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Antibiotic Resistance Markers in *Burkholderia pseudomallei* Strain Bp1651 Identified by Genome Sequence Analysis

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ABSTRACT *Burkholderia pseudomallei* Bp1651 is resistant to several classes of antibiotics that are usually effective for treatment of melioidosis, including tetracyclines, sulfonamides, and β-lactams such as penicillins (amoxicillin-clavulanic acid), cephalosporins (ceftazidime), and carbapenems (imipenem and meropenem). We sequenced, assembled, and annotated the Bp1651 genome and analyzed the sequence using comparative genomic analyses with susceptible strains, keyword searches of the annotation, publicly available antimicrobial resistance prediction tools, and published reports. More than 100 genes in the Bp1651 sequence were identified as potentially contributing to antimicrobial resistance. Most notably, we identified three previously uncharacterized point mutations in *penA*, which codes for a class A β-lactamase and was previously implicated in resistance to β-lactam antibiotics. The mutations result in amino acid changes T147A, D240G, and V261I. When individually introduced into select agent-excluded *B. pseudomallei* strain Bp82, D240G was found to contribute to ceftazidime resistance and T147A contributed to amoxicillin-clavulanic acid and imipenem resistance. This study provides the first evidence that mutations in *penA* may alter susceptibility to carbapenems in *B. pseudomallei*. Another mutation of interest was a point mutation affecting the dihydrofolate reductase gene *folA*, which likely explains the trimethoprim resistance of this strain. Bp1651 was susceptible to aminoglycosides likely because of a frameshift in the *amrB* gene, the transporter subunit of the AmrAB-OprA efflux pump. These findings expand the role of *penA* to include resistance to carbapenems and may assist in the development of molecular diagnostics that predict antimicrobial resistance and provide guidance for treatment of melioidosis.

KEYWORDS *Burkholderia pseudomallei*, antimicrobial resistance, genome sequence, *penA*

Burkholderia pseudomallei is a Gram-negative, rod-shaped, motile bacterium that is widespread in soil and water in Southeast Asia and tropical northern Australia. This organism infects humans and animals through broken skin, by ingestion, or by inhalation and is responsible for a life-threatening disease, melioidosis (1). On rare occasions, melioidosis is diagnosed in patients in the United States, usually after the infection is acquired during travel to areas of endemicity (2). *B. pseudomallei* is included in the U.S. Department of Human and Health Services and the U.S. Department of Agriculture Select Agents and Toxins List as a tier 1 agent (3).

The recommended therapy for melioidosis includes two stages of treatment: an acute stage that involves intravenous therapy with β-lactam antibiotics, including ceftazidime (CAZ) or a carbapenem (imipenem [IPM] or meropenem [MEM]) for at least 14 days, followed by an eradication stage consisting of oral therapy with trimethoprim-sulfamethoxazole (SXT), doxycycline (DOX), or amoxicillin-clavulanic acid (AMC), for up

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to 6 months (4, 5). Although treatment of melioidosis that follows the current recommendations improves the outcome, failure rates remain high (5, 6). The treatment failures result in part from the intrinsic and acquired resistance of *B. pseudomallei* to antibiotics via some classical mechanisms, such as reduced permeation, drug efflux, enzymatic drug inactivation, or target mutations (7). Resistance encoded on plasmids has not been reported. Other contributing factors, such as an intracellular lifestyle and the ability to remain dormant in the host for many years, may contribute to the failure of clinical therapy and/or disease relapse (7–10).

The mechanisms of resistance to β-lactam antibiotics in *B. pseudomallei* have been described as drug target modification and drug inactivation. Loss of a gene required for synthesis of a penicillin-binding protein 3 (PBP3; locus tag BPSS1219 in the genome sequence of strain K96243) resulted in CAZ resistance in *B. pseudomallei* (11, 12). Specific point mutations within *penA*, which codes for a class A β-lactamase, resulting in amino acid changes C69Y (13) and P167S (14) have been implicated in CAZ resistance and clavulanic acid resistance, respectively (15, 16). (Numbering of these amino acid residues is in accordance with the standard scheme for class A β-lactamases [17]. PenA exhibits 39% identity to the prototypical canonical class A enzyme TEM-1.) Overproduction of PenA due to changes in its promoter region may also increase resistance to CAZ (18).

Outer membrane porins (OMPs) may play a role in decreased susceptibility to β-lactam antibiotics in *B. pseudomallei*, although this role is under investigation (19, 20). Although expression of *B. pseudomallei* outer membrane porin *Bps*Omp38 decreases susceptibility to IPM and CAZ in an *Escherichia coli* strain that is deficient in native major porins, the proteolysosomes containing *Bps*Omp38 were still permeable to these antibiotics (19).

Like other Gram-negative organisms, there are multiple efflux pumps encoded in the *B. pseudomallei* genome, but only two are known to generate clinically significant resistance. The AmrAB-OprA multidrug efflux pump provides intrinsic resistance to aminoglycosides and macrolides, and it is due to the constitutive expression of this pump that these antibiotics are ineffective for treatment of *B. pseudomallei* infections (7, 21). Although expressed only in regulatory mutants, the BpeEF-OprC efflux pump appears to be the most clinically significant pump identified so far. When expressed, it confers multidrug resistance (MDR) in *B. pseudomallei*, including resistance to chloramphenicol, fluoroquinolones, tetracyclines, and trimethoprim and low-level resistance to SXT (7, 21, 22). A third pump, BpeAB-OprB, is expressed only in *bpeR* mutants and confers low-level resistance to chloramphenicol, fluoroquinolones, macrolides, and tetracyclines (23, 24). These three efflux systems belong to the resistance-nodulation-cell division (RND) superfamily, which is known to play a major role in antibiotic efflux in various bacteria (21, 25).

Recent advances in whole-genome sequencing technologies have resulted in the generation of 59 complete genome sequences for *B. pseudomallei* that were publicly available on the NCBI website as of March 2017. This species has two circular chromosomes. The larger, chromosome 1, is approximately 4 Mbp, and the smaller, chromosome 2, is approximately 3 Mbp. Both chromosomes have a relatively high G+C content of 65 to 68%. Analysis of sequenced genomes can facilitate the identification of factors that potentially contribute to antimicrobial resistance, although the mere presence of a resistance gene is not always an accurate predictor of resistance. In this study, we report the results from an analysis of the genome sequence of *B. pseudomallei* strain Bp1651 (26), an isolate that is resistant to AMC, CAZ, DOX, IPM, and SXT.

Strain Bp1651 was isolated from a patient with cystic fibrosis (CF). The infection was chronic, and *B. pseudomallei* was isolated from this patient repeatedly for several years. Although the strain was isolated in the United States, the patient had a history of prior travel to Australia, where the infection is likely to have been acquired. The patient traveled only as far north as the subtropical region of Queensland, which is south of the usual tropical area of endemicity. However, there are case reports, although rare, of melioidosis acquired in that area (27, 28).

TABLE 1 Antimicrobial susceptibility profiles of *B. pseudomallei* strains analyzed in this work[a]

Antimicrobial agent	CLSI susceptibility breakpoint (μg/ml)[b]			Susceptibility of indicated *B. pseudomallei* strain (MIC, μg/ml)[c]			
	S	I	R	Bp1651 (26)	MSHR1655 (29, 30)	1026b (22, 24)	K96243 (31)
AMC	≤8/4	16/8	≥32/16	64/32 (R)	32/16 (R)	1/0.5 (S)	S
CAZ	≤8	16	≥32	>128 (R)	2 (S)	4 (S)	S
DOX	≤4	8	≥16	16 (R)	16 (R)	0.5 (S)	NR
IPM	≤4	8	≥16	32 (R)	16 (R)	0.38 (S)	S
SXT	≤2/38		≥4/76	>32/608 (R)	2/38 (S)	0.25/4.75 (S)	R

[a]Breakpoints and MICs are shown for the indicated antibiotics (AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; DOX, doxycycline; IPM, imipenem; SXT, trimethoprim-sulfamethoxazole). MICs were determined after 16 to 20 h for Bp1651 and after 48 h for MSHR1655 using an in-house prepared BMD panel. Antibiotic susceptibility data for strains 1026b and K96243 were previously published. Only the interpretations of susceptibility test results were published for K96243.
[b]S, susceptible; I, intermediate; R, resistant.
[c]NR, not reported. References are provided for genome sequence and characterization.

RESULTS

Phenotypic characterization of Bp1651. Bp1651 is an atypical isolate of *B. pseudomallei*. Bp1651 failed to grow on Ashdown's agar, a common selective medium for *B. pseudomallei* (32, 33). It was also negative in the latex agglutination test, which is reported to have 99.1% specificity for *B. pseudomallei* (34). However, the strain was proved to be *B. pseudomallei* by whole-genome sequencing (see "Whole-genome sequencing of Bp1651"). It had a single nucleotide polymorphism (SNP) that is characteristic of *B. pseudomallei* based on the highly specific real-time PCR allelic discrimination assay BurkDiff (35). This SNP is located at position 2970369 on chromosome 2 (26). The strain also had a C at position 75 in all four copies of its 16S rRNA genes, which can be used to discriminate *B. pseudomallei* from *Burkholderia mallei* (36). The multilocus sequence type (MLST) is ST880 (37). The antibiotic resistance profile for Bp1651 is presented in Table 1 in comparison with the susceptibility profiles of three other *B. pseudomallei* strains with publicly available genome sequences. The selected comparison strains are known to be susceptible to some or all of the antibiotics tested, and their genome sequences were compared to determine whether a gene or a mutation identified in Bp1651 may be responsible for its antimicrobial resistance.

Whole-genome sequencing of Bp1651. The genomic DNA of strain Bp1651 was sequenced using PacBio (26) and Illumina MiSeq (this work) technologies. PacBio sequences were assembled into two contigs that corresponded to the two chromosomes of *B. pseudomallei*, and the assembled sequence was annotated and deposited at NCBI GenBank (26). The MiSeq data for Bp1651 generated 3,300,740 sequences in pairs with an average length of 240.5 nucleotides (nt). When the MiSeq reads were mapped to the PacBio assembly, 92% of the reads aligned to the two assembled chromosomes with an average depth of coverage of 99×. Across the two chromosomes, less than 1% of the genome was not covered by MiSeq reads. Since 36,183 nucleotides of the PacBio assembly were not covered by MiSeq reads and editing would be incomplete, we chose not to correct the PacBio assembly. Only 17 discrepancies (minimum variant frequency of 0.65, minimum coverage of 30×, with no multiple sequence differences within the same MiSeq read compared to the reference) ranging from 1 to 3 nucleotides between the MiSeq reads and the HGAP.3 reference were identified, indicating that the PacBio assembly was of good quality.

Predicted antibiotic resistance genes. The complete list of >100 genes potentially contributing to antibiotic resistance in *B. pseudomallei* Bp1651 is available in Table S1 in the supplemental material. Two online tools with regularly updated databases were employed to predict antimicrobial resistance genes and mutations in the Bp1651 genome: the Comprehensive Antibiotic Resistance Database (CARD) (38) at http://arpcard.mcmaster.ca and ResFinder (39) at http://cge.cbs.dtu.dk/services/ResFinder/. ResFinder identified only one gene at settings of 60% selected identity threshold and 60% selected minimum length. However, while CARD, which does not have an option of selecting these parameters, found more genes, these genes constituted only a

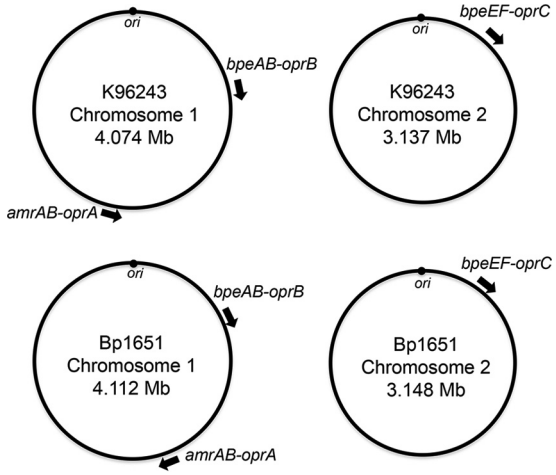

FIG 1 Genomic locations of three major RND efflux pump operons in strains K96243 and Bp1651. Arrows indicate the approximate locations and transcriptional orientations of operons encoding the three major characterized RND efflux pumps, AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC, on chromosomes 1 and 2. Although RND operon distribution is conserved among diverse *B. pseudomallei* strains, the locations and orientations on the two chromosomes with respect to the origins of replication (*ori*) vary because of differences in chromosome sizes and/or rearrangements. Gene and *ori* chromosome locations are derived from published data (26, 31).

fraction of the genes identified by a manual keyword search of the annotation. The manual search was accomplished by searching annotation files for the words "resistance," "antibiotic," "drug," "lactamase," "penA," "tet," and "macrolide." Literature and BLAST searches were also employed to find additional candidates for resistance genes in *B. pseudomallei* and to verify that the genes identified by the manual search of the annotation and by the antibiotic prediction online tools were likely to be involved in resistance. Potential antibiotic resistance genes were grouped based on the class of antibiotics to which they produce resistance. Locus tags for the Bp1651 gene annotation are identified as TR70_XXXX. Selected resistance mechanisms investigated in this study are described below.

Efflux of antimicrobial agents. Multiple efflux systems were predicted in the genome of Bp1651, including all 10 of the previously reported RND efflux systems identified in the reference strain *B. pseudomallei* K96243 (40). Involvement in antibiotic resistance in Bp1651 was predicted for 7 of these 10 RND systems (Table S1). Only three of these systems, AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC, have been at least partially characterized in *B. pseudomallei* (21). The genomic locations and orientations of the operons encoding these systems with respect to the chromosomal origin of replication vary in diverse *B. pseudomallei* strains because chromosomal rearrangements are not uncommon in this species. Compared to strain K96243, the *amrAB-oprA* and *bpeAB-oprB* operons of strain Bp1651 differ in location and orientation in chromosome 1, whereas the relative locations of the *bpeEF-OprC* operon on chromosome 2 are similar in strains K96243 and Bp1651 (Fig. 1).

The AmrAB-OprA efflux system mediates aminoglycoside, macrolide, and ketolide resistance in *B. pseudomallei* (41–43). In Bp1651, this system is encoded by genes in locus tags TR70_0535 (AmrA), TR70_0536 (AmrB), and TR70_0538 (OprA) located on chromosome 1. The gene for TR70_0536 (AmrB) contains a deletion of two nucleotides (G760 and C761) that are present in *amrB* of *B. pseudomallei* strains K96243 and 1026b. The deletion is predicted to result in a frameshift at codon 253 and in premature translation termination after codon 300. This change would render the efflux system nonfunctional and would explain why Bp1651 failed to grow on Ashdown's selective medium, which contains the aminoglycoside gentamicin (GEN). The repressor for the *amrAB-oprA* operon is encoded by the *amrR* gene (TR70_0534), which is located upstream of *amrAB-oprA*. Compared to the prototype K96243 and 1026b sequences,

the *amrR* gene was found to contain a 2-bp deletion (G384 and C385) that also resulted in a frameshift at codon 128 and premature termination after codon 187.

BpeAB-OprB is a broad-spectrum drug efflux pump. However, some discrepancies in this pump's substrate specificity which may be strain dependent have been described (23, 24). The typical substrates of the pump are chloramphenicol, fluoroquinolones, macrolides, and tetracyclines. The coding sequences that correspond to this efflux system in Bp1651, those of locus tags TR70_3285 (BpeA), TR70_3286 (BpeB), and TR70_3287 (OprB), are located on chromosome 1. BpeAB-OprB in *B. pseudomallei* is expressed only in regulatory mutants affecting BpeR, a TetR family repressor encoded by a gene located immediately upstream of the *bpeAB-oprB* operon. Since such mutants have not yet been observed in clinical isolates and the levels of resistance bestowed on these mutants are generally low, the contribution of the BpeEB-OprB pump to the resistance of *B. pseudomallei* to clinically relevant antibiotics remains unclear. The Bp1651 BpeR (TR70_3284) amino acid sequence is identical to *B. pseudomallei* BpeR sequences from strains K96243 and 1026b (21, 44).

Bp1651 homologs to BpeEF-OprC are encoded by genes in locus tags TR70_5659 (BpeE), TR70_5660 (BpeF), and TR70_5661 (OprC) located on chromosome 2. BpeEF-OprC is a multidrug efflux pump that transports compounds as diverse as chloramphenicol, fluoroquinolones, tetracyclines, and trimethoprim (21, 44). The BpeEF-OprC efflux pump is expressed only in regulatory mutants that contain mutations affecting BpeT (TR70_5657), which is encoded by a gene located upstream of the *bpeEF-oprC* operon. Examination of the Bp1651 *bpeT* gene revealed no such mutations in comparison to other *B. pseudomallei* strains, including K96243 and 1026b.

Resistance to β-lactams. (i) *pbp3*. Chromosomal deletions of a region encoding PBP3 are known to lead to high-level CAZ resistance in *B. pseudomallei* (MIC, >256 μg/ml) and are associated with severe growth defects (11, 12). The PBP3 gene in the Bp1651 genome sequence was annotated as TR70_4140, and the gene had no nonsense mutations that would produce a truncated form of PBP3. Bp1651 had only one amino acid substitution, T584A, in PBP3 compared to the three CAZ-susceptible strains, K96243, MSHR1655, and 1026b. In addition, there were no large deletions or gene rearrangements upstream of this gene in Bp1651, compared to the susceptible strains, that would predict loss of expression. Bp1651 grew on blood agar and in Mueller-Hinton broth, media that did not support growth of *pbp3* deletion mutants (11), indicating that *pbp3* in Bp1651 is functional. Two additional proteins were annotated as PBPs in Bp1651. These proteins were designated by locus tags TR70_2008 and TR70_4161. They have ~50% amino acid identity to PBP3.

(ii) *penA*. The name *penA* is commonly used in the *B. pseudomallei* literature as a designation for the β-lactamase gene investigated in this study, and we will continue to use this nomenclature. The protein encoded by this gene is actually classified as an extended-spectrum β-lactamase (ESBL), PenI (45). Several point mutations within *penA* have been implicated in conferring resistance to β-lactam antibiotics in *B. pseudomallei* (13, 14). Mutations that result in the amino acid changes C69Y and P167S, known to confer CAZ resistance, were not present in the Bp1651 *penA* (TR70_6344) sequence (Fig. 2). However, Bp1651, like strain MSHR1655, contained a known S72F substitution that resulted in resistance to AMC (Table 1). Bp1651 also contained three previously undescribed PenA amino acid substitutions, T147A, D240G, and V261I, compared to analogous sequences in the β-lactam-susceptible strains, 1026b and K96243. To determine the contribution, if any, of these mutations to β-lactam resistance, a plasmid was generated that carried the *penA* gene sequence with one of the point mutations and each plasmid was introduced individually into *B. pseudomallei* strain Bp82. MIC results were determined by broth microdilution (BMD), which is the standard antimicrobial susceptibility testing (AST) method for *B. pseudomallei* in the United States and which has interpretive criteria published by the CLSI (46) and by Etest, which is routinely used for *B. pseudomallei* testing in Australia and Southeast Asia (such as Malaysia and Thailand), where this bacterium is endemic (47–49). Both tests revealed that D240G

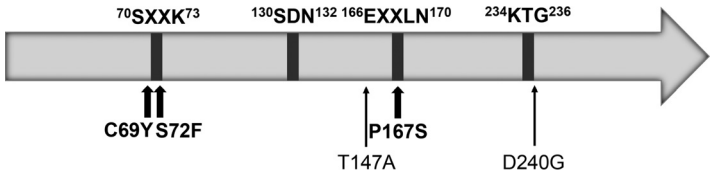

FIG 2 Diagram of the *B. pseudomallei* PenA gene and relative locations of amino acid changes leading to clinically significant antibiotic resistance. The positions of conserved regions and amino acid substitutions leading to altered β-lactam resistance phenotypes are numbered according to the Ambler scheme (17). Previously identified amino acid changes leading to CAZ resistance, i.e., C69Y and P167S, and AMC resistance, i.e., S72F, are indicated with thick arrows. Two new substitutions leading to increased resistance to CAZ (D240G) and to AMC and IPM (T147A) are indicated by thin arrows. The role of D240G was established by both expression of $penA_{D240G}$ from a multicopy plasmid in *B. pseudomallei* Bp82 and introduction of the D240G allele into *penA* on the Bp82 chromosome. The role of T147A in increased AMC and IPM resistance was determined by introducing a multicopy plasmid expressing $penA_{T147A}$ into *B. pseudomallei* Bp82. In addition to the indicated point mutations within *penA*, the upstream region contains a previously reported mutation (see the text for details).

contributed to CAZ resistance and that T147A contributed to AMC and IPM resistance (Table 2). The conservative change V261I did not significantly impact the MICs of these β-lactams (Table 2). To ensure that overexpression of *penA* on a multicopy plasmid, pUCP28T (50, 51), did not contribute to increased resistance in the Bp82 strain, the mutation leading to the D240G substitution was inserted into *penA* on chromosome 2 of strain Bp82. The BMD MIC of CAZ increased for the Bp82 $penA_{D240G}$ mutant (Table 2), confirming that D240G contributed to CAZ resistance. Another mutation with a potential impact on the resistance profile of Bp1651 was a G-to-A SNP at nucleotide 78 (G-78A) upstream of the ATG initiation codon of *penA*. This mutation changed a putative weak −10 sequence (5′-TACGCT-3′) that is present in *B. pseudomallei* strains such as K96243 and 1026b to 5′-TACACT-3′, a sequence that is closer to consensus (TATAAT). The G-78A SNP has been identified in several CAZ-resistant clinical isolates (16, 22). In a laboratory-selected CAZ-resistant *Burkholderia thailandensis* mutant, this mutation leads to increased *penA* transcription (18).

(iii) *oxa-59*. Class D β-lactamases encoded by *oxa* genes can confer resistance to penicillins, carbapenems, and cephalosporins in bacteria (52). *B. pseudomallei* genomes have various homologs for *oxa* genes (53). Strain Bp1651 has the *oxa-59* variant (TR70_4977) identical to *oxa* homologs of MSHR1655 and K96243. The *oxa* gene in strain 1026b has single nucleotide polymorphisms that result in two amino acid substitutions, in comparison to OXA-59, and the protein was identified by the ResFinder tool as having 99.75% identity to OXA-57.

Resistance to tetracyclines: *tet*. Since the BpeEF-OprC and the BpeAB-OprB efflux pumps were not likely expressed in Bp1651 due to intact sequences of their known transcriptional repressors, other potential tetracycline resistance genes were analyzed.

TABLE 2 Antimicrobial susceptibility in the *B. pseudomallei* Bp82 parent and its derivative strains containing *penA* variant alleles[a]

B. pseudomallei strain	Plasmid	Source of *penA* promoter	Source of *penA* gene	MIC (μg/ml) determined by: BMD AMC	BMD CAZ	BMD IPM	Etest AMC	Etest CAZ	Etest IPM
Bp82	None	None	None	4/2	1	≤2	2/1	1.5	0.75
	pUCP28T	None	None	4/2	1	≤2	2/1	1.5	0.75
	pJB037	Bp1651	Bp1651	32/16	32	8	32/16	>256	>32
	pJB038	Bp82	Bp82	16/8	8	4	8/4	6	8
	pJB041	Bp82	Bp82 T147A	32/16	4	8	12/6	16	16
	pJB042	Bp82	Bp82 D240G	4/2	32	≤2	3/1.5	64	8
	pJB043	Bp82	Bp82 V261I	16/8	8	4	12/6	6	8
Intrachromosomal $penA_{D240G}$ mutant					16				

[a]MICs (μg/ml) were determined by BMD (custom manufactured for AMC and IPM, in-house for CAZ) and Etest for Bp82 with and without plasmids carrying *penA* variant alleles. The MIC of CAZ for the intrachromosomal mutant was determined by the in-house BMD test.

Three genes in the genome were annotated by Prokka as *tet* genes. *tetA* (TR70_4192) was predicted to code for a class C tetracycline (TET) resistance protein, a TET efflux mechanism commonly found in *B. pseudomallei*. Although another *tetA* (TR70_3586) was predicted to produce a class B TET resistance protein, the similarity of this protein to tetracycline resistance proteins was for only half of the length of the protein when it was compared by BLAST to TetA proteins available in the NCBI database. Another predicted protein was annotated as TetD (TR70_4158) based on its similarity to other proteins designated TetD. However, only one-third of the protein was similar. Due to their limited similarity, the role of the last two proteins in tetracycline resistance in *B. pseudomallei* is unclear.

Sulfonamide/trimethoprim resistance: *folP* and *folA*. Mutants of dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), the essential enzymes involved in the synthesis of folates in bacteria, can result in resistance to sulfonamide and trimethoprim, respectively (54). The DHPS (FolP) protein encoded by the gene at locus tag TR70_0185 in the SXT-resistant strain Bp1651 was identical to the corresponding proteins in strains 1026b and K96243, which were susceptible to SXT. *B. pseudomallei* strain MSHR1655 has a single amino acid substitution, A162V, compared to FolP of the three other strains. This substitution is not in the conserved region of the protein (55) but still can potentially affect the properties of the protein. There were two substitutions in DHFR (FolA) in Bp1651 TR70_1420, I99L and A145T, in comparison to three other strains. The I99L substitution at the equivalent position occurs in trimethoprim-resistant *Streptococcus pneumoniae* (I100L) and is sufficient to confer trimethoprim resistance in that species (56–58). This substitution is localized to the predicted active site of the DHFR enzymes (59) and may therefore be responsible for trimethoprim resistance in *B. pseudomallei*.

DISCUSSION

The genome of *B. pseudomallei* Bp1651 was analyzed to identify genes and mutations that are responsible for the MDR phenotype of this strain. More than 100 predicted genes with potential to contribute to antimicrobial resistance were identified in the annotated genome sequence. This resistance gene prediction approach relied on database tools or keyword searches within annotations and is based on homology. Therefore, this approach would not identify entirely new resistance genes. As a result, the limited number of predicted genes from the Bp1651 genome sequence may be due to the restricted number of well-characterized antibiotic resistance markers available for bacteria with high G+C contents that are currently described in publicly available databases. Novel genes and mutations within a gene or a promoter that may contribute to resistance can be detected by comparing the genome sequences of susceptible and resistant strains. However, all of these predictions are hypothetical, and the findings must be confirmed experimentally by phenotypic methods. Furthermore, antimicrobial resistance prediction based solely on the detection of a resistance gene sequence may be inherently flawed because the presence of a gene does not necessarily correspond to expression of the encoded protein or, if expressed, the product may not be a functional protein. For example, efflux-mediated resistance in bacteria is rarely accompanied by changes in the efflux pump protein subunits (60) but rather is most often due to mutations affecting regulatory proteins. To be comprehensive, genetic analyses should also include sequence analyses of any regulatory genes known to control resistance determinants.

For these reasons, some of the predicted genes may not necessarily be responsible for antimicrobial resistance. For example, *oxa-59* was identified by three different search tools, Prokka annotation, CARD, and ResFinder, and it was the only antibiotic resistance gene identified by ResFinder. However, its role in resistance in *B. pseudomallei* remains unclear. Although it was previously reported that *oxa* expression is increased in CAZ-resistant strains of *B. pseudomallei* (62), its role in CAZ resistance has never been demonstrated, and no CAZ or IPM hydrolytic activity has been shown for *B. pseudomallei* OXA enzymes (53, 62).

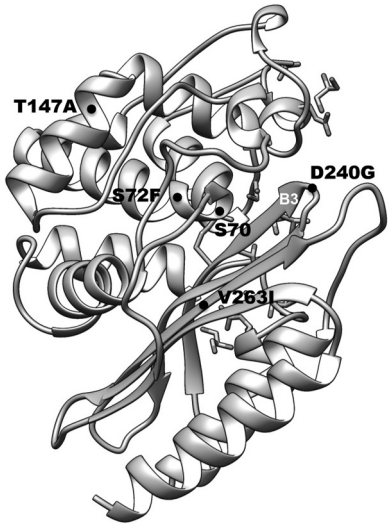

FIG 3 Crystal structure of *B. pseudomallei* beta-lactamase PenA (61) and location of amino acid changes leading to antimicrobial resistance. The previously known amino acid change S72F which causes AMC resistance in *B. pseudomallei* is located near the active-site serine at position 70. The amino acid substitution T147A which increased AMC and IPM resistance in Bp1651 is located near the surface of the enzyme. The D240G substitution which increased Bp1651 resistance to CAZ in located adjacent to the B3 strand of the enzyme. The V261I substitution which did not have a major effect on Bp1651 susceptibility is located distantly from the active site of the enzyme. More details are provided in Discussion.

For the most meaningful antimicrobial resistance determinant predictions, sequence analysis should be accompanied by evidence of gene expression and enzymatic activity. We therefore experimentally confirmed the role in susceptibility for novel mutations identified within *penA*. Introducing each mutation into the chromosome of a bacterial cell is preferred over cloning a mutated version of a gene on a plasmid, since the latter generates results that may depend on copy number and/or stability of the plasmid in a cell. However, the plasmid-based approach produces rapid preliminary results and can be useful for the first screening.

The Bp1651 *penA* region contained four mutations resulting in amino acid sequence changes in comparison to more susceptible strains such as K96243 and 1026b. Three of these substitutions are located within or next to the conserved Ambler domains that are important for PenA β-lactamase activity (45). As reported for other *B. pseudomallei* strains, amino acid change S72F (14) is located inside the ^{70}SXXK^{73} motif (Fig. 2), near the active-site serine at position 70 (Fig. 3). The S72F change is the likely cause of AMC resistance of Bp1651 because it is present in other AMC-resistant clinical isolates, and genetic and biochemical evidence with purified protein showed its involvement in AMC resistance (14, 45). T147A amino acid substitution that increased AMC and IPM resistance in Bp1651 is located between the conserved ^{130}SDN^{132} loop and the Omega loop (amino acids 164 to 179) (Fig. 2), but from its location near the surface of the enzyme (Fig. 3), it is not readily evident how this amino acid substitution may affect the PenA extended-spectrum activities and/or inhibitory properties. The D240G substitution that increased resistance to CAZ in Bp1651 is close to the ^{234}KTG^{236} motif (Fig. 2) and located adjacent to the B3 strand of PenA (Fig. 3). The D240G substitution is a frequent change in class A CTX-M β-lactamases that acquire the ability to hydrolyze CAZ (63). The role of the D240G substitution in these enzymes has been studied biochemically and structurally previously (64). The authors concluded that increased flexibility of the B3 β-strand that parallels the active site is correlated with higher activity against ceftazidime. Curiously, it was previously shown that in a *B. pseudomallei* C69F variant, CAZ interacted with D240 via a unique hydrogen bond formation not seen in wild-type PenA (65). The V261I substitution did not affect the susceptibility profile of Bp1651, which could be explained by the conservative amino acid change and location, which

is distant from the active-site motifs (Fig. 3). A common feature of CAZ-resistant *B. pseudomallei* clinical isolates is the combination of the G-to-A mutation in the *penA* upstream region and mutations affecting PenA structure (16), as was also found in Bp1651.

A mutation in *folA* changes the amino acid sequence of the trimethoprim target and thus can explain the high trimethoprim resistance of strain Bp1651. Associated drug target mutations are rarely seen, because expression of the BpeEF-OprC drug efflux pump is the dominant trimethoprim resistance mechanism in *B. pseudomallei* and is known to be responsible for the widespread resistance of this species to the drug (66). However, BpeEF-OprC is expressed only in BpeT regulatory mutants, and because the *bpeT* gene does not contain any mutations compared to prototype strains, this pump is most likely not expressed in Bp1651. Although RND pump-mediated efflux, especially via BpeEF-OprC, is the only known tetracycline resistance mechanism in *B. pseudomallei*, it is likely not the only one. Expression of TetA or some other *tet* resistance determinant may contribute to this phenotype, but this has yet to be ascertained.

The mutations identified in Bp1651 can also explain its failure to grow on Ashdown's agar, a GEN-containing selective medium routinely used for the identification of *B. pseudomallei* (32, 33). This failure is likely due to the deletions of nucleotides G760 and C761 in *amrB*, resulting in the nonfunctional efflux pump AmrAB-OprA and susceptibility of Bp1651 to GEN. A majority of clinical isolates of *B. pseudomallei* found in Sarawak, Malaysian Borneo, are also GEN susceptible (67). Some of this susceptibility is attributed to a nonsynonymous SNP, C1102G, within *amrB* (67). We previously showed that the GEN-susceptible phenotypes of GEN-susceptible Thai isolates were due to either deletion of the *amrAB-oprA* operon or regulatory mutations affecting its expression (42). Our findings further support the notion that GEN susceptibility may not be the rare exception in *B. pseudomallei*, as was previously thought. Because such isolates are obviously missed when Ashdown's agar is used, the diagnostic must be supplemented with alternative methods for a definite identification of *B. pseudomallei*.

One basis of inherent drug resistance in many Gram-negative bacteria is the composition of the outer membrane. High-level drug resistance often evolves in synergy between a resistance determinant, e.g., efflux, and the exclusionary properties of the outer membrane, and *B. pseudomallei* is no exception (68). Outer membrane porins have evolved to facilitate the diffusion or transport of nutrients across the outer membrane, and mutations in porin genes can result in reduced porin permeability. Thus, increased resistance to a drug will occur if it relies on that particular porin for entry into the cell. A total of 31 coding regions were predicted by Prokka analysis to code for porins in Bp1651 (see Table S1 in the supplemental material). Locus tag TR70_6273 corresponds to the porin *Bps*Omp38 that was previously characterized in its involvement in susceptibility to CAZ and IPM (19).

Unlike many other Gram-negative species, the mechanism for acquiring (or losing) antimicrobial resistance in *B. pseudomallei* is more likely due to point mutations or large-scale deletions than to horizontal transfer by mobile genetic elements such as plasmids. Very few plasmids have been demonstrated in *B. pseudomallei*, and none were shown to contain antibiotic resistance determinants (30, 31, 69). The Bp1651 strain contained point mutations but no deletions. The primary mechanism for acquiring resistance by point mutations is similar to that in *Mycobacterium tuberculosis*, a slow-growing bacterium that can persist in the host for years and is known to accumulate point mutations in response to inadequate drug therapy (70). Like *M. tuberculosis*, *B. pseudomallei* can produce chronic infections with repeated relapse after withdrawal of antibiotic treatment, and multidrug therapy for eradication is recommended (1). The CF context that requires frequent antimicrobial use is consistent with the accumulation of multiple mutations leading to resistance in strain Bp1651.

This targeted sequence analysis of the genome of an MDR strain of *B. pseudomallei* furthers our understanding of the mechanisms of resistance and their acquisition and may contribute to improving strategies for the selection of more effective treatments and prevention of relapsed melioidosis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. pseudomallei* strains used in this work are listed in Table 1. *Burkholderia thailandensis* E264 is an avirulent strain that was used as a control in the latex agglutination assay. *Burkholderia* strains were grown at 35°C on tryptic soy agar II (TSAII)-5% sheep blood plates (Becton, Dickinson and Company, Franklin Lakes, NJ) unless indicated otherwise. Ashdown's agar (32) was used as a selective medium to evaluate the growth of *B. pseudomallei* Bp1651. All media for culture of *B. pseudomallei* Bp82 and its derivatives were supplemented with adenine (71). NEB Turbo *Escherichia coli* (New England BioLabs, Ipswich, MA) was the host strain used for intermediate cloning steps. *E. coli* was cultured using Difco LB broth or agar (Becton, Dickinson and Company, Franklin Lakes, NJ).

Biosafety. *B. pseudomallei* strains Bp1651, MSHR1655, and 1026b are virulent strains and therefore were handled in a U.S. Federal Select Agent Program (FSAP)-registered, biosafety level 3 (BSL3) laboratory. Procedures were performed in a class II type A2 biological safety cabinet by trained personnel wearing personal protective equipment, including a powered air-purifying respirator (PAPR). Strain Bp82 is a Δ*purM* mutant of strain 1026b. This strain is highly attenuated (71) and is designated a select agent-excluded strain by the FSAP. Bp82 and its derivatives were handled in a BSL2 laboratory. Genetic manipulations of the avirulent strain Bp82 performed in this study were approved by the Institutional Biosafety Committees at the CDC and the University of Florida.

Latex agglutination. Latex agglutination was performed in accordance with a previously published procedure (34). Briefly, isolates were cultured on LB agar for 18 to 24 h at 37°C. Single colonies were selected and added to 10 μl of the latex suspension on a glass microscope slide. The glass slide containing the latex suspension with the suspended colony was subjected to gentle rocking for 2 min, after which time the reaction was recorded as either positive (agglutination) or negative (no agglutination). *B. pseudomallei* 1026b was used as the positive control and *B. thailandensis* E264 was used as the negative control each time isolates were tested.

Real-time PCR allelic discrimination assay. BurkDiff, a real-time PCR allelic discrimination assay employing a *B. pseudomallei*-specific TaqMan single nucleotide polymorphism probe (see Table S2 in the supplemental material) was performed in accordance with the published procedure (35).

AST. Susceptibility to antibiotics was determined by broth microdilution (BMD) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (46), using either 96-well plates prepared in-house with cation-adjusted Mueller-Hinton broth (CAMHB; Becton, Dickinson and Company, Sparks, MD) or custom-manufactured Sensititre 96-well plates (TREK Diagnostic Systems Ltd., England) and cation-adjusted Mueller-Hinton broth with TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (CAMHBT; Thermo Fisher Scientific, Lenexa, KS) as recommended by the manufacturer. The antimicrobial susceptibility profiles of strain Bp82 and the strains expressing extrachromosomal derivatives of *penA* were also determined by Etest (bioMérieux, St. Louis, MO). For both antimicrobial susceptibility testing (AST) methods, bacterial cells from colonies cultured on TSAII with 5% sheep blood at 35°C for 16 to 24 h were suspended to a concentration equivalent to a 0.5 McFarland density standard (measured with a MicroScan turbidity meter [Siemens]). The bacterial suspension was then diluted to a final concentration of 2×10^5 to 8×10^5 CFU/ml and inoculated into 96-well plates containing antibiotics for BMD. Alternatively, the cell suspension was spread onto a Difco Mueller-Hinton agar plate (Becton, Dickinson and Company, Franklin Lakes, NJ) for AST by Etest. Susceptibility test results were read after 16 to 20 h of incubation at 35°C in ambient air (46), with one exception. For the BMD test, MSHR1655 was incubated for 48 h due to insufficient growth at 20 h.

DNA isolation and sequencing. *B. pseudomallei* genomic DNA was prepared using a MasterPure complete DNA and RNA purification kit (Epicentre, Madison, WI) in accordance with the manufacturer's DNA purification protocol. Plasmid DNA was prepared using a HiSpeed plasmid midi kit (Qiagen) or QIAprep Spin miniprep kit (Qiagen). The Bp1651 genome sequence was generated with the Illumina MiSeq system (251 × 251 paired end run) using TruSeq chemistry (Illumina, San Diego, CA). Sequence reads were analyzed using CLC Genomics Workbench (CLC Inc., Denmark) and Geneious (Biomatters Ltd., New Zealand). The region containing cloned *penA* and its promoter (described below in "Generating extrachromosomal *penA* variants") was sequenced by the Sanger method using an Applied Biosystems 3130xl genetic analyzer with primers M13 forward (−20) (Life Technologies), M13 reverse (Life Technologies), penA_seq_F, and penA_seq_R (Table S2). If a reference is not provided for the primer sequence, the primer was designed for this study. Primers P1687 and P1712 (Table S2) were used to sequence the *penA* region in *B. pseudomallei* strain Bp82.315 and the plasmids constructed for its generation (described below in "Generating intrachromosomal *penA* variants").

Predicting antibiotic resistance genes. The finished, annotated sequences of the two chromosomes of Bp1651 were generated previously using the PacBio RSII (26) and deposited in NCBI GenBank under accession numbers CP012041 and CP012042. In the present study, genes and mutations that may contribute to antimicrobial resistance were identified by several methods, including search within publicly available databases, keyword search within the annotation, review of relevant publications, and comparison with available genome sequences of documented antimicrobial-susceptible *B. pseudomallei* strains 1026b (22, 24) and K96243 (31) and strain MSHR1655 (29, 30), which is susceptible to some but not all of the clinically relevant antibiotics (Table 1). Specific sequences of interest in the assembled PacBio genome of Bp1651 were verified by mapping with the MiSeq reads.

Generating extrachromosomal *penA* variants. The Bp1651 gene encoding PenA (plus 130 nucleotides [nt] upstream of its ATG translation start codon, to include a potential promoter [18] and 132 nt downstream of the TGA stop codon) was amplified from genomic DNA (gDNA) using primers Bp1651_Bp82_penA_5′_F and Bp1651_penA_3′_R (Table S2). From strain Bp82, the analogous sequence,

but extending to 144 nt downstream from the TGA stop codon, was amplified from gDNA using primers Bp1651_Bp82_penA_5′_F and Bp82_penA_3′_R (Table S2). Both amplicons were cloned individually into *E. coli* plasmid pCR-Blunt II-TOPO (Invitrogen). The *penA* insert and its orientation were verified by DNA sequence analysis before each insert was recloned into the *E. coli-B. pseudomallei* shuttle vector pUCP28T (50, 51) by using sites HindIII and XbaI (NEB). The resulting *penA* insert was in the opposite orientation of the *lacZ*α gene of pUCP28T, allowing *penA* to be expressed from its own promoter and not the *lacZ*α promoter. The plasmid containing *penA* of Bp1651 was designated pJB037, and the plasmid containing *penA* from Bp82 was designated pJB038. Site-directed mutagenesis was performed on plasmid pJB038 using QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) and the following primers (sequences are available in Table S2): penA_T147A_F and penA_T147A_R to introduce the T147A substitution, penA_D240G_F and penA_D240G_R to introduce the D240G substitution, and penA_V261I_F and penA_V261I_R to introduce the V261I substitution. Plasmid pJB041 expressed $penA_{T147A}$, plasmid pJB042 expressed $penA_{D240G}$, and pJB043 expressed $penA_{V261I}$. The *penA* region and its predicted promoter in all plasmids were verified by Sanger sequencing. Plasmids pJB037, pJB038, pJB041, pJB042, and pJB043 were each introduced into competent Bp82 by electroporation as described by Choi et al. (72). Transformants containing the plasmids were selected on 100 μg/ml trimethoprim (Sigma-Aldrich, St. Louis, MO) in agar.

Generating intrachromosomal *penA* variants. *B. pseudomallei* strain Bp82.315 expressing $penA_{D240G}$ was generated by allelic replacement using pEXKm5-based mutagenic plasmids, by employing kanamycin for merodiploid selection and sucrose counterselection for merodiploid resolution as previously described (73). Construction of plasmid pPS3257, used to generate Bp82.315, was achieved in several steps. First, a 1,296-bp fragment containing *penA* was PCR amplified using Bp82 genomic DNA and Q5 high-fidelity polymerase (NEB) and primers P1687 and P1712 (Table S2). Second, A-tailing of this fragment was performed using *Taq* polymerase (NEB) in accordance with the manufacturer's protocol. The resulting 1,298-bp fragment was then cloned into pGEM-T Easy (Promega, Madison, WI) to form plasmid pPS3250. This plasmid was used as the template for site-directed mutagenesis with mutagenic primer P2594 (Table S2) and the QuikChange II kit (Agilent Technologies, Santa Clara, CA) and resulted in pPS3256. This plasmid contains a *penA* gene with an A-to-G change (in bold in the P2594 sequence) at *penA* nucleotide 734, resulting in a D240G amino acid change. A 1,320-bp EcoRI fragment containing $penA_{D240G}$ was then subcloned into the EcoRI site of pEXKm5, which created pPS3257. All plasmids were verified by restriction enzyme digestion and DNA sequence analysis. After transformation into the *E. coli* mobilizer strain RHO3, pPS3257 was introduced into Bp82 Δ*penA* (Bp82.11) (45) by conjugation, followed by merodiploid selection on medium containing 1,000 μg/ml kanamycin (Sigma-Aldrich). The merodiploids were then resolved using sucrose counterselection, and generated strain Bp82.315 was confirmed by Sanger sequencing.

Accession number(s). MiSeq reads were deposited in the NCBI Sequence Read Archive (SRA) under accession number SRR2102060.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00010-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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