactamase. FEBS Lett. **581**:3904–3908.
20. **Song H, et al.** 2010. The early stage of bacterial genome-reductive evolution in the host. PLoS Pathog. **6**:e1000922. doi:10.1371/journal.ppat.1000922.
21. **Thibault FM, Hernandez E, Vidal DR, Girardet M, Cavallo JD.** 2004. Antibiotic susceptibility of 65 isolates of *Burkholderia pseudomallei* and *Burkholderia mallei* to 35 antimicrobial agents. J. Antimicrob. Chemother. **54**:1134–1138.
22. **Tribuddharat C, Moore RA, Baker P, Woods DE.** 2003. *Burkholderia*

pseudomallei class a beta-lactamase mutations that confer selective resistance against ceftazidime or clavulanic acid inhibition. Antimicrob. Agents Chemother. **47**:2082–2087.
23. **Vakulenko SB, Toth M, Taibi P, Mobashery S, Lerner SA.** 1995. Effects of Asp-179 mutations in TEMpUC19 beta-lactamase on susceptibility to beta-lactams. Antimicrob. Agents Chemother. **39**:1878–1880.
24. **Wang X, Minasov G, Shoichet BK.** 2002. Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. J. Mol. Biol. **320**:85–95.
25. **Wong W-T, et al.** 2011. Increased structural flexibility at the active site of a fluorophore-conjugated β-lactamase distinctively impacts its binding toward diverse cephalosporin antibiotics. J. Biol. Chem. **286**:31771–31780.
26. **Wuthiekanun V, Peacock SJ.** 2006. Management of melioidosis. Expert Rev. Anti Infect. Ther. **4**:445–455.