Supplemental Material

Conservation of resistance nodulation cell division efflux pump mediated antibiotic resistance in *Burkholderia cepacia* complex and *Burkholderia pseudomallei* complex species

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METHODS

Media and growth conditions

Lennox lysogeny broth (LB) containing 5 g/l NaCl (Fisher BioReagents™, Global Chemicals Thermo Fisher Scientific, Pittsburgh, PA) was used for routine growth of bacteria and cation-adjusted Mueller Hinton II broth (cMHB) (Becton Dickinson and Company, Sparks, MD) was employed for assessment of resistance gene induction by antibiotics. Cation-adjusted Mueller Hinton II agar (cMHA) (Becton Dickinson and Company) was used for minimal inhibitory concentration (MIC) assays using Etest® strips.

Antimicrobial susceptibility testing

MIC assays were performed using Etest® strips and following manufacturer's (AB Biomérieux, Marcy l'Etoile, France) guidelines.

R**everse-transcription quantitative PCR (RT-qPCR)**

Expression levels of mRNAs of target genes, specifically *bpeF*, were determined in *B. latens* strains as previously described for *B. ubonensis* (1). Briefly, bacteria were grown at 37°C in LB Lennox Broth medium to mid-log phase (OD_{600nm} = 0.6 to 0.8). Total RNA was extracted using the RNeasy Protect Bacteria mini kit (Qiagen, Valencia, CA) and cDNA synthesis was performed as previously described, employing 23SRNA as the housekeeping control (2, 3). The primer sets used were Bp23S_F (5'-GTAGACCCGAAACCAGGTGA) and Bp23S_R (5'-CACCCCTATCCACAGCTCAT) previously designed for the *B. pseudomallei* 23S rRNA housekeeping control (2), and P3533 (5'-TCGGAATATCCGGAAGTCGT) and P3534 (5'- GTCCTCGACGCCGTTGATCT) for $bpeF_{BI}$. GraphPad Prism (GraphPad Software, La Jolla, CA) was used for data analysis and presentation.

RNA-seq

Expression levels of *B. latens* mRNAs were also assessed using RNA-seq. Three biological replicates were grown in cMHB for ~18 hours at 37°C with shaking. Fresh cMHB broth (5 mL) was inoculated with 50-100 μl of the overnight culture and incubated at 37°C while shaking until an OD_{600nm} of 1.0 was reached. Cells were then either treated with antibiotic or left untreated, and then incubated for another two hours. One ml of culture was pelleted at 10,000x*g* for 5 min and the supernatant was removed. The pellet was re-suspended in 200 μl of fresh lysis buffer (20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2% Triton-X100, 10 mg/mL lysozyme), flash frozen in liquid nitrogen, and stored at -80°C until used for RNA extractions. RNA was isolated using the QIAGEN RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. DNA was depleted using the Ambion ThermoFisher DNA-free DNA Removal kit (ThermoFisher Scientific, Waltham, MA). The initial RNA and DNA depleted RNA concentration and integrity was accessed using Invitrogen ThermoFisher QuBit RNA BR and with Agilent Fragment Analyzer using the DNF-471 RNA Kit (15 nt) (Agilent, Santa Clara, CA) respectively. All subsequent RNA products had concentration and integrity accessed using Invitrogen ThermoFisher QuBit RNA HS and with Agilent Fragment Analyzer using the DNF-472 HS RNA Kit (15 nt) respectively. Two rounds of ribosomal RNA depletion were performed on all RNA samples using siTOOLs BIOTECH riboPool5 for Pan Prokaryote (Universal Microbe) 003 (siTOOLs BIOTECH, Planegg, Germany) following the manufacturer's recommendations. The Zymo RNA Clean and Concentrator-5 was used to deplete 5S ribosomal RNA (Zymo, Irvine, CA) and manufacturer's recommendations were followed except that flow-through was discarded to remove the smaller rRNAs (<200 nucleotides). RNA libraries were prepared using Illumina TruSeq® Stranded mRNA Library Prep Kit (Illumina, San Diego, CA) following the manufacturer's recommendations. Final libraries were quantified using KAPA Library Quantification Kit (ROX Low) (Roche KAPA Biosystems, Wilmington, DE), and then pooled in

equimolar concentrations. Whole transcriptome sequencing was performed using Illumina NextSeq 550 platform with the 500/550 High Output KT v2.3 (300 cycles) and 2x150 bp read lengths. For completed reference genomes, coding region sequences (CDSs) were pulled from Prokka v1.14.6 annotations (4). RNA-seq reads were aligned against CDSs with Kallisto v0.45.0 (5, 6) and processed with DE-Seq v1.30.0 (7), which also normalized read counts.

Data availability

Bioproject accession PRJNA717300 (www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA717300) provides links to all unpublished Sequence Read Archive data generated in this study.

Table S1. Efflux pump regulatory and structural genes and locus tags

	Regulator	Pump			Regulator	Pump			Regulators		Pump		
	AmrR	**AmrAB-OprA**			**BpeR**	**BpeAB-OprB**			**BpeT**	**BpeS**	**BpeEF-OprC**		
***Burkholderia pseudomallei* K96243**[1]													
Gene name	*amrR*	*amrA*	*amrB*	*oprA*	*bpeR*	*bpeA*	*bpeB*	*oprB*	*bpeT*	*bpeS*	*bpeE*	*bpeF*	*oprC*
Locus tag	BPSL1805	BPSL1804	BPSL1803	BPSL1802	BPSL0812	BPSL0814	BPSL0815	BPSL0816	BPSS0290	BPSL0731	BPSS0292	BPSS0293	BPSS0294
Chromosome	1	1	1	1	1	1	1	1	2	1	2	2	2
***Burkholderia thailandensis* E264**													
Gene name	*amrR*	*amrA*	*amrB*	*oprA*	*bpeR*	*bpeA*	*bpeB*	*oprB*	*bpeT*	*bpeS*	*bpeE*	*bpeF*	*oprC*
Locus tag	BTH_I2446	BTH_I2445	BTH_I2444	BTH_I2443	BTH_I0679	BTH_I0680	BTH_I0681	BTH_I0682	BTH_II2108	BTH_I0632	BTH_II2106	BTH_II2105	BTH_II2104
Chromosome	1	1	1	1	1	1	1	1	2	1	2	2	2
***Burkholderia ubonensis* Bp8955**[2]													
Gene name	*amrR*	*amrA*	*amrB*	*oprA*	*bpeR*	*bpeA*	*bpeB*	*oprB*	*bpeT*	*bpeS*	*bpeE*	*bpeF*	*oprC*
Locus tag	CJO66_ RS00480	CJO66_ RS00475	CJO66_ RS00470	CJO66_ RS00465	CJO66_ RS9860	CJO66_ RS09870	CJO66_ RS09875	CJO66_ RS09880	CJO66_ RS28035	CJO66_ RS09640	CJO66_ RS28025	CJO66_ RS28020	CJO66_ 28015
Chromosome[3]	1	1	1	1	1	1	1	1	2	1	2	2	2
***Burkholderia cenocepacia* J2315**[4]													
Gene name	*amrR**[5]	*amrA*	*amrB*	*oprA*	*bpeR*	*bpeA*	*bpeB*	*oprB*	*bpeT*	*bpeS*	*bpeE*	*bpeF*	*oprC*
Alternative gene name[6]	N/A[7]	RND-3 MFP	RND-3 TP	RND-3 OMP	N/A	RND-4 MFP	RND-4 TP	RND-4 OMP *oprM*	*ceoR*	N/A	*ceoA* RND-10 MFP	*ceoB* RND-10 TP	*opcM*
Locus tag	BCAL1672	BCAL1674	BCAL1675	BCAL1676	BCAL2823	BCAL2822	BCAL2821	BCAL2820	BCAM2554	BCAL0946	BCAM2551	BCAM2550	BCAM2549
Chromosome	1	1	1	1	1	1	1	1	2	1	2	2	2
***Burkholderia multivorans* BAA-247**													
Gene name	*amrR*	*amrA*	*amrB*	*oprA*	*bpeR*	*bpeA*	*bpeB*	*oprB*	*bpeT*	*bpeS*	*bpeE*	*bpeF*	*oprC*
Locus tag	NP80_ RS20925	NP80_ RS20930	NP80_ RS20935	NP80_ RS20940	NP80_ RS26110	NP80_ RS26105	NP80_ RS26100	NP80_ RS26095	NP80_ RS12210	NP80_ RS26315	NP80_ RS12200	NP80_ RS12195	NP80_ RS12190
Chromosome	1	1	1	1	1	1	1	1	2	1	2	2	2
***Burkholderia latens* AU17928**													
Gene name	*amrR*	*amrA*	*amrB*	*oprA*	*bpeR*	*bpeA*	*bpeB*	*oprB*	*bpeT*	*bpeS*	*bpeE*	*bpeF*	*oprC*
Locus tag	WK25_ RS07990	WK25_ RS07995	WK25_ RS08000	WK25_ RS08005	WK25_ RS12665	WK25_ RS12660	WK25_ RS12655	WK25_ RS12650	WK25_ RS28540	WK25_ RS12910	WK25_ RS28530	WK25_ RS28525	WK25_ RS28520
Chromosome	1	1	1	1	1	1	1	1	2	1	2	2	2

[1]Assembly accession numbers are: *B. pseudomallei* K96243 GCF_000011545.1; *B. thailandensis* E264 GCF_000012365.1; *B. ubonensis* Bp8955 GCF_002276145.1; *B. cenocepacia* J2315 GCF_000009485.1; *B. multivorans* BAA-247 GCF_000959525.1; and *B. latens* AU17928 GCF_001718795.1.

[2]Also known as Bu278 (1).

[3]Chromosome locations for genes in *B. ubonensis* Bp8955 are not yet known, but due to overall conservation in *Burkholderia* species are likely as shown.

[4]J2315 has two *bpeAB-oprB* gene clusters allocated, one on chromosome 1 and one on chromosome 3 (see **Fig. S3**). Only the most conserved and experimentally supported cluster is shown in this table.

[5]*amrR**, pseudogene encoding a truncated non-functional AmrR.

[6]Sources: *Burkholderia* Genome Database version 9.1 (www.burkholderia.com) (8).

[7]Abbreviations: N/A, not applicable; MFP, membrane fusion protein; OMP, outer membrane protein; TP, transporter protein.

Table S2. Identification of AmrAB-OprA and induction of its expression by chloramphenicol in *B. latens*

Cells of the indicated strains were grown at 37°C in cMHB to an OD_{600nm} of ~1.0. Cultures then were either supplemented with 16 μg/ml chloramphenicol (+CHL) or remained untreated (-CHL). After incubation for an additional 2 hours (OD_{600nm} of ~1.2) cells were pelleted and stored frozen until RNA isolation.

Transporter component annotation		**Normalized read count**[1]		
		***B. latens* (susceptible)**[2]	***B. latens* (resistant)**[2]	
Original	**Revised**	**AU2934**[3] -CHL	**AU0505** -CHL	**AU0505** + 16 μg/ml CHL
MDR[4] protein MexA	AmrA	2,702.67 ± 576.72	4,583.67 ± 531.17	16,899.00 ± 3,213.41
MD[5] efflux pump subunit AcrB	AmrB	4,741.00 ± 615.38	7,879.67 ± 769.40	24,612.33 ± 4,281.46

[1]The ± indicate standard deviations from the mean of three technical replicates.
[2]Susceptible or resistant to chloramphenicol (CHL), ciprofloxacin (CIP), trimethoprim (TMP), and TMP + sulfamethoxazole (SXT). Strains AU0505 and AU2934 were isolated from sputum of cystic fibrosis patients.
[3]AU2934 was not induced with CHL because it does not grow in the presence of the 16 μg/ml CHL used for induction in the resistant strain AU0505.
[4]MDR, multidrug resistance.
[5]MD, multidrug.

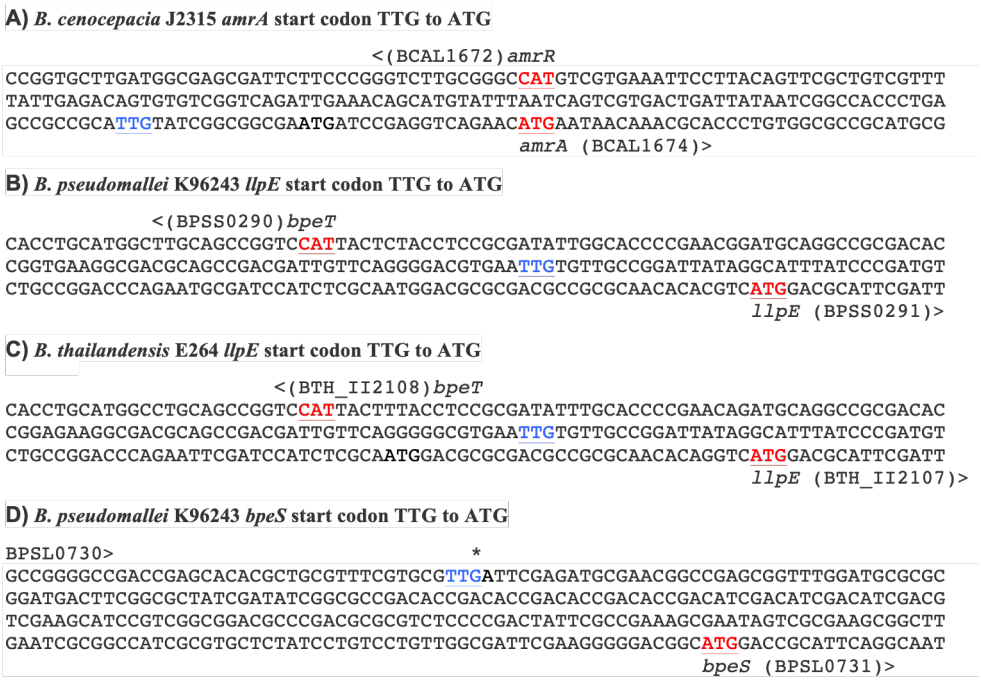

Figure S1. Revised translation start sites for operons encoding efflux pump components and transcriptional regulatory genes. Shown are the upstream regions of: (**A**) the *amrA* gene of the *amrAB-oprA* operon of *B. cenocepacia* J2315. Note that *amrR* is a frame-shifted pseudogene in J2315 (see **Fig. 1A** in main text); (**B**) and **(C)** the *llpE* genes of the *llpE-bpeE-bpeF-oprC* operons of *B. pseudomallei* K96243 and *B. thailandensis* E264; and (**D**) the upstream region of the *bpeS* regulatory gene of *B. pseudomallei* K96243. Originally annotated start codons are from the *Burkholderia* Genome Database version 9.1 (www.burkholderia.com) and are indicated in blue type. The currently annotated TTG start of *bpeS* from *B. pseudomallei* K96243 overlaps with the TGA stop codon (asterisk) of BPSL0730 (**D**). Revised start codons are indicated in red type. Revised start sites were derived after performing DNA sequence alignments with the corresponding genes from other *Burkholderia* species (see **Figs. 1A to C** in main text). CLUSTAL multiple sequence alignment by MUSCLE (3.8) were performed online on the EMBL-EBI server (https://www.ebi.ac.uk/Tools/msa/clustalo/).

A)

Bp K96243	100.00	95.22	72.09	72.40	72.56	72.65	
Bt E264	95.22	100.00	72.73	74.16	73.68	73.68	
Bu Bp8955	72.09	72.73	100.00	90.23	89.77	87.91	**AmrR**
Bm BAA-247	72.40	74.16	90.23	100.00	91.16	87.33	
Bc K56-2	72.56	73.68	89.77	91.16	100.00	92.09	
Bl AU17928	72.65	73.68	87.91	87.33	92.09	100.00	

Bp K96243	100.00	98.58	81.99	80.09	81.52	81.04	
Bt E264	98.58	100.00	81.99	80.09	81.52	81.04	
Bc J2315	81.99	81.99	100.00	94.81	92.45	93.87	**BpeR**
Bl AU17928	80.09	80.09	94.81	100.00	91.98	92.45	
Bu Bp8955	81.52	81.52	92.45	91.98	100.00	93.40	
Bm BAA-247	81.04	81.04	93.87	92.45	93.40	100.00	

Bp K96243	100.00	95.06	85.19	81.99	83.95	84.57	
Bt E264	95.06	100.00	83.64	81.68	83.33	84.88	
Bu Bp8955	85.19	83.64	100.00	82.30	86.11	85.49	**BpeS**
Bm BAA-247	81.99	81.68	82.30	100.00	86.34	86.34	
Bc J2315	83.95	83.33	86.11	86.34	100.00	93.52	
Bl AU17928	84.57	84.88	85.49	86.34	93.52	100.00	

Bp K96243	100.00	97.90	91.32	91.02	91.32	93.41	
Bt E264	97.90	100.00	90.72	91.02	90.42	92.81	
Bu Bp8955	91.32	90.72	100.00	93.11	94.01	94.01	**BpeT**
Bc J2315	91.02	91.02	93.11	100.00	93.71	95.21	
Bl AU17928	91.32	90.42	94.01	93.71	100.00	95.81	
Bm BAA-247	93.41	92.81	94.01	95.21	95.81	100.00	

B)

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                                                                HTH
                                                          Probability
Bp K96243   MARKTREESLNTKNRILDAAELVLLEKGVGQTAMADIAEAAGMSRGAVYGHFNGKIEVCV    100%
Bt E264     MARKTREESLNTKNRILDAAELVLLERGVGQTAMADIAEAAGMSRGAVYGHFKGKIEVCV    100%
Bu Bp8955   MARKTREESLAIKHRILDAAELVLLEKGVAQTAMADLAEAAGMSRGAVYGHYKNKMEVCL     90%   AmrR
Bm BAA-247  MARKTREESLAIKHRILDAAELVLLDKGVAQTAMADLAEAAGMSRGAVYGHYRNKMEVCL    100%
Bc K56-2    MARKTREESLAIKHRILDAAELVLLERGVAQTAMADLAEAAGMSRGAVYGHYRNKMEVCL    100%
Bl AU17928  MARKTREESLAIKHRILDAAELVPLEKGVAQTAMADLAEAAGMSRGAVYGHYRNKMEVCL    100%
            **********  *:********* *:.**.******:**************:..*:***:

Bp K96243   MARRTKEEALATRDRILDAAEHVFFEKGVSHTSLADIAQHAGVTRGAIYWHFASKSELFD    100%
Bt E264     MARRTKEEALATRDRILDAAEHVFFEKGVSHTSLADIAQHAGVTRGAIYWHFASKSELFD    100%
Bc J2315    MVRRTKEEALETRNRILDAAEHVFFEKGVSHTSLADIAQHAGVTRGAIYWHFANKSELFD    100%   BpeR
Bl AU17928  MVRRTKEEALETRNRILDAAEHVFFEKGVSHTSLADIAQHAGVTRGAIYWHFANKSELFD    100%
Bu Bp8955   MVRRTKEEALATRNGILDAAEHVFFEKGVSHTSLADIAHHAGVTRGAIYWHFANKSELFD    100%
Bm BAA-247  MVRRTKEEALETRNRILDAAEHVFFEKGVSHTSLADIAQHAGVTRGAIYWHFANKSDLFD    100%
            *.******** **: ************************:**************.**:***

Bp K96243   MDRIQAMEVFTRVVDANSFTRAAETLGMPRASVTTIIQNLEALLGVRLMHRTTRRLSLTP     50%
Bt E264     MDRIQAMEVFTRVVEANSFTRAAETLGMPRASVTTIIQNLEALLGARLMHRTTRRLSLTP     50%
Bu Bp8955   MDRIQAMEVFTRVVDANSFTRAADTLTMPRASVTTIIQNLEALLGVRLLHRTTRRLSLTP      NS   BpeS
Bm BAA-247  MDRIQAMEVFTRVVDANSFTRAAETLAMPRASVTTIIQNLEALLGVRLMHRTTRRLSLTP      NS
Bc J2315    MDRIQAMEVFTRVVDANSFTRAADTLAMPRASVTTIIQNLEALLGVRLMHRTTRRLSLTP      NS
Bl AU17928  MDRIQAMEVFTRVVDANSFTRAAETLAMPRASVTTIIQNLEALLGVRLMHRTTRRLSLTP      NS
            **************:********:** ******************.**:***********

Bp K96243   MDRLQAMQVFTRVVDTNSFTKAAETLGLPRASVTTIIQNLEAFLGVRLMHRTTRRLSLTP     50%
Bt E264     MDRLQAMQVFTRVVDTNSFTKAAETLGLPRASVTTIIQNLEAFLGVRLMHRTTRRLSLTP     50%
Bu Bp8955   MDRLQAMQVFTRVVDTSSFTKAAETLGLPRASVTTIIQNLEAFLGVRLMHRTTRRLSLTP     50%   BpeT
Bc J2315    MDRLQAMQVFTRVVDTSSFTKAAETLSLPRASVTTIIQNLEAFLGVRLMHRTTRRLSLTP      NS
Bl AU17928  MDRLQAMQVFTRVVDTSSFTKAAETLGLPRASVTTIIQNLEAFLGVRLMHRTTRRLSLTP     50%
Bm BAA-247  MDRLQAMQVFTRVVDTSSFTKAAETLGLPRASVTTIIQNLEAFLGVRLMHRTTRRLSLTP     50%
            ****************.*********.**********************************
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Figure S2 (previous page). Conservation of transcriptional efflux pump regulators in Bcc and Bpc species. The analysis included the two TetR family regulators AmrR and BpeR, and the LysR transcriptional regulator (LTTR) family regulators BpeT and BpeS (see **Fig. 1** in main text and **Fig. S4**). (**A**) **Percent identity matrices.** CLUSTAL multiple sequence alignment by MUSCLE (3.8) were performed online on the EMBL-EBI server (https://www.ebi.ac.uk/Tools/msa/muscle/). After alignment, identity matrices were created by Clustal 2.1 integral to MUSCLE. (**B**) **Helix-turn-helix (HTH) DNA-binding domains.** Potential HTH DNA-binding domains (highlighted in yellow) and their probabilities were predicted using the Rhone-Alpes Bioinformatic Pole Gerland Site (https://npsa-prabi.ibcp.fr). HTH probability scores for BpeS and BpeT LTTRs are significantly lower or not significant (NS) than those observed with AmrR and BpeR. Data obtained with *B. pseudomallei* BpeT and BpeS support the functionality of the predicted HTH domains in this bacterium, even though the HTH probability in both proteins is only 50% (9, 10). Data used for analyses shown in (**A**) and (**B**) were from the *Burkholderia* Genome Database version 9.1 (www.burkholderia.com).

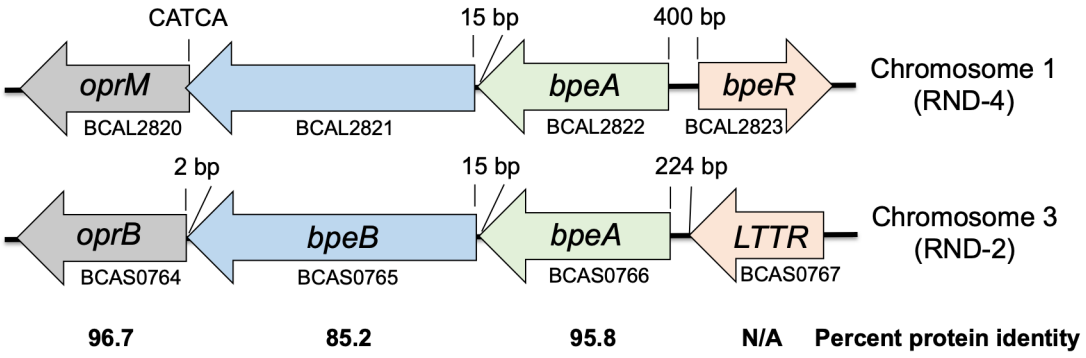

Figure S3. Two related *bpeAB-oprA* loci annotated in *Burkholderia* cenocepacia J2315. Although the proteins that constitute the pump structural components of the two annotated efflux pumps are very closely related (85 to 97% identity), the pumps are clearly different. For instance, in all Bcc and Bpc species analyzed the *bpeAB-oprB* operon is located on chromosome 1 (**Fig. 1B** in main text). Its expression is regulated by the TetR-family transcriptional regulator, BpeR, whose structural gene is located adjacent to and transcribed divergently from the *bpeAB-oprB* operon. In contrast, the annotated *bpeAB-oprB* operon in *B. cenocepacia* J2315 is located on chromosome 3. Its expression may be regulated by a LysR-type transcriptional regulator (LTTR). This LTTR is encoded by a gene that is located upstream and transcribed in the same direction as the *bpeAB-oprB* operon. RND-2 and RND-4 are alternate descriptors of the efflux pumps encoded by BCAS0764-0766 and BCAL2820-2822. Original data used for these graphs were from the *Burkholderia* Genome Database version 9.1 (www.burkholderia.com).

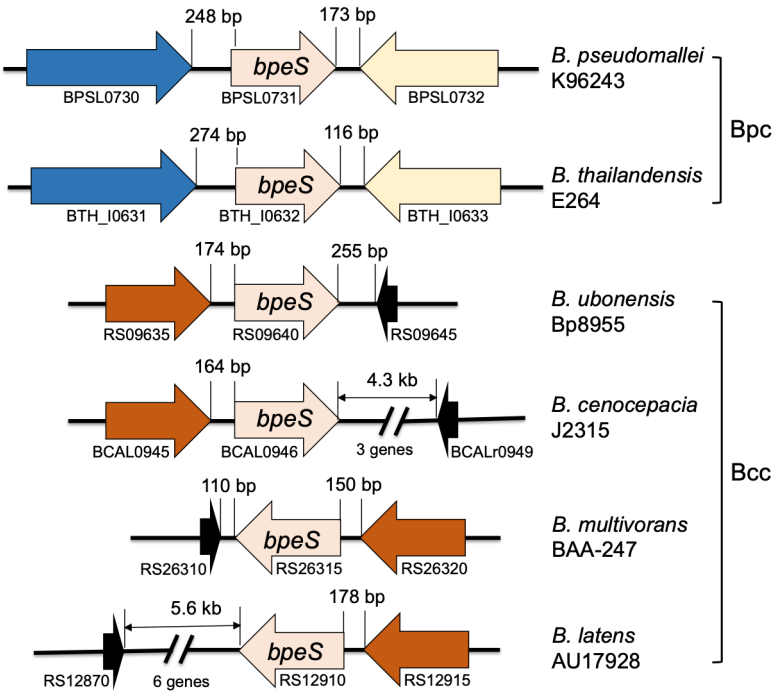

Figure S4. Genetic maps of the chromosome 1 region of *Burkholderia* Bpc and Bcc species containing *bpeS*, a LysR-type transcriptional regulator of the BpeEF-OprC efflux pump. Putative *bpeS* genes were identified as described in the **Figure S2** legend using the known and partially characterized *B. pseudomallei* BpeS (9, 10) as query. In Bpc the *bpeS* gene is flanked upstream by a gene that is predicted to encode a peptidase/amidase (blue arrows) and downstream by a gene predicted to code for a histidine sensor kinase (yellow arrows). In Bcc, the predicted *bpeS* gene is flanked upstream by a gene that is either annotated as AraC-family (*B. cenocepacia* and *B. multivorans*) or GlxA-family (*B. latens* and *B. ubonensis*) transcriptional regulators (brown arrows). GlxA transcriptional regulators are frequently noted as AraC-family regulators due to high similarity. (The AraC- and GlxA-family proteins shown here are between 84.8 and 91.9% identical.) A common feature downstream of Bcc *bpeS* is a tRNA locus (black arrows) annotated as tRNA-Met in *B. cenocepacia* J2315. In *B. ubonensis* Bp8955 and *B. multivorans* BAA-247 this tRNA locus is located adjacent to *bpeS*. However, in *B. cenocepacia* J2315 and *B. latens* AU17928 the tRNA locus is located 4.3 kb and 5.6 kb, respectively, from *bpeS*. These intervals contain several (3 and 6, respectively) genes and/or pseudogenes coding for diverse unrelated functions. Some of these genes that are annotated as putative plasmid related recombination enzyme and putative phage related integrase (*B. cenocepacia* J2315) or integrase and IS3 family transposase (*B. latens* AU17928) hint at past integration events downstream of *bpeS*, perhaps phage integration at the tRNA locus. Distances between genes are indicated in base pairs (bp) or kilo base pairs (kb). Partial locus tags (numbers prefixed by RS) are provided for *B. ubonensis*, *B. multivorans* and *B. latens*; complete locus tags for these species are prefixed with CJO66_, NP80_ and WK25_, respectively (see **Table S1**). Original data used for these graphs are from the *Burkholderia* Genome Database version 9.1 (www.burkholderia.com). *B. ubonensis* strain Bp8955 is also known as Bu278 (1). **Note:** While this paper was under review, a manuscript was posted on the *Antimicrobial Agents and Chemotherapy* journal website under *AAC Accepted Manuscripts* that showed a schematic representation of the chromosome 1 location of *bpeS* from *B. multivorans* BAA-247 using NCBI accession tags and annotation (11).

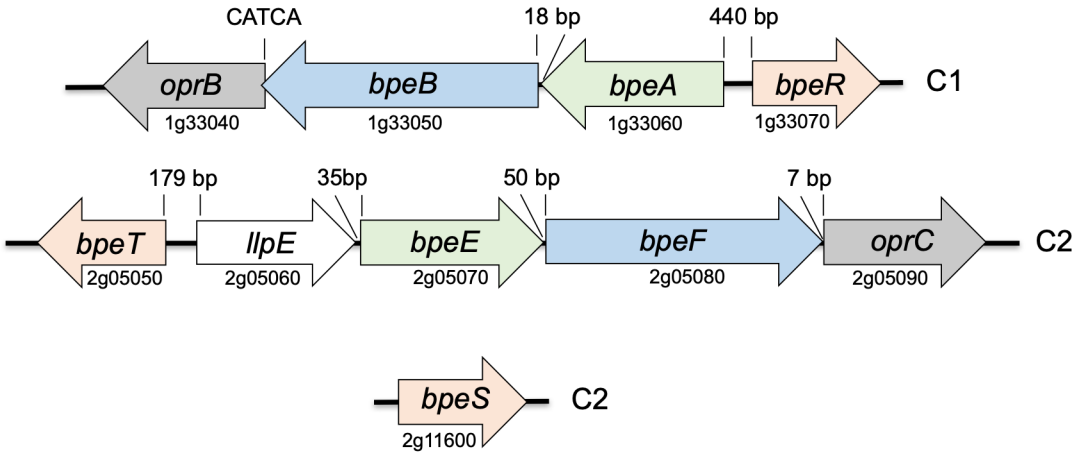

Figure S5. BpeEF-OprB and BpeEF-OprC encoding genetic loci *Burkholderia gladioli* BSR3. DIAMONDP BLAST searches did not reveal AmrAB-OprA encoding loci in whole genome sequences of several *B. gladioli* species. However, such searches and additional parameters such as characteristics of associated regulatory proteins revealed BpeAB-OprB and BpeEF-OprC encoding loci and genes coding for associated regulatory proteins. As in Bcc and Bpc species, *bpeAB-oprB* and *bpeEF-oprC* and associated *bpeR* and *bpeT* are located on chromosome 1 (C1) and 2 (C2), respectively. As in all *Burkholderia* analyzed to date, the *bpeEF-oprC* genes are co-transcribed with *llpE*, which encodes a putative lipase/esterase of unknown function. *B. gladioli* also has a putative *bpeS* regulatory gene locus, although it is located on chromosome 2 and in a different genetic context than in Bcc and Bpc. Letters and numbers below genes indicate abbreviated locus tags (complete locus tags for 1lg33040 and 2g05050 are bgla_1g33040 and bgla_2g05050, respectively). Original data used for these graphs were from the *Burkholderia* Genome Database version 9.1 (www.burkholderia.com).

A)

	MIC (μg/ml)[1,2]			
B. latens	**CHL**	**CIP**	**TMP**	**SXT**
AU17928	32	4	>32	8/152
AU0505	64	4	>32	8/152
AU2934	<4	2	4	<1/19

[1]CHL, chloramphenicol; CIP, ciprofloxacin; TMP, trimethoprim; SXT, trimethoprim+sulfamethoxazole
[2]Resistance cutoffs (μg/ml): CHL, ≥32 (resistant, R); CIP, 2 (I) ≥4 (R); TMP, 4 (S), ≥16 (R); SXT, <1/19 (S), ≥4/76 (R).

B)

Transporter component annotation		Normalized read count[1]	
Original	**Revised**	***B. latens* AU2934 (susceptible)[2]**	***B. latens* AU0505 (resistant)[2]**
MDR[3] resistance protein MtdE	BpeE	33.33 ± 0.94	1,264.67 ± 186.73
MDR efflux RND transporter OqxB7	BpeF	113.33 ± 17.17	4,429.67 ± 700.24

[1]The ± indicate standard deviations from the mean of three technical replicates.
[2]Susceptible or resistant to CHL, CIP, TMP, and SXT (see panel **A**).
[3]MDR, multidrug resistance.

C)

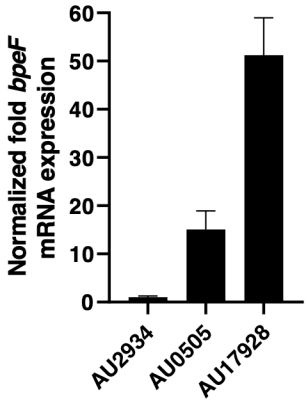

Figure S6. Demonstration of BpeEF-OprC expression in *B. latens*. (**A**) **Multidrug resistance due to BpeEF-OprC substrates.** Using Etest, susceptibility profiles for CHL, CIP, TMP, and SXT were established for a susceptible strain (AU2934) and for a resistant strain (AU0505) (both of these strains are cystic fibrosis patient sputum isolates). For comparative purposes strain AU17928 (cystic fibrosis patient maxillary sinus isolate) was included in our studies. It exhibited the same resistance pattern as AU0505 indicating that the strain may express the BpeEF-OprC efflux pump. (**B**) and (**C**) **MDR resistant strains constitutively express BpeEF-OprC.** In panel (**B**), RNA-seq analysis was performed on mRNA isolated from cells of susceptible strain AU2934 and resistant strain AU0505 grown in cMHB in the absence of antibiotics. RNA-seq identified two RND efflux pump components annotated as MDR resistance protein MdtE and MDR transporter OqxB7. Closer analysis showed that the proteins correspond to BpeE and BpeF, the membrane fusion protein and RND transporter components of BpeEF-OprC. Substantially higher normalized read counts observed with the resistant strain AU0505 when compared to the susceptible strain AU2934 show that BpeEF-OprC is expressed in the resistant strain but not in the susceptible strain. BpeEF-OprC expression in AU0505 is constitutive since the RNA was isolated from cells grown in the absence of antibiotics that induce expression, e.g. CML and TMP in *B. pseudomallei* (9). In panel (**C**), cells of susceptible strain AU2934, and resistant strains AU0505 and AU17928 were grown in Lennox LB medium (5 g/l NaCl) in the absence of antibiotics and total RNA was isolated. The *bpeF* mRNA levels were determined by RT-qPCR. Expression levels are shown relative to susceptible strain

AU2934. Error bars indicate standard deviations from the mean of three biological and technical replicates. RT-qPCR analysis verified the RNA-seq results obtained with strains AU2934 and AU0505, and confirm constitutive expression of BpeEF-OprC in AU17928 and its susceptibility profile. We do not at present understand strain's AU17928 BpeEF-OprC hyper-expression when compared to AU0505. This hyper-expression does not affect relative resistance levels (see panel **A**).

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