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Burkholderia pseudomallei Class A β-Lactamase Mutations That Confer Selective Resistance against Ceftazidime or Clavulanic Acid Inhibition

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***Burkholderia pseudomallei*, the causative agent of melioidosis, is inherently resistant to a variety of antibiotics including aminoglycosides, macrolides, polymyxins, and β-lactam antibiotics. Despite resistance to many β-lactams, ceftazidime and β-lactamase inhibitor–β-lactam combinations are commonly used for treatment of melioidosis. Here, we examine the enzyme kinetics of β-lactamase isolated from mutants resistant to ceftazidime and clavulanic acid inhibition and describe specific mutations within conserved motifs of the β-lactamase enzyme which account for these resistance patterns. Sequence analysis of regions flanking the *B. pseudomallei penA* gene revealed a putative regulator gene located downstream of *penA*. We have cloned and sequenced the *penA* gene from *B. mallei* and found it to be identical to *penA* from *B. pseudomallei*.**

Burkholderia pseudomallei is the causative agent of melioidosis, an endemic disease of Southeast Asia and Northern Australia (6). The severity of the disease can vary from asymptomatic infection to a severe form leading to acute sepsis and death. *B. pseudomallei* is a facultative intracellular pathogen which is able to survive inside phagocytic cells and thereby escape the host's humoral response. The disease can be reactivated after a very long remission (3, 5, 12). Currently, prolonged antibiotic treatment is advised to ensure complete eradication of the organism. Unfortunately, this practice creates a strong positive selection for antibiotic resistant strains resulting in many cases of treatment failure. Many reports have described successful treatment using a combination of β-lactam antibiotics and a β-lactamase inhibitor, such as amoxicillin plus clavulanic acid (19). Livermore, et al. described a clavulanic acid-inhibitable β-lactam resistance phenotype of *B. pseudomallei* (13), and recently, the cloning of *B. pseudomallei* class A and D β-lactamases has been reported (4, 14).

Godfrey et al. described three different phenotypes of clinical isolates from three patients which had undergone antibiotic treatment, and demonstrated that the resistance was due to derepressed β-lactamase production and structural mutations in the enzyme (10). Here, we examine the *B. pseudomallei penA* gene encoding a class A β-lactamase in the clinical isolates of *B. pseudomallei* described by Godfrey et al. and from *B. mallei* ATCC 23344. We have identified point mutations in two of the isolates which likely account for their altered phenotypes. Finally, the enzyme kinetics of these mutants were compared to the wild type enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmid used in this study are shown in Table 1. *B. pseudomallei* strains used in this study were collected from blood and urine samples from melioidosis patients both before and during antibiotic treatment at Sappasitprasong Hospital, Ubon Ratchatani, Thailand, between 1986 and 1989 and have been described previously (7, 10). All bacterial strains were grown at 37°C on Luria-Bertani (LB) agar or in LB broth. Media used for growing *B. mallei* were supplemented with 4% glycerol. When used, antibiotics were added at the indicated concentrations.

PCR amplification and cloning of PCR products. PCR products were generated in a 100-μl reaction mixture using the following cycling program: 95°C, 5 min; 95°C 1 min, 55°C 1 min, and 72°C 1 min for 30 cycles; and 72°C 10 min. Mixtures were then held at 4°C. Primers used in the amplification of the 580-bp *penA* from *B. pseudomallei* 1026b were (i) 5′-GCAGCACATCCAAGATGATG C-3′ and (ii) 5′-GCCGATCGTGTTCATCGTCTA-3′. The primers used in reverse PCR to amplify flanking regions of *penA* using *Xho*I-digested and ligated chromosomal DNA of 1026b were (i) 5′out (5′-GCATCATCTTGGATGTGCT GC-3′) and (ii) 3′out (5′-GCCGATCGTGTTCATCGTCTA-3′). The primers used to amplify the entire *penA* gene were (i) 5′penA (5′-GAGAGCTGATAC GCTAGCGAG-3′) and (ii) 3′penA (5′-GCGGCTTCCGGAAGGTTCA-3′). The zeocin resistance gene was amplified with (i) Zeo1 (5′-TGGCCTTTTGCT CACATGTGT-3′) and (ii) Zeo2 (5′-TCTAGAGTCGACCTGCAGGCA-3′).

Cloning of β-lactamase genes from *B. pseudomallei* and *B. mallei*. The *penA* gene was amplified from various *B. pseudomallei* mutants and from *B. mallei* using the 5′ penA and the 3′ penA primers. The PCR products were subsequently cloned into pCR2.1-TOPO (Invitrogen) as per the manufacturer's instructions. The cloned *penA* genes were transferred from the pCR2.1-TOPO cloning vector to pUCP31T (17) for MIC testing and to pT7Zeo (Invitrogen) for β-lactamase expression. Restriction enzymes and T4 ligase were purchased from BRL/Invitrogen. Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen), and chromosomal DNA was prepared using a Wizard DNA purification kit (Promega). When required, PCR products were purified using GenElute PCR DNA purification kit (Sigma).

MIC determination. MICs were determined using agar dilution or E-test strips (AB Biodisk, Solna, Sweden). For agar dilution, Mueller-Hinton agar plates were prepared containing twofold dilutions of antibiotic ranging from 1 to 256 μg/ml for ampicillin, amoxicillin, and cefazolin; from 0.25 to 128 μg/ml for amoxicillin plus clavulanic acid (8:1 ratio), cefoxitin, ceftriaxone, and aztreonam; and from 0.25 to 128 μg/ml for ceftazidime and imipenem. Plates were spotted with approximately 10^4 organisms diluted from overnight liquid cultures and examined after overnight incubation. E-test strips were used as per the manufacturer's instruction.

DNA sequencing and sequence analysis. DNA sequencing was performed by University Core DNA Services (University of Calgary). The CLUSTAL W program (20) was used to align *penA* genes and their translated protein sequences.

Purification of β-lactamase and analysis of enzyme kinetics. The β-lactamase enzyme from *B. pseudomallei* 316a, 316c, and 392f was purified in the following manner. *Escherichia coli* BL21(DE3) (Invitrogen) cells were transformed with p316aT7Z or p392fT7Z, and from the transformants periplasmic proteins were obtained using an osmotic shock procedure.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX*74 *deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
E. coli BL21 (DE3)	F$^-$ *ompT hsdS$_B$* ($r_B^-m_B^-$) *gal dcm* (DE3) pLysS (Cam^r)	Invitrogen
E. coli BL21 (DE3)pLysS	BL21(DE3)pLysS: F$^-$ *ompT hsdS$_B$* ($r_B^-m_B^-$) *gal dcm* (DE3) pLysS (Cam^r)	Invitrogen
B. cepacia K56-2	CF isolate, Toronto, Canada	8
B. mallet ATCC 23344	Type strain (human isolate)	USAMRIID[a]
B. pseudomallei 316a	Clinical isolate, wild-type phenotype	8
B. pseudomallei 316c	Clinical isolate, selectively resistant to ceftazidime	8
B. pseudomallei 365c	Clinical isolate, wild-type phenotype	8
B. pseudomallei 365a	Clinical isolate, derepressed phenotype	8
B. pseudomallei 392a	Clinical isolate, wild-type phenotype	8
B. pseudomallei 392f	Clinical isolate, decreased susceptibility to clavulanic acid inhibition	8
B. thailandensis ATCC 700388	Type strain (soil isolate)	16
Plasmids		
pCR2.1-TOPO	Topoisomerase-mediated cloning vector: Ap^r Km^r	Invitrogen
pEM7/Zeo	Expression vector, Zeo^r	Invitrogen
pUCP31T	Broad-host-range vector. OriT pRO1600 ori; Gm^r	16
p316a31T	pUCP31T containing cloned penA from 316a	This study
p316c31T	pUCP31T containing cloned penA from 316c	This study
p365a31T	pUCP31T containing cloned penA from 365a	This study
p365c31T	pUCP31T containing cloned penA from 365c	This study
p392a31T	pUCP31T containing cloned penA from 392a	This study
p392f31T	pUCP31T containing cloned penA from 392f	This study
pJES307	pT7-7 derivative, expression vector with T7 promoter	15
pT7Zeo	pJES307 derivative with disrupted bla_{TEM-1}, Zeo^r	This study
p316aT7Z	pT7Zeo containing cloned penA from 316a	This study
p316cT7Z	pT7Zeo containing cloned penA from 316c	This study
p392fT7Z	pT7Zeo containing cloned penA from 392f	This study

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For *B. pseudomallei* 316c β-lactamase purification, *E. coli* BL21(DE3)LysS (Invitrogen) was used in an effort to obtain higher β-lactamase expression. For osmotic shock, four liters of each *E. coli* transformant was grown overnight, and cells were harvested by centrifugation and resuspended in 30 to 50 ml of 0.5 M sucrose for approximately 15 min. Periplasmic proteins were released by gently resuspending centrifuged cells in 20 ml of sterile distilled water. The periplasmic protein extracts were filter-sterilized and adjusted to 40 mM Tris-HCl, pH 8.5, to a final volume of 10 ml. The adjusted extracts were loaded into a Q-Sepharose fast flow 16/20 chromatography column. The *B. pseudomallei* β-lactamase enzyme was collected in the pass-through fraction. The pass through fraction was then concentrated to 2 ml with a Centriprep Centricon-10 and loaded into a MonoS HR 5/5 fast-performance liquid chromatography column (Amersham Pharmacia). The β-lactamase fraction was then eluted with a 0 to 2 M NaCl gradient to obtain a pure fraction of the enzyme. The pH of the enzyme extract was adjusted to 7.0, and this material was used for kinetic studies.

Enzyme purity was assessed by SDS–14% PAGE (data not shown). The kinetic analysis of β-lactam hydrolysis was performed with a Beckman DU640 spectrophotometer using 0.1 M phosphate buffer, pH 7.0.

Competition assays were performed in a total volume of 500 μl of buffer in 5- or 10-mm path length quartz cuvettes. Reporter (nitrocefin) was added to a final concentration of 100 μ M and inhibitor to a concentration of 50 or 100 μM. The extinction coefficients (Δε) and UV absorption wavelength of each antibiotic used in this study were as follows: nitrocefin, +15,000 M^{-1} cm^{-1} and 482 nm; ampicillin −1,100 M^{-1} cm^{-1} and 232 nm; amoxicillin, −1,100 M^{-1} cm^{-1} and 232 nm; cefazolin, −7,900 M^{-1} cm^{-1} and 260 nm; cefoxitin, −7,700 M^{-1} cm^{-1} and 260 nm; ceftriaxone, −9,400 M^{-1} cm^{-1} and 260 nm; ceftazidime, −8,660 M^{-1} cm^{-1} and 260 nm; aztreonam, −640 M^{-1} cm^{-1} and 318 nm; and imipenem, −9000 M^{-1} cm^{-1} and 300 nm.

K_m and V_{max} were calculated using nonlinear regression analysis by Prism software. The k_{cat} was obtained using the known amount of enzyme measured by bicinchoninic acid protein assay (Pierce, Rockford, Ill.). K_i was obtained using the method described by Galleni et al. (9) and was used as K_m when the hydrolysis rate could not be measured.

Nucleotide sequence accession numbers. The *penA* sequences were submitted to GenBank under accession numbers AY032868, AY032869, AY032870, AY032871, AY032872, AY032873, and AY032874.

RESULTS

Reverse PCR and flanking region of the *penA* gene. Reverse PCR was performed in order to obtain the sequence of flanking regions of the *penA* gene. The orientation of *penA* and flanking genes are shown in Fig. 1. The *penA* gene is downstream of the *nlpD* gene which is presumably involved in lipoprotein synthesis and is upstream of a putative regulator gene, *penR*. The *nlpD*, *penA*, and *penR* genes have the same orientation. The intergenic region between *nlpD* and *penA* and that between *penA* and *penR* are approximately 150 and about 700 bp, respectively.

DNA sequence analysis. The PCR amplified *penA* gene from six different isolates of *B. pseudomallei* and *B. mallei* ATCC 23344 was sequenced and compared using the CLUSTAL W program (Fig. 2). The DNA sequences of these seven strains

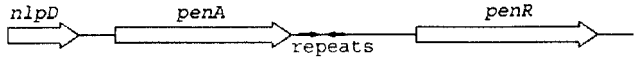

FIG. 1. Orientation of *penA* and *penR* in *B. pseudomallei* and *B. mallei*. *nlpD*, putative enzyme involved in lipoprotein synthesis; *penA*, class A β-lactamase gene; repeats, inverted repeats (not to scale); *penR*, putative regulator gene.

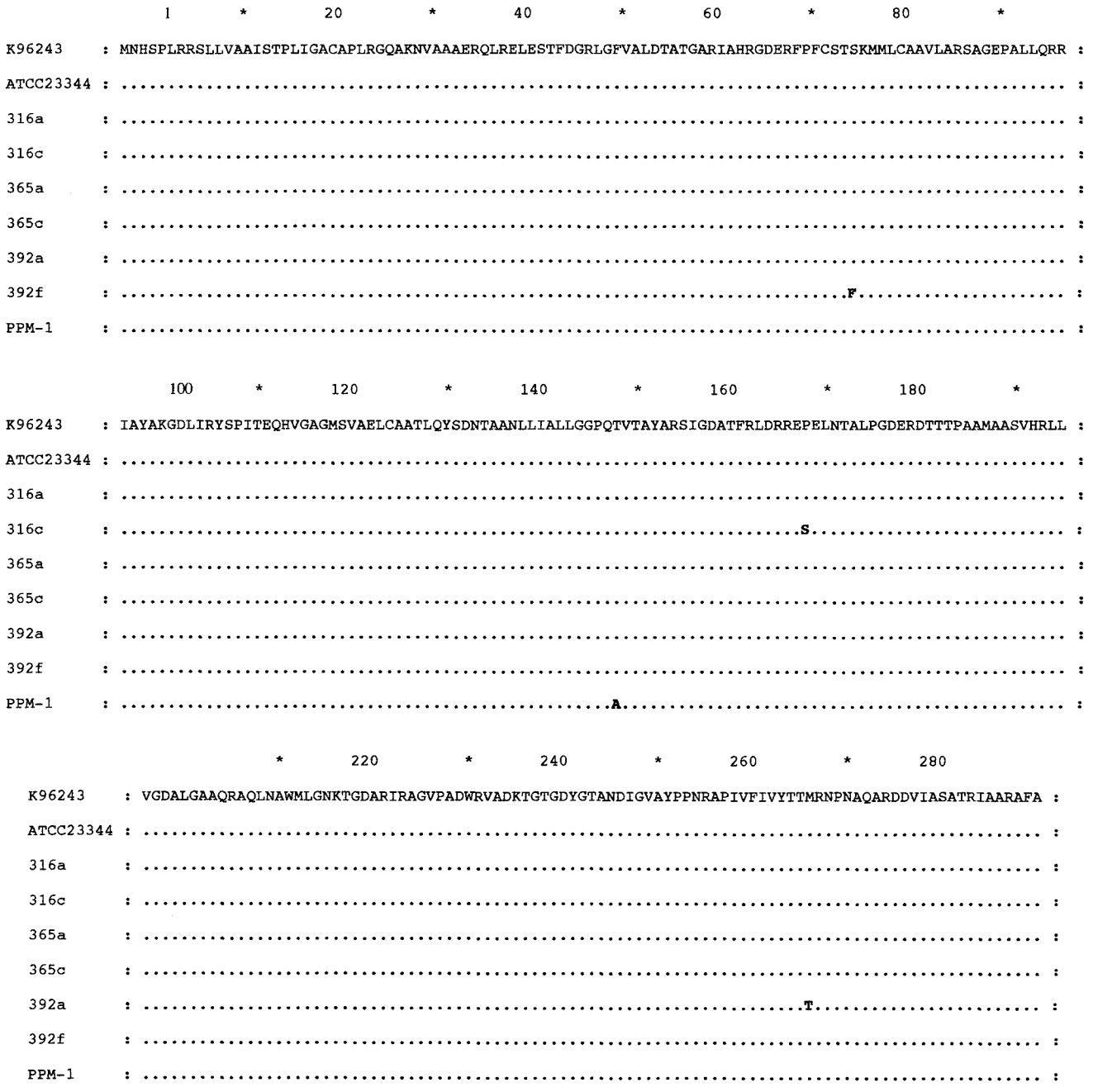

FIG. 2. CLUSTAL W alignment of nine β-lactamases from *B. pseudomallei* and *B. mallei*. K96243, *B. pseudomallei* K96243; ATCC 23344, *B. mallei* ATCC 23344; PPM-1, *B. pseudomallei* Hong Kong strain (4). Dots indicate amino acids identical to those in the sequence for strain K96243. Letters indicate changed amino acids. Amino acids are numbered in accordance with the Ambler (ABL) numbering scheme (Ambler et al., letter).

were almost identical in that only a few single base changes were identified. The presumptive translated protein sequences were identical between *B. mallei* and *B. pseudomallei* strains 316a (wild-type phenotype), 365a (derepressed), and 365c (wild-type phenotype). When strain 316c (ceftazidine resistant) was compared to strain 316a (wild-type phenotype), a single nucleotide change (C to T) was found resulting in a change of proline to serine at position 167 (P167S, ABL numbering scheme [R. P. Ambler, A. F. Coulson, J. M. Frere, J. M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley, Letter, Biochem. J. **276:**269-270, 1991]). The clavulanic acid resistant strain 392f had a single nucleotide change (C to T) at S72F. Nucleotide changes in both 316c and 392f resulted in amino acid changes within conserved regions of class A β-lactamases. A single base change (T to C) in strain 392a (wild-type phenotype) resulted in a substitution of methionine with threonine (M266T) and was outside of the conserved regions of class A β-lactamases. Sequence comparison

TABLE 2. MICs (μg/ml) of different β-lactams for *E. coli, B. pseudomallei,* and *B. mallei*

Strain	MIC[a] (μg/ml)								
	AMP	AMX	AMC	CFZ	CRO	FOX	CAZ	ATM	IPM
TOP10	4	4	4	2	<1	4	0.25	<1	0.25
TOP10(p316a31T)	>256	>256	128	256	4	4	2	8	0.25
TOP10(p316c31T)	256	>256	>128	>256	4	>128	4	2	1
TOP10(p365a31T)	>256	>256	32	256	16	8	4	8	0.25
TOP10(p365c31T)	256	>256	32	128	16	8	4	8	0.25
TOP10(p392a31T)	128	>256	8	128	4	8	2	8	0.25
TOP10(p392f31T)	128	>256	16	64	4	8	1	8	0.25
ATCC 23344	64	128	8	>256	8	>128	1	32	0.25
316a	64	128	8	>256	8	>128	4	32	0.5
316c	64	128	8	>256	8	>128	64	32	0.5
365a	>256	>256	128	>256	128	>128	16	>256	2
365c	64	128	128	>256	128	>128	16	>256	0.5
392a	64	128	16	>256	4	>128	2	32	0.5
392f	64	128	32	>256	8	>128	4	64	0.5

[a] Abbreviations for antibiotics: AMP, ampicillin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; CFZ, cefazolin; CRO, ceftriaxone; FOX, cefoxitin; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem.

of the putative regulator region in all of the strains examined did not reveal any differences at the amino acid level (data not shown).

MIC determination in parental strains and their corresponding clones. The MICs of ten different β-lactam antibiotics were determined and compared in pairs of *B. pseudomallei* and *E. coli* TOP10 containing the corresponding *penA* clone. The results are shown in Table 2. The MICs of ceftazidime for both 316c and TOP10(p316c31T) were relatively high, although that for TOP10(p316c31T) was not significantly higher than many of the other *E. coli* Top10 *penA* clones. Also, both 392f and TOP10(p392f31T) showed a small but consistent decrease in susceptibility to clavulanic acid inhibition compared with 392a or TOP10(p392a31T).

β-Lactamase purification and kinetic parameters. Enzyme obtained from periplasmic extracts and subjected to ion exchange column purification yielded β-lactamase with greater than 90% purity. The enzyme preparations were used to examine the kinetics of β-lactam hydrolysis in 3 of the *B. pseudomallei* strains. In general, the β-lactams used in this study could be divided into five groups. The first group consisted of "good" substrates, such as nitrocefin, cefazolin and ceftriaxone, which exhibited high k_{cat}/K_m. The second group represented poor substrates, such as ampicillin and aztreonam. The third group were very poor substrates, such as amoxicillin, in that the hydrolysis rate could not be measured but could be derived by using the competitive hydrolysis method. The fourth group consisted of nonsubstrates as the enzyme could not recognize those β-lactams either as substrates or inhibitors. The last group was the inhibitor group and consisted of a single substrate, clavulanic acid. The hydrolysis rates of nine different β-lactams, representing the five groups described above, were examined using the "good" substrate nitrocefin as a reporter and the K_i obtained from these competitive analysis experiments was used as a K_m for comparison. The kinetic parameters obtained from β-lactamases from three of the *B. pseudomallei* strains are shown in Table 3. Ceftazidime was not recognizable by 316a and 392f enzymes; however, it was a substrate for the 316c enzyme, in that it was recognizable via competitive hydrolysis, albeit very poorly. Ampicillin and aztreonam were very poor substrates for the 316c enzyme yet good substrates for 316a and 392f. The K_m (K_i) of 392f for clavulanic acid was about fivefold higher than that of 316a indicating lower affinity of the 392f enzyme for clavulanic acid; however, the 316c enzyme had the highest K_m for clavulanic acid among the three strains.

DISCUSSION

This study examines the *penA* gene and the class A β-lactamase enzyme which it encodes from several *B. pseudomallei* clinical isolates. In addition, we have sequenced the *penA* gene from *B. mallei* and have found it to be identical to that found in *B. pseudomallei*. We have shown that the β-lactamase resistant phenotype in *B. pseudomallei* can be attributed to amino acid changes in conserved regions of the β-lactamase enzyme.

Although the sequence of *B. pseudomallei penA* has recently been reported (4), reverse PCR experiments revealed a unique arrangement of the *penA* structural gene with a putative regulator downstream and in the same orientation. Sequence analysis of the putative regulator region did not reveal any differences between all *B. pseudomallei* strains examined at the amino acid level suggesting that the observed derepressed phenotypes are not a result of mutations within this region and that other factors contribute to the elevated enzyme levels in these strains.

The approximately 700-bp region which separates the *penA* gene and the putative regulator contains repeats and inverted repeats and may possibly contain unknown regulatory features. This region remains a target for further studies aimed at understanding the regulation of the β-lactamase enzyme.

The *penA* gene in *Burkholderia spp.* encodes a class A β-lactamase which is susceptible to clavulanic acid inhibition. The predicted protein sequence contains all four conserved motifs found in other class A enzymes—namely, SXXK, SDN, omega loop (EXXLN), and KTG motifs (11)—and according to its activity, *penA* would be classified in the Bush group 2e (2, 4). The enzymes from two strains, 316c and 392f, had mutations that resulted in amino acid changes within the conserved motifs of the catalytic site. The mutation of 316c at the omega

TABLE 3. Kinetic parameters of *B. pseudomallei* β-lactamases[a]

Substrate	PenA	V_{max}	K_m	k_{cat}	k_{cat}/K_m
Nitrocefin	316a	159 ± 7.4	10.9 ± 2.1	4.91	446
	316c	67.6 ± 4.2	3.34 ± 0.7	1.01	303
	392f	351 ± 7.2	38 ± 2.1	46.2	1,214
AMP	316a	1,316 ± 114	126 ± 29.5	0.18	1.45
	316c	NM	13.8	0.04	2.74*
	392f	4,087 ± 601	1,051 ± 237	37.2	35.4
AMX	316a	NM	2.22*	NM	NM
	316c	NM	0.88*	0.09	96.9*
	392f	NM	74.6*	NM	NM
CFZ	316a	1,024 ± 106	59.5 ± 17.8	21.35	359
	316c	168 ± 5.9	12.6 ± 1.8	0.63	50
	392f	2,390 ± 383	255 ± 52.8	78.6	307
FOX	316a	NH	NR	NH	NH
	316c	NM	15.4*	0.02	1.02*
	392f	NH	NR	NH	NH
CRO	316a	1,514 ± 389	287 ± 117	63.1	219
	316c	253 ± 29.4	29.4 ± 8.5	0.95	32.4
	392f	802 ± 128	138 ± 34.4	26.3	191
CAZ	316a	NM	NR	NM	NM
	316c	NM	10.3*	0.02*	2.33*
	392f	NM	NR	NM	NM
ATM	316a	1,645 ± 211	179 ± 61.4	3.2	17.9
	316c	NM	48.1*	0.15*	3.2*
	392f	1,939 ± 144	336 ± 51.9	17.6	52.5
IPM	316a	NH	NR	NH	NH
	316c	NH	NM**	NH	NM**
	392f	NH	NR	NH	NH
CLA	316a	ND	0.61*	ND	ND
	316c	ND	17.7*	ND	ND
	392f	ND	3.18*	ND	ND

[a] Units are μMs^{-1} for Vmax, μM for Km, s^{-1} for kcat, and $mM^{-1}s^{-1}$ for kcat/Km. CLA; clavulanic acid. Other antibiotic abbreviations are as listed in Table 1. NR, antibiotic was not recognized by that particular enzyme; NM, the rate of hydrolysis is too slow to be measured accurately; ND, not determined; NH, hydrolysis was not detected after 30 minutes. *, Ki was used as Km ; **, the enzyme was completely inactivated by imipenem and no nitrocefin hydrolysis was detected.

loop (P167S) may explain the observed ceftazidime resistance as this mutation has been shown to be associated with ceftazidine resistance in *K. pneumoniae* (15). Although the rates of ceftazidime hydrolysis by β-lactamase from 316c and 316a were not directly measurable, the 316c enzyme could recognize ceftazidime as competitive substrate and thereby allowed calculation of K_i, k_{cat}, and k_{cat}/K_m ratio values. It is likely that the increased affinity for ceftazidime of the 316c enzyme may account for the increased resistance of this strain to this antibiotic.

The 392f enzyme also contained a point mutation in a conserved motif resulting in a S72F mutation. Although the phenylalanine at this position can be found in many β-lactamases, it is convincing that in this case, the change resulted in a decreased susceptibility to clavulanic acid inhibition in both *B. pseudomallei* strain 392f and in *E. coli* TOP10(p392f31T) as the K_i for clavulanic acid was higher for the 392f enzyme than the wild type, 316a enzyme. The higher K_i would indicate decreased affinity of clavulanic acid, resulting in a higher MIC for amoxicillin plus clavulanic acid for strain 392f and *E. coli* TOP10(p392f31T) compared to strain 392a and TOP10 (p392a31T).

The K_i obtained for clavulanic acid and strain 316c was higher than for strains 392f and 316a, which may explain decreased susceptibility to clavulanic acid inhibition. However, the MIC of amoxicillin/clavulanic acid in 316c was not higher than 392f. This may be explained by the fact that 316c enzyme hydrolyzed amoxicillin more poorly than the wild type enzyme, so the decreased susceptibility to clavulanic acid inhibition could not raise the MIC of amoxicillin/clavulanic acid.

The MICs for *B. pseudomallei penA* genes cloned into *E. coli* TOP10 may not accurately reflect actual enzyme activity in *B. pseudomallei*. Efflux mechanisms and/or differences in outer membrane permeability may alter periplasmic β-lactam concentrations and thus may affect apparent enzyme activity.

While this work was being reviewed the cloning of a class D β-lactamase from *B. pseudomallei* was reported (14). The authors reported increased transcription of the class D β-lactamase gene in laboratory generated ceftazidime resistant mu-

tants. However, extracts of *E. coli* carrying the cloned gene from parent and mutant showed no detectable ceftazidime or imipenem hydrolyzing activity. Thus, the role of the *B. pseudomallei* class D β-lactamase in β-lactam resistance remains unclear.

Other factors outside the coding region of the *penA* gene may also contribute to highly resistant phenotypes as seen in 365a and 365c. Currently, we are studying the function of *penR*, the putative regulator and the intergenic 700bp region on the expression of β-lactamase in *B. pseudomallei*.

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