



The Mla Pathway Plays an Essential Role in the Intrinsic Resistance of *Burkholderia cepacia* Complex Species to Antimicrobials and Host Innate Components

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ABSTRACT Antibiotic resistance is a threat to our modern society, and new strategies leading to the identification of new molecules or targets to combat multidrug-resistant pathogens are needed. Species of the genus *Burkholderia*, including the *Burkholderia cepacia* complex (Bcc), *Burkholderia pseudomallei*, and *Burkholderia mallei*, can be highly pathogenic and are intrinsically resistant to multiple classes of antibiotics. Bcc species are nonetheless sensitive to extracellular products released by *Pseudomonas aeruginosa* in interspecies competition. We screened for *Burkholderia* transposon mutants with increased sensitivity to *P. aeruginosa* spent medium and identified multiple mutants in genes sharing homology with the Mla pathway. Insertional mutants in representative genes of the Bcc Mla pathway had a compromised cell membrane and were more sensitive to various extracellular stresses, including antibiotics and human serum. More precisely, *mla* mutants in the Bcc species *Burkholderia cenocepacia* and *Burkholderia dolosa* were more susceptible to Gram-positive antibiotics (i.e., macrolides and rifampin), fluoroquinolones, tetracyclines, and chloramphenicol. Genetic complementation of *mlaC* insertional mutants restored cell permeability and resistance to Gram-positive antibiotics. Importantly, Bcc *mla* mutants were not universally weaker strains since their susceptibilities to other classes of antibiotics were unaffected. Although cell permeability of homologous *mla* mutants in *Escherichia coli* or *P. aeruginosa* was also impaired, they were not more sensitive to Gram-positive antibiotics or other antimicrobials as was observed in Bcc *mla* mutants. Together, the data suggest that the Mla pathway in *Burkholderia* may play a different biological role, which could potentially represent a *Burkholderia*-specific drug target in combination therapy with antibiotic adjuvants.

IMPORTANCE The outer membrane of Gram-negative bacteria acts as an effective barrier against toxic compounds, and therefore compromising this structure could increase sensitivity to currently available antibiotics. In this study, we show that the Mla pathway, a system involved in maintaining the integrity of the outer membrane, is genetically and functionally different in *Burkholderia cepacia* complex species compared to that in other proteobacteria. Mutants in *mla* genes of *Burkholderia cenocepacia* or *Burkholderia dolosa* were sensitive to Gram-positive antibiotics, while this effect was not observed in *Escherichia coli* or *Pseudomonas aeruginosa*. The Mla pathway in *Burkholderia* species may represent an ideal genus-specific target to address their intrinsic antimicrobial resistances.

KEYWORDS *Burkholderia*, *Burkholderia cenocepacia*, *Burkholderia dolosa*, antibiotic resistance, outer membrane

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Bacteria belonging to the genus *Burkholderia* represent a wide range of metabolically diverse species (1). Members of the *Burkholderia cepacia* complex (Bcc), known to chronically colonize cystic fibrosis (CF) airways, and the level 3 pathogens *Burkholderia pseudomallei* and *Burkholderia mallei* (2) are the most common *Burkholderia* species associated with deadly infections in humans (1). One hallmark of *Burkholderia* species is their high intrinsic level of resistance to antibiotics, including to polymyxins (3), which are often used as last-resort antimicrobials against multidrug-resistant Gram-negative pathogens (4). The intrinsic resistance of *Burkholderia* to several classes of antibiotics is primarily attributed to the very low permeability of its outer membrane (OM), the presence of several efflux pumps, and its unique lipopolysaccharide (LPS) structure (3). For all Gram-negative bacteria, including *Burkholderia*, the low permeability is the result of the lipid asymmetry of the OM, with LPS at the outer leaflet and phospholipids at the inner leaflet. This particular arrangement is critical for maintaining barrier function of the OM and protection toward extracellular stresses, including antibiotics (5). Furthermore, the atypical LPS structure of *Burkholderia* directly confers resistance to polymyxins, including colistin (6).

As antibiotic resistance is becoming a pandemic threat, the development of innovative strategies leading to the identification of new molecules or targets against multidrug-resistant pathogens is required (7, 8). We and others have shown that Bcc species are highly susceptible to *Pseudomonas aeruginosa* toxins, including the secondary metabolites called phenazines (9, 10), which are antibiotics (11). Due to chemical gradients occurring in multispecies communities (12), we speculated that Bcc organisms must have intrinsic resistance mechanisms for tolerating subinhibitory concentrations of *P. aeruginosa* toxins that should also be required to protect against clinically relevant antibiotics.

Herein, we wanted to take advantage of the natural competitive interactions between *P. aeruginosa* and the Bcc to identify new drug targets in *Burkholderia* that are potentially associated to its “intrinsic resistome” (5). We performed a chemical genomics approach to identify hypersensitive transposon mutants of *Burkholderia cenocepacia* strain K56-2 to toxins/antibiotics (i.e., spent medium) of *P. aeruginosa* strain PA14.

RESULTS

Identification of *B. cenocepacia* mutants susceptible to subinhibitory concentrations of *P. aeruginosa* spent medium. To identify genes or bacterial components associated with the intrinsic resistance of Bcc species to antimicrobials and bacterial toxins, we screened for hypersensitive *Burkholderia* mutants using *P. aeruginosa* PA14 supernatant as a source of toxins targeting sensitive *B. cenocepacia* K56-2 mutants. We made a small random transposon library of ~3,800 *B. cenocepacia* K56-2 mutants and visually compared the growth of each mutant colony to that of their wild-type (WT) parent on agar containing spent medium (25%, vol/vol) extracted from *P. aeruginosa* planktonic cultures (24 h in lysogeny broth [LB]). At this concentration (25%, vol/vol), the growth of WT K56-2 colonies on agar was similar to that without *Pseudomonas* supernatant (Fig. 1A; also see Fig. S1 in the supplemental material). We identified 20 mutants with growth defects (24 h at 37°C and 72 h at room temperature) specific to agar containing *P. aeruginosa* spent medium that were not observed on LB agar alone (Table 1 and Fig. S1). Transposon (Tn) insertions affecting genes involved in regulation/signaling (BCAL0499, BCAM1948, and BCAM2426), transport (BCAL0302, BCAL0305, and BCAM2618), lipoprotein (BCAM2620), type IV secretion system (BCAM0324), hypothetical genes, and mobile elements were identified (Table 1). Although we tested a small number of Tn mutants, our mutagenesis was effective, as demonstrated by the selection of three loci with different Tn insertions in multiple neighboring genes (BCAL1079 to BCAL1082, BCAL0302 to BCAL0305, and BCAM2618 to BCAM2620) and three genes with multiple insertions (BCAL0179, BCAL0305, and BCAL3252).

Genes coding for proteins with diverse functions may be required by *B. cenocepacia* K56-2 to tolerate sublethal concentrations of *P. aeruginosa* toxins on an agar surface. Among the selected Tn mutants, different patterns of growth were observed on agar

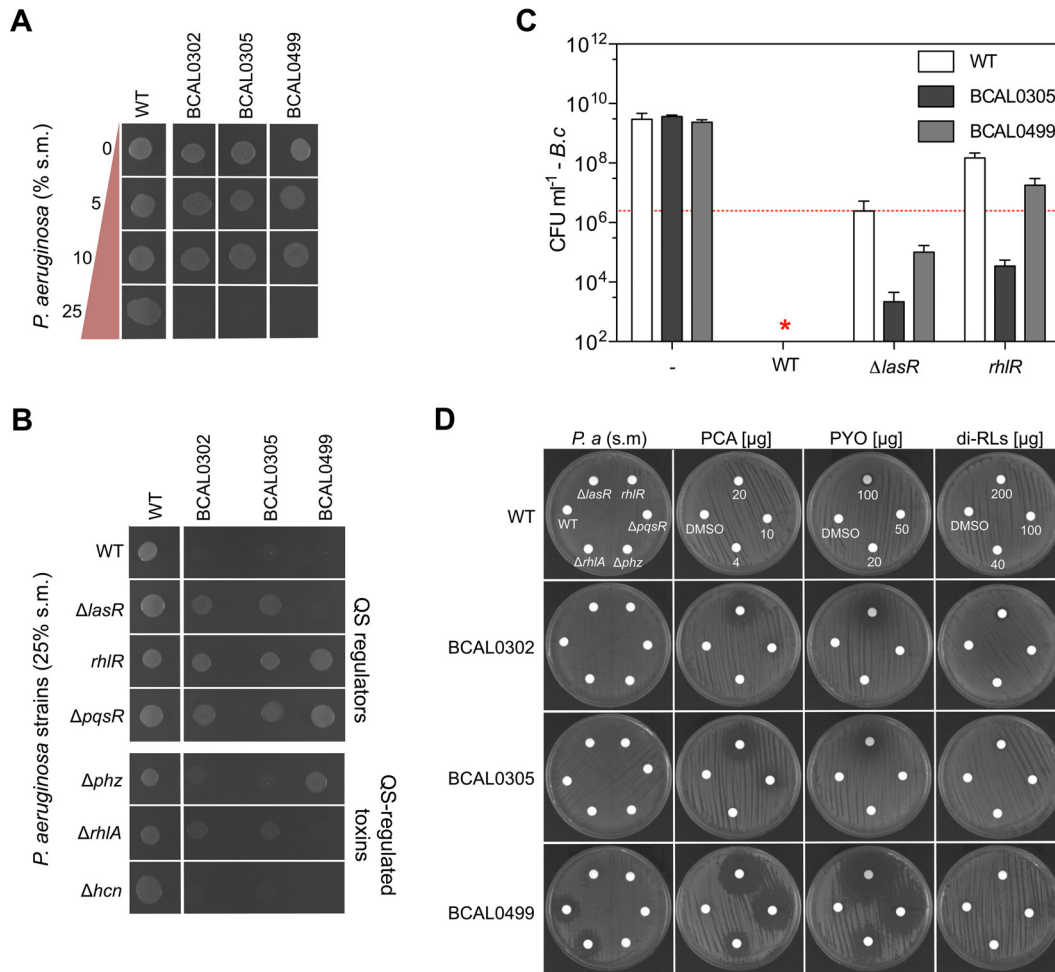


FIG 1 Sensitivity of *B. cenocepacia* K56-2 mutants to *P. aeruginosa* spent medium (s.m.). Killing of *B. cenocepacia* K56-2 mutants by *P. aeruginosa* PA14 supernatant is concentration dependent (A) and involves quorum-sensing-regulated molecules on agar (B and D) and in liquid cocultures (C). Panels A, B, and D show representative pictures from at least three experiments. Data from panel C represent means \pm standard deviations (SD) from three replicates of coculture competition experiments done in large flasks (see Materials and Methods). The red line in panel C represents CFU of *B. cenocepacia* WT and mutant strains at time zero ($\sim 2 \times 10^6$ CFU \cdot ml $^{-1}$). The star in panel C indicates that no *B. cenocepacia* CFU were recovered from the cocultures or that they were below the detection limits (~ 200 CFU).

containing *P. aeruginosa* spent medium. First, the majority of the Tn mutants grew like their WT parent for the first 24 h; however, unlike WT K56-2, the opacity of their colonies faded over time (Fig. S1). On the other hand, the growth of three Tn mutants affecting genes in a locus coding for different ABC transporters (Tn mutant 25E5 [BCAL0302] and Tn mutants 2C5 and 30G10 [BCAL0305]) was severely impaired within the first 24 h, but the opacity of their colonies increased over time (Fig. S1). Lastly, we had one Tn mutant in a two-component regulatory system (Tn mutant 12C7 [BCAL0499]) that was unable to grow over a period of 96 h (Fig. S1). These three genes (BCAL0302, BCAL0305, and BCAL0499) were further characterized in this study.

Insertional mutants (i.e., plasmid insertion via single crossover recombination) in the ABC transporter genes BCAL0302 and BCAL0305, as well as in the two-component response regulator gene BCAL0499, were made in *B. cenocepacia* strain K56-2. Their hypersensitivity profiles to *P. aeruginosa* toxins (i.e., spent medium) on agar were confirmed (Fig. 1A), thereby supporting our initial genetic results. Growth of the insertional mutants (now referred to as mutant or mutants for the rest of the text) on *Pseudomonas* spent medium-containing agar could be rescued by either reducing the concentration of WT spent medium to 10% (vol/vol) or by adding supernatant (25%, vol/vol) extracted from the less toxic *P. aeruginosa* quorum-sensing (QS) regulator

TABLE 1 *B. cenocepacia* K56-2 mutants selected for their sensitivity to *P. aeruginosa* spent medium on agar surface

Mutant ID ^b	Tn insertion or J2315 identifier ^a	Description or function (gene)
15G6	BCAL0179	Hypothetical protein
36D7	BCAL0179	Hypothetical protein
37A8	BCAL0182	Putative plasmid recombinase
25E5	BCAL0302	ABC transport permease (<i>miaE</i>)
30G10	BCAL0305	ABC transport (<i>miaC</i>)
2C5	BCAL0305	ABC transport (<i>miaC</i>)
12C7	BCAL0499	Two-component response regulator
14C3	BCAL1048	Putative alpha/beta hydrolase
37B7	BCAL1172	Hypothetical protein
28H12	BCAL1246	Putative malonyl coenzyme A-acyl carrier protein
37H7	(BCAL3225)	Transposase upstream of capsular polysaccharide
2D10	(BCAL3252)	Transposase
35H10	(BCAL3252)	Transposase
4E2	BCAL3336	Purine ribonucleotide biosynthesis (<i>purH</i>)
8H11	(BCAM0275a)	Hypothetical protein
13F4	(BCAM0324)	Type IV secretion system (<i>virB1</i>)
16H4	BCAM1948	Redox-sensitive transcriptional regulator (<i>soxR</i>)
24C3	(BCAM2426)	c-di-GMP phosphodiesterase
17F3	BCAM2618	ABC transport periplasmic protein (<i>argT</i>)
33C6	BCAM2620	Lipoprotein (<i>yaeC</i>)

^aTransposon (Tn) insertion mapping was analyzed using the complete genome sequence and annotation of *Burkholderia cenocepacia* strain J2315 (13), which is closely related to strain K56-2 (14) and available in the *Burkholderia* Genome Database (15). Genes in parentheses represent the open reading frames likely affected by the Tn inserted in the intergenic region.

^bID, identification.

mutants in $\Delta lasR$, *rhIR*, and $\Delta pq sR$ (9). More specifically, the three *B. cenocepacia* mutants could grow on agar containing supernatant extracted from *rhIR* and $\Delta pq sR$ mutants, while growth on agar with spent medium from the $\Delta lasR$ mutant was still toxic for BCAL0499 mutant but not for the BCAL0302 and BCAL0305 mutants (Fig. 1B). Furthermore, BCAL0305 and BCAL0499 mutants were more sensitive than WT K56-2 in liquid cocultures with the less toxic *P. aeruginosa* $\Delta lasR$ and *rhIR* mutants (Fig. 1C) demonstrating that the hypersensitivity phenotype was exhibited on agar surfaces and in liquid cultures.

Phenazines, rhamnolipids, and hydrogen cyanide (HCN) are known QS-regulated toxins involved in Bcc killing in liquid cocultures (9, 10). Growth on agar containing supernatant extracted from a *P. aeruginosa* PA14 HCN biosynthesis mutant ($\Delta hcnABC$) was still blocked, suggesting that the *B. cenocepacia* hypersensitive mutants were susceptible to toxins other than HCN (Fig. 1B). In fact, growth of the BCAL0499 mutant was restored on agar supplemented with spent medium extracted from a phenazine-null mutant of *P. aeruginosa* (Δphz), while supernatant from a rhamnolipid-null mutant ($\Delta rhIA$) partially restored growth of BCAL0302 and BCAL0305 mutants on agar (Fig. 1B). A disk diffusion assay on agar with phenazine-1-carboxylic acid (PCA) and pyocyanin (PYO), two *P. aeruginosa* phenazines (11), confirmed that the BCAL0499 mutant was highly susceptible to phenazines compared to WT K56-2 but was not more susceptible to dirhamnolipids (Fig. 1D). Zones of inhibition around disks containing supernatants extracted from WT *P. aeruginosa* PA14 or different isogenic mutants ($\Delta lasR$, *rhIR*, $\Delta pq sR$, Δphz , and $\Delta rhIA$ mutants) were only observed with BCAL0499 mutant exposed to pigmented spent media (blue-green color; PYO), namely, that from WT and the $\Delta rhIA$ mutants (Fig. 1D). Mutants BCAL0302 and BCAL0305 mutants were more sensitive to PCA and PYO than was the WT but were less sensitive than the BCAL0499 mutant (Fig. 1D). However, as suggested by their growth on $\Delta rhIA$ agar (Fig. 1B), BCAL0302 and BCAL0305 mutants were more sensitive to dirhamnolipids, as demonstrated by a small zone of inhibition with 200 μ g that was not present with WT K56-2 (Fig. 1D).

Altogether, our chemical genetic screen using *P. aeruginosa* spent medium as a source of small molecules was demonstrated to be an effective strategy in identifying key components of *B. cenocepacia* K56-2 for tolerating sublethal concentrations of *P.*

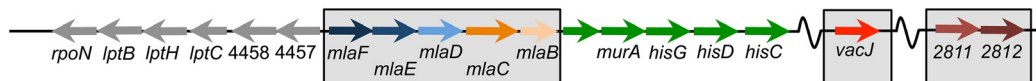
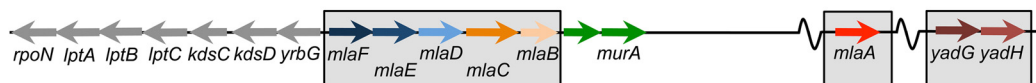
Burkholderia* spp.**Pseudomonas aeruginosa******Escherichia coli***

FIG 2 Genetic organization of the Mla pathways in different bacterial species. Comparison between *Burkholderia* species and *P. aeruginosa* strain PAO1 and *E. coli* K-12 strain MG1655. Colors indicate homologous genes between species based on reciprocal BLASTP and BLASTX analyses. An asterisk represents the likely *mIaB* homologue in *Burkholderia*, since no homology was shared with *P. aeruginosa* or *E. coli* homologues, but all three genes have STAS domains. Genes with numbers in *Burkholderia* use the J2315 identifiers, for which “BCAL” was not included on the figure. As for *P. aeruginosa*, the numbers are based on the PAO1 identifiers and “PA” was also not included on the figure.

aeruginosa-derived toxins. Among those findings, we identified a two-component regulatory system and ABC transporters required for tolerating phenazines and/or rhamnolipids.

The Mla pathway plays a critical role in the intrinsic resistance of *B. cenocepacia* and *Burkholderia dolosa* to antibiotics. To determine whether the selected *B. cenocepacia* K56-2 transposon mutants (Table 1) were also sensitive to clinically relevant antimicrobials, we first evaluated antibiotics that are not typically used as first line therapy against Bcc species (16) using a disk assay on LB agar.

We tested azithromycin, cefepime, imipenem, and tobramycin, representing the antibiotic classes of macrolides, cephalosporins, carbapenems, and aminoglycosides, respectively. Tn mutants in the ABC transporter genes BCAL0302 (Tn mutant 25E5) and BCAL0305 (Tn mutants 2C5 and 30G10) were sensitive to azithromycin, while the WT parent and all of the other Tn mutants were resistant (see Table S1 in the supplemental material). None of the Tn mutants were more sensitive to the other tested antibiotics than the WT (Table S1). The two-component response regulator BCAL0499 mutant was not more sensitive than the WT to any tested antibiotics (see Table S2 in the supplemental material). We then decided to only investigate further mutants/genes (i.e., BCAL0302 and BCAL0305) where a clear difference in zone of clearing was observed.

Genes BCAL0302 and BCAL0305 are part of an eight-gene locus coding for ABC transporter systems that share high sequence identities at both gene and protein levels with the Mla pathway (Fig. 2 and Table 2) found in various Gram-negative bacteria, including *Escherichia coli*, *Shigella flexneri*, and *P. aeruginosa* (17–19). Reciprocal BLASTP analyses showed that only MlaB from *E. coli* K-12 or PA4452 from *P. aeruginosa* strain PAO1 had no homologue in *B. cenocepacia* (Table 2). However, based on the genetic organization (Fig. 2) and the presence of sulfate transporter and anti- σ factor antagonist (STAS) domains in BCAL0306, MlaB, and PA4452, we determined that BCAL0306 was the most likely *Burkholderia* MlaB homologue (Fig. 2 and Table 2). Although genetic similarities are observed between the three species, all *Burkholderia* genes are in a single and continuous genetic locus, whereas *mIaA* and *vacJ* are located elsewhere in the genomes of *E. coli* and *P. aeruginosa*, respectively (Fig. 2). For the rest of the study, BCAL0302 and BCAL0305 genes will be referred to as *mIaE* and *mIaC*, respectively.

Using the *mIaE* and *mIaC* mutants, we further demonstrated their increased sensitivity to tetracyclines (tetracycline, doxycycline, and tigecycline), chloramphenicol, macrolides (azithromycin, clarithromycin, and erythromycin), fluoroquinolones (cipro-

TABLE 2 Similarities between the Mla pathway genes/proteins of *Burkholderia*, *E. coli* and *P. aeruginosa*

Bcc ^a	Protein (% identity by BLASTP)		Predicted function
	<i>P. aeruginosa</i> ^b	<i>E. coli</i> K-12	
BCAL0301	PA4456 (55)	MlaF (55)	ATP-binding protein
BCAL0302	PA4455 (66)	MlaE (61)	Permease
BCAL0303	PA4454 (47)	MlaD (48)	Periplasmic substrate-binding protein
BCAL0304	VacJ (44)	MlaA (39)	Lipoprotein
BCAL0305	PA4453 (24)	MlaC (26)	Periplasmic binding protein
BCAL0306	PA4452 (0) ^c	MlaB (0) ^c	Protein with STAS domain
BCAL0307	PA2812 (42)	YadG (42)	ATP-binding protein
BCAL0308	PA2811 (35)	YadH (38)	Membrane protein

^aThe organization of the described ABC transporters (Fig. 2) is the same among all Bcc strains within the database (15). For simplicity, we therefore used genes or locus tags of *B. cenocepacia* strain J2315.

^bLocus tags of *P. aeruginosa* strain PAO1 are represented.

^cVery low similarity at the amino acid level, but proteins from the three species have a STAS domain.

floxacin and levofloxacin), and rifampin (Tables 3 and S2). Importantly, the sensitivity of *mlaE* and *mlaC* mutants to penicillins, cephalosporins, carbapenems, monobactams, aminoglycosides, oxazolidinones, lincosamides, and fosfomycin remained the same as that of WT K56-2 (Tables 2 and S2). These results strongly suggest that mutations in the Mla pathway did not necessarily lead to generally weak mutants susceptible to all antimicrobials, but rather to those susceptible to class-specific antibiotics.

In a recent study, we identified a *vacJ* Tn mutant of *B. dolosa* strain PC543 that was unable to modify its morphotype or to swarm upon exposure to small amounts of rhamnolipids (20). Here, we showed that the *vacJ* Tn mutant and a newly made *mlaC*

TABLE 3 Antibiotic resistance profile of *B. cenocepacia* K56-2 *mla* mutants^a

Antibiotic target	Antibiotic category	Antibiotic class	Antibiotic	Antibiotic amt (μg)	Resistance profile of <i>B. cenocepacia</i> K56-2 ^b				
					WT	<i>mlaE</i>	<i>mlaC</i>		
Cell wall synthesis	Beta-lactams	Penicillins	Ampicillin	25	R	R	R		
			Amoxicillin-clavulanate	30	R	R	R		
			Piperacillin-tazobactam	110	S	S	S		
		Cephalosporins	Cefepime	30	S	S	S		
			Cefoxitin	30	R	R	R		
			Ceftazidime	30	S	S	S		
			Ceftriaxone	30	S	S	S		
			Carbapenems	Imipenem	10	S	S	S	
		Monobactams	Meropenem	10	S	S	S		
			Aztreonam	30	S	S	S		
		Protein synthesis	30S	Aminoglycosides	Tobramycin	10	R	R	R
					Amikacin	30	R	R	R
Tetracyclines	Tetracycline			30	S	↑ S	↑ S		
	Doxycycline			30	S	↑ S	↑ S		
	Tigecycline			15	S	↑ S	↑ S		
	Oxazolidonones			Linezolid	30	R	R	R	
50S	Chloramphenicol		30	S	↑ S	↑ S			
	Macrolides		Azythromycin	15	R	↑ ↑ S	↑ ↑ S		
			Clarithromycin	15	R	↑ ↑ S	↑ ↑ S		
Lincosamides	Erythromycin		15	R	↑ ↑ S	↑ ↑ S			
	Clindamycin		2	R	R	R			
DNA topoisomerases	Fluoroquinolones		Ciprofloxacin	5	S	↑ S	↑ S		
		Levofloxacin	5	S	↑ S	↑ S			
mRNA synthesis	Other	Rifampin	5	R	↑ ↑ S	↑ ↑ S			
		Fosfomycin	50	R	R	R			

^aSummary of the raw data with zones of clearing measurements (mm) is shown in Table S2.

^bBacterial strains were considered resistant (R) when no zone of clearing was observed, while any clearing was recorded as sensitive (S). When the susceptibility of *mlaE* or *mlaC* mutant strains was greater than that of the WT (i.e., greater zone of clearing), their hypersensitivity was labeled as ↑ S. On the other hand, *mlaE* or *mlaC* mutant strains that were sensitive to antibiotics while the WT was resistant were labeled as ↑ ↑ S.

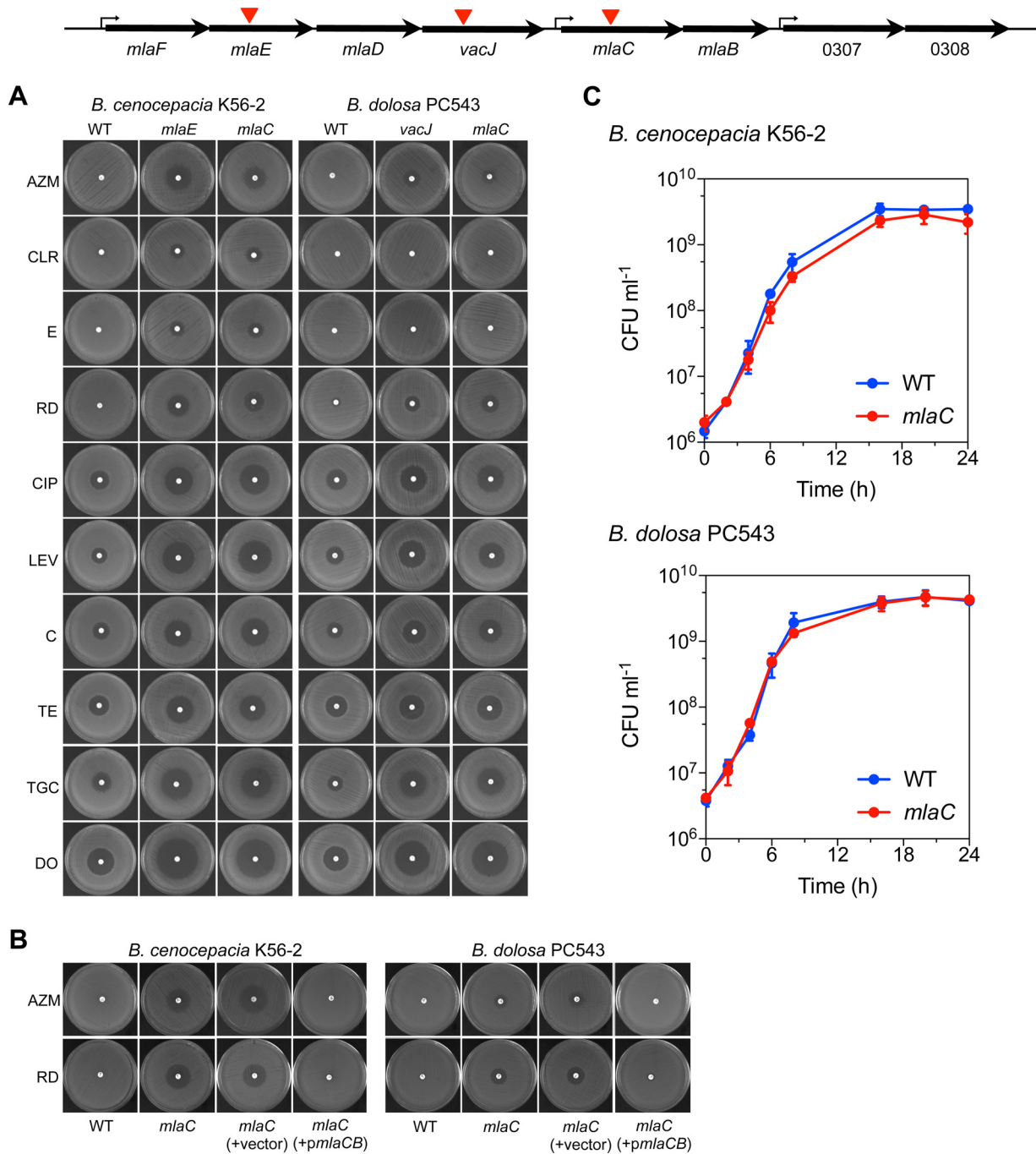


FIG 3 The impact of the Bcc Mia pathway on antibiotic resistance. (A) Disk diffusion assay demonstrating the increased susceptibility of the *miaC* and *miaE* insertional mutants or the *vacJ* Tn mutant of *B. cenocepacia* K56-2 or *B. dolosa* PC543, indicated by the red triangles in the Mia genetic region map inset, to 10 antibiotics representing 5 different classes (macrolide, rifampin, fluoroquinolone, chloramphenicol, and tetracycline). (B) Genetic complementation of the *miaC* insertional mutants with pHERD26T-*miaCB* (*pmlaCB*) or with the empty pHERD26T (vector) using the Gram-positive antibiotics azithromycin and rifampin. (C) *miaC* does not impact growth of *B. cenocepacia* K56-2 or *B. dolosa* PC543 in large shaken LB cultures. Panels A and B show representative pictures from at least three experiments. Data from panel C represent means \pm SD from three replicates. AZM, azithromycin; CLR, clarithromycin; E, erythromycin; RD, rifampin; CIP, ciprofloxacin; LEV, levofloxacin; C, chloramphenicol; TE, tetracycline; TGC, tigecycline; and DO, doxycycline.

insertional mutant in *B. dolosa* PC543 had similar increased sensitivity to macrolides, tetracyclines, fluoroquinolones, and rifampin (Fig. 3A). Those results were recapitulated in cultures of *B. cenocepacia* K56-2 and *B. dolosa* PC543 *miaC* mutants compared to those of the WT (Table 4). The growth in liquid culture over 24 h of the *miaC* mutants was comparable to that of their respective WT parent (Fig. 3C). The lack of general

TABLE 4 Impact of *mlaC* on the antibiotic sensitivity of *B. cenocepacia* and *B. dolosa* in liquid cultures

Antibiotic	MIC ($\mu\text{g} \cdot \text{ml}^{-1}$) for:			
	<i>B. cenocepacia</i> K56-2		<i>B. dolosa</i> PC543	
	WT	<i>mlaC</i>	WT	<i>mlaC</i>
Azythromycin	256	16	>256	64
Erythromycin	>256	16	>256	128
Rifampin	32	1	32	4
Ciprofloxacin	2	0.5	2	0.5
Chloramphenicol	16	4	32	8
Tetracycline	8	2	4	2
Ceftazidime	>256	>256	>256	>256
Tobramycin	>256	>256	>256	>256

growth defect by the mutants could not explain the increased sensitivity to specific antibiotics. The *mlaCB* genes are predicted to be transcribed as a 2-gene operon (Fig. 2 and 3) (15). Consequently, genetic complementation of the *mlaC* mutants in *B. cenocepacia* K56-2 and *B. dolosa* PC543 was carried out with a plasmid containing the *mlaCB* genes under the control of their native promoter. Complementation of the *mlaC* mutants restored the resistance to rifampin and azithromycin to WT level in both *B. cenocepacia* K56-2 and *B. dolosa* PC543 (Fig. 3B). These data genetically demonstrate that the Mla pathway participates in the intrinsic resistance of *Burkholderia* to Gram-positive antibiotics.

Notably, our data demonstrate that mutations in the Mla pathway render Bcc sensitive to typical Gram-positive antibiotics, such as macrolides and rifampin. We rescreened the Tn library in *B. cenocepacia* K56-2 and the 34 *B. dolosa* PC543 Tn mutants, which were previously selected for their inability to differentiate morphologically upon exposure to rhamnolipids (20) and identified BCAM2829 and *bamC* as genes also required for azithromycin and rifampin resistance (Fig. S2). BCAM2829 is a VacJ-like lipoprotein (VacJ2), while BamC is a predicted lipoprotein that participates in the assembly and insertion of outer membrane β -barrel proteins in *E. coli* (21). At the amino acid level, VacJ2 is 54% identical to the VacJ lipoprotein of the Mla pathway.

Together, our data demonstrate that the Mla pathway is critical for the intrinsic antibiotic resistance to macrolides, rifampin, fluoroquinolones, tetracyclines, and chloramphenicol in both *B. cenocepacia* and *B. dolosa*. On the other hand, the antibiotic resistance profile of the two-component regulatory system mutant BCAL0499 was similar to that of its WT parent, demonstrating that this mutation primarily affected tolerance to small molecules like phenazines but not to antibiotics (Table S2). Although *vacJ2* and *bamC* were directly selected with azithromycin, our original chemical genetic screen using *P. aeruginosa* supernatant directly led to the identification of the Mla pathway as a major determinant of the Bcc intrinsic resistome.

Bcc mutants in the Mla pathway have a defective cell permeability. Studies in *E. coli* have shown that the Mla pathway is involved in lipid homeostasis in the outer leaflet and is consequently responsible for maintaining the barrier function of the outer membrane (OM) in Gram-negative bacteria (18, 22–24). Consistent with those findings, the sensitivity of Bcc *mla* or *vacJ* mutants to Gram-positive antibiotics, such as macrolides and rifampin (Fig. 3A), would also suggest a potential outer membrane defect.

As an indicator of cell permeability, we performed an SDS-EDTA sensitivity assay to determine whether the Mla pathway played a role in the OM permeability of members of the Bcc. By chelating cations, EDTA destabilizes LPS interactions, and bacterial death occurs upon exposure to the detergent SDS when the OM is compromised. *B. cenocepacia* K56-2 mutants (i.e., *mlaC* and *mleE* mutants) were highly sensitive to SDS (0.025%, wt/vol) (Fig. 4A). In *B. dolosa* PC543 background, the *mlaC* mutant and the *vacJ* Tn mutant were resistant to 0.025% (wt/vol) SDS, but unlike their WT parent, they were unable to grow on SDS-containing agar in the presence of EDTA (Fig. 4A). Genetic complementation of the *mlaC* mutant with a plasmid containing the *mlaCB* genes

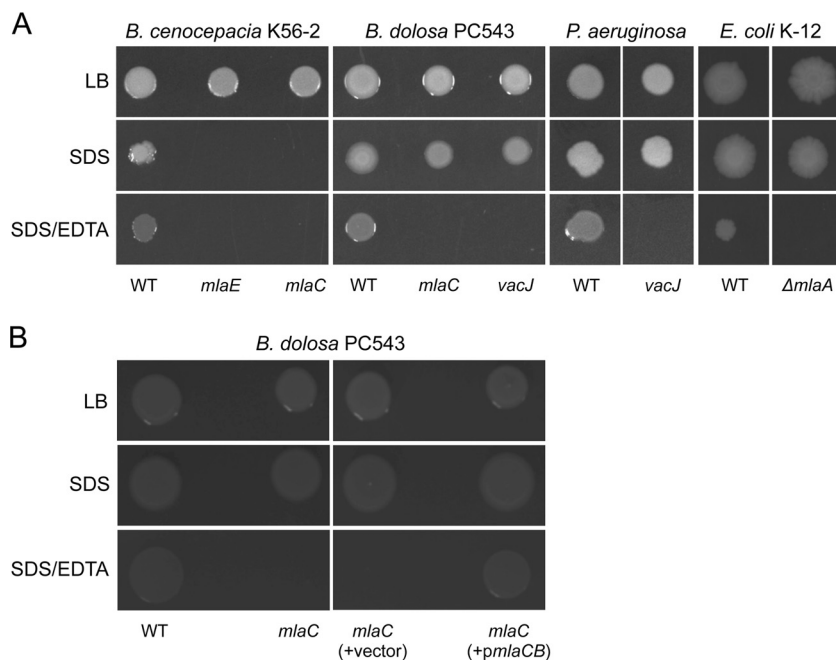


FIG 4 Mutants in the Mla pathway have impaired permeability. (A) Outer membrane permeability was assessed by the SDS-EDTA sensitivity assay using representative *mla* or *vacJ* mutants in different species, including *B. cenocepacia* K56-2, *B. dolosa* PC543, *P. aeruginosa* PA14, and *E. coli* K-12 (strain BW25113). (B) Genetic complementation of the *mlaC* insertional mutant in *B. dolosa* PC543 with pHERD26T-*mlaCB* (*pmlaCB*) or with the empty pHERD26T (vector). The growth or absence of growth from 2 μ l of undiluted cultures spotted on agar is represented. The final concentrations of SDS and EDTA were 0.025% (vol/vol) and 0.55 mM, respectively. Pictures were taken after 48 h at 37°C.

restored the ability of the mutant strain to grow on SDS-EDTA agar like its WT PC543 parent (Fig. 4B). The *vacJ2* Tn mutant had an intermediate sensitivity to SDS-EDTA, with a 1.5-log reduction in survival compared to that of its WT K56-2 parent. On the other hand, the SDS-EDTA sensitivity of the *bamC* Tn mutant in *B. dolosa* PC543 could not be evaluated, since it was highly sensitive to SDS like *B. cenocepacia* K56-2 *mla* mutants (data not shown).

As suggested by the SDS-EDTA sensitivity results (Fig. 4A), mutations in the Mla pathway disrupt the OM permeability of the Bcc, *P. aeruginosa*, and *E. coli*, but they do not have the same effect on antibiotic susceptibility. For instance, *mla* or *vacJ* mutants in *E. coli* or *P. aeruginosa* were not more sensitive to macrolides, rifampin, fluoroquinolones, chloramphenicol, and tetracyclines (see Tables S3 and S4 in the supplemental material). Altogether, our data strongly suggest that mutations in the *Burkholderia* Mla pathway or in *vacJ2* increase OM permeability (Fig. 4A) and likely enhance susceptibility to several classes of antibiotics (Fig. 3 and Table 3).

Bcc mutants in the Mla pathway have impaired motility and colony development on agar. We previously showed that *B. dolosa* PC543 and *B. cenocepacia* K56-2 exhibited different colony morphotypes and swarming behaviors when exposed to *P. aeruginosa* spent medium or rhamnolipids (20). As a *vacJ* Tn mutant failed to demonstrate these phenotypes (20), we next showed that *mlaC* and *mlaE* mutants also did not differentiate morphologically (Fig. 5A) and could not swarm (Fig. 5B) upon exposure to *P. aeruginosa* supernatant-containing rhamnolipids. In addition, colonies of *mlaC*, *mlaE*, or *vacJ* mutants were smaller in size compared to those of their respective WT parents (Fig. 5C). Interestingly, homologue mutants of *vacJ* in *P. aeruginosa* PA14 or $\Delta mlaA$ in *E. coli* K-12 did not have smaller colonies like those observed in *B. cenocepacia* K56-2 or *B. dolosa* PC543 (Fig. 5C). Data on antibiotic resistance, motility, and colony size tend to show that the Mla pathway has a different impact in the biology of *Burkholderia* species compared to that in *P. aeruginosa* and *E. coli*.

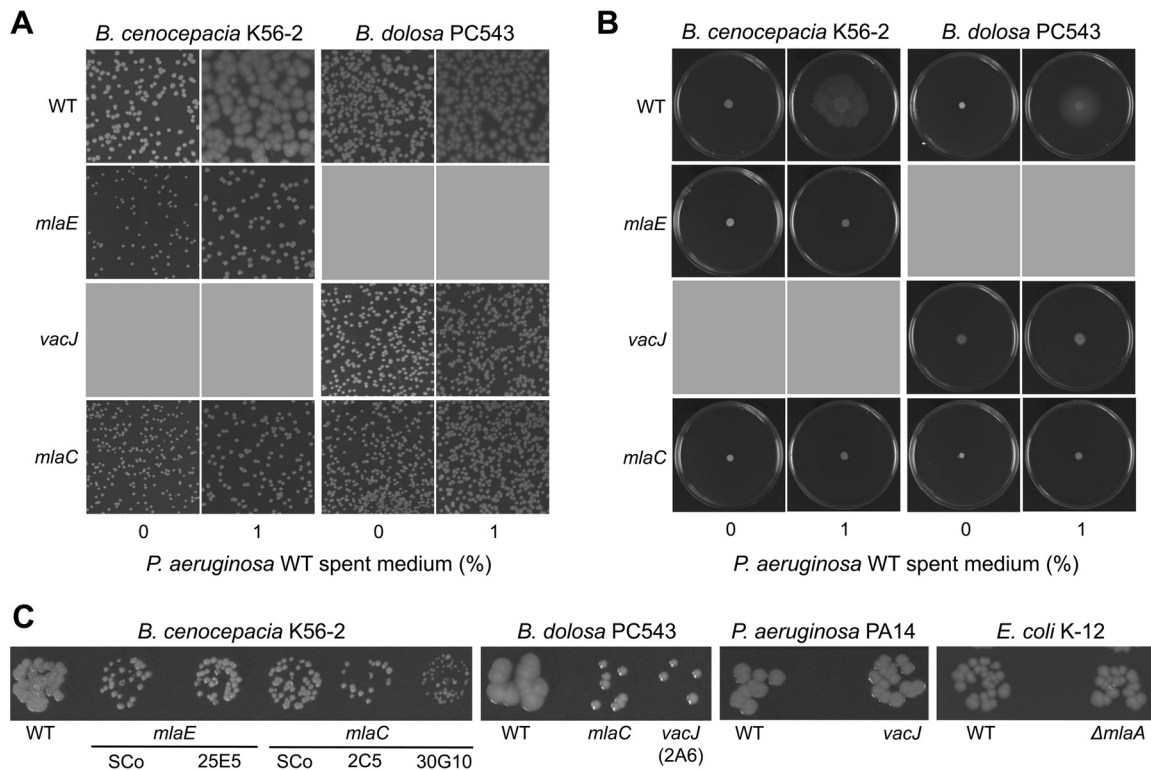


FIG 5 Bcc *mla* or *vacJ* mutants have impaired colony morphotype, colony size, and motility. (A and B) Colony morphotype (A) and swarming motility (B) upon exposure to *P. aeruginosa* PA14 spent medium of different *mla* or *vacJ* mutant representative strains in *B. cenocepacia* K56-2 or *B. dolosa* PC543. (C) *mla* or *vacJ* mutants have smaller colonies on LB agar compared to WT in *B. cenocepacia* K56-2 and *B. dolosa* PC543 but not in *P. aeruginosa* PA14 or *E. coli* K-12 K-12 (strain BW25113). Tn mutant numbers are labeled below *mlaE*, *mlaC*, or *vacJ*. All pictures were taken after 48 h at 37°C. SCo, single crossover mutant.

The Bcc Mla pathway is required for host innate resistance. Some studies have demonstrated that the Mla pathway is important for resisting the bactericidal effect of human serum in various bacterial pathogens (19, 25–27). To determine whether a similar role exists in the Bcc, we collected and pooled serum from five healthy donors (4 females and 1 male) and tested the killing activity of the serum over a period of 2 h at a final concentration of 30%.

Prior to evaluating Bcc mutants in the Mla pathway, we first evaluated serum resistance of WT *B. cenocepacia* K56-2 and *B. dolosa* PC543 in comparison to that of the sensitive *E. coli* K-12 strain BW25113. *B. cenocepacia* K56-2 was resistant to human serum, as previously reported (28, 29), while *B. dolosa* PC543 was as sensitive as *E. coli* K-12 (Fig. 6A). Consequently, *mlaC* and *vacJ* mutants in *B. dolosa* PC543 were not evaluated due to the high serum sensitivity of WT PC543. Mutants *mlaE* and *mlaC* in *B. cenocepacia* K56-2 were highly sensitive to human serum, as no viable colonies could be recovered after 2 h (Fig. 6B). On the other hand, the two-component regulatory system mutant BCAL0499 was resistant, while the *vacJ2* Tn mutant selected for its sensitivity to azithromycin was slightly sensitive to serum, with a close to 1-log-unit reduction in survival (Fig. 6B). Heat inactivation of the serum restored bacterial viability of *mlaE*, *mlaC*, and *vacJ2* mutants to WT levels (Fig. 6C) suggesting that heat-labile components of human serum, such as complement, are likely involved in the killing activity. Although *P. aeruginosa* PA14 was more sensitive to serum than was *B. cenocepacia* K56-2, the impact of a compromised Mla pathway on serum resistance was greater in *B. cenocepacia* (Fig. 6B) compared to that in *P. aeruginosa* (Fig. 6D).

In addition to serum and/or complement, cationic antimicrobial peptides (AMPs) represent another nonspecific innate host response toward pathogens. Here, we evaluated the impact on the resistance to AMPs of the *mlaC* mutation in both *B.*

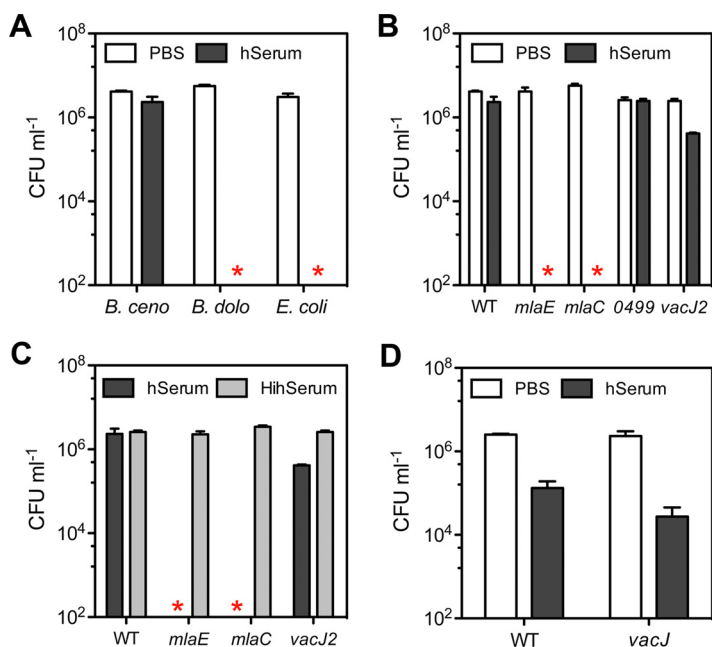


FIG 6 The role of the Mla pathway in serum resistance. (A) Resistance to 30% human serum (hSerum) over a period of 2 h was evaluated for *B. cenocepacia* K56-2 (*B. ceno*), *B. dolosa* PC543 (*B. dolo*), and *E. coli* K-12 strain BW25113 (*E. coli*). (B) Resistance to 30% hSerum of the *miaC*, *miaE*, and BCAL0499 insertional mutants and the *vacJ2* Tn mutant in comparison to WT *B. cenocepacia* K56-2. (C) The killing activity of 30% heat-inactivated human serum (HihSerum) was tested with *miaC* and *miaE* insertional mutants, the *vacJ2* Tn mutant, and WT *B. cenocepacia* K56-2 and compared to that of hSerum. (D) Resistance to 30% hSerum of a *vacJ* mutant compared to its WT *P. aeruginosa* PA14 parent strain. Stars represent the absence of recovered CFU or below the detection limits (~200 CFU). All data represent means ± SD from three replicates.

cenocepacia K56-2 and *B. dolosa* PC543. We used polymyxin B (PMB) and colistin as proxies of host AMP susceptibility since the resistance profile of commensal and pathogenic bacteria to PMB or colistin is similar to that to mammalian-derived AMPs (30). *Burkholderia* species are highly resistant to AMPs (6), and disruption of *miaC* did not lead to complete killing in both *B. cenocepacia* and *B. dolosa* but rather led to a dose-dependent growth inhibition for both PMB and colistin (Fig. 7). Although WT *B. dolosa* PC543 is intrinsically more resistant to PMB than WT *B. cenocepacia* K56-2, the reduced growth of the *miaC* mutant had the same trend in both genetic backgrounds in the presence of increasing concentrations of PMB or colistin (Fig. 7).

Altogether, our results suggest that the Bcc Mla pathway is likely required to effectively tolerate the bactericidal action of nonspecific host innate immune responses, such as complement and AMPs.

DISCUSSION

The intrinsic resistance of many Gram-negative bacteria to antibiotics involves several mechanisms, including low OM permeability and multidrug efflux pumps (5, 31). A large proportion of essential genes in *Burkholderia* species are involved in maintaining the integrity of the cell envelope, representing potential new drug targets (32). Here, we applied a simple chemical genetic screen to identify components of the intrinsic resistome of *Burkholderia* to subinhibitory concentrations of *P. aeruginosa* toxins. Among the determinants identified as being required to tolerate *Pseudomonas* toxins and antibiotics in *B. cenocepacia* K56-2 is the Mla pathway, a system shown to maintain the lipid asymmetry of the OM in *E. coli* and consequently its primary function as a barrier to toxic compounds (18, 23, 33). Although the Mla pathway is not essential in *B. cenocepacia* K56-2 (32), it was proposed to be essential in *B. cenocepacia* strain J2315 (34). Even though these differences could be attributed to strain-specific factors,

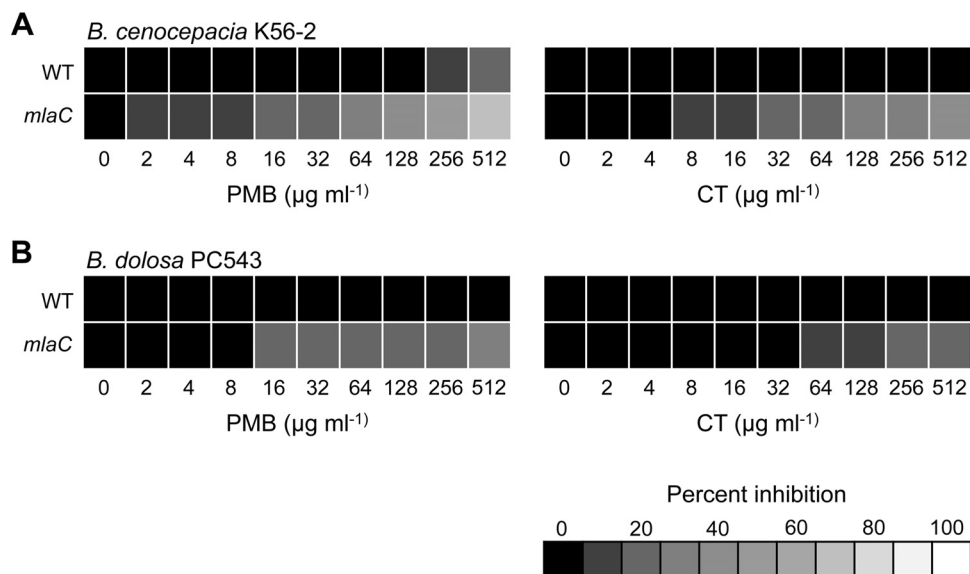


FIG 7 The impact of the Mla pathway on resistance to polymyxins. MIC determination in liquid cultures (LB) for WT and *mlaC* mutants in *B. cenocepacia* K56-2 (A) and *B. dolosa* PC543 (B) genetic background against polymyxin B (PMB) and colistin (CT).

it further demonstrates that the Mla pathway plays an essential role in the biology of *Burkholderia* and could be used as a potential drug target.

Our study showed that the genetic organization (Fig. 2) and phenotypes associated with the Mla pathways, such as antibiotic resistance, differed greatly between bacterial species. For example, although *mla* or *vacJ* mutants in *E. coli* or *P. aeruginosa* have increased cell permeability (18, 19, 35), they are not more sensitive to Gram-positive antibiotics (see Tables S3 and S4 in the supplemental material) (18), as described in this study for Bcc species (Fig. 3 and Table 3). Similar to the Bcc, *vacJ* mutants in *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* are more sensitive to various antibiotics, including tilmicosin (27, 36), a macrolide antibiotic used in veterinary medicine. Together, these differences in antibiotic susceptibility suggest that factors other than cell permeability that would be species specific might be required by Gram-negative bacteria to be resistant to Gram-positive antibiotics. Supporting this possibility, synergy between efflux pumps and OM diffusion, which is antibiotic and species specific, occurs in Gram-negative bacteria (31, 37). In *E. coli*, MlaA interacts with the OmpF/C porins (22), demonstrating that Mla proteins can physically interact with other systems in the OM that are not necessarily involved in transport of phospholipids. Could the Mla pathway interact with efflux pumps in *Burkholderia* species and therefore reduce antibiotic efflux in the absence of Mla proteins, which would then be necessary for proper anchoring of pump-associated proteins in the OM?

Burkholderia species harbor several efflux pumps, of which only a few have been partially characterized (38). The BpeAB-OprB efflux pump in *Burkholderia pseudomallei* strain 1026b is known to impact multiple antibiotics (i.e., macrolides, tetracyclines, fluoroquinolones, chloramphenicol, and rifampin) (39), and the *mla* mutants in *B. cenocepacia* and *B. dolosa* were found to be more sensitive to these antibiotics. A BpeAB-OprB homologue (BCAL2020 to BCAL2022) in *B. cenocepacia* strain J2315 is required for resistance to macrolides and fluoroquinolone but also to tobramycin and aztreonam (40), two antibiotics for which *mla* mutants in *B. cenocepacia* K56-2 were unaffected (Table 3). These differences could be strain specific, as was previously described in *B. pseudomallei* (39, 41), or involved more than one efflux pump systems if they are indeed interacting with Mla proteins. Interestingly, another copy of the *bpeAB-oprB* gene (BCAS0764 to BCAS0766) is present in *B. cenocepacia* and a few other Bcc species but not in *B. dolosa* (15). If this pump interacts with the Mla pathway, it might explain the high sensitivity of *mla* mutants to SDS in *B. cenocepacia* K56-2 but not

in *B. dolosa* PC543. Whether efflux pumps truly interact with the Mla pathway in *Burkholderia* or in other species remains an unanswered question that would require further investigation. If true, it may help to elucidate differences in antibiotic resistance between species in *mIa* null strains.

In most species, the organization of the *mIa* genes is discontinuous, with the *mIaFEDCB* genes located together, while *mIaA* or *vacJ* is located elsewhere on the genome (Fig. 2). In *Burkholderia*, the *mIa* genes are all continuous, with the *mIaA* or *vacJ* homologue located between *mIaD* and *mIaC*. Besides this difference, two additional genes, BCAL0307 and BCAL0308, are located downstream of *mIaFEDACB* (Fig. 2), suggesting that they may be part of the Mla pathway and possibly interacting with the other Mla proteins. Supporting this possibility, the BCAL0308 homologue protein in *E. coli*, YadH, was recently shown to interact and form a protein complex with MlaA, and it participates in phospholipid trafficking in order to maintain OM lipid asymmetry (33). Like the $\Delta mIaA$ mutant, a *yadH* mutant was hyperpermeable and not more sensitive to erythromycin (33), which is in agreement with our data (see Table S3 in the supplemental material). Although we do not have any experimental evidence in *Burkholderia*, we can speculate that BCAL0307 and BCAL0308 are possibly interacting with Mla proteins like YadH in *E. coli*.

In addition to OM permeability and antibiotic resistance, genetic studies in several species have shown that the Mla pathway also affects biofilm formation, outer membrane vesicle formation, cell morphology, survival, and pathogenesis in animals (17, 19, 27, 35, 36, 42–44). Here, we showed that it also affects swarming motility (Fig. 5B) as well as colony size (Fig. 5C), demonstrating the pleiotropic role of the Mla pathway in the biology of *Burkholderia*. In addition to the phenotypes stated above, we also showed that *mIaC* or *mIaE* was fully required for serum resistance, while *vacJ2* was partially required in the serum-resistant strain *B. cenocepacia* K56-2 (Fig. 6). These results are in agreement with those of other studies, in which *vacJ* or *mIa* genes conferred various levels of protection against human serum components (e.g., complement) in different species, including *P. aeruginosa*, *Haemophilus influenzae*, *Actinobacillus pleuropneumoniae*, *B. pseudomallei*, and *Burkholderia thailandensis* (19, 25–27). Capsule polysaccharides and extracellular polysaccharides (EPS) are bacterial factors that can interfere with the complement cascade (45), but the presence of EPS was not associated with serum resistance in Bcc strains (29). Although mechanisms of serum resistance in the Bcc are not fully understood, a few genetic studies have identified determinants conferring resistance to serum killing in *B. cenocepacia* K56-2, such as a complete LPS structure (28) and a trimeric autotransporter (46). The impaired growth of the *mIa* mutants in the presence of PMB or colistin (Fig. 7) might suggest a potential LPS defect, since the loss of lipid A-core oligosaccharide heptoses and not that of O antigen was shown to reduce PMB resistance (47). However, the *mIa* mutants were still viable with PMB or colistin at concentrations up to $512 \mu\text{g} \cdot \text{ml}^{-1}$, which suggests that the LPS structure was minimally affected or that other unknown resistance mechanisms were still intact to maintain a high level of polymyxin resistance, considering that the MIC of enteropathogens toward polymyxins is typically below $1 \mu\text{g} \cdot \text{ml}^{-1}$ (30).

Overall, our genetic study suggests that the Mla pathway may represent a *Burkholderia*-specific target to potentiate current antibiotics (48–50). The fact that the Mla pathway is present and is genetically similar in more than 675 *Burkholderia* sequenced strains (15), including the level 3 pathogens *B. mallei* and *B. pseudomallei*, for which the functionality seems similar to that of the Bcc (25), increases the value of developing a combination therapy against these multidrug-resistant pathogens by exploiting the Mla pathway. Therefore, targeting the Mla pathway with an antibiotic adjuvant (e.g., nonantibiotic molecule) in combination with macrolides, for example, should enhance *Burkholderia* killing and avoid enhanced killing of commensal bacteria, such as *E. coli*. In addition to characterizing the mode of action of potential nonantibiotic molecules used in combination therapy, Bcc *mIa* mutant strains could also be used as a high-throughput screening tool to find new antimicrobial compounds that could be further developed for treating *Burkholderia* infections.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are described in Table S5 in the supplemental material. Bacterial strains were routinely grown in LB-Miller broth or on 1.5% LB-Miller agar (EMD Chemicals Inc., Gibbstown, NJ), supplemented with antibiotics when appropriate and incubated at 37°C. M9 minimal medium (Becton, Dickinson and Company [BD], Sparks, MD) containing 0.5% Casamino Acids (M9_{C_{AA}}) was used for swarming motility assays, as previously described (20). Antibiotics were added to the culture medium of *Pseudomonas aeruginosa* background strains, when appropriate, at the following concentrations: tetracycline (Tet) at 150 $\mu\text{g} \cdot \text{ml}^{-1}$ and gentamicin (Gm) at 50 $\mu\text{g} \cdot \text{ml}^{-1}$. Trimethoprim (Tp) was added at 100 $\mu\text{g} \cdot \text{ml}^{-1}$ for strains of the Bcc and *Escherichia coli*. Gm and kanamycin (Km) were used at 10 and 50 $\mu\text{g} \cdot \text{ml}^{-1}$, respectively, for *E. coli*. Tet was added at 5 $\mu\text{g} \cdot \text{ml}^{-1}$ for *E. coli*, while between 50 to 150 $\mu\text{g} \cdot \text{ml}^{-1}$ was added for *Burkholderia mlaC* mutants carrying Tet^r plasmids (see “Cloning of *malCB* and complementation of the *mlaC* insertional mutants,” below). Bacterial stocks were frozen and stored at -80°C in 10% skim milk (BD). Phenazine-1-carboxylic acid (PCA) was purchased from Princeton BioMolecular Research (catalogue no. PBMRO30094; Princeton, NJ) and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 2 mg $\cdot \text{ml}^{-1}$. Pyocyanin (PYO) was purchased from Sigma-Aldrich (catalogue no. P0046; St. Louis, MO) and dissolved in DMSO at a final concentration of 10 mg $\cdot \text{ml}^{-1}$. All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Extraction of bacterial spent medium. Bacterial spent medium was extracted as previously described (9, 20). Briefly, bacterial cells from overnight cultures (4 ml of LB medium in borosilicate glass test tubes) were spun down (2 min at 8,000 rpm) and washed once in phosphate-buffered saline (PBS). Flasks containing 25 ml of culture medium were inoculated with 80 μl of washed cells (1:300 dilution) and incubated for 24 h at 37°C with shaking (175 rpm). Bacterial cells were subsequently spun down, and the resulting supernatant was filtered using 0.2- μm filters (Millipore) and stored at 4°C.

Genetic manipulations. Restriction enzymes, T4 DNA ligase, and Phusion high-fidelity DNA polymerase were obtained from New England BioLabs (Ipswich, MA).

(i) Construction of single crossover insertional mutants in *B. cenocepacia* K56-2 and *B. dolosa* PC543. Insertional mutants in *mlaE* (BCAL0302), *mlaC* (BCAL0305), and BCAL0499 were carried out using the suicide vectors pMQ87Tp-*mlaE*, pMQ87Tp-*mlaC*, and pMQ87Tp-BCAL0499, respectively (Table S5). These vectors were created using the primer pairs BCAL0302_5L-3L, BCAL0305_5L-3L, and BCAL0499_5L-3L, respectively (see Table S6 in the supplemental material). An internal DNA fragment (~500 to 700 bp) from the gene to be mutated was PCR amplified from *B. cenocepacia* strain K56-2 and subsequently cloned into pMQ87Tp (linearized with SmaI) via homologous recombination in *Saccharomyces cerevisiae*, as previously described (51). To take advantage of the efficient homologous recombination of *S. cerevisiae*, PCR primers were 60 bp long each, with 40 bp matching to the DNA sequence flanking each side of the SmaI site used to linearize pMQ87Tp. In addition to a bacterial origin of replication, this plasmid also carries an origin of replication for *S. cerevisiae*. Auxotrophy to uracil is used as a selection marker in yeast for the recombined plasmid. The resulting plasmid in yeast was isolated and then transformed into *E. coli* DH5 α cells, for which newly extracted plasmid was sequenced (MOBIX Lab, McMaster University, Hamilton, ON, Canada), using the universal primers M13 forward and reverse (see Table S6 in the supplemental material) for verification. Bcc strains were then conjugated by triparental mating, using pRK2013 as the mobilizing plasmid (52). Transconjugants were selected on *Pseudomonas* isolation agar (PIA; BD) containing 100 $\mu\text{g} \cdot \text{ml}^{-1}$ of Tp.

(ii) Cloning of *mlaCB* and complementation of the *mlaC* insertional mutants. A 1,367-bp DNA fragment containing the *mlaCB* genes and the upstream region was PCR amplified from *B. cenocepacia* K56-2 genomic DNA using Phusion high-fidelity DNA polymerase with the primers *mlaCB*-For and *mlaCB*-Rev (Table S6). The PCR product was then subcloned into pCR2.1 using the TA cloning kit (Invitrogen). Since the Phusion enzyme does not leave 3' A overhangs, an extra extension cycle of 10 min at 72°C with Taq DNA polymerase (Invitrogen) was performed prior to the subcloning step into *E. coli* DH5 α cells. The resulting plasmid, pCR2.1-*mlaCB*, was then sequenced (MOBIX Lab), using the universal primers M13 forward and reverse (Table S6) for verification. Then, a 1,321-bp KpnI-EcoRI fragment from pCR2.1-*mlaCB* was cloned into pHERD26T (53) digested with the same enzymes in the opposite direction of the arabinose-inducible promoter to generate pHERD26T-*mlaCB*. *E. coli* strains carrying pHERD26T and pHERD26T-*mlaCB* were then conjugated into *Burkholderia* strains by triparental mating, using pRK2013 as the mobilizing plasmid (52). Tet was used at a concentration of 50 $\mu\text{g} \cdot \text{ml}^{-1}$ in *mlaC* mutants of *B. cenocepacia* K56-2 and *B. dolosa* PC543 carrying pHERD26T, while 150 $\mu\text{g} \cdot \text{ml}^{-1}$ Tet was used with pHERD26T-*mlaCB*.

(iii) Transposon mutant libraries in *B. cenocepacia* K56-2. Random transposon mutagenesis was performed as previously described (54, 55). Briefly, plasmid pSC ρ haBout (55) was conjugated into *B. cenocepacia* K56-2 by triparental mating, using pRK2013 as the mobilizing plasmid (52). Transconjugants were selected on LB agar plates containing the required antibiotics (100 and 50 $\mu\text{g} \cdot \text{ml}^{-1}$ of Tp and Gm, respectively). Transposon mutants were transferred to 96-well microtiter plates containing 100 μl per well of LB (with Tp at 100 $\mu\text{g} \cdot \text{ml}^{-1}$) and allowed to grow at 37°C for 16 to 20 h. Glycerol was then added to each well at a final concentration of approximately 15% before plates were sealed and stored at -80°C .

(iv) Screening of the transposon mutant libraries. The transposon library (40 plates) was used to identify mutants with impaired ability to grow upon exposure to sublethal concentrations (25%, vol/vol) of *P. aeruginosa* (strain PA14) metabolites. Briefly, microtiter plates containing 100 μl per well of LB (with Tp at 100 $\mu\text{g} \cdot \text{ml}^{-1}$) were inoculated directly from each plate of the frozen library using a 96-pin replicator and incubated with shaking (175 rpm) at 37°C overnight. Overnight cultures were then

transferred with a 96-pin replicator onto LB agar plates containing filtered spent medium from *P. aeruginosa* (strain PA14) at a final concentration of 25% and incubated for 24 h at 37°C and room temperature for an extra 72 h. Growth of transposon mutants was monitored daily and those impaired in growth on spent medium agar were retested at least 2 more times.

(v) Mapping of transposon insertion sites. Transposon insertion mapping was performed as previously described (54, 55). Briefly, genomic DNA was extracted from overnight cultures of selected transposon mutants by using the Promega Wizard genomic DNA purification kit (Promega, Madison, WI), digested with NotI or XhoI restriction enzymes (NEB), self-ligated using T4 DNA ligase (Rapid DNA ligation kit, Roche, Germany), and transformed into *E. coli* DH5 α competent cells. Transformants were selected on LB agar plates containing 100 $\mu\text{g} \cdot \text{ml}^{-1}$ of Tp. Plasmids from these respective transformants were extracted and sequenced (MOBIX Lab) using primer 824 (55). Sequences obtained were then compared using BLAST to sequences from the complete genome of *B. cenocepacia* strain J2315 (13) at the *Burkholderia* Genome Database (www.burkholderia.com) (15).

Monoculture and coculture growth. Competitive experiments and bacterial growth curves were performed as previously described (9). Briefly, overnight cultures were spun down (2 min at 8,000 rpm) and washed once in phosphate-buffered saline (PBS) and used for inoculating liquid cultures in shaken flasks.

(i) Monoculture and coculture. Flasks containing 25 ml of culture medium were inoculated with 80 μl of washed cells (1:300 dilution) and incubated for 24 h at 37°C with shaking (175 rpm).

(ii) Bacterial survival quantification. Viable cell counts were used to determine bacterial growth or survival of both monoculture and coculture growth. Bacterial cultures were serially diluted and plated on agar-containing medium with a detection limit of 200 CFU $\cdot \text{ml}^{-1}$. To accurately quantify *B. cenocepacia* viable counts in cocultures, LB agar was supplemented with Gm (50 $\mu\text{g} \cdot \text{ml}^{-1}$) to counter select *P. aeruginosa*.

Antibiotic susceptibility assays. Antibiotic sensitivity was performed using the disk diffusion assay and the broth microdilution method.

(i) Disk diffusion. LB agar plates were entirely streaked with cotton swabs that were previously immersed into LB medium containing test bacteria that were diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 from an overnight culture. Antibiotic disks or disks containing *Pseudomonas* toxins with concentrations stated in the text (i.e., PCA, PYO, or dirhamnolipids) were then deposited on inoculated agar and incubated at 37°C for 24 to 48 h.

(ii) Broth microdilution method. MIC values of selected antibiotics was determined by macrodilutions in LB as previously described (56), with minor modifications. Briefly, overnight cultures were diluted to an OD₆₀₀ of 0.1 and added to 96-well plates at a final dilution of 1:200 (vol/vol). The final volume per well was 100 μl , including the tested antibiotic serially diluted. Inoculated plates were sealed with a sticky, breathable membrane (catalogue no. 9123-6100; Breathe-Easy, USA Scientific) and incubated for 24 h at 37°C with shaking (175 rpm). The last dilution without visual growth was considered the MIC value.

Colony morphotype assay. Morphology assays were performed as previously described (20). Briefly, bacterial cells from overnight cultures were pelleted for 2 min at 8,000 rpm and washed with PBS, and a final 10⁻⁶-fold dilution was plated on agar-containing spent medium at a final concentration of 1% (vol/vol) and incubated at 37°C for 24 to 48 h.

Swarming motility assays. Assays were performed as previously described (20). Briefly, 2 μl of an overnight culture was spotted in the middle of swarm plates allowed to dry for 1 h at room temperature and then incubated at 37°C for 24 to 48 h. Swarm plates were made of M9_{CAA} supplemented with agar at 0.5% (wt/vol) supplemented or not supplemented with 1% (vol/vol) bacterial spent medium.

SDS-EDTA sensitivity assay. Susceptibility to SDS-EDTA was performed as previously described (18), with a few modifications. Briefly, 2- μl aliquots overnight cultures in LB were spotted on agar and incubated at 37°C for 48 h. Growth of spotted colonies on regular LB agar was then compared to growth on agar containing 0.55 mM EDTA, 0.025% SDS, or 0.025% SDS–0.55 mM EDTA.

Serum bactericidal assay. Heparinized blood was collected from 5 healthy volunteers (4 females and 1 male), as approved by the Hamilton Integrated Research ethics board. Once coagulated, collecting tubes were centrifuged for 10 min, and human serum (hSerum) was frozen at –80°C. Resistance to hSerum was performed as previously described (57), with a few modifications. Prior to use, sera from all volunteers were pooled equally and diluted with PBS to a final concentration of 30% (vol/vol). Briefly, overnight cultures in LB were washed in PBS and 1 μl ($\sim 1 \times 10^6$ to 3×10^6 CFU $\cdot \text{ml}^{-1}$) was added to 100 μl of 30% hSerum and incubated for 2 h at 37°C. Incubation in PBS was performed in parallel as the negative control. Serum was heat inactivated (HihSerum) at 65°C for 30 min. Viable bacteria at time zero and after 2 h were serially diluted and spotted on agar for enumeration.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00156-18>.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

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