Investigation of aminoglycoside resistance inducing conditions and a putative AmrAB-OprM efflux system in *Burkholderia vietnamiensis*

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**Abstract**

**Background:** *Burkholderia cepacia* complex (BCC) bacteria are highly virulent, typically multidrug-resistant, opportunistic pathogens in cystic fibrosis (CF) patients and other immunocompromised individuals. *B. vietnamiensis* is more often susceptible to aminoglycosides than other BCC species, and strains acquire aminoglycoside resistance during chronic CF infection and under tobramycin and azithromycin exposure *in vitro*, apparently from gain of antimicrobial efflux as determined through pump inhibition. The aims of the present study were to determine if oxidative stress could also induce aminoglycoside resistance and provide further observations in support of a role for antimicrobial efflux in aminoglycoside resistance in *B. vietnamiensis*.

**Findings:** Here we identified hydrogen peroxide as an additional aminoglycoside resistance inducing agent in *B. vietnamiensis*. After antibiotic and hydrogen peroxide exposure, isolates accumulated significantly less [³H] gentamicin than the susceptible isolate from which they were derived. Strains that acquired aminoglycoside resistance during infection and after exposure to tobramycin or azithromycin overexpressed a putative resistance-nodulation-division (RND) transporter gene, *amrB*. Missense mutations in the repressor of *amrB*, *amrR*, were identified in isolates that acquired resistance during infection, and not in those generated *in vitro*.

**Conclusions:** These data identify oxidative stress as an inducer of aminoglycoside resistance in *B. vietnamiensis* and further suggest that active efflux via a RND efflux system 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*. Furthermore, the repressor AmrR is likely just one regulator of the putative AmrAB-OprM efflux system in *B. vietnamiensis*.

**Keywords:** *Burkholderia vietnamiensis*, Aminoglycoside, Azithromycin, Hydrogen peroxide, Efflux, AmrB, AmrR

**Findings**

Members of the *Burkholderia cepacia* complex (BCC) can cause severe respiratory infections in individuals with cystic fibrosis (CF) [1]. Furthermore, many strains are highly and intrinsically resistant to various antimicrobials, including aminoglycosides [2], ribosome-targeting antibiotics important in the treatment of CF respiratory disease [3].

*B. cenocepacia* studies suggest that resistance-nodulation-division (RND) efflux systems are involved in BCC resistance to aminoglycosides [4-6]. The MexXY-OprM RND pump is the predominant determinant of aminoglycoside resistance in CF isolates of *Pseudomonas aeruginosa* [7], and aminoglycoside susceptibility in *B. pseudomallei* results from loss of AmrAB-OprA [8]. At subinhibitory concentrations, ribosome-targeting antibiotics and oxidative stress induce *mexXY* expression [9,10]. *mexXY* is under the control of the MexZ repressor [11], and *mexZ* mutations are common in pan-aminoglycoside resistant isolates [12].

We previously reported that *B. vietnamiensis* isolates are often aminoglycoside-susceptible and strains acquire resistance during chronic CF infection and under tobramycin exposure as determined through pump inhibition. The aims of the present study were to determine if oxidative stress could also induce aminoglycoside resistance and provide further observations in support of a role for antimicrobial efflux in aminoglycoside resistance in *B. vietnamiensis*. Here we identified hydrogen peroxide as an additional aminoglycoside resistance inducing agent in *B. vietnamiensis*. After antibiotic and hydrogen peroxide exposure, isolates accumulated significantly less [³H] gentamicin than the susceptible isolate from which they were derived. Strains that acquired aminoglycoside resistance during infection and after exposure to tobramycin or azithromycin overexpressed a putative resistance-nodulation-division (RND) transporter gene, *amrB*. Missense mutations in the repressor of *amrB*, *amrR*, were identified in isolates that acquired resistance during infection, and not in those generated *in vitro*. Furthermore, the repressor AmrR is likely just one regulator of the putative AmrAB-OprM efflux system in *B. vietnamiensis*. These data identify oxidative stress as an inducer of aminoglycoside resistance in *B. vietnamiensis* and further suggest that active efflux via a RND efflux system 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*. Furthermore, the repressor AmrR is likely just one regulator of the putative AmrAB-OprM efflux system in *B. vietnamiensis*.

**Keywords:** *Burkholderia vietnamiensis*, Aminoglycoside, Azithromycin, Hydrogen peroxide, Efflux, AmrB, AmrR

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and azithromycin pressure in vitro [13]. Decreased access of aminoglycosides to their target resulted from apparent gain of antimicrobial efflux via a RND pump, the latter determined with an inhibitor [13].

**B. vietnamiensis develops aminoglycoside resistance under hydrogen peroxide pressure in vitro**

Aminoglycoside resistance can be induced in susceptible CF isolates of *B. vietnamiensis* following serial exposure to tobramycin (Table 1: C8395TE, D0072TE) or a single exposure to subinhibitory concentrations of azithromycin [13]. To characterize resistance inducing antimicrobial pressures further, after serial passage in cation-adjusted Mueller-Hinton broth (CAMHB) containing azithromycin, meropenem, ceftazidime, and co-trimoxazole at doubling concentrations as described previously [13] the drug susceptibility of C8395 was evaluated. Triplicate minimum inhibitory concentrations (MICs) were determined using broth microdilution methods [14], and their stability confirmed after 20 passages on antibiotic-free media. *P. aeruginosa* and non-Enterobacteriaceae breakpoints were used in the absence of *B. cepacia* breakpoints. Only serial exposure of C8395 to azithromycin resulted in notable (≥4 fold) increases in aminoglycoside MICs (Table 1). By previously described methods [9], but with selective agar containing tobramycin at 2.5 times the MIC, serial exposure of C8395 to hydrogen peroxide at half the MIC resulted in a 16-fold stable increase in aminoglycoside MIC for C8395PE (Table 1). Other acquired resistance was also observed: after passage with all antimicrobials the MICs of the respective agents against C8395 increased greatly (Table 1: C8395AE, C8395ME, C8395CE, and C8395SE), and some other cross-resistance, most notably between the β-lactams antibiotics, was also seen.

Hydrogen peroxide is, therefore, an additional inducer of aminoglycoside resistance in *B. vietnamiensis in vitro*, a particularly important finding because CF airways are rich in reactive oxygen species [15]. Moreover, *B. vietnamiensis* can acquire resistance after exposure to other antimicrobials used in treating BCC-infected CF patients, namely meropenem, ceftazidime, and co-trimoxazole [16].

The aminoglycoside-resistant derived isolates C8395TE and C8395PE accumulated 2.65 and 3.50 times less [3H] gentamicin than C8395, respectively (P = 0.0118, one-way ANOVA) (data not shown). Accumulation was determined in triplicate in Luria-Bertani (LB) medium as previously used to show the late, aminoglycoside-resistant isolate D0774 accumulates less gentamicin than C8395 [13]. There were no significant differences in the CFU/ml between C8395 and the comparison isolates at starting time (data not shown). Decreased access of aminoglycosides to their intracellular target is, therefore, responsible for the observed in vitro antibiotic and oxidative stress-induced resistance.

**Analysis of putative efflux system genes in clinical and in vitro stress exposed *B. vietnamiensis* isolates**

Of the 11 putative RND transporters that the sequenced environmental *B. vietnamiensis* isolate G4 (accession

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**Table 1 Antimicrobial susceptibilities of *B. vietnamiensis* after serial exposure to antibiotics or hydrogen peroxide**

| Isolate* | AMK (μg/ml) | GEN | KAN | TOB | AZM | MEM | CAZ | SXT | CIP |
|----------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Clinical CF | | | | | | | | | |
| 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 | | | | | | | | | |
| C8395TE (TOB) | >128 | >128 | 128 | >128 | 64 | 1 | 4 | 4/20 | 4 |
| C8395AE (AZM) | 32 | 16 | 16 | 16 | 2048 | 2 | 16 | 8/40 | 4 |
| C8395ME (MEM) | 16 | 8 | 8 | 8 | 32 | 16 | 64 | 4/20 | 16 |
| C8395CE (CAZ) | 8 | 8 | 4 | 4 | 32 | 8 | 16 | 2/10 | 16 |
| C8395SE (SXT) | 8 | 8 | 2 | 2 | 32 | 0.5 | 4 | >64/320 | 8 |
| C8395PE (peroxide) | 32 | 64 | 32 | 32 | 32 | 4 | 16 | 4/20 | 4 |
| C8395PC (control) | 8 | 8 | 4 | 8 | 32 | 4 | 1 | 1/5 | 1 |
| D0072TE (TOB) | 32 | 32 | 16 | 16 | >32 | 1 | 2 | 2/10 | 1 |

*Patient identification numbers and bacterial isolation dates are noted in brackets. Abbreviations: TE, TOB exposed; AE, AZM exposed; ME, MEM exposed; CE, CAZ exposed; SE, SXT exposed; PE, hydrogen peroxide exposed; PC, passage control.

*AMK, aminoglycoside and azithromycin MICs for C8395 and D0774, and tobramycin and azithromycin MICs for D0072, D2910, C8395TE, and D0072TE were previously published to some extent [13] and are shown here for comparison. MICs represent susceptibility after 3 passages on antibiotic-free media. Abbreviations: AMK, amikacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin; AZM, azithromycin; MEM, meropenem; CAZ, ceftazidime; SXT, co-trimoxazole; CIP, ciprofloxacin.*
NC_009256.1) contains (determined as previously [17]), following sequence alignment only Bcep1808_1575 showed high identity, 71%, 85%, and 92%, with the characterized transportersMexY (accession NC_008463.1) and AmrB (accession NC_007434.1), and their homologue BCAL1675 in B. cenocepacia (accession NC_011000.1), respectively. These transporters are part of an operon also encoding a repressor, membrane fusion protein, and outer membrane channel [7]. PCR product analysis revealed that B. vietnamiensis clinical isolates C8395, D0774, D0072, and D2910 contained these efflux system genes in the same order (data not shown).

To evaluate the expression of RND pump genes in B. vietnamiensis, triplicate overnight cultures were diluted 1:100 into CAMHB, LB medium, or synthetic cystic fibrosis sputum medium (SCFM) [18] with or without 1:100 into CAMHB, LB medium, or synthetic cystic fibrosis sputum medium (SCFM) [18] with or without 1:100 into CAMHB, LB medium, or synthetic cystic fibrosis sputum medium (SCFM) [18].

AmrB (accession NC_009256.1) contains (determined as previously [17]), the same order (data not shown). Compared with C8395, D0774 also overexpressed amrR (P < 0.01) (Figure 1A). In another set of se- quential isolates, the late, aminoglycoside-resistant isolate C8395 and D0774 expressed 11.4-, 9.6-, and 8.0-fold more amrR, owing to D0774 expressing less amrB (P < 0.01) (Figure 1A). amrB overexpression was not sufficient to cause resistance to non-aminoglycoside antibiotics, supporting the notion that they are not substrates for the putative AmrAB-OprA efflux system [13]. Lastly, there was no association between amrR expression and aminoglycoside resistance or amrB expression, as is also true for mexZ [20].

To determine if mutations in amrR were responsible for the observed overexpression of amrB in B. vietnamiensis, sequences of the putative repressor were examined. DNA isolation, PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, USA) with specific primers (5′-TTCAAAGGAGGTGCGCCAGGA-3′, 5′-CCGAAACGACACCAGATAGACG-3′), and 16S (for normalization) (5′-CAGCGTTCGGCAGG-3′), and product ana- lysis by agarose gel electrophoresis were done using standard protocols [21]. PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega) and cloned into One Shot TOP10 E. coli cells with a Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Plasmid DNA was isolated using a QIAprep Miniprep Kit (Qiagen), and M13 primers amplified amrR. Resultant products were sequenced at the UBC Centre for Molecular Medicine and Therapeutics.

C8395 and D0774 amrR differed from that of G4 by two silent mutations (data not shown). The late, aminoglycoside-resistant isolate D0774 also contained a substitution at position 425 (T → C), that at residue 142 of the protein, in the suggested ligand binding alpha helix region [22], replaces a leucine with a proline. D0072 and D2910 amrR sequences also differed: at position 156, or amino acid residue 52 amid the predicted DNA and
C-terminal ligand binding domains [22], there was a ~2000 bp insertion in the late, aminoglycoside-resistant D2910. Only silent mutations were observed in amrR among C8395, C8395TE, C8395AE, C8395PE, and C8395PC (data not shown).

The amrR mutations identified likely influenced the expression of the putative B. vietnamiensis amrB transporter gene. The change in D0774 AmrR may indirectly affect DNA binding to the transcription factor [22], while the large insertion within D2910 amrR likely inactivates the repressor altogether. As per the in vitro derived isolate findings, aminoglycoside-resistant P. aeruginosa isolates overexpressing mexXY without mutations in mexZ also exist [23,24].

In conclusion, in B. vietnamiensis, oxidative stress can induce aminoglycoside resistance, while active efflux via the putative AmrAB-OprM efflux system is likely involved in clinical and in vitro antimicrobial-induced...
aminoglycoside resistance. Such elucidation of resistance inducing conditions and resistance factors may improve therapeutic regimes against infection with this species. Additional mechanisms of aminoglycoside resistance should be investigated next. The contribution of resistance determinants to aminoglycoside inefficacy may explain the observed varied degrees of resistance.

Availability of supporting data
The data supporting the results of this study is included within the article.

Abbreviations
BCC: Burkholderia cepacia complex; CF: Cystic fibrosis; RND: Resistance-nodulation-division; CAMHB: Cation-adjusted Mueller-Hinton broth; MIC: Minimum inhibitory concentration; LB: Luria-Bertani; SCFM: Synthetic cystic fibrosis sputum medium; OD$_{600}$: Optical density at 600 nm.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ANJ designed the study, performed and analyzed experiments not done by CMF, and wrote the manuscript. CMF serially exposed C8395 to hydrogen peroxide and performed most of the susceptibility and expression tests. DPS participated in the design and coordination of the study and critically reviewed the manuscript. All authors read and approved the final manuscript.

Authors' information
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