AMINOGLYCOSIDE SUSCEPTIBILITY AND ACQUIRED RESISTANCE IN *BURKHOLDERIA VIETNAMIENSIS*

by

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Abstract

The *Burkholderia cepacia* complex (BCC) group of Gram-negative bacteria are highly virulent, opportunistic pathogens in cystic fibrosis (CF) patients and other immunocompromised individuals. It is the current dogma that all species of the complex are highly and intrinsically resistant to polycationic antimicrobials, including aminoglycosides, and that this resistance is due to unusual characteristics of the lipopolysaccharide (LPS). Cationic agents enter Gram-negative bacteria through LPS-mediated uptake, relying on anionic lipid A binding sites. Here we observed that environmental and clinical isolates of *B. vietnamiensis* were more often susceptible to aminoglycosides than those of other BCC species, but were not inhibited by other cationic agents (natural and synthetic cationic antimicrobial peptides, polymyxin B). Furthermore, *B. vietnamiensis* strains acquired aminoglycoside resistance during chronic CF infection, and *in vitro* under tobramycin, azithromycin, and hydrogen peroxide pressure. *B. vietnamiensis* strains also displayed enhanced catalase activity and became less mucoid. Gentamicin and tobramycin time-kill assays revealed drug concentrations up to 8 × the minimum inhibitory concentration were unable to kill a susceptible *B. vietnamiensis* CF isolate. Aminoglycoside resistant *B. vietnamien*sis CF isolates accumulated significantly less [^{3}H]gentamicin than susceptible isolates. Aminoglycoside resistance, however, was not correlated with LPS chemotype, and mass spectrometry revealed the presence of lipid A-associated 4-amino-4-*deoxy*-L-arabinose moieties, residues that neutralize anionic lipid A binding sites, in aminoglycoside-susceptible and -resistant *B. vietnamiensis* isolates. Furthermore, permeability to the fluorescent hydrophobic probe 1-*N*-phenylnapthylamine was not enhanced following incubation with gentamicin or tobramycin in any *B. vietnamiensis* isolates. Aminoglycoside-resistant *B.*

vietnamiensis isolates overexpressed a putative resistance-nodulation-division (RND) efflux system transporter gene, *amrB.* After serial exposure to tobramycin and azithromycin, but not hydrogen peroxide, *amrB* expression was induced in an aminoglycoside-susceptible *B. vietnamiensis* CF isolate. Moreover, inhibition of the putative efflux system enhanced *B. vietnamiensis* susceptibility to aminoglycosides. These data suggest that active efflux via a RND efflux system, not LPS modification, impairs aminoglycoside accumulation in clinical *B. vietnamiensis* strains that have acquired aminoglycoside resistance, and in those exposed to tobramycin and azithromycin, but not hydrogen peroxide, *in vitro*. These new insights may help in the design of improved therapeutic regimens against *Burkholderia* species.

Preface

Ethics approval was obtained for the collection of patient data from the University of British Columbia (UBC)-Providence Health Care Research Institute (ethics certificate #H07-01396). [^{3}H]gentamicin was used under the UBC Radioisotope license PAED-3258.

Parts of Chapter 3, 4, and 5 were published in *Antimicrobial Agents and Chemotherapy* on May 1 2011 (accepted for publication on February 1 2011;). The study was designed by AN Jassem with assistance from DP Speert. Data in Table 4 and Table 5 were generated in part by JEA Zlosnik and DA Henry. Cationic peptides used to generate data in Table 6 and Table 7 were provided by REW Hancock. Patient data in Figure 2 and Table 11 were collected by JEA Zlosnik. Lipopolysaccharide in Figure 14a was extracted by RK Ernst. Lipid A in Figure 15 was extracted and analyzed by RK Ernst. Figure 15 was produced in part by RK Ernst. Data in Figure 16 were generated by AN Jassem in REW Hancock's laboratory. Data in Table 17 were generated by O Lomovskaya. AN Jassem performed all remaining experiments, analyzed the data, and wrote the manuscript.

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The remaining parts of Chapter 3, 4, and 5 were designed by AN Jassem with assistance from DP Speert. Selection of bacterial isolates after exposure to hydrogen peroxide at half the

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List of Symbols and Abbreviations

α: alpha

β: beta

γ: gamma

[^{3}H]: tritiated

ABC: ATP-binding cassette

AhpC: alkyl hydroperoxide reductase subunit C

ANOVA: analysis of variance

Ara4N: 4-amino-4-*deoxy*-L-arabinose

ATP: adenosine triphosphate

BC: British Columbia

BCC: *Burkholderia cepacia* complex

BLAST: Basic Local Alignment Search Tool

BCSA: *Burkholderia cepacia* selective agar

CBCCRRR: Canadian *Burkholderia cepacia* complex Research and Referral Repository

CCCP: carbonyl cyanide m-chlorophenylhydrazone

cci: *Burkholderia cenocepacia* island

CFU: colony forming units

CGD: chronic granulomatous disease

CF: cystic fibrosis

CFTR: cystic fibrosis transmembrane conductance regulator

CFF: Cystic Fibrosis Foundation

CMMT: Centre for Molecular Medicine and Therapeutics

DNA: deoxyribonucleic acid

EPS: exopolysaccharide

LB: Luria-Bertani

LPS: lipopolysaccharide

MALDI-TOF: matrix-assisted laser desorption ionization–time of flight

MATE: multidrug and toxic compound extrusion

MF: major facilitator

MH: Mueller Hinton

MIC: minimum inhibitory concentration

NADPH: nicotinamide adenine dinucleotide phosphate

NCBI: National Center for Biotechnology Information

NPN: 1-*N*-phenylnaphthylamine

OD_{600}: optical density at 600 nm

PAGE: polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

PFGE: pulsed-field gel electrophoresis

Q RT-PCR: real-time reverse transcription-PCR

RAPD: random amplified polymorphic DNA

RNA: ribonucleic acid

rRNA: ribosomal RNA

RND: resistance-nodulation-division

ROS: reactive oxygen species

SCFM: synthetic CF sputum medium

SDS: sodium dodecyl sulfate

SOD: superoxide dismutase

SMR: small multidrug resistance

UBC: University of British Columbia

UK: United Kingdom

US: United States

YEM: yeast extract medium

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Chapter 1: INTRODUCTION

The *Burkholderia cepacia* complex (BCC) is a group highly versatile, closely related Gram-negative, non-spore forming bacilli divided into 17 species (1). BCC species are distributed in soil, rhizosphere, and aquatic environments of natural ecosystems, where they can facilitate beneficial processes, but are also capable of causing plant disease (2, 3). They have also been identified as contaminants in clinical and industrial settings (1, 3). As human opportunistic pathogens, members of the BCC most notably cause severe respiratory infections in individuals with chronic granulomatous disease (CGD) or cystic fibrosis (CF) (4, 5). In CGD patients, BCC infections result in pneumonia and sepsis, and are the leading cause of bacterial death (6-8). BCC infection in CF patients is a significant risk factor for morbidity and mortality (9-22).

BCC species are capable of person-to-person spread, can survive within phagocytes and respiratory epithelial cells, and produce a number of virulence factors including lipopolysaccharide, an immunogenic component of the bacterial cell surface, biofilms, communities of bacteria that are better protected against external factors, and quorum sensing molecules that facilitate bacterial cell-to-cell communication (5, 23). Treatment of BCC infections is greatly impaired by the high intrinsic resistance of most strains to a broad range of antimicrobials, including aminoglycosides (24-26), a widely used group of antibiotics that has had a major impact on the treatment of bacterial infections for half a century (27) and is particularly important in the management of CF (28, 29). The goal of this thesis is to gain a better understanding of the antimicrobial susceptibilities of BCC species, as well as the induction and mechanisms of aminoglycoside resistance specifically. Antibiotic resistance is a major threat

to public health, and tackling this problem will depend in part on increasing our knowledge of resistance prevalence and bacterial factors involved (30-33).

1.1 The *Burkholderia cepacia* complex

1.1.1 Taxonomy

BCC species have had many names (1). Walter H. Burkholder first described *Pseudomonas cepacia* as the causative agent of onion rot in 1950 (34). Based on 16S ribosomal RNA sequences, DNA-DNA homology values, and phenotypic characteristics, in 1992, *P. cepacia* was transferred to the new genus *Burkholderia*, named in honour of W. H. Burkholder (35). In 1997, *B. cepacia* strains were divided into five phenotypically similar but genetically distinct genomovars, with only *B. vietnamiensis* and *B. multivorans* formally designated as species, and the group was collectively referred to as the BCC (36). In the years that followed, the development of novel differentiation tests lead to the identification of additional species within the BCC (37-44), for a current total of 17 (1), as well as the subdivision of *B. cenocepacia* on basis of *recA* gene sequencing (41, 45) (Table 1).

1.1.2 Genomics

Lessie *et al.* (46) first described the large, insertion sequence rich genomes of BCC bacteria. Since then, the sequences of *B. cenocepacia* strains J2315, H12424, AU1054, and MC0-3, *B. multivorans* strain 17616, *B. vietnamiensis* strain G4, *B. ambifaria* strains AMMD and MC40-6, and *B. lata* strain 383 have become available online. Each of these strains has three chromosomes and most an additional plasmid, with genomes ranging from 7 to 9 Mb, among the largest observed in Gram-negative bacteria (5). Only *B. vietnamiensis* G4 contains more than one plasmid, with five. In *B. cenocepacia* J2315, chromosome 1 contains genes mainly encoding

Table 1. Overview of the *B. cepacia* complex
(adapted from Vandamme and Dawyndt (47), and Vial *et al.* (48))

Species	Habitat	Relevant characteristics
B. ambifaria	Human (CF, non-CF), soil, rhizosphere	Plant growth promotion, major biocontrol agent
B. anthina	Human (CF), turtle, soil, rhizosphere, river water, plant, hospital contaminant	
B. arboris	Human (CF, non-CF), soil, rhizosphere, river water, industrial contaminant	
B. cenocepacia	Human (CF, non-CF), soil, rhizosphere, plant, river water, industrial contaminant	Major CF pathogen, epidemic strains described in CF, plant pathogen (banana), IIIA-D subgroups
B. cepacia	Human (CF, non-CF), soil, rhizosphere, plant, river water	Plant pathogen (onion)
B. contaminans	Human (CF, non-CF), sheep, contaminant, plant	
B. diffusa	Human (CF, non-CF), soil	
B. dolosa	Human (CF), plant, rhizosphere	Epidemic strains described in CF
B. latens	Human (CF)	
B. lata	Human (CF, non-CF), soil, rhizosphere, plant, river water, industrial contaminant	
B. metallica	Human (CF)	
B. multivorans	Human (CF, non-CF), soil, rhizosphere, plant, river water, contaminant	Major CF and CGD pathogen, epidemic strains described in CF
B. pyrrocinia	Human (CF, non-CF), soil, rhizosphere, river water	
B. seminalis	Human (CF, non-CF), soil, rhizosphere, plant	Plant pathogen (apricot)
B. stabilis	Human (CF, non-CF), hospital contaminant, plant	
B. ubonensis	Human (non-CF), soil	
B. vietnamiensis	Human (CF, non-CF), soil, rhizosphere, plant, river water, industrial contaminant	Plant growth promotion, nitrogen-fixation, major bioremediation agent

"housekeeping" functions, such as cell division and metabolism, while chromosomes 2 and 3 contain a greater proportion of genes encoding accessory functions, such as protective responses and horizontal gene transfer, and genes of unknown function (47). Indeed, as demonstrated in cured mutants, in several BCC species chromosome 3 functions like a large plasmid, encoding virulence, secondary metabolism, and other accessory functions (48). The large, multireplicon, insertion rich genomes confer extreme metabolic capacity and genome plasticity, that likely contribute to the adaptable nature of BCC strains (5, 46).

The best characterized genomic island of the BCC is the 44 kb *B. cenocepacia* island (cci) on chromosome 2 (49). This island is most often associated with IIIA strains and contains the *B. cepacia* epidemic strain marker used to identify a lineage of virulent *B. cenocepacia* strains that infect persons with CF (47, 49). Functions attributed to the island include arsenic and antibiotic resistance, ion and sulfate transport, stress response, fatty acid metabolism, quorum sensing, and virulence in the rat agar bead model (49). More recently, a genomic island encoding genes involved in antibacterial production on chromosome 3 of *B. ambifaria* strain AMMD was described (50).

1.2 BCC in the environment

BCC species are distributed widely in the natural environment (Table 1), where they have beneficial interactions with plants but are also capable of inducing plant disease (1-4). *B. cepacia* is the causative agent of soft onion rot (3, 34). Onion maceration in *B. cepacia* is dependent on a plasmid-encoded endopolygalacturonase, PehA (51). However, multiple BCC species, including *B. cenocepacia*, colonize the onion rhizosphere and have the potential to

cause onion disease (52, 53). *B. cenocepacia* is also responsible for banana finger tip rot in Taiwan (54), and *B. seminalis* causes apricot fruit rot in China (55, 56).

Although members of the BCC are known phytopathogens, most interactions between BCC bacteria and plants are beneficial to the host (2). *B. cepacia*, *B. cenocepacia*, *B. ambifaria*, *B. pyrrocinia*, and *B. contaminans* produce antifungal compounds that can protect commercially valuable plants from fungal diseases such as root rot and dampening-off of seedlings, and brown patch disease of lawn grass (57-60). BCC strains also protect plants by producing antimicrobial compounds that are effective against other environmental bacteria and plant parasites (58, 61). The ability of BCC species to suppress plant disease has applications in biocontrol; for example, the growth promotion of maize, cucumber, soybean, and pepper by *B. ambifaria* (58), and grape vine by *B. cepacia* (62). Other plant growth promoting properties in the BCC include the production of the phytohormone auxin (indoleacetic acid) (61), and the ability of *B. vietnamiensis* to fix atmospheric nitrogen to boost important food crops such as rice and sugarcane (63-66). Owing to their extensive metabolic capacity, members of the BCC are also effective bioremediation agents capable of breaking down man-made toxins, such as those found in herbicides and pesticides (5, 58). *B. vietnamiensis* in particular is effective in degrading the environmental contaminants trichloroethylene (67) and toluene (68). Indeed, field-trials with an isolate derived from *B. vietnamiensis* G4 showed a dramatic reduction of chlorinated solvents in groundwater (69). However, the ecological use of BCC isolates has been limited by the ability of species to cause human disease (70, 71).

1.3 BCC as opportunistic human pathogens

Members of the BCC do not normally infect healthy individuals, but they are highly virulent in some immunocompromised hosts (5). Respiratory and invasive BCC infections are predominantly seen in patients with CGD or CF (5, 72), but nosocomial infections are being reported with increased frequency (73). BCC species are not normally carried as commensal organisms; infection is thought to be acquired from either clinical settings (nosocomially) or the natural environment (5).

1.3.1 Nosocomial infections

Hospital acquired BCC infections occur worldwide and are usually the result of point-source outbreaks (74-98). These infections often occur in vulnerable populations, for example, in immunocompromised cancer patients (99-103). BCC hospital outbreaks in non-CF patients are most often associated with the contamination of water sources (75, 78, 82, 89, 91, 95), inhaled/ingested solutions or devices (74, 77, 79, 81, 83, 86-88, 90, 92, 97, 98), topically applied gels/moisturizers (76, 80, 84), and disinfectants (89, 93, 94, 96, 101). BCC infections acquired in clinical settings can lead to lethal bacteremia; for example, one retrospective study reported that 55% of non-CF patients died shortly after the onset of BCC bacteremia (104).

1.3.2 Chronic granulomatous disease

CGD is a rare genetic disorder with X-linked and autosomal recessive forms, and an estimated incidence in the United States (US) of 1:250,000 live births, with similar rates observed in other countries (6, 105). CGD is caused by mutations in genes encoding

components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme that catalyzes the production of superoxide from oxygen (7, 105, 106). NADPH is assembled from its components on the plasma and phagosomal membranes of professional phagocytes of the immune system, such as neutrophils and macrophages, but is also found in nonphagocytic cells, such as B lymphocytes and endothelial cells (106). Mutations in the membrane-bound and phagocyte-specific gp91^{phox} account for approximately 65% of CGD cases, with the remainder caused by mutations in p47^{phox} (~25%), p67^{phox} and p22^{phox} (105). Defects in gp91^{phox} (X-linked CGD) are associated with diagnosis at an early age, the most severe disease phenotype, and the highest mortality rate of the different genotypes (6, 107).

The basic defect in superoxide production results in impaired microbial killing and inflammatory complications in CGD patients (7, 105). The most prevalent site of disease is the lungs, with approximately 80% of patients affected, followed by the lymph nodes, liver, skin, gastro-intestinal and genitourinary system, head and neck, and central nervous system (6, 8, 105, 107). The most common clinical manifestation of CGD is pneumonia caused by infections with catalase-positive microorganisms, most often fungal species of *Aspergillus* (~41%) and the Gram-positive bacterium *Staphylococcus aureus* (~11%) (6, 8, 107). BCC organisms are the next most commonly isolated species, and thus the major cause of CGD pneumonia among Gram-negative bacteria, at about 7%, although it is recognized that this could be an underestimate because infections caused by the BCC may have been reported as *Pseudomonas* in older reports (6, 8, 105). The remainder of lung infections are mainly attributed to *Nocardia* species, *Serratia marcenscens*, *Klebsiella* species, and *Mycobacterial* species (6, 105). Infections with these fungal and bacterial pathogens, as well as additional organisms such as *Candida*, *Paecilomyces*, and *Salmonella* species, also result in abscesses,

adenitis, osteomyelitis, cellulitis, meningitis, and encephalitis in CGD patients, and can spread into the bloodstream causing bacteremia/fungemia (6, 8).

BCC infections in CGD are remarkably aggressive, and are the leading causes of bacterial sepsis and death (6-8). Indeed, BCC species are resistant to phagocytic non-oxidative killing mediated by cationic peptides (108). A recent study of BCC species distribution in CGD patients in the US suggests that *B. multivorans* is the most prevalent, although a broad representation of species existed in this relatively small set of patients and furthermore, multiple infecting species/strains were identified (109). Strains were not however shared by several patients, suggesting they are not spread person-to-person, but rather are acquired from the environment (109).

CGD patients are treated with prophylactic agents, along with therapies directed at specific infections and conditions as they occur (7, 105). The antibiotic trimethoprim/sulfamethoxazole and azole antifungals are often used as prophylactic treatments in CGD (7, 105). Following the introduction of azole agents as prophylactic antifungals, mortality rates in CGD significantly decreased, and CGD patients are now expected to live into adulthood (105). The prophylactic cytokine interferon-γ is administered to reduce the number and severity of infections in CGD patients (7, 105). Steroids and tumor necrosis factor-α blocking agents are used to treat the inflammatory complications of CGD, such as inflammatory bowel disease (105). Bone marrow transplantation is able to provide a definitive cure of the molecular defect of CGD (105).

1.3.3 Cystic fibrosis

CF is the most common lethal genetic disease in Caucasian populations, with an

estimated incidence of 1 in 2500-3000 live births (110, 111). The median survival age has improved substantially over the past 25 years, from 25 years in 1984 to 47 years in 2009 in Canada (112), with similar life expectancy improvements noted in the US and United Kingdom (UK) (111). CF is an autosomal recessive disorder caused by mutations in a single gene on chromosome 7 that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) (113). CFTR is expressed in epithelial and blood cells, and functions primarily as an ion channel that regulates liquid volume on epithelial surfaces through its effects on chloride secretion and sodium absorption (110, 111). Over 1500 CFTR mutations have been identified, but the most common is the deletion of phenylalanine at position 508 (known as phe508del or ΔF508), which occurs in about 70% of the CF population and results in a protein trafficking defect (110, 111). Some rare mutations result in milder forms of the disease, likely owing to residual activity of the protein that is able to reach the cell membrane (110, 111).

The most widely accepted explanation of how CFTR dysfunction leads to the phenotypic disease is the ‘low volume’ hypothesis: a reduced volume of surface liquid leads to the systemic production of thick, dehydrated mucus, which subsequently results in pancreatic insufficiency, intestinal obstruction, infertility, and lung disease (110, 111). In the lungs, cilia are compressed and the mucus can harbour microbes that would normally be cleared, allowing them to establish infections in the otherwise sterile lower airways (110, 111). Persistent lung infection is the leading cause of morbidity and mortality in CF patients, accounting for at least 80% of deaths (110, 111, 114). Defects in CFTR are also thought to contribute to the excessive inflammatory response that characterizes CF disease (110, 111). Early CF airway infections are usually caused by *S. aureus* and *Haemophilus influenzae*,

common human pathogens, while later-life infections are attributed to opportunistic bacterial and fungal pathogens, such as *P. aeruginosa*, BCC organisms, *Stenotrophomonas maltophilia*, *Streptococcus milleri*, *Burkholderia gladioli*, *Achromobacter xylosoxidans*, *Aspergillus* species, and nontuberculous mycobacteria (114, 115). *P. aeruginosa* is the most common bacterial species involved in infectious airway disease in CF (112, 114). For example, in 2008 in the US (114) and in 2009 in Canada (112), respectively, 52.5% and 42.4% of patients included in the national CF patient registries had positive cultures for the organism. Interestingly, the prevalence of *S. aureus* in CF has increased steadily over the last 15-25 years both in the US and in Canada, and is now similar to that observed for *P. aeruginosa* (112, 114). The prevalence of BCC infection in CF patients is relatively low, and has ranged between 3% and 4% in the US for many years (114), while it has declined from 8.7% to 4.3% in the Canadian CF population from 1984 to 2009 (112). Based on the most recent patient registry report from the European Cystic Fibrosis Society, between 2 to 6 % of CF patients are infected with BCC strains in European countries (116).

BCC infections in CF patients, however, are associated with enhanced morbidity and mortality and are known independent risk factors for death, most notably in recipients of lung transplants (9-22). Furthermore, in a subset of patients, BCC infections can spread into the bloodstream causing 'cepacia syndrome', characterized by rapid clinical deterioration, septicaemia, and death, a complication not observed with *P. aeruginosa* infections (117, 118). *B. cenocepacia* appears to be particularly virulent *in vivo* (9, 13, 17, 119-121). Of the 17 species in the complex, all but *Burkholderia ubonensis* have been isolated from patients with CF (1, 114). *B. cenocepacia* and *B. multivorans* however, cause the vast majority of BCC infections in CF patients, with *B. cepacia*, *B. dolosa*, and *B. vietnamiensis* causing a

small but significant number of other BCC CF infections (114, 122, 123). Although *B. cenocepacia* was historically the most prevalent of the BCC species in CF populations, in the last decade, *B. multivorans* has surpassed *B. cenocepacia* in prevalence in the US (114), the UK (122), and in Canada (DP Speert, personal communication). BCC strains can be transmitted from person to person through CF patient contact (124, 125). Indeed, several epidemic strains have been described, most belonging to *B. cenocepacia*, and the decline in their incidence over the years has been attributed to the implementation of strict infection control practices (114). The acquisition of BCC strains in CF populations also occurs from the natural environment. In fact, the incidence of an epidemic in the US caused by strain PHDC is considered to be related to the wide recovery of PHDC from agricultural soil, but the majority of BCC infected CF patients harbour genotypically distinct strains, suggesting different environmental reservoirs (114).

With the recent first ever approval of a drug that corrects the root cause of cystic fibrosis, the G551D CFTR mutation specifically (126), now more than ever it seems a reversal of the defect for all types of CF is within reach. Indeed, a search for novel small molecule correctors and potentiators of CFTR accounts for much of CF research (127). In the meantime, however, and in the absence of gene therapy, treatment of airway obstruction, infection and inflammation in CF is critical (111, 128). Physical airway clearance techniques, inhaled hypertonic saline, and dornase alfa, an aerosolized deoxyribonuclease, are recommended for the breakdown/prevention of airway obstruction (128). Prophylactic antibiotic administration to prevent infection is not currently standard practice in North America; eradicating infection once it occurs is the antimicrobial strategy of choice (128, 129). Notably, inhaled tobramycin, an aminoglycoside antibiotic, is particularly important in

the treatment of early and chronic *P. aeruginosa* infections in CF patients (28, 128), and targeting exacerbations - flares of CF lung disease - aggressively with intravenous antibiotics is crucial to patient well-being (111, 128). Eradication of BCC strains in CF patients is notoriously difficult owing to their innate resistance to most antibiotics, including tobramycin (24-26, 128, 130). Ibuprofen and azithromycin, a macrolide antibiotic that also has direct effects on the host immune system, are most often used in the management of inflammation in CF patients (111, 128). Lastly, proper nutrition and supplementation with pancreatic enzymes and vitamins is beneficial to CF patient health, and lung transplantation remains as a final therapeutic option for patients with end stage lung disease (111).

1.3.4 Interactions with epithelial and phagocytic cells

Interactions between BCC strains and cells of the host immune system are thought to play a key role in the pathogenesis of human disease (23). Airway epithelial cells are the first cells to be challenged by airborne pathogens like the BCC, and are instrumental in maintaining mucosal integrity and modulating the innate immune response (23, 131). *B. cenocepacia* is internalized into epithelial cells via membrane-bound vacuoles and interferes with the normal endocytic pathway; bacteria escape from late endosomes and lysosomes to enter authophagosomes and ultimately replicate (132). BCC species can also induce apoptosis in airway epithelial cells (133, 134).

BCC isolates survive with minimal or no replication within macrophages, resident phagocytic cells of the lung that mediate the early response to infection by recruiting and activating other inflammatory cells and are involved in the clearance of apoptotic cells (23, 135). Intracellular *B. cenocepacia* causes a delay in the maturation of the macrophage

phagosome; macrophage engulfed bacteria reside in vacuoles which exhibit a pronounced delay in fusion with lysosomes and subsequently in acidification (136). *B. cenocepacia* also delays the assembly of a functional NADPH oxidase complex on the macrophage vacuole membranes, which is associated with impaired superoxide production (137). These *B. cenocepacia*-induced effects are enhanced in CFTR-defective cells (137, 138).

B. cenocepacia enhances apoptosis in neutrophils, fundamental phagocytes of the innate immune system that are crucial for effective microbe killing (23, 139). Furthermore, *B. cenocepacia* induces neutrophil necrosis in CGD cells, where reactive oxygen species (ROS) production is compromised (139). Neutrophils that die of necrosis, instead of apoptosis, release their toxic contents in an uncontrolled fashion, which results in exacerbated inflammation and tissue damage (140, 141).

Dendritic cells capture, process, and present microbial components to orchestrate immune responses, and are crucial in bridging innate and adaptive immunity (142). *B. cenocepacia*, but not *B. multivorans*, reduces the expression of co-stimulatory molecules and induces necrosis in dendritic cells (143).

1.3.5 Virulence factors

BCC species produce an extensive arsenal of virulence factors that have a potential role in the pathogenesis of human disease (5). A few of the best characterized virulence factors are briefly discussed here.

Lipopolysaccharide (LPS), composed of lipid A, core oligosaccharide, and O-antigen, is an immunogenic component of the cell surface of Gram-negative bacteria; indeed, BCC LPS induces a strong proinflammatory response from cells of the immune system, such as

monocytes, that likely contributes to host tissue damage (144-146). Furthermore, a *B. cenocepacia* mutant lacking the O-antigen portion of LPS is susceptible, by minimum inhibitory concentration (MIC) testing, to cationic peptides (147), defective in survival in the rat agar bead model of lung infection (147), and attenuated in the *C. elegans* and *G. mellonella* infection models (148). The O-antigen of *B. cenocepacia* is required for phagocytosis by macrophages and adhesion to epithelial cells (149). Notably, changes in lipid A acylation patterns can impact the ability of BCC strains to stimulate immune cells (150-152).

BCC species produce several different types of exopolysaccharide (EPS), literally external sugar coatings which endow bacteria with a mucoid phenotype, the most common being cepacian (153-156). In BCC strains, the EPS production is significantly correlated with lung function in CF patients (157) and may play a role bacterial persistence in mouse models of infection (158, 159). BCC EPS inhibits cationic peptide antimicrobial activity (160), is involved in the formation of biofilms (161, 162), and can interfere with neutrophil chemotaxis and ROS production (163).

BCC species produce several types of siderophores, iron chelators, to facilitate the acquisition of iron which is essential for bacterial growth: salicylic acid, ornibactin, pyochelin, malleobactin, cepaciachelin, and cepabactin (61). Ornibactin in particular is an important virulence factor in a rat model of chronic respiratory infection (164-166), as well as in *C. elegans* and *G. mellonella* infection models (148).

In the BCC, the best characterized adhesion molecules are the cable pilus and its associated 22 kDa adhesin; adhesin-mediated bacterial attachment to eukaryotic cell surfaces is a key step in pathogenesis (23). The 22 kDa adhesin binds to epithelial mucin (167) and

cytokeratin 13 (168), the latter being highly expressed in CF airway epithelial cells (169). Both the cable pili and 22 kDa adhesin are required for *B. cenocepacia* interaction, invasion, and transmigration across squamous epithelial cells (170, 171), epithelial cell cytotoxicity (134, 170), and contribute to *B. cenocepacia* persistence in a mouse model of infection (172). The cable pilus also facilitates the formation of diffuse cell networks in *B. cenocepacia*, preventing cell aggregation (173). More recently, glycolipid receptors have been implicated in BCC invasion of epithelial cells (174).

BCC species produce catalase, superoxide dismutase (SOD), alkyl hydroperoxide reductase subunit C (AhpC), and a melanin-like pigment to resist oxidative damage (23). Catalase and SOD are enzymes responsible for the breakdown ROS (175), and, in *B. cenocepacia*, SOD also contributes to bacterial survival within macrophages (176). Loss of AhpC is associated with increased sensitivity to oxidative stress (177, 178). A brown, melanin-like pigment produced by *B. cenocepacia* scavenges exogenous superoxide (179) and protects the bacterium from intracellular sources of oxidative stress (180).

BCC species produce two metalloproteases, enzymes that degrade proteins whose catalytic mechanism involves a metal (61). The *B. cenocepacia* zinc metalloprotease ZmpA is capable of cleaving several biologically relevant substrates including type IV collagen, fibronectin, α-1 proteinase inhibitor, α2-macroglobulin, and gamma interferon (181). The *B. cenocepacia* zinc metalloprotease ZmpB is also able to cleave type IV collagen, fibronectin, α-1 proteinase inhibitor, and α2-macroglobulin, but additionally cleaves lactoferrin, transferrin, and human immunoglobins, and is involved in virulence in the rat agar bead model of chronic infection (182). Furthermore, both ZmpA and ZmpB can digest cationic

antimicrobial peptides, decreasing *B. cenocepacia* susceptibililty to peptide-mediated killing (183).

B. cenocepacia has several known secretion systems, which enable bacteria to secrete effector molecules directly into or in the proximity or host cells (secretion systems are not well defined in other BCC species) (23, 184). A type III secretion system is required for *B. cenocepacia* virulence in a mouse model of infection (185). A type IV secretion system contributes to the intracellular survival of *B. cenocepacia* in epithelial cells and macrophages (186). A type VI secretion system in *B. cenocepacia* mediates actin rearrangements in macrophages (187-189), prevents the recruitment of the NADPH oxidase complex subunits to bacteria-containing macrophage vacuoles (188), and is required for virulence in the rat agar bead model of chronic lung infection (187, 190).

BCC species produce two types of flagella, long filaments that endow bacteria with motility (191). Flagella are involved in the proinflammatory epithelial cell response to *B. cenocepacia* (192, 193). Mutations in *B. cenocepacia* flagellar components result in reduced motility (194), reduced invasiveness of respiratory epithelial cells (194), and attenuation in a mouse model of chronic infection (192).

BCC species are capable of producing biofilms, communities of bacteria that have enhanced protection against external factors (195). The effect of BCC biofilms on antibiotic susceptibility is unclear. Caraher *et al.* (196) found that BCC biofilm inhibitory concentrations to β-lactams and piperacillin-tazobactam, but not for tobramycin and amikacin, were considerably higher than the corresponding minimum inhibitory concentrations (MICs) of planktonic cells, while Peeters *et al.* (25) found MICs of several antibiotics to be similar between exponentially growing planktonic cells and freshly adhered

sessile cells. Differences in results are attributed to methodology (195). Antibiotic MICs change dramatically during growth phase in both planktonic and biofilm grown *B. cepacia*, and no major differences exist between stationary-phase planktonic cultures and mature biofilms (197). Growth of BCC isolates in biofilms results in an enhanced resistance to the antimicrobial actitivty of disinfectants (198-201).

In the BCC, sigma factors that initiate transcription are thought to be involved in the modulation of gene expression in response to environmental cues encountered in the eukaryotic host (23). Indeed, in *B. cenocepacia*, the production of ornibactin in response to iron starvation requires transcription dependent on the sigma factor OrbS (202) and bacterial motility and biofilm are dependent on the sigma factor RpoN (203). Furthermore, both RpoN and RpoE are required for the delay of fusion between bacteria-containing vacuoles with lysosomes that allows *B. cenocepacia* to survival within macrophages (203, 204).

Quorum-sensing systems involving *N*-acyl homoserine lactone signaling molecules are widespread among BCC species, and facilitate cell-to-cell communication and control the expression of various virulence factors (195). The CepIR quorum sensing system is found in all BCC species (195) and regulates several genes and functions, including biofilm formation (205), ornibactin production (206, 207), the transcription of zinc metalloprotease genes (208-210), flagellar-associated genes (209, 210), catalase genes (210), the nematocidal protein AidA (209), iron transport genes (209), and sigma factor expression (211). Furthermore, in *B. cenocepacia*, the CepIR system contributes to virulence in *C. elegans* (148), *G. mellonella* (148), and in two different murine models of infection (208). Other quorum systems that have been described in the BCC include the CciR, CepR2, and BviIR systems (195). The CciR system is only present in *B. cenocepacia* strains containing the genomic island cci (49),

and regulates protease activity (212), swarming motility (212), as well as the expression of a number of genes, including those associated with flagella, iron transport, and oxidative stress (209). CepR2 influences the expression of CepIR and CccIR regulated genes in *B. cenocepacia* (213). The BviIR system is unique to *B. vietnamiensis* (214, 215).

During the course of a chronic infection, phenotypic changes in virulence determinants and other traits can occur in BCC strains, a testament to their ability to adapt to new environments. Clonal sequential isolates can differ in EPS production (158, 216-218), swimming and swarming motility (216, 217, 219), colony morphology (216, 219), fatty acid composition (216), lipid A structure (150, 152), their ability to grow under iron (216, 219), oxygen (217) or nutrient (217) limited conditions, form biofilms (158, 216, 217, 219), resist oxidative stress (178), and cause disease in model organisms (158, 217). In fact, proteomic (178, 219) and transcriptomic (178, 217, 220) studies have revealed a number of proteins/genes to be differentially produced/expressed between sequential isolates.

1.3.6 Antimicrobial resistance

Antibiotic resistance can be intrinsic, i.e. a naturally occurring trait arising from the biology of the organism, or can be acquired by mutations resulting in changes in the organism's biology or by the acquisition of new resistance-encoding DNA from other organisms (221). Clinically, there is a link between antibiotic exposure and bacterial resistance (222). Bacterial antibiotic resistance mechanisms can be classified into four basic biochemical types: (i) enzymatic inactivation of antibiotics, (ii) restriction of target site binding by alteration or competitive inhibition, (iii) the use of "bypass" pathways not

targeted by antibiotics, and (iv) reduced intracellular accumulation of antibiotics, the latter of which can result from decreased uptake or increased efflux (221) (Figure 1).

Bacterial enzymes are able to degrade or chemically modify antibiotics to inactivate them (223). Bacterial target alterations result from mutations or chemical modification via methylation (224). In CF patients, when the internal production of folic acid - essential for bacterial DNA synthesis - is inhibited in *S. aureus* by trimethoprim/sulfamethoxazole, the organism uses external thymidine provided by destroyed eukaryotic cells to synthesize folic acid, an example of using a "bypass" pathway to overcome antibiotic effects (225). Decreased antimicrobial uptake results from modifications in porins (226) and (in Gram-negative bacteria) LPS (151). Bacterial efflux systems capable of accommodating antimicrobials fall into five classes: the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (227, 228). Chromosomally encoded RND efflux systems are the most clinically relevant multidrug pumps in Gram-negative bacteria because they span both the inner and outer membranes (227, 228). BCC species are intrinsically highly resistant to a number of antimicrobials and disinfectants (229) and difficult to eradicate *in vivo* (130). BCC resistance to antibiotics in the context of CGD and CF, with a focus on polycationic agents (polymyxins, cationic peptides, and aminoglycosides), is discussed here.

1.3.6.1 Resistance to chloramphenicol

Chloramphenicol binds to the 50S ribosomal subunit to inhibit bacterial protein

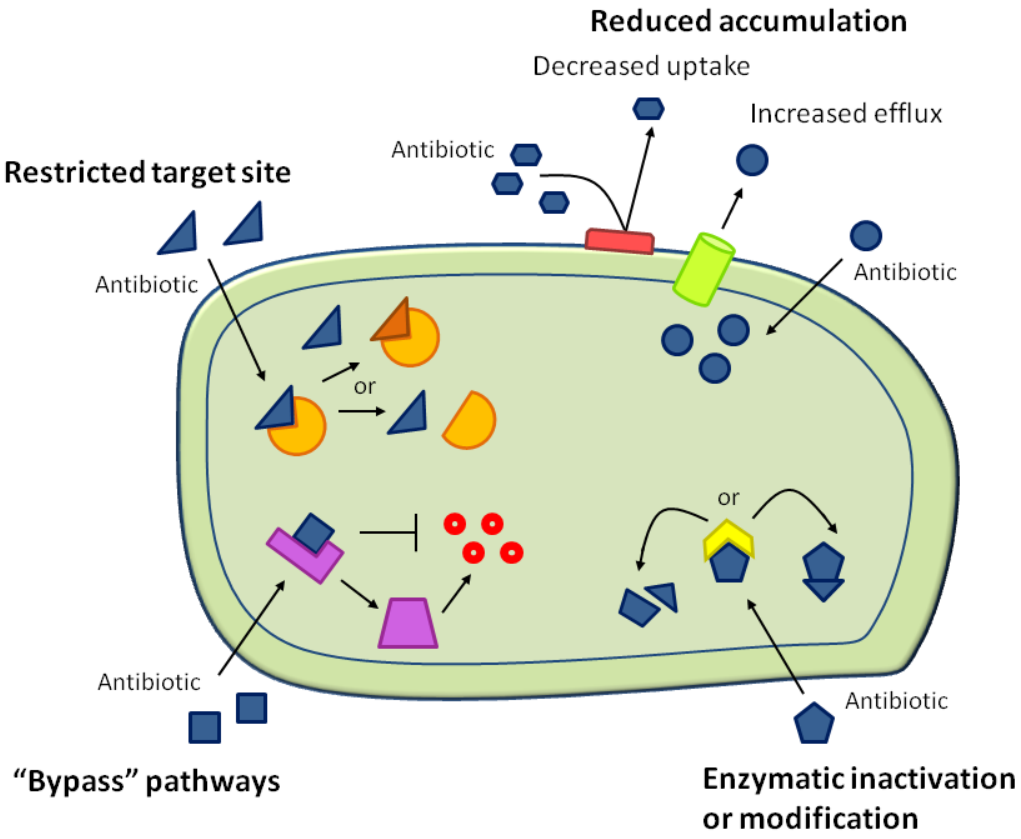

Figure 1. The four major biochemical mechanisms of bacterial antibiotic resistance.
Adapted from Hawkey (1998) (226).

synthesis (230). Chloramphenicol is used occasionally in the management of lung disease in CF (231) and in the management of BCC infections (130). Chloramphenicol resistance is attributed to enzymatic inactivation by acetyltransferases or phosphotransferases, target alteration via mutation, active efflux through MF and RND pumps, and impermeability owing to outer membrane protein modifications (228, 230). Based on antimicrobial MICs and established *B. cepacia* breakpoints (232), most BCC strains, including those of *B. vietnamiensis*, are not susceptible to chloramphenicol (they grow in the presence of ≤16 μg/ml) (26). Chloramphenicol resistance in the BCC is associated with porin-mediated impermeability (233, 234) and RND efflux systems (235-239).

1.3.6.2 Resistance to trimethoprim/sulfamethoxazole

Sulfonamides and trimethoprim block the bacterial pathway for folic acid synthesis, which ultimately inhibits DNA synthesis (240). The combination of trimethoprim and sulfamethoxazole, also known as co-trimoxazole, is an important prophylactic agent for the management of bacterial and fungal infections in CGD (241), and is a drug of choice against BCC infections (130). Efflux systems (240) and the use of “bypass” pathways (225) result in bacterial resistance to trimethoprim. Based on antimicrobial MICs and established *B. cepacia* breakpoints (232), BCC, including those of *B. vietnamiensis*, strains are often resistant to trimethoprim/sulfamethoxazole, although to a lesser degree compared with other antibiotics (25, 26). Trimethoprim resistance in the BCC is associated with porin-mediated impermeability (233, 234) and RND system-mediated efflux (236, 238, 239). Furthermore, BCC resistance due to the production of a trimethoprim-resistant dihydrofolate reductase, the drug target, has been reported (242).

1.3.6.3 Resistance to tetracyclines

Tetracyclines bind to the 30S ribosomal subunit to inhibit bacterial protein synthesis (243). Tigecycline belongs to the new glycylcycline class of antibiotics derived from tetracyclines (243). Unlike other tetracyclines, tigecycline is resistant to the two major mechanisms of tetracycline resistance, efflux and ribosomal protection (243). Owing to its enhanced spectrum of activity, tigecycline is likely to be used increasingly in the management of multidrug resistant infections, such as those in CF patients (231). Tetracyclines are used in the management of BCC infections (130). Bacterial resistance to tetracyclines is caused by active efflux via pumps of the MF, RND, and MATE families, target protection by competitive inhibition, enzymatic inactivation through monohydroxylation, target modification by mutation, and decreased uptake through porins (226, 228, 243). Growth of BCC strains is generally not inhibited by <16 μg/ml of tetracycline or tigecycline, although lower tetracycline concentrations are more active against *B. vietnamiensis* compared with other BCC species (24, 26, 244). Based on the use of the RND pump inhibitor MC-207110 (245), cloning of an MF pump into *Escherichia coli* (246), and the decreased tetracycline MIC for a *B. cenocepacia* RND system mutant (239), efflux systems are thought to be involved in tetracycline resistance in the BCC.

1.3.6.4 Resistance to quinolones

Quinolones inhibit bacterial DNA replication, and subsequently transcription and translation, by targeting DNA gyrase (topoisomerase II) and DNA topoisomerase IV (247). The fluoroquinolone (contains a fluorine atom) ciprofloxacin is important in the treatment of infections caused by Gram-positive and Gram-negative bacteria in CGD patients (241), and

is used in the management of infectious lung disease in CF (231). Ciprofloxacin is used often in the treatment of BCC infections (130), as, based on antimicrobial MICs and established non-*Enterobacteriaceae* breakpoints (232), BCC strains, including those of *B. vietnamiensis*, are often susceptible to ciprofloxacin (24, 25). Quinolone resistance can result from mutations or the production of proteins that interfere with target binding, enzymatic inactivation via acetylation, porin-mediated impermeability, and efflux via MF, ABC, SMR, RND, and MATE pumps (226, 228, 247). BCC resistance to fluoroquinolones, including ciprofloxacin, results from porin-mediated impermeability (233, 234), active efflux through RND pumps (236-238), and is associated with mutations in the topoisomerase genes *gyrA* and *parC* (248).

1.3.6.5 Resistance to β-lactams

β-lactams are a large class of antimicrobials that inhibit cell wall synthesis by interfering with peptidoglycan synthesis, and include penicillins, penems, carbapenems, cephalosporins, and monobactams (249). The usual therapy of *P. aeruginosa*-infected CF patients includes β-lactam antibiotics (128, 231). Ceftazidime, a cephalosporin, is extremely important in the treatment of BCC infections; in a systematic review of case reports and cohort studies, 33.3% of BCC infected patients had received ceftazidime-based regimens, and in 73.7% of those, the infecting strain was eradicated (130). Furthermore, many BCC infected patients are treated with other β-lactam antibiotics, most notably meropenem and penicillins, and in most cases these treatments are associated with clinical improvements and/or strain eradication (130). Bacterial resistance to β-lactam antibiotics results from active efflux via RND or ABC transporters (228), porin-mediated impermeability (226),

modifications in peptidoglycan or its production (224), penicillin-binding proteins (224), and the production of β-lactamases, enzymes that hydrolyze the β-lactam bond to inactivate the antibiotics (249, 250). Although many BCC strains are resistant to β-lactam antibiotics based on antimicrobial MICs and established non-*Enterobacteriaceae* or *B. cepacia* breakpoints (232), BCC species, including *B. vietnamiensis*, are more often susceptible to some β-lactams, specifically ceftazidime and meropenem (24-26). BCC resistance to β-lactams is attributed to porin-mediated impermeability (233, 234, 251) and the production of β-lactamases (252-258). Furthermore, 8-fold decreases in aztreonam MIC, a monobactam (237), and ampicillin MIC (259) were observed between *B. cenocepacia* mutants in RND efflux systems and their parents.

1.3.6.6 Resistance to macrolides

Macrolides bind to the 50S ribosomal subunit to inhibit bacterial protein synthesis (260). The indirect effects of macrolides on *P. aeruginosa* include the inhibition of adherence to epithelial cells, virulence factor production, biofilm formation, and quorum sensing (260). Macrolide antibiotics, notably azithromycin, can also modulate functions of the human immune system to downregulate hyperimmunity or hyperinflammation without impairing defenses against infection (260). Azithromycin is a safe and effective treatment of *P. aeruginosa*-infected CF patients; improvements in lung function and reductions in pulmonary exacerbations have been demonstrated (261, 262). Furthermore, the emergence of other bacteria, including BCC species, is not associated with azithromycin treatment (262). The clinical impact of azithromycin treatment on CF patients infected with BCC species has not yet been investigated, but azithromycin is used in the management of BCC infections (130).

The US Cystic Fibrosis Foundation (CFF) recommends chronic azithromycin therapy for *P. aeruginosa*-infected patients with CF, 6 years of age and older (28). The two main mechanisms responsible for macrolide resistance are ribosomal target modification via methylation or mutation and active efflux through transporters of the MF, ABC, or RND families (228, 260). BCC strains are often not inhibited by <512 µg/ml of azithromycin or clarithromycin, however synergistic effects between these macrolides and other conventional antibiotics have been demonstrated (263, 264). The mechanism of macrolide resistance in the BCC has not been described.

1.3.6.7 Resistance to polymyxins and cationic antimicrobial peptides

Polymyxin B and colistin (polymyxin E) are bactericidal, pentacationic cyclic lipodecapeptides that permeabilize the Gram-negative bacterial outer membrane (they are inactive against Gram-positive bacteria) (265). Their effectiveness is however, often overshadowed by the associated nephrotoxicity (265). With the emergence of multidrug-resistant Gram-negative bacteria, polymyxins have been used increasingly (266), especially inhaled colistin for therapy of respiratory *P. aeruginosa* infections (28, 128, 267). Polymyxins generally have no antimicrobial activity against BCC strains, including those of *B. vietnamiensis* ; large-scale studies of antimicrobial susceptibility involving multiple BCC species (≥38 isolates and ≥4 antibiotics tested) found polymyxin B and colistin not to be inhibitory against ≥87.9 % (24, 26, 268, 269) and 100% (270-272) of isolates, respectively.

Short cationic amphiphilic peptides are key constituents of virtually every host defense system, and hence are also referred to as host-defense peptides (273). They have broad-spectrum antimicrobial activity and are able to modulate the mammalian immune

response (273). In the last two decades, cationic antimicrobial peptides have become appealing as potential new therapeutic agents for a variety of conditions. Although cationic peptides display promising activity against *P. aeruginosa* and other CF pathogens (274), they generally do not have inhibitory activity against BCC strains (108, 275-278).

Bacterial resistance to polycationic antimicrobials is often attributed to outer membrane impermeability resulting from LPS modifications. 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 (279). The interaction relies on the availability of phosphate groups at the lipid A domain. Several organisms, including CF strains of *P. aeruginosa* (280, 281), have modifications in their lipid A structure with the addition of polar groups such as 4-amino-4-*deoxy*-L-arabinose (Ara4N) (151). Ara4N neutralizes the negative charge of the phosphate residue to which polycationic antimicrobials bind, thereby reducing bacterial susceptibility to cationic agents (151, 282). In *P. aeruginosa* this modification, and the subsequent resistance, is carried out by the products of the *arnBCADTEF-ugd* locus and is dependent on regulation by the two-component systems PhoPQ (283, 284), PmrAB (284-286), and ParRS (282, 287).

BCC intrinsic resistance to the antimicrobial activity of peptides is attributed to LPS modifications (229). BCC lipid A contains at least one Ara4N residue (144, 150, 152), and peptides, specifically polymyxin B and protegrin-1, bind poorly to whole BCC bacteria and to purified BCC LPS (288, 289). Notably, in *B. cenocepacia*, the biosynthesis of Ara4N is essential for viability (290, 291). Furthermore, mutations in the biosynthetic pathway for the assembly of the core oligosaccharide of LPS in *B. cenocepacia* results in 3.5- to 64-fold

reductions in the polymyxin B MIC required to inhibit the growth of 50% of bacteria (147, 292). Moreover, recent evidence has demonstrated that hopanoids, analogues of eukaryotic sterols involved in membrane stability and barrier function, are involved in polymyxin resistance in *B. cenocepacia* (293, 294). Lastly, a polymyxin B-susceptible mutant of *B. vietnamiensis* was generated by transposon mutagenesis of the *norM* gene encoding a MATE efflux protein (295) and zinc metalloproteases have been suggested to contribute to cationic peptide resistance in *B. cenocepacia* (183).

1.3.6.8 Resistance to aminoglycosides

Aminoglycosides are bactericidal, polycationic amino-modified sugars that target the 30S subunit of bacterial ribosomes and exert pleiotropic effects on cells, including interference with protein synthesis and disruption of membrane integrity (27, 296, 297). They are broad-spectrum antibiotics that are valuable in the treatment of various infectious conditions, even with their associated nephrotoxicity and ototoxicity, and have been in use for nearly 70 years (27). The useful characteristics of aminoglycosides include concentration-dependent bactericidal activity, postantibiotic effect (they continue to kill bacteria after the drug has been removed following a short incubation with the organism), and synergy with other antibiotics (27). Indeed, aminoglycoside antibiotics are often administered intravenously in combination with other antibacterial agents, such as β-lactams (often the case in CF patients), to treat serious infections caused by aerobic Gram-negative bacteria (27, 128). Aminoglycosides are mainly administered parenterally, but to increase the concentration of the antibiotic at the site of infection and decrease the risk of toxicity, they are also given in aerosolized solutions, for example tobramycin for respiratory tract

infections in CF patients, or, more recently, encapsulated in liposomes, particularly of value for penetrating cells to target intracellular organisms (27, 29, 128).

Based on a systematic review of trials assessing the use of aerosolized tobramycin in patients with established *P. aeruginosa* infection and moderate to severe airway disease, as well as those with asymptomatic or mild airway disease, chronic inhaled tobramycin therapy is recommended by the CFF for treatment of persistent *P. aeruginosa* pulmonary infections in CF patients ≥ 6 years of age with any type of disease (28). Inhaled tobramycin has been shown to decrease pulmonary exacerbations and improve lung function in CF patients infected with *P. aeruginosa* (28). Tobramycin Inhalation Powder is a new form of aerosolized tobramycin, offering advantages over the traditional nebulized Tobramycin Inhalation Solution (298). Long-term prophylaxis with inhaled gentamicin can delay the colonization with *P. aeruginosa* in children with CF (299). The current evidence on aerosolized gentamicin is, however, insufficient for the CFF to recommend its routine use (28). Aminoglycosides have been used in the clinical management of BCC infections (130).

The prevalence of aminoglycoside resistance is increasing; for example, in the US, up to 30% of CF *P. aeruginosa* isolates are now resistant to tobramycin (29). BCC species are intrinsically resistant to aminoglycosides; based on antimicrobial MICs and established non-*Enterobacteriaceae* breakpoints (232), large-scale studies of antimicrobial susceptibility involving multiple BCC species (≥8 isolates and ≥4 antibiotics tested) found amikacin, gentamicin, and tobramycin not to be inhibitory against 53.8-100% (268-270, 272, 300-304), 85.5-98.5% (269, 270, 272, 303), and 82.5-100% (24, 25, 268, 269, 303-306) of isolates, respectively. Notably, 256 µg/ml of tobramycin, a high concentration that can be achieved through inhaled solutions (307), inhibited 45-89.5 % of strains (25, 308). Mechanisms of

bacterial resistance to aminoglycosides include decreased antibiotic uptake, increased antibiotic efflux, modification of the ribosomal target, and enzymatic modification of the drug (27).

Aminoglycosides penetrate aerobically growing bacteria in three consecutive steps: ionic binding to LPS in Gram-negative bacteria (self-promoted uptake described above) or to teichoic acids in Gram-positive bacteria; energy-dependent phase I of uptake through the cytoplasmic membrane (microorganisms with deficient electron transport systems, such as anaerobes, are resistant); and, following the subsequent insertion of misfolded proteins generated into the cytoplasmic membrane, energy-dependent phase II of uptake, where additional aminoglycosides are transported across the damaged membrane (27, 297).

The major mechanism of aminoglycoside resistance in clinical isolates is enzymatic modification of the amino or hydroxyl groups via acetyl-CoA-dependent acetylation, phosphorylation, and adenylylation (27, 223). The enzymes classically involved are phosphotransferases, acetyltransferases, and nucleotidyltransferases, but more recently, bifunctional enzymes with two different aminoglycoside-modifying activities have been identified (27, 223). Mutations in the 16S ribosomal RNA (rRNA) gene that confer resistance to aminoglycosides have been identified most often in *Mycobacterium* species (27, 223). 16S rRNA methylation is an emerging aminoglycoside resistance mechanism (309), and pan-aminoglycoside resistance-promoting 16S rRNA methylases have been described in *P. aeruginosa* (310). Modified aminoglycosides or ribosomes result in poor binding of the drug to its target, and the subsequent failure to trigger energy-dependent phase II. These mechanisms of aminoglycoside resistance have not yet been investigated in the BCC.

BCC resistance to aminoglycosides is often attributed to reduced drug uptake owing to structural features of the LPS that inhibit their passive transport through the bacterial outer membrane (229) as described for peptide resistance above. BCC lipid A contains Ara4N modifications (150, 152, 311-315) that are involved in aminoglycoside resistance in *P. aeruginosa* (282, 316), and, in general, contribute to outer membrane impermeability (151). Specifically, ParRS activation of the lipopolysaccharide modification operon *arnBCADTEF* increases resistance to tobramycin and gentamicin in *P. aeruginosa* (282). Furthermore, in *P. aeruginosa*, mutations in several genes within the operon responsible for LPS O polysaccharide synthesis were identified in a tobramycin resistance screen (317).

More recent studies suggest that RND efflux systems are involved in aminoglycoside resistance in *B. cenocepacia* (239, 259, 318). Efflux systems that accommodate aminoglycosides have been identified in several organisms, including *E. coli*, *Burkholderia pseudomallei*, and *P. aeruginosa*, and generally belong to the RND class and are chromosomally-encoded (228, 319). The MexXY-OprM aminoglycoside-accommodating efflux system of *P. aeruginosa* is the predominant mechanism of aminoglycoside resistance in CF isolates (228, 320-322), and rare aminoglycoside susceptibility in *B. pseudomallei* is attributed to the loss of expression of its major aminoglycoside-accommodating efflux system AmrAB-OprA (323). The MexXY system is encoded by the *mexXY* operon that is under the control of the MexZ repressor (324), and mutations in *mexZ* are common in pan-aminoglycoside resistant CF isolates of *P. aeruginosa* (310). The expression of this efflux locus is also under regulation of a house-keeping gene named *PA5471* (325, 326) and the ParRS two-component regulatory system (282, 287). At subinhibitory concentrations, ribosome-targeting antibiotics, including aminoglycosides and macrolides, are capable of

inducing the expression of *mexX* (327) and *mexY* (328). Furthermore, serial exposure to hydrogen peroxide at half the MIC induces *mexX* expression (329). Deletion of the *mexXY-OprM* system homologue in *B. cenocepacia* did not change tobramycin and gentamicin MICs in one study (237), while tobramycin and gentamicin MICs decreased from 512 to 8 µg/ml and >1024 to 8 µg/ml, respectively, in another study (239). Deletion of other putative RND efflux systems in *B. cenocepacia* resulted in 4-fold tobramycin (from 1000 to 250 µg/ml) (318) and gentamicin (2048 to 512 µg/ml) (259) MIC reductions. Aminoglycoside efflux has not been investigated in other BCC species.

1.4 Hypothesis

The BCC is notorious for its high intrinsic resistance to many antimicrobials, including aminoglycosides. It is hypothesized that within the BCC, *B. vietnamiensis* is more often susceptible to aminoglycosides, and acquired aminoglycoside resistance is caused by decreased intracellular drug accumulation.

1.5 Overall goal

The overall goal of this thesis is to provide a better understanding of antimicrobial susceptibilities of the species within the BCC, and the induction and mechanisms of resistance. Novel insights may help in the design of improved antimicrobial therapeutic regimens against *B. vietnamiensis* infections and the re-evaluation of the use of this organism in bioremediation and plant growth promoting processes.

Chapter 2: MATERIALS AND METHODS

2.1 Strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. Isolates were selected from the Canadian *Burkholderia cepacia* complex Research and Referral Repository (CBCCRRR) (University of British Columbia, Vancouver, BC) or the *Burkholderia cepacia* complex (BCC) experimental strain panel (330). Bacteria were stored at -80°C in Mueller Hinton (MH) II Broth (cation-adjusted) (pH 7.3) with 8% (vol/vol) dimethyl sulfoxide (DMSO) and plated out twice before any testing. Cultures were routinely grown at 37°C in MH II Broth with aeration by shaking (250 rpm) after subculture on MH II Agar, unless otherwise indicated. For evaluation of exopolysaccharide (EPS) production cultures were grown on yeast extract medium (YEM) (0.5 g/liter yeast extract, 4g/liter mannitol, supplemented with 15 g/liter agar). For [^{3}H]gentamicin assays cultures were grown in Luria-Bertani (LB) medium (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter sodium chloride) (pH 7.1) after subculture on LB agar (LB supplemented with 15 g/liter agar). For RNA extraction cultures were grown in MH II Broth, LB medium and synthetic cystic fibrosis sputum medium (SCFM) (pH 6.8) (331). With the exception of sodium chloride (Fisher Scientific, Pittsburgh, PA) and mannitol (BDH/VWR International, Radnor, PA), MH II media and LB media components were purchased from BD (Franklin Lakes, NJ). SCFM components were purchased from EMD Chemicals (Gibbstown, NJ), Sigma-Aldrich (Oakville, ON), Gibco (Invitrogen, Carlsbad, CA), MP Biomedicals (Irvine, CA), BDH (VWR International), and Fisher Scientific. Overnight cultures were grown in 3 ml of media. Isolates used for study of chronic infections (Table 8, Table 9, Table 10, Figure 2) were chosen on the basis that infections spanned a minimum of 2 years with at least 3 isolates with

Table 2. Bacterial strains used in this study

Strain or plasmid	Description[a]	Reference[b]
BCC from Table 4, Table 5[c]		
B. cepacia		
CEP1318	CF respiratory isolate	B. Lui
C9139	CF respiratory isolate	CBCCRRR
FC1450	Blood isolate	BCCH
CEP1255	Non-CF respiratory isolate	BCCH
CEP1334	CF respiratory isolate	H. Semeniuk
CEP1311	CF respiratory isolate	Y. Yau
CEP1136	CF respiratory isolate	A. Rendina
D3119	CF respiratory isolate	CBCCRRR
CEP0534	Onion isolate	P. C. Taylor
CEP0190	Soil isolate	J. R. W. Govan
CEP0128	Onion isolate	J. R. W. Govan
LMG 17993	Urine isolate	P. Vandamme
CEP0842	Neck isolate	A. Rendina
B. multivorans		
D2094	CF respiratory isolate	CBCCRRR
D2095	CF respiratory isolate	CBCCRRR
D1533	CF respiratory isolate	CBCCRRR
C5568	CF respiratory isolate	CBCCRRR
D0089	CF respiratory isolate	CBCCRRR
D1782	CF respiratory isolate	CBCCRRR
C4297	CF respiratory isolate	CBCCRRR
D1396	CF respiratory isolate	CBCCRRR
C6126	CF respiratory isolate	CBCCRRR
D0155	CF respiratory isolate	CBCCRRR
C5274	CF respiratory isolate	CBCCRRR
CEP0155	Soil isolate	J. R. W. Govan
ATCC 17616	Soil isolate	J. R. W. Govan (330)
CEP0157	Soil isolate	J. R. W. Govan
CEP0159	Soil isolate	J. R. W. Govan
CEP0161	Soil isolate	J. R. W. Govan
CEP0208	Soil isolate	J. R. W. Govan
FC0646	Environmental isolate	CBCCRRR
FC0647	Environmental isolate	CBCCRRR
CEP0601	Cerebrospinal fluid isolate	J. L. Burns
CEP0935	Non-CF respiratory isolate	P. Ackerman
CEP1129	Urine isolate	A. Matlowe
CEP1336	Lymph node isolate	IWKHC
B. cenocepacia		
CEP1310	CF respiratory isolate	C. Goswell
D1478	CF respiratory isolate	CBCCRRR
C5424	CF respiratory isolate	CBCCRRR
C6159	CF respiratory isolate	CBCCRRR
J2315	CF respiratory isolate	J. R. W. Govan
C3921	CF respiratory isolate	CBCCRRR
C8963	CF respiratory isolate	CBCCRRR
C9343	CF respiratory isolate	CBCCRRR

Strain or plasmid	Description[a]	Reference[b]
C6433	CF respiratory isolate	CBCCRRR
CEP1279	Blood isolate	BCCH
FC0114	Blood isolate	BCCH
CEP1267	Dialysate isolate	BCCH
CEP0107a	Radish isolate	Genentech
CEP0162	Bark isolate	J. R. W. Govan
CEP0163	Bark isolate	J. R. W. Govan
FC0666	Environmental isolate	USDA
FC0668	Environmental isolate	USDA
D1817	CF respiratory isolate	CBCCRRR
FC0120	Clinical urine isolate	BCCH
B. stabilis		
CEP0970	CF respiratory isolate	P. C. Taylor
CEP1270	CF respiratory isolate	L. Cote
C8389	CF respiratory isolate	CBCCRRR
C6061	CF respiratory isolate	CBCCRRR
B. vietnamiensis		
CEP0339	CF respiratory isolate	H. Dick
CEP0974	CF respiratory isolate	P. C. Taylor
CEP0175	CF isolate	J. R. W. Govan
CEP0213	CF isolate	J. R. W. Govan
CEP1223	CF respiratory isolate	L. Cote
CEP1225	CF isolate	L. Cote
CEP1224	CF isolate	L. Cote
CEP0626	CF respiratory isolate	J. L. Burns
D0072	CF respiratory isolate	CBCCRRR
D0779	CF respiratory isolate	CBCCRRR
D1389	CF respiratory isolate	CBCCRRR
C9287	CF respiratory isolate	CBCCRRR
SQ004C	CF respiratory isolate	BCCH
CEP0041	CF isolate	J. L. Burns
CEP0043	CF isolate	J. L. Burns
CEP0033	CF isolate	J. L. Burns
CEP1110	CF respiratory isolate	C. Lavelle
D0775	CF respiratory isolate	CBCCRRR
LMG 10823	Soil isolate	P. Vandamme
LMG 16232	CF isolate	P. Vandamme
LMG 16233	CF isolate	P. Vandamme
LMG 16234	CF isolate	P. Vandamme
CEP1312	CF respiratory isolate	H. Senay
C8766	CF respiratory isolate	CBCCRRR
CEP0255	CF respiratory isolate	L. Wilcox
CEP1262	CF respiratory isolate	MUHSC
CEP1322	CF respiratory isolate	E. Tullis
CEP1323	CF respiratory isolate	E. Tullis
D0099	CF respiratory isolate	CBCCRRR
D0718	CF respiratory isolate	CBCCRRR
D1181	CF respiratory isolate	CBCCRRR
D1212	CF respiratory isolate	CBCCRRR
D1632	CF respiratory isolate	CBCCRRR
D2075	CF respiratory isolate	CBCCRRR
D2074	CF respiratory isolate	CBCCRRR
C8395	CF respiratory isolate	CBCCRRR

Strain or plasmid	Description[a]	Reference[b]
C8644	CF respiratory isolate	CBCCRRR
C8952	CF respiratory isolate	CBCCRRR
C9178	CF respiratory isolate	CBCCRRR
C9177	CF respiratory isolate	CBCCRRR
C9710	CF respiratory isolate	CBCCRRR
D0247	CF respiratory isolate	CBCCRRR
D0774	CF respiratory isolate	CBCCRRR
FC0622	CF isolate	J. J. LiPuma
CEP1172	CF respiratory isolate	R. Pierce
FC0373	CF isolate	J. J. LiPuma
CEP0480	CF isolate	J. L. Burns
CEP0982	CF blood isolate	P. C. Taylor
CEP0649	CF isolate	M. Roe
CEP0706	CF respiratory isolate	D. Rennie
CEP0087	CF respiratory isolate	Genentech
CEP0639	CF respiratory isolate	J. L. Burns
CEP0086	CF respiratory isolate	Genentech
CEP0504	CF isolate	E. Bingen
CEP1236	CF respiratory isolate	P. Roy
CEP1325	CF respiratory isolate	B. Lui
FC0441	CGD respiratory isolate	BCCH
CEP0126	Soil isolate	J. R. W. Govan
FC0656	Water isolate	T. Lessie
FC0654	Soil isolate	T. Lessie
FC1006	Sewage isolate	B. Conway
LMG 16230	CF respiratory isolate	P. Vandamme
LMG 17988	Urine isolate	P. Vandamme
LMG 10929t	Rice isolate	P. Vandamme
FC0434	Environmental isolate	J. J. LiPuma
CEP0106	Soil isolate	Genentech
CEP1291	Urine isolate	A. Matlowe
CEP0865	Renal isolate	M. A. Valvano
CEP0143	Clinical non-CF isolate	J. R. W. Govan
CEP0192	Clinical non-CF isolate	J. R. W. Govan
CEP0196	Clinical non-CF isolate	J. R. W. Govan
LMG 06998	Blood isolate	P. Vandamme
LMG 06999	Neck abscess isolate	P. Vandamme
C9371	CF respiratory isolate	CBCCRRR
CEP0047	Lymph node isolate	P. Ferriera
D1767	CF respiratory isolate	CBCCRRR
CEP0160	Hospital environmental isolate	J. R. W. Govan
CEP0149	Hospital environmental isolate	J. R. W. Govan
CEP0233	Hospital environmental isolate	J. R. W. Govan
D2448	CF respiratory isolate	CBCCRRR
CEP0084	Soil isolate	Genentech
BCC from Table 9		
B. multivorans sequential isolates		
C6558	CF respiratory early isolate (Bm1, 26/05/1995)	CBCCRRR
D2095	CF respiratory late isolate (Bm1, 01/06/2006)	CBCCRRR
C8814	CF respiratory early isolate (Bm2, 03/10/1999)	CBCCRRR
D0998	CF respiratory late isolate (Bm2, 23/01/2004)	CBCCRRR
C7117	CF respiratory early isolate (Bm3, 04/06/1996)	CBCCRRR

Strain or plasmid	Description[a]	Reference[b]
D1310	CF respiratory late isolate (Bm3, 30/09/2004)	CBCCRRR
D1506	CF respiratory early isolate (Bm4, 16/03/2005)	CBCCRRR
D3823	CF respiratory late isolate (Bm4, 19/05/2010)	CBCCRRR
D1285	CF respiratory early isolate (Bm5, 23/09/2004)	CBCCRRR
D3677	CF respiratory late isolate (Bm5, 13/01/2010)	CBCCRRR
C9876	CF respiratory early isolate (Bm6, 23/10/2001)	CBCCRRR
D3928	CF respiratory late isolate (Bm6, 14/09/2010)	CBCCRRR
D1533	CF respiratory early isolate (Bm7, 07/04/2005)	CBCCRRR
D3532	CF respiratory late isolate (Bm7, 21/09/2009)	CBCCRRR
C7463	CF respiratory early isolate (Bm8, 11/02/1997)	CBCCRRR
D2446	CF respiratory late isolate (Bm8, 02/05/2007)	CBCCRRR
C9165	CF respiratory early isolate (Bm9, 13/06/2000)	CBCCRRR
D4032	CF respiratory late isolate (Bm9, 16/03/2011)	CBCCRRR
C3168	CF respiratory early isolate (Bm10, 21/02/1989)	CBCCRRR
C6957	CF respiratory late isolate (Bm10, 23/02/1996)	CBCCRRR
C7062	CF respiratory early isolate (Bm11, 16/04/1996)	CBCCRRR
C8777	CF respiratory late isolate (Bm11, 09/09/1999)	CBCCRRR
D1407	CF respiratory early isolate (Bm12, 15/12/2004) (also Figure 9)	CBCCRRR
D3220	CF respiratory late isolate (Bm12, 05/12/2008) (also Figure 9)	CBCCRRR
C8298	CF respiratory early isolate (Bm13, 22/09/1998) (also Figure 9)	CBCCRRR
D2156	CF respiratory late isolate (Bm13, 01/08/2006) (also Figure 9)	CBCCRRR
C6398	CF respiratory early isolate (Bm14, 21/02/1995)	CBCCRRR
D2889	CF respiratory late isolate (Bm14, 10/03/2008)	CBCCRRR
C6396	CF respiratory early isolate (Bm15, 21/02/1995)	CBCCRRR
D0913	CF respiratory late isolate (Bm15, 20/11/2003)	CBCCRRR
C9861	CF respiratory early isolate (Bm16, 19/10/2001)	CBCCRRR
D1268	CF respiratory late isolate (Bm16, 09/08/2004)	CBCCRRR
D0400	CF respiratory early isolate (Bm17, 01/12/2002)	CBCCRRR
D3556	CF respiratory late isolate (Bm17, 25/09/2009)	CBCCRRR
D1858	CF respiratory early isolate (Bm18, 25/11/2005)	CBCCRRR
D3738	CF respiratory late isolate (Bm18, 15/03/2010)	CBCCRRR
D2187	CF respiratory early isolate (Bm19, 25/09/2006)	CBCCRRR
D3250	CF respiratory late isolate (Bm19, 28/01/2009)	CBCCRRR
C0514	CF respiratory early isolate (Bm20, 03/03/1983) (also Figure 9)	CBCCRRR
C5449	CF respiratory late isolate (Bm20, 06/10/1993) (also Figure 9)	CBCCRRR
D2494	CF respiratory early isolate (Bm21, 06/06/2007) (also Figure 9)	CBCCRRR
D3752	CF respiratory late isolate (Bm21, 03/04/2010) (also Figure 9)	CBCCRRR
B. cenocepacia early isolates		
C1258	CF respiratory isolate (also Figure 9)	CBCCRRR
C2303	CF respiratory isolate (also Figure 9)	CBCCRRR
C2864	CF respiratory isolate	CBCCRRR
C3868	CF respiratory isolate	CBCCRRR
C3938	CF respiratory isolate	CBCCRRR
C4053	CF respiratory isolate	CBCCRRR
C4364	CF respiratory isolate	CBCCRRR
C4414	CF respiratory isolate (also Figure 9)	CBCCRRR
C4526	CF respiratory isolate	CBCCRRR
C4629	CF respiratory isolate	CBCCRRR
C4914	CF respiratory isolate	CBCCRRR
C5424	CF respiratory isolate	CBCCRRR
C5605	CF respiratory isolate	CBCCRRR
C5876	CF respiratory isolate	CBCCRRR
C5967	CF respiratory isolate	CBCCRRR

Strain or plasmid	Description[a]	Reference[b]
C6006	CF respiratory isolate (also Figure 9)	CBCCRRR
C6114	CF respiratory isolate	CBCCRRR
C6432	CF respiratory isolate	CBCCRRR
C6483	CF respiratory isolate	CBCCRRR
C6956	CF respiratory isolate	CBCCRRR
C7261	CF respiratory isolate	CBCCRRR
D0960	CF respiratory isolate	CBCCRRR
D1903	CF respiratory isolate	CBCCRRR
B. vietnamiensis from Figure 2		
Patient Bv1		
C8395	CF respiratory isolate (03/11/1998)	CBCCRRR
C8414	CF respiratory isolate (04/12/1998)	CBCCRRR
C8644	CF respiratory isolate (18/05/1999)	CBCCRRR
C8952	CF respiratory isolate (07/12/1999)	CBCCRRR
C9178	CF respiratory isolate (27/06/2000)	CBCCRRR
C9177	CF respiratory isolate (27/06/2000)	CBCCRRR
C9710	CF respiratory isolate (26/06/2001)	CBCCRRR
D0247	CF respiratory isolate (12/07/2002)	CBCCRRR
D0774	CF respiratory isolate (25/07/2003)	CBCCRRR
Patient Bv2		
D0099	CF respiratory isolate (23/04/2002)	CBCCRRR
D0278	CF respiratory isolate (15/08/2002)	CBCCRRR
D0718	CF respiratory isolate (19/06/2003)	CBCCRRR
D1181	CF respiratory isolate (15/07/2004)	CBCCRRR
D1212	CF respiratory isolate (21/07/2004)	CBCCRRR
D1211	CF respiratory isolate (21/07/2004)	CBCCRRR
D1476	CF respiratory isolate (03/03/2005)	CBCCRRR
D1632	CF respiratory isolate (16/06/2005)	CBCCRRR
D2074	CF respiratory isolate (18/05/2006)	CBCCRRR
D2075	CF respiratory isolate (18/05/2006)	CBCCRRR
D2178	CF respiratory isolate (15/09/2006)	CBCCRRR
D2273	CF respiratory isolate (04/01/2007)	CBCCRRR
D2459	CF respiratory isolate (03/05/2007)	CBCCRRR
D2455	CF respiratory isolate (03/05/2007)	CBCCRRR
D2460	CF respiratory isolate (03/05/2007)	CBCCRRR
D2605	CF respiratory isolate (13/09/2007)	CBCCRRR
Patient Bv3		
D0072	CF respiratory isolate (15/03/2002)	CBCCRRR
D0121	CF respiratory isolate (26/04/2002)	CBCCRRR
D0439	CF respiratory isolate (29/11/2002)	CBCCRRR
D0779	CF respiratory isolate (01/08/2003)	CBCCRRR
D0780	CF respiratory isolate (01/08/2003)	CBCCRRR
D1389	CF respiratory isolate (06/12/2004)	CBCCRRR
D1767	CF respiratory isolate (26/09/2005)	CBCCRRR
D2448	CF respiratory isolate (30/04/2007)	CBCCRRR
D2910	CF respiratory isolate (31/03/2008)	CBCCRRR
Additional BCC		
B. cepacia		

Strain or plasmid	Description[a]	Reference[b]
ATCC 25416^T	Onion isolate	(330)
ATCC 17759	Soil isolate	(330)
CEP509	CF respiratory isolate	(330)
LMG 17997	UTI isolate	(330)
B. multivorans		
C5393		CBCCRRR
	CF respiratory isolate	(330)
LMG 13010^T	CF isolate	(330)
C1576	CF isolate	(330)
CF-A1-1	CF isolate	(330)
JTC	CGD isolate	(330)
C1962	Brain abscess isolate	(330)
249-2	Laboratory isolate	(330)
D2240	CF respiratory isolate	CBCCRRR
D2685	CF respiratory isolate	CBCCRRR
D2855	CF respiratory isolate	CBCCRRR
C2158	CF respiratory isolate	CBCCRRR
C4785	CF respiratory isolate	CBCCRRR
D1443	CF respiratory isolate	CBCCRRR
D1459	CF respiratory isolate	CBCCRRR
D1948	CF respiratory isolate	CBCCRRR
D1949	CF respiratory isolate	CBCCRRR
D2324	CF respiratory isolate	CBCCRRR
B. cenocepacia		
BC7	CF isolate	(330)
K56-2	CF isolate	(330)
C1394	CF isolate	(330)
PC184	CF isolate	(330)
CEP511	CF isolate	(330)
J415	CF isolate	(330)
ATCC 17765	UTI isolate	(330)
C8747	CF respiratory isolate (Figure 9)	CBCCRRR
D3002	CF respiratory isolate (Figure 9)	CBCCRRR
C5594	CF respiratory isolate (Figure 9)	CBCCRRR
C5491	CF respiratory isolate (Figure 9)	CBCCRRR
B. stabilis		
LMG 14294	CF isolate	(330)
LMG 14086	Respiratory isolate	(330)
LMG 18888	Blood isolate	(330)
B. vietnamiensis		
PC259	CF USA	(330)
G4TR	G4 serially passaged in tobramycin (tobramycin resistant)	This study
G4PC	G4 serially passaged in media alone (passage control)	This study
D0072TR	D0072 serially passaged in tobramycin (tobramycin resistant)	This study
C8395TR	C8395 serially passaged in tobramycin (tobramycin resistant)	This study
C8395AR	C8395 serially passaged in azithromycin (azithromycin resistant)	This study
C8395MR	C8395 serially passaged in meropenem (meropenem resistant)	This study
C8395CR	C8395 serially passaged in ceftazidime (ceftazidime resistant)	This study
C8395SR	C8395 serially passaged in septra (septra resistant)	This study
C8395HP1	C8395 serially passaged in hydrogen peroxide (hydrogen peroxide passage), first pick	This study

Strain or plasmid	Description[a]	Reference[b]
C8395HP2	C8395 serially passaged in hydrogen peroxide (hydrogen peroxide passage), second pick	This study
C8395HP3	C8395 serially passaged in increasing concentrations of hydrogen peroxide (hydrogen peroxide passage), first pick	This study
C8395HP4	C8395 serially passaged in increasing concentrations of hydrogen peroxide (hydrogen peroxide passage), second pick	This study
C8395PC	C8395 serially passaged in media alone (passage control)	This study
C8395PC2	Second C8395 serially passaged in media alone (passage control)	This study
P. aeruginosa		
ATCC 27853	CLSI quality control strain	(232)
E. coli		
ATCC 25922	CLSI quality control strain	(232)
E. faecalis		
ATCC 29212	CLSI quality control strain	(232)
S. aureus		
ATCC 29213	CLSI quality control strain	(232)

[a] Patient identification numbers and bacterial isolation dates (day/month/year) are noted in parentheses for serial clinical isolates.

[b] Abbreviations: BCC, *Burkholderia cepacia* complex; CF, cystic fibrosis; CGD, chronic granulomatous disease; UTI, urinary tract infection; CLSI, Clinical and Laboratory Standards Institute; CBCCRRR, Canadian BCC Research and Referral Repository; BCCH, British Columbia Children's Hospital; IWKHC, IWK Health Centre; USDA, U.S. Department of Agriculture; MUHSC, McMaster University Health Sciences Centre.

[c] Isolates used in Table 5 only are listed first.

the same strain identification having been collected locally, from British Columbia (BC) Children's Hospital or St Paul's Hospital, during that time. Early isolates were defined as the first isolates of the infecting strain to be cultured from a cystic fibrosis (CF) patient. Late isolates were defined as the last isolates to be cultured from a CF patient.

2.2 Strain typing

Bacterial isolates from the CBCCRRR were previously speciated and previously evaluated for strain type by random amplified polymorphic DNA (RAPD) analysis at the Speert Laboratory (University of British Columbia) (332). *B. vietnamiensis* CF isolates from patients Bv1, Bv2, and Bv3 were further typed for this study by pulsed-field gel electrophoresis as described previously (333). Briefly, overnight cultures suspended in agarose (Sigma-Aldrich) were treated with pronase (Roche Applied Science, Laval, QC) and subsequently digested with Spe1 (New England Biolabs Inc., Ipswich, MA) prior to gel-electrophoresis. RAPD analysis of *in vitro* passaged isolates was done using DNA extracted by boiling cells for 15 minutes in the presence of 5% chelex beads (Bio-Rad, Hercules, CA) and previously described *B. cepacia* primers (332). Polymerase chain reaction (PCR) was performed using *Taq* DNA Polymerase (Invitrogen) in a MyCycler Thermal Cycler (Bio-Rad). Reactions contained 1x PCR buffer, 0.25 mM dNTPs, 0.04 µM of each primer, 4% DMSO, 40 ng of template DNA, and 1.25 units of polymerase in a 25 µl total volume. An initial PCR cycle of 94°C for 5 minutes, 36°C for 5 minutes, and 72°C for 5 minutes was performed, followed by 30 cycles as follows: 1 minute of denaturation at 94°C, 1 minute of annealing at 36°C, elongation at 72°C for 1 minute. A final 6 minute elongation at 72°C followed. PCR products were analyzed with a 2100 Bioanalyzer (Aligent Technologies,

Cedar Creek, TX) at the Centre for Molecular Medicine and Therapeutics DNA Sequencing Core Facility (University of British Columbia).

2.3 Growth analysis and antimicrobial time-kill assays

Overnight cultures were first diluted to an optical density at 600 nm (OD_{600}) of 1.0, then further diluted 1:50 (to approximately 2×10^7 colony forming units (CFU)/ml) in 25 or 50 ml MH II Broth, and the OD_{600} was read every hour up to 12 hours and at 24 hours of growth using a SpectraMax Plus384 Microplate Reader (Molecular Devices, Inc., Sunnyvale, CA). Twenty µl samples were also taken at these time points and additionally at an OD_{600} of 0.5, serially diluted 10-fold up to 9 times in phosphate-buffered saline (Fisher Scientific), and 10 µl drops were plated in triplicate on MH II Agar. Viable counts were obtained after overnight growth at the minimal dilution where distinct, accurately countable colonies were present and taken. Differences in CFU/ml among isolates were analyzed using the Student's t-test or one-way analysis of variance (ANOVA). Antimicrobial time-kill assays measuring growth in the presence of gentamicin (Sigma-Aldrich) and tobramycin (Sandoz, Boucherville, QC) at 1, 2, 4, and 8 × the minimum inhibitory concentration (MIC) were done using the same method, with *P. aeruginosa* ATCC 27853 as a control organism, except starting cultures were set up by first growing overnight cultures diluted 1:50 to an OD_{600} 0.5, then diluting those cultures 1:1000 (to approximately 5×10^5 CFU/ml). Growth of *in vitro* passaged isolates was analyzed using a Bioscreen C (Growth Curves, Piscataway, NJ). Overnight cultures were diluted to 1×10^7 CFU/ml in 300 µl MH II Broth, and incubated with shaking for 24 hours, with OD_{600} readings taken every 15 minutes.

2.4 Antimicrobial susceptibility testing

Antimicrobial MICs were determined using established MH II Agar dilution and MH II Broth microdilution methods (334), with the exception of cationic peptide MICs, which were determined based on a previously described modified broth microdilution method (335). Briefly, peptide solutions were prepared in 96-well polypropylene microtiter plates with 0.2% bovine serum albumin (Sigma-Aldrich) and 0.01% acetic acid (Fisher Scientific). Agar dilution MICs were used as screens and determined only once. Broth microdilution MIC testing was done in triplicate unless otherwise stated. *P. aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *S. aureus* ATCC 29213 were used as quality control organisms. Established *P. aeruginosa* and non-*Enterobacteriaceae* breakpoints were used to analyze susceptibility to aminoglycosides, imipenem, cefepime, and ciprofloxacin. Established *B. cepacia* breakpoints were used to analyze susceptibility to meropenem, ceftazidime, and trimethoprim/sulfamethoxazole. Antibiotics were purchased from Sigma-Aldrich, MP Biomedicals, EMD Chemicals, Sandoz, AstraZeneca (Mississauga, ON), Pfizer (Kirkland, QC), Pharmaceutical Partners of Canada Inc. (Richmond Hill, ON), and GlaxoSmithKline (Mississauga, ON). Cationic peptides were provided by REW Hancock (University of British Columbia). Susceptibility testing data with the efflux pump inhibitor MP 601384 were generously provided by O. Lomovskaya (Mpex Pharmaceuticals, San Diego, CA).

2.5 Patient data

Forced expiratory volume in 1 second and antimicrobial therapy data were extracted

from hospital charts for CF patients chronically infected with *B. vietnamiensis*, from the time of their initial colonization until their death or most recent isolate up to August 2008, as reported previously (157). Ethical approval was obtained from the University of British Columbia, BC Children's and Women's Hospital, and Providence Health Services Authority research ethics boards.

2.6 *In vitro* selection of bacterial isolates

2.6.1 *In vitro* selection of antibiotic-resistant *B. vietnamiensis*

Overnight cultures were diluted 1 in 10 in 25 or 50 ml MH II Broth containing tobramycin (Sandoz) or ceftazidime (Pharmaceutical Partners of Canada Inc.) at half the MIC, or azithromycin (Pfizer), meropenem (AstraZeneca), or trimethoprim/sulfamethoxazole (GlaxoSmithKline) at a quarter of the MIC. Resistant isolates were selected following serial 24 hour inoculations into broth containing serially doubling concentrations of antibiotic. Inoculations were repeated until early isolates grew at antibiotic concentrations representing half the MICs found for late isolates, with the exception of the exposure of *B. vietnamiensis* C8395 to azithromycin up to 2048 μg/ml (the precise MIC for the late isolate D0774 was not determined). Antimicrobial MICs were determined after each 24 hour passage. Resultant isolates were plated out on antibiotic-free MH II Agar three successive times, after which antimicrobial MICs were determined. Aminoglycoside MICs and MICs of the agent to which the isolates were exposed were also determined after 10 and 20 passages on antibiotic-free agar after culture freezing. The environmental *B. vietnamiensis* isolate G4 was serially exposed to tobramycin over 8 days using the same protocol. Overnight cultures were also

diluted 1 in 10 in 50 ml broth containing 1, 2, 4, 8, or 16 µg/ml azithromycin. After 24 hours, aminoglycoside MICs were determined.

2.6.2 *In vitro* selection of *B. vietnamiensis* after serial exposure to hydrogen peroxide

The early *B. vietnamiensis* isolate C8395 was serially exposed to hydrogen peroxide (Safeway, Calgary, AB) based on previously described methods (329). Briefly, an overnight culture was diluted 1 in 49 in 50 ml of MH II Broth, grown for 2 hours, after which peroxide was added at half the MIC three times at 2 hour intervals. This was repeated every 24 hours over 8 days, at which time serial dilutions of the resultant isolate were plated on antibiotic-free MH II Agar and agar supplemented with tobramycin at 2.5 × the MIC. An 8-day unexposed C8395 control was processed in parallel. Tobramycin resistance frequencies were calculated relative to growth on non-antibiotic agar. Randomly selected tobramycin-resistant and passage-control colonies were subsequently picked, passaged 8 times on antibiotic-free agar, and then evaluated for antimicrobial susceptibility by MIC testing. In addition, C8395 was serially exposed to serially doubling and gradually (0.25 mM increases daily) increasing concentrations of peroxide using the same methods, starting at a concentration of half the MIC, over 8 days or until growth could no longer be observed. Peroxide MICs were determined using the same method for antimicrobial broth microdilution MIC testing (334).

2.7 Catalase activity testing

Single colonies grown on MH II Agar were picked into 40 µl drops of hydrogen peroxide (Safeway) on glass cover slips. For the screen involving many isolates of multiple species, colonies were first spread onto glass cover slips before being covered in whole by

peroxide. The immediate formation of many bubbles indicated catalase activity. Isolates were termed weakly positive for catalase activity if only a few, 10 or fewer small bubbles were observed. *B. vietnamiensis* D0774 and *B. cenocepacia* J2315 were used as positive controls.

2.8 Exopolysaccharide production analysis

After bacterial growth on YEM, the capacities of isolates to elaborate EPS was determined based on a previously defined scoring method: nonmucoid (-), no evidence of EPS production and colonies are dry and matte; partially mucoid (+), evidence of EPS production in the confluent growth region but the plate contains predominately nonmucoid bacteria; frankly mucoid, both the confluent area and single colonies are mucoid in appearance (++), EPS production overwhelms the streaked-out area and raised areas are observed (+++), same as +++ but EPS drips onto the lid of the plate (+++d) (218).

2.9 Measurement of aminoglycoside cellular accumulation

The accumulation of [^{3}H]gentamicin in bacterial cells was determined as described previously (336), with the following modifications. Overnight cultures were diluted in 50 ml of LB medium, grown to an OD_{600} of 0.5 to 0.65, and adjusted to an OD_{600} of 0.5 to 0.55 if necessary. 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 (Sigma-Aldrich) to a final concentration of 5 or 20 µg/ml, with a specific activity of 88 dpm/ng. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added to a final concentration of 50 µM. 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 (Sigma-Aldrich)-50 mM KPO_4 (made from Sigma KH_2P0_4 and EMD Chemicals K_2HP0_4), pH 7.0, air dried, and used for determination of radioactivity in a Beckman LS 6000IC liquid scintillation counter (Beckman Coulter Inc., Indianapolis). 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 antimicrobial MIC testing and growth curve analysis in the presence of the antibiotics. *P. aeruginosa* ATCC 27853 was used as a control organism. Differences in [^{3}H]gentamicin accumulation among isolates were analyzed with one- and two-way ANOVA.

2.10 Lipopolysaccharide purification and analysis

After growth in LB medium, lipopolysaccharide (LPS) was isolated with hot water-phenol extraction (337) from sequential *B. vietnamiensis* isolates C8395, C8952, and D0774, and purified to remove potential Toll-like receptor 2 activating proteins as described previously (338). This work was carried out by RK Ernst (University of Washington, Seattle, WA). LPS was also analyzed from proteinase K (Fisher Scientific) digested lysates based on a previously described method (339), with the following modifications. Overnight cultures grown in LB medium were diluted to an OD_{600} of 0.5, and 1.5 ml was harvested by centrifugation at 10,000 × g for 1.5 minutes. Cells were heated for 10 minutes at 100°C in

100 µl of lysing buffer containing 2% sodium dodecyl sulfate (SDS) (Fisher Scientific), 4% 2-mercaptoethanol (EMD Chemicals), 10% glycerol (MP Biomedicals), 0.004% bromophenol blue (Sigma-Aldrich), and 1 M Tris-HCl (Fisher Scientific) (pH6.8). Proteins were digested for 5 hours with 25 µg of proteinase K and solubilized in 10 µl of lysing buffer. Twenty micrograms of purified LPS or 5 µl of LPS fraction were analyzed by polyacrylamide (Bio-Rad) gel electrophoresis using the Laemmli buffer system with 4% and 12.5% stacking and separating gels, respectively, that did not contain SDS (340). Electrophoresis was done at 60 V with Tris-glycine (EMD Chemicals) buffer (pH 8.3) plus 1% SDS for approximately 4 hours. Separating gels were stained with silver nitrate by use of a Pierce silver stain kit (Thermo Fisher Scientific, Waltham, MA) as per the manufacturer's protocol. Controls included smooth LPS from *E. coli* 0111 (Invitrogen) and from *B. multivorans* C5568 and *B. cenocepacia* C6433 (previously extracted (341)).

2.11 Lipid A isolation and mass spectrometry

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 (342). Lipid A was extracted by SDS-based hydrolysis (343). Negative-ion matrix-assisted laser desorption ionization–time offlight (MALDI -TOF) mass spectrometry was performed as described previously (280). Experiments were performed using a Bruker Autoflex II MALDI -TOF mass spectrometer (Bruker Daltonics Inc., Billerica, MA), and each spectrum was an average of 300 shots. This work was carried out by RK Ernst (University of Washington).

2.12 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 based on a previously described method (344). Briefly, overnight cultures were diluted 1 in 50 ml of MH II Broth, grown with shaking at 200, 225, or 250 rpm to an OD_{600} between 0.4 and 0.6, harvested by centrifugation at 3000 × g for 10 minutes, and washed and resuspended in 5 mM sodium HEPES buffer (Sigma-Aldrich) (pH 7.2) containing 5 µM CCCP (Sigma-Aldrich) and 5 mM glucose (EMD Chemicals) to an OD_{600} of 0.5. Incubation of cells with CCCP prior to the addition of NPN ensured that fluorescence was optimal by trapping it in the hydrophobic compartment. NPN was added to a final concentration of 10 µM. Gentamicin and tobramycin were added to final concentrations of 1, 2, 4, 8, 16, 32, 64, and 128 µg/ml. 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, Waltham, MA). NPN was added to cells 30 seconds after initiation of readings, and antibiotic was added 30 to 90 seconds later. *P. aeruginosa* ATCC 27853 and *B. multivorans* 26D7 (345) were used as control organisms. Supplementary experiments were carried out in buffer containing 50 µM CCCP, sodium azide (at 0.1, 10, or 20 mM) as an alternative for CCCP, or in the absence of inhibitors of energy-dependent processes or glucose. All experiments were performed in REW Hancock's laboratory. Differences in the associated NPN fluorescence among isolates were analyzed with one-way ANOVA.

2.13 Analysis of resistance-nodulation-division (RND) efflux system genes

2.13.1 Bioinformatic analysis

Sequences were retrieved from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) protein or gene databases. The search for multidrug RND transporters was done using conserved amino acid sequences (346) and the Basic Local Alignment Search Tool (BLAST) program provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd/) was used for protein classification. Sequence similarity searches between potential efflux system proteins and proteins in *B. cenocepacia*, *B. pseudomallei*, and *P. aeruginosa* were done using the BLAST program within NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein sequences were aligned using DNAMAN sequence analysis software (Lynnon Corporation, Pointe-Claire, QB). *B. vietnamiensis* Bcep1808_1575 structure/function was predicted using the TMHMM program (347) within the Center for Biological Sequence Analysis website (http://www.cbs.dtu.dk/services/TMHMM/) for prediction of transmembrane domains, the Phyre server (348) within the Structural Bioinformatics Group website (http://www.sbg.bio.ic.ac.uk/~phyre/) for prediction of the 3-dimentional structure, and the InterProScan Sequence Search program (349) within the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/pfa/iprscan/) for functional analysis.

2.13.2 Genomic DNA isolation and quantification

Genomic DNA for polymerase chain reaction was isolated based on a previously described protocol (350). One loop full of cells was suspended in Chelex 100 Resin (Bio-

Rad) diluted to 5% in water, and boiled for 15 minutes. Cellular debris was removed by centrifugation at 4500 × g for 15 minutes. DNA concentration in the supernatant was quantified using a NanoDrop 100 Spectrophotometer (Thermo Scientific), diluted to 20 and 5 ng/µl, and stored in short-term at 4°C.

2.13.3 Polymerase chain reaction

Oligonucleotide primers were designed by Primer Quest within the Integrated DNA Technologies website (http://www.idtdna.com/scitools/applications/primerquest/) using sequences retrieved from NCBI and synthesized by Integrated DNA Technologies (Coralville, IA) (Table 3). PCR was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc.) in a MyCycler Thermal Cycler (Bio-Rad). Reactions contained 1x Phusion HF buffer, 0.4 mM dNTPs, 0.5 µM of each primer, 4% DMSO, 10 or 40 ng of template DNA, and 0.5 units of polymerase in a 25 µl total volume. Denaturation was performed for 3 minutes at 98°C followed by 30 cycles as follows: 30 seconds of denaturation at 98°C, 30 seconds of annealing at 66°C for *amrB, amrAB,* and *amrB-OprA*, elongation at 72°C for 30 seconds for *amrAB* and *amrB-OprA*, and 1 minute for *amrB*. A final 10 minute elongation at 72°C followed. PCR products were analyzed by agarose gel electrophoresis on a 0.9 % gel for *amrB* and 1.2% gel for *amrAB* and *amrB-OprA*.

2.14 Quantification of RND transporter *amrB* expression

Overnight cultures were diluted 1:100 into 50 ml MH II broth, LB medium, or SCFM, with or without tobramycin and ceftazidime at half the MIC, or azithromycin, meropenem, and trimethoprim/sulfamethoxazole at a quarter of the MIC, and grown to an

Table 3. Oligonucleotide primers used in this study

Gene	Forward (5' to 3')	Reverse (5' to 3')
For Bv PCR[a]		
amrB	TGATCGCGCTGTTCATCCTG	AATGCGAACCCTCCATCGTC
amrAB	AGCACGACGTCACGGTCA	ACGTTCGCCGACGCGTATT
amrB-OprA	CAAGGGCAGGCTGCTGTTCA	TGGATGTTGTCCGCTGCCTTCT
16S	TGCGGGACTTAACCCAACATCTCA	ACCGGAAGAATAAGCACCGGCTAA
For QRT-PCR[b]		
Bv *amrB*	CCGAACGACATCTACTTCAAGGTCGG	ATCCTTCGCGACTTCGACGATCAG
Bc *amrB*	GTGCGCGTATCGATGAACAAGGTC	CGCAGGTTCTGCATGAACAGGAAC
16S	CACGCTTTACGCCCAGTAATTCCG	CCGGAAGAATAAGCACCGGCTAAC

[a] Primers were designed with PrimerQuest and generated by Integrated DNA Technologies based on the *B. vietnamiensis* G4 sequence (http://www.ncbi.nlm.nih.gov/nuccore/CP000614.1).
[b] Primers were designed with PrimerQuest and generated by Alpha DNA based on the *B. vietnamiensis* G4 sequence (http://www.ncbi.nlm.nih.gov/nuccore/CP000614.1) or *B. cenocepacia* J2315 sequence (http://www.sanger.ac.uk/Projects/B_cenocepacia/private/).
[c] Abbreviations: Bv, *B. vietnamiensis*; Bc, *B. cenocepacia.*

OD_{600} of 0.3, 0.5, or 0.8. Total bacterial RNA was extracted from 0.5 ml of cultures using the Qiagen RNeasy Plus Mini kit (Qiagen, Toronto, ON), and treated with Rnase-free Dnase (Promega, San Luis Obispo, CA) (1 U enzyme/µg of RNA for 60 min at 37°C, followed by 15 min at 65°C). Reverse transcription was performed using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The resultant copy DNA was quantified in a 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) in the presence of SybrGreen (Invitrogen), with primers designed by Primer Quest using sequences retrieved from NCBI or the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/about/), and synthesized by Alpha DNA (Montreal, QB) (Table 3). To ensure samples were free of genomic DNA contamination, non-reverse transcribed RNA was also quantified by PCR. Differences in *amrB* expression between isolates and growth conditions were analyzed using technical means and the Student's t-test or one-way ANOVA.

Chapter 3: CHARACTERIZATION OF AMINOGLYCOSIDE SUSCEPTIBILITY AND ACQUIRED RESISTANCE IN *B. VIETNAMIENSIS*

3.1 Summary

It is the current dogma that all species of the *Burkholderia cepacia* complex (BCC) are highly and intrinsically resistant to the inhibitory effects of a number of antimicrobials, including polycationic agents such as aminoglycosides and polymyxins (229). Previous studies, however, have identified *B. vietnamiensis* isolates that are susceptible to several antibiotics, including aminoglycosides, but not to polymyxins (24, 26). The purpose of this study was to gain a better understanding of antimicrobial susceptibility within the BCC, as well as the frequency and induction of aminoglycoside resistance in *B. vietnamiensis* specifically. We observed that clinical and environmental isolates of *B. vietnamiensis* were more often susceptible to a number of antimicrobials, including aminoglycosides, than those of other BCC species, based on antimicrobial minimum inhibitory concentrations (MICs) and established breakpoints (232), but were not inhibited by other cationic agents (natural and synthetic cationic antimicrobial peptides and polymyxin B). *B. vietnamiensis* strains acquired aminoglycoside resistance during chronic infection in cystic fibrosis (CF) patients, and *in vitro* under tobramycin, azithromycin, and hydrogen peroxide pressure. *B. vietnamiensis* isolates displayed enhanced catalase activity and became less mucoid, alongside the acquisition of aminoglycoside resistance. Gentamicin and tobramycin concentrations up to 8 × the MIC were unable to kill a susceptible *B. vietnamiensis* CF isolate.

3.2 Introduction

Large-scale studies of antimicrobial susceptibility involving multiple BCC species (≥38 isolates and≥4 antibiotics tested) have, for the most part, not distinguished among species (263, 269-272, 300-306, 308, 351) or have not included large numbers of isolates for every species (24, 25, 268, 352), making susceptibility comparisons among BCC species difficult. The exception is a study published by Vermis *et al.* (2003) who tested 14 to 20 isolates per species (for each of the 9 that were part of the complex at that time). The authors concluded that, based on antimicrobial MICs and established breakpoints (232), *B. vietnamiensis* was most susceptible to ticarcillin, imipenem, tetracycline, and amikacin, the latter an aminoglycoside, yet isolates were not inhibited by polymyxin B (26). Acquired aminoglycoside resistance has not been investigated in the BCC, given that the current dogma is that all BCC species are intrinsically resistant to aminoglycosides (229). However, fluctuations in tobramycin, gentamicin, and/or amikacin MICs during chronic infection with strains from various BCC species have been noted (216, 219, 304, 352), although not from *B. vietnamiensis* specifically.

The specific objectives were:

1. To determine the antimicrobial susceptibility of *B. vietnamiensis* in comparison to other BCC species.
2. To determine the frequency and induction of aminoglycoside resistance in *B. vietnamiensis*.

3.3 Results

3.3.1 *B. vietnamiensis* is more susceptible to aminoglycoside and carbapenem antibiotics than other *B. cepacia* complex species

Agar dilution antimicrobial MIC testing of 140 isolates, using established methods and breakpoints (232), identified *B. vietnamiensis* as the species most often susceptible to aminoglycosides of the five investigated (Table 4). At concentrations that define antimicrobial susceptibility breakpoints for *P. aeruginosa* and non- *Enterobacteriaceae* (334) (aminoglycoside breakpoints for BCC species are not defined), 58.0%, 12.3%, and 18.5% of *B. vietnamiensis* isolates were inhibited at or less than 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. The *B. stabilis* sample size was too small for definitive comparison. Within *B. vietnamiensis*, environmental isolates were most often susceptible to aminoglycosides, while CF isolates were most often resistant. The aminoglycoside MICs for the first isolates for six patients, however, were markedly different from the aminoglycoside MICs for all CF isolates combined: 2 and all 6 of the first isolates were inhibited by 16 µg/ml amikacin, respectively; 1, 3, and all 6 of the first isolates were inhibited by 4, 16, and 64 µg/ml gentamicin, respectively; 5 and all 6 of the first isolates were inhibited by 4 and 16 µg/ml kanamycin, respectively; and 3 and all 6 of the first isolates were inhibited by 4 and 16 µg/ml tobramycin, respectively. *B. vietnamiensis* isolates were also more often susceptible to the carbapenem antibiotics imipenem and meropenem than isolates from the other BCC species (Table 5). MIC ranges for all sources and antimicrobials were extensive.

Table 4. Antimicrobial susceptibilities of *B. cepacia* complex species to aminoglycosides[b]

Species[a] (n)	Test agent	MIC range (μg/ml)	Number (%) inhibited at concentrations (μg/ml)			
			1	4	16	64
B. vietnamiensis						
Clinical CF (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 (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;	AMK	8	0	0	3 (100.0)	3 (100.0)
Hospital (3)	GEN	32	0	0	0	3 (100.0)
	KAN	4	0	3 (100.0)	3 (100.0)	3 (100.0)
	TOB	8	0	0	3 (100.0)	3 (100.0)
Environmental (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 (81)	AMK	1 – >128	4 (4.9)	12 (14.8)	47 (58.0)	69 (85.2)
	GEN	≤0.5 – >128	6 (7.4)	10 (12.3)	21 (25.9)	58 (71.6)
	KAN	≤0.5 – >128	9 (11.1)	29 (35.8)	62 (76.5)	77 (95.1)
	TOB	≤0.5 – >128	7 (8.6)	15 (18.5)	54 (66.7)	75 (92.6)
B. cepacia	AMK	4 - >128	0	1 (7.7)	1 (7.7)	5 (38.5)
All sources (13)	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	AMK	16 – >128	0	0	1 (4.3)	12 (52.2)
All sources (23)	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	AMK	32 - >128	0	0	0	4 (21.1)
All sources (19)	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)
B. stabilis	AMK	4 – >128	0	1 (25)	1 (25)	1 (25)
All sources (4)	GEN	≤0.5 – >128	1 (25)	1 (25)	1 (25)	1 (25)
	KAN	≤0.5 – >128	1 (25)	1 (25)	1 (25)	3 (75)
	TOB	≤0.5 – >128	1 (25)	1 (25)	1 (25)	2 (50)

[a] *B. cepacia* sources: 6 CF, 4 clinical non-CF, 3 environmental; *B. multivorans* sources: 11 CF, 4 clinical non-CF, 8 environmental; *B. cenocepacia* sources: 10 CF, 4 clinical non-CF, 5 environmental.

[b] Abbreviations: CF, cystic fibrosis; MIC, minimum inhibitory concentration; AMK, amikacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin.

Table 5. Antimicrobial susceptibilities of *B. cepacia* complex species to non-aminoglycoside antibiotics[b]

Species (n[a])	Test agent	MIC range (μg/ml)	Number (%) inhibited at concentrations (μg/ml)			
			1	4	16	64
B. vietnamiensis (79)	ERY	16 – >64	0	0	2 (2.5)	54 (68.4)
	CLR	8 – >64	0	0	21 (26.6)	64 (81.0)
	IPM	≤0.5 – >32	51 (64.6)	71 (89.9)	73 (92.4)	n/a
	MEM	≤0.5 – >32	29 (36.7)	72 (91.1)	77 (97.5)	n/a
	CAZ	≤0.5 – 64	40 (50.6)	70 (88.6)	77 (97.5)	79 (100.0)
	FEP	1 – >64	10 (12.7)	46 (58.2)	66 (83.5)	76 (96.2)
	CIP	≤0.5 – >32	3 (3.7)	58 (71.6)	69 (85.2)	n/a
B. cepacia (4)	ERY	64 – >64	0	0	0	1 (25)
	CLR	64 – >64	0	0	0	1 (25)
	IPM	8 – >32	0	0	2 (50)	n/a
	MEM	1 – 16	1 (25)	1 (25)	4 (100)	n/a
	CAZ	≤0.5 – 16	1 (25)	1 (25)	4 (100)	4 (100)
	FEP	2 – 64	0	1 (25)	1 (25)	4 (100)
	CIP	≤0.5 – >32	3 (23.1)	12 (92.3)	12 (92.3)	n/a
B. multivorans (11)	ERY	64 – >64	0	0	0	3 (27.3)
	CLR	32 – >64	0	0	0	7 (63.6)
	IPM	≤0.5 – >32	1 (9.1)	1 (9.1)	1 (9.1)	n/a
	MEM	1 – 32	1 (9.1)	4 (36.4)	10 (90.9)	n/a
	CAZ	1 – 32	4 (36.4)	7 (63.6)	8 (72.7)	11 (100)
	FEP	1 – >64	2 (18.2)	7 (63.6)	7 (63.6)	10 (90.9)
	CIP	1 – 16	3 (13.0)	19 (82.6)	23 (100)	n/a
B. cenocepacia (12)	ERY	32 – >64	0	0	0	1 (8.3)
	CLR	16 – >64	0	0	1 (8.3)	5 (41.7)
	IPM	16 – >32	0	0	3 (25)	n/a
	MEM	4 – >32	0	3 (25)	9 (75)	n/a
	CAZ	2 – 64	0	7 (58.3)	10 (83.3)	12 (100)
	FEP	4 – >64	0	1 (8.3)	3 (25)	9 (75)
	CIP	1 – >32	2 (10.5)	9 (47.4)	14 (73.7)	n/a
B. stabilis (4)	ERY	>64	0	0	0	0
	CLR	16 – >64	0	0	1 (25)	1 (25)
	IPM	8 – >32	0	0	3 (75)	n/a
	MEM	4 – 16	0	2 (50)	4 (100)	n/a
	CAZ	2 – 8	0	2 (50)	4 (100)	4 (100)
	FEP	16 – >64	0	0	1 (25)	3 (75)
	CIP	2 – >32	0	1 (25)	2 (50)	n/a

[a] Except with CIP where n = 81, 13, 23, and 19 for *B. vietnamiensis, B. cepacia, B. multivorans, B. cenocepacia,* and *B. stabilis* respectively.

[b] Abbreviations: MIC, minimum inhibitory concentration; ERY, erythromycin; CLR; clarithromycin; IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin.

3.3.2 *B. cepacia* complex species are resistant to the inhibitory activity of cationic antimicrobial peptides and polymyxin B

To determine if aminoglycoside-susceptible *B. vietnamiensis* isolates could also be inhibited by cationic antimicrobial peptides and polymyxin B, the activities of these agents against a subset of isolates were evaluated by broth microdilution antimicrobial MIC testing (Table 6). The activities of natural and synthetic cationic peptides against the BCC experimental strain panel (330) were also determined (Table 7). Virtually all *B. vietnamiensis* isolates were highly resistant to the antimicrobial activity of cationic antimicrobial peptides and to polymyxin B, with the majority of MICs being >128 µg/ml and >75 µg/ml, respectively. Within *B. vietnamiensis* isolates, the greatest inhibitory activity was CP26 against CEP0106 (MIC of 8 µg/ml). The cationic peptides were also inactive against isolates from other BCC species, with the exception of a lab strain, *B. multivorans* 249-2, which was inhibited by 8 µg/ml CP26 and 4 µg/ml CP29. Of the peptides, CP29 had the greatest inhibitory activity against BCC species.

3.3.3 *B. vietnamiensis* acquires aminoglycoside resistance *in vivo*

Further evaluation of aminoglycoside susceptibility in sequential CF isolates C8395, C8952, and D0774 from patient Bv1 and D0099 and D2075 from patient Bv2, by broth microdilution antimicrobial MIC testing using established methods and non-*Enterobacteriaceae* breakpoints (232), 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 8). Further evaluation of other select isolates by broth microdilution MIC testing confirmed that *B.*

Table 6. Antimicrobial susceptibilities of *B. vietnamiensis* to cationic peptides and polymyxin B

	MIC (μg/ml) for indicated agent[a]										
Isolate	Bac2a	K24	E2	E6	CP26	CP29	IND	LL-37	PMI	P7	PMB
Clinical CF											
D1389	>128	>128	>128	>128	>128	32	>128	>128	64	>128	>75
C8395	>128	>128	>128	>128	>128	64	>128	>128	>128	>128	>75
C8952	>128	>128	>128	>128	>128	64	>128	>128	128	>128	>75
D0774	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
D0099	>128	>128	>128	>128	>128	>128	>128	>128	128	>128	>75
D2075	>128	>128	>128	>128	>128	>128	>128	>128	128	>128	>75
Clinical non-CF											
LMG 06999	>128	>128	>128	>128	>128	16	>128	>128	128	>128	>75
Environmental											
FC0656	>128	>128	>128	>128	128	32	>128	>128	128	>128	37.5
CEP0106	>128	>128	>128	>128	8	16	>128	>128	16	>128	>75
LMG 10929^T	>128	>128	>128	>128	>128	>128	>128	>128	128	>128	>75
G4	>128	>128	>128	>128	>128	32	>128	>128	128	>128	>75

[a] Abbreviations: CF, cystic fibrosis; MIC, minimum inhibitory concentration; Bac2A, K24, E2 and E6, synthetic derivatives of a bovine bactenecin; CP26 and CP29, analogues based on the insect cecropin-bee melittin hybrid peptide; IND, bovine indolicidin; LL-37, human cathelicidin; PMI, horseshoe crab polyphemusin I; P7, inactive peptide.

Table 7. Antimicrobial susceptibilities of the *B. cepacia* complex strain panel to cationic peptides and polymyxin B

Strain	MIC (μg/ml) for indicated agent[a]									
	Bac2a	K24	E2	E6	CP26	CP29	IND	LL-37	PMI	PMB
B. cepacia										
ATCC 25416^T	>128	>128	>128	>128	>128	>128	>128	>128	128	>75
ATCC 17759	>128	>128	>128	>128	>128	>128	>128	>128	128	>75
CEP509	>128	>128	>128	>128	>128	>128	>128	>128	128	>75
LMG 17997	>128	>128	>128	>128	>128	64	>128	>128	128	>75
B. multivorans										
C5393	>128	>128	>128	>128	>128	>128	>128	>128	128	>75
LMG 13010^T	>128	>128	>128	>128	>128	>128	>128	>128	128	>75
C1576	>128	>128	>128	>128	>128	>128	>128	>128	128	>75
CF-A1-1	>128	>128	>128	>128	>128	64	>128	>128	>128	>75
JTC	>128	>128	>128	>128	>128	>128	>128	>128	128	>75
C1962	>128	>128	>128	>128	>128	>128	>128	>128	128	>75
ATCC 17616	>128	>128	>128	>128	>128	64	>128	>128	>128	>75
249-2	>128	>128	128	>128	8	4	64	>128	16	18.75
B. cenocepacia										
J2315	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
BC7	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
K56-2	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
C5424	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
C6433	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
C1394	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
PC184	>128	>128	>128	>128	>128	64	>128	>128	128	>75
CEP511	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
J415	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
ATCC 17765	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
B. stabilis[b]										
LMG 14294	>128	>128	>128	>128	>128	>128	>128	>128	>128	>75
LMG 14086	>128	>128	>128	>128	128	64	>128	>128	128	>75
LMG 18888	>128	>128	32	>128	128	32	>128	>128	64	>75
B. vietnamiensis										
PC259	>128	>128	>128	>128	>128	>128	>128	>128	128	>75
LMG 16232	>128	>128	>128	>128	>128	128	>128	>128	128	>75
FC441	>128	>128	>128	>128	128	32	>128	>128	128	>75
LMG 10929^T	>128	>128	>128	>128	>128	>128	>128	>128	128	>75

[a] Abbreviations: MIC, minimum inhibitory concentration; Bac2A, K24, E2 and E6, synthetic derivatives of a bovine bactenecin; CP26 and CP29, analogues based on the insect cecropin-bee melittin hybrid peptide; IND, bovine indolicidin; LL-37, human cathelicidin; PMI, horseshoe crab polyphemusin I; P7, inactive peptide.
[b] Antimicrobial MICs for *B. stabilis* C7322 were not obtained because the isolate could not be cultured.

Table 8. Antimicrobial susceptibilities of select *B. vietnamiensis* isolates to aminoglycosides

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

[a] Patient identification numbers and bacterial isolation dates are noted in brackets.

[b] Abbreviations: CF, cystic fibrosis; MIC, minimum inhibitory concentration; AMK, amikacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin.

vietnamiensis is however often susceptible to aminoglycosides; based on established breakpoints (232), an early isolate from a third CF patient, patient Bv3, a non-CF clinical isolate, and environmental isolates of *B. vietnamiensis* were all susceptible to aminoglycosides (Table 8). To determine if *B. multivorans* and *B. cenocepacia* could also acquire aminoglycoside resistance during the course of a chronic CF infection, we tested the tobramycin susceptibilities of early and late isolates or just early isolates, respectively, by agar dilution using established methods and non-*Enterobacteriaceae* breakpoints (232) (Table 9). None of the *B. cenocepacia* early isolates were susceptible to tobramycin and therefore did not merit further investigation. Tobramycin MIC evaluation of *B. multivorans* sequential isolates revealed two instances where tobramycin MICs for early isolates were ≤4 μg/ml and for late isolates were ≥16-fold higher than those. Further susceptibility analysis by broth microdilution antimicrobial MIC testing of all *B. multivorans* isolates recovered from these chronic infections, however, did not identify any cases of acquired aminoglycoside resistance (where MICs were initially ≤4 μg/ml and later at least 3-fold higher), but did identify a unique infection where the strain remained tobramycin susceptible and may represent a heterogeneous population (Table 10).

To examine the phenomenon of acquired aminoglycoside resistance in *B. vietnamiensis* further, patient antimicrobial therapy data and the tobramycin MICs for all sequential isolates were analyzed for three chronically infected patients, from the time of their initial colonization until their death or most recent isolate (Figure 2). Lung function data were included as a general indicator of patient health. Patients received 19 (Figure 2a), 9 (Figure 2b), or no (Figure 2c) courses of tobramycin treatment while infected with *B. vietnamiensis*. The tobramycin MICs for infecting strains increased from 2 to 128 μg/ml

Table 9. Tobramycin susceptibilities of *B. multivorans* and *B. cenocepacia* CF isolates [a]

Species (n)	MIC range (μg/ml)	Number (%) inhibited at concentrations (μg/ml)			
		1	4	16	64
B. multivorans					
Early isolates (21)	2 – >128	0	2 (9.5)	5 (23.8)	15 (71.4)
Late isolates (21)	16 - >128	0	0	2 (9.5)	12 (57.1)
B. cenocepacia					
Early isolates (23)	32 - >128	0	0	0	3 (13.0)

[a] Abbreviations: CF, cystic fibrosis; MIC, minimum inhibitory concentration.

Table 10. Tobramycin susceptibilities of *B. multivorans* sequential isolates

Isolate[a]	MIC (μg/ml)[b]
D2187 (Bm1, 25/09/2006)	64
D2240 (Bm1, 15/11/2006)	128
D2685 (Bm1, 25/10/2007)	>128
D2855 (Bm1, 14/02/2008)	>128
D3250 (Bm1, 28/01/2009)	>128
C0514 (Bm2, 03/03/1983)	64
C2158 (Bm2, 22/01/1987)	64
C4785 (Bm2, 21/07/1992)	128
C5449 (Bm2, 06/10/1993)	32
D1407 (Bm3, 15/12/2004)	4
D1443 (Bm3, 15/12/2004)	2
D1459 (Bm3, 10/02/2005)	2
D1948 (Bm3, 02/02/2006)	128
D1949 (Bm3, 02/02/2006)	2
D2324 (Bm3, 24/01/2007)	≤1
D3220 (Bm3, 05/12/2008)	4

[a] Patient identification numbers and bacterial isolation dates are noted in brackets.

[b] Tobramycin MICs for early and late isolates were done is triplicate, and in duplicate for middle isolates from patients Bm1 and Bm2. The lower MIC is shown if there was a 2-fold difference between duplicates. Abbreviations: MIC, minimum inhibitory concentration.

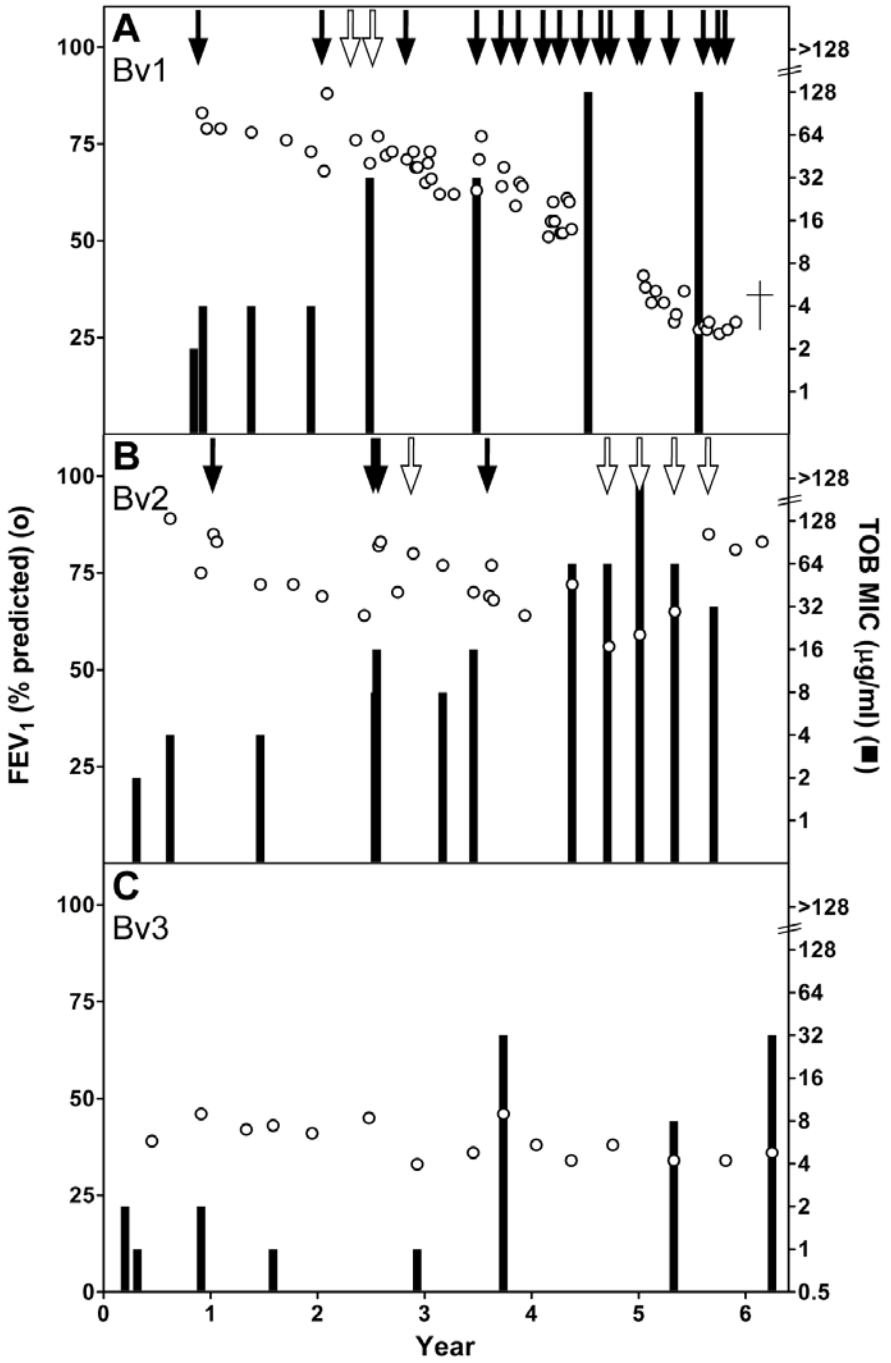

Figure 2. *B. vietnamiensis* acquisition of aminoglycoside resistance *in vivo*.
Chronic *B. vietnamiensis* infections in cystic fibrosis patients Bv1 (A), Bv2 (B), and Bv3 (C) were evaluated based on clinical chart data. Patient FEV_1 data are shown as open circles. Tobramycin MICs for the infecting strains are shown as bar graphs. 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 treatment. Open arrows indicate start dates of inhaled tobramycin treatment. A cross refers to the time of patient death. Abbreviations: TOB, tobramycin; FEV_1, forced expiratory volume in 1 second.

(Figure 2a), from 2 to >128 µg/ml (Figure 2b), and from 1 to 32 µg/m1 (Figure 2c). Tobramycin MIC fluctuations occurred for strains infecting patients Bv2 (Figure 2b) and Bv3 (Figure 2c). Patients Bv1 and Bv2 were co-infected 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 (cloxacillin, piperacillin, imipenem, meropenem, aztreonam, cephalexin, ceftazidime, cefotaxime, and cefuroxime), ciprofloxacin, colistin, chloramphenicol, azithromycin, and trimethoprim/sulfamethoxazole (Table 11). Select isolates were used to confirm strain type by pulsed-field gel electrophoresis (PFGE) (Figure 3). Isolates are considered genetically indistinguishable if their restriction patterns are identical, and closely related if PFGE patterns differ by ≤ three bands because single genetic events, such as point mutations or insertions, typically result in two or three band differences (353). Sequential isolates from patients Bv1, Bv2, and Bv3 are therefore considered clonal because PFGE patterns between them differed by ≤ three bands.

3.3.4 *B. vietnamiensis* acquires aminoglycoside resistance under tobramycin, azithromycin, and hydrogen peroxide pressure *in vitro*

3.3.4.1 *In vitro* selection of antibiotic-resistant *B. vietnamiensis*

To determine if tobramycin alone could induce acquired aminoglycoside resistance in *B. vietnamiensis*, tobramycin MICs using established methods and non-*Enterobacteriaceae* breakpoints (232) were evaluated under antibiotic pressure *in vitro* (Figure 4). After serial passage in Mueller Hinton (MH) II Broth (cation-adjusted, pH 7.3) containing doubling tobramycin concentrations, early, aminoglycoside-susceptible isolates from patients Bv1 and

Table 11. Non-aminoglycoside antimicrobial therapy given to CF patients chronically infected with *B. vietnamiensis*

	Number of treatment courses		
Antimicrobial[a]	Bv1	Bv2	Bv3
CLX	3	0	0
PIP	4	2	0
IPM	1	0	0
MEM	7	4	0
ATM	2	0	0
LEX	2	2	0
CAZ	6	1	0
CTX	0	0	2
CXM	0	0	2
CIP	10	14	2
CST	2	2	0
CHL	1	0	0
AZM[b]	Cont.	Cont.	Cont.
SXT	2	1	1

[a] Abbreviations: CLX, cloxacillin; PIP, piperacillin; IPM, imipenem; MEM, meropenem; ATM, aztreonam; LEX, cephalexin; CAZ, ceftazidime; CTX, cefotaxime; CXM, cefuroxime; CIP, ciprofloxacin; CST, colistin; CHL, chloramphenicol; AZM, azithromycin; SXT, trimethoprim/sulfamethoxazole.

[b] Azithromycin was given continuously.

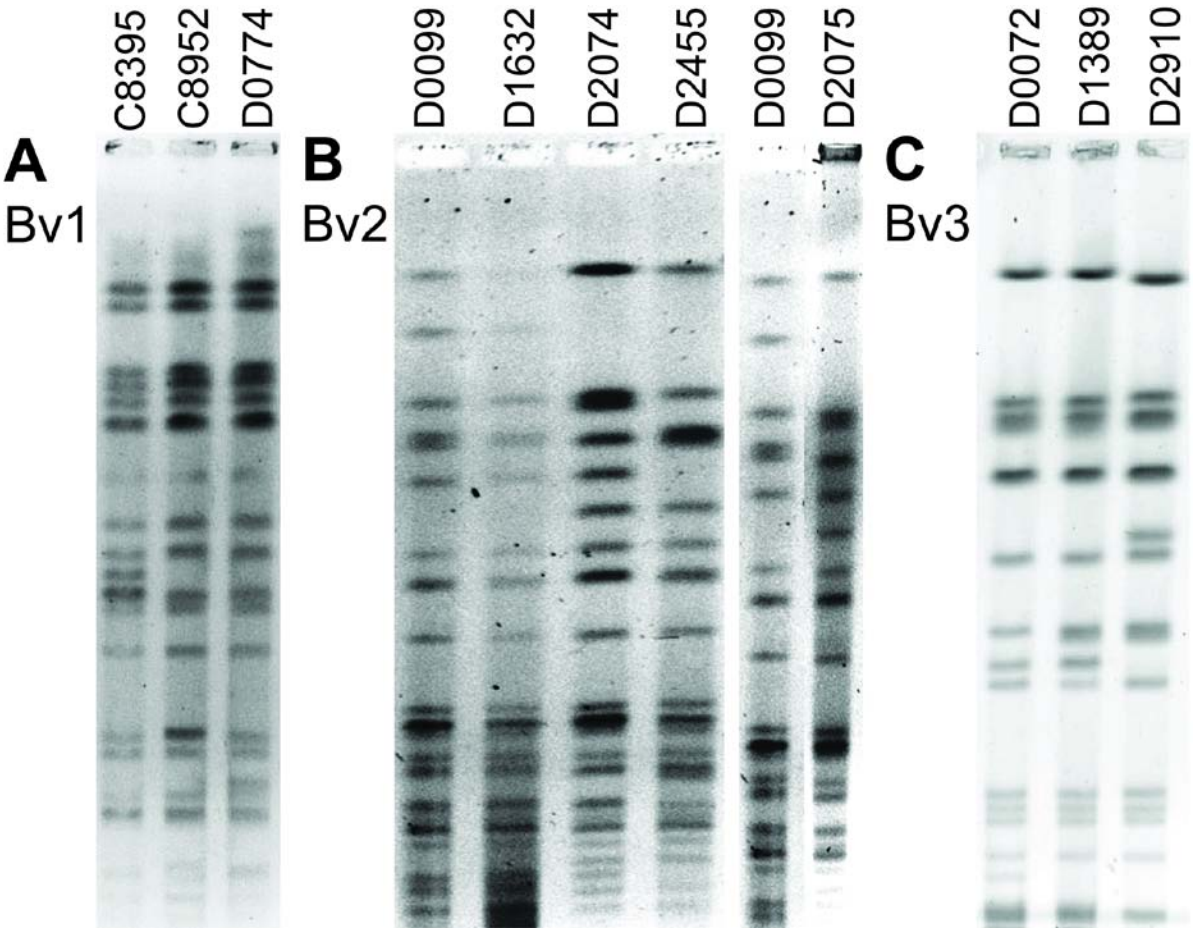

Figure 3. Strain typing of isolates from *B. vietnamiensis* infections.
Select isolates from chronic *B. vietnamiensis* infections in cystic fibrosis patients Bv1 (A), Bv2 (B), and Bv3 (C) were strain typed by pulsed field gel electrophoresis. Isolate D2075 was typed on a different day than the other isolates from patient Bv2; D0099 was used as an internal control.

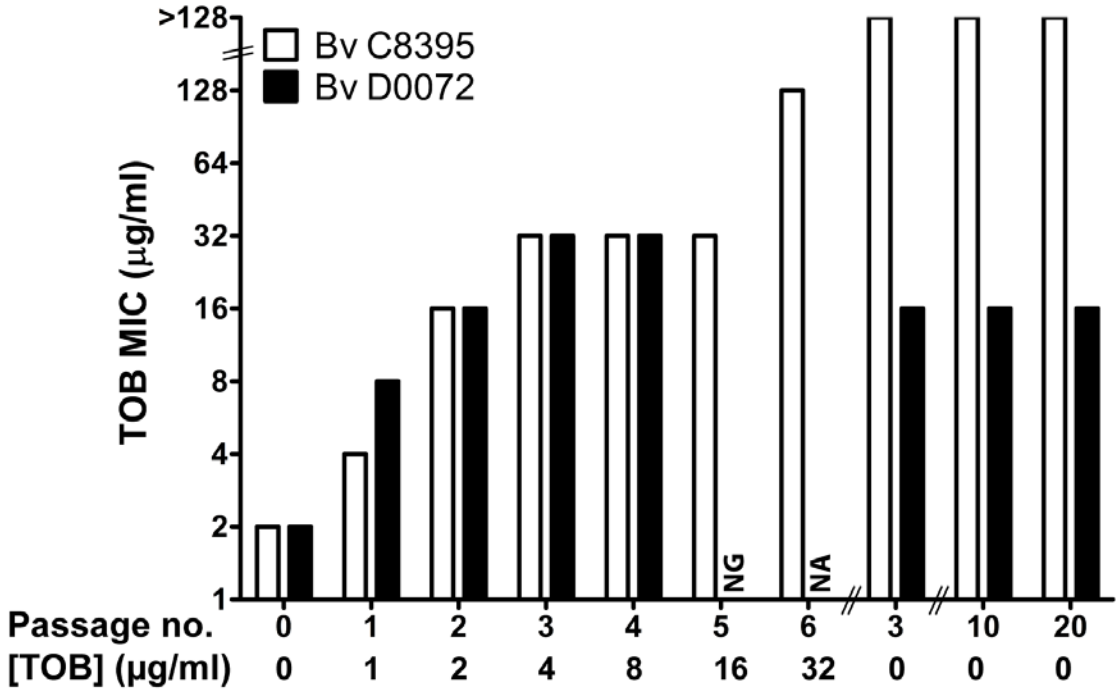

Figure 4. *B. vietnamiensis* acquisition of aminoglycoside resistance under tobramycin pressure *in vitro.*

Selection of aminoglycoside resistance in early isolates from cystic fibrosis patients Bv1 and Bv3 was done under chronic tobramycin pressure *in vitro*. Isolates were serially passaged every 24 hours in medium containing the antibiotic, to concentrations that represent half the MICs found for late isolates (16 µg/ml for D0072 and 64 µg/ml for C8395), at which time tobramycin MIC testing was performed. MIC data for isolates grown at half the MICs found for late isolates are not shown due to their lack of viability during analysis. The x-axis break lines separate the initial passages done under tobramycin pressure and those subsequently done on antibiotic-free media, before and after isolate freezing, respectively. Abbreviations: MIC, minimum inhibitory concentration; NG, no growth; NA, not applicable.

Bv3 - C8395 and D0072, respectively - acquired tobramycin resistance to the level of late isolates, i.e., tobramycin MICs of 128 and 32 µg/ml, respectively, representing ≥16 -fold increases. Tobramycin resistance was stable after passage on antibiotic-free medium, although 2-fold differences in the tobramycin MIC were observed. Amikacin, gentamicin, and kanamycin resistance was also acquired (Table 12), and gentamicin resistance was also stable after 20 passages on antibiotic-free media. Azithromycin, meropenem, ceftazidime, trimethoprim/sulfamethoxazole, and ciprofloxacin MICs for the resultant isolates were generally unchanged or changed only 2-fold, and where MIC increases were observed they remained lower than those found for late isolates. Non-aminoglycoside antibiotic MIC changes were also inconsistent between tobramycin-passaged isolates, with the exception of azithromycin MICs which increased in both cases. After serial passage in broth containing doubling tobramycin concentrations, the aminoglycoside-susceptible, environmental isolate G4 also acquired aminoglycoside resistance. Tobramycin and gentamicin MICs after passage were 64 and >128 µg/ml, respectively, representing ≥256-fold increases. Only the gentamicin MIC for the passage control G4 isolate increased 4-fold, to 2 µg/ml. The derived isolates described above were named C8395TR, D0072TR, and G4TR for their acquired tobramycin resistance, and G4PC for the G4 passage control. Aminoglycoside MICs for the early isolates (C8395 and D0072) were also examined after a single, 24 hour exposure to 1, 2, 4, 8, or 16 µg/ml azithromycin (Table 13). All patients received azithromycin therapy, even in the absence of aminoglycoside treatment. Tobramycin and gentamicin MICs for C8395 increased 2-fold and 4-fold, respectively, after exposure to 2, 4, 8, and 16 µg/ml azithromycin. Tobramycin MICs for D0072 increased 2-fold after exposure to 2 µg/ml azithromycin and 8 fold after exposure to 16 µg/ml azithromycin. Gentamicin MICs for

Table 12. Antimicrobial susceptibilities of *B. vietnamiensis* after serial exposure to antibiotics or hydrogen peroxide

	MIC (µg/ml)[b]								
Isolate[a]	AMK	GEN	KAN	TOB	AZM	MEM	CAZ	SXT	CIP
Clinical CF isolates									
C8395 (3/11/1998, Bv1)	2	4	2	2	32	1	4	2/10	1
D0774 (25/7/2003, Bv1)	>128	128	128	128	>2048	128	128	64/320	>32
D0072 (15/03/2002, Bv3)	2	4	1	2	32	0.5	2	2/10	1
D2910 (31/03/2008, Bv3)	128	32	64	32	>32	2	4	1/5	16
In vitro exposed									
C8395TR	**>128**	**>128**	**128**	**>128**	64	1	4	4/20	**4**
C8395AR	**32**	16	**16**	16	**2048**	2	**16**	**8/40**	**4**
C8395MR	16	8	8	8	32	**16**	**64**	4/20	**16**
C8395CR	8	8	4	4	32	**8**	**16**	2/10	**16**
C8395SR	8	8	2	2	32	0.5	4	**>64/320**	**8**
C8395HP2	**32**	**64**	**32**	**32**	32	**4**	**16**	4/20	**4**
C8395PC	8	8	4	8	32	1	4	1/5	1
D0072TR	32	32	16	16	>32	1	2	2/10	1

[a] Patient identification numbers and bacterial isolation dates are noted in brackets. Abbreviations: TR, TOB resistant; AR, AZM resistant; MR, MEM resistant; CR, CAZ resistant; SR, SXT resistant; HP2, hydrogen peroxide resistant second pick; PC, passage control.

[b] ≥3-fold antimicrobial MIC increases for C8395 after serial *in vitro* passage in broth containing antibiotics or hydrogen peroxide, and relative to the passage control (C8395PC), are shown in bold. MICs shown represent susceptibility after 3 passages on antibiotic-free media. Abbreviations: CF, cystic fibrosis; MIC, minimum inhibitory concentration; AMK, amikacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin; AZM, azithromycin; MEM, meropenem; CAZ, ceftazidime; SXT, trimethoprim/sulfamethoxazole, CIP, ciprofloxacin.

Table 13. Antimicrobial susceptibilities of *B. vietnamiensis* after a single 24 hour exposure to azithromycin

	Exposure to azithromycin (µg/ml)									
	1	2	4	8	16	1	2	4	8	16
Isolate[a]	TOB MIC (µg/ml)					GEN MIC (µg/ml)				
C8395 (3/11/1998, Bv1)	2	4	4	4	4	4	16	16	16	16
D0072 (15/03/2002, Bv3)	2	4	2	ND[b]	16	8	8	8	ND[b]	64

[a] Patient identification numbers and bacterial isolation dates are noted in brackets.

[b] Abbreviations: TOB, tobramycin; GEN; gentamicin; ND; not determined.

D0072 increased 2-fold after exposure to 1, 2, and 4 µg/ml azithromycin and 16-fold after exposure to 16 µg/ml azithromycin. Aminoglycoside MICs were not initially determined for D0072 after a 24 hour exposure to 8 µg/ml azithromycin because the culture color changed from light brown to red brown, indicating that this concentration may have other effects on the bacterium. In an additional experiment not shown, the aminoglycoside MICs at this concentration were found to be decreased 2-fold.

To confirm that azithromycin could induce acquired aminoglycoside resistance in *B. vietnamiensis,* and to determine if meropenem, ceftazidime, and trimethoprim/sulfamethoxazole could do the same, the early isolate C8395 was first serially exposed to the agents *in vitro* (Figure 5), after which antimicrobial MICs were evaluated (Table 12). Ribosome-targeting agents, such as aminoglycosides and macrolides, but not other antimicrobials, are capable of inducing aminoglycoside resistance determinants in *P. aeruginosa* (328). After serial passage in MH II Broth containing azithromycin, meropenem, ceftazidime, and trimethoprim/sulfamethoxazole, the MICs required to inhibit C8395 growth increased >64-, 8-, 8-, and 32-fold, respectively, but only azithromycin and trimethoprim/sulfamethoxazole MICs increased to the levels required to inhibit the late isolate D0774. The observed MIC increases were stable after passage on antibiotic-free medium, although 2-fold differences were observed. Only serial exposure of C8395 to azithromycin resulted in notable increases in aminoglycoside MICs, although resistance was not acquired to the level of the late isolate D0774. Other effects were observed: exposure to azithromycin resulted in increased ceftazidime, trimethoprim/sulfamethoxazole, and ciprofloxacin MICs; exposure to meropenem resulted in increased ceftazidime and ciprofloxacin MICs; exposure to ceftazidime resulted in increased meropenem and

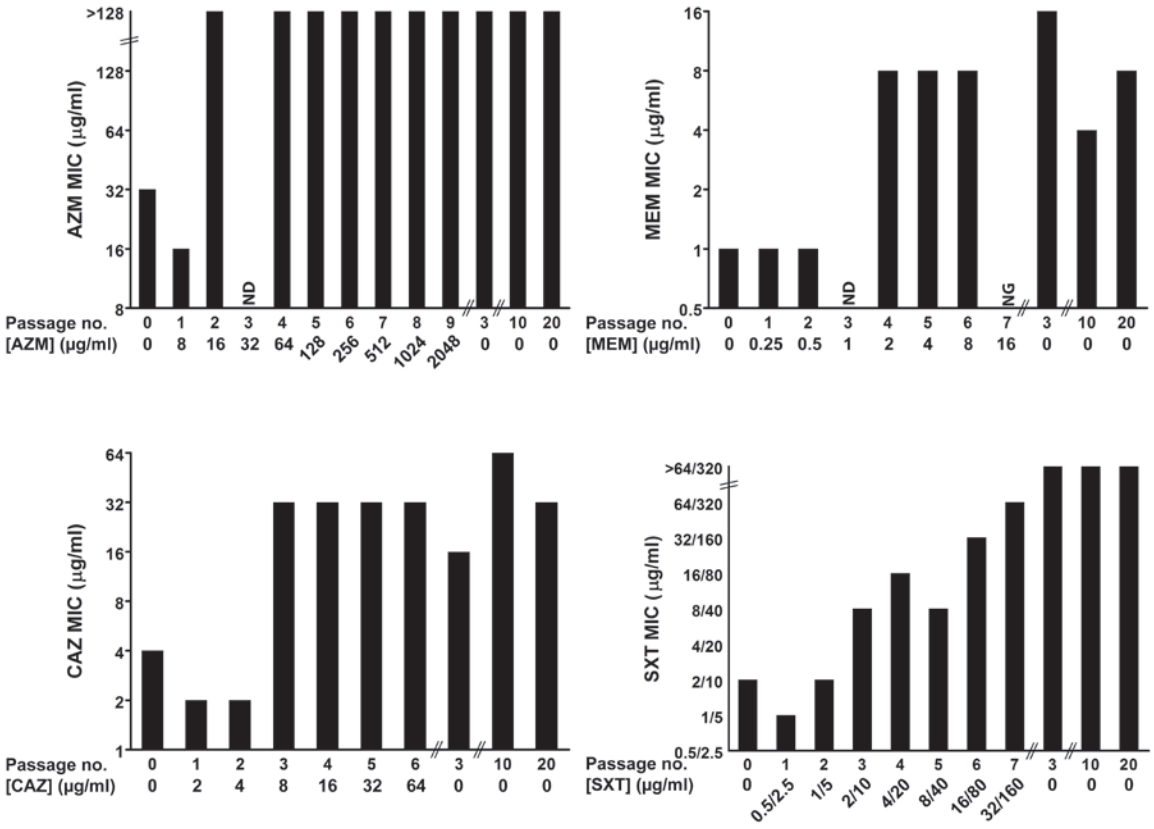

Figure 5. *B. vietnamiensis* C8395 decreased susceptibility to azithromycin, meropenem, ceftazidime, and trimethoprim/sulfamethoxazole after exposure to them *in vitro*.
Selection of resistance in the early isolate C8395 from cystic fibrosis patient Bv1 was done under chronic azithromycin (A), meropenem (B), ceftazidime (C), and trimethoprim/sulfamethoxazole (D) pressure *in vitro*. Isolates were serially passaged every 24 hours in medium containing the antibiotics, to concentrations that represent half the MICs found for the late isolate or to 2048 µg/ml azithromycin, at which time antimicrobial MIC testing was performed. The azithromycin MIC at passage 9, and after 3 and 20 passages on antibiotic-free media was actually >2048, 2048, and 2048 µg/ml, respectively. MIC data in some cases could not be determined due to insufficient bacterial viability during analysis (ND). The x-axis break lines separate the initial passages done under antibiotic pressure and those subsequently done on antibiotic-free media, before and after isolate freezing, respectively. Abbreviations: MIC, minimum inhibitory concentration; AZM, azithromycin; MEM, meropenem; CAZ, ceftazidime; SXT, trimethoprim/sulfamethoxazole; ND, not determined.

ciprofloxacin MICs; exposure to trimethoprim/sulfamethoxazole resulted in increased ciprofloxacin MICs. The derived isolates described above were named C8395AR, C8395MR, C8395CR, and C8395SR, for their acquired azithromycin, meropenem, ceftazidime, and trimethoprim/sulfamethoxazole resistance, respectively.

3.3.4.2 *In vitro* selection of *B. vietnamiensis* after serial exposure to hydrogen peroxide

To determine if hydrogen peroxide alone could induce acquired aminoglycoside resistance in *B. vietnamiensis*, based on antimicrobial MICs and established non-*Enterobacteriaceae* breakpoints (232), MICs were evaluated after oxidative pressure *in vitro* (Table 12). CF airways are rich in reactive oxygen species (ROS) (354, 355) and peroxide treatment enhances the recovery of aminoglycoside resistant mutants of *P. aeruginosa* (329). Peroxide MICs were determined to be 0.5 mM and 1 mM for the early, aminoglycoside-susceptible isolate C8935 and the late, aminoglycoside-resistant isolate D0774, respectively. The early isolate C8395 from patient Bv1 was exposed to three doses of peroxide at half the MIC in MH II Broth over 8 days and subsequently plated on antibiotic-free agar and agar supplemented with tobramycin at 2.5 × the MIC. An 8-day unexposed culture was processed in parallel. After growth on agar supplemented with tobramycin, the tobramycin resistance frequencies were enumerated for the peroxide exposed and unexposed isolates relative to their growth on antibiotic-free agar: 2.86×10^{-1} and 2.06×10^{-2} colony forming units (CFU)/ml, respectively. Two colonies from the peroxide exposed isolate grown on agar with tobramycin and one passage control colony from antibiotic-free agar were randomly selected and passaged 8 times, following which antimicrobial and peroxide MICs were assessed by

broth microdilution. Chronic *in vitro* exposure of C8395 to hydrogen peroxide resulted in a 16-fold stable aminoglycoside MIC increase for one of the progenies, C8395HP2. Relative to the passage control, where aminoglycoside susceptibility decreased 2- or 4-fold, the increase was ≥4-fold. Four-fold meropenem, ceftazidime, and ciprofloxacin MIC increases were also observed for C8395HP2. There were no significant aminoglycoside MIC changes for the other C8395 progeny, C8395HP1; tobramycin, gentamicin, and amikacin MICs were 4, 4, and 8 μg/ml, respectively. Interestingly, reduced susceptibility to non-aminoglycoside antibiotics was not observed in the passage control. The peroxide MICs for all picked colonies were unchanged. The derived isolates described above were named C8395HP1 and C8395HP2 for their pick number, first or second, after passage in hydrogen peroxide, or C8395PC for the passage control.

The early isolate C8395 was also serially exposed to doubling or gradually (0.25 mM daily increments) increasing concentrations of hydrogen peroxide, starting with a concentration representing half the MIC, over 8 days or until growth could no longer be observed. After exposure to doubling peroxide concentrations, minimal growth was observed in 2 mM peroxide, and no growth in 4 mM peroxide. After gradually increasing peroxide exposure, the resultant isolate was plated from broth containing 2 mM peroxide onto antibiotic-free agar and agar supplemented with tobramycin at 2.5 × the MIC. An 8-day unexposed culture was processed in parallel. After growth on agar supplemented with tobramycin, the tobramycin resistance frequencies were enumerated for the peroxide exposed and unexposed isolates relative to their growth on antibiotic-free agar: 6.14×10^{-4} and 8.59×10^{-4} CFU/ml, respectively. The tobramycin, gentamicin, and peroxide MICs for two randomly selected colonies from the peroxide exposed isolate grown on agar with

tobramycin were immediately assessed by broth microdilution. Aminoglycoside and peroxide MICs increased 8- and 2-fold, respectively. The tobramycin, gentamicin, and peroxide MICs for one randomly selected passage control colony were also assessed. Gentamicin and tobramycin MICs increased 4- and 2-fold, respectively, and peroxide MICs decreased 2-fold. The derived isolates described above were named C8395HP3 and C8395HP4 for their pick in sequence with previously derived isolates after passage in hydrogen peroxide, or C8395PC2 for the second passage control.

3.3.4.3 Strain typing of *B. vietnamiensis* exposed to antibiotics or hydrogen peroxide *in vitro*

To ensure that *in vitro* serially passaged isolates were clonal, they were typed by random amplified polymorphic DNA analysis and compared to the original isolates from which they were derived (Figure 6). All isolates passaged *in vitro* maintained the same banding patterns as the early, aminoglycoside-susceptible isolates, and hence are considered genetically identical (332).

3.3.4.4 Growth analysis of *B. vietnamiensis* exposed to antibiotics or hydrogen peroxide *in vitro*

The growth curves for *in vitro* selected isolates were determined in MH II Broth and compared to the early, aminoglycoside-susceptible isolates from which they were derived, as well as the late, aminoglycoside-resistant isolates from the same patient (Figure 7). The late, aminoglycoside-resistant isolates grew slower than the early, aminoglycoside- susceptible isolates for both of the sets. Growth rates of the *in vitro* antibiotic or peroxide exposed

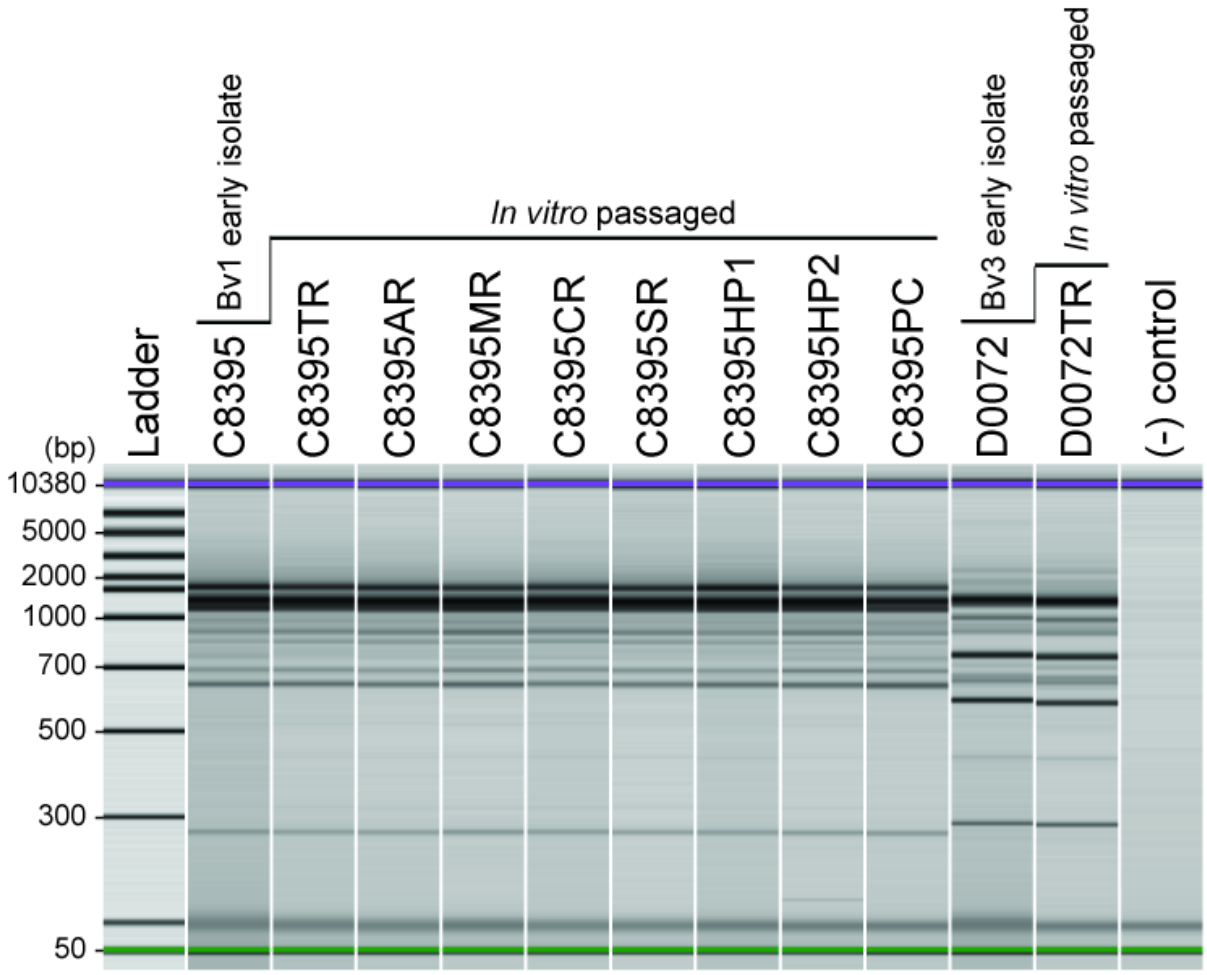

Figure 6. Random amplified polymorphic DNA (RAPD) analysis of early and *in vitro* passaged isolates of *B. vietnamiensis*.

Random amplified polymorphic DNA analysis of early isolates from cystic fibrosis patients Bv1 and Bv3, C8395 and D0772, respectively, and their derivatives was done using a 2100 Bioanalyzer at the Centre for Molecular Medicine and Therapeutics DNA Sequencing Core Facility (University of British Columbia). DNA was extracted from cells boiled in the presence of 5% chelex beads. Abbreviations: TR, tobramycin resistant; AR, azithromycin resistant; MR, meropenem resistant; CR, ceftazidime resistant; SR, trimethoprim/sulfamethoxazole resistant; HP1, hydrogen peroxide passage first pick; HP2, hydrogen peroxide passage second pick; PC, passage control.

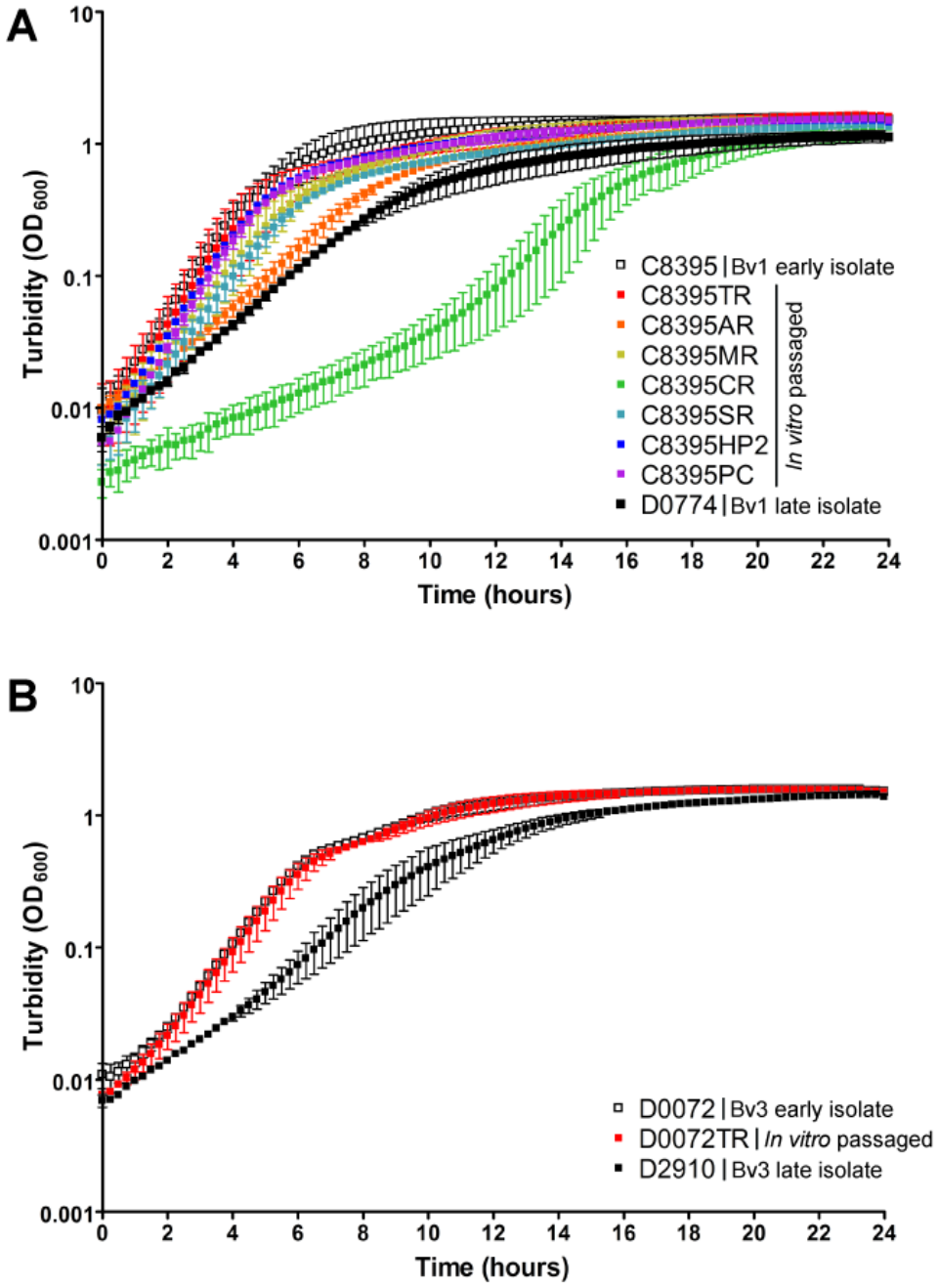

Figure 7. Growth curves of early, late, and *in vitro* passaged isolates of *B. vietnamiensis*.
Growth analysis of early and late isolates from cystic fibrosis patients Bv1 (A) and Bv2 (B) and their derivatives was done using a Bioscreen C with starting cultures of 1×10^7 CFU/ml in 300 µl. OD_{600} readings were taken every 15 minutes. The averages of five technical repeats were taken for each biological replicate. Data points represent the averages for two biological replicates ± standard deviations. Abbreviations: OD_{600}, optical density at 600 nm; TR, tobramycin resistant; AR, azithromycin resistant; MR, meropenem resistant; CR, ceftazidime resistant; SR, trimethoprim/sulfamethoxazole resistant; HP2, hydrogen peroxide passage second pick; PC, passage control.

isolates derived from the early isolate from patient Bv1, C8395, were intermediate between those observed for the early and late isolates from that patient, with the exception of C8395CR which exhibited extremely delayed growth. The *in vitro* tobramycin exposed isolate D0072TR derived from the early isolate from patient Bv3, D0072, grew at a similar rate as the early isolate.

3.3.5 *B. vietnamiensis* acquires catalase activity and becomes less mucoid during chronic infection

Catalase activity and exopolysaccharide (EPS) production were measured in *B. vietnamiensis* isolates and compared with other BCC species to gain a better understanding of phenotypic changes that can occur in *B. vietnamiensis* during chronic infection alongside the acquisition of antimicrobial resistance. All BCC species produce catalase, an enzyme that degrades hydrogen peroxide, though the degree of production and activity varies among isolates (175), and a link between EPS production and oxidative stress resistance in *B. cenocepacia* has recently been described (178). Furthermore, in *P. aeruginosa*, exposure to hydrogen peroxide upregulates the expression of catalases (356, 357) and aminoglycoside resistance determinants (329). We devised a scoring method to semi-quantitatively measure catalase production after the addition of bacteria, grown on MH II Agar, to peroxide, based on the principle that when catalase catalyzes the decomposition of hydrogen peroxide into water and oxygen, bubbles are immediately formed. Catalase activity was scored as – to ++, and the criteria for the scoring are described in the legend to Figure 8, with examples shown in Figure 8a. Notably, if enough bacteria were added, catalase activity could be measured from every isolate tested (Figure 8b). EPS production was measured using a previously

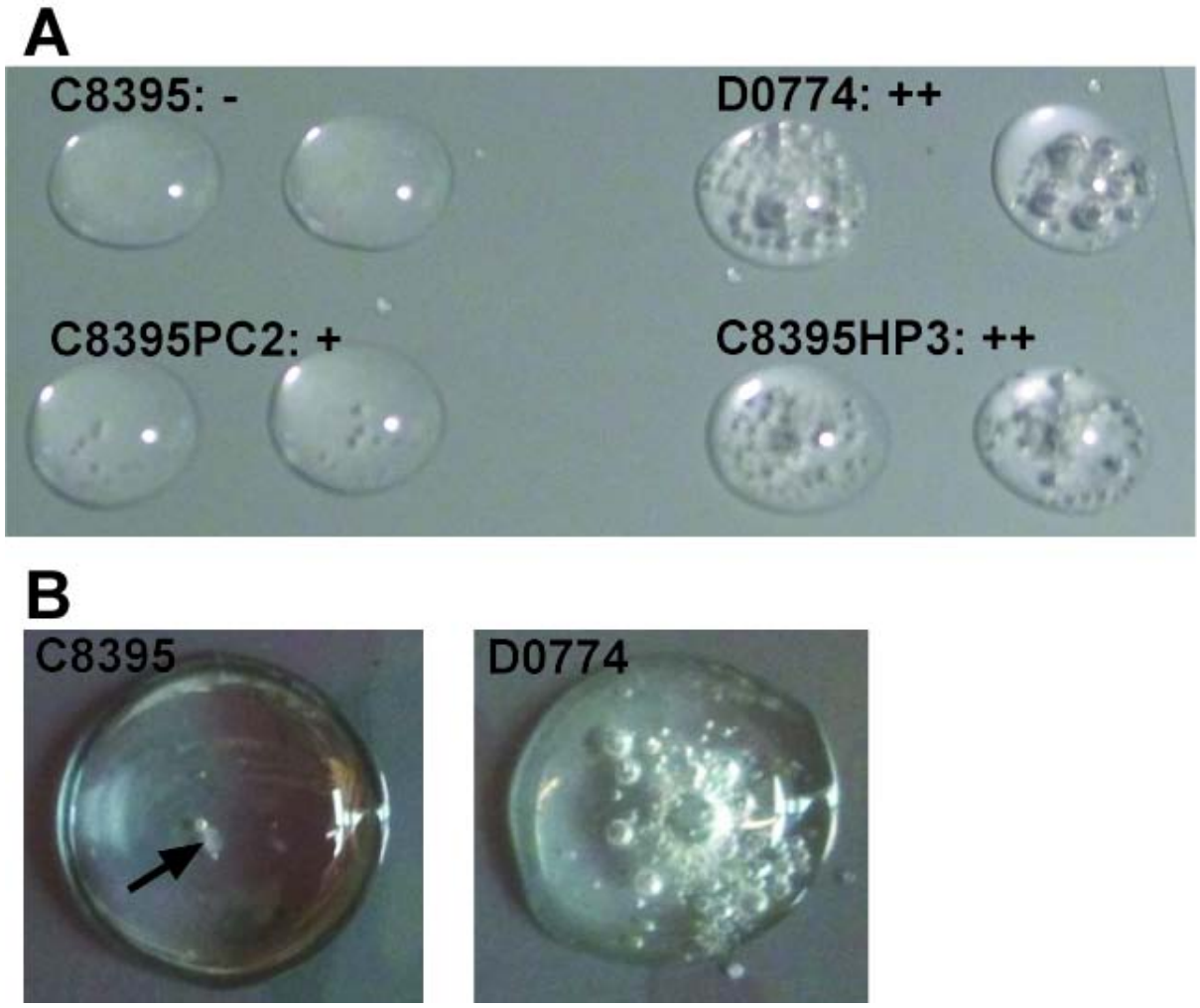

Figure 8. Catalase activity in *B. vietnamiensis*.
(A) Catalase activity after the addition of bacteria to hydrogen peroxide was defined as negative (-), weakly positive (+), and frankly positive (++): -, no immediate formation of bubbles was observed; +, ≤10 small bubbles were immediately formed; ++, many small and large bubbles were immediately formed. (B) Notably, if enough bacteria were used, minimal catalase activity could be measured, as seen with C8395 and compared to D0774. The arrow points to visible bacterial colonies. Abbreviations: PC2, second passage control; HP3, hydrogen peroxide passage in increasing concentrations, first pick.

developed scoring system based on bacterial growth on yeast extract medium (YEM), and scored as – to +++d (218). Note, EPS production on MH II Agar was not evident; the isolates tested varied in shine on MH II Agar, however there was no phenotypic correlation with EPS production on YEM medium.

All 10 isolates that were frankly positive for catalase activity produced no or minimal amounts of EPS, while no or minimal catalase activity was observed for all 14 frankly mucoid isolates (Figure 9). The observed association between catalase activity and EPS production was significant by Fisher's Exact Test ($P < 0.001$). In all but one *B. cenocepacia* sequential pair, C6006 and D3002, catalase activity increased between the early and late isolates; of the 11 early isolates tested 6, 4, and 1 were -, +, and ++, respectively, while 0, 2, and 9 of the 11 late isolates tested were -, +, and ++, respectively. In all but one *B. vietnamiensis,* one *B. cenocepacia,* and one *B. multivorans* sequential pair, D0072 and D2910, C4414 and C5594, and D2494 and D3752, respectively, EPS production decreased between the early and late isolates; of the 11 early isolates tested 1, 2, 5, 1, and 2 were -, +, ++, +++, and +++d, respectively, while 8, 2, 0, 0, and 1 of the 11 late isolates tested were -, +, ++, +++, and +++d, respectively. *B. cenocepacia* was the most nonmucoid of the three species tested, confirming previous findings (5), and also the most frankly positive for catalase activity. Within *B. vietnamiensis,* none of the non-CF *B. vietnamiensis* isolates were frankly positive for catalase activity, and only one environmental isolate, CEP0106, was weakly positive. Of the 10 aminoglycoside-susceptible *B. vietnamiensis* isolates, none were frankly positive for catalase activity and 7 were frankly mucoid. Of the 3 aminoglycoside-resistant *B. vietnamiensis* isolates, all were frankly positive for catalase activity and all produced no or minimal amounts of EPS.

Catalase activity	Exopolysaccharide production: -/+ Nonmucoid (15)	Exopolysaccharide production: ++/+++/+++d Frankly mucoid (14)
-/+	5 (33.3%)	14 (100.0%)
++	10 (66.7%)	0

Figure 9. Exopolysaccharide production vs. catalase activity in *B. vietnamiensis*, *B. multivorans*, and *B. cenocepacia*.

Catalase activity after the addition of bacteria to hydrogen peroxide was defined as follows: -, no immediate formation of bubbles was observed; +≲10 small bubbles were immediately formed; ++, many small and large bubbles were immediately formed. EPS production after bacterial growth on yeast extract medium was defined as follows: -, no evidence of EPS production and colonies are dry and matte; +, some evidence of EPS production but predominantly nonmucoid bacteria; ++, flat, mucoid growth throughout; +++, EPS overwhelms growth with raised areas; +++d, same as +++ except EPS drips on the lid of the plate. Sources: *B. vietnamiensis*, 8 clinical CF, 1 clinical non-CF, 4 environmental (same as in Table 8); *B. multivorans*, 8 clinical CF; *B. cenocepacia*, 8 clinical CF.

To determine if differences in catalase activity were observed simply by chance, we screened 100 colonies each of the early *B. vietnamiensis* isolate C8395 and the late *B. vietnamiensis* isolate D0774. All 100 C8395 colonies were negative for catalase activity while all 100 D0774 colonies were frankly positive. After serial exposure to antibiotics C8395 remained negative for catalase activity. Interestingly, after exposure to gradually increasing concentrations of hydrogen peroxide, but not after serial exposure to peroxide at half the MIC, C8395 became frankly positive for catalase activity (Figure 8a). Only the passage control isolate processed simultaneously with the former acquired some catalase activity (Figure 8a).

3.3.6 The rate and extent of aminoglycoside killing in a susceptible *B. vietnamiensis* isolate

Growth in MH II Broth in the presence of gentamicin and tobramycin at 1, 2, 4, and 8 × the MIC was analyzed for the aminoglycoside-susceptible *B. vietnamiensis* isolate D1389 from CF patient Bv3 to examine the rate and extent of aminoglycoside killing in this species (Figure 10b and c). Both aminoglycosides reduced bacterial growth in a dose-dependent manner; growth inhibition occurred more rapidly and to a greater extent with higher drug concentrations up to a 5 fold log reduction in CFU after 4 hours with a concentration of 8 × the MIC. Notably, the isolate was not eradicated under any of the conditions tested, and displayed an increase in growth after the initial CFU drop. The rate and extent of aminoglycoside killing observed for D1389 is contrary to the rapid killing observed for *P. aeruginosa* (Figure 10a) (358, 359). At 4 and 8 × the gentamicin MIC, no CFU were detected at 2 hours and up to 24 hours post-incubation of the aminoglycoside-susceptible *P.*

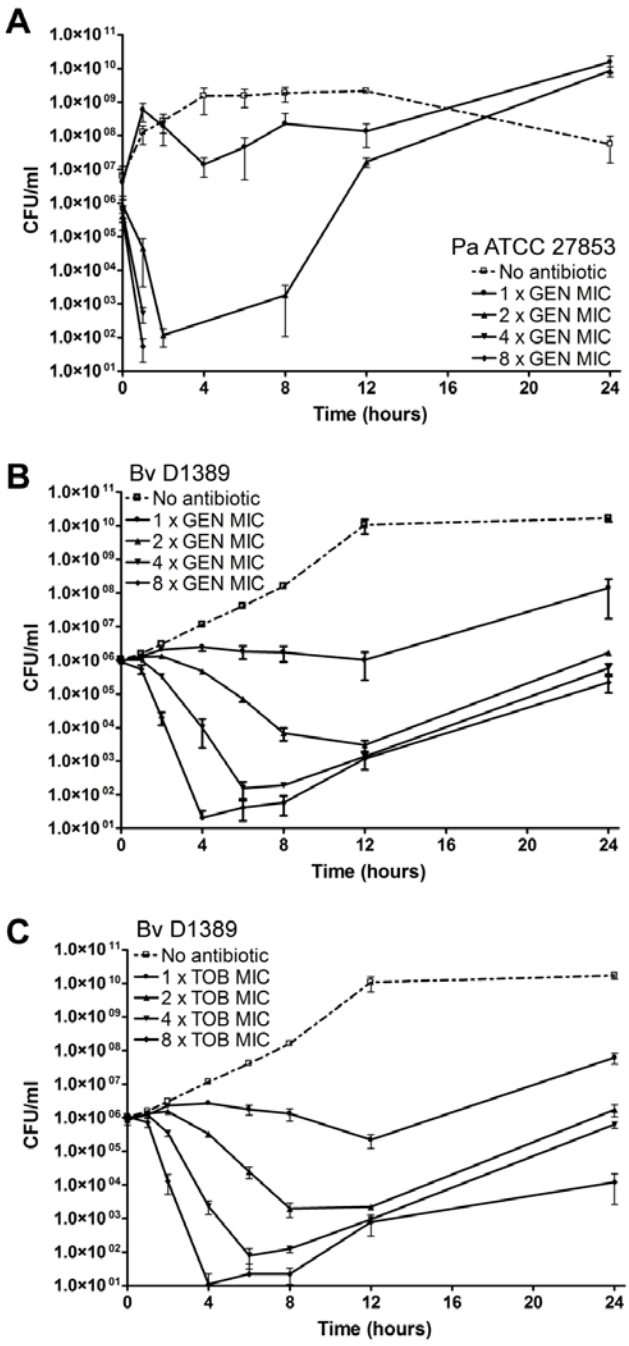

Figure 10. The rate and extent of aminoglycoside killing in a susceptible *B. vietnamiensis* isolate compared with *P. aeruginosa*.

The aminoglycoside-susceptible *B. vietnamiensis* isolate D1389 from cystic fibrosis patient Bv3 was grown in the presence of 1, 2, 4, and 8 × the gentamicin (B) and tobramycin (C) MIC. Twenty µl samples were taken at 0, 1, 2, 4, 6, 8, 12, and 24 hours of growth, serially diluted, and plated in triplicate on agar. Viable counts were obtained after overnight growth at the minimal dilution where distinct, accurately countable colonies were present. The aminoglycoside-susceptible *P. aeruginosa* ATCC 27853 was used as a control organism (A). Data points represent the averages for 3 biological replicates ± standard errors. Abbreviations: MIC, minimum inhibitory concentration; Pa, *P. aeruginosa*; Bv, *B. vietnamiensis*; CFU, colony forming units; GEN, gentamicin; TOB, tobramycin.

aeruginosa ATCC 27853 with gentamicin. Furthermore, after growth under antibiotic pressure a subpopulation of D1389 large colony variants was often observed upon plating on solid media (Figure 11). Variants could be observed under all drug concentrations tested, at 2 hours post-incubation with antibiotic and up to 24 hours, and represented 1 to 10% of the population when they were seen. After re-inoculation on antibiotic-free MH agar they reverted back to their original phenotype and subsequent MIC experiments revealed that they were as aminoglycoside susceptible as the original population.

3.1 Discussion

Members of the BCC are important opportunistic pathogens that are capable of resisting therapeutic interventions (5). Intrinsic resistance to the inhibitory activity of polymyxins and aminoglycosides is considered to be a characteristic of the BCC so much so that polymyxin B and gentamicin, along with vancomycin, are used as diagnostic ingredients in *Burkholderia cepacia* selective agar (BCSA) (360). Current antimicrobial options for therapy of BCC infections are therefore limited, and eradication of the organisms from patients with CF, chronic granulomatous disease (CGD), or other immunocompromised individuals, is a major challenge (130).

In agreement with other large-scale studies of antimicrobial susceptibility in the BCC (24, 25, 263, 268-272, 300-306, 308, 351, 352), and based on established MIC breakpoints (232), our susceptibility data emphasize the high level of resistance of the BCC to a number of antimicrobials used to treat respiratory infections in CF (130) and CGD patients (241), including aminoglycosides, and confirms that ceftazidime, meropenem, and ciprofloxacin are among the most inhibitory antimicrobials against multiple species *in vitro*. Furthermore,

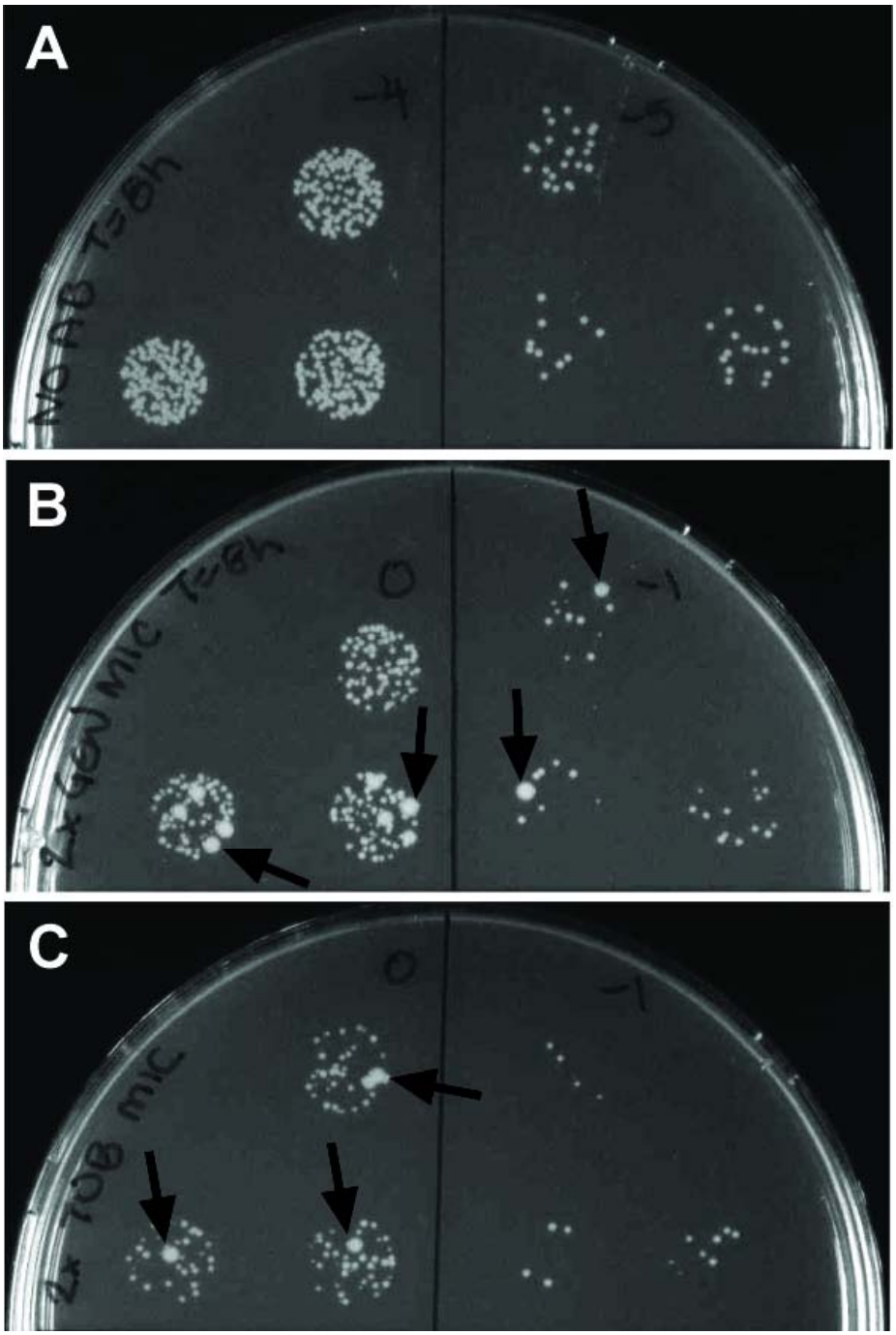

Figure 11. *B. vietnamiensis* large colony variants after growth in aminoglycosides.
Examples of D1389 large colony variants observed after growth of the aminoglycoside-susceptible isolate from CF patient Bv3 in gentamicin (B) and tobramycin (C) at 2 × the MIC for 8 hours, compared with colonies recovered after 8 hours of growth in the absence of antibiotics (A). D1389 was grown in the presence of 1, 2, 4, and 8 × the gentamicin and tobramycin MIC. Twenty µl samples were taken at 0, 1, 2, 4, 6, 8, 12, and 24 hours of growth, serially diluted, and plated in triplicate on agar. Viable counts were obtained after overnight growth at the minimal dilution where distinct, accurately countable colonies were present. Arrows point to variants, numbers on the right corners of plate quadrants refer to dilutions.

consistent with previous findings, our data highlight the inactivity of polymyxin B (24, 26, 268, 269, 361) and cationic antimicrobial peptides (108, 276-278, 362-365) against all BCC species. Notably, CP29, a synthetic antimicrobial peptide derived from an insect cecropin (366), had slightly better inhibitory activity than the other peptides tested, suggesting that increasing the amphipathic content of α-helical peptides has the potential to increase their efficacy against the BCC. We did not test colistin (polymyxin E) against the BCC in the study, the polymyxin actually administered in clinical practice to CF patients infected with *P. aeruginosa* (28). We expect that the colistin MIC data would be similar to those which were obtained with polymyxin B (270-272).

Based on antimicrobial MICs and established non-*Enterobacteriaceae* breakpoints (232), aminoglycoside-susceptible isolates of the BCC have been noted and were found to belong to the species *B. vietnamiensis* (24, 26, 367). In *in vitro* studies of susceptibility, these isolates were inhibited by several antimicrobials, but not by polymyxin B (24, 26). Our results show that *B. vietnamiensis* is in fact more often susceptible to aminoglycosides and carbapenems than other BCC species, suggesting that existing drugs recommended to treat lung disease in CF (128) and CGD (241) patients may be more effective at treating *B. vietnamiensis* infections than previously thought. Indeed, Magalhães *et al.* (367) reported successful treatment with ciprofloxacin, trimethoprim/sulfamethoxazole, and amikacin of an amikacin-susceptible strain of *B. vietnamiensis*, but not of an amikacin-resistant *B. cenocepacia* strain, in a case of a double infection in a child with CF (based on antimicrobial MICs and established breakpoints (232), both strains were susceptible to ciprofloxacin and trimethoprim/sulfamethoxazole). However, *B. vietnamiensis* isolates in our study were resistant to the inhibitory activity of other polycationic agents, cationic antimicrobial peptides

and polymyxin B, indicating that peptides specifically remain of limited value as antimicrobial monotherapy against BCC infections, despite their promise as therapeutic agents against other CF pathogens (274). Importantly, MIC ranges for some antimicrobials were wide, stressing the need for large sample sizes in susceptibility studies.

From an epidemiological perspective, our finding of a common aminoglycoside-susceptible phenotype of *B. vietnamiensis* cautions that this organism may be underrepresented in clinical and environmental samples if BCSA is used as the primary isolation medium, and considering that in clinical settings it may be rapidly eradicated following aminoglycoside treatment targeted at *P. aeruginosa* or other BCC species. Indeed, Vermis *et al.* (26) found that an antimicrobial susceptible CF isolate of *B. vietnamiensis* failed to grow on BCSA. A *B. vietnamiensis* infection therefore may go unnoticed, especially given that CF and CGD lungs are often colonized with multiple opportunistic pathogens (8, 115). If the infection is not appropriately treated from the start, the infecting strain is given the chance to acquire antibiotic resistance, establish a chronic infection, and consequently cause tissue damage in the host. BCSA is the only selective media used in the isolation of BCC species that contains an aminoglycoside antibiotic; others contain tetracycline, bacitracin, ticarcillin, and/or polymyxin B (368-371).

The antimicrobial susceptible phenotype of *B. vietnamiensis* also has implications outside the clinic. In basic scientific research, an aminoglycoside-susceptible isolate of *B. vietnamiensis* could be used in the study of BCC intracellular replication using traditional gentamicin protection assays, which is currently not possible without the creation of aminoglycoside-susceptible mutant strains (239). Furthermore, despite their capacity to promote plant growth and degrade pollutants (2, 4, 58), the use of BCC isolates in

biotechnological applications is cautioned against because of their associated health risks (70, 71). *B. vietnamiensis* specifically is capable of promoting the growth of rice (63), sugarcane (65), and grass (66), presumably through its ability to fix atmospheric nitrogen (64), and is an effective bioremediation agent, capable of degrading the environmental contaminants trichloroethylene (67) and toluene (68). As Nzula *et al*. (24) have noted in the past, in the risk assessment of candidate biopesticide strains, it has been argued that clinical features such as the frequency of human colonization, the transmissibility rate, and the susceptibility to available antimicrobials be taken into consideration (71). Therefore, based on the current susceptibility data, and given the low incidence of *B. vietnamiensis* in CF and non-CF patients in Canada (123, 218), the US (109, 114, 372, 373), and in other parts of the world (122, 303, 374-388), *B. vietnamiensis* could be reassessed for its biotechnological potential in these regions. However, the potential for *B. vietnamiensis* to acquire resistance, discussed below, should also be taken into consideration.

In general, BCC isolates from clinical sources are resistant to a larger number of antimicrobials and to a higher degree than those isolated from the environment (24, 26, 268), and clinical isolates recovered from CF patients have been found to be more often resistant than those from non-CF patients (26). Consistent with these previous findings, our antimicrobial MIC results suggest that *B. vietnamiensis* often exists in an aminoglycoside-susceptible state in its natural environmental niche. Environmental isolates were most often susceptible, though this certainly is not always the case - one environmental isolate was extremely resistant to all aminoglycosides tested, with aminoglycoside MICs >128 µg/ml. The inhabitation of a unique environmental niche by *B. vietnamiensis* may explain why *B. vietnamiensis* environmental isolates are more often susceptible to aminoglycosides than

those of other BCC species. *B. vietnamiensis* is the only species within the complex that has been found to promote plant growth by fixing atmosphereic nitrogen (3), suggesting that it may be more intimately associated with plant roots in the rhizosphere and, consequently, may not be exposed to the same environmental stresses as the other BCC species. A better understanding of the natural habitats of BCC species is necessary to examine this hypothesis.

The observation that CF isolates of *B. vietnamiensis* were more often resistant to aminoglycosides than environmental and other clinical isolates, suggests that the CF host environment in particular selects for an aminoglycoside resistant phenotype of the bacterium, a notion further supported by the lower aminoglycoside MICs for the early CF isolates of *B. vietnamiensis* when compared to the collective group of CF isolates. Indeed, in this study we report for thefirst time the acquisition of aminoglycoside resistance in *B. vietnamiensis* in chronically infected CF patients, inferred from the study of sequential isolates, a phenomenon that has been described for *P. aeruginosa* (389, 390). Notably, these infections were not eradicated during our study period, despite the aggressive antibiotic treatment regimens administered to the patients. Even when therapy is guided by susceptibility testing, eradication of BCC strains is often not achieved (130), and in this case may be at least partially attributed to the ability of *B. vietnamiensis* to acquire antibiotic resistance. Fluctuations in tobramycin MIC between sequential isolates of the infecting *B. vietnamiensis* strains were observed, and can be attributed to the range of experimental error (334), or alternatively to the state of disease at the time of isolate acquisition, as BCC isolates retrieved during exacerbations can be less susceptible to antibiotics, including tobramycin (304, 352). The acquisition of aminoglycoside resistance was not apparent in *B. cenocepacia* or *B. multivorans*. All early *B. cenocepacia* and *B. multivorans* isolates were already resistant to

tobramycin, with the exception of one *B. multivorans* isolate representing a strain that remained susceptible throughout a chronic infection. The lack of access within our repository to sequential isolates from chronic BCC infections with other species limited our ability to investigate the acquisition of aminoglycoside resistance to the few species which are most commonly recovered from patients with CF.

In *P. aeruginosa*, the clinical acquisition of aminoglycoside resistance in CF patients has been linked to tobramycin therapy (389, 390), and serial exposure to amikacin increases aminoglycoside MICs in *B. cenocepacia* (391). Despite having observed acquired aminoglycoside resistance in all three *B. vietnamiensis* infected CF patients in our study, only two of them received aminoglycoside treatment, in the form of tobramycin, indicating that selective pressures other than the exposure to aminoglycosides are able to induce aminoglycoside resistance in *B. vietnamiensis*. We identified tobramycin, azithromycin, and hydrogen peroxide as inducers of aminoglycoside resistance in *B. vietnamiensis in vitro*. Tobramycin pressure lead to the biggest changes in aminoglycoside MIC; only exposure of the early *B. vietnamiensis* isolate C8395 to tobramycin resulted in the high level aminoglycoside resistance that was observed for the late isolate D0774.

These results correlate with our findings in *B. vietnamiensis*-infected CF patients; strains isolated from the two patients receiving tobramycin therapy, patients Bv1 and Bv2, acquired resistance to a higher level than the strain from the patient Bv3, who did not receive aminoglycoside therapy but was administered azithromycin (we assume all strains were exposed to oxidative stress in the lung environment (354, 355)). The degree of resistance is an important consideration since higher *in vivo* antibiotic concentrations are achieved with inhaled than parenteral tobramycin therapy (392-394). The susceptibility breakpoint adapted

for BCC to nebulized tobramycin is 256 µg/ml (25, 308). Tobramycin sputum concentration following tobramycin inhalation therapy is approximately 1000 µg/ml, but in cystic fibrosis patients about 50% of the drug is thought to be bound to mucin, which renders it biologically inactive (307). Notably, tobramycin exposure also induced aminoglycoside resistance in an environmental isolate of *B. vietnamiensis*, suggesting that all *B. vietnamiensis* have the capacity to acquire aminoglycoside resistance.

Exposure to ribosome-targeting antibiotics such as aminoglycosides and macrolides (327, 328, 395, 396) and oxidative stress in the form of hydrogen peroxide (329) induces the expression of genes involved in aminoglycoside efflux in *P. aeruginosa*. Tobramycin can also activate a general *P. aeruginosa* membrane stress response, resulting in increased aminoglycoside resistance (397). Consistent with these findings, exposure to non-ribosome targeting antibiotics, meropenem, ceftazidime, and trimethoprim/sulfamethoxazole specifically, did not alter aminoglycoside MICs for *B. vietnamiensis*. In *P. aeruginosa*, polymyxins and cationic peptides can also induce aminoglycoside resistance (282, 287). Although inhaled colistin is used for therapy of respiratory *P. aeruginosa* infections (28, 267), and cationic antimicrobial peptides, key constituents of host defense systems, are found in abundance in CF airways (398), the effects of peptide exposure on the induction of aminoglycoside resistance in *B. vietnamiensis* was not investigated, because of the lack of interaction of *Burkholderia* species with these cationic agents (288, 289). Other triggers of resistance, such as pH, anaerobiosis, ion concentration, carbon source, and polyamines (399), could have implications in the general clinical and environmental acquisition of antibiotic resistance in *B. vietnamiensis*. Moreover, our finding that serial passage alone could result in 2- to 4-fold increases in aminoglycoside MICs, suggests that aminoglycoside resistance

determinants in *B. vietnamiensis* are involved in general environmental adaptations. Mutations in energy metabolism contribute to low-level aminoglycoside resistance in *P. aeruginosa* (317), and multiple aminoglycoside resistance determinants in *P. aeruginosa* are controlled by the ParRS (282, 287) or AmgRS (397) two-component regulatory systems which could be activated by various environmental cues.

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 (28), and since CF patients can be co-infected with these two organisms (115) (our study). Furthermore, azithromycin is administered in the absence of *P. aeruginosa* infection, as seen with patient Bv3 in our study. The beneficial effects of tobramycin (390, 400, 401) and azithromycin (262) treatment on clinical outcomes in CF patients infected with the BCC and/or *P. aeruginosa* however, may outweigh their potential to decrease antibiotic susceptibility, and therefore usage should not necessarily be terminated. Antimicrobial courses and doses should be cautiously designed with the potential drug MIC increases in mind, especially since, to our knowledge, the clinical impact of azithromycin treatment on CF patients infected with BCC species has not yet been investigated. Oxidative stress induction of stable aminoglycoside resistance in *B. vietnamiensis* is also worrisome, as CF airways are rich in ROS owing to a robust inflammatory response (354, 355), and emphasizes the need for anti-inflammatory therapy in CF (128).

Moreover, we demonstrate the ability of *B. vietnamiensis* to acquire resistance to other antimicrobials used in clinical practice to treat BCC-infected CF patients (meropenem,

ceftazidime, and trimethoprim/sulfamethoxazole) (130) (our study) after exposure to them *in vitro*. Interestingly, changes in the MICs of different classes of antimicrobials were observed after *B. vietnamiensis* exposure to these antibiotics, azithromycin, tobramycin, and hydrogen peroxide. Two-component regulatory systems have the potential to trigger the acquisition of resistance to several unrelated classes of antibiotics; ParRS interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-lactams in *P. aeruginosa* through its effects on multiple genes (287). The resistance profiles of isolates that acquired aminoglycoside resistance, C8395TR, C8395AR, and C8395HP2, were different however, suggesting that mechanisms of resistance induced by tobramycin, azithromycin and hydrogen peroxide differ.

A key finding in our *in vitro* induction studies was that once the antibiotic resistant phenotypes were selected in *B. vietnamiensis*, they were stably maintained - unsurprisingly, as it is often the case (221). As Sass *et al.* (2011) explain after observing amikacin induced increases in aminoglycoside MICs for *B. cenocepacia* (391), resistance determinants are therefore likely stably inherited and do not revert, suggesting that clinical changes in antibiotic therapy, resulting in the removal of inducing conditions, will not reverse resistance. Finally, and also not surprisingly, antibiotic resistant *B. vietnamiensis* isolates grew at slower rates than susceptible isolates. It is well documented that a resistant phenotype can affect the fitness of bacteria (402, 403), including in *P. aeruginosa* (404), and slower growth rates in the BCC have previously been associated with isolates that are resistant to a larger number of antimicrobials and at higher levels (405).

In an investigation of other features that have the potential to change over time during the course of a chronic infection alongside the acquisition of antimicrobial resistance, we

found that in *B. vietnamiensis* EPS production and catalase activity were correlated with aminoglycoside MICs; most susceptible isolates were frankly mucoid and did not demonstrate any catalase activity, whereas the opposite was true for aminoglycoside resistant isolates. In *P. aeruginosa*, oxidative stress results in the upregulation of catalases (356, 357) as well as the aminoglycoside-accommodating efflux system MexXY-OprM (329), and a link between the loss of EPS production and an increase in oxidative stress resistance has recently been described in *B. cenocepacia* (178). Furthermore, we established a novel correlation between mucoid phenotype and catalase activity in the BCC, a finding, which to our knowledge, has not been previously shown in bacteria; all *B. vietnamiensis*, *B. cenocepacia*, and *B. multivorans* isolates that were frankly positive for catalase activity produced no or minimal amounts of EPS, while no or minimal catalase activity was observed for all the frankly mucoid isolates. Moreover, in general, catalase activity increased between early and late sequential CF isolates, while EPS production decreased.

Taken together, these findings confirm that EPS production can vary between clonal sequential isolates of the BCC (158, 216-218), identify catalase activity as an additional phenotypic change that can occur during chronic infection, and suggest that switches in these two bacterial features occur in tandem with the acquisition of antimicrobial resistance in *B. vietnamiensis*, and likely reflect necessary adaptations for survival of this organism in the antimicrobial and ROS rich CF lung environment. Indeed, after exposure to increasing concentrations of hydrogen peroxide *B. vietnamiensis* displayed enhanced catalase activity. Likewise, Peeters *et al.* (406) reported increases in catalase expression after *B. cenocepacia* exposure to hydrogen peroxide. Given the correlation between EPS production and catalase activity found in our study, it is unclear whether or not there is an actual difference in

catalase production in the BCC bacteria or whether catalase is scavenged by bacterial EPS before it can act on hydrogen peroxide. This is the basis of current investigations in our lab.

Kill curve experiments showed that, despite the *in vitro* susceptibility of the *B. vietnamiensis* CF isolate D1389 to aminoglycosides, it could not be eradicated in the presence of tobramycin or gentamicin up to 8 × the MIC. Growth with gentamicin or tobramycin at 2, 4, and 8 × the MIC initially resulted in large CFU reductions after 8, 6, and 4 hours, respectively, however, population re-growth was observed. Furthermore, exposure of D1389 to gentamicin or tobramycin resulted in colony morphology changes; approximately 10% of the population were large-colony variants. Notably, after re-inoculation on antibiotic-free agar, the population reverted back to its original phenotype and was as aminoglycoside-susceptible as the original population, suggesting resistance determinants were not acquired. There are several known mechanisms of non-inherited adaptive drug resistance (407). In our study, the surviving fraction of the bacterial population may represent persister cells, a sub-population portion that exhibits tolerance to aminoglycosides (407, 408). The existence of this sub-population could explain the inability of aminoglycosides to eradicate a susceptible *B. vietnamiensis* isolate in our *in vitro* growth studies as well as *in vivo*, in the chronically infected patients described above. The morphology changes exhibited by *B. vietnamiensis* under aminoglycoside pressure may be a result of bacterial responses to antimicrobial stress conditions. In *P. aeruginosa* (397, 409, 410) and in *B. cenocepacia* (391), single exposures to lethal and/or subinhibitory concentrations of aminoglycosides alters the expression of genes involved in general bacterial stress responses. Regardless of the mechanisms involved, the fact that *B. vietnamiensis* can tolerate a single exposure of aminoglycosides, along with its ability to

acquire aminoglycoside resistance, described above, is frightening in terms of its capacity to survive and subsequently cause significant morbidity and mortality in CF patients.

Chapter 4: ROLE OF OUTER MEMBRANE PERMEABILITY IN *B. VIETNAMIENSIS* ACQUIRED AMINOGLYCOSIDE RESISTANCE

4.1 Summary

It is widely believed that *Burkholderia cepacia* complex (BCC) resistance to the inhibitory activity of polycationic antimicrobials is due to unusual characteristics of the lipopolysaccharide (LPS) (229). Cationic agents enter Gram-negative bacteria through LPS-mediated self-promoted uptake, relying specifically on anionic lipid A binding sites, which in resistant bacteria are blocked with polar residues such as A-associated 4-amino-4-*deoxy*-L-arabinose (Ara4N) (151). Furthermore, gross LPS structural features have been implicated in aminoglycoside resistance in *P. aeruginosa* (317). The purpose of this study was to determine if acquired aminoglycoside resistance in *B. vietnamiensis* is due to reduced intracellular drug accumulation owing to LPS characteristics. *B. vietnamiensis* cystic fibrosis (CF) isolates accumulated significantly less [^{3}H]gentamicin than susceptible isolates. Aminoglycoside resistance, however, was not correlated with LPS chemotype, and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry revealed the presence of lipid A-associated Ara4N in aminoglycoside-susceptible and -resistant *B. vietnamiensis* isolates. Furthermore, permeability to the fluorescent hydrophobic probe 1-*N*-phenylnapthylamine (NPN), a measure of self-promoted uptake, was not enhanced following incubation with gentamicin or tobramycin in any *B. vietnamiensis* isolates.

4.2 Introduction

Resistance to aminoglycosides in the BCC is often attributed to reduced uptake owing to LPS structural features that are thought to interfere with the passive transport of the antibiotics through the bacterial outer membrane (229), despite the lack of definitive evidence supporting this theory. BCC lipid A contains Ara4N modifications (144, 150, 152) responsible for aminoglycoside resistance in *P. aeruginosa* (282, 316), and general impermeability-type resistance in other bacteria (151). Mutations in the Ara4N biosynthetic pathway in *B. cenocepacia* however affect cell viability (290, 291). Furthermore, in *P. aeruginosa*, the O polysaccharide portion of LPS is involved in resistance to aminoglycosides (317). *B. cenocepacia* studies on the impact of lipopolysaccharide chemotype on antimicrobial minimum inhibitory concentrations (MICs), however, have only reported polymyxin B and cationic peptide susceptibility data (147, 292).

The specific objectives were:

1. To determine the intracellular accumulation of an aminoglycoside antibiotic in susceptible and resistant *B. vietnamiensis* isolates.
2. To examine lipopolysaccharide features in susceptible and resistant *B. vietnamiensis* isolates.

4.3 Results

4.3.1 Growth analysis of aminoglycoside-susceptible and -resistant *B. vietnamiensis* and *B. cenocepacia*

The growth curves of BCC isolates used for further study were determined in 25 or 50 ml MH II Broth (Figure 12). The late, aminoglycoside-resistant *B. vietnamiensis* isolate

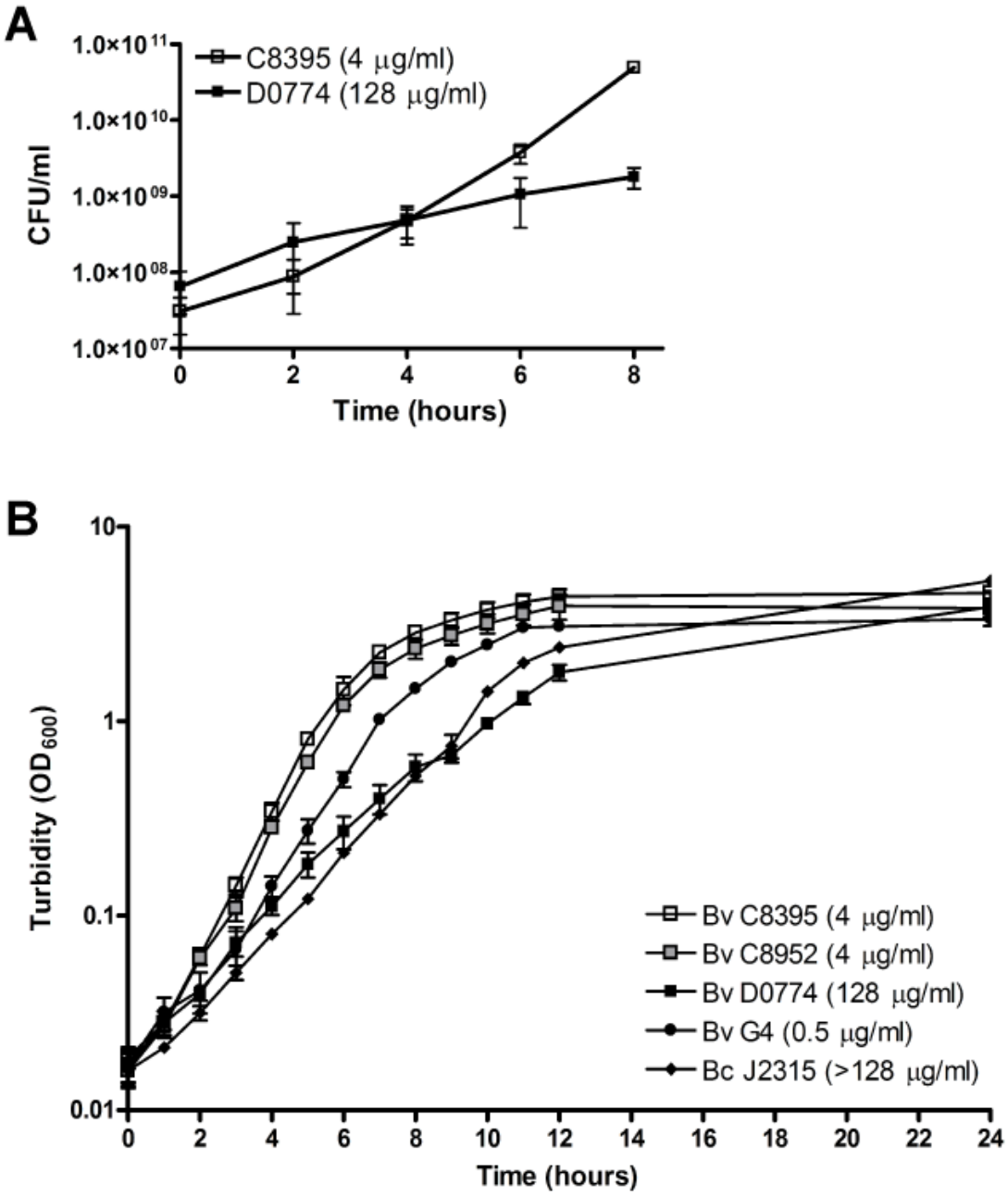

Figure 12. Growth curves of aminoglycoside-susceptible and -resistant BCC isolates used for further study.

Isolates were grown in broth starting from an OD_{600} of 0.02. Twenty µl samples were diluted and plated on agar, from which viable counts were taken (A). OD_{600} readings were taken every hour up to 12 hours and at 24 hours of growth (B). Gentamicin minimum inhibitory concentrations are shown in parentheses. Data points represent the averages for at least three biological replicates ± standard errors. Abbreviations: CFU, colony forming units; OD_{600}, optical density at 600 nm.

D0774 from patient Bv1 grew slower than the early, aminoglycoside-susceptible isolates C8395 and C8952. Growth rates of *B. vietnamiensis* G4 and *B. cenocepacia* J2315 were intermediate between those observed for C8395 and D0774. Similar growth rates were observed in Luria-Bertani (LB) medium: it took 4 vs. 6 hours for C8395 and D0774, respectively, to reach an optical density at 600 nm (OD_{600}) of 0.5 (data not shown). The colony forming units (CFU)/ml at OD_{600} of 0.5 was also determined for early, late, and *in vitro* derived *B. vietnamiensis* isolates (Table 14) to ensure that a similar number of cells were used in accumulation and permeability assays. In theory, the number of cells used experimentally would impact the results if all cells retaining [3H]gentamicin or NPN were saturated to the same extent. There were no significant differences in CFU/ml between isolates grown in MH II Broth, by unpaired Student's t-test, or in LB medium, with the exception of C8395TR vs. D0774 (P <0.05), as assessed with Bonferroni's Multiple Comparison test after one-way analysis of variance (ANOVA).

4.3.2 Aminoglycoside-resistant *B. vietnamiensis* accumulates significantly less gentamicin than aminoglycoside-susceptible *B. vietnamiensis*

To determine if decreased drug accumulation is involved in *B. vietnamiensis* acquired aminoglycoside resistance, the cellular accumulation of [^{3}H]gentamicin was measured in the serial clinical isolates C8395 and D0774 from patient Bv1, and isolates derived from C8395 after exposure to tobramycin, C8395TR, or hydrogen peroxide, C8395HP2 (Figure 13). Aminoglycoside-susceptible *P. aeruginosa* ATCC 27853 was used as a positive control, based on previous reports of aminoglycoside accumulation in this species (337, 411). In the aminoglycoside-susceptible early isolate C8395, [^{3}H]gentamicin accumulated at a slower rate

Table 14. *B. vietnamiensis* viable counts at a turbidity of OD_{600} 0.5

	Isolates in LB Medium					Isolates in MH II Broth	
CFU/ml	C8395	C8395TR	C8395AR	C8395HP2	D0774	C8395	D0774
Mean	5.23×10^8	3.88×10^8	6.30×10^8	4.80×10^8	7.97×10^8	4.98×10^8	7.98×10^8
SD	0	1.01×10^8	7.07×10^7	1.08×10^8	8.49×10^7	7.31×10^7	9.55×10^7

Abbreviations: OD_{600}, optical density at 600 nm; CFU, colony forming units; LB, Luria-Bertani; MH, Mueller Hinton; TR, tobramycin resistant; AR, azithromycin resistant; HP2, hydrogen peroxide resistant second pick; SD, standard deviation of two replicates.

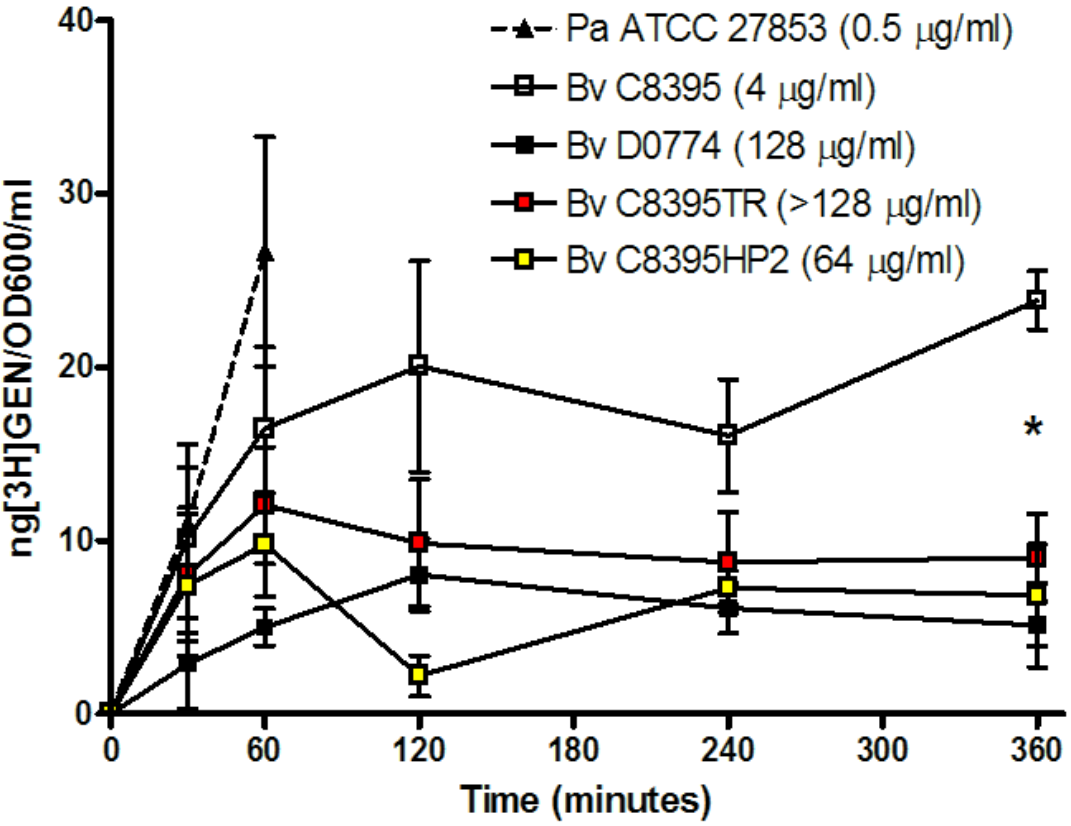

Figure 13. Accumulation of 20 μg/ml [^{3}H]gentamicin by *B. vietnamiensis* and 5 μg/ml [^{3}H]gentamicin by *P. aeruginosa* ATCC 27853.

Baseline accumulation was set as 0. Gentamicin minimum inhibitory concentrations are shown in parentheses. Data points represent the averages of three biological replicates ± standard errors. *, $P < 0.05$ (two-way analysis of variance). Abbreviations: Bv, *B. vietnamiensis*; Pa, *P. aeruginosa*; GEN, gentamicin; OD600, optical density at 600 nm.

than that in *P. aeruginosa* and reached a maximum of 23.85 ng/optical density at 600 nm (OD_{600})/ml at 6 hours before a plateau was noted; experiments were performed up to 8 hours. Under the same conditions, the aminoglycoside-resistant late isolate D0774 accumulated [^{3}H]gentamicin minimally, 4.7 times less than C8395 at 6 hours. The aminoglycoside-resistant *in vitro* derived isolates C8395TR and C8395HP2 also accumulated less [^{3}H]gentamicin than C8395, specifically 2.6 and 4.2 times less at 6 hours, respectively. The differences observed between isolates at 6 hours were significant (P = 0.0118) by one-way ANOVA. Bonferroni's Multiple Comparison Test identified significant differences between C8395 and D0774 (P <0.05), and C8395 and C8395HP2 (P <0.05). Differences observed in [^{3}H]gentamicin accumulation over time were significant (P = 0.0369) between isolates by two-way ANOVA. To confirm that aminoglycoside-susceptible isolates of *B. vietnamiensis* accumulate [^{3}H]gentamicin, the susceptible isolate D1389 from patient Bv3 was also tested; accumulation after 6 hours reached 31.0 ng/OD_{600}/ml (standard error of 10.5) (data not shown). Addition of carbonyl cyanide m-chlorophenylhydrazone (CCCP) to the resistant *B. vietnamiensis* isolate D0774 prior to the accumulation experiments did not enhance the cellular retention of [3H]gentamicin over 6 hours (data not shown). In fact, levels of [3H]gentamicin remained equivalent to background. CCCP and other inhibitors of energy dependent processes are used to block transporter-mediated energy dependent drug efflux in accumulation assays to determine the mechanism of resistance (412).

4.3.3 LPS modifications are not responsible for acquired aminoglycoside resistance in *B. vietnamiensis*

4.3.3.1 Aminoglycoside resistance does not correlate with LPS chemotype

To determine if LPS modifications are involved in acquired aminoglycoside resistance in *B. vietnamiensis*, LPS chemotypes were compared between susceptible and resistant isolates (Figure 14). Polyacrylamide gel electrophoresis (PAGE) analysis of 20 µg of purified LPS revealed no gross differences among serial clinical isolates C8395, C8952, and D0774 from patient Bv1; all had rough LPS (LPS lacking O antigen) (Figure 14a). 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). The serial clinical isolates D0099 and D2075 from patient Bv2 also had rough LPS, as did the additional aminoglycoside-susceptible clinical and environmental isolates tested, although D1389, LMG 06999, and G4 had some faint banding at higher molecular weights (Figure 14b).

4.3.3.2 Aminoglycoside-susceptible and -resistant *B. vietnamiensis* contain aminoarabinose residues at lipid A

The lipid A portion of LPS was analyzed by MALDI-TOF mass spectrometry from the aminoglycoside-susceptible and -resistant *B. vietnamiensis* isolates listed in Table 8 (Figure 15). Consistent with previous reports (144, 150), lipid A structures were a blend of tetra- and penta-acylated molecules, identified by mass-to-charge ratios (m/z) 1444, 1468, 1469, and 1495, and m/z 1670, 1695, and 1696, respectively. The environmental isolates LMG 10929^T and G4 had additional unique peaks of higher mass. Tetra- and penta-acylated lipid A structures of aminoglycoside-susceptible and -resistant isolates were positive for

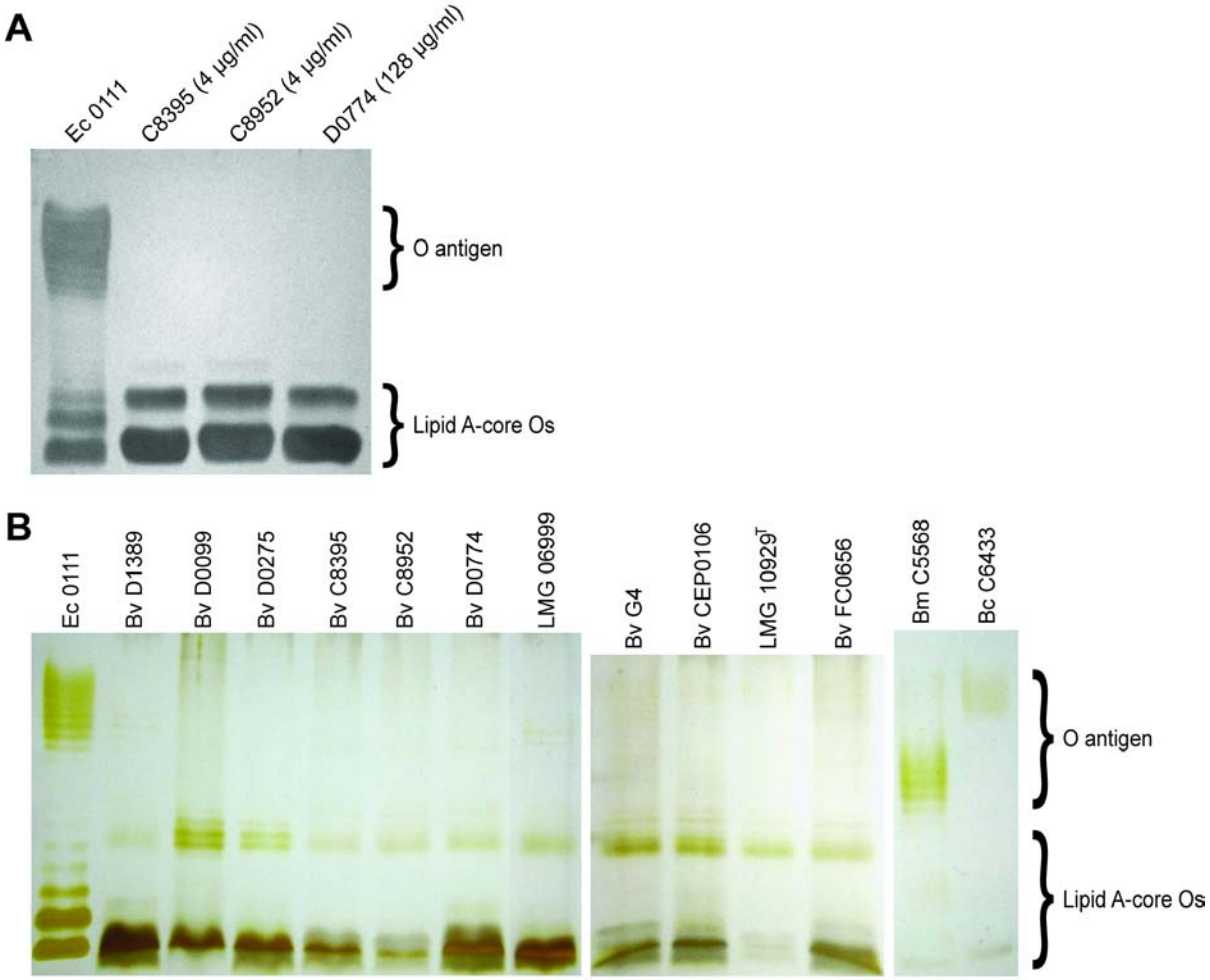

Figure 14. Detection of *B. vietnamiensis* lipopolysaccharide by silver stain.
Twenty micrograms of LPS extracted by hot water-phenol (A) or 5µl of lipopolysaccharide fraction extracted by proteinase K digestion (B) were electrophoresed on 12.5% sodium dodecyl sulfate-polyacrylamide gels. Gentamicin minimum inhibitory concentrations are shown in brackets (A). Ec, *E. coli*; Bv, *B. vietnamiensis*; Bm, *B. multivorans*; Bc, *B. cenocepacia*; O, oligosaccharide.

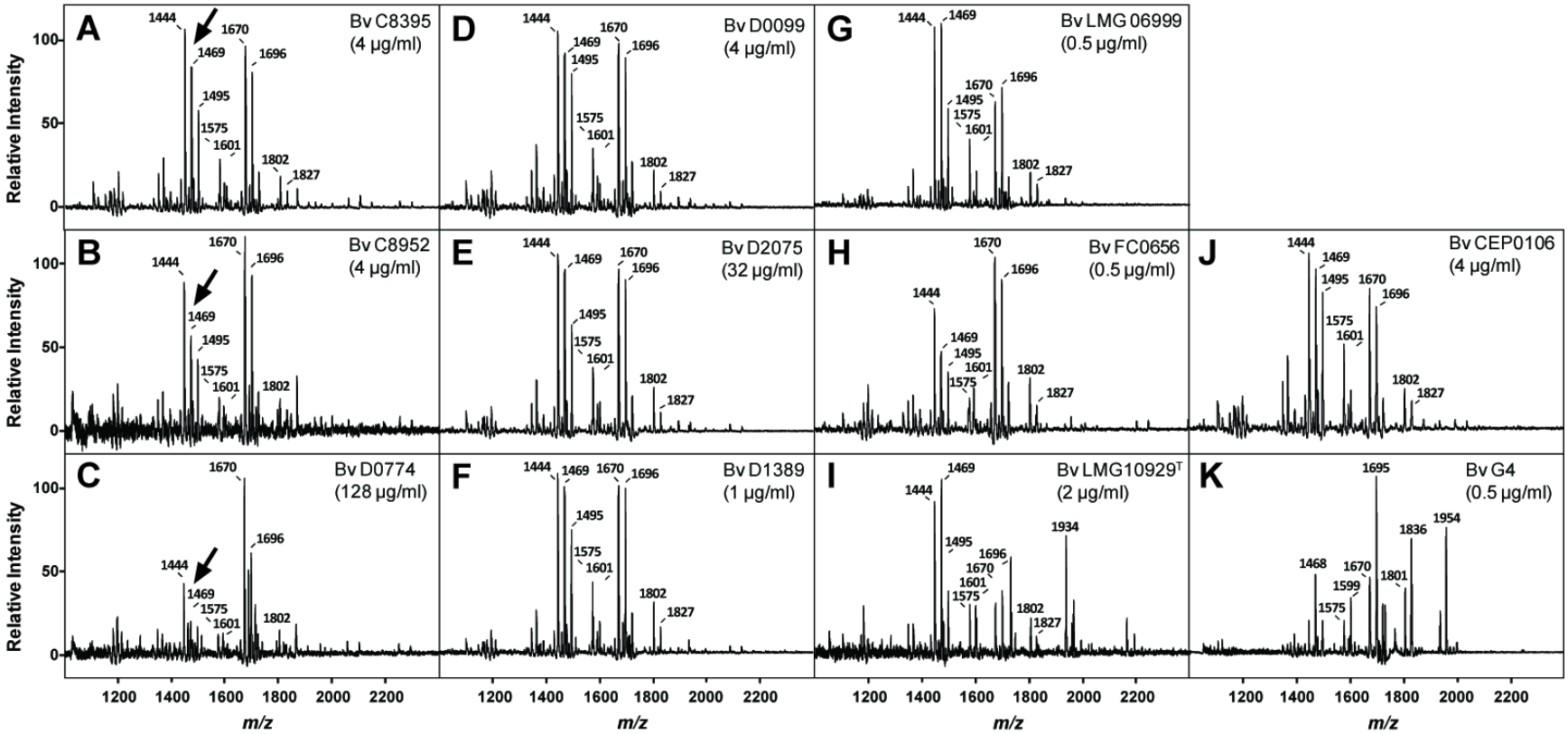

Figure 15. *B. vietnamiensis* lipid A structural analysis.
(A-C), sequential isolates from patient Bv1; (D-E), sequential isolates from patient Bv2; (F), isolate from patient Bv3; (G), clinical non-CF isolate; (H-K), environmental isolates. Gentamicin minimum inhibitory concentrations are shown in parentheses. Purified lipid A was analyzed by negative-ion matrix-assisted laser desorption ionization–time of flight mass spectrometry. Tetra- and penta-acylated molecules are identified by m/z 1444, 1468, 1469, and 1495 and m/z 1670, 1695, and 1696, respectively. Lipid A moieties containing Ara4N are identified by m/z 1575, 1599, 1601, 1801, 1802, and 1827. Arrows point to changes in acylation between sequential isolates. Bv, *B.vietnamiensis*; m/z, mass-to-charge ratio.

Ara4N, identified on spectra as 1575, 1599, 1601, 1801, 1802, and 1827, based on the Ara4N m/z of 131. Notably, these findings included lipids of serial clinical isolates for strains that had acquired aminoglycoside resistance: C8395, C8952, and D0774 from patient Bv1 (Figure 15a-c) and D0099 and D2075 from patient Bv2 (Figure 15d-e). Furthermore, differences in lipid A acylation patterns were observed among sequential isolates C8395, C8952, and D0774; acylation increased with time, with the lipids becoming enriched for penta-acylated molecules (Figure 15a-c).

4.3.3.3 Aminoglycoside-susceptible and -resistant outer membranes are not permeabilized by aminoglycosides

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 1-*N*-phenylnaphthylamine (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 (344) (Figure 16). A lack of NPN fluorescence therefore results from the presence of LPS features that inhibit this drug interaction. The addition of the proton gradient uncoupler CCCP to the reaction buffer ensures fluorescence is optimal. The outer membranes of aminoglycoside-susceptible and -resistant *B. vietnamiensis* isolates were not permeabilized by up to 128 µg/ml gentamicin (Figure 16), as inferred from the lack of a significant increase in NPN fluorescence and assessed by Bonferroni's Multiple Comparison test after one-way ANOVA. Similar results

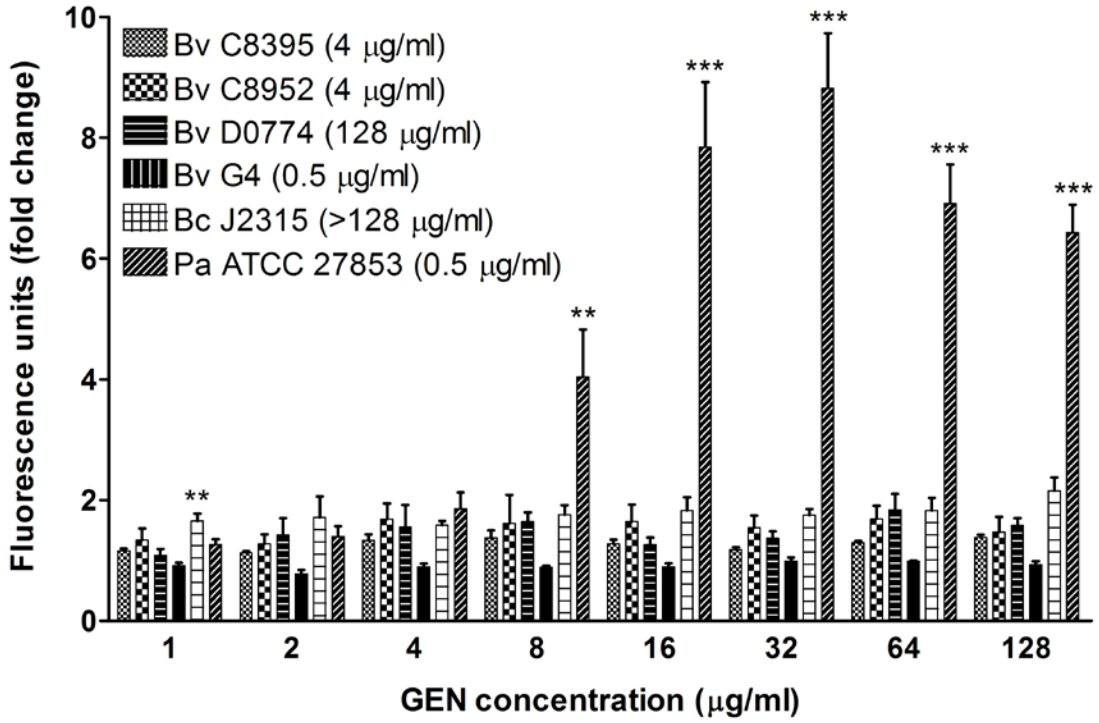

Figure 16. Permeabilizing effects of gentamicin on *B. vietnamiensis, B. cenocepacia,* and *P. aeruginosa* ATCC 27853.

1-*N*-phenylnaphthylamine (NPN) was added to cells 30 seconds after initiation of fluorescence readings; antibiotic was added 30 to 90 seconds later. Final values were taken as averages of those recorded from 200 to 500 seconds, when a plateau in fluorescence was observed. Fluorescence was measured at least every 10 seconds. Baseline NPN fluorescence was set to 1. Gentamicin minimum inhibitory concentrations are shown in parentheses. Data points represent the averages for at least three biological replicates plus standard errors. **, $P < 0.05$; ***, $P<0.001$ (Bonferroni's Multiple Comparison test after one-way analysis of variance). Bv, *B. vietnamiensis*; Bc, *B. cenocepacia*; Pa, *P. aeruginosa*; GEN, gentamicin.

were found for *B. cenocepacia* J2315 (Figure 16), a BCC isolate that also contains lipid A-associated Ara4N residues (313). The *B. vietnamiensis* G4 outer membrane was also not permeabilized by 2, 16, or 128 µg/ml tobramycin; NPN fluorescence was 1.08 fold higher than background at most, while *P. aeruginosa* ATCC 27853 exposure to 16 and 128 µg/ml tobramycin resulted in 4.6 and 12.5 fold higher levels, respectively (data not shown).

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 (344) (data not shown), the assay was extended to 20 min, but no increase in NPN fluorescence was observed after exposure of G4 to gentamicin at 2, 16, or 128 µg/ml (data not shown). In addition, no increases in NPN fluorescence were observed after G4 exposure to gentamicin at concentrations ranging between 2 to 128 µg/ml in the absence of glucose, in the absence of CCCP, in the presence of 50 µM CCCP (10 times the concentration), or in the presence of sodium azide (0.1, 10, and 20mM) as an alternative of CCCP (data not shown, permeability to NPN was enhanced in P. aeruginosa). No increase in NPN fluorescence was observed after G4 exposure to tobramycin at 16 or 128 µg/ml in the absence of glucose (data not shown, permeability to NPN was enhanced in P. aeruginosa).

4.4 Discussion

Aminoglycoside resistance in the BCC is often attributed to reduced drug uptake owing to structural features of the LPS that inhibit their passive transport through the bacterial outer membrane (229), though definitive evidence is lacking. BCC lipid A contains Ara4N modifications (150, 152, 311-315) that are involved in aminoglycoside resistance in

P. aeruginosa (282, 316), and, in general, contribute to outer membrane impermeability in bacteria (151). Our findings suggest that decreased access of aminoglycosides to their intracellular antimicrobial target is involved in the acquired aminoglycoside resistance observed during chronic CF infection by *B. vietnamiensis*, as reflected in differential intracellular accumulation of [^{3}H]gentamicin between susceptible and resistant sequential CF isolates. Furthermore, induced *B. vietnamiensis* aminoglycoside resistance under tobramycin or hydrogen peroxide pressure *in vitro*, also results in decreased [^{3}H]gentamicin accumulation. From these data however, it is impossible to determine if the cause of the decreased accumulation in resistant *B. vietnamiensis* isolates is due to decreased influx or increased efflux of the drug, as apparent failure of drug accumulation can result from either.

Classically, the proton gradient uncoupler CCCP, or an alternative inhibitor of energy dependent processes, is used to block transporter-mediated energy dependent drug extrusion in accumulation assays to determine the mechanism of resistance (412). Addition of CCCP to a resistant isolate of *B. vietnamiensis* prior to the accumulation experiments however, did not increase the cellular retention of [^{3}H]gentamicin. In fact, we found no association between [^{3}H]gentamicin and the resistant isolate D0774, whereas without CCCP present, there was some accumulation of the antimicrobial in this isolate. The data could be interpreted to mean that outer membrane permeability is involved in acquired aminoglycoside resistance in *B. vietnamiensis*, however, the decrease in overall association of [^{3}H]gentamicin in the presence of CCCP would not be explained.

Similar findings with streptomycin accumulation experiments were reported in *B. pseudomallei* (413) and *P. aeruginosa* (414), where active efflux proved to be involved in aminoglycoside resistance. The unexpected results were attributed to the presumed inhibitory

effects of CCCP on the active inward transport of aminoglycosides across the cytoplasmic membrane as well as their efflux (414). In theory however, in the presence of CCCP, aminoglycosides should still accumulate in the bacterial periplasmic space in resistant organisms, given that aminoglycoside uptake is thought to occur passively in Gram-negative bacteria, through the displacement of divalent cations that cross-bridge anionic LPS molecules, in a process termed self-promoted uptake (297). We therefore argue that CCCP, through its action on the cytoplasmic membrane potential, could also disrupt aminoglycoside transport through the outer membrane in *B. vietnamiensis*, if it is an energy-dependent process mediated by a protein complex. The mechanism for aminoglycoside uptake in the BCC has not yet been explained.

In *P. aeruginosa*, upregulation of the *arn* LPS modification operon, responsible for the addition of Ara4N to lipid A phosphate groups (151), results in decreased aminoglycoside MICs (282, 316). Ara4N reduces bacterial susceptibility to cationic agents by neutralizing the negative charge of lipid A-associated phosphate residues required for self-promoted uptake, thereby decreasing permeability to the antimicrobials (151). Furthermore, the disruption of LPS O polysaccharide assembly in *P. aeruginosa* results in enhanced aminoglycoside resistance and reduced aminoglycoside-mediated outer membrane permeabilization (317). Poor uptake owing to LPS modifications is not responsible for the acquisition of aminoglycoside resistance in *B. vietnamiensis*, however, since susceptible and resistant isolates showed the presence of lipid A-associated Ara4N residues and had similar or identical (in the case of sequential isolates) rough LPS chemotypes. The sequential isolates from patient Bv1 also all displayed core oligosaccharide banding to the same degree, as observed by PAGE analysis. A complete LPS inner core oligosaccharide is required for *B.*

cenocepacia resistance to antimicrobial peptides and polymyxin B (147, 292). Lastly, 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* (290, 291). Indeed, all BCC lipid A structures studied to date contain Ara4N modifications (150, 152, 311-315).

These findings contradict the current dogma that the lack of LPS anionic binding sites is sufficient to cause resistance to aminoglycosides (229). Along with our and previous (24, 26) susceptibility data of antibiotic-susceptible but cationic peptide and polymyxin B resistant *B. vietnamiensis*, these findings also suggest that resistance mechanisms can differ for different classes of polycationic antimicrobials, and that the determinant(s) that results in the resistance of *B. vietnamiensis* to the inhibitory effects of peptides is not sufficient to cause resistance to aminoglycosides or other classes of antibiotics.

Indeed, polymyxin susceptible mutants of *B. multivorans* remain resistant to aminoglycosides (293). The presence of Ara4N residues at lipid A of *B. vietnamiensis* may account for the observed resistance to the inhibitory effects of cationic antimicrobial peptides and polymyxin B, as well as low-level aminoglycoside resistance. 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 passive self-promoted uptake. Based on these observations however, it is still unclear if outer membrane permeability is involved in *B. vietnamiensis* acquired aminoglycoside resistance. A description of aminoglycoside uptake in *B. vietnamiensis* will be necessary to answer this

question, as alterations in specific properties involved in uptake have the potential to result in acquired resistance (151, 226).

The altered production or function of outer membrane porins results in bacterial resistance to various classes of antimicrobials, most notably β-lactams and fluoroquinolones, owing to the role of porins in the active uptake of antibiotics (226). Indeed, porins have been implicated in BCC resistance to β-lactams and trimethoprim/sulfamethoxazole (233, 234, 251, 415). Porin-mediated uptake of aminoglycosides seems unlikely, based on the large size and hydrophobic nature of the antimicrobials. In fact, to date there are no reports of aminoglycoside influx through bacterial porins. It has been suggested however, that aminoglycosides can interact with a divalent cation binding site on the outside of the OmpF porin in *E. coli* (416). In *B. vietnamiensis*, where anionic LPS binding regions are unavailable, the importance of these types of sites in aminoglycoside uptake may be of greater importance than in other Gram-negative bacteria where self-promoted uptake is possible. The potential role of porins in bacterial uptake of aminoglycosides, and consequently their possible involvement in impermeability-type resistance, should be considered, and could explain the slow rate of aminoglycoside uptake observed for *B. vietnamiensis* in our study (passive aminoglycoside uptake in *P. aeruginosa* is nearly instantaneous (337, 344, 411)). Further evaluation of the interaction between aminoglycosides and bacterial outer membrane porins is necessary.

Importantly, differences noted in *B. vietnamiensis* lipid A acylation patterns among sequential CF isolates may impact their ability to stimulate host immune cells, such as monocytes (150-152). BCC lipid A is composed of a blend of tetra-and penta-acylated species, but can vary in the degree of acylation, with some structures shown to be more or

less tetra-acylated (150, 152, 311-315). Furthermore, BCC strains have the capacity to modify lipid A acylation patterns during the course of a chronic CF infection (150, 152), with *B. vietnamiensis* lipid A specifically becoming more penta-acylated. Indeed, we found that the degree of lipid A acylation increased over time in the *B. vietnamiensis* strain infecting CF patient Bv1, with lipids becoming enriched for penta-acylated molecules. In general, a higher degree of lipid A acylation is associated with more potent proinflammatory activity (151), a phenomenon that has been described in CF strains of *P. aeruginosa* (417, 418) and the BCC, *B. vietnamiensis* included (150, 152, 315). *B. vietnamiensis* may modify its lipid structure as an adaptive response to the CF lung environment; however, the potential effects of the modified structure on inflammation could be detrimental to the host. The correlation between BCC lipid A structure and biological activity should be examined in future studies.

Chapter 5: ROLE OF ACTIVE EFFLUX IN *B. VIETNAMIENSIS* ACQUIRED AMINOGLYCOSIDE RESISTANCE

5.1 Summary

Despite the dogma that *Burkholderia cepacia* complex (BCC) resistance to the inhibitory effects of polycationic antimicrobials is due to unusual characteristics of the lipopolysaccharide (LPS) (229), recent studies have demonstrated the involvement of resistance-nodulation-division (RND) efflux systems in *B. cenocepacia* resistance to aminoglycosides (237, 239, 259). RND efflux systems accommodate aminoglycosides in a number of clinically important organisms, including *P. aeruginosa* and *B. pseudomallei* (228). The purpose of this study was to determine if acquired aminoglycoside resistance in *B. vietnamiensis* is due to reduced intracellular drug accumulation owing to increased efflux via a RND transporter. Aminoglycoside-resistant *B. vietnamiensis* isolates expressed more of a putative *amrB* efflux system transporter gene than susceptible isolates. After serial exposure to tobramycin and azithromycin, but not hydrogen peroxide, *amrB* expression was induced in an aminoglycoside-susceptible *B. vietnamiensis* cystic fibrosis (CF) isolate. Inhibition of the putative efflux system enhanced *B. vietnamiensis* susceptibility to aminoglycosides.

5.2 Introduction

Recent studies have noted the involvement of RND efflux systems in *B. cenocepacia* aminoglycoside resistance (237, 239, 259). Notably, all of these studies were based on whole operon deletions, and therefore did not access the involvement of a RND transporter alone in antimicrobial resistance. Furthermore, complementation analysis of the generated mutants

was not performed. Based on Koch's Molecular Postulates, after loss of function resulting from gene deletion, the subsequent reintroduction of the gene and restoration of function (complementation) is essential in determining the role of a bacterial gene (419). RND efflux systems are major determinants of aminoglycoside resistance in Gram-negative bacteria (228), and rare aminoglycoside susceptibility in clinical isolates of *B. pseudomallei* is attributed to the loss of expression of its major aminoglycoside accommodating RND efflux pump (323).

The specific objectives were:

1. To identify and characterize a homologue of known aminoglycoside-accommodating efflux systems in *B. vietnamiensis*.
2. To determine the expression of a putative transporter of aminoglycosides in susceptible and resistant *B. vietnamiensis* isolates.

5.3 Results

5.3.1 Homologues of characterized efflux system proteins responsible for aminoglycoside resistance in *P. aeruginosa* and *B. pseudomallei* exist in *B. vietnamiensis*

To identify potential *B. vietnamiensis* drug efflux transporters belonging to the RND family, *B. vietnamiensis* G4 (accession no. NC_009256.1) predicted proteins were scanned for the presence of four highly conserved amino acid sequences of multidrug RND transporters, as was done previously for *B. cenocepacia* (235): motif A (G x s x v T v x F x x g t D x x x A q v q V q n k L q x A x p x L P x x V q x q g x x v x k), motif B (a l v l s a V F l P m a f f g G x t G x i y r q f s i T x v s A m a l S v x x v a l t l t P A l c A), motif C (x x x G k x l x e A x x x a a x x R L R P l L M T s L a f i l G v l P l a i a t G x A G a), and motif D (S i

N t l T l f g l v l a i G L l v D D A l V v V E N v e R v l a e), where x is any amino acid, capital letters are amino acids that occur in >70% of sequences, and lowercase letters are amino acids that occur in >40% of sequences (346). G4 is the only *B. vietnamiensis* sequenced to date. Eleven putative RND transporters were identified (expect values $\leq$ 0.05) in G4 with this search (Table 15). Functional domain analysis with the National Center for Biotechnology Information Conserved Domain Database classified all but one of the putative transporters as multidrug efflux proteins. The exception based on the best multi-domain hits was Bcep1808_7176, which was classified as a metal efflux protein.

Previously characterized RND transporters involved in aminoglycoside resistance, *P. aeruginosa* MexY, *B. pseudomallei* AmrB, as well as their homologue in *B. cenocepacia*, BCAL1675, were aligned against *B. vietnamiensis* G4 protein sequences to identify regions of homology. Only the G4 (accession no. NC_009256.1) putative protein Bcep1808_1575 showed high identity (>51%) with the characterized transporters of *P. aeruginosa* (accession no. NC_008463.1), *B. pseudomallei* 1710b (accession no. NC_007434.1), and *B. cenocepacia* (accession no. NC_011000.1), with identities of 71%, 85%, and 92%, respectively (Table 16). Enzyme function is well conserved when sequence identity is above 40% (420). Sequence alignments showing regions of homology are presented in Figure 17. In *P. aeruginosa* and *B. pseudomallei* these transporters are part of an efflux system operon also encoding a repressor protein, MexZ or AmrR, respectively, a membrane fusion protein, MexA or AmrA, respectively, and, in *B. pseudomallei*, an outer membrane channel, OprA (*P. aeruginosa* OprM is located downstream of the operon). The putative *B. vietnamiensis* G4 (accession no. NC_009256.1) proteins encoded by regions adjacent to *Bcep1808_1575* were aligned against characterized repressor, fusion, and channel protein sequences of *P.*

Table 15. Predicted multidrug RND transporters in *B. vietnamiensis* G4[a]

Protein (chromosome/plasmid)	Location of RND conserved motifs	E-value[b]
Bcep1808_2722 (1)	86-131 (A), 446-494 (B), 953-997 (C), 389-422 (D)	6E-12
Bcep1808_1575 (1)	85-132 (A), 445-493 (B), 947-991 (C), 388-421 (D)	4E-10
Bcep1808_4956 (2)	86-131 (A), 445-493 (B), 953-997 (C), 389-422 (D)	2E-09
Bcep1808_4759 (2)	86-131 (A), 446-494 (B), 974-1018 (C), 389-422 (D)	2E-09
Bcep1808_3579 (2)	88-133 (A), 448-496 (B), 961-1005 (C), 392-425 (D)	6E-09
Bcep1808_6402 (3)	978-1022 (C), 383-416 (D)	8E-08
Bcep1808_5403 (2)	446-494 (B), 934-978 (C), 390-423 (D)	2E-07
Bcep1808_1112 (1)	439-487 (B), 938-982 (C), 383-416 (D)	8E-06
Bcep1808_7176 (pBVIE02)	961-1005 (C), 387-420 (D)	5E-05
Bcep1808_1111 (1)	439-487 (B), 1002-1046 (C), 383-416 (D)	5E-05
Bcep1808_1684 (1)	952-997 (C), 391-424 (D)	0.03

[a] Abbreviations: RND, resistance-nodulation-division.

[b] The expect value (E-value) describes the number of hits that can be expected by chance.

Table 16. Homology between *B. vietnamiensis* putative proteins and proteins of *P. aeruginosa, B. pseudomallei*, and *B. cenocepacia*[a]

	Repressor identities (%)			Fusion protein identities (%)			Transporter identities (%)			Channel identities (%)		
Bv G4 putative proteins	Pa MexZ	Bp AmrR	Bc 1672[b]	Pa MexX	Bp AmrA	Bc 1674[b]	Pa MexY	Bp AmrB	Bc 1675[b]	Pa OprM	Bp OprA	Bc 1676[b]
Bcep1808_1573	58	76	90	-	-	-	-	-	-	-	-	-
Bcep1808_1574	-	-	-	65	79	89	-	-	-	-	-	-
Bcep1808_1575	-	-	-	-	-	-	71	85	92	-	-	-
Bcep1808_1576	-	-	-	-	-	-	-	-	-	45	56	71

[a] *B. vietnamiensis* (Bv) G4 (accession no. NC_009256.1); *P. aeruginosa* (Pa) UCBPP-PA14 (accession no. NC_008463.1); *B. pseudomallei* (Bp) 1710b (accession no. NC_007434.1); *B. cenocepacia I* (Bc) J2315 (accession no. NC_011000.1) for BCAL1674, BCAL1675, and BCAL1676, *B. cenocepacia* AU1054 (NC_008060.1) for BCAL1672.

[b] BCAL precedes number in protein full name.

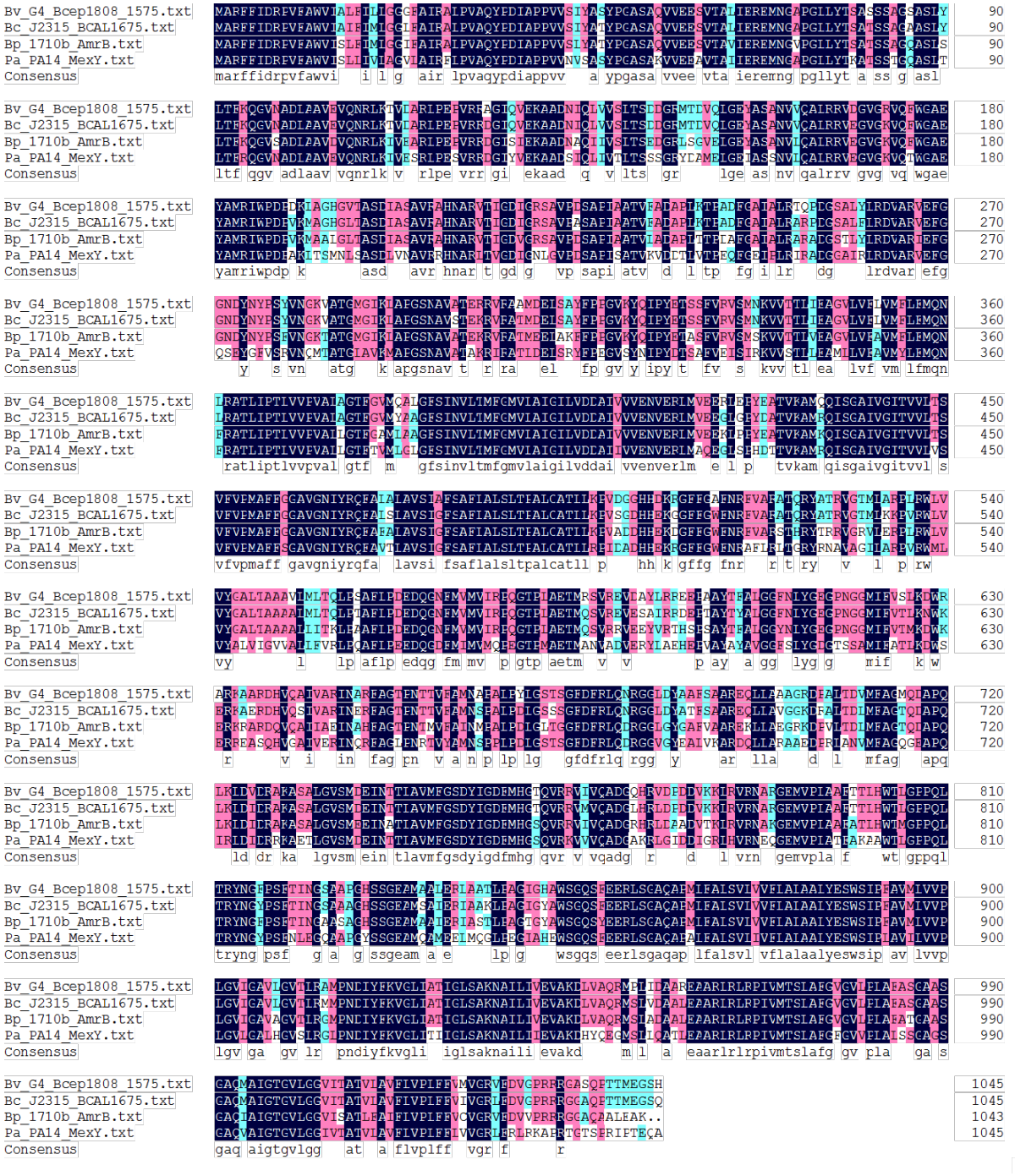

Figure 17. Multiple alignment of Bcep1808_1575 with RND transporter homologues.
Bcep1808_1575 of *B. vietnamiensis* (Bv) G4 (accession no. NC_009256.1) is aligned with BCAL1675 of *B. cenocepacia* (Bc) J2315 (accession no. NC_011000.1), AmrB of *B. pseudomallei* (Bp) 1710b (accession no. NC_007434.1), and MexY of *P. aeruginosa* (Pa) UCBPP-PA14 (accession no. NC_008463.1). The alignment was generated using DNAMAN software. Amino acid homology is shaded as follows: navy blue, 100%; pink, ≥75%; light blue, ≥50%.

aeruginosa (accession no. NC_008463.1) and *B. pseudomallei* (accession no. NC_007434.1) encoded by corresponding regions, as well as their homologues in *B. cenocepacia* (accession no. NC_011000.1 or NC_008060.1, where the former sequence was unavailable). High identity (≥65%) was seen for the membrane fusion protein alignments; repressor and outer membrane channel proteins show less, but some, homology, ≥58, and ≥45% identities, respectively. All alignment scores generated from protein sequence comparisons are shown in Table 16.

The predicted structure and function of *B. vietnamiensis* G4 Bcep1808_1575 was analyzed further, as was done previously in *B. cenocepacia* (235), as this putative protein was a likely candidate for an aminoglycoside-accommodating RND transporter. RND transporters have 12 transmembrane segments (TMS), with large loops between TMS1 and TMS2, and between TMS7 and TMS8 (346). These structural features appear to be conserved in the hypothetical Bcep1808_1575 as predicted by the TMHMM program. Structural modeling with the Phyre server predicted Bcep1808_1575 as a membrane protein of the AcrB multidrug efflux transporter family. To obtain a putative function for Bcep1808_1575, analysis for signature features of protein families and functional sites was performed using the InterProScan Sequence Search program. Bcep1808_1575 contains ACRIFLAVINRP, a 9-element print signature of members of the acriflavine resistance protein family including AcrB of *E. coli*. Bcep1808_1575 contains two transmembrane functional domains (amino acids 295-497, 801-1026) that are signatures of the multidrug efflux transporter AcrB family, as well as the four pore subdomains of AcrB, PN1, PN2, PC1, and PC2 (amino acids 38-133, 134-329, 564-667). Bcep1808_1575 also contains the

two subdomains of the AcrB TolC docking domain, DN and DC (181-272, 719-806) (TolC being the outer membrane channel component of the efflux system involving AcrB).

Based on these homology and predictive studies, from this point onwards Bcep1808_1573, Bcep1808_1574, Bcep1808_1575, and Bcep1808_1576 will be referred to as AmrR, AmrA, AmrB, and OprA, respectively.

5.3.2 Clinical CF isolates of *B. vietnamiensis* contain genes of a putative aminoglycoside-accommodating efflux system

The presence of the putative RND transporter gene *amrB* in early and late *B. vietnamiensis* isolates from CF patients Bv1 (C8395 and D0774), Bv2 (D0099 and D2075), and Bv3 (D0072 and D2910) was determined by polymerase chain reaction (PCR) and compared to the sequenced environmental isolate G4. *16S* was used as a positive control. All isolates contained *amrB*, and presumably in its full size, as determined by gel electrophoresis of the 3095 base pair (bp) amplified products (G4 *amrB* is 3138 bp long) (Figure 18a). By designing primers to amplify regions of *amrB* as well as regions of *amrA* or *OprA*, the genes located on either side of *amrB* in the sequenced isolate G4, it was also determined that the clinical isolates contained *amrA* and *OprA*, and that these genes, along with *amrB*, were organized in an operon in the same way as in G4 (Figure 18b).

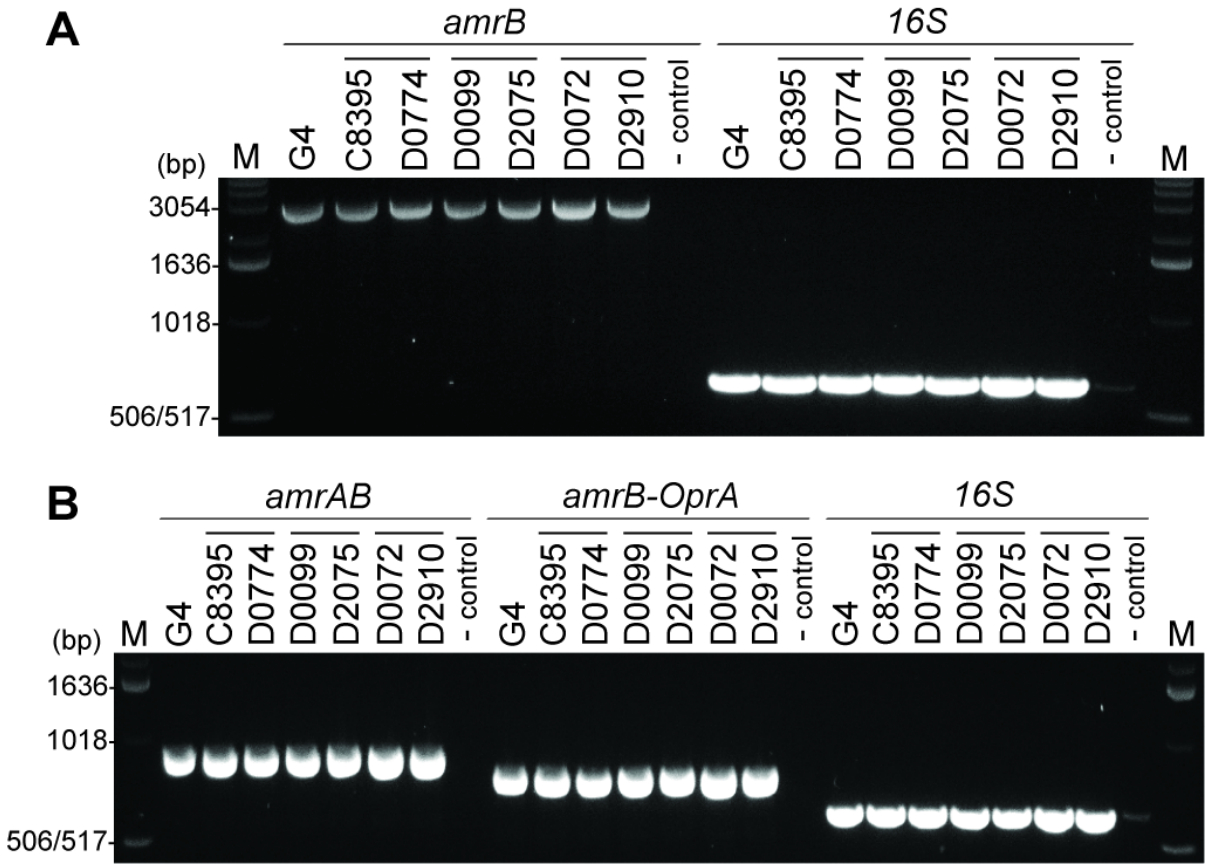

Figure 18. Amplification of putative efflux genes in early, aminoglycoside-susceptible and late, aminoglycoside-resistant CF isolates of *B. vietnamiensis*.
Polymerase chain reaction amplification of (A) *amrB*, (B) *amrAB*, and *amrB-OprA* in sequential isolates from cystic fibrosis patients Bv1 (C8395 and D0774), Bv2 (D0099 and D2075), and Bv3 (D0072 and D2010), was compared with amplification of the ribosomal subunit gene *16S* as well as the amplification of these genes in the sequenced isolate G4.

5.3.3 Expression of the putative RND transporter gene *amrB* in clinical CF and *in vitro* antibiotic or hydrogen peroxide exposed *B. vietnamiensis*

5.3.3.1 Aminoglycoside-resistant *B. vietnamiensis* expresses significantly more *amrB* than aminoglycoside-susceptible *B. vietnamiensis*

Transcriptome analysis by real-time (Q) reverse transcription (RT) PCR revealed that the late, aminoglycoside-resistant isolate from patient Bv1, D0774, expressed significantly more of the putative RND transporter gene *amrB* than the early, aminoglycoside-susceptible isolate C8395, independent of the growth conditions tested, as determined by unpaired Student's t-tests: 11.4 ($P < 0.0001$), 9.6 ($P < 0.0001$), 3.5 ($P < 0.01$), and 8.0 ($P < 0.01$) fold increases were observed between D0774 and C8395 after growth to an optical density at 600 nm (OD_{600}) of 0.8 in Mueller-Hinton (MH) II Broth, to an OD_{600} 0.8 in Luria-Bertani (LB) medium, to an OD_{600} of 0.5 in LB medium, and to an OD_{600} of 0.8 in synthetic CF sputum medium (SCFM), respectively (Figure 19a). By one-way analysis of variance (ANOVA), there were no significant differences in *amrB* expression for either C8395 or D0774 when grown in MH II Broth, LB medium, or SCFM. The aminoglycoside-resistant D0774 did however express significantly less *amrB* ($P < 0.01$ by unpaired Student's t-test) when grown in LB to an OD_{600} of 0.5 instead of 0.8 (Figure 19a). No significant differences were observed between C8395 grown in LB to an OD_{600} of 0.5 vs. 0.8. To confirm the observation of differential expression in another set of sequential isolates, *amrB* expression was evaluated by Q RT-PCR in the early, aminoglycoside-susceptible isolate from patient Bv3, D0072, and the late, aminoglycoside-resistant isolate, D2910, after growth in MH II Broth to an OD_{600} of 0.8: a 5.4 (standard error (SE) 0.8) fold increase was observed, although it was not significant by an unpaired Student's t-test ($P = 0.0645$), likely owing to the variability in expression (SE

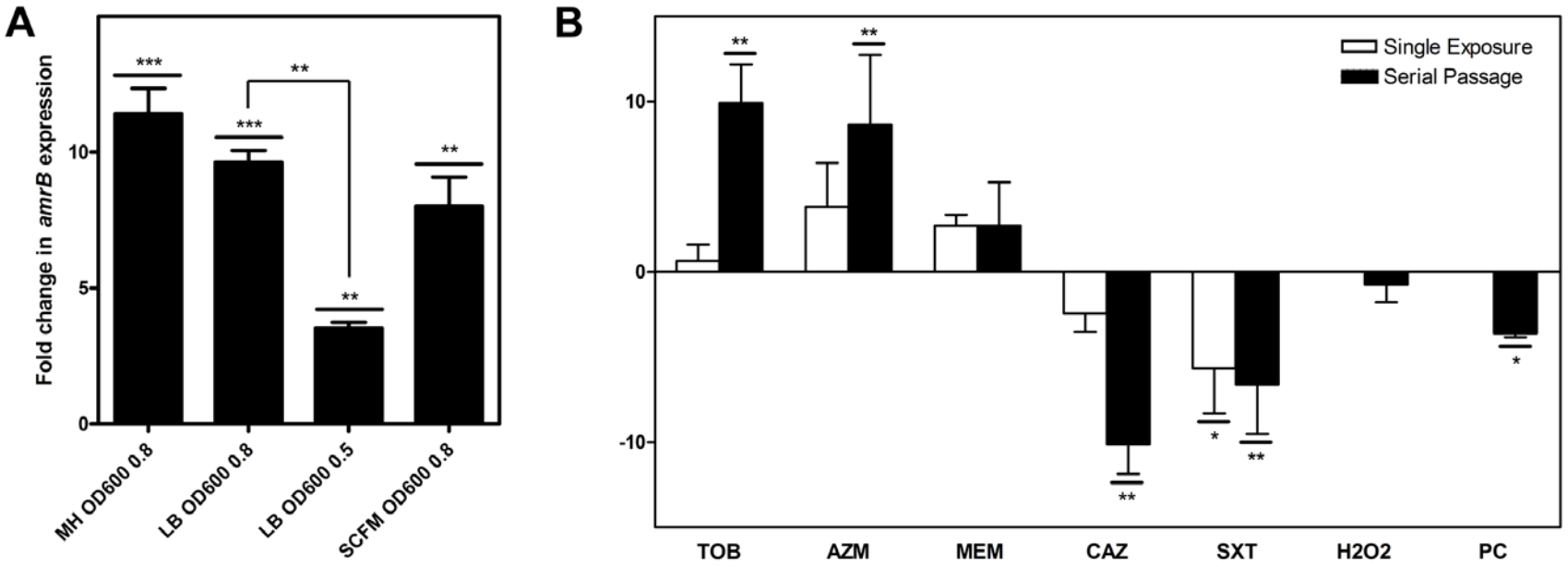

Figure 19. Expression of the putative RND transporter gene *amrB* in clinical CF and *in vitro* antibiotic or hydrogen peroxide exposed *B. vietnamiensis* isolates.

Expression was determined by real-time reverse transcription polymerase chain reaction and compared (A) between the early, aminoglycoside-susceptible isolate from cystic fibrosis patient Bv1, C8395, and the late, aminoglycoside-resistant D0774, in various types of media and stages of growth, and (B) between the early, aminoglycoside susceptible C8395 before and after its exposure to various antimicrobials, peroxide, or passage alone. The averages of three technical repeats were taken for each biological replicate. Fold change means were calculated by comparing the mean *amrB* expression in C8395 to each biological replicate of (A) D0774 or (B) condition. Data points represent the averages of three biological replicates ± standard errors. (A) **, P <0.01; *** P < 0.001 by unpaired Student's t-test. (B) *, P < 0.05; **, P <0.01 by Dunnett's Multiple Comparison Test after one-way analysis of variance. Abbreviations: RND, resistance-nodulation-division; MH, Mueller Hinton II Broth; LB, Luria-Bertani medium; SCFM, synthetic cystic fibrosis sputum medium; OD600, optical density at 600 nm; TOB, tobramycin; AZM, azithromycin; MEM, meropenem; CAZ, ceftazidime; SXT, trimethoprim/sulfamethoxazole; H2O2, hydrogen peroxide; PC, passage control.

0.9 cycles) between biological replicates of D0072 (data not shown). *B. vietnamiensis* J2315 was used as a quality control organism. J2315 expresses more *amrB* after a single exposure to chloramphenicol (235), which we found quantitatively to be 2.2 times more after growth in LB to an OD_{600} of 0.8 (standard deviation of 0.1, n = 2) and 1.5 times more after growth in LB to an OD_{600} of 0.3 (n = 1) (data not shown).

5.3.3.2 Serial exposure to aminoglycoside and macrolide antibiotics, but not to hydrogen peroxide, induces the expression of *amrB* in *B. vietnamiensis*

In *P. aeruginosa*, a single exposure to antibiotics that target the bacterial ribosome, such as aminoglycosides and macrolides, leads to the induction of *mexY* expression (328), and furthermore, oxidative stress in the form of hydrogen peroxide exposure induces *mexX* expression (329) (MexXY-OprM is the RND efflux system responsible for the extrusion of aminoglycosides in *P. aeruginosa*). After a single exposure of the early, aminoglycoside susceptible isolate from patient Bv1, C8395, to subinhibitory concentrations of tobramycin, azithromycin, meropenem, ceftazidime, or trimethoprim/sulfamethoxazole in MH II Broth, transcriptome analysis by Q RT-PCR did not reveal any significant expression increases in the putative RND transporter gene *amrB* by Dunnett's Multiple Comparison Post-Test, while exposure to trimethoprim/sulfamethoxazole resulted in a 5.7 ($P < 0.05$) fold decrease in *amrB* expression (Figure 19b). After exposure of C8395 to serially doubling concentrations of tobramycin from half the minimum inhibitory concentration (MIC) to 32 × the MIC (C8395TR) and azithromycin from a quarter of the MIC to 64 × the MIC (C8395AR), *amrB* expression was 9.9 ($P < 0.01$) and 8.6 times ($P < 0.01$) times higher, respectively, as determined by Dunnett's Multiple Comparison Post-Test (Figure 19b). Exposure of the

aminoglycoside-susceptible C8395 to serially doubling concentrations of meropenem did not change *amrB* expression, while exposure to serially doubling concentrations of ceftazidime and trimethoprim/sulfamethoxazole resulted in significant decreases in *amrB* expression, 10.1 ($P < 0.01$) and 6.6 ($P < 0.01$) times less, respectively (Figure 19b). After serial exposure of C8395 to hydrogen peroxide at half the MIC in MH II Broth (isolate C8395HP2), no significant change in *amrB* expression was observed by Dunnett's Multiple Comparison Post-Test (Figure 19b). Notably, the passage control isolate C8395PC expressed 3.6 ($P < 0.05$) times less *amrB* than C8395 (Figure 19b). The overall differences observed in *amrB* expression after exposure of the early, aminoglycoside susceptible isolate C8395 to various antimicrobials, peroxide, or by passage alone were significant ($P < 0.0001$) by one-way ANOVA.

5.3.4 Inhibition of a putative RND efflux system increases the susceptibility of *B. vietnamiensis* to aminoglycosides

To determine the involvement of the putative AmrAB-OprA 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 (421). In the presence of 20 µg/ml of MP 601384, aminoglycoside (amikacin, gentamicin, arbekacin, tobramycin) MICs for susceptible and resistant isolates decreased 2- to >32-fold (Table 17). The inhibitor had no consistent effects on the MICs of other antimicrobials (Table 17).

Table 17. Antimicrobial susceptibilities of *B. vietnamiensis* to aminoglycosides in the presence of a RND efflux pump inhibitor

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

[a] Patient identification numbers and bacterial isolation dates are noted in brackets for serial clinical isolates.

[b] Abbreviations: RND, resistance-nodulation-division; MIC, minimum inhibitory concentration; AMK, amikacin; GEN, gentamicin; ABK, arbekacin; TOB, tobramycin; LVX, levofloxacin; CIP, ciprofloxacin; TGC, tigecycline; MIN, minocycline.

5.1 Discussion

Efflux systems that accommodate aminoglycosides have been identified in a number of organisms, including *P. aeruginosa* and *B. pseudomallei,* and generally belong to the RND family (228, 319). In CF isolates of *P. aeruginosa*, the MexXY-OprM RND efflux system is the predominant mechanism of aminoglycoside resistance (228, 320-322), and rare aminoglycoside susceptibility in *B. pseudomallei* is attributed to the loss of expression of its major aminoglycoside-accommodating RND transporter, AmrB (323). In *B. cenocepacia*, deletion of genes encoding putative RND efflux systems results in aminoglycoside MIC decreases (239, 259, 318). We identified a putative aminoglycoside-accommodating RND system in *B. vietnamiensis* G4, the only *B. vietnamiensis* isolate sequenced to date, based on homology studies with the characterized systems from *P. aeruginosa* and *B. pseudomallei*, MexXY-OprM and AmrAB-OprA, respectively, and using structure and function predictive programs. After determining that clinical *B. vietnamiensis* isolates contained the operon homologous to *amrAB-OprA*, we showed that late, aminoglycoside-resistant CF isolates expressed more of the putative *amrB* transporter gene than early, aminoglycoside-susceptible CF isolates, irrespective of growth medium, with apparent transcriptomic differences in MH II Agar, LB, and SCFM, the latter mimicking nutritional conditions of the CF airways (331). Furthermore, aminoglycoside MICs for *B. vietnamiensis* decreased in the presence of an inhibitor, MP 601384, specific to MexXY-type efflux systems. This study therefore suggests that active efflux is involved in the observed decreased access of aminoglycosides to their antimicrobial target, and subsequently bacterial resistance, in *B. vietnamiensis*, and also shows that *B. vietnamiensis* RND pump expression can change during the course of a chronic CF infection, as previously reported in *P. aeruginosa* (321) and *B. cenocepacia* (178, 220).

Notably, transcriptome analysis revealed that *amrB* expression in *B. vietnamiensis* is dependent on growth phase; the late, aminoglycoside-resistant CF isolate D0774 expressed significantly more *amrB* when grown to an OD_{600} of 0.8 than when grown to an OD_{600} of 0.5. In *P. aeruginosa*, *mexXY-OprM* (328) and *mexAB-OprM* (422) expression is growth phase regulated, reaching a maximum at the onset of the stationary phase. Considering that at the start of our [^{3}H]gentamicin accumulation assays, D0774 was in mid-log phase, at an OD_{600} of 0.5, the growth dependent expression of *amrB* may be responsible for the initial observed drug accumulation during the first 2 hours of the assay.

We were unable to determine protein production levels of AmrB in *B. vietnamiensis*. To our knowledge, an anti-*Burkholderia* AmrB antibody has not yet been developed, and despite numerous attempts, we were unable to unambiguously detect AmrB in *B. vietnamiensis* using an anti-MexY antibody designed by Hocquet *et al.* (395) and generously provided by Dr. P. Plésiat (Université de Franche-Comté). Although sequence analysis suggested the antibody would be specific to *B. vietnamiensis* AmrB, we found a large amount of non-specific cross-reactivity to *B. vietnamiensis* protein preps (data not shown). *P. aeruginosa* ATCC 27853 exposed to gentamicin was used as a positive control (395).

We were also unable to generate an *amrB* deletion mutant in the aminoglycoside-resistant CF isolate D0774, to provide the strongest evidence that the putative transporter is involved in acquired aminoglycoside resistance in *B. vietnamiensis.* The use of Koch's Molecular Postulates, i.e. the inactivation/deletion of a gene resulting in the loss of function and the subsequent restoration of function upon reintroduction of the gene, is the classical method for determining the role of a bacterial gene (419). Manipulating the BCC genome, particularly in clinical isolates, is notoriously difficult, and previous studies, including those

deleting the putative *amrAB-OprA* operon in *B. cenocepacia* (423), have had to adopt non-conventional methods to do so. In our study specifically, the suicide plasmid pEX18Tc containing 500 base pair cloned fragments of either end of *amrB* ligated together was unsuccessfully mobilized into D0774, and G4 as a control, via tri-parental mating. No single-cross over mutants were obtained after multiple attempts. Subsequent experiments will involve modifications in this protocol, and/or a modified method for the construction of gene deletions in the BCC described by Flannagan *et al*. (424) and based on the endonuclease I-SceI. This method has recently been used to delete the putative *amrAB-OprA* operon in *B. cenocepacia* (237, 239, 318).

Although not the major determinant of resistance to these antimicrobials, in *P. aeruginosa,* MexXY-OprM has also been implicated in observed reduced susceptiblity to β-lactams, fluoroquinolones and tetracyclines (228, 310). Moreover, in *B. pseudomallei*, AmrAB-OprA can also accommodate macrolides (413). It is reasonable therefore to speculate that *B. vietnamiensis* AmrAB-OprA has the potential to contribute to resistance to other classes of antimicrobials. Indeed, for late, aminoglycoside-resistant CF isolates the azithromycin (a macrolide antibiotic), ciprofloxacin (a fluoroquinolone antibiotic), and meropenem and ceftazidime (β-lactam antibiotics) MICs were higher than those for early, aminoglycoside-susceptible CF isolates. Fluoroquinolones and tetracycline antibiotics specifically however, are not likely substrates of the putative AmrB transporter in *B. vietnamiensis,* since no general change in levofloxacin, ciprofloxacin, tigecycline, and minocycline MIC was observed for after efflux inhibition with MP 601384.

In *P. aeruginosa,* a single exposure to subinhibitory concentrations of antibiotics that target the bacterial ribosome, such as aminoglycosides and macrolides, but not to antibiotics

that act on other cellular targets, induces *mexY* expression (328). Of the antimicrobials tested, no single antibiotic exposure induced expression of the putative *amrB* transporter gene in an aminoglycoside-susceptible *B. vietnamiensis* isolate. However, consistent with the observations published in *P. aeruginosa*, exposure to serially doubling concentrations of tobramycin and azithromycin, but not to meropenem, ceftazidime, or trimethoprim/sulfamethoxazole, resulted in increased *amrB* expression in the early isolate C8395, notably, to levels equivalent to those observed in its sequential isolate, the late, aminoglycoside-resistant isolate D0774. The observed increases in *amrB* expression coincide with the acquisition of aminoglycoside resistance in the *in vitro* generated isolates C8395TR and C8395AR, after serial exposure to tobramycin and azithromycin, respectively, as well as the reduced uptake of [^{3}H]gentamicin in C8395TR. These findings suggest that *in vitro* acquisition of aminoglycoside resistance after exposure to tobramycin or azithromycin is at least in part a result of increased expression of the putative *amrB* transporter gene, and therefore the presence of these antimicrobials at the site of infection could promote efflux gene expression in *B. vietnamiensis*, rendering it resistant to aminoglycosides by reducing the intracellular drug concentration. These findings also emphasize the importance of experimental design; the efflux-inducing capacity of an agent could be missed if only single bacterial exposures are performed. Moreover, serial exposures of bacteria to antibiotics are more physiologically relevant to CF lung disease than a single exposure, since antibiotics are administered chronically (28, 130). Indeed, and as an example, aminoglycoside resistance levels and *amrB* expression were higher when *B. vietnamiensis* C8395 was exposed serially to doubling concentrations of azithromycin than when it was exposed to a subinhibitory concentration of the drug a single time. In future studies, it would also be worth evaluating

bacterial properties after serial exposure to subinhibitory antibiotic concentrations. Notably, when Sass *et al.* (391) serially exposed *B. cenocepacia* to amikacin at 2 × the MIC, they did not find any changes in RND efflux gene expression, despite a reported increase in aminoglycoside MICs. Their study isolate however was already highly resistant to aminoglycosides at the onset of the study, prior to drug exposure, and therefore the expression of any RND efflux systems involved in this resistance may already have been maximal.

Serial exposure to hydrogen peroxide at half the MIC induces *mexX* expression in *P. aeruginosa* (329). Such exposure to peroxide did not result in increased expression of the putative *amrB* transporter gene in *B. vietnamiensis*, despite its induction of aminoglycoside resistance and the reduced uptake of [^{3}H]gentamicin in the generated isolate, C8395HP2. Peeters *et al.* (406) report similar findings; no changes in RND efflux gene expression were observed after exposure of *B. cenocepacia* to hydrogen peroxide, although compared with our study the peroxide concentration used was higher and exposure time shorter, and drug susceptibility was not reported, making comparisons difficult. These findings suggest that oxidative stress in the CF airways could select for an aminoglycoside-resistant phenotype of *B. vietnamiensis* that is not dependent on the activation of this particular RND efflux system, and therefore also imply that other aminoglycoside resistance determinants exist in *B. vietnamiensis*. Likewise, overexpression of *amrB* was not sufficient to cause azithromycin, meropenem, ceftazidime, trimethoprim/sulfamethoxazole, or ciprofloxacin MIC increases; no correlation was observed between susceptibility to these antimicrobials and *amrB* expression following the *in vitro* passage of C8395 under antibiotic or oxidative stress, further supporting the notion that non-aminoglycoside antibiotics are not substrates for *B.*

vietnamiensis AmrAB-OprA. The observed acquisition of aminoglycoside resistance and reduced accumulation of [^{3}H]gentamicin independent of AmrAB-OprA in *B. vietnamiensis* may result from impermeability-type resistance, as discussed in Chapter 4.

B. vietnamiensis upregulation of the putative RND transporter gene *amrB* in response to agents that directly target the bacterial ribosome suggests that this is a bacterial response to the subsequent downstream effects that result from this interaction and not to antibiotics *per se*. Bacterial multidrug efflux pumps are known to have other functions (425, 426). Aminoglycoside resistance through upregulation of the MexXY-OprM efflux system in *P. aeruginosa* specifically, is thought to result indirectly from bacterial stress responses to aberrant hybrid proteins generated from errors in translation (328, 426, 427) or the oxidation of polypeptides (329). Indeed, drug-accommodating pumps are known to function for toxic waste disposal (428). Moreover, the decreased expression of *amrB* following exposure to ceftazidime and trimethoprim/sulfamethoxazole, as well as after passage alone, implies its regulation in response to general environmental adaptations. However, the elimination of mistranslation products generated by tobramycin, and the associated acquisition of aminoglycoside resistance, can also result from efflux-independent mechanisms, specifically though membrane protease activity (329, 429).

Notably, our evaluation of efflux involvement in acquired aminoglycoside resistance in *B. vietnamiensis* was limited to one RND transporter, the homologue of *P. aeruginosa* MexXY-OprM and *B. pseudomallei* AmrAB-OprA, and it is conceivable that other RND transporters could contribute to antimicrobial resistance in this organism. The RND family of transporters are the major aminoglycoside-accommodating pumps (228), and we identified several putative RND drug transporters in *B. vietnamiensis*, including a homologue of *B.*

pseudomallei Bpe, a transporter the role of which is currently controversial because it has been shown to be involved in aminoglycoside efflux in one strain (430) but not in another (431). Deletion of the putative *bpeAB-OprB* operon in *B. cenocepacia* however, does result in a 2- to 4-fold decrease in aminoglycoside MICs (318). Furthermore, other potential mechanisms of aminoglycoside resistance, such as ribosomal modification which has recently been shown to be involved in aminoglycoside resistance in clinical isolates of *P. aeruginosa* (310), were beyond the scope of this thesis, although certainly may be involved in acquired aminoglycoside resistance in *B. vietnamiensis*, and should be investigated in future studies. The contribution of different resistance determinants to aminoglycoside inefficacy may explain the observed differences in the level of acquired resistance among *B. vietnamiensis* isolates.

Elucidation of factors involved in drug resistance in *B. vietnamiensis* may aid in the design of improved antimicrobial therapeutic regimens against infections with strains from the *Burkholderia* genus. Indeed, by establishing a link between the putative RND efflux system amrAB-OprA and aminoglycoside resistance in *B. vietnamiensis*, it is already evident that natural or synthetic efflux pump inhibitors may be useful in treating infections caused by this organism. The combinational use of efflux pump inhibitors with antibiotics is expected to increase the activity of antimicrobials that are substrates of pumps, owing to an increase in their intracellular concentration, and reduce the emergence of acquired antimicrobial resistance (319, 432, 433).

Chapter 6: CONCLUSIONS AND FUTURE DIRECTIONS

Burkholderia cepacia complex (BCC) species are highly virulent opportunistic pathogens, most notably in persons with chronic granulomatous or cystic fibrosis (CF), and are difficult to eradicate *in vivo* in part because they are intrinsically resistant to most available antibiotics (5, 130). **Major thesis findings:** Based on minimum inhibitory concentrations and established breakpoints (232), we found that one species within the BCC, *B. vietnamiensis*, is more often susceptible to carbapenems and aminoglycosides than the others, the latter class of antibiotics being widely used (27) and particularly important in the management of CF (28, 29). Furthermore, *B. vietnamiensis* strains acquired aminoglycoside resistance during chronic infection in CF patients, and *in vitro* under tobramycin, azithromycin, and hydrogen peroxide pressure. Notably, *B. vietnamiensis* is able to persist in broth containing gentamicin and tobramycin at concentrations up to 8 × the MIC. Active efflux via a resistance-nodulation-division (RND) efflux system, not lipopolysaccharide characteristics, is responsible for decreased cellular drug accumulation in clinical CF *B. vietnamiensis* strains that have acquired aminoglycoside resistance, and in those exposed to tobramycin and azithromycin, but not hydrogen peroxide, *in vitro*.

Antibiotic resistance is a major threat to public health, and tackling this problem will depend on increasing our knowledge of resistance prevalence and bacterial factors involved, as well as increased government involvement and laboratory support, public and professional education, and preventative measures (1, 30-33). It is hoped that our novel insights will help in the design of improved antimicrobial therapeutic regimens against *B. vietnamiensis* infections and the re-evaluation of the use of this organism in bioremediation and plant growth promoting processes. Indeed, a better understanding of how bacteria resist

aminoglycoside treatment has resulted in the pharmaceutical development of efflux pump inhibitors (319, 432) and liposome-encapsulated aminoglycosides (434) for example, each of which has been shown to have inhibitory activity against BCC isolates (245, 421, 435, 436) (our study). Efflux pump inhibitors, specifically, are not yet available clinically as they have their shortcomings - target specificity is a challenge for example (319). However, as discussed in Chapter 5, their use in combination with antibiotics is promising as a novel therapeutic strategy.

There are some limitations of our study. Because we were unable to generate a *B. vietnamiensis* mutant lacking a putative RND transporter, our study lacks irrefutable evidence of efflux involvement in acquired aminoglycoside resistance in *B. vietnamiensis*. Furthermore, we did not investigate other biochemical mechanisms of aminoglycoside resistance in *B. vietnamiensis*, such as target modification, enzymatic inactivation, or membrane protease involvement (27, 429), or the acquired resistance to non-aminoglycoside antibiotics, as these topics were beyond the scope of the thesis. Despite the administration of aggressive antibiotic treatment regimens guided by susceptibility testing, eradication of BCC strains is often not achieved (130). Antibiotic treatment of BCC strains is thought to fail because of factors independent of bacterial susceptibility as well, such as inadequate antibiotic concentrations at the site of infections, inactivation of the antibiotic in sputum, impaired host defenses, *in vivo* growth rate of the organisms, and bacterial biofilm formation, none of which was addressed in this thesis (25, 197). Moreover, treatment of BCC-infected patients is often based on combination therapy, with two or three antibiotics showing synergistic activity (123, 300, 308).

Future studies should further examine the role of efflux in *B. vietnamiensis*. Bacterial RND efflux pumps have physiological roles apart from conferring drug resistance that are relevant in bacterial pathogenicity (425, 426), and mutations in the repressor of these efflux systems are often responsible for their enhanced expression (310). Future studies should also address the limitations discussed above, focus on the regulation of resistance determinants, and extend the study to all members of the BCC.

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