

The BpeEF-OprC Efflux Pump Is Responsible for Widespread Trimethoprim Resistance in Clinical and Environmental *Burkholderia pseudomallei* Isolates

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Trimethoprim-sulfamethoxazole (co-trimoxazole) is the primary drug used for oral eradication therapy of *Burkholderia pseudomallei* infections (melioidosis). Here, we demonstrate that trimethoprim resistance is widespread in clinical and environmental isolates from northeast Thailand and northern Australia. This resistance was shown to be due to BpeEF-OprC efflux pump expression. No dihydrofolate reductase target mutations were involved, although frequent insertion of ISBma2 was noted within the putative *folA* transcriptional terminator. All isolates tested remained susceptible to trimethoprim-sulfamethoxazole, suggesting that resistance to trimethoprim alone in these strains probably does not affect the efficacy of co-trimoxazole therapy.

Burkholderia pseudomallei is a saprophytic Gram-negative bacterium found mostly in soil and water in many subtropical and tropical regions of the world, including northern Australia and southeast Asia (1, 2). *B. pseudomallei* is the etiologic agent of the multifaceted disease melioidosis (2–6). Treatment of melioidosis is complicated by the intrinsic resistance of *B. pseudomallei* to many classes of antimicrobials (7, 8). The current recommended therapy includes an initial intensive phase followed by a lengthy eradication phase to prevent relapse (6, 9, 10). Most patients require at least 10 to 14 days of parenteral ceftazidime or a carbapenem followed by 12 to 20 weeks of oral trimethoprim-sulfamethoxazole with or without doxycycline. Trimethoprim and sulfamethoxazole inhibit the folic acid biosynthetic pathway by targeting dihydrofolate reductase (FolA) and dihydropteroate synthase (FolP), respectively (11). The synergistic trimethoprim-sulfamethoxazole combination, co-trimoxazole, has a potent antimicrobial effect. *B. pseudomallei* co-trimoxazole resistance was previously documented in regions where the disease is endemic (12–16), and rates range from 2.5% in Australia (13) to 13 to 16% in Thailand (12, 14). Previous studies have identified and characterized trimethoprim resistance mechanisms including resistant dihydrofolate reductases in other organisms, such as *Escherichia coli* (11, 17), but in *B. pseudomallei*, trimethoprim resistance has been studied only indirectly in surrogate (18) or closely related (19) bacteria, showing that efflux could play an important role in resistance. The objective of this study was to identify and characterize the mechanism responsible for trimethoprim resistance in clinical and environmental *B. pseudomallei* isolates from northeast Thailand and northern Australia.

MATERIALS AND METHODS

Bacterial strains. *B. pseudomallei* strain 1026b was used as a prototype strain for all experiments in this study (20, 21). Additionally, a collection of 30 clinical and 30 environmental isolates from Thailand (isolated in 2001 and 1990 to 2001, respectively) and 4 clinical isolates and 1 environmental isolate from Australia (isolated between 1994 and 1997) were examined. All procedures involving *B. pseudomallei* were performed in select-agent-approved biosafety level 3 (BSL3) facilities in the Rocky Mountain Regional Biosafety Laboratory at Colorado State University, using approved select-agent-compliant procedures and protocols.

Antimicrobial susceptibility testing. Trimethoprim, sulfamethoxazole, and co-trimoxazole susceptibilities were assessed by determining MICs using the Etest method according to the manufacturer's instructions (AB bioMérieux, Marcy l'Etoile, France). Briefly, strains were grown to mid-log phase (optical density at 600 nm [OD₆₀₀] = 0.6 to 0.8) and diluted to a 0.5 McFarland standard in 0.85% sterile saline. The resulting bacterial cell suspension was then used to swab Mueller-Hinton II agar plates (Becton, Dickinson and Company, Sparks, MD) to which the Etest strips were applied. MIC results were determined following 16 to 20 h of incubation at 37°C. Results were read at 80% inhibition, again according to the manufacturer's guidelines. Since there are no established breakpoints for non-*Enterobacteriaceae*, the following *Enterobacteriaceae* MIC cutoffs were used to define susceptibility and resistance for trimethoprim alone (≤ 8 $\mu\text{g/ml}$ for susceptible and > 8 $\mu\text{g/ml}$ for resistant) according to CLSI guidelines (see Table 2A in reference 22). The non-*Enterobacteriaceae* sulfonamide cutoffs were used for sulfamethoxazole alone (≤ 256 $\mu\text{g/ml}$ for susceptible and > 256 $\mu\text{g/ml}$ from resistant) according to CLSI guidelines (see Table 2B-5 in reference 22), while CLSI standard MIC cutoffs for *B. pseudomallei* were used for co-trimoxazole (trimethoprim-sulfamethoxazole, $\leq 2/38$ $\mu\text{g/ml}$ for susceptible and $> 2/38$ $\mu\text{g/ml}$ for resistant) (see Table 2K in reference 22).

Amplification and sequencing of *folA*. The *folA* coding sequence was PCR amplified in four independent PCRs from genomic DNA isolated with PureGene Core kit A (Qiagen, Valencia, CA), using primers P1966 (5'-CTTCCGGCCTCTTTCTTTC) (Integrated DNA Technologies, Coralville, IA) and P1967 (5'-GTGCTGATCGAGCAGATGAC) and Platinum Taq DNA Polymerase High Fidelity (Life Technologies Corporation, Grand Island, NY). The PCR products were pooled for each strain and purified from agarose gels by using the GenElute gel extraction kit (Sigma-Aldrich, St. Louis, MO). The PCR products were sequenced using P1966 and P1967 at the Colorado State University Proteomics and

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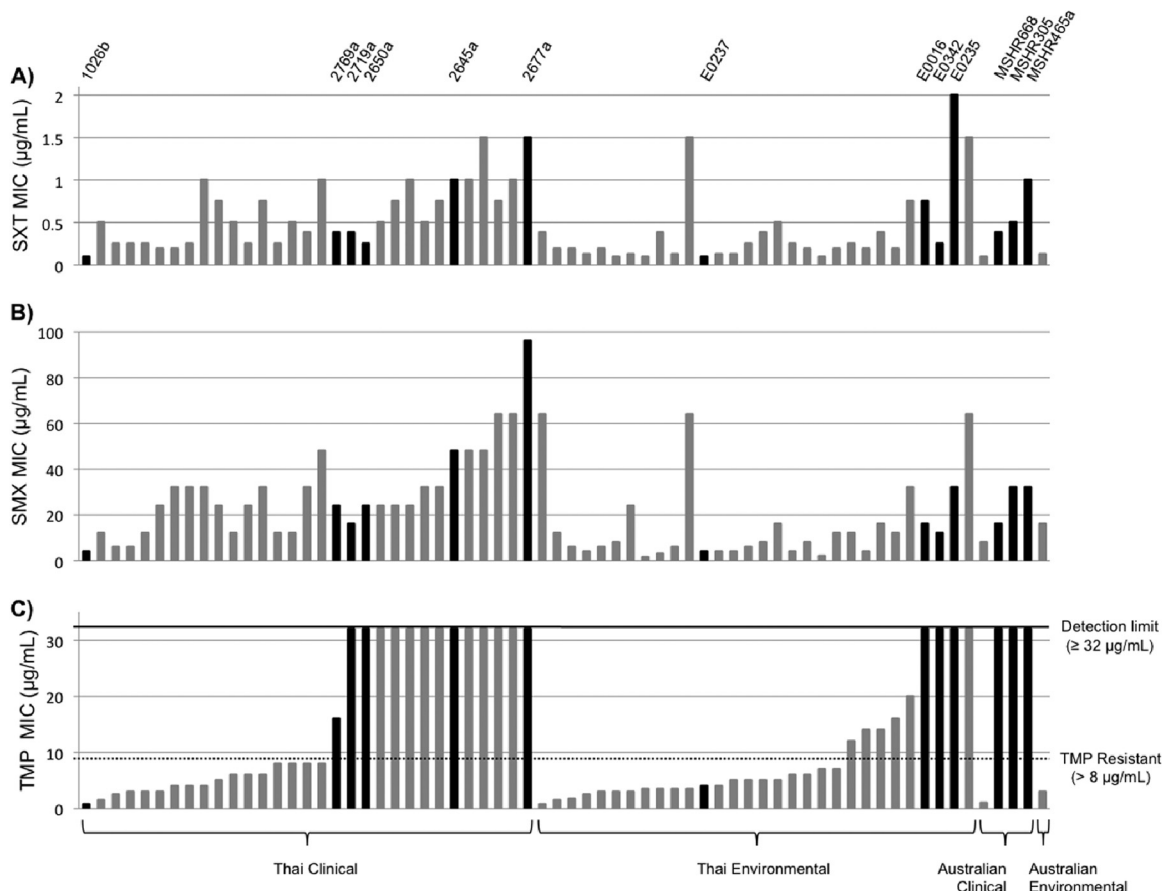


FIG 1 Distribution of trimethoprim, sulfamethoxazole, and trimethoprim-sulfamethoxazole susceptibilities in *B. pseudomallei* clinical and environmental isolates. MICs of the trimethoprim-sulfamethoxazole drug combination (SXT) (A), sulfamethoxazole (SMX) (B), and trimethoprim (TMP) (C) were determined for a collection of 35 clinical and 31 environmental isolates from Thailand and Australia. All 66 isolates tested were susceptible to trimethoprim-sulfamethoxazole (A) and sulfamethoxazole (B). However, isolates in panel C with values above the dotted line are classified as trimethoprim resistant, while others below the dotted line are trimethoprim susceptible. Several isolates from this collection were selected for further studies (black bars), and the names of these 13 strains are indicated at the top of panel A.

Metabolomics facility. Alignments of *folA* sequences from experimental samples and comparison with the 1026b *folA* sequence were performed by using ClustalW2 (23).

Multiplex ISBma2 PCR. Primers P2578 (5'-CCAACGATTCACGTACGC), P2569 (5'-CCGTACAGCACGACCAATC), and P2579 (5'-GACGTTGACCTGGACCTCAC) were designed and used in a multiplex PCR to determine the orientation of ISBma2 in the clinical and environmental strains. P2578, P2569, and P2579 were all added at final concentrations of 0.6 pmol/µl, and standard *Taq* polymerase (New England BioLabs, Waltham, MA) was used. PCR conditions were an initial denaturation step at 95°C for 2 min and 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min, followed by a final extension step at 72°C for 10 min.

Markerless deletion of *bpeEF-oprC*. A 4,314-bp region of the *bpeEF-oprC* operon was deleted in several of the clinical and environmental isolates by allelic exchange using the pEXKm5 vector system and sucrose counterselection, as described previously (24).

Complementation of *bpeEF-oprC* deletions. Genetic complementation was accomplished by utilizing the mini-Tn7 system, which allows for stable and site-specific single-copy insertions into the *B. pseudomallei* genome at three possible *glmS*-associated sites (25). The inducible *E. coli lac* operon *P_{tac}* promoter was used to express the *bpeEF-oprC* operon, which originated from strain 1026b (18). BpeEF-OprC expression in the complemented strains was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Gold Biotechnology, St. Louis, MO) at a final concentration of 1 mM.

RT-qPCR. Expression levels of *bpeF* and *bpeT* mRNAs were analyzed in bacteria grown to mid-log phase ($OD_{600} = 0.6$ to 0.8) in Lennox Luria broth (Mo Bio Laboratories, Carlsbad, CA), at which point *bpeEF-oprC* expression either remained uninduced or was induced for 1 h by the addition of trimethoprim to a final concentration of 32 µg/ml. RNA extraction, cDNA synthesis, and reverse transcription-quantitative PCR (RT-qPCR) were done as previously described (26, 27), except that the RNeasy Protect Bacteria minikit (Qiagen, Valencia, CA) was used for RNA extraction. 23S rRNA was used as the housekeeping control. The primer sets used were Bp23S_F and Bp23S_R for 23S rRNA, *bpeF*-F1_RT and *bpeF*-R1_RT for *bpeF* (26), and *bpeT*_RT_for (P1814) (5'-GAGCTTTCAGGTCAACAACC) and *bpeT*_RT_rev (P1815) (5'-GTGAGTGGAA TTCGAGAG) for *bpeT*.

RESULTS AND DISCUSSION

To evaluate the innate frequency of trimethoprim, sulfamethoxazole, and co-trimoxazole resistance in *B. pseudomallei* clinical and environmental isolates, MICs were determined for a collection of 60 clinical and environmental isolates from northeast Thailand and 5 clinical isolates and 1 environmental isolate from northern Australia. Using the susceptibility criteria described in Materials and Methods, MIC testing of strains from Thailand showed that 47% of clinical strains (14/30 strains) and 30% of environmental strains (9/30) were trimethoprim resistant, with MICs ranging

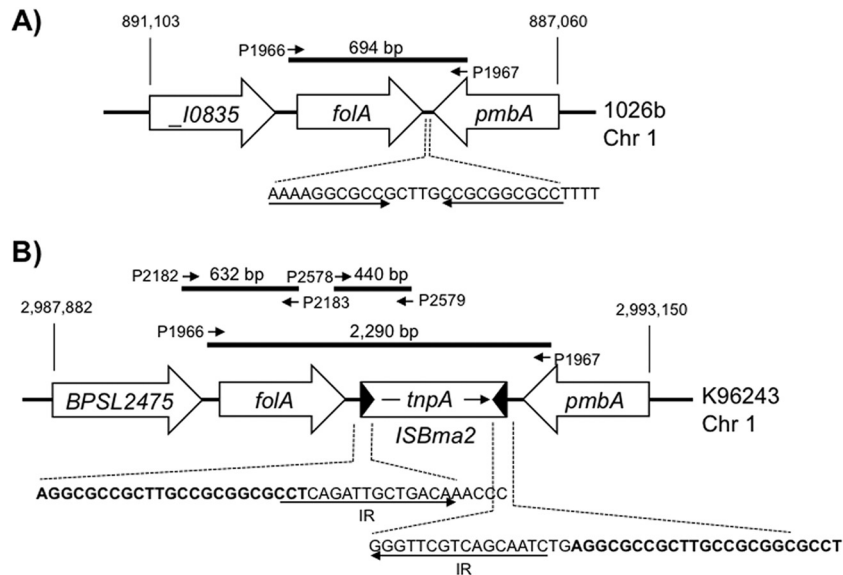


FIG 2 Genomic location and organization of the *folA* region of *B. pseudomallei* in the two sequenced prototypes 1026b (A) and K96243 (B). Chromosome 1 sequence coordinates and gene nomenclatures are taken from the GenBank entries for strains 1026b (accession number NC_017831.1) and K96243 (accession number NC_006350.1). Gene annotations are as follows: *_I0835* (short for *BP1026B_I0835*) and *BPSL2475*, σ 54-dependent transcriptional regulators; *folA*, dihydrofolate reductase; *pmbA*, protein belonging to the peptidase U62 family; *tnpA*, transposase. The same sequences and gene organizations were confirmed for Thai isolates E0016 (1026b-like) and 2650a (K96243-like). Horizontal black lines indicate PCR fragments obtained with the indicated primers. Primers pairs used were P1966 and P1967, which amplify the *folA* gene with and without *ISBma2*, and P2578 and P2579, which amplify a fragment containing the *folA*-*ISBma2* junction sequences. In isolates where *ISBma2* is in the opposite orientation, primer set P2578 and P2579 is replaced by P2578 and P2569, and this combination yields a 647-bp fragment containing the *folA*-*ISBma2* junction sequences (not shown). All primer sequences are given in the text. The *folA* upstream region and 5' coding sequences can be amplified by using primer pair P2182 (5'-CTGTATCGGCTGATGGTGTGTC) and P2183 (5'-AGGCCTTCCTCGTACAGTTG). In panel A, the sequence with underlined inverted repeats indicates the putative *folA* transcriptional terminator. In panel B, sequences that compose the 5' and 3' *ISBma2* inverted repeats (IR) (underlined) and the 23-bp duplicated genomic DNA segments (in boldface type) are indicated. The sequences of *ISBma2* elements and their organization as well as those of the duplicated target DNA segments are consistent with previously reported data on *ISBma2* (28).

from 12 μ g/ml to the detection limit of ≥ 32 μ g/ml (Fig. 1). None of these isolates were resistant to sulfamethoxazole or co-trimoxazole. The frequency of trimethoprim resistance in Australian isolates was 60% (3/5 isolates), and again, these 5 isolates were all susceptible to sulfamethoxazole and co-trimoxazole. These data show that although trimethoprim resistance was highly prevalent in our collection of *B. pseudomallei* isolates, none of the 65 strains tested showed clinically significant co-trimoxazole resistance. We would therefore expect treatment with the standard regimen to still be effective for infections caused by the strains investigated in this study.

Mutations affecting the trimethoprim target dihydrofolate reductase (FolA) can cause resistance to this antibiotic. We selected nine Thai isolates (eight of which are trimethoprim resistant) to compare to the trimethoprim-susceptible 1026b reference strain (20, 21). Two mutations, V77A and A144T, were found in five of the eight trimethoprim-resistant isolates compared to reference strain 1026b. However, these conservative mutations were not linked to trimethoprim resistance in these isolates.

While attempting to PCR amplify the *folA* region in the nine isolates, we noticed that four strains (2665a, 2769a, E0016, and E0342) yielded the same 694-bp DNA fragment as reference strain 1026b (Fig. 2A). The remaining four isolates (2650a, 2677a, 2719a, and E0235), however, showed a substantially larger (≈ 2.3 -kb) fragment when using the same primers. DNA sequencing of this fragment with P1967 revealed the presence of an *ISBma2* insertion sequence (28). Further analyses were conducted by using representative strains E0016 (694-bp fragment) and 2650a (≈ 2.3 -kb

fragment). Employing genomic DNAs as templates and PCR amplification with the subset of primers shown in Fig. 2A for E0016 and Fig. 2B for 2650a followed by sequencing confirmed for E0016 the identical sequence and gene order found in 1026b. In strain 2650a, the 1,596-bp *ISBma2* sequence was inserted into the 130-bp *folA*-*pmbA* intergenic region in a palindromic sequence located between nucleotides 47 and 75 downstream of *folA* that most likely serves as this gene's transcriptional terminator. The insertion site and orientation of *ISBma2* were identical to those found in the genome of strain K96243 (29). In both 1026b and K96243, the *folA* gene and neighboring genes are located on chromosome 1 but in different locations and in reverse order (Fig. 2). The two PCR amplification patterns observed for the isolates analyzed in this study indicate two distinct populations of strains, one similar to 1026b and another similar to K96243. The frequency of the *ISBma2* insertion at this site was determined for the 65 clinical and environmental isolates using PCR amplification of the *folA* region with P1966 and P1967. This analysis showed that 38% of clinical and 45% of environmental isolates contain the *ISBma2* insertion at this locus. Additionally, DNA sequencing of this region in strain MSHR305 and comparison to the whole-genome shotgun sequence (30) revealed that the *ISBma2* element was in the opposite orientation, relative to K96243, and thus can insert at this site in either orientation. For the strains that contained *ISBma2*, a multiplex PCR was used to determine the orientation of the insertion element. There was a near-equal distribution of *ISBma2* orientation, with 52% having the same orientation as K96243, resulting in a 440-bp product, and 48% having the

TABLE 1 Trimethoprim, sulfamethoxazole, and trimethoprim-sulfamethoxazole susceptibilities of selected clinical and environmental isolates with and without the BpeEF-OprC efflux pump^a

Strain	MIC ($\mu\text{g/ml}$)		TMP			
	SXT	SMX	$bpeE^+F^+-oprC^+$		$\Delta(bpeEF-oprC)$, complemented, ^b uninduced	$\Delta(bpeEF-oprC)$, complemented, ^b +1 mM IPTG
	($bpeE^+F^+-oprC^+$)	($bpeE^+F^+-oprC^+$)	$bpeE^+F^+-oprC^+$	$\Delta(bpeEF-oprC)$		
1026b	0.094/1.79	4	0.75	0.5	0.5	6
Thailand Clinical						
2650a	1/19	8	≥ 32	1.5	1.5	≥ 32
2665a	1/19	16	≥ 32	1.5	1.5	16
2677a	1.5/28.5	24	≥ 32	2	2	≥ 32
2719a	1/19	12	≥ 32	1	1	16
2769a	0.75/14.25	12	16	1.5	2	≥ 32
Thailand environmental						
E0016	0.75/14.25	12	≥ 32	1	1.5	16
E0235	1.5/28.5	24	≥ 32	1.5	3	≥ 32
E0237	0.5/9.5	8	4	1	2	16
E0342	0.75/14.25	24	≥ 32	1	1.5	16
Australia clinical						
MSHR305	0.5/9.5	32	≥ 32	2	2	16
MSHR668	0.38/7.22	16	≥ 32	2	2	32
MSHR465a	1/19	32	≥ 32	3	2	≥ 32

^a Abbreviations: TMP, trimethoprim; SMX, sulfamethoxazole; SXT, trimethoprim-sulfamethoxazole.

^b The respective strains containing the $\Delta(bpeEF-oprC)$ mutation have a mini-Tn7T-P_{lac}- $bpeE^+F^+-oprC^+$ element integrated at the chromosomal *glmS2* locus, except for the MSHR668 derivative, which has the same element integrated at *glmS3*. The $bpeE^+F^+-oprC^+$ operon contained on this mini-Tn7 element is derived from prototype strain 1026b (18).

opposite orientation, resulting in a 647-bp product. There was a slight bias in the orientation when environmental isolates were compared to clinical isolates, where 64% of environmental and 38% of clinical isolates were in the same orientation as in strain K96243. However, a significantly larger sample size would be needed to determine the true orientation bias. The location of *ISBma2* most likely does not affect *folA* expression but has implications regarding the design of PCR primers for *folA* amplification for diagnostic purposes.

The BpeEF-OprC efflux pump is encoded by the *bpeEF-oprC* genes, which are the distal genes of the *llpE-bpeEF-oprC* operon located on *B. pseudomallei* chromosome 2 (26, 29). Expression of this operon is governed by the LysR-type regulator BpeT, which is encoded by a gene located immediately upstream of the *llpE-bpeEF-oprC* operon. BpeEF-OprC has been shown to efflux trimethoprim when expressed in *Pseudomonas aeruginosa* (18), in *Burkholderia thailandensis* isolates that are resistant to chloramphenicol (19), and in *B. pseudomallei* isolates that express BpeEF-OprC as a result of a BpeT truncation (21). To investigate a potential contribution of this efflux pump to trimethoprim resistance in clinical and environmental *B. pseudomallei* isolates, a 4,314-bp segment of the *llpE-bpeEF-oprC* operon was deleted from 11 trimethoprim-resistant isolates and trimethoprim-susceptible reference strains 1026b and E0237. Deletion of *bpeEF-oprC* from the trimethoprim-resistant strains resulted in susceptible mutant derivatives with at least 10-fold decreases in MIC. A lower trimethoprim MIC was observed for E0237, but the susceptibility of 1026b remained unchanged (Table 1). No significant trimethoprim susceptibility changes were observed in uninduced

complemented strains, but induction of BpeEF-OprC expression resulted in significant MIC increases such that all of the isolates, except for 1026b, became trimethoprim resistant (Table 1). Similar results were observed with strains containing deleted and complemented *bpeT*, respectively (data not shown). These results suggest that expression of the BpeEF-OprC pump is required for trimethoprim resistance in these isolates.

To assess whether mutations in structural pump components were contributing to trimethoprim resistance, we sequenced the *bpeT-llpE-bpeE* and *oprC* regions in nine Thai isolates and the *bpeF* gene in three of those isolates using a primer-walking strategy. These analyses showed that the DNA sequence of the entire region was highly conserved, with few synonymous and conservative mutations. For example, compared to the 1026b sequence, the following mutations were found in $\leq 22\%$ of the sequenced trimethoprim-resistant isolates: A401T in BpeE and V78A, A207Q, and T508A in OprC. There were only a few conservative mutations in the *bpeT* gene and no mutations in the *bpeT-llpE* intergenic region containing the putative promoter regions for *bpeT* and the *llpE-bpeEF-oprC* operon. Given the conservative nature of the observed mutations and the results of the complementation study which showed that the 1026b operon sequence was sufficient for the trimethoprim-resistant phenotype, it is unlikely that changes in structural BpeEF-OprC components are root causes for resistance in any of the tested isolates.

To gauge expression levels of the BpeT regulator and BpeEF-OprC, RT-qPCR was used to determine the relative *bpeT* and *bpeF* mRNA expression levels. Interestingly, in uninduced cells, *bpeF* mRNA levels were very similar between the nine Thai isolates

tested and 1026b. However, when induced with 32 µg/ml trimethoprim, isolates with trimethoprim MICs of ≥32 µg/ml had increased *bpeF* mRNA levels, with fold increases ranging from 3.2 to 6.6. In contrast, two isolates, E0237 and 2769a, with respective MICs of 4 µg/ml and 16 µg/ml, and 1026b exhibited no increase in *bpeF* expression levels when induced with 32 µg/ml of trimethoprim. As this high concentration of trimethoprim may adversely affect strains with MICs lower than 32 µg/ml, the experiment was repeated by performing induction with trimethoprim at one-half the respective MICs for 1026b, 2769a, and E0237 for 1 h. However, even when using one-half the MICs of trimethoprim for induction, no increases in *bpeF* expression levels were observed. These data suggest that high-level trimethoprim resistance results from overexpression of the BpeEF-OprC efflux pump. Repetition of these experiments for *bpeT* showed no changes in regulatory gene expression both with and without trimethoprim induction. Since DNA sequencing revealed only conservative mutations in *bpeT* and RT-qPCR analysis indicated no changes in expression levels of this regulator, we conclude that BpeEF-OprC expression in the trimethoprim-resistant clinical and environmental isolates tested is governed by a to-date-undefined regulatory mechanism(s).

This is the first study aimed at elucidation of the molecular mechanisms governing trimethoprim resistance in clinical and environmental *B. pseudomallei* isolates. The results show that resistance to trimethoprim alone is frequent in *B. pseudomallei* strains. However, resistance to sulfamethoxazole and co-trimoxazole was not detected in any of these clinical and environmental isolates tested. In all of the Australian and Thai isolates assessed, trimethoprim resistance was attributed to expression of BpeEF-OprC but not changes in the dihydrofolate reductase target, indicating that efflux is the predominant trimethoprim resistance mechanism in *B. pseudomallei*. Increased transcription of the *bpeEF-oprC* structural genes in the presence of trimethoprim led to high-level resistance, with MICs above the detection limit of 32 µg/ml. The regulatory mechanism(s) governing BpeEF-OprC expression in these isolates remains unknown. In the course of these studies, we discovered that the genomic location and organization of genes in the immediate *folA* region follow a distinct pattern that, in reference to three sequenced prototypes, allows grouping of strains into K96243-like, MSHR305-like, and 1026b-like strains.

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