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In Vitro Susceptibility of *Burkholderia vietnamiensis* to Aminoglycosides▽

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***Burkholderia cepacia* complex (BCC) bacteria are opportunistic pathogens that can cause severe disease in cystic fibrosis (CF) patients and other immunocompromised individuals and are typically multidrug resistant. Here we observed that unlike other BCC species, most environmental and clinical *Burkholderia vietnamiensis* isolates were intrinsically susceptible to aminoglycosides but not to cationic antimicrobial peptides or polymyxin B. Furthermore, strains acquired aminoglycoside resistance during chronic CF infection, a phenomenon that could be induced under tobramycin or azithromycin pressure *in vitro*. In comparing susceptible and resistant *B. vietnamiensis* isolates, no gross differences in lipopolysaccharide structure were observed, all had lipid A-associated 4-amino-4-*deoxy*-L-arabinose residues, and all were resistant to the permeabilizing effects of aminoglycosides, a measure of drug entry via self-promoted uptake. However, susceptible isolates accumulated 5 to 6 times more gentamicin than a resistant isolate, and aminoglycoside susceptibility increased in the presence of an efflux pump inhibitor. *B. vietnamiensis* is therefore unusual among BCC bacteria in its susceptibility to aminoglycosides and capacity to acquire resistance. Aminoglycoside resistance appears to be due to decreased cellular accumulation as a result of active efflux.**

The *Burkholderia cepacia* complex (BCC) is a group of Gram-negative bacteria that can cause severe respiratory disease in individuals with cystic fibrosis (CF) or chronic granulomatous disease (36). BCC infections in CF patients are associated with enhanced morbidity and mortality compared to infections caused by the more common organism *Pseudomonas aeruginosa* (9), and in a subset of patients, can lead to rapid clinical deterioration characterized by bacteremia (26). Of the 17 species in the complex, all but *Burkholderia ubonensis* have been isolated from patients with CF (50, 51). Treatment of BCC infections is greatly impaired by the high intrinsic resistance of most strains to a broad range of antimicrobials, including polycationic agents such as aminoglycosides and polymyxins (39, 41, 52). The distribution of this resistance and the mechanisms involved have not been fully elucidated in the BCC.

Aminoglycosides target bacterial ribosomes and exert pleiotropic effects on cells, including interference with protein synthesis and disruption of membrane integrity (17, 18). Inhaled tobramycin is currently recommended by the Cystic Fibrosis Foundation for treatment of persistent *P. aeruginosa* pulmonary infections in CF patients 6 years of age and older (15). With the emergence of multidrug-resistant Gram-negative bacteria, polymyxins have been used increasingly, especially inhaled colistin for therapy of respiratory *P. aeruginosa* infections (15). In the last 2 decades, cationic antimicrobial peptides have become appealing as potential new therapeutic agents for a variety of conditions (20). Although cationic peptides display promising activity against *P. aeruginosa* and other CF pathogens (56), they are generally ineffective against members of the BCC (3, 45, 46, 49).

Bacterial resistance to polycationic antimicrobials is often attributed to outer membrane impermeability resulting from lipopolysaccharide (LPS) modifications or to active efflux. In Gram-negative bacteria, cationic agents competitively displace divalent cations that cross-bridge anionic LPS molecules to destabilize the outer membrane and promote their own entry into the cell, a process termed self-promoted uptake (18, 19). The interaction relies on the availability of phosphate groups at the lipid A domain. Several organisms, including CF strains of *P. aeruginosa* (13), modify their lipid A structure with the addition of polar groups such as 4-amino-4-*deoxy*-L-arabinose (Ara4N) (43). Ara4N neutralizes the negative charge of the phosphate residue to which it binds, thereby reducing bacterial susceptibility to cationic antimicrobials (14, 43). Moreover, mutations in genes that code for proteins involved in the assembly of the O polysaccharide (44) and core oligosaccharide (34) portions of LPS can contribute to increased resistance to tobramycin and cationic peptides, respectively. BCC lipid A contains at least one Ara4N residue (10, 23–25), and furthermore, polymyxin and protegrin-1, a cationic peptide, bind poorly to whole BCC bacteria and to purified BCC LPS (1, 38). Efflux systems that accommodate aminoglycosides have been identified in a number of organisms, including *P. aeruginosa* and *Burkholderia pseudomallei* (42). Homologues of these have been reported for the BCC (5, 11, 16). Deletion of genes encoding putative resistance-nodulation-division (RND)

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transporters and affiliated proteins causes enhanced aminoglycoside susceptibility in *Burkholderia cenocepacia* (11, 16).

We observed that *Burkholderia vietnamiensis* is unusual among BCC organisms in its susceptibility to aminoglycosides, yet it remains resistant to other cationic agents. We report here an investigation of the intrinsic susceptibility and acquired resistance of *B. vietnamiensis* to aminoglycosides, using patient data and *in vitro* assays.

(Part of this work was presented at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 12 to 15 September 2009 [28] and at the 24th North American Cystic Fibrosis Conference, Baltimore, MD, 21 to 23 October 2010 [27].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Isolates were selected from the BCC experimental strain panel (35), the Canadian BCC Research and Referral Repository (University of British Columbia), or the CF Foundation *Burkholderia cepacia* Research Laboratory and Repository (University of Michigan). A complete strain list is available upon request. Sequential clinical isolates were evaluated for strain type by random amplified polymorphic DNA analysis using established methods (47). *B. vietnamiensis* CF isolates from patients Bv1 (C8395, C8952, and D0774), Bv2 (D0099, D1632, D2074, D2075, and D2455), and Bv3 (D0072, D1389, and D2910) were further typed by pulsed-field gel electrophoresis as described previously (47). Bacteria were stored at −80°C in Mueller-Hinton (MH) broth with 8% (vol/vol) dimethyl sulfoxide. After subculture on MH agar or Luria-Bertani (LB) agar (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter sodium chloride, 15 g/liter agar), a single colony was grown at 37°C in cation-adjusted MH broth (CAMHB) (pH 7.3) or LB medium (pH 7.1), respectively, with aeration by shaking. Growth curves in CAMHB were determined for C8395, C8952, D0774, D1389, and G4. Briefly, cultures were grown to exponential phase and diluted to 5×10^5 CFU/ml in 25 or 50 ml of CAMHB. Samples were taken at 0, 1, 2, 4, 6, 8, 12, and 24 h, serially diluted 10-fold up to 9 times in phosphate-buffered saline, and plated in triplicate on MH agar. Viable counts were obtained after overnight growth at the minimal dilution where distinct, accurately countable colonies were present.

Patient data. Forced expiratory volume in 1 s (FEV_1) and antimicrobial therapy data were extracted from hospital charts for patients chronically infected with *B. vietnamiensis*, from the time of their initial colonization until their death or most recent isolate, as reported previously (57). Ethical approval for this study was obtained from the University of British Columbia, BC Children's and Women's Hospital, and Providence Health Services Authority research ethics boards.

Antimicrobial susceptibility testing. MICs were determined using established agar dilution and broth microdilution methods (8), with the exception of cationic peptide MICs, which were determined based on a previously described modified broth microdilution method (54). Briefly, peptide solutions were prepared in 96-well polypropylene microtiter plates with 0.2% bovine serum albumin and 0.01% acetic acid. Susceptibility testing data with the efflux pump inhibitor MP 601384 were generously provided by O. Lomovskaya (Mpex Pharmaceuticals, San Diego, CA). *P. aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* ATCC 25922 were used as quality control organisms.

***In vitro* selection of aminoglycoside-resistant bacteria.** Overnight cultures were diluted 1 in 10 in 25 ml of CAMHB containing 1 μg/ml tobramycin. Resistant bacteria were selected following serial inoculations into 25 ml of CAMHB containing exponentially increasing concentrations of tobramycin. Passages were done every 24 h, at which time aminoglycoside MICs of the cultures were determined. Inoculations were repeated until the tobramycin MICs for bacteria derived from susceptible early isolates matched those for resistant late isolates. Resultant cultures were plated out on antibiotic-free agar three successive times, after which aminoglycoside MICs were evaluated. Isolates were frozen, and aminoglycoside MICs of the frozen cultures were determined after 10 and 20 passages on antibiotic-free agar. Susceptibility of the resultant bacteria to nonaminoglycoside antibiotics was also determined. In addition, overnight cultures were diluted 1 in 10 in 50 ml of CAMHB containing 1, 2, 4, 8, or 16 μg/ml azithromycin. After 24 h, aminoglycoside MICs of the cultures were determined.

LPS purification and analysis. LPS was isolated from bacterial cells grown in LB using the proteinase K digestion method (22) and, additionally, the hot water-phenol extraction method for sequential isolates C8395, C8952, and D0774 (53). After the latter, purification was performed to remove potential Toll-like receptor 2 activating proteins (21). LPS fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels (22) stained with silver nitrate by use of a Pierce silver stain kit (Thermo Fisher Scientific) per the manufacturer's protocol. Controls included smooth LPS from *E. coli* 0111 (Invitrogen) and from *Burkholderia multivorans* C5568 and *B. cenocepacia* C6433 (previously extracted [7]).

Lipid A isolation and mass spectroscopy. LPS was isolated from overnight cultures grown in LB medium supplemented with 1 mM $MgCl_2$ by a rapid small-scale method for mass spectrometry analysis (55). Lipid A was isolated by SDS-based hydrolysis (6). Negative-ion matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry was performed as described previously (13). Experiments were performed using a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics), and each spectrum was an average of 300 shots.

Aminoglycoside outer membrane interaction studies. The hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN) (Sigma-Aldrich) was used to study the permeabilizing effects of aminoglycosides on bacterial cells as described previously (31). Briefly, cultures were prepared in 5 mM sodium HEPES buffer (pH 7.2) containing 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to an optical density at 600 nm (OD_{600}) of 0.5. Incubation of cells with CCCP prior to the addition of NPN ensured that fluorescence was optimized by trapping it in the hydrophobic compartment. NPN was added to a final concentration of 10 μM. Excitation and emission wavelengths were set at 350 and 420 nm, respectively. Fluorescence was measured using a Perkin Elmer LS 50B fluorescence spectrophotometer (Perkin Elmer). *P. aeruginosa* ATCC 27853 was used as a control organism.

Measurement of aminoglycoside cellular accumulation. The accumulation of [^{3}H]gentamicin in bacterial cells was determined as described previously (4), with the following modifications. Overnight cultures were diluted in 50 ml of LB medium and grown to an OD_{600} of 0.5. Ten-milliliter cell suspensions were incubated for another 10 min prior to the addition of a mixture of [^{3}H]gentamicin (1 mCi/ml; American Radiolabeled Chemicals Inc., St. Louis, MO) and unlabeled gentamicin to a final concentration of 5 or 20 μg/ml, with a specific activity of 88 dpm/ng. Two-hundred-microliter samples were removed at various time points, diluted in 2 ml of LB medium, and filtered through 0.22-μm-pore-size membrane filters that had been presoaked with 5 or 20 μg/ml gentamicin to prevent nonspecific binding of labeled antibiotic. Filters were then washed with 5 ml of ice-cold 0.1 M LiCl-50 mM K_2HPO_4, pH 7.0, air dried, and used for determination of radioactivity in a Beckman LS 6000IC liquid scintillation counter (Beckman Coulter). Treatment of filters with LiCl removes aminoglycosides not internalized by bacteria due to the high binding affinity of the chemical for the antibiotic. LB medium containing only [^{3}H]gentamicin was used as a control. Bacterial growth was determined by measurement of the OD_{600}. Control experiments showed that there was no difference in killing between the mixture of [^{3}H]gentamicin and unlabeled gentamicin, as determined by broth microdilution MIC testing and growth curve analysis in the presence of the antibiotics. *P. aeruginosa* ATCC 27853 was used as a control organism. The difference in [^{3}H]gentamicin accumulation between C8395 and D0774 was analyzed using means and the unpaired Student *t* test.

RESULTS

Many *B. vietnamiensis* isolates are susceptible to aminoglycosides but not to cationic antimicrobial peptides or polymyxin B. Agar dilution MIC testing of 133 isolates identified *B. vietnamiensis* as the most susceptible to aminoglycosides of the four species investigated (Table 1). *B. vietnamiensis* isolates were also more susceptible to macrolide and β-lactam antibiotics, most notably imipenem and meropenem (data not shown). At concentrations that represent antimicrobial susceptibility breakpoints for *P. aeruginosa* (8) (aminoglycoside breakpoints for BCC species are not defined), 56.4%, 12.8%, and 19.2% of *B. vietnamiensis* isolates were inhibited at 16 μg/ml amikacin, 4 μg/ml gentamicin, and 4 μg/ml tobramycin, respectively, while considerably fewer *B. cepacia* and *B. multivorans* isolates and no *B. cenocepacia* isolates were inhibited at these antimicrobial concentrations (Table 1). *B. vietnamiensis* environmental isolates were particularly susceptible to amino-

TABLE 1. Antimicrobial susceptibilities of *Burkholderia cepacia* complex species to aminoglycosides

Species and type of isolates[a] (*n*)	Test agent[b]	MIC range (μg/ml)	No. (%) of isolates inhibited at indicated concn (μg/ml)			
			1	4	16	64
B. vietnamiensis						
Clinical CF isolates (58)	AMK	2–>128	0	5 (8.6)	26 (44.8)	47 (81.0)
	GEN	≤0.5–>128	2 (3.4)	4 (6.9)	11 (19.0)	36 (62.1)
	KAN	1–>128	4 (6.9)	15 (25.9)	40 (69.0)	55 (94.8)
	TOB	≤0.5–>128	2 (3.4)	7 (12.1)	33 (56.9)	53 (91.4)
Clinical non-CF isolates (10)	AMK	1–32	1 (10.0)	2 (20.0)	9 (90.0)	10 (100.0)
	GEN	≤0.5–64	1 (10.0)	2 (20.0)	3 (30.0)	10 (100.0)
	KAN	1–16	2 (20.0)	5 (50.0)	10 (100.0)	10 (100.0)
	TOB	≤0.5–32	2 (20.0)	3 (30.0)	9 (90.0)	10 (100.0)
Environmental isolates (10)	AMK	1–>128	3 (30.0)	5 (50.0)	9 (90.0)	9 (90.0)
	GEN	≤0.5–>128	3 (30.0)	4 (40.0)	7 (70.0)	9 (90.0)
	KAN	≤0.5–>128	3 (30.0)	6 (60.0)	9 (90.0)	9 (90.0)
	TOB	≤0.5–>128	3 (30.0)	5 (50.0)	9 (90.0)	9 (90.0)
Total (78)	AMK	1–>128	4 (5.1)	12 (15.4)	44 (56.4)	66 (84.6)
	GEN	≤0.5–>128	6 (7.7)	10 (12.8)	21 (26.9)	55 (70.5)
	KAN	≤0.5–>128	9 (11.5)	26 (33.3)	59 (75.6)	74 (94.9)
	TOB	≤0.5–>128	7 (9.0)	15 (19.2)	51 (65.4)	72 (92.3)
B. cepacia (all sources) (13)	AMK	4–>128	0	1 (7.7)	1 (7.7)	5 (38.5)
	GEN	≤0.5–>128	1 (7.7)	1 (7.7)	1 (7.7)	3 (23.1)
	KAN	2–>128	0	1 (7.7)	2 (15.4)	6 (46.2)
	TOB	≤0.5–>128	1 (7.7)	1 (7.7)	1 (7.7)	6 (46.2)
B. multivorans (all sources) (23)	AMK	16–>128	0	0	1 (4.3)	12 (52.2)
	GEN	16–>128	0	0	1 (4.3)	10 (43.5)
	KAN	4–>128	0	1 (4.3)	9 (39.1)	16 (69.6)
	TOB	8–>128	0	0	8 (34.8)	14 (60.9)
B. cenocepacia (all sources) (19)	AMK	32–>128	0	0	0	4 (21.1)
	GEN	32–>128	0	0	0	4 (21.1)
	KAN	8–>128	0	0	2 (10.5)	5 (26.3)
	TOB	16–>128	0	0	1 (5.3)	6 (31.6)

[a] *B. cepacia* sources: 6 CF isolates, 4 clinical non-CF isolates, and 3 environmental isolates. *B. multivorans* sources: 11 CF isolates, 4 clinical non-CF isolates, and 8 environmental isolates. *B. cenocepacia* sources: 10 CF isolates, 4 clinical non-CF isolates, and 5 environmental isolates. Isolates were selected from the Canadian BCC Research and Referral Repository (University of British Columbia) and the CF Foundation *Burkholderia cepacia* Research Laboratory and Repository (University of Michigan).

[b] AMK, amikacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin.

glycosides, while CF isolates were most resistant (Table 1). The MIC ranges for the first isolates for six patients, however, were markedly different from the MIC ranges for all CF isolates combined: 2 to 8 versus 2 to >128 μg/ml for amikacin, 4 to 32 versus ≤0.5 to >128 μg/ml for gentamicin, 1 to 8 versus 1 to >128 μg/ml for kanamycin, and 2 to 8 versus ≤0.5 to >128 μg/ml for tobramycin (Table 2 and data not shown). MIC ranges for all sources were extensive (Table 1).

To determine if aminoglycoside-susceptible *B. vietnamiensis* isolates were also susceptible to cationic antimicrobial peptides and polymyxin B, the activities of these agents against a subset of isolates were evaluated by broth microdilution (Table 2 and data not shown). The activities of natural and synthetic cationic peptides against the BCC experimental strain panel (35) were also determined (data not shown). Virtually all *B. vietnamiensis* isolates were highly resistant to the cationic antimicrobial peptides and to polymyxin B, with the majority having MICs of >128 μg/ml and >75 μg/ml, respectively (Table 2 and data not shown). Only *B. vietnamiensis* CEP0106 was moderately susceptible to one peptide, CP26, with a MIC of 8 μg/ml. Isolates from other BCC species were also highly resistant to the cationic peptides, with the exception of a lab strain, *B. multivorans* 249-2, which was relatively susceptible to CP26 and CP29, with MICs of 8 and 4 μg/ml, respectively (data not shown). Of the peptides, CP29 had the greatest antimicrobial activity against BCC species (data not shown).

***B. vietnamiensis* acquires aminoglycoside resistance *in vivo* and under antibiotic pressure *in vitro*.** Evaluation of aminoglycoside susceptibility in sequential CF isolates C8395, C8952, and D0774 from patient Bv1 and D0099 and D2075 from patient Bv2 revealed that *B. vietnamiensis* acquired resistance to aminoglycosides during chronic infection; sequential isolates from the two patients showed ≥32-fold and ≥4-fold increases in aminoglycoside MICs, respectively (Table 2). Increases in macrolide, β-lactam, and ciprofloxacin MICs were also observed (data not shown). To examine the phenomenon of acquired aminoglycoside resistance further, patient antimicrobial therapy data and the tobramycin susceptibilities of all sequential isolates were analyzed for three chronically *B. vietnamiensis*-infected patients, from the time of their initial colonization until their death or most recent isolate (Fig. 1A to C). Lung function data were included as a general indicator of

TABLE 2. Antimicrobial susceptibilities of *Burkholderia vietnamiensis* isolates to aminoglycosides, cationic antimicrobial peptides, and polymyxin B

Isolate[a]	MIC (μg/ml)[b]						
	AMK	GEN	KAN	TOB	Bac2a	LL-37	PMB
Clinical CF isolates							
C8395 (Bv1, 3/11/1998)	2	4	2	2	>128	>128	>75
C8952 (Bv1, 7/12/1999)	2	4	2	4	>128	>128	>75
D0774 (Bv1, 25/7/2003)	>128	128	128	128	>128	>128	>75
D0099 (Bv2, 23/4/2002)	8	4	4	2	>128	>128	>75
D2075 (Bv2, 18/5/2006)	32	32	16	32	>128	>128	>75
D1389 (Bv3, 6/12/2004)	0.5	1	0.5	1	>128	>128	>75
Clinical non-CF isolate							
LMG 06999	0.5	0.5	0.25	0.5	>128	>128	>75
Environmental isolates							
FC0656	0.25	0.5	0.25	0.25	>128	>128	37.5
CEP0106	4	4	4	4	>128	>128	>75
LMG 10929^T	2	2	2	4	>128	>128	>75
G4	0.25	0.5	0.25	0.25	>128	>128	>75

[a] Patient identification numbers and bacterial isolation dates (day/month/year) are noted in parentheses for serial clinical isolates. Isolates were selected from the Canadian BCC Research and Referral Repository (University of British Columbia).

[b] Peptides tested but not included in the table included the following: K24, E2, and E6, synthetic derivatives of a bovine bactenecin; CP26 and CP29, analogues based on the insect cecropin-bee melittin hybrid peptide; bovine indolicidin; and horseshoe crab polyphemusin I. Abbreviations: AMK, amikacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin; Bac2A, synthetic derivative of a bovine bactenecin; LL-37, human cathelicidin; PMB, polymyxin B.

patient health. Patients received 19, 9, or no courses of tobramycin treatment while infected with *B. vietnamiensis* (Fig. 1A to C). The tobramycin MICs of infecting strains increased from 2 to 128 μg/ml, from 2 to >128 μg/ml, and from 1 to 32 μg/m1 (Fig. 1A to C). MIC reversions occurred in strains infecting patients Bv2 and Bv3 (Fig. 1A to C). Patients Bv1 and Bv2 were coinfected with *P. aeruginosa*, against which tobramycin therapy may have been directed. None of the patients were treated with any other aminoglycoside antibiotics. Patients did receive a number of other antimicrobial treatments, including courses with various β-lactam antibiotics, ciprofloxacin, colimycin, chloramphenicol, azithromycin, and septra (data not shown).

To determine if tobramycin alone could induce acquired aminoglycoside resistance in *B. vietnamiensis*, tobramycin susceptibility was evaluated under antibiotic pressure *in vitro* (Fig. 1D). After serial passage in broth containing tobramycin at exponentially increasing concentrations, early isolates from patients Bv1 and Bv3, namely, C8395 and D0072, respectively, acquired tobramycin resistance to the level of late isolates, i.e., MICs of 128 and 32 μg/ml, respectively (Fig. 1D). Tobramycin resistance was stable after passage on antibiotic-free medium, although 2-fold differences in the MIC were observed (Fig. 1D). Gentamicin resistance was also acquired and stable (data not shown). Susceptibilities of the resultant cultures to ciprofloxacin, meropenem, and ceftazidime were generally unchanged (where differences were noted, they were 2- or 4-fold differences and were inconsistent between isolates, and MICs remained lower than those of late isolates), while azithromycin MICs increased to levels comparable to those for late isolates (32 to >32 μg/ml) (data not shown). Aminoglycoside susceptibility was also examined after bacterial exposure to azithromycin. All patients received azithromycin therapy, and macrolide antibiotics are capable of inducing aminoglycoside resistance determinants (29). C8395 gentamicin and tobramycin MICs increased 4-fold and 2-fold, respectively, after exposure to 2, 4, 8, and 16 μg/ml azithromycin (data not shown). D0072 gentamicin and tobramycin MICs increased 2-fold after exposure to 2 and 4 μg/ml azithromycin and 8-fold after exposure to 16 μg/ml azithromycin (data not shown). No changes in aminoglycoside MICs were observed for D0072 after a 24-h exposure to 8 μg/ml azithromycin; however, the culture color changed from light brown to red brown, indicating that this concentration may have other effects on the bacterium.

LPS modifications are not responsible for aminoglycoside resistance in *B. vietnamiensis*. To determine if LPS modifications are involved in aminoglycoside resistance in *B. vietnamiensis*, LPS compositions were compared between susceptible and resistant isolates. SDS-PAGE analysis of isolated LPS molecules revealed no gross differences among serial clinical isolates C8395, C8952, and D0774 from patient Bv1 and D0099 and D2075 from patient Bv2; all had rough LPS (LPS lacking O antigen) (data not shown). Overloading gels with up to 50 μg of LPS did not show the presence of O antigen in any of these isolates (data not shown). Lipid A species from the *B. vietnamiensis* isolates listed in Table 2 were analyzed by mass spectroscopy, and representative spectra are shown in Fig. 2A to F. Consistent with previous reports (25), lipid A structures were a blend of tetra- and penta-acylated molecules (Fig. 2A to F and data not shown). Lipid A structures of aminoglycoside-susceptible and -resistant *B. vietnamiensis* isolates were positive for Ara4N, identified on spectra by mass-to-charge ratios of 1,575, 1,601, 1,802, and 1,827 (Fig. 2A to F and data not shown). Notably, these findings included lipids of serial clinical isolates for strains that had acquired aminoglycoside resistance: C8395, C8952, and D0774 from patient Bv1 (Fig. 2A to C) and D0099 and D2075 from patient Bv2 (Fig. 2D and E). Furthermore, differences in lipid A acylation patterns were observed among sequential isolates C8395, C8952, and D0774: acylation in-

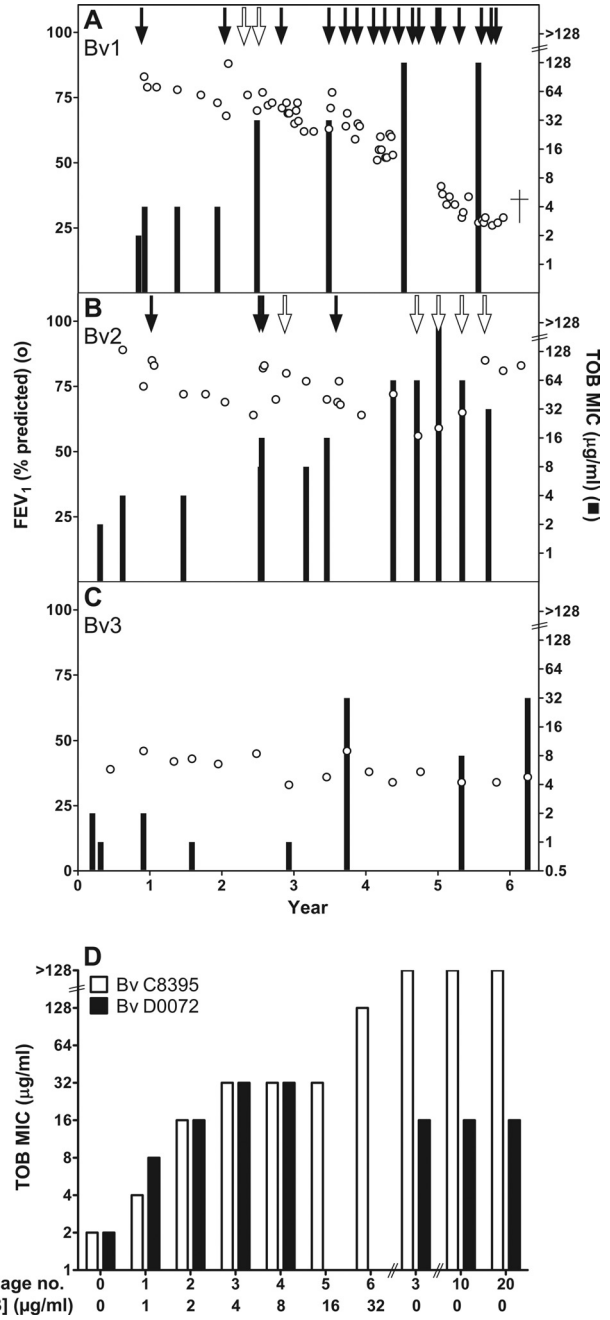

FIG. 1. *B. vietnamiensis* acquisition of aminoglycoside resistance *in vivo* and *in vitro*. Chronic *B. vietnamiensis* infections in CF patients Bv1 (A), Bv2 (B), and Bv3 (C) were evaluated based on clinical chart data. Patient FEV_1 data are shown as open circles. MICs for the infecting *B. vietnamiensis* strains are shown as bar graphs and represent triplicate experiments. In cases where multiple isolates from the same day were tested, only the highest MIC is shown (where there was a difference, it was 2-fold). Filled arrows indicate start dates of intravenous tobramycin (TOB) treatment. Open arrows indicate start dates of inhaled tobramycin treatment. A cross refers to the time of patient death. (D) Selection of aminoglycoside resistance in early isolates from patients Bv1 and Bv3 was done under tobramycin pressure *in vitro*. Isolates were serially passaged every 24 h in medium containing the antibiotic, to concentrations that represent half the MICs of late isolates (16 μg/ml for D0072 and 64 μg/ml for C8395). Susceptibility data for isolates grown at half the MICs of late isolates are not shown due to their lack of viability during analysis.

creased with time, with the lipids becoming enriched for pentaacylated molecules (Fig. 2A to C).

To confirm that differences in LPS structure that could account for differences in aminoglycoside susceptibility did not exist among the *B. vietnamiensis* isolates, we examined the interaction of the fluorescent probe NPN with the outer membranes of aminoglycoside-susceptible and -resistant isolates. Upon LPS-mediated disruption of the *P. aeruginosa* outer membrane during aminoglycoside self-promoted uptake, NPN enters the membrane hydrophobic space, with the attendant increase in fluorescence being a function of aminoglycoside-induced permeability (31). A lack of NPN fluorescence therefore results from the presence of LPS features that inhibit this drug interaction. The outer membranes of resistant and susceptible *B. vietnamiensis* isolates were not permeabilized by gentamicin (Fig. 3) or tobramycin (data not shown), as inferred from the lack of NPN fluorescence. Because the association of aminoglycosides with *B. vietnamiensis* cells may take longer than that with *P. aeruginosa*, where at high concentrations of antimicrobial an increase in NPN fluorescence is nearly instantaneous (31), the assay was extended to 20 min, but no increase in NPN fluorescence was observed (data not shown).

Efflux systems may be responsible for the decreased aminoglycoside accumulation observed in resistant *B. vietnamiensis* bacteria. To determine if decreased drug accumulation is involved in *B. vietnamiensis* aminoglycoside resistance, the cellular accumulation of [^{3}H]gentamicin was measured in the susceptible isolate D1389 and the serial clinical isolates C8395 and D0774 (Fig. 4). In aminoglycoside-susceptible isolates of *B. vietnamiensis*, [^{3}H]gentamicin accumulated at a lower rate than that in *P. aeruginosa* (4) and reached a maximum at 6 h before a plateau was noted (Fig. 4 and data not shown). Under the same conditions, the aminoglycoside-resistant isolate D0774 accumulated [^{3}H]gentamicin minimally, 5 times less than C8395 ($P < 0.05$) (Fig. 4). Differences in rates of accumulation are attributed to differences in bacterial growth rates, since aminoglycosides target dividing cells; however, these may also result from differences in drug uptake mechanisms.

To examine the involvement of an RND efflux system in *B. vietnamiensis* aminoglycoside resistance, antimicrobial MICs of susceptible and resistant isolates were determined in the presence of the efflux inhibitor MP 601384. MP 601384 has specificity toward aminoglycoside-accommodating RND efflux systems, such as MexXY-OprM of *P. aeruginosa*, and is nontoxic to bacteria (32). In the presence of 20 μg/ml of MP 601384, aminoglycoside MICs for susceptible and resistant *B. vietnamiensis* isolates decreased 2- to 6-fold (Table 3). The inhibitor had no effect on susceptibilities to other antimicrobials (data not shown).

DISCUSSION

Members of the BCC are important opportunistic pathogens that are capable of resisting therapeutic interventions (36). Current antimicrobial options for therapy of BCC infections are limited, and eradication of the organisms from patients with CF is a major challenge (2). In this study, we investigated resistance to polycationic antimicrobials in one specific species within the BCC, *B. vietnamiensis*. Though they are rare, aminoglycoside-susceptible isolates of the BCC have been noted and

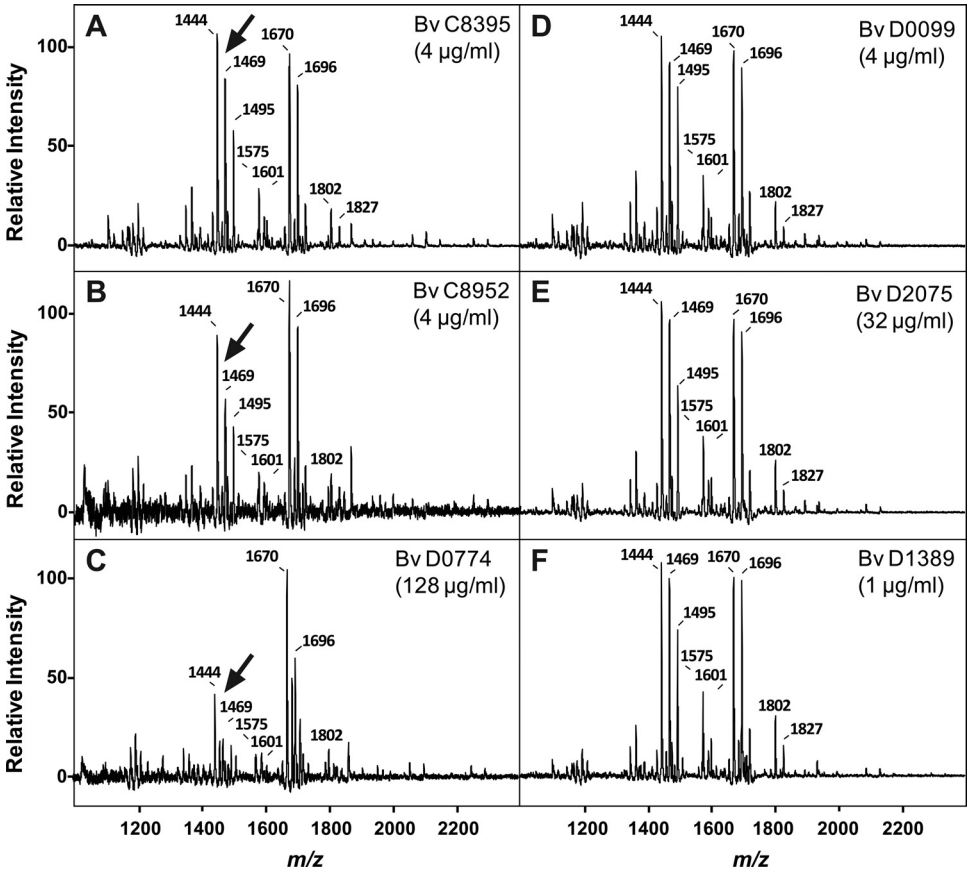

FIG. 2. *B. vietnamiensis* lipid A structural analysis. C8395 (A), C8952 (B), and D0774 (C) are sequential isolates from patient Bv1. D0099 (D) and D2075 (E) are sequential isolates from patient Bv2. (F) D1389 is an isolate from patient Bv3. MICs of gentamicin are shown in parentheses. Purified lipid A was analyzed by MALDI-TOF mass spectroscopy. Tetra- and penta-acylated molecules are identified by *m/z* 1,444, 1,469, and 1,495 and *m/z* 1,670 and 1,696, respectively. Lipid A moieties containing Ara4N are identified by *m/z* 1,575, 1,601, 1,802, and 1,827. Arrows point to changes in acylation between sequential isolates. Bv, *B. vietnamiensis*; *m/z*, mass-to-charge ratio.

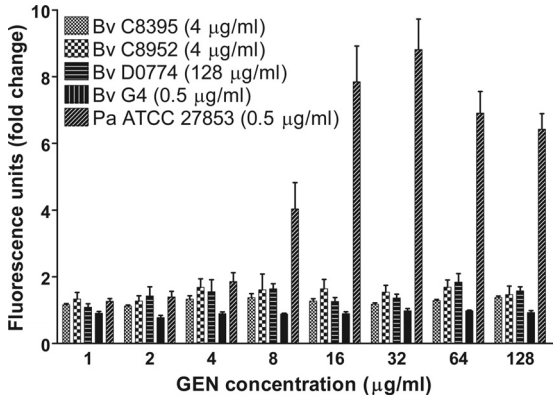

FIG. 3. Permeabilizing effects of gentamicin on *B. vietnamiensis* and *P. aeruginosa* ATCC 27853. NPN was added to cells 30 s after initiation of fluorescence readings; antibiotic was added 30 to 90 s later. Final values were taken as averages of those recorded from 200 to 500 s, when a plateau in fluorescence was observed. Fluorescence was measured at least every 10 s. Baseline NPN fluorescence was set to 1. MICs of gentamicin are shown in parentheses. Data points represent the averages for at least three biological replicates plus standard errors. Bv, *B. vietnamiensis*; GEN, gentamicin.

were found to be *B. vietnamiensis* (39, 52). Our results show that *B. vietnamiensis* is in fact often susceptible to aminoglycosides, and within the BCC, it is uniquely susceptible to a broad range of antimicrobials, suggesting that existing drugs may be more effective at treating *B. vietnamiensis* infections than previously thought. However, *B. vietnamiensis* bacteria were highly resistant to other polycationic agents (cationic antimicrobial peptides and polymyxin B), indicating that these antimicrobials specifically remain of limited value as monotherapy against BCC infections and that resistance mechanisms can differ for different classes of polycationic antimicrobials. These results suggest that unlike other BCC species, *B. vietnamiensis* often exists in an aminoglycoside-susceptible state in its natural environment (presumably soil), though this certainly is not always the case; one environmental isolate was extremely resistant to all aminoglycosides tested, with MICs of >128 μg/ml. Little is known about environmental factors affecting antibiotic resistance in bacteria; however, it is reasonable to hypothesize that environmental cues can select for aminoglycoside-resistant strains. In *P. aeruginosa*, cationic peptides (14) and polyamines (30) can induce aminoglycoside resistance by affecting the expression of genes involved in modifying lipid A and two-component regulatory systems. Further

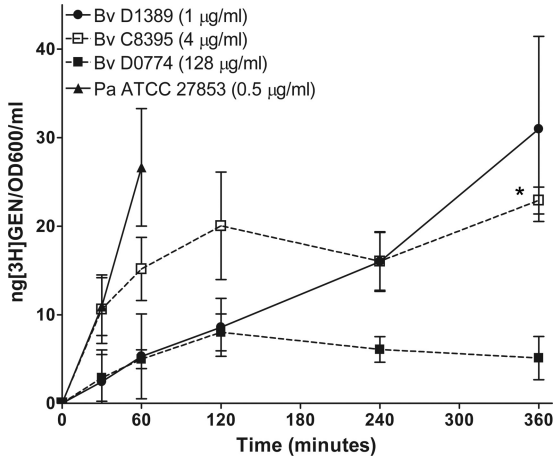

FIG. 4. Accumulation of 20 μg/ml [^{3}H]gentamicin by *B. vietnamiensis* and 5 μg/ml [^{3}H]gentamicin by *P. aeruginosa* ATCC 27853. Baseline accumulation was set as 0. MICs of gentamicin are shown in parentheses. Data points represent the averages for at least three biological replicates ± standard errors. *, $P < 0.05$ compared with *B. vietnamiensis* D0774 (unpaired Student *t* test). Bv, *B. vietnamiensis*; Pa, *P. aeruginosa*; GEN, gentamicin.

study of the natural habitat of BCC bacteria is necessary to elucidate natural inducers of antibiotic resistance determinants, as well as other virulence factors.

Furthermore, we report for the first time the acquisition of aminoglycoside resistance in *B. vietnamiensis* in chronically infected CF patients, a phenomenon that has been described for *P. aeruginosa* (12). We also identified tobramycin and azithromycin as possible sources of selective pressure for the acquisition of aminoglycoside resistance both *in vivo* and *in vitro*. At subinhibitory concentrations, ribosome-targeting antibiotics, including aminoglycosides and macrolides, are capable of inducing the expression of MexX (37) and MexY (29), components of the aminoglycoside-accommodating efflux system in *P. aeruginosa*. Azithromycin induction of aminoglycoside resistance may explain why acquired resistance in *B. vietnamiensis* was observed in the absence of aminoglycoside treatment in patient Bv3. Moreover, as mentioned above, cationic peptides, which are present in abundance in CF host cells and tissues, can also contribute to an increase in aminoglycoside resistance (14). Regardless of the mechanisms involved, the fact that *B. vietnamiensis* exposure to tobramycin and azithromycin can induce resistance to aminoglycosides is of particular concern, since both antibiotics are recommended for the treatment of CF patients who are 6 years of age and older and have persistent *P. aeruginosa* infections (15) and since CF patients can be coinfected with these two organisms (our study). Furthermore, azithromycin is administered in the absence of *P. aeruginosa* infection, as seen with patient Bv3 in our study.

In this paper, we report that LPS modifications previously associated with aminoglycoside resistance in Gram-negative bacteria (34, 43, 44) are not responsible for acquired resistance in *B. vietnamiensis*. The addition of Ara4N to lipid A via induction of LPS modification genes results in enhanced aminoglycoside resistance in *P. aeruginosa* (14, 30). The disruption of LPS O polysaccharide assembly has also been implicated in *P. aeruginosa* aminoglycoside resistance (44). Poor uptake owing to LPS modifications was not responsible for the acquisition of aminoglycoside resistance in our study of *B. vietnamiensis* isolates, since susceptible and resistant isolates showed the presence of lipid A-associated Ara4N residues and had the same LPS chemotypes. In addition, all *B. vietnamiensis* isolates tested were resistant to the permeabilizing effects of aminoglycosides, independent of aminoglycoside susceptibility, confirming that susceptible and resistant isolates contain LPS features that inhibit aminoglycoside-mediated outer membrane disruption. The biosynthesis of Ara4N residues may be essential for *B. vietnamiensis* viability, as is the case in *B. cenocepacia* (33, 40). These findings contradict the current dogma that the lack of LPS anionic binding sites is sufficient to cause resistance to polycationic antimicrobials and suggest that a mechanism of aminoglycoside entry other than self-promoted uptake exists in *B. vietnamiensis.* Notably, the presence of Ara4N residues at lipid A of *B. vietnamiensis* may still account for the observed resistance to cationic antimicrobial peptides and polymyxin B, as well as low-level aminoglycoside resistance. Importantly, differences noted in lipid A acylation patterns among sequential clinical isolates may impact their ability to stimulate immune cells such as monocytes, as found previously for this organism and others (25, 43).

The present study does suggest that decreased access of aminoglycosides to their antimicrobial target is involved in adaptive aminoglycoside resistance in *B. vietnamiensis*, as reflected in differential intracellular accumulation of [^{3}H]gentamicin between susceptible and resistant isolates. These findings also reveal that aminoglycosides can enter bacterial cells in the presence of lipid A-associated Ara4N, confirming that aminoglycoside entry in *B. vietnamiensis* does not occur via self-promoted uptake. The basis of the observed differential accumulation of the aminoglycoside antibiotic is currently unclear. Recent studies have noted the involvement of RND transporters in *B. cenocepacia* aminoglycoside resistance (5, 11, 16), and furthermore, rare aminoglycoside susceptibility in *B. pseudomallei* is attributed to the loss of expression of its major

TABLE 3. Antimicrobial susceptibilities of *Burkholderia vietnamiensis* to aminoglycosides in the presence of an RND efflux pump inhibitor

Isolate[a]	MIC (μg/ml) with (+) and without (−) addition of MP 601384[b]							
	AMK		GEN		ABK		TOB	
	−	+	−	+	−	+	−	+
Clinical CF isolates								
C8395 (Bv1, 3/11/1998)	8	1	32	1	16	1	8	≤0.5
C8952 (Bv1, 7/12/1999)	8	2	16	4	16	2	8	1
D0774 (Bv1, 25/7/2003)	>32	8	>32	4	>32	4	>32	1
D0099 (Bv2, 23/4/2002)	4	≤0.5	16	1	8	≤0.5	4	1
D2075 (Bv2, 18/5/2006)	>32	4	>32	4	>32	4	32	2
D1389 (Bv3, 6/12/2004)	2	1	4	2	2	1	1	≤0.5
Clinical non-CF isolate								
LMG 06999	2	1	2	1	2	≤0.5	1	≤0.5

[a] Patient identification numbers and bacterial isolation dates (day/month/year) are noted in parentheses for serial clinical isolates. Isolates were selected from the Canadian BCC Research and Referral Repository (University of British Columbia).

[b] Abbreviations: RND, resistance-nodulation-division; AMK, amikacin; GEN, gentamicin; ABK, arbekacin; TOB, tobramycin.

aminoglycoside-accommodating efflux pump (48). Indeed, we found that *B. vietnamiensis* aminoglycoside susceptibility increased in the presence of an inhibitor specific to known efflux systems, suggesting that an RND efflux pump is involved in aminoglycoside resistance in *B. vietnamiensis* and may be responsible for the observed apparent impermeability of resistant bacteria to gentamicin. We are currently evaluating the role of aminoglycoside efflux in BCC organisms to gain a better understanding of constitutive and acquired antimicrobial resistance in this group of opportunistic pathogens. Future investigations will also include the study of regulatory mechanisms involved in the induction of aminoglycoside resistance determinants in the BCC. Novel insights may help in the design of improved antimicrobial therapeutic regimens against BCC infections.

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