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MECHANISMS OF RESISTANCE

Burkholderia ubonensis High-Level Tetracycline Resistance Is Due to Efflux Pump Synergy Involving a Novel TetA(64) Resistance Determinant

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ABSTRACT *Burkholderia ubonensis*, a nonpathogenic soil bacterium belonging to the *Burkholderia cepacia* complex (Bcc), is highly resistant to some clinically significant antibiotics. The concern is that *B. ubonensis* may serve as a resistance reservoir for Bcc or *B. pseudomallei* complex (Bpc) organisms that are opportunistic human pathogens. Using a *B. ubonensis* strain highly resistant to tetracycline (MIC, $\geq$256 μg/ml), we identified and characterized *tetA*(64) that encodes a novel tetracycline-specific efflux pump of the major facilitator superfamily. TetA(64) and associated TetR(64) regulator expression are induced by tetracyclines. Although TetA(64) is the primary tetracycline and doxycycline resistance determinant, maximum tetracycline and doxycycline resistance requires synergy between TetA(64) and the nonspecific AmrAB-OprA resistance nodulation cell division efflux pump. TetA(64) does not efflux minocycline, tigecycline, and eravacycline. Comprehensive screening of genome sequences showed that TetA(64) is unequally distributed in the Bcc and absent from the Bpc. It is present in some major cystic fibrosis pathogens, like *Burkholderia cenocepacia*, but absent from others like *Burkholderia multivorans*. The *tetR*(64)-*tetA*(64) genes are located in a region of chromosome 1 that is highly conserved in *Burkholderia* sp. Because there is no evidence for transposition, the *tetR*(64)-*tetA*(64) genes may have been acquired by homologous recombination after horizontal gene transfer. Although *Burkholderia* species contain a resident multicomponent efflux pump that allows them to respond to tetracyclines up to a certain concentration, the acquisition of the single-component TetA(64) by some species likely provides the synergy that these bacteria need to defend against high tetracycline concentrations in niche environments.

KEYWORDS *Burkholderia*, tetracycline, resistance, efflux pump, *tetA*(64), synergy

Members of the genus *Burkholderia* are known to occupy remarkably diverse ecological niches (1). A notable feature of *Burkholderia* species is that they possess a multipartite genome structure consisting of at least two chromosomes and, in some species, large plasmids (2–5). The ensuing large coding capacity explains at least in part the genus' metabolic versatility and potential, as well as adaptation to and survival in diverse ecological niches, including adversarial environments (1). A common, yet not very well explored, property is the intrinsic antimicrobial resistance (AMR) of *Burkholderia* species. This is especially problematic with species that are opportunistic pathogens, which are found in two major *Burkholderia* complexes, the *Burkholderia cepacia* complex (Bcc) and the *Burkholderia pseudomallei* complex (Bpc) (6, 7). Species from both complexes afflict mostly compromised individuals, for instance immunocompromised and

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cystic fibrosis (CF) patients. Of the opportunistic Bcc bacteria known to be capable of causing serious disease, the two most clinically relevant species are *Burkholderia cenocepacia* and *Burkholderia multivorans*, which account for the vast majority of CF infections caused by Bcc bacteria (8). The only known Bpc opportunistic pathogens are *B. pseudomallei* and *Burkholderia mallei*, which cause melioidosis and glanders, two diseases with high mortality (9–11). It should be noted that *B. mallei* is a mammalian host-adapted clone of *B. pseudomallei* and is incapable of existing outside the host environment for extended periods of time (12). While *B. pseudomallei* is known to infect individuals with CF (13), the major risk factor for melioidosis is diabetes (11, 14). Bcc and Bpc nonpathogenic and pathogenic bacteria frequently coinhabit soil and aquatic environments in geographic areas where they both exist, mostly in tropical and subtropical regions of the world (15, 16). *Burkholderia ubonensis* is a nonpathogenic Bcc bacterium that coinhabits soil environments around the globe with *B. pseudomallei*, e.g., Australia, Thailand, and the Caribbean islands (17). It is highly resistant to some clinically significant antibiotics (17, 18). The concern is that *B. ubonensis* may serve as a reservoir for horizontal gene transfer of AMR determinants to pathogenic Bcc and Bpc species.

Tetracycline antibiotics have been in clinical use since the first report of chlortetracycline in 1948 (19). In attempts to keep pace with emerging resistance, early naturally occurring tetracyclines, including tetracycline itself that was discovered in 1953, were replaced in time with semisynthetic derivatives (e.g., doxycycline and minocycline, discovered in 1967 and 1972, respectively; the history of discovery of tetracyclines is reviewed in reference 20). After a lengthy pause, efforts were made to identify and develop semisynthetic compounds like tigecycline (discovered in 1993) with efficacy against emerging multidrug-resistant (MDR) organisms (21). The fully synthetic eravacycline is one of the latest derivatives to be introduced into clinical use (22).

For over 70 years, tetracycline antibiotics have been used extensively for management of bacterial infections in human and veterinary medicine and growth promotion in the cattle and poultry industries, as well as treatment of fruit trees for prevention of bacterial diseases (23). The first report of resistance to tetracyclines was in 1953, 5 years after the first clinical deployment of chlortetracycline (24). Resistance mechanisms include tetracycline-specific mechanisms (active efflux from the cell via single-component transporters, production of ribosomal protection proteins, enzymatic degradation, and 16S RNA target mutations) (20, 25) and also nonspecific mechanisms, especially in Gram-negative bacteria, which can include active efflux from the cell via multicomponent transporters and decreased cell-envelope permeability (reviewed in references 20, 25, and 26). The most common tetracycline-specific resistance mechanism is active efflux via single-component pumps belonging to the major facilitator superfamily (MFS) (reviewed in references 20 and 25). At the time of writing the manuscript, there were 35 distinct bacterial tetracycline-specific pumps registered (faculty.washington.edu/marilynr; 20 February 2020 update). Nonspecific multicomponent efflux pumps that actively extrude tetracyclines from Gram-negative bacteria belong to the resistance nodulation cell division (RND) family. Examples are the MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM pumps from *Pseudomonas aeruginosa* (reviewed in reference 26); the AdeABC pump from *Acinetobacter baumannii* (27); and the AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC pumps from *B. pseudomallei* and its closely related near neighbor *Burkholderia thailandensis* (28–34).

The aggregate of all tetracycline resistance mechanisms discovered and characterized in pathogenic and environmental organisms that are not normally associated with disease is an example of what Gerry Wright and colleagues defined as the resistome for this class of antibiotics (25). According to the resistome concept, it is paramount to investigate resistance in nonpathogenic environmental bacteria since there is ample evidence for them being reservoirs for resistance determinants that can be transmitted to pathogens by horizontal gene transfer (35–37).

TABLE 1 *Burkholderia ubonensis* strains used in this study

Strain	Description	Source
Bu278[a]	Wild type; soil isolate, Puerto Rico	17
Bu333	Bu278 *amrB*::T23	18
Bu424	Bu278 *tetA*(64)::T23	This study
Bu431	Bu278 Δ*tetA*(64)	This study
Bu436	Bu431::mini-Tn*7*T-TMP[b]	This study
Bu434	Bu431::mini-Tn*7*T-TMP-$P_{tetA(64)}$-*tetA*(64)$^+$	This study
Bu437	Bu278 Δ*amrB*	This study
Bu441	Bu278::mini-Tn*7*T-TMP	This study
Bu448	Bu437::mini-Tn*7*T-TMP	This study
Bu450	Bu437::mini-Tn*7*T-TMP-$P_{amrAB\text{-}oprA}$-*amrA*$^+$*B*$^+$-*oprA*$^+$	This study
Bu439	Bu278 Δ*amrB* Δ*tetA*(64)	This study
Bu452	Bu278 Δ*tetR*(64)	This study

[a]Bu278 is also known as Bp8955 (18).
[b]Mini-Tn*7*T-TMP insertions in Bu278 derivatives confer TMP^r and are located at *glmS3* (18).

We recently defined chromosomally encoded carbapenem resistance mechanisms in the environmental Bcc bacterium *B. ubonensis*, which has never been associated with animal or human disease (18). During these studies, we noted that the bacterium was highly resistant to tetracycline (TET; MIC ≥256 μg/ml) and doxycycline (DOX; MIC = 32 μg/ml). In this study, we show that the high-level TET resistance is due to synergy between a nonspecific RND efflux pump and a previously unreported tetracycline resistance determinant, *tetA*(64), that encodes a tetracycline-specific single-component MFS efflux pump, which is transcriptionally regulated by TetR(64). We provide evidence for the presence of *tetA*(64) in the genomes of some but not all Bcc species and its absence in Bpc species.

RESULTS

Identification of a *B. ubonensis* mutant with reduced tetracycline susceptibility. We recently described construction of a random transposon T23 mutant library of *B. ubonensis* strain Bu278 (*B. ubonensis* strains used in this study are listed in Table 1) (18). To identify the resistance determinant(s) governing the high (MIC, ≥256 μg/ml) TET resistance (TET^r) of strain Bu278, ~2,000 mutants of this library were screened for increased TET susceptibility as defined by no growth on Lennox broth (LB) plates containing 60 μg/ml TET. We identified one mutant, Bu424, that could no longer grow at this TET concentration, and the MIC of TET for this strain was reduced from ≥256 μg/ml to 16 μg/ml (Table 2). The T23 insertion also caused an 11-fold reduction in the DOX MIC (32 μg/ml for Bu278 versus 3 μg/ml for Bu424), but its effect on the minocycline (MIN) MIC was minimal (3 μg/ml for Bu278 versus 2 μg/ml for Bu424).

TABLE 2 MICs for tetracyclines against *B. ubonensis* efflux pump mutants

		MIC (μg/ml)[a] for:								
Strain	Relevant genotype	TET	DOX	MIN	ERV	TGC	CHL	CIP	GEN	TMP
Bu278	Wild type	≥256	32	3	3	6	48	2	128	8
Bu424	Bu278 *tetA*(64)::T23	16	3	2	ND[b]	ND	ND	ND	ND	ND
Bu431	Bu278 Δ*tetA*(64)	16	3	3	3	6	48	1.5	128	8
Bu441	Bu278::mini-Tn*7*T-TMP	≥256	32	3	3	6	ND	ND	ND	ND
Bu436	Bu431::mini-Tn*7*T-TMP	12	2	2	ND	ND	ND	ND	ND	ND
Bu434	Bu431::mini-Tn*7*T-TMP-$P_{tetA(64)}$-*tetA*(64)$^+$	≥256	32	2	ND	ND	ND	ND	ND	ND
Bu333	Bu278 *amrB*::T23	96	2	0.5	ND	ND	ND	ND	ND	ND
Bu437	Bu278 Δ*amrB*	96	3	0.5	0.19	0.38	24	1.5	0.5	8
Bu448	Bu437::mini-Tn*7*T-TMP	96	3	0.75	0.19	0.38	ND	ND	ND	ND
Bu450	Bu437::mini-Tn*7*T-TMP-$P_{amrAB\text{-}oprA}$-*amrA*$^+$*B*$^+$-*oprA*$^+$	≥256	12	1.5	1.0	3	ND	ND	ND	ND
Bu439	Bu278 Δ*tetA*(64) Δ*amrB*	1	0.38	0.5	0.19	0.38	24	1.5	0.5	8

[a]MIC was determined using the Etest method performed in triplicate on three separate days, and values are reported as the mode of the readings.
[b]ND, not done.

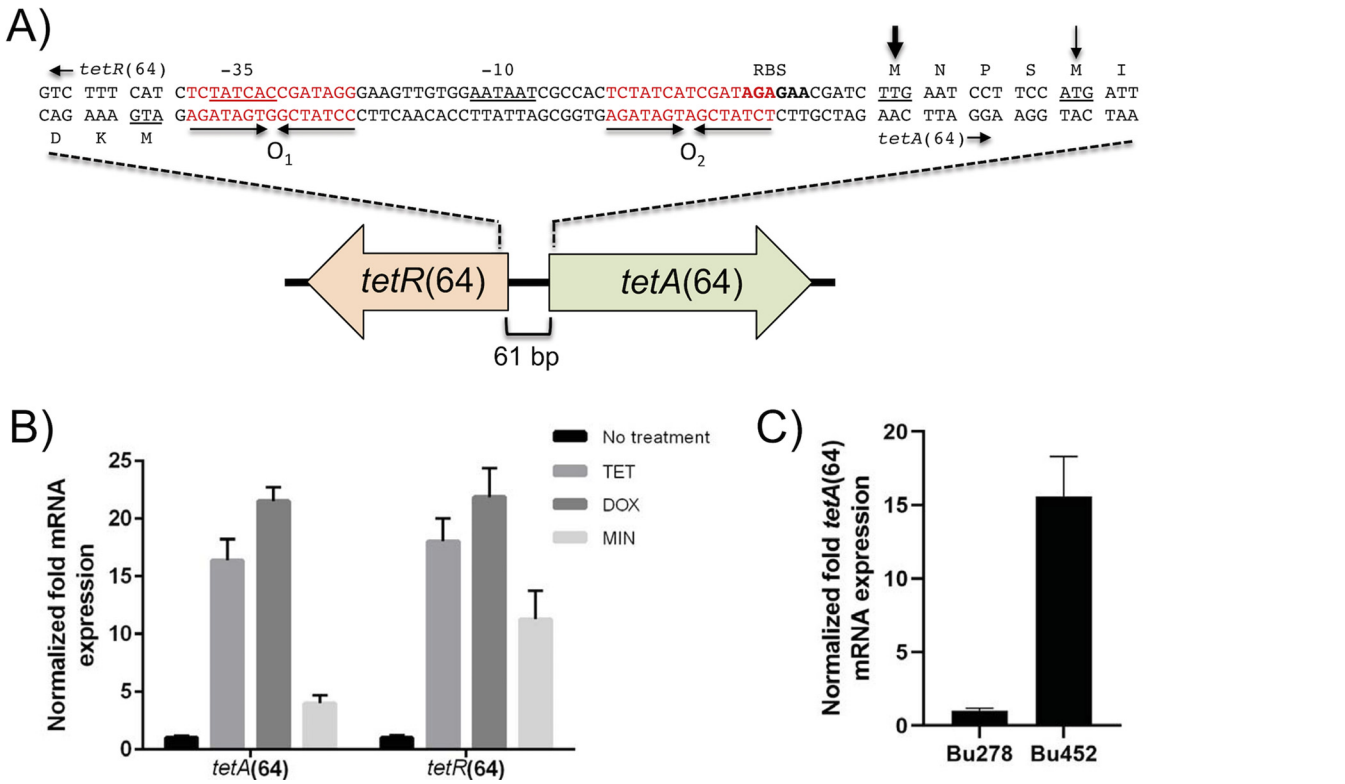

FIG 1 Genetic organization and functional analysis of *B. ubonensis* Bu278 *tetR*(64)-*tetA*(64) resistance locus. (A) Gene organization and sequence of intergenic region. The *tetA*(64) gene encoding the TetA(64) MFS efflux pump and the *tetR*(64) gene encoding the TetR (64) regulatory protein are separated by the 61-bp intergenic region (IR). The thin arrow indicates the TetA(64) ATG start codon (underlined) annotated in the *B. ubonensis* genome sequences. The more likely TTG start codon (underlined) that is preceded by a putative ribosome-binding site (RBS; bolded) is marked with a thick arrow. The IR contains two regions of imperfect dyad symmetry (inverted arrows), the putative O_1 and O_2 operator sites, whose sequences are in red type. The predicted −35 and −10 regions of the *tetA*(64) promoter that share significant homology with bacterial σ70 promoters are underlined. The *tetR*(64)-*tetA*(64) region of Bu278 was extracted from GenBank assembly accession no. GCA_002276145.1 and is available via GenBank accession no. MW052058. (B) Transcription of *tetA*(64) and *tetR*(64) is induced by tetracyclines. Cells of Bu278 were grown in LB medium in the absence or presence of the indicated tetracyclines, and total RNA was isolated. The *tetA*(64) and *tetR*(64) mRNA levels were determined by RT-qPCR. Expression levels are shown relative to uninduced cells. Error bars indicate the standard deviation of comparisons between three biological replicates, of which each was performed in technical triplicate. (C) Transcription of *tetA*(64) is constitutive in a Δ*tetR* (64) mutant. Cells of Bu278 and its Δ*tetR*(64) derivative Bp452 were grown in LB medium, and total RNA was isolated. The *tetA*(64) mRNA levels were determined by RT-qPCR. Expression levels are shown relative to LB-grown uninduced Bu278 cells. Error bars indicate the standard deviation of comparisons between three biological replicates, of which each was performed in technical triplicate.

Characterization of the tetracycline resistance locus. The transposon insertion site in strain Bu424 was mapped to locus CJO66_RS05975 (GenBank assembly accession GCA_002276145.1) (38), which is annotated as a Tet(A)/Tet(B)/Tet(C) family tetracycline MFS transporter (Fig. 1A). The gene encodes a predicted 392-amino acid, 40-kDa protein. BLAST searches showed that the protein was most similar to TetA(41) from environmental *Serratia marcescens* strain FMC 1-23-O (GenBank accession no. AY264780) (39), exhibiting 70% amino acid identity. Since the amino acid identity of the predicted *B. ubonensis* protein with any known Tet resistance protein was less than 80%, it was officially assigned the name TetA(64) in accordance with established nomenclature of tetracycline resistance determinants (23).

A subsequent closer examination of the annotated region upstream of *tetA*(64) predicts no suitable ribosome-binding site (RBS) ahead of the annotated ATG start codon but instead a putative RBS upstream of a TTG located four codons anterior to the ATG (Fig. 1A). This assignment is supported by the observation that the translation of tetracycline MFS efflux transporters is frequently initiated at a TTG start codon. Examples are *S. marcescens* TetA(41) and TetA(64) homologs from other *Burkholderia* species,

e.g., *B. cenocepacia* K56-2 whose TetA(64) is 88% identical to the extended *B. ubonensis* TetA(64). Other than increasing the *tetA*(64) gene length from 1,179 to 1,191 bp, shortening of the *tetR*(64)-*tetA*(64) intergenic region to 61 bp, and increasing the total amino acid residue count from 392 to 396, the ATG-to-TTG start codon change has no bearing on important TetA(64) parameters, such as percent identity to the nearest tetracycline resistance protein (remains at 70%) and 12 transmembrane α helix domains (see below). In the remainder of the manuscript, we used the TetA(64) gene and protein coordinates that reflect the TTG translation start site.

Mapping of the T32 insertion site in Bu424 showed that the transposon had inserted between nucleotides 287 and 288 in codon 96 of *tetA*(64). A mutant containing an unmarked 1,179-bp deletion of *tetA*(64) was constructed, and the resulting Δ*tetA*(64) strain Bu431 exhibited the same TET, DOX, and MIN susceptibility pattern as the *tetA*(64)::T23 strain Bu424 (Table 2). Furthermore, TetA(64) did not transport the glycylcycline tigecycline (TGC) and the fluorocycline eravacycline (ERV), of which both were previously shown to not be exported by tetracycline-specific efflux pumps (20). The wild-type Bu278 TET and DOX resistance pattern were restored when Δ*tetA*(64) was complemented in single copy with wild-type *tetA*(64) (strain Bu434) but not in strain Bu436 containing the empty mini-Tn*7* vector control (Table 2). The results show that TetA(64) is the main TET and DOX resistance determinant of *B. ubonensis* strain Bu278 but that this MFS transporter does not efflux MIN, ERV, and TGC. TetA(64) is specific for tetracyclines because it does not efflux antibiotics belonging to other classes, including ciprofloxacin (CIP), chloramphenicol (CHL), gentamicin (GEN), and trimethoprim (TMP) (Table 2).

As mentioned above, BLAST searches showed that *B. ubonensis* TetA(64) is most closely related to *S. marcescens* TetA(41), which, in turn, was most similar to *Acinetobacter* sp. Tet(39) (GenBank accession no. AAW66497), a tetracycline efflux pump found in diverse Gram-negative bacteria. Like Tn*10* TetA, and the more closely related TetA(41) and Tet(39), TetA(64) belongs to the group 1 family of tetracycline efflux proteins, and it was predicted to contain 12 transmembrane α helices, which is characteristic of this efflux protein family.

Located adjacent to and divergently transcribed from the 1,191-bp *tetA*(64) gene is locus CJO66_RS05970 (Fig. 1A). This 633-bp gene encodes a predicted TetR family transcriptional regulator, TetR(64) (40). The TetR(64) protein (210 amino acids; 22.8 kDa) is most closely related (62.4% identity) to TetR(41) from *S. marcescens* strain FMC 1-23-O (GenBank accession no. AY264780) (39) and is predicted to contain a helix-turn-helix DNA-binding domain (amino acids 29 to 50). The genetic *tetR-tetA* arrangement and the presence of two operator sites in the *tetR*(64)-*tetA*(64) intergenic region with strong homology to the TetR consensus-binding sites hints at TetR(64)-mediated transcriptional regulation of *tetA*(64) and *tetR*(64) by tetracycline(s). This has been repeatedly observed with other TetR-TetA tetracycline resistance determinants, including the most closely related *S. marcescens tetR*(41)-*tetA*(41) (39). Reverse transcription-quantitative PCR (RT-qPCR) analysis confirmed that compared with untreated Bu278, *tetA*(64) expression is significantly induced by the TetA(64) substrates TET (16-fold) and DOX (22-fold) and to a lesser extent by the nonsubstrate MIN (4-fold) (Fig. 1B). This finding is paralleled by *tetR*(64) expression, which is significantly induced by TET (18-fold) and DOX (22-fold) and to a lesser extent by the nonsubstrate MIN (11-fold). RT-qPCR analyses also showed that *tetA*(64) expression is 15.6-fold higher in uninduced strain Bu452 [Bu278 Δ*tetR*(64)] than baseline levels in uninduced cells of wild-type Bu278 (Fig. 1C). Collectively, the data confirm that TetR(64) is a repressor of *tetA*(64) expression.

Maximum tetracycline and doxycycline resistance requires TetA(64)-AmrAB-OprA efflux pump synergy. We previously identified a GEN-susceptible strain of Bu278, Bu333, which had a T23 insertion in the RND transporter component of the AmrAB-OprA efflux pump (18). This pump is ubiquitously present and expressed in *Burkholderia* species. While best studied in *B. pseudomallei*, published data suggest that it is responsible for the intrinsic aminoglycoside resistance of *Burkholderia* species in general (28, 41, 42). Because AmrAB-OprA is known to accommodate tetracycline

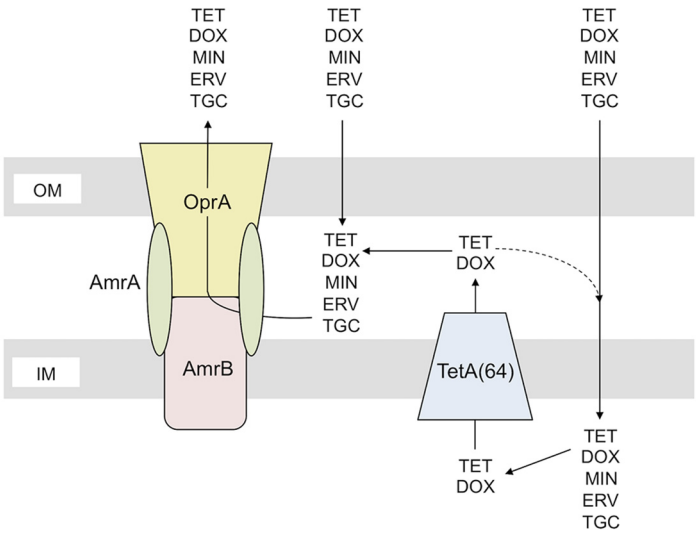

FIG 2 Model of synergistic interaction between single-component and multicomponent efflux pumps in *B. ubonensis* resistance to tetracycline antibiotics. The single-component MFS transporter TetA(64) removes TET and DOX from the cytoplasm and transports them to the periplasm. The multicomponent AmrAB-OprA RND efflux pump then captures TET and DOX from the periplasm and extrudes the drugs unto the extracellular milieu. A portion of periplasmic TET and DOX may reenter the cytoplasm (dashed line). TetA(64) and AmrAB-OprA efflux pump synergy is required to achieve maximum levels of TET and DOX resistance. In contrast, AmrAB-OprA is an intrinsic resistance determinant for MIN, ERV, and TGC, which are not effluxed by TetA(64).

antibiotics, we also tested the TET, DOX, and MIN susceptibility of the *amrB*::T23 strain Bu333. The MIC of TET for strain Bu333 was reduced from ≥256 μg/ml to 96 μg/ml (Table 2). The T23 insertion also caused a 16-fold reduction in the DOX MIC (32 μg/ml for Bu278 versus 2 μg/ml for Bu333) and a 6-fold reduction in the MIN MIC (3 μg/ml for Bu278 versus 0.5 μg/ml for Bu333). The same numbers were obtained when MIC determinations were repeated with Δ*amrB* strain Bu437. Furthermore, use of this strain showed that *B. ubonensis* AmrAB-OprA also effluxes ERV and TGC; compared with Bu278, the MICs of these antibiotics for Bu437 dropped 16-fold for ERV (from 3 μg/ml to 0.19 μg/ml) and for TGC (from 6 μg/ml to 0.38 μg/ml). The wild-type Bu278 TET MIC was fully restored and DOX, MIN, ERV, and TGC MICs were partially restored when Δ*amrB* was complemented in single copy with the wild-type *amrAB-oprA* operon expressed from its native promoter (strain Bu450) but not in strain Bu448 containing the empty mini-Tn*7* vector control (Table 2). Deletion of both *tetA*(64) and *amrB* had the most profound effect on the resistance to all tetracycline antibiotics tested, especially TET and DOX, whose MICs were lowest in the Δ*tetA*(64) Δ*amrB* strain Bu439 (1 μg/ml and 0.38 μg/ml for TET and DOX, respectively). These data are consistent with the synergistic action of the single-component TetA(64) efflux pump and the multicomponent AmrAB-OprA efflux pump to accomplish efflux of TET and DOX from the cytoplasm to the extracellular space (Fig. 2) (43, 44).

Distribution of TetA(64)-TetR(64) resistance determinants in *Burkholderia* species. To our knowledge, MFS tetracycline-specific efflux transporters and their cognate transcriptional regulators have not yet been definitively demonstrated in *Burkholderia* species. We therefore used BLAST searches to assess the distribution of TetA(64) and its gene *tetA*(64), as well as TetR(64) and its gene *tetR*(64) in the available genomes from over 3,200 Bcc and Bpc bacteria. The sequences were curated to include only those with a full species name and by focusing on nine Bcc species and four Bpc species.

The total number of genome sequences analyzed for the presence of *tetA*(64) using the 80% identity rule was 2,893, of which 1,600 were *B. pseudomallei* sequences. We found that *tetA*(64) is unequally distributed in Bcc bacteria and absent from Bpc bacteria (Table 3). The gene is present in 99.7% of 306 analyzed *B. ubonensis* genomes and is

TABLE 3 Prevalence of *tetA*(64) orthologs in the genomes of Bcc and Bpc organisms[a]

	No. of genome sequences analyzed for presence of *tetA*(64)									
	Burkholderia cepacia complex[b]						*Burkholderia pseudomallei* complex			
Presence of *tetA*(64)	*B. ubonensis* (*n* = 306)	*B. cepacia* (*n* = 129)	*B. cenocepacia* (*n* = 300)	*B. multivorans* (*n* = 211)	*B. stagnalis* (*n* = 100)	*B. vietnamiensis* (*n* = 49)	*B. pseudomallei* (*n* = 1,600)	*B. mallei* (*n* = 82)	*B. thailandensis* (*n* = 52)	*B. oklahomensis* (*n* = 8)
Positive	305 (99.7%)	128 (99.2%)	276 (92%)	None	100 (100%)	None	None	None	None	None
Negative	1 (0.3%)	1 (0.8%)	24 (8%)	211 (100%)	None	49 (100%)	1,600 (100%)	82 (100%)	52 (100%)	8 (100%)

[a]Species identification was done from a core genome phylogeny.

[b]Other Bcc bacteria analyzed were *B. diffusa* (*n* = 12; 100% negative), *B. territori* (*n* = 34; 100% negative), and *B. ambifaria* (*n* = 10; 9 positive, 1 negative).

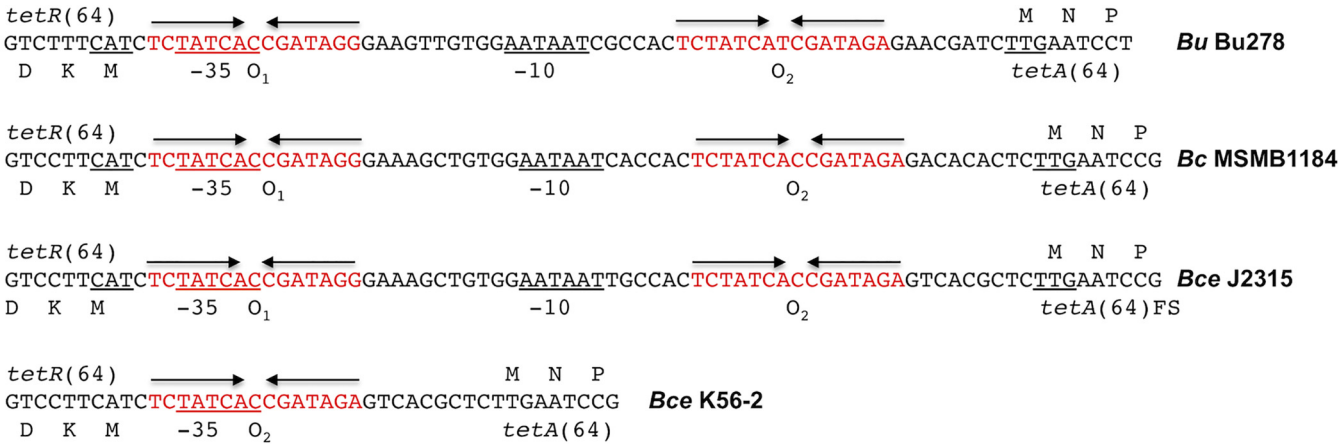

FIG 3 Conservation of the *tetR*(64)-*tetA*(64) intergenic region (IR) in Bcc bacteria. Shown are sequences that are representative of wild-type *tetR*(64)-*tetA*(64) IRs (*B. ubonensis* Bu278, *B. cepacia* MSMB1184WGS, and *B. cenocepacia* J2315) and a mutant IR (*B. cenocepacia* K56-2). Features shown include the predicted amino-terminal amino acid sequence of the *tetR*(64) and *tetA*(64) genes on the left and right, respectively, the predicted −35 and −10 regions of the *tetA* (64) promoter, and the putative O_1 and O_2 operator sites. The *B. cepacia* MSMB1184WGS and *B. cenocepacia* J2315 IRs are identical in length (63 nucleotides), compared with 61 nucleotides for Bu278. The *B. cenocepacia* K56-2 IR is only 25 nucleotides long and only contains an O_2 operator site and a putative −35 sequence but no −10 sequence. FS, frame-shifted and truncated TetA(64) in *B. cenocepacia* J2315.

also present in a few other analyzed Bcc organisms, e.g., 100% *Burkholderia stagnalis* (100 analyzed), 99.2% *B. cepacia* (129 analyzed), and 92% *B. cenocepacia* (300 analyzed). It is absent from other Bcc bacteria, e.g., *B. multivorans* ($n = 211$), *Burkholderia vietnamiensis* ($n = 49$), *Burkholderia diffusa* ($n = 12$), and *Burkholderia territorii* ($n = 34$). The *tetA* (64) gene is also absent from the four Bpc species analyzed, i.e., *B. pseudomallei* ($n = 1,600$), *B. mallei* ($n = 82$), *B. thailandensis* ($n = 52$), and *Burkholderia oklahomensis* ($n = 8$) (Table 3).

Although the TetR proteins are not as conserved as the TetA proteins, the *tetA*(64) data are mirrored by *tetR*(64) data. Compared with *B. ubonensis* TetR(64), TetR proteins are present in TetA(64)-containing Bcc species with identities of 85% to 87% (*B. stagnalis*), 77% to 78% (*B. cepacia*), and 76% to 77% (*B. cenocepacia*). Conversely, TetR proteins are absent from Bcc (e.g., *B. multivorans*, *B. vietnamiensis*, *B. diffusa*, and *B. territorii*) and Bpc (e.g., *B. pseudomallei*, *B. mallei*, *B. thailandensis*, and *B. oklahomensis*) species lacking TetA(64).

Notable Bcc bacteria containing *tetA*(64) are K56-2 and J2315, two well-studied *B. cenocepacia* cystic fibrosis patient isolates of the highly transmissible ET12 lineage (5, 45). It should be noted that *tetA*(64) is a pseudogene in J2315 due to a deletion of nucleotide T941 (5, 38). This mutation causes a frameshift and production of a 344-residue truncated TetA(64) mutant protein that lacks helices 11 and 12. K56-2 is likely not expressing TetA (64) because it is missing the predicted −10 region of the putative *tetA*(64) promoter due to a deletion of 38 bp from the *tetR*(64)-*tetA*(64) intergenic region caused by a recombination event between the two operator sites (Fig. 3). The latter could also possibly occur in *B. ubonensis*, *B. cepacia*, and *B. cenocepacia* since the intergenic regions are highly conserved in length and sequence across the three species. The *B. cepacia* MSMB1184WGS and *B. cenocepacia* J2135 intergenic region (IR) sequences are 94% identical, and both sequences are 92% identical to the IR sequence from *B. ubonensis* Bu278.

Genomic localization of the *tetR*(64)-*tetA*(64) locus. Examination of the genomic location of the *tetR*(64)-*tetA*(64) resistance determinants revealed that, when present, they are located on chromosome 1 in a conserved location either upstream or downstream of the *miaA* gene, dependent on chromosomal orientation (Fig. 4). The *tetR*(64) gene is always oriented toward *miaA* and *tetA*(64) toward *purM*. In *B. ubonensis* Bu278, *B. stagnalis* MSMB1512WGS, and *B. cepacia* MSMB1184WGS, *tetA*(64) is located directly adjacent to *purM*. In *B. cenocepacia* K56-2, *purM* and *tetA*(64) are separated by a 396 bp open reading frame (ORF) that encodes a 131-residue protein. Similar sized ORFs (393 to 402 bp) encoding similar sized proteins (130 to 133 residues) are present in the

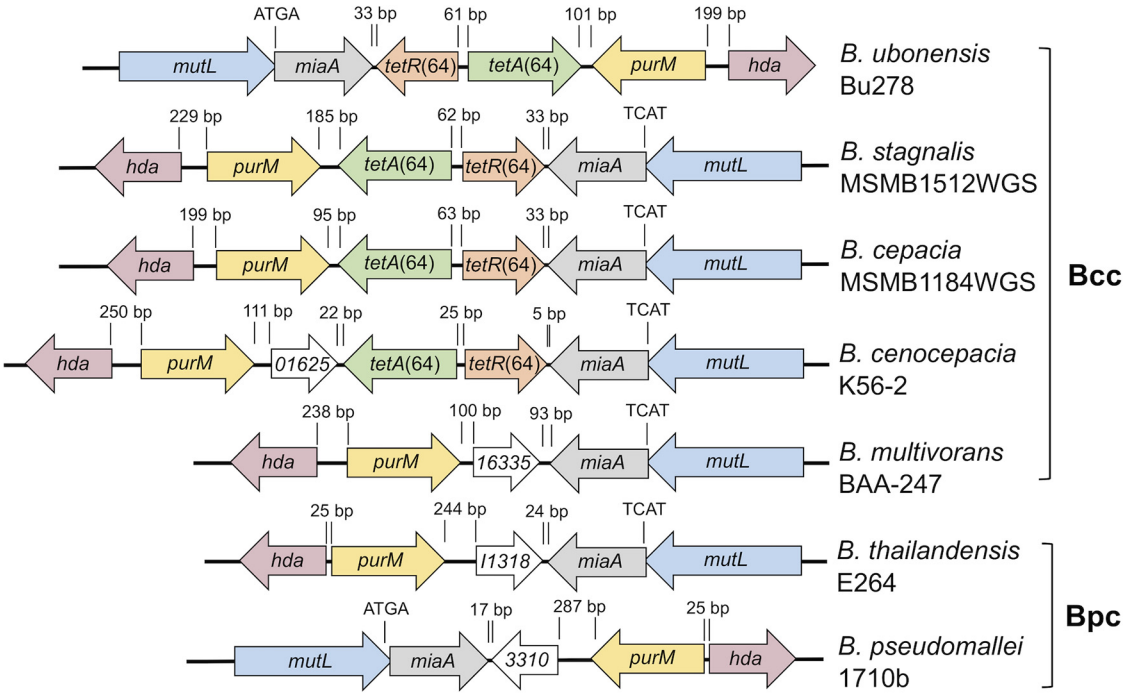

FIG 4 Genetic maps of the chromosome 1 region containing the *tetR*(64)-*tetA*(64) resistance determinants in representative Bcc and Bpc strains. In *B. ubonensis* Bu278, *B. stagnalis* MSMB1512WGS, and *B. cepacia* MSMB1184WGS, the *tetR*(64) and *tetA*(64) genes are inserted between the *purM* and *miaA* genes, with *tetR*(64) oriented toward *miaA* and *tetA*(64) toward *purM*. *B. cenocepacia* K56-2 represents a variant where a gene annotated as *01625* (short for locus tag BURCENK562V_RS01625) is inserted between *purM* and *tetA*(64). Orthologs of this gene are also present in the same location in Bcc bacteria (e.g., *B. multivorans* BAA-247) or Bpc bacteria (e.g., *B. thailandensis* and *B. pseudomallei*) that do not contain the *tetR*(64)-*tetA*(64) locus. Gene annotations are as follows: *hda*, DNA regulatory inactivator Hda; *purM*, phosphoribosylformylglycinamidine cyclo-ligase; *miaA*, tRNA (adenosine[37]-N6)-dimethylallyl transferase; *mutL*, DNA mismatch repair protein; *01625*, *16335* (locus tag NP80_RS16335), *I1318* (locus tag BTH_I1318), and *3310* (locus tag BURPS1710b_3310), orthologs that are either annotated as ketosteroid isomerase or hypothetical protein. Distances between genes are indicated in base pairs; ATGA (and reverse) indicates the overlap of the *miaA* and *mutL* start and stop codons, respectively.

same location in other *Burkholderia* species that lack *tetR*(64) and *tetA*(64), e.g., *B. multivorans* BAA-247, *B. thailandensis* E264, and *B. pseudomallei* 1710b. The small proteins are very closely related; the K56-2 protein shares 96.6%, 78.5%, and 76.3% identity with the respective proteins from *B. multivorans* BAA-274, *B. thailandensis* E264, and *B. pseudomallei* 1710b. The proteins are either annotated as a ketoisomerase or protein of unknown function. The latter is likely correct since the *Burkholderia* proteins are missing the active site residues present in other small ketoisomerases, such as the 131-residue ketoisomerase from a *Pseudomonas putida* species that only shares 22.5% identity with the 131-residue protein from *B. cenocepacia* K56-2 (46). Except for different chromosomal orientation and some variations due to insertion elements, the genetic makeup of the chromosome 1 regions shown in Fig. 4 for representative strains of the Bcc and Bpc is conserved in the respective species.

The intergenic sequences between *tetR*(64) and *tetA*(64) and their respective neighboring genes are short, ranging from 22 to 185 bp on the *tetA*(64) side and 5 to 33 bp on the *tetR*(64) side in the 4 examples more closely analyzed in this study. These intergenic regions provide no evidence for transposition, i.e., no insertion elements or other significant repeat sequences.

DISCUSSION

Although *Burkholderia* species are frequently cited as being intrinsically resistant to antibiotics, clinically significant tetracycline resistance in *Burkholderia* species has to our knowledge been studied and described in any detail only in *B. pseudomallei*. Historically, TET and more recently DOX have been included in the regimen for oral

eradication therapy of *B. pseudomallei* infections, and DOX remains an important option in instances where other primary (trimethoprim + sulfamethoxazole) or secondary (amoxicillin + clavulanic acid) drugs are contraindicated or ceased because of adverse effects (34, 47). Clinically significant acquired DOX resistance (MIC, ≥16 μg/ml) is rare but has been reported (48), most notably after DOX was used in primary therapy for high-burden *B. pseudomallei* infection (34). Previous studies with *B. pseudomallei* and *B. thailandensis* showed that DOX is exported via the AmrAB-OprA, BpeAB-OprB, and BpeEF-OprA efflux pumps, although tetracyclines are poor RND efflux pump substrates in wild-type strains (28–34). A subsequent study with an isogenic pair of patient isolates of which the second isolate was DOX resistant confirmed an AmrAB-OprA contribution to resistance. However, DOX resistance in this patient isolate was attributed to synergy of loss of methyltransferase function and increased AmrAB-OprA pump efflux activity (34), similar to the synergy noted for TET in this study.

Despite that several Bcc organisms, e.g., *B. cenocepacia* and *B. multivorans*, are clinically significant pathogens in CF, very little is known about tetracycline resistance in these species. Tetracyclines such as TET, MIN, and TGC have been included in studies comparing antibiograms for diverse Bcc bacteria, and the studies showed that even newer tetracyclines like TGC are scarcely active (45, 49–51). No tetracyclines were included in recent proposed treatment protocols for Bcc in CF patients (52–54). There are some published data for tetracycline resistance mechanisms in Bcc bacteria (33, 75), and it was shown that the *B. cenocepacia* RND-4 efflux pump extrudes MIN (55). Based on tetracycline resistance gene amplification by PCR, the Tet(O) ribosomal protection mechanism was proposed for a *B. cepacia* forest soil isolate (56). The same investigators also proposed a Tet(D) efflux resistance determinant for what is called a *B. pseudomallei* forest isolate. This is likely a misidentification given the global geographic distribution of *B. pseudomallei* in subtropical and tropical regions. To our knowledge, neither of these tetracycline resistance determinants have been characterized in detail.

When studying AMR mechanisms in *B. ubonensis*, we noted that the bacterium exhibited high-levels of resistance to TET (MIC, ≥256 μg/ml) and DOX (MIC, 32 μg/ml) but was considerably more susceptible to MIN (MIC = 3 μg/ml), ERV (MIC = 3 μg/ml), and TGC (MIC = 6 μg/ml). Random transposon mutagenesis of wild-type Bu278 identified a tetracycline-specific efflux pump belonging to the major facilitator superfamily as the primary TET resistance determinant. Although closely related to *S. marcescens* TetA(41) (70% identity), the *B. ubonensis* resistance determinant was sufficiently different to warrant assignment of a novel name, TetA(64). Deletion of *tetA*(64) had a significant effect on TET and DOX resistance, but MIN, ERV, and TGC susceptibility levels were unaffected. Not surprisingly, all tetracyclines tested were substrates of the AmrAB-OprA RND efflux pump, but unlike *B. pseudomallei* where for instance DOX is a poor AmrAB-OprA substrate, it is a good substrate in *B. ubonensis*. The Bu439 Δ*tetA*(64) Δ*amrB* double mutant was highly susceptible (MICs, 0.19 to 1 μg/ml) to all tetracyclines tested, confirming that tetracycline resistance in *B. ubonensis* is primarily due to efflux. The double-mutant MIC data also confirmed that TetA(64) pump and AmrAB-OprA efflux pump synergy is required to achieve maximum levels of TET and DOX resistance. This finding is in agreement with that of previous studies with *Escherichia coli* and *Pseudomonas aeruginosa*, which showed that synergy between single-component efflux pumps that export antibiotics into the periplasm and multicomponent efflux pumps that then accomplish their efflux into the external medium resulted in resistance levels that were significantly higher than those conferred by each of the pumps operating separately (43, 44). In the present case, the effect for both TET and DOX is multiplicative. While synergistic interactions between pumps of different types have been demonstrated before, the recruitment of a tetracycline-specific resistance determinant in addition to a resident nonspecific tetracycline resistance determinant present in all *Burkholderia* species to enhance overall resistance levels is a novel observation.

A comprehensive genome analysis from 2,893 Bcc and Bpc organisms using a

threshold of 80% amino acid identity over the entire protein length revealed an uneven distribution of the TetA(64) resistance determinant in the Bcc, i.e., either its presence in 92% to 100% of some Bcc species or complete absence in others. The *B. stagnalis* TetA(64) is most closely related to *B. ubonensis* TetA(64), which is consistent with the two species being phylogenetically most closely related among the Bcc (57). *B. stagnalis* has been isolated from the environment and from the respiratory tract of patients (57). Of interest is that of the 24 *B. cenocepacia* strains whose TetA proteins fell below the 80% identity threshold, 20 were in a cluster with J2315. Eighteen of these strains had identical truncated TetA amino acid sequences as J2315. For two strains, no TetA sequences were available. TetA(64) was found to be absent from the Bpc, including 1,600 *B. pseudomallei* strains. The presence of TetA (64) in 50% of the Bcc species we examined indicates that its acquisition may be facilitated by these organisms encountering high tetracycline concentrations in the environment.

The data from the present study suggest that it is worth revisiting the sensitivity of individual Bcc pathogens when looking for therapeutic options for infections in CF patients because while resistance to TET and DOX may be present, in some cases the pathogen may be sensitive to MIN, ERV, and TGC.

The synthesis of most Gram-negative Tet efflux pumps is regulated by a repressor protein that is encoded by the *tetR* gene located adjacent to and transcribed divergently from *tetA* (58–60). In the presence of tetracycline, the repressor binds the antibiotic and is released from the operators that control *tetA* and *tetR* expression (40, 60, 61). *B. ubonensis* shows a *tetR*(64)-*tetA*(64) genetic organization observed in other Gram-negative, including *Burkholderia*, *tetR-tetA* systems. The *B. ubonensis* genes are separated by a 61-bp IR. The IR contains operators that are predicted to govern *tetR* (64) and *tetA*(64) expression via TetR(64). In this study, we show that *tetA*(64) and *tetR* (64) expression are indeed significantly induced in the presence of the TetA(64) substrates TET and DOX and to a lesser extent also by MIN that is not a substrate of TetA (64). We also showed that TetR(64) is a repressor of *tetA*(64). Constitutive expression of tetracycline-specific efflux pumps strongly reduces fitness (62). To avoid undue fitness costs, bacteria like *B. ubonensis* have evolved two mechanisms: (i) tight regulation by TetR and (ii) reduction of translation initiation efficiency by utilization of a TTG start codon for TetA translation. In this context, it is tempting to speculate that the observed mutations in the two *B. cenocepacia* CF patient isolates J2315 (and at least 18 other strains) and K56-2, which express a truncated TetA(64) or contain a truncated regulatory region, respectively, may reflect *tetA*(64) purifying selection due to a lack of selective pressure since transitioning from the environment to a human infection.

Most tetracycline-specific MFS efflux pumps are encoded by transposons or plasmids, but some are encoded by chromosomal genes. In *B. ubonensis*, the *tetR*(64)-*tetA* (64) genes are located in a conserved region of chromosome 1 between the *miaA* (tRNA modification enzyme) and *purM* (purine metabolism enzyme) genes. Since we could not find any evidence for transposition, the *tetR*(64)-*tetA*(64) genes may have been acquired by homologous recombination between conserved flanking housekeeping genes after horizontal gene transfer from a yet unknown source.

In conclusion, we describe the first tetracycline-specific resistance determinant in *Burkholderia* species TetA(64), which is accompanied by the cognate TetR(64) transcriptional regulator. The *tetR*(64)-*tetA*(64) genes are located in the same region on chromosome 1 in all Bcc bacteria that carry them. At present, we do not know where the very closely related, but not identical, *tetR*(64)-*tetA*(64) genes observed in various Bcc bacteria originate and how they get integrated into the chromosomes. This begs the question as to whether the resistance genes can be transferred between *Burkholderia* species. The short *tetR*(64)-*tetA*(64)-adjacent gene intergenic regions provide no evidence for transposition, i.e., no insertion elements or other significant repeat sequences. Curiously, in each of the examples examined, the *tetR*(64)-*tetA*(64) genes are always oriented with *tetA*(64) toward *purM*, which is consistent with site- and orientation-specific

TABLE 4 Primers used in this study

Primer	Sequence (5′–3′)	Purpose
P3754	CCTGTTATCCCTACCCGGGCAATTCCTCGACGGCGCGA	*tetA*(64) deletion
P3755	CGGATTCCGCGGAAGGATTCAAGATCGTTCTCTATCG	*tetA*(64) deletion
P3756	GAATCCTTCCGCGGAATCCGGCCGCCCG	*tetA*(64) deletion
P3757	GGGATAACAGGGTAATCCCGCTCGTGCAGGGCGCCGAG	*tetA*(64) deletion
P3769	CCGTGCTGCTCGCGTCG	*tetA*(64) RT-qPCR
P3770	CGTTCGCGCCCGTGATG	*tetA*(64) RT-qPCR
P3773	CCTGTTATCCCTACCCGGGCCCAGCTCGACTACGCGAC	*amrB* deletion
P3774	TTCGCAACCTATGAAGATGGCGATCACCC	*amrB* deletion
P3775	CCATCTTCATAGGTTGCGAAGGATCTCG	*amrB* deletion
P3776	GGGATAACAGGGTAATCCCGGTCCGACAGGCTCTTCAC	*amrB* deletion
P3777	CTCGATCATGCATGAGCTCACGCGCCCGTGTCTTTCATC	*tetA*(64) complementation
P3778	TTCGCGAGGTACCGGGCCCAATTCCGCTTACGCGGCCG	*tetA*(64) complementation
P3853	CTCGATCATGCATGAGCTCAGTTGCGAGATTCCTTACGTTTTGCTGTC	*amrB* complementation
P3854	CTGCGGCCGATGTCGCCCACCGTCACGC	*amrB* complementation
P3855	GTGGGCGACATCGGCCGCAGCGCGGTGC	*amrB* complementation
P3856	CACGACGAGCACCGACAGCGCGAACAGCATCGG	*amrB* complementation
P3857	CGCTGTCGGTGCTCGTCGTGTTCCTTGC	*amrB* complementation
P3858	TTCGCGAGGTACCGGGCCCATCACACGTCGGCTTCCGC	*amrB* complementation
P3887	ACGAGGTCGGCATCAATG	*tetR*(64) RT-qPCR
P3888	CATGATCGCTTCCGCCA	*tetR*(64) RT-qPCR
P3889	CCTGTTATCCCTACCCGGGCCTGTACTACAAGGCGCTG	*tetR*(64) deletion
P3890	CACGGTCCTGGTGTTCAAGTGCGGCATC	*tetR*(64) deletion
P3891	ACTTGAACACCAGGACCGTGTCGCGCGT	*tetR*(64) deletion
P3892	GGGATAACAGGGTAATCCCGTCGACCAGCCGAAATGCGC	*tetR*(64) deletion

transfer of a DNA fragment containing *tetR*(64)-*tetA*(64) and neighboring genes. This could happen via homologous recombination between conserved neighboring genes but does not preclude other mechanisms.

Resistance gene transfer between nonpathogenic and pathogenic species is always of concern, especially with organisms like *Burkholderia* that already contain a formidable armamentarium of AMR determinants (63). Obviously, *tetR*(46)-*tetA*(46) have already been acquired by the opportunistic pathogens *B. stagnalis*, *B. cepacia*, and *B. cenocepacia*. The question of whether these resistance genes could be acquired by a high-consequence Bpc pathogen like *B. pseudomallei* in environments where it frequently coexists with Bcc bacteria like *B. ubonensis* remains to be answered.

MATERIALS AND METHODS

Strains, media, and growth conditions. *Burkholderia ubonensis* strains used in this study are listed in Table 1. *Escherichia coli* strain DH10B (ThermoFisher Scientific, Waltham, MA) was used for cloning and expression experiments. RHO3 was used for conjugal transfer of plasmids into *B. ubonensis* (64). Lennox broth (LB) containing 5 g/liter NaCl was used for routine growth of bacteria, and cation-adjusted Mueller-Hinton II agar (MHA; Becton, Dickinson and Company, Sparks, MD) was used for MIC assays. For plasmid selection in *E. coli*, media were supplemented with 100 μg/ml ampicillin (AMP), 35 μg/ml kanamycin (KAN), or 100 μg/ml trimethoprim (TMP). For use in plasmid maintenance or merodiploid or mini-Tn*7* integrant selection in *B. ubonensis*, KAN and TMP were used at 1,000 μg/ml and 100 μg/ml, respectively.

Antimicrobial susceptibility testing. MIC assays were performed using Etest strips and following the manufacturer's (AB bioMérieux, Marcy l'Etoile, France) guidelines.

Deletion mutant construction. Gene replacement plasmids were constructed by the Gibson assembly method (65). Briefly, overlap sequences for the target plasmid and insert were designed using the NEBuilder assembly tool v.2.2.7 (New England BioLabs, Ipswich, MA). *B. ubonensis* Bu278 (Bp8955) genomic DNA served a source for PCR-amplified templates and was isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI). Primers were purchased from IDT Technologies (Coralville, IA), and their sequences are provided in Table 4.

For construction of a Bu278 derivative containing a 1,179-bp Δ*tetA*(64), 936 bp of upstream and 854 bp of downstream sequences of the *tetA*(64) flanking regions were PCR amplified using primer pair 3754 and 3755 and pair 3756 and 3757, respectively. Similarly, for construction of a Bu278 derivative containing a 2,765-bp Δ*amrB*, 768 bp of upstream and 787 bp of downstream flanking regions of Bu278 Δ*amrB* were PCR amplified using primer pair 3773 and 3774 and primer pair 3775 and 3776, respectively.

TABLE 5 Plasmids used in this study

Name	Description[a]	Source
pEDL1005	TMP^r; allelic exchange vector; sucrose or I-SceI counterselection	18
pBADSce-Km	KAN^r; *araC*-P_{BAD}-*I-sceI* expression vector with pRO1600(Ts) replicon	Lab collection
pPS3551	TMP^r; pEDL1005 with 1,179 bp Δ*tetA*(64) from ATG start to stop codon	This study
pPS3556	TMP^r; pEDL1005 with 2,765 bp Δ*amrB* (nt 57–2,821 of 3,138-nt ORF)	This study
pPS3596	TMP^r; pEDL1005 with 519 bp Δ*tetR*(64) (nt 43–561 of 633-nt ORF)	This study
pPS1897	TMP^r, AMP^r; pUC18T-mini-Tn*7*T-TMP (GenBank accession no. DQ493875)	18
pPS3553	TMP^r, AMP^r; pUC18T-mini-Tn*7*T-TMP-P_{tetA}-*tetA*(64)	This study
pPS3592	TMP^r, AMP^r; pUC18T-mini-Tn*7*T-TMP-$P_{amrAB\text{-}oprA}$-*amrA*$^+$*B*$^+$-*oprA*$^+$	This study

[a]nt, nucleotide(s); Ts, temperature-sensitive.

For construction of a Bu278 derivative containing a 519-bp Δ*tetR*(64), 835 bp of upstream and 753 bp of downstream flanking regions of Bu278 Δ*tetR*(64) were PCR amplified using primer pair 3891 and 3892 and primer pair 3889 and 3890, respectively. DNA fragments were assembled with NotI, and EcoRI linearized pEDL1005 using NEBuilder high-fidelity (hifi) DNA assembly master mix (New England BioLabs), creating pPS3551 [Δ*tetA*(64)], pPS3556 (Δ*amrB*), and pPS3596 [Δ*tetR*(64)] (plasmids used in this study are listed in Table 5).

Plasmid DNA was isolated using the Nucleospin plasmid kit (Macherey-Nagel, Düren, Germany), and plasmid integrity was analyzed by Sanger sequencing. The gene replacement plasmids were transferred to Bu278 by conjugation from *E. coli* RHO3 (64), and TMP-resistant merodiploids were resolved using I-SceI counter selection as previously described (64). The presence of the desired deletions was verified by PCR amplification and Sanger sequencing.

The resulting mutants are Bu431 [Bu278 Δ*tetA*(64)], Bu437 (Bu278 Δ*amrB*), and Bu452 [Bu278 Δ*tetR* (64)]. The Bu278 Δ*tetA*(64) Δ*amrB* mutant Bu452 was obtained by conjugally transferring pPS3556 into strain Bu431 and following the verification steps outlined above for the single mutants.

Deletion mutant complementation. Mutant complementation was achieved using the mini-Tn*7* system, which enables stable and site-specific single-copy insertions into at least two *glmS*-associated sites in the *B. ubonensis* genome (18, 66). Delivery plasmids containing mini-Tn*7* elements expressing wild-type *tetA*(64) and *amrAB-oprA* from their native promoters were constructed by Gibson assembly of PCR fragments amplified from Bu278 genomic DNA.

For pPS3553, a 1,317-bp fragment containing *tetA*(64) and its promoter was PCR amplified using P3777 and P3778. Purified DNA fragments were assembled with SpeI, and HindIII linearized pPS1897 using NEBuilder hifi DNA assembly master mix (New England BioLabs), creating pPS3553. Plasmid DNA was isolated and its integrity analyzed by Sanger sequencing.

For pPS3592, three DNA fragments with 10- to 20-bp overlaps and the *amrAB-oprA* operon promoter were PCR amplified using primers P3853 and P3854 (2,058 bp), P3855 and P3856 (1,992 bp), and P3857 and P3858 (1,977 bp), respectively. PCR fragments were assembled with SpeI, and HindIII linearized pPS1897 using NEBuilder hifi DNA assembly master mix, creating pPS3592. Plasmid integrity was analyzed by plasmid next-generation sequencing (MGH CCIB DNA Core, Massachusetts General Hospital, Cambridge, MA).

The mini-Tn*7* delivery plasmids pPS3553, pPS3592, and pPS1897 containing the empty mini-Tn*7* vector were transferred to Bu278 Δ*tetA*(64) strain Bu431 (pPS3553 or pPS1897) or the Bu278 Δ*amrB* strain Bu437 (pPS3592 or pPS1897) by conjugation from *E. coli* RHO3 (64) or by electroporation (67), along with the transposase-encoding helper pTNS3. Insertions at *glmS*-associated *att*Tn*7* sites were identified using PCR as previously described (18). Complemented deletion mutants are listed in Table 1.

Reverse transcription-quantitative PCR (RT-qPCR). Expression levels of mRNA levels of target genes were determined in Bu278 grown at 37°C in Lennox Luria Broth medium to mid-log phase (optical density at 600 nm [OD_{600}], 0.4 to 0.6), at which point *tetA*(64) expression either remained uninduced or was induced for 1 h with 150 μg/ml TET, 16 μg/ml DOX, or 1.5 μg/ml MIN (these concentrations are subinhibitory and correspond to approximately one-half of the respective MIC). Total RNA was extracted using the RNeasy protect bacteria minikit (Qiagen, Valencia, CA), and cDNA synthesis was performed as previously described (30, 68). The primer sets used were Bp23S_F and Bp23S_R previously designed for the *B. pseudomallei* 23S rRNA housekeeping control (68), P3769 and P3770 for *tetA*(64), and P3887 and P3888 for *tetR*(64) (Table 4). Prism (GraphPad Software, La Jolla, CA) was used for data analysis and presentation.

Sequence analysis. SnapGene software v4.3.9 (GSL Biotech, Chicago, IL) was used to perform general DNA analyses. DNA and protein sequence alignments and similarity predictions were performed using Clustal Omega software (69) on EMBL-EBI (70) (https://www.ebi.ac.uk/Tools/msa/clustalo/). Helix-turn-helix (HTH) DNA-binding domains were predicted by the Rhone-Alpes Bioinformatic Pole Gerland Site (https://npsa-prabi.ibcp.fr) (71). The TMHMM Server 2.0 online at https://services.healthtech.dtu.dk/service.php?TMHMM-2.0 was used for the prediction of transmembrane helices in proteins. For TetA and TetR alignments, the respective sequences for *tetA*(64) (BW23_977) and *tetR*(64) (BW23_976) from *B. ubonensis* were aligned against 3,273 *Burkholderia* genomes with TBLASTN v2.5.0+ (72) in conjunction with LS-BSR v1.2.0 (73). The blast score ratio (BSR) (74) was calculated by dividing the query bit score by the reference self-alignment bit score. A BSR value of 0.80 is equivalent to 80% peptide identity over 100% of the peptide length.

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