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Burkholderia pseudomallei acquired ceftazidime resistance due to gene duplication and amplification

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ABSTRACT

Ceftazidime (CAZ) is the antibiotic of choice for the treatment of *Burkholderia pseudomallei* infection (melioidosis). The chromosomally-encoded PenA β-lactamase possesses weak cephalosporinase activity. The wild-type *penA* gene confers clinically significant CAZ resistance only when overexpressed due to a promoter mutation, transcriptional antitermination or by gene duplication and amplification (GDA). Here we characterise a reversible 33-kb GDA event involving wild-type *penA* in a CAZ-resistant *B. pseudomallei* clinical isolate from Thailand. We show that duplication arises from exchanges between short (<10 bp) chromosomal sequences, which in this example consist of 4-bp repeats flanked by 3-bp inverted repeats. GDA involving β-lactamases may be a common CAZ resistance mechanism in *B. pseudomallei*.

1. Introduction

The introduction of ceftazidime (CAZ) into clinical practice for the treatment of *Burkholderia pseudomallei* infection (melioidosis) halved the mortality of affected patients compared with individuals receiving the previous therapeutic regimen [1]. Despite an often lengthy treatment regimen, acquired CAZ resistance is still rare but does exist [2]. Although penicillin-binding protein 3 (PBP3) target deletion has been demonstrated as a cause of clinically significant CAZ resistance [3], the main cause of CAZ resistance in *B. pseudomallei* is the chromosomally-encoded PenA class A β-lactamase [4,5]. In 1991, Godfrey et al. noted that increased resistance to β-lactam antibiotics developed fairly rapidly during treatment with CAZ [6]. This was either due to increased expression of what is now known as PenA β-lactamase or its evolution into an enzyme capable of better CAZ hydrolysis. Since then, it has been shown that the most common cause of CAZ resistance in clinical isolates is mutations causing PenA amino acid changes that extend the substrate spectrum of this β-lactamase to cephalosporins [4,7–11]. Amino acid changes can cause high-level CAZ resistance. More recently, CAZ-resistant (CAZ^R) isolates were described that exhibit (i) *penA* overexpression due to a promoter mutation [10–12], which is likely synonymous with what was previously described as β-lactamase 'derepression' [6], and (ii) *penA* gene amplification due to gene duplication and amplification (GDA) [13]. Wild-type PenA is a weak cephalosporinase, therefore increased transcription and an increased copy number via GDA will result in lower level, yet clinically significant CAZ resistance. In combination with PenA structural mutations, GDA results in high-level CAZ resistance [11,13]. Documented GDA events in isolates from Australian cystic fibrosis patients included: (i) a 30-fold duplication and amplification of a 7.5-kb region containing a $PenA_{C69Y}$ mutant variant in an isolate from patient CF6; and (ii) a 10-fold duplication and amplification of a 36.7-kb genomic segment containing wild-type *penA* in an isolate from patient CF11 [13]. Both of these GDA events resulted in CAZ resistance, being high-level resistance in CF6 [minimum inhibitory concentration (MIC) $\geq$256 µg/mL] and intermediate resistance (MIC = 12 µg/mL) in CF11. CAZ resistance in CF6 cannot be attributed to GDA because the $PenA_{C69Y}$ mutation alone is a known mutation for high-level CAZ resistance [4,8,10]. Here we describe a GDA event in a *B. pseudomallei* isolate from a Thai patient that leads to clinically significant CAZ resistance, and

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provide molecular details about the sequences that likely caused the initial duplication event in this isolate.

2. Materials and methods

2.1. Bacterial strains and growth media

Burkholderia pseudomallei strains used in this study were prototype strains 1026b [14] and K96243 [15] as well as the 1026b Δ*penA* derivative Bp319 [4]. Moreover, strain 5041c was isolated in 2008 from a melioidosis patient undergoing acute-phase treatment with CAZ at Sunpasitthiprasong Hospital (Ubon Ratchathani, Northeast Thailand). The strain was minimally passaged in the laboratory in the absence of selection between patient isolation, freezer stock and shipping sample preparation in Thailand (three passages) and then again for freezer stock preparation in the USA (two passages). Low passage captures more reliably the state of the organism at the time of isolation compared with numerous passages. Unless otherwise indicated, strains were grown in LB medium (Lennox) (5 g/L NaCl) (Thermo Fisher Scientific, Waltham, MA). All experiments with virulent *B. pseudomallei* strains were performed at BSL-3 in Select Agent-certified laboratory facilities at the University of Florida (Gainesville, FL) and employing compliant standard operating procedures approved by the Institutional Biosafety Committee.

2.2. Antimicrobial susceptibility assays

β-Lactam susceptibility assays were performed using the broth microdilution (BMD) method and cation-adjusted Mueller–Hinton II broth (Becton Dickinson & Co., Sparks, MD) following Clinical and Laboratory Standards Institute (CLSI) guidelines [16].

2.3. β-Lactamase assay

A nitrocefin assay was employed to detect β-lactamase activity in bacterial cultures. Briefly, after removing an aliquot for bacterial cell counts, 20 µL of bacterial culture grown in LB medium to log phase [optical density at 600 nm (OD_{600}) = 0.3–0.7] was transferred to the wells of a microtitre plate containing 0.2 mL of 0.5 mg/mL nitrocefin (Santa Cruz Biotechnology, Dallas, TX) in 100 mM $NaPO_4$ (pH 7). The plates were incubated at 37°C and the increase in absorbance at 486 nm (ΔA_{486}) was recorded with a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT). The ΔA_{486}/min was calculated from the linear portion of the recording. β-Lactamase specific activity units were calculated as ($\Delta A_{486}/10^6$ cells × min) × 10^5.

2.4. Determination of the frequency of ceftazidime susceptibility

To determine the percentage of CAZ-susceptible (CAZ^S) bacteria present in the Schweizer laboratory −80°C stock culture, a 100 µL aliquot was removed and was diluted with phosphate-buffered saline (Thermo Fisher Scientific) to achieve ca. 2×10^4 cells/mL. Then, 200 µL aliquots of this cell suspension were plated onto a 24 cm × 24 cm Q Tray (Molecular Devices, San Jose, CA) agar plates to yield ca. 3000 colonies per plate. Plates were incubated overnight at 37°C, followed by an overnight incubation at room temperature. Colonies were picked and arrayed into 96-well plates containing LB + 10% glycerol using a QPix2 colony-picking robot (Molecular Devices) and were incubated overnight at 37°C. To determine the frequency of CAZ^S bacteria, cells from individual wells were replicated onto freshly prepared LB plates and LB plates + 8 µg/mL CAZ (Sigma-Aldrich, St Louis, MO) using a 96-pin replicator.

2.5. Purification of PenA and anti-PenA polyclonal antibodies

For purification of PenA with a carboxy-terminal hexahistidine tag (PenA-His_6), the PenA coding sequence minus the amino-terminal 89 bp coding for the twin-arginine transport and lipidation signals was amplified by PCR from 1026b genomic DNA prepared using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI), Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA), and primers P3210 and P3211 [purchased from Integrated DNA Technologies, Inc. (IDT), Coralville, IA] (Table 1). Gibson assembly® of the resulting 844-bp PCR fragment with *Hin*dIII- and *Nde*I-digested pET24a(+) DNA employing the NEBuilder® DNA Assembly Master Mix (New England Biolabs) yielded plasmid pPS3397 encoding PenA-His_6. This plasmid was transformed into competent *Escherichia coli* BL21(DE3) (New England Biolabs) for expression and purification. PenA-His_6 was purified to near homogeneity using HisPur™ Ni-NTA Resin (Thermo Fisher Scientific) and the manufacturer's procedure for purification of native His_6-tagged proteins using a gravity flow column. Buffer exchange and protein concentration were achieved using an Amicon® Ultracel-3K centrifugal filter (Merck Millipore, Burlington, MA). The protein (>98% pure) was stored in 20 mM $NaPO_4$, 300 mM NaCl (pH 7.4). Anti-PenA rabbit polyclonal antibodies were produced by Antibody Research Corporation (St Charles, MO).

2.6. Western blot analysis

For western blot analysis, whole-cell extracts were prepared by growing bacteria in LB medium (Lennox) at 37°C. After reading the OD_{600}, 0.2 mL of each culture (OD_{600} = 0.8–0.9) was transferred to a microcentrifuge tube and was harvested by centrifugation for 1 min at 12 000 × *g*. After removing 0.19 mL of the supernatant, the cell pellet was suspended in a total of 0.1 mL of LB medium (0.01 mL residual + 0.09 mL fresh LB). The cell suspension was then incubated at 110°C for 15 min to kill the bacteria. Following a brief centrifugation, a 0.01 mL aliquot was checked for sterility by plating on LB agar followed by incubation at 37°C for 48 h. The heat-killed bacteria were then mixed with an equal volume of 2 × sample Laemmli loading buffer. The samples were boiled for 3 min, were centrifuged and equal volumes were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 0.1% SDS–12% polyacrylamide gel. Western blot analysis was performed as described previously [17], except that anti-PenA polyclonal primary antibodies were detected with horseradish peroxidase using goat anti-rabbit IgG (H+L) Superclonal™ secondary antibody (Thermo Fisher Scientific) and 1-Step Ultra TMB-Blotting Solution (Thermo Fisher Scientific).

2.7. Genome sequencing

Genomic DNA was isolated from strain 5041c grown in LB medium (Lennox). Paired-end sequencing on an Illumina MiSeq platform (Illumina Inc., San Diego, CA) was performed at the University of Florida Interdisciplinary Center for Biotechnology Research (UF-ICBR). Library preparation, sequencing and data analysis were performed as previously described [18]. The genome sequence and gene annotations of strain K96243 were used as a reference [15,19]. The primary genome sequencing data for strain 5041c have been deposited in the NCBI BioProject database under accession no. PRJNA412633.

2.8. Determination of penA copy number

Digital droplet PCR was used to assess the *penA* copy number in strain 5041c in comparison with strains 1026b and K96243. Genomic DNA was isolated from these strains grown in LB medium

Table 1
Primers used in the study.

Primer name	Primer sequence[a,b,c]
PenA carboxy-terminal hexahistidine tag expression vector construction	
P3210	5′-CTTTAAGAAGGAGATATACATatgaaaaacgtcgccgccgccgagc (*Nde*I)
P3211	5′-GGTGCTCGAGTGCGGCCGCAAGCTTggcgaacgcccgcgcggc (*Hin*dIII)
Determination of *penA* upstream and coding sequence	
P127	5′-TCAATCCGATGCCGTATCTG
P1712	5′-aagcttATACCGGCATCGTTTCGCTG (*Hin*dIII)
Determination of *penA* copy number	
P1-*penA*	5′-CAATTGCGCGAACTCGAATC
P2-*penA*	5′-CAGCGCAAAGCATCTTG
P3-*penA*	5′-6-FAM/TGCAGAACG/ZEN™/GGAAACGCTCGTC/3IABkQ
P1-*narK*	5′-CGTACGGCGGCTTCTTTAT
P2-*narK*	5′-GACACGTAGAACGCGATGAA
P3-*narK*	5′-HEX/AAGAGTTTC/ZEN™/GGCACGTCGCTCG/3IABkFQ
Determination of *bpss0960* and *bpss0934* junction sequences	
P3109	5′-GCGTTATGACGAGCGCGG
P3110	5′-AGGTATGCCGCCCCAGCG
P3111	5′-AAGTCGGGGACGTGACCG
P3112	5′-CGTAGAGGAACTGCTGTATT

[a] Lower case letters indicate *penA* coding sequence and upper case letters indicate pET24a(+) His_6 tag fusion and expression vector (EMD Millipore).
[b] Underlining indicates a newly generated restriction enzyme cleavage site.
[c] 6-FAM, 6-carboxyfluorescein; ZEN™, proprietary Integrated DNA Technologies (IDT) quencher; 3IABkFQ, Iowa Black quencher; 5′-HEX, hexafluorescein.

Table 2
β-Lactam antimicrobial susceptibilities of clinical *Burkholderia pseudomallei* isolates and mutant derivatives.

Strain	Relevant genotype	MIC (μg/mL)[a]				
		AMX	CAZ	CAZ + AVI	MEM	IPM
1026b	Prototype	256	2	1	1	1
Bp319	1026b Δ*penA*	2	0.5	1	1	0.25
K96243	Prototype	256	2	1	1	0.5
5041c	*penA* GDA[b]	>256	32	1	1	0.5
Bp954.3	CAZ^S 5041c revertant	128	2	1	0.5	0.25
Bp954.4	CAZ^S 5041c revertant	64	1	0.5	1	0.25

MIC, minimum inhibitory concentration; AMX, amoxicillin; CAZ, ceftazidime; AVI, avibactam; MEM, meropenem; IPM, imipenem; S, susceptible.
[a] The MIC was determined by the broth microdilution method performed in triplicate and on three separate occasions. For CAZ + AVI, AVI was maintained at 4 μg/mL in all wells.
[b] Gene duplication and amplification (GDA).

(Lennox) and was used for digital droplet PCR analysis performed by UF-ICBR using a QX200™ Droplet Digital™ PCR System (Bio-Rad, Hercules, CA). The primers and probe used for *penA* analysis were P1-*penA*, P2-*penA* and P3-*penA*, respectively. The reference locus was *narK*, one of the multilocus sequence typing (MLST) markers [20]. The primers and probe used for *narK* analysis were P1-*narK*, P2-*narK* and P3-*narK*, respectively.

3. Results and discussion

3.1. Phenotypic and genetic strain characterisation

Burkholderia pseudomallei 5041c is a CAZ^R sputum isolate obtained in 2008 from a melioidosis patient who was diagnosed 15 days earlier with a culture-confirmed infection by CAZ^S *B. pseudomallei*. CAZ therapy was initiated at the time of diagnosis.

BMD confirmed that strain 5041c was CAZ^R (MIC = 32 μg/mL) but was susceptible to meropenem and imipenem (Sigma-Aldrich) (Table 2). The CAZ resistance of 5041c was completely reversed when the β-lactamase inhibitor avibactam (Advanced ChemBlocks, Burlingame, CA) was co-administered with CAZ in the BMD assay, indicating that the resistance was mediated by a β-lactamase. A quantitative nitrocefin assay showed that 5041c expressed significantly higher levels of PenA β-lactamase activity (Fig. 1A) compared with the CAZ^S control strains 1026b and K96243.

Analysis of the 5041c laboratory −80°C freezer stock culture revealed that it consisted of a mixture of 99.72% CAZ^R and 0.28% CAZ^S colonies (8 of 2849 analysed single colonies were CAZ^S). Single-colony purified CAZ^S derivatives Bp954.3 and Bp954.4 exhibited low CAZ MICs (1–2 μg/mL) (Table 2). Nine single colonies obtained from the freezer stock grown in the absence of selection (LB) for ≥24 h had identical CAZ MICs (32 μg/mL) by BMD, confirming that heterogeneity in the number of *penA* locus copies in the samples is not a major issue. The CAZ^S derivatives Bp954.3 and Bp954.4 expressed low β-lactamase levels (Fig. 1A), similar to the 1026b and K96243 prototype strains. Consistent with PenA β-lactamase activity patterns, PenA protein levels were significantly higher in strain 5041c in comparison with 1026b, K96243 and the two CAZ^S 5041c derivatives Bp954.3 and Bp954.4 (Fig. 1B).

Using targeted PCR and DNA sequencing with primers P127 and P1712, we next assessed whether strain 5041c harboured either of the two types of *penA*-associated mutations that are known to confer a CAZ^R phenotype: (i) the presence of a conserved G→A mutation at position −78 relative to the first *penA* start codon nucleotide in the *penA* upstream region that causes a promoter-up mutation; and (ii) known mutations affecting the PenA amino acid sequence, including C69Y, P167S and D240G, that affect CAZ substrate binding. These analyses revealed presence of *penA* and fewer mutations compared with the 1026b prototype strain. First, four mutations in the 174-bp *penA* untranslated upstream sequence were identified (−139T, −71A, −61T and −41T in 5041c versus −139C, −71G, −61C

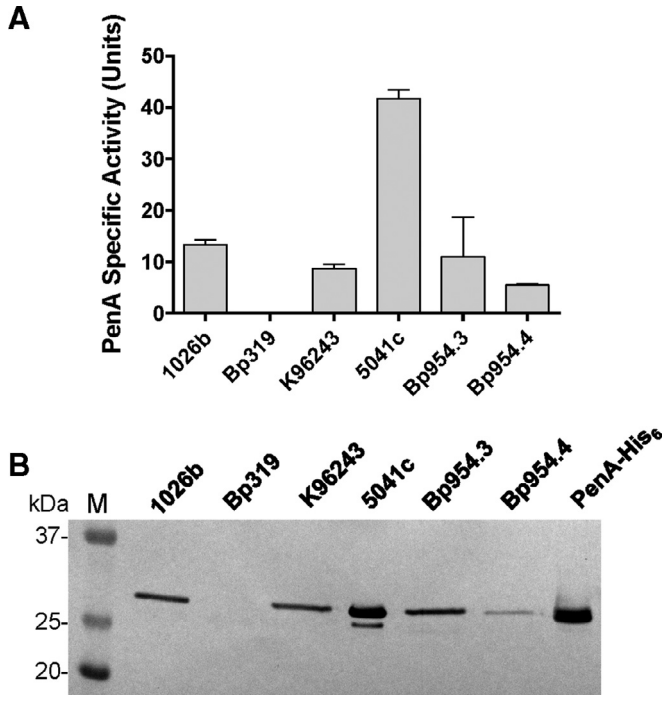

Fig. 1. PenA expression in *Burkholderia pseudomallei* strains. (A) PenA β-lactamase specific activity. The bacteria analysed were prototype strain 1026b, its Δ*penA* derivative Bp319, prototype strain K96243, clinical isolate 5041c and the ceftazidime-susceptible 5041c revertants Bp954.3 and Bp954.4. β-Lactamase activity was measured and the specific activity units were calculated as described in Section 2.3. PenA β-lactamase specific activity units were derived by subtracting the activity observed with Δ*penA* strain Bp319. The data show a representative example of experiments conducted on separate days with separate cell preparations. (B) Western blots of whole cells using anti-PenA antibodies. PenA in whole-cell extracts from the indicated strains or purified hexahistidine-tagged PenA (PenA-His_6) was detected using anti-PenA polyclonal antibodies. Lane M, Precision Plus Protein™ Dual Color Standards (Bio-Rad, Hercules, CA) molecular size markers; the size in kilodaltons (kDa) of the three pertinent markers is indicated on the left. The molecular size of the mature PenA lipoprotein in whole cells is ca. 29 800 Da [17], and that of PenA-His_6 missing the lipoprotein signal sequence is a calculated 29 870 Da.

and –41C in 1026b). Three of these differences (positions –71, –61 and –41) also occur in other CAZ^R and CAZ^S strains and do not affect *penA* expression [12]. The mutation at position –139 has not yet been observed in other strains but is in a region that has no effect on CAZ susceptibility [12]. The promoter-up –78G→A transition that increases *penA* transcription and is present in some CAZ^R strains was also absent [12]. Second, a C versus T at nucleotide 55 of *penA* was identified, which conserves a leucine codon but uses the CTG (5041c and most other *B. pseudomallei* strains) instead of the less common TTG found in 1026b. These same mutations were present in the *penA* upstream and coding regions of the CAZ^S strains Bp954.3 and Bp954.4. MLST determinations showed that 5041c and its CAZ^S derivatives belonged to the identical rare sequence type ST949 [20]. Aside from 5041c, there are currently only two other isolates of ST949 in the database (1027a and 1131a) (https://pubmlst.org/bpseudomallei/). Like 5041c, both are human sputum isolates from Ubon Ratchathani in Thailand.

Collectively, the phenotypic and molecular data are consistent with the conclusions that: (i) CAZ resistance in strain 5041c is due to increased wild-type PenA expression that is not caused by a promoter-up mutation; and (ii) the single-colony purified CAZ^S derivatives Bp954.3 and Bp954.4 isolated from the laboratory stock culture are spontaneous clonal revertants of 5041c that exhibit properties of strains with a single copy of *penA* and indicate a genetically unstable mechanism of CAZ resistance.

3.2. Identification and characterization of gene duplication and amplification (GDA)

Since PCR amplification and sequencing of the *penA* gene and its upstream region from strain 5041c revealed no *penA*-associated mutations known to be involved in CAZ resistance, and we could not demonstrate plasmids in preparations obtained via different commercial and non-commercial methods, including a method optimised for plasmid isolation from *B. pseudomallei* [21], whole-genome sequencing (WGS) was employed to investigate potential mechanisms of CAZ resistance. The sequence of the *penA* gene upstream untranslated and coding regions obtained by WGS was identical to that obtained by directed PCR amplification and Sanger sequencing. Furthermore, the amino acid sequences of the CAZ PBP3 target were identical in strains 5041c and 1026b, and differed from the K96243 sequence by only 2 conserved amino acid changes within the last 19 amino acids of the carboxy-terminus. The only notable sequence feature was that the read depth of an ca. 33-kb region encompassing genes *bpss0934* and *bpss0960* of chromosome 2 containing *penA* and 23 other genes was on average ca. 11 times higher than that of adjacent regions (Fig. 2A). One of the Illumina short reads captured a junction sequence between genes *bpss0934* (1665 bp; annotated as encoding a hypothetical 60 821 Da protein) and *bpss0960* (4626 bp; annotated as a 171 947 Da Rhs-related membrane protein) (Fig. 2B). Digital droplet PCR showed that whereas reference strains 1026b and K96243 each contained 1 copy of *penA*, the 5041c genome contains ca. 10–11 copies of this gene (Fig. 2C). This notion is supported by the higher relative levels of PenA β-lactamase specific activity and elevated protein levels in 5041c compared with 1026b and K96243 (Fig. 1). We noted that the repeatedly determined 5041c CAZ MIC (32 μg/mL) is considerably higher than the previously reported 12 μg/mL for an Australian strain harbouring ca. 10 copies of PenA in a similarly sized GDA [11,13]. This may be partly due to different methodology (BMD versus Etest) or strain differences because the overall CAZ^R phenotype is multifactorial and possibly strain-dependent [10–12]. In summary, these data show a GDA event that generated ≥11 copies of a 33 051-bp region of chromosome 2, which includes wild-type *penA* and 23 other genes; and the increased *penA* copy number is sufficient for bestowing clinically significant CAZ resistance.

The presence or absence of the junction between *bpss0960* and *bpss0934* was confirmed in CAZ^R and CAZ^S strains by PCR using the primer pairs P3109 and P3110, P3111 and P3112, and P3109 and P3112 designed for amplification of internal regions of *bpss0960* (560 bp), *bpss0934* (516 bp) and the *bpss0960–bpss0934* junction (584 bp), respectively (approximate locations of primer-binding sites are indicated in Fig. 2B). The results showed that all strains contained intact *bpss0960* and *bpss0934* genes flanking the amplified 33-kb region as indicated by the presence of 560-bp and 516-bp PCR products (Fig. 3A,B), but the 584-bp *bpss0960–bpss0934* junction product was only evident with strain 5041c (Fig. 3C). These data confirm the presence of GDA in CAZ^R strain 5041c and its absence in CAZ^S strains 1026b and K96243 as well as the spontaneously emerged CAZ^S 5041c derivatives Bp954.3 and Bp954.4.

Since no data are yet available that would illuminate the genetic drivers for *penA* region GDA in *B. pseudomallei*, next we more closely examined gene junction sequences for possible clues. Sequence analysis of the junction product showed that *bpss0960–bpss0934* gene fusion involved the first 1912 bp of *bpss0960* and the first 648 bp of *bpss0934* (Fig. 3D). Aside from the presence of a TTCG sequence that is common to both genes, of which only one copy is present after junction formation, and short trinucleotide inverted repeats flanking the TTCG tetranucleotide, the junction sequence region did not reveal any features such as insertion sequences, palindromes or repeat sequences that

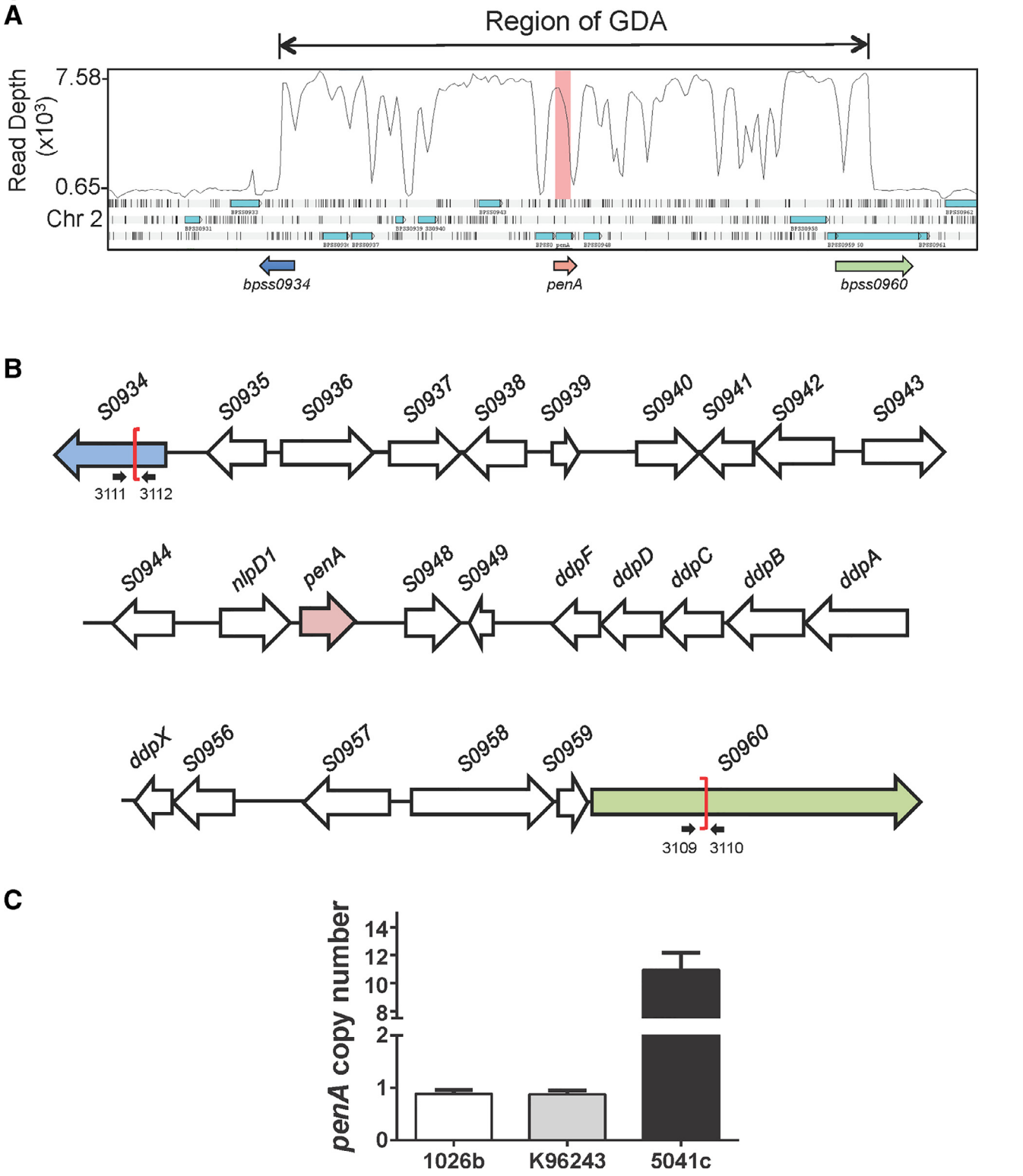

Fig. 2. Region of chromosome 2 that is amplified in *Burkholderia pseudomallei* clinical isolate 5041c. (A) The *penA*-containing region that is gene duplicated and amplified (GDA) between genes *bpss0934* (blue) and *bpss0960* (green) containing *penA* (light red) is indicated by the horizontal bidirectional arrow. The Illumina sequence read depth of this region is ca. 11 higher than the average genome coverage. (B) The extent of the 33 051-bp amplified region is indicated by red brackets located in *bpss0934* (blue) and *bpss0960* (green). The *penA* gene is shown in light red. Other annotated genes encode proteins that may partake in peptidoglycan recycling or metabolism: *nlpD1*, outer membrane lipoprotein; *ddpX*, D-alanyl-D-alanine dipeptidase; *ddpABCDF*, an operon encoding a putative dipeptide transport system with its periplasmic dipeptide-binding protein (DdpA), dipeptide permease subunits (DdpB and DdpC) and ATP-binding protein subunits (DdpD and DdpF) [22]. Annotations of genes with no assigned names follow the K96243 genome sequence annotation for chromosome 2 (GenBank accession no. NC_006351.1 and reference [19]). The approximate locations of primers used to assess the presence of intact *bpss0960* (3109 and 3110), *bpss0934* (3111 and 3112) or the *bpss0934–bpss0960* fusion junction (3109 and 3112) are indicated by the inverted black arrows. (C) Determination of *penA* copy number using digital droplet PCR. The *penA* copy number was determined in ceftazidime-susceptible reference strains 1026b and K96243 as well as the ceftazidime-resistant clinical isolate 5041c. The reference locus was *narK*, which was present in one copy in all strains. QuantaSoft™ software (Bio-Rad, Hercules, CA) was used for data analysis. Error bars indicate the standard deviation from the mean.

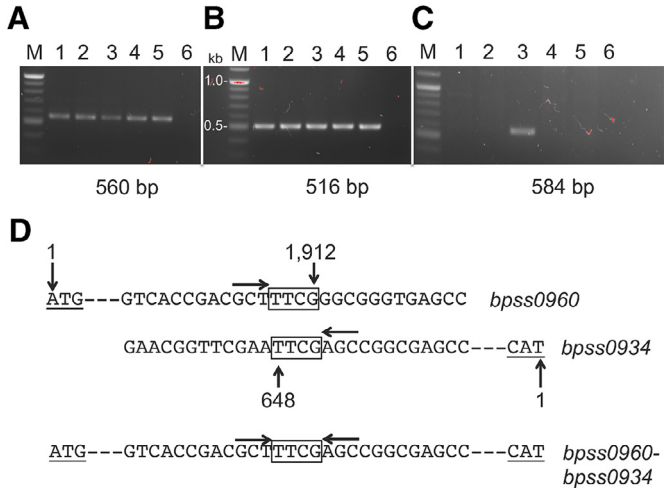

Fig. 3. Presence and molecular features of junction sequences. (A–C) PCR verification of the presence of intact genes and junction sequences in *Burkholderia pseudomallei* clinical isolate 5041c and ceftazidime-susceptible (CAZ^S) strains. The panels show the presence of intact *bpss0960* (A), intact *bpss0934* (B) and the *bpss0960–bpss0934* junction sequence (C). The respective PCR fragment sizes are indicated below each panel. Intact *bpss0960* and *bpss0934* are present in all strains; the *bpss0960–bpss0934* junction sequence is only present in strain 5041c. Lane M, Quick-Load® 100-bp DNA ladder (New England Biolabs, Ipswich, MA); lane 1, strain K96243; lane 2, strain 1026b; lane 3, strain 5041c; lane 4, strain Bp954.3; lane 5, strain Bp954.4; and lane 6, sterile Milli-Q® water (negative control). Strains K96243 and 1026b are CAZ^S reference strains, and Bp954.3 and Bp954.4 are spontaneous CAZ^S derivatives of 5041c. (D) Molecular features of the *bpss0960–bpss0934* junction sequence. The junction sequence involves the first 1912 nucleotides of *bpss0960* (top sequence) and the first 648 nucleotides of the divergently transcribed *bpss0934* (middle sequence). Start codons for both genes are underlined. The boxed TTCG sequence is common to both genes, but only one copy is retained after fusion (bottom sequence). This tetranucleotide sequence is flanked by short trinucleotide inverted repeats that are marked with horizontal arrows and contributed by *bpss0960* and *bpss0934*, respectively.

may be catalysts for the duplication events. This observation is consistent with the finding that despite studying GDA events for many decades, mechanisms of gene duplication events remain uncertain [23]. Duplications frequently occur between very short (ca. 10 bp) repeat sequences and others show no repeats [23]. We have previously shown that large-scale chromosomal deletion events resulting in generation or loss of antimicrobial resistance also exhibit unremarkable junction sequences [3,24].

4. Conclusions

These experiments show conclusively that the CAZ resistance observed in *B. pseudomallei* strain 5041c is solely due to a reversible GDA event that amplifies the *penA*-containing region of chromosome 2 by ca. 11-fold. GDA has been well documented in the laboratory as a means of acquiring readily reversible antimicrobial resistance in Gram-negative and Gram-positive bacteria (reviewed in [25]). Presumably because of their usually rapid loss after selective pressure removal, it has been less frequently demonstrated as a cause for clinical resistance [26,27]. Until very recently, GDA had not been described in *B. pseudomallei* and even then no details were provided about the involved sequences [13]. Because of its transient nature and its difficulty to detect using traditional diagnostic assays, GDA may be an underestimated contributing factor to the recalcitrance of *B. pseudomallei* to antibiotic treatment and its resistance development during infection. Strain 5041c was obtained as a CAZ^R sputum isolate 15 days after obtaining a CAZ^S sputum isolate from the same site in the same patient. The time between observed CAZ resistance development in this patient as a result of CAZ therapy is consistent with previously published data that indicated 15 days as the median duration of treatment prior to detection of resistance [2]. Several lines of evidence indicate that the GDA in strain 5041c may be fairly stable: (i) presence of a low percentage of bacteria after at least five rounds of laboratory passage in the absence of CAZ selection; and (ii) non-detectable *penA* copy number heterogeneity in MIC susceptibility assays. Further investigations with additional CAZ^R clinical isolates will have to be conducted to assess the scope of GDA in *B. pseudomallei* CAZ resistance and to elucidate possible mechanisms of GDA and its stability in this bacterium.

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Competing interests

None declared.

Ethical approval

Not required.

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