Substrate spectrum extension of PenA in *Burkholderia thailandensis* with a single amino acid deletion Glu168del

Hyojeong Yi§, Karan Kim§, Kwang-Hwi Cho2, Oksung Jung2, and Heenam Stanley Kim1*

1Department of Medicine, College of Medicine, Korea University, Anam-Dong, Seongbuk-Gu, Seoul 136-705, Korea; 2School of Systems Biomedical Science and Research Center for Integrative Basic Science, Soongsil University, Seoul 156-743, Korea

Running Title: Glu168del in a β-lactamase PenA

Key Words: antibiotic resistance, class A β-lactamase, substrate spectrum, *Burkholderia*, PenA, ceftazidime, Glu168del

§These authors contributed equally to this work.

*All correspondence to:
Dr. Heenam Stanley Kim
Tel:82-2-920-6422
E-mail: hstanleykim@korea.ac.kr
Abstract

We describe a deletion mutation in a class A β-lactamase PenA in *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). The 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 (CF) or chronic granulomatous disease, and in other immuno-compromised patients (10). The antibiotic regimen used to treat infections by these bacteria generally includes ceftazidime (10, 26). To date, only a few cases of ceftazidime resistance in *Burkholderia* spp. have been reported and resistance mostly pointed to a single gene, *penA*. Two single amino acid substitutions, Pro167Ser and Cys69Tyr (amino acid residue numbering following 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 7 and 2 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 extending substrate spectrum to ceftazidime in *penA*
from *B. thailandensis* (3) (BTH_II1450 from *B. thailandensis* strain E264), which is closely
related to PenAs in the pathogenic species *B. pseudomallei, B. mallei*, and the *Burkholderia*
cepacia complex (Bcc) (13, 17, 22). The resistant isolate arose against a high level of
ceftazidime (5 μg/ml) in LB medium. The MIC for ceftazidime of this isolate was measured to
be 48, which was significantly higher than 1.75 of the wild type (Fig. 1). By PCR-amplifying
*penA* from its genomic DNA using the primers *penA*-F (5’-CGTCAATCCGATGCAGTACC-3’)
and *penA*-R (5’-GCCGTTATCGCACCTTTATC-3’), and sequencing the amplicon using
a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) in both directions, 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* by replacing a region spanning 196 bp in the middle of
the coding region with a *tetR* cassette, which was obtained from a broad host range vector
pRK415K (9). The *penA* null mutant was verified by PCR using a primer pair *penA*-LF (5’-
AACAGATCGCCGAGATGG-3’) and *penA*-LR (5’-GCGAACGTTGCCCCGATAC-3’) that
hybridize to the genomic regions outside *penA*. The mutant strain with inactivated *penA-
Glu168del* (that is, with Δ*penA*-Glu168del) lost resistance to ceftazidime, having an MIC
comparable to the level of the wild-type strain with Δ*penA*-WT (Fig. 1). The MIC values were
measured by the E-test (8), following the manufacturer’s instructions (AB Biodisk, Solna,
Sweden). When a mutant strain with Δ*penA*-Glu168del was provided with an intact copy of
the 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 a deletion of an amino acid residue located in the omega loop, a structural domain constituting part of the active-site pocket (7). Specifically, this deleted a residue Glu168, 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 166EXXLN170 (numbering following 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 (PDBID: 3BFE, 54% of AA identity to PenA) (12) as a template with SYBYL-X (Tripos Inc., St. Louis, MO, USA). 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. Then, energy minimization was performed using SYBYL-X (Tripos Inc., St. Louis, MO, USA) with a Tripos force field until the energy gradient reached 0.001 kcal/(mol·A). We chose SED-1 for the simulation because it does not hydrolyze ceftazidime similar to the wild-type PenA 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 the 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 residues 163 and 174 and between 164 and 173) increased in the mutated PenA compared to the wild type PenA, 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. Then, this flexibility would in turn relieve steric hindrance between the omega loop and the bulky 7β-side chain of ceftazidime, thereby increasing accessibility of ceftazidime to the binding pocket (25).

To investigate the extent to which the structural changes in the PenA that were adjusted to ceftazidime affect the enzyme activity towards other β-lactam antibiotics, we measured the MICs for selected β-lactam antibiotics. These antibiotics included the four that the wild-type enzyme was able to hydrolyze and a carbapenem antibiotic (meropenem) and a β-lactamase inhibitor (clavulanic acid with amoxicillin). The mutated PenA exhibited decreased levels of resistance to the original substrates as observed in many mutants of the class A β-lactamase that acquired activity to 3rd generation cephalosporins (24) (Table 1). The hydrolytic activity of the mutated PenA 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 the high potential for the increased emergence of ceftazidime.
resistance in these bacterial groups. In this regard, pre-determination of possible mutations in PenA capable of altering the substrate spectrum of the enzyme is significant. Furthermore, this data has substantial value, as it suggests 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.

**Acknowledgments**

This work was supported by grants 20090058514 from the Basic Research Program and 20110016847 from the Core Research Program, both of which are from the Ministry of Education, Science & Technology in the Republic of Korea. Additional support was provided by the Korea Foundation for International Cooperation of Science & Technology (KICOS) through a grant provided by the Korean Ministry of Science & Technology (MOST) in K20903001812-11E0100-01700 to H.S.K.

**Figure Legends**

Figure 1. A single amino acid deletion mutation in PenA. The PenA protein is represented by an arrow, on which four conserved domains of sequences and the omega loop common to class A \( \beta \)-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 their MIC levels for ceftazidime are shown in the bar graph.
Strains noted “complemented” contain the penA alleles, corresponding to the disrupted genes, carried by a plasmid pRK415K.

Figure 2. Altered α-helix structure and the internal space in the omega loop in PenA. Glu168del resulted in the disruption of the α-helix structure and expansion in the internal space in the omega loop. The measured distances are denoted A, B, and C in the simulated 3D omega loop structure. The distance between positions 164 and 179, where the ionic bond is present (at least in the wild type), of the PenA-Glu168del is compared to that of the wild type in a bar graph. The distances between positions 164-173 and 163-174 in the omega loop representing the internal space of the omega loop are also compared to those of the wild type in the bar graph.

Table 1. MICs for various β-lactams with B. thailandensis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMX</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>E264 (penA-WT)</td>
<td>36</td>
</tr>
<tr>
<td>Mutant</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>E264 (penA-Glu168del)</td>
<td>4</td>
</tr>
<tr>
<td>penA-null mutant strains</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>E264 (ΔpenA-WT)</td>
<td>4</td>
</tr>
<tr>
<td>E264 (ΔpenA-Glu168del)</td>
<td>3</td>
</tr>
<tr>
<td>penA-null mutants complemented by penA alleles carried by pRK415K</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>E264 (ΔpenA-WT, complemented)</td>
<td>&gt;256</td>
</tr>
<tr>
<td>E264 (ΔpenA-Glu168del, complemented)</td>
<td>10</td>
</tr>
</tbody>
</table>

*MICs were measured by the E-test.
Abbreviations: AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CTXM, cefotaxime; CRX, ceftriaxone; CEF, cefepime; MER, meropenem.

Literature cited


