*J Antimicrob Chemother* 2014; **69**: 2690–2698
doi:10.1093/jac/dku192 Advance Access publication 10 June 2014

Journal of Antimicrobial Chemotherapy

# Mechanisms of reduced susceptibility and genotypic prediction of antibiotic resistance in *Prevotella* isolated from cystic fibrosis (CF) and non-CF patients

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*Received 12 March 2014; returned 2 April 2014; revised 1 May 2014; accepted 2 May 2014*

**Objectives:** To investigate mechanisms of reduced susceptibility to commonly used antibiotics in *Prevotella* cultured from patients with cystic fibrosis (CF), patients with invasive infection and healthy control subjects and to determine whether genotype can be used to predict phenotypic resistance.

**Methods:** The susceptibility of 157 *Prevotella* isolates to seven antibiotics was compared, with detection of resistance genes (*cfxA*-type gene, *ermF* and *tetQ*), mutations within the CfxA-type β-lactamase and expression of efflux pumps.

**Results:** *Prevotella* isolates positive for a *cfxA*-type gene had higher MICs of amoxicillin and ceftazidime compared with isolates negative for this gene ($P<0.001$). A mutation within the CfxA-type β-lactamase (Y239D) was associated with ceftazidime resistance ($P=0.011$). The UK CF isolates were 5.3-fold, 2.7-fold and 5.7-fold more likely to harbour *ermF* compared with the US CF, UK invasive and UK healthy control isolates, respectively. Higher concentrations of azithromycin ($P<0.001$) and clindamycin ($P<0.001$) were also required to inhibit the growth of the *ermF*-positive isolates compared with *ermF*-negative isolates. Furthermore, *tetQ*-positive *Prevotella* isolates had higher MICs of tetracycline ($P=0.001$) and doxycycline ($P<0.001$) compared with *tetQ*-negative isolates. *Prevotella* spp. were also shown, for the first time, to express resistance nodulation division (RND)-type efflux pumps.

**Conclusions:** This study has demonstrated that *Prevotella* isolated from various sources harbour a common pool of resistance genes and possess RND-type efflux pumps, which may contribute to tetracycline resistance. The findings indicate that antibiotic resistance is common in *Prevotella* spp., but the genotypic traits investigated do not reflect phenotypic antibiotic resistance in every instance.

**Keywords:** resistance genes, β-lactamases, efflux pumps

## Introduction

*Prevotella* spp. are opportunistic Gram-negative obligate anaerobic pathogens that are associated with polymicrobial infections in a multitude of sites throughout the body, including the respiratory tract.[1] Although the role of these bacteria in the pathogenesis of chronic pulmonary infection is unclear, this genus has been repeatedly detected as a member of the respiratory microbiota in patients with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) or bronchiectasis using both culture and molecular methods.[2–9] Antibiotics routinely used in the management of these chronic lung infections such as β-lactam, macrolide/lincosamide/streptogramin B (MLS) and tetracycline antibiotics have activity against these anaerobic bacteria. However, a recent

study by our group detected resistance to antibiotics in each of these classes among *Prevotella* spp. from a range of sources.[10]

Antimicrobial susceptibility testing of *Prevotella* spp. takes several days to complete and the delay in reporting the susceptibility results may have negative clinical implications for the patient. Therefore, it is essential to better understand the mechanisms underlying resistance in *Prevotella* isolates and to determine whether the genotype can be used to predict phenotypic resistance in this genus. These data may enable clinicians to more rapidly select the most appropriate antibiotic if *Prevotella* spp. are to be treated as part of a polymicrobial infection such as that present in chronic pulmonary disease.

Three putative antibiotic resistance genes have been previously detected in *Prevotella* spp. cultured from non-CF patients: (i) a *cfxA*-type gene encoding a β-lactamase, which hydrolyses the β-lactam bond in some penicillin and cephalosporin antibiotics; (ii) *ermF*, encoding a methylase, which alters the binding site of MLS antibiotics; and (iii) *tetQ*, encoding a ribosomal protection protein, which prevents the action of tetracyclines.[11,12] It is also recognized that mutations within β-lactamase-encoding genes may extend the enzyme's spectrum of activity;[13] however, it has not yet been determined whether similar mutations exist within CfxA-type β-lactamases in *Prevotella* spp. Furthermore, bacterial efflux may contribute to resistance to multiple structurally diverse antimicrobial agents.[14] Efflux pumps associated with clinically significant resistance in Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Bacteroides fragilis*, belong to the resistance nodulation division (RND) family.[14,15] The type of efflux pump expressed by *Prevotella* spp. is currently unknown.

In this study we investigated mechanisms of resistance to β-lactam, MLS and tetracycline antibiotics in a large number of clinical *Prevotella* isolates, including for the first time CF-associated *Prevotella*. We also determined whether the detection of specific genes, mutations within a β-lactamase-encoding gene and the expression of efflux pumps could predict phenotypic resistance.

## Materials and methods

### *Clinical Prevotella isolates*

The *Prevotella* isolates ($n=157$) analysed in this study have been previously described in detail.[10] Briefly, the isolates were split into four groups depending on their source (UK CF, $n=57$; US CF, $n=23$; UK invasive, $n=50$; and UK healthy controls, $n=27$). The CF isolates were cultured from adult (≥18 years) CF patients attending the adult CF clinic, Belfast, UK (sputum, $n=53$; and plaque, $n=4$) or paediatric (<18 years) CF patients attending the CF clinic, University of North Carolina at Chapel Hill, USA (sputum, $n=9$; and bronchoalveolar lavage, $n=14$). The UK invasive isolates were cultured from non-CF patients (of unknown age) with various infections including brain abscesses, kidney ulcers and infections identified on blood culture ($n=50$) and were kindly provided Dr Valerie Hall, Anaerobe Reference Laboratory, Cardiff, UK. The remaining isolates were from UK adult (≥18 years) healthy control subjects (induced sputum, $n=27$). Isolates were identified by 16S rRNA sequencing (Table S1, available as Supplementary data at *JAC* Online). Where available, information was obtained on azithromycin prescription (currently prescribed or not currently prescribed) at the time of sample collection.

**Table 1.** Primers used for PCR, qRT-PCR and sequencing experiments

| Target gene | Primer sequence (5′–3′) | Amplicon size (bp) | Reference |
|---|---|---|---|
| Antibiotic resistance genes | | | |
| *cfxA*-type gene | AGCTGCTATCCTATCTACACC, CCACACTCATTCCTCGTTC | 214 | this study |
| *ermF* | CGGGTCAGCACTTTACTATTG, GGACCTACCTCATAGACAAG | 466 | 16 |
| *tetQ* | CATGGATCAGCAATGTTCAATATCGG, CCTGGATCCACAATGTATTCAGAGCGG | 460 | 17 |
| Amplification and sequencing of the full-length *cfxA*-type gene | | | |
| *cfxA*-type gene | GAAAAAAACAGAAAAAAACAAATC, TTAAGATTTTACTGAAGTTTG | 966 | 11 |
| 5′ end of gene and 3′ end of gene | ACGCAGCAAATCTCTCACTG, TACGGAAGAGGAAATGTCGG | used for sequencing only | this study |
| Species-specific efflux pumps (PCR and/or qRT-PCR) | | | |
| *P. melaninogenica* pump 1 (PCR and qRT-PCR) | GGTGGTAGCGGTCAGATTGT, TCGCCCATTTTAAGACCACT | 200 | this study |
| *P. melaninogenica* pump 2 (PCR and qRT-PCR) | CATCCAGAACGCTTTTCACA, TGTTGGCATTGCTAAAGACG | 151 | this study |
| *P. salivae* pump 1 (PCR) | CTCCTTGGCTGACGCTAAAG, GACTTATCAGGCAGCCGTTC | 211 | this study |
| *P. salivae* pump 2 (PCR) | CTTTTCCGCATTTCGATTGT, CATTGAGCTTCGCTTTAGCC | 157 | this study |
| *P. salivae* pump 1 (qRT-PCR) | TCCCATCATTCTGTGGGTTT, ATCCCGAACGCATATTGAAG | 186 | this study |
| *P. salivae* pump 2 (qRT-PCR) | ACAGGCCACGTATGAAGAGG, TCATGCACTTTCTGCACTCC | 174 | this study |
| *P. histicola* pump 1 (PCR and qRT-PCR) | GGCTCAAGCAAAGGCTAATG, CTTGTCGAACACCTGCTTCA | 248 | this study |
| *P. histicola* pump 2 (PCR and qRT-PCR) | TAAGGGAAAGACGCTTGTGC, TTGTTGGTGCTGACGAAGAG | 192 | this study |
| *P. nigrescens* pump 1 (PCR and qRT-PCR) | GCCCTAAACTCTGCACAAGC, CCTGAATGGGCAGTTTGAAT | 179 | this study |
| *P. denticola* pump 1 (PCR and qRT-PCR) | GATTACCGTAGACCCGCAGA, TTTCTCCGATTTCCTGATGG | 166 | this study |
| 16S rRNA reference gene (qRT-PCR) | TAAGCATCCCACCTGGGGAG, GCTGACGACAACCATGCAGC | 197 | this study |

### PCRs

Genomic DNA extraction was performed using a ZR Fecal DNA Kit™ (Zymo Research, Cambridge Biosciences, UK) in accordance with the manufacturer's instructions. Primer pairs for PCR assays are described elsewhere[11,16,17] or were designed in Primer3 software (Table 1) and were purchased from Eurofins MWG Operon, UK. Each PCR was performed using the JumpStart™ *Taq* DNA Polymerase Kit with 10× reaction buffer without $MgCl_2$ (Sigma-Aldrich, UK). The final reaction mixture (50 μL) contained 1× PCR buffer, 200 μM of each dNTP, 0.5 μM of each forward and reverse primer and 1.25 U of *Taq* polymerase. $MgCl_2$ was added at the concentration specified in Table S2 (available as Supplementary data at *JAC* Online). DNA template (5 μL) was added and sterile DEPC-treated water (Ambion®, UK) was used to bring the reaction volume to 50 μL. Sterile DEPC-treated water (Ambion®, UK) replacing bacterial DNA was used in all PCR assays as a negative control. PCR was performed using a Veriti® Thermal Cycler (Applied Biosystems, UK). Table S2 provides details of the cycling parameters used. Amplicons were analysed by agarose gel electrophoresis and the resulting bands were visualized by UV luminescence (Gel Doc™, Bio-Rad, UK) using Quantity One software (Bio-Rad).

### Detection of antibiotic resistance genes

PCR screening assays were used for detection of the *cfxA*-type gene (penicillin and cephalosporin resistance), *ermF* (MLS resistance) and *tetQ* (tetracycline resistance). *Prevotella pallens* NCTC 13042 (*cfxA2* and *tetQ*) and *Prevotella bivia* R24779 (*ermF*) were used as positive controls. Where available, MIC and susceptibility category (provided that anaerobic breakpoints were approved by the CLSI) of a range of antibiotics (amoxicillin, ceftazidime, co-amoxiclav, azithromycin, clindamycin, doxycycline and tetracycline), determined previously,[10] were compared in the presence and absence of the relevant antibiotic resistance gene in the clinical isolates.

### Analysis of the CfxA-type β-lactamase in clinical Prevotella isolates

Amplification of the full-length β-lactamase-encoding gene (966 bp) was carried out with 20 clinical isolates of *Prevotella* and *P. pallens* NCTC 13042, which were positive for the 214 bp fragment of the *cfxA*-type gene. PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen, UK) in accordance with the manufacturer's instructions. Both strands of DNA were sequenced by Eurofins MWG Operon using appropriate primers (Table 1). The CfxA-type protein sequence was determined from a consensus DNA sequence. The deduced protein sequences of the CfxA-type β-lactamase from the clinical *Prevotella* isolates and *P. pallens* NCTC 13042 were compared between isolates deposited in GenBank and correlated with amino acid substitutions. The MICs of amoxicillin, ceftazidime and co-amoxiclav determined previously[10] were also compared between isolates with amino acid substitutions within the β-lactamase protein sequence.

### Detection of species-specific RND-type efflux pumps

Protein sequences of RND efflux pumps in *P. aeruginosa* PAO1 (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) and *B. fragilis* ATCC 25285 (BmeABC1–8) were searched for homologies within five *Prevotella* genomes (*Prevotella denticola* F0289, *Prevotella nigrescens* ATCC 33563, *Prevotella melaninogenica* ATCC 25845, *Prevotella histicola* F0411 and *Prevotella salivae* DSM 15606) using protein BLAST. Species-specific primer pairs were designed to amplify the gene encoding the linker protein, a periplasmic membrane fusion protein,[14] of the efflux pump operon found in the *Prevotella* genomes (Table 1). Clinical *Prevotella* isolates ($n=87$) that belonged to these five species (*P. denticola*, $n=13$; *P. nigrescens*, $n=12$; *P. melaninogenica*, $n=34$; *P. histicola*, $n=20$; and *P. salivae*, $n=8$) and two type strains (*P. denticola* NCTC 13067 and *P. melaninogenica* ATCC 25845) were selected and screened for the presence of species-specific RND-type efflux pumps.

### Transcription levels of species-specific RND-type efflux pumps

A detailed description of the method used to prepare total RNA from stationary growth (optical density at 550 nm of 0.8–1.3) cultures ($n=26$) is provided elsewhere.[18] Clinical isolates were selected randomly. RNA integrity was verified on a 1% agarose gel. The quantitative real-time PCR (qRT-PCR) procedure was carried out as previously described,[18] with several modifications for use with *Prevotella* spp. as follows: (i) 500 ng of total RNA was used for cDNA generation; (ii) the cycling conditions used were 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C; and (iii) real-time PCR was performed in a final reaction volume of 20 μL containing 2.5 μL of cDNA (1:10 dilution), 10 μL of FastStart® Universal SYBR green master mix with ROX (Roche, UK), 0.2 μL of each forward and reverse primer (10 μM) and 7.1 μL of DEPC-treated water (Ambion®). The species-specific efflux pump primers that were used for qRT-PCR are listed in Table 1 and were designed from the gene encoding the linker protein of the efflux pump operon found in the *Prevotella* genomes. The comparative threshold cycle ($C_T$) method ($\Delta\Delta C_T$) was used to determine the relative transcription levels using the 16S rRNA gene as the endogenous control. Data were analysed using Applied Biosystems 7500 software.

### Inhibition of efflux pumps

Under strict anaerobic conditions clinical *Prevotella* isolates ($n=25$) were inoculated onto supplemented brucella blood agar with or without an efflux pump inhibitor (EPI), Phe-Arg β-naphthylamide dihydrochloride (Sigma-Aldrich), added uniformly to the agar at a final concentration of 80 mg/L.[19] The MICs of amoxicillin, azithromycin, ceftazidime, clindamycin, co-amoxiclav, doxycycline and tetracycline were then determined in the presence or absence of the EPI by Etest® (bioMérieux, France) according to the manufacturer's instructions.

### Statistical analysis

MICs were compared in isolates that were positive versus negative for: (i) the *cfxA*-type gene (amoxicillin, ceftazidime and co-amoxiclav); (ii) *ermF* (azithromycin and clindamycin); and (iii) *tetQ* (tetracycline and doxycycline) using the Mann–Whitney test. Isolates were separated into groups depending on a Y239D substitution in the CfxA-type β-lactamase and amoxicillin, ceftazidime and co-amoxiclav MICs compared using the Mann–Whitney test. A relative risk (RR) analysis was performed to determine the risk of harbouring *ermF* in UK CF isolates relative to US CF, UK invasive and UK healthy control isolates. Isolates were grouped according to patient (UK CF, US CF and healthy control) and prescription of azithromycin (currently prescribed versus not currently prescribed) and a $\chi^2$ test with Yates correction was used to determine whether there was evidence of an association between *ermF* and current azithromycin prescription. The Wilcoxon signed rank test was used to compare the MICs before and after inhibition of the efflux pumps. All statistical analyses were carried out using an SPSS software package (SPSS Version 19; Chicago, IL, USA). A two-tailed $P$ value $<0.05$ was considered statistically significant.

## Results

### The cfxA-type gene was common among Prevotella isolates from a range of sources and was associated with high MICs of β-lactam antibiotics

The *cfxA*-type gene codes for a class 2e β-lactamase[13] and was detected in 70/155 (45%) isolates with similar proportions in

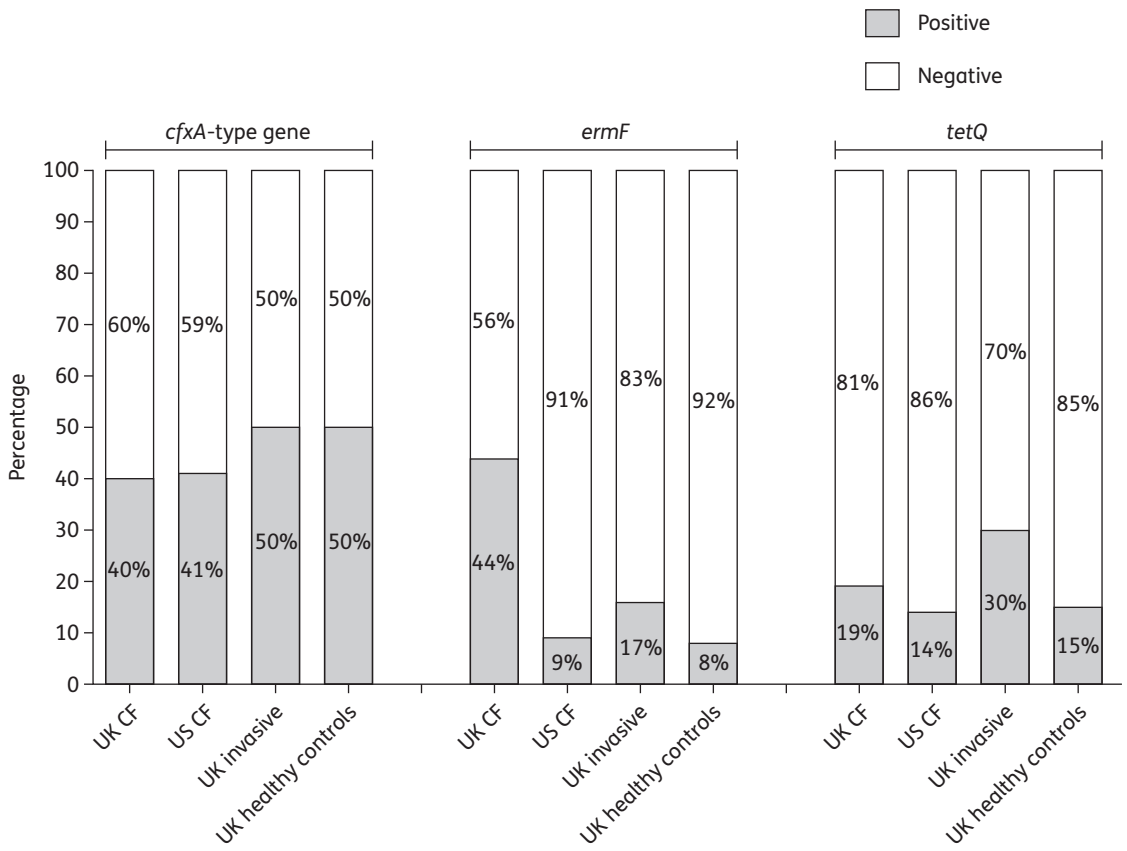


**Figure 1.** Prevalence of resistance genes (*cfxA*-type gene, *ermF* and *tetQ*) in each of the *Prevotella* groups.

each *Prevotella* group positive for the gene (Figure 1). The isolates positive for the *cfxA*-type gene had significantly higher MICs of amoxicillin and ceftazidime compared with isolates negative for the *cfxA*-type gene ($P<0.001$, Mann–Whitney test) (Figure 2). Although this β-lactamase is typically inhibited by clavulanic acid,[13] isolates positive for the *cfxA*-type gene also had significantly higher MICs of co-amoxiclav compared with isolates negative for the *cfxA*-type gene ($P<0.001$; Figure 2). However, only 16/69 (23%) isolates positive for the *cfxA*-type gene were also categorized as resistant or intermediately resistant to co-amoxiclav.

### *Prevotella isolates with a Y239D substitution in the CfxA-type β-lactamase protein sequence were more resistant to ceftazidime*

The deduced amino acid sequences of the CfxA-type β-lactamase in 20 clinical isolates and one type strain (*P. pallens* NCTC 13042) were >98% similar. Several amino acid substitutions were observed when compared with CfxA from *Bacteroides vulgatus* (GenBank protein accession number AAB17891) (Table 2). Each of the deduced amino acid sequences possessed the K272E substitution common to CfxA2, and an additional substitution at residue 239 (Y239D/C/S) was observed more than once (Table 2). Given that the Y239D substitution was most common (Table 2; CfxA3), the MICs of amoxicillin, ceftazidime and co-amoxiclav were compared between isolates with this substitution (Table 2). Those clinical isolates with an aspartic acid (D) at this residue ($n=6$) had significantly higher MICs of ceftazidime compared with those with tyrosine (Y) at this position ($n=11$) ($P=0.011$, Mann–Whitney test). No difference in the MICs of amoxicillin and co-amoxiclav was detected between the two groups.

### *The UK CF isolates were more likely to harbour ermF compared with the other groups*

The *ermF* gene was detected most frequently in the UK CF *Prevotella* isolates (44%; Figure 1). There was a significant difference in the proportion of *ermF*-positive isolates between the UK CF group and the US CF (RR 5.26, 95% CI 1.35–20.49, $P=0.005$), UK invasive (RR 2.74, 95% CI 1.36–5.52, $P=0.004$) and UK healthy control (RR 5.70, CI 1.46–22.29, $P=0.003$) groups. The risk of UK CF isolates harbouring *ermF* was increased 5.3-fold, 2.7-fold and 5.7-fold relative to the US CF, UK invasive and UK healthy control isolates, respectively. Significantly higher concentrations of both azithromycin ($P<0.001$, Mann–Whitney test) and clindamycin ($P<0.001$) were required to inhibit the *ermF*-positive isolates compared with the *ermF*-negative isolates (Figure 2). Furthermore, 28/37 (76%) *ermF*-positive isolates were categorized as resistant to clindamycin. A significant association between a current patient prescription of azithromycin and the presence of *ermF* among clinical *Prevotella* isolates was also detected ($\chi^2=31.534$, $P<0.001$); 11/27 (41%) and 9/46 (20%) isolates from patients currently and not currently prescribed azithromycin, respectively, were positive for the gene.

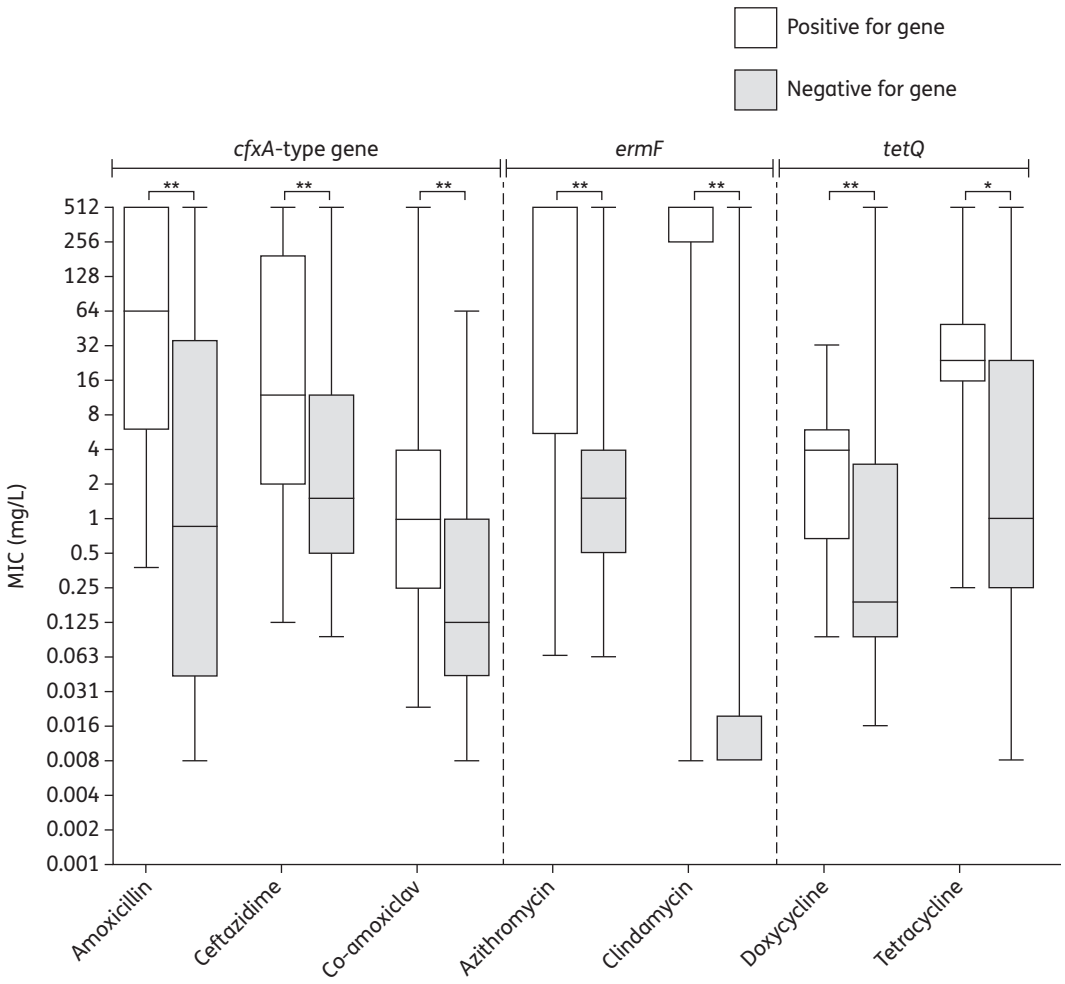


**Figure 2.** Comparison of susceptibility (MICs) in isolates positive and negative for associated resistance genes. In the box and whisker plots, the top and bottom boundaries of each box indicate the 75th and 25th quartile values, respectively, with the line inside the box representing the median (50th quartile). The ends of the whiskers indicate the range. Any isolates recorded as having an MIC greater than the maximum concentration (>256 mg/L) or lower than the minimum concentration (<0.016 mg/L) on the Etest® strip are shown as double the maximum concentration or half the lower concentration, respectively. *$P<0.05$ and $\geq 0.001$; **$P<0.001$.

### *The tetQ gene was detected in similar proportions in each group and was associated with high MICs of tetracycline antibiotics*

Thirty-two of 151 (21%) *Prevotella* isolates were positive for *tetQ*. This gene was detected in similar proportions in each group (Figure 1). Significantly higher concentrations of tetracycline ($P=0.001$, Mann-Whitney test) and doxycycline ($P<0.001$) were required to inhibit the growth of *tetQ*-positive compared with *tetQ*-negative isolates (Figure 2). Among the *tetQ*-positive isolates, 19/23 (83%) were categorized as resistant or intermediately resistant to tetracycline.

### *A high percentage of Prevotella isolates tested harboured one or more antibiotic resistance genes*

Of the 151 *Prevotella* isolates tested for the three antibiotic resistance genes, 93 (62%) were positive for one or more of the antibiotic resistance genes by PCR (Figure S1, available as Supplementary data at *JAC* Online). Twenty-six isolates carried two resistance genes [*cfxA*-type gene and *ermF*, 13/26 (50%); *cfxA*-type gene and *tetQ*, 11/26 (42%); and *ermF* and *tetQ*, 2/26 (8%)] and 6/151 (4%) isolates of *Prevotella* were found to harbour all three resistance genes (Figure S1).

### *Prevotella spp. express RND-type efflux pumps, which may contribute to tetracycline resistance*

Genes encoding a linker protein, inner membrane protein and outer membrane protein were detected within each of the five *Prevotella* genomes searched *in silico* and were organized as an operon similar to RND-type efflux pumps in other bacteria.[14] At the time of our analysis a single efflux pump was identified in *P. denticola* F0289 and *P. nigrescens* ATCC 33563 and two efflux pumps were detected in *P. melaninogenica* ATCC 25845, *P. histicola* F0411 and *P. salivae* DSM 15606 (Table S3, available as Supplementary data at *JAC* Online).

**Table 2.** Amino acid sequence analysis of 20 clinical *Prevotella* isolates and a type strain, *P. pallens* NCTC 13042, compared with CfxA from *B. vulgatus* AAB17891[23]

| Source | MIC (mg/L) | | | Amino acid substitutions in CfxA | CfxA-type β-lactamase | Protein accession number of identical sequence |
|---|---|---|---|---|---|---|
| | amoxicillin | ceftazidime | co-amoxiclav | | | |
| UK CF | 6 | 2 | 1.5 | K272E, L189F | NA | NA |
| UK CF | >256 | >256 | 64 | K272E, Y239S | NA | NA |
| UK CF | >256 | 256 | >256 | K272E, Y239S | NA | NA |
| UK CF | >256 | 128 | 3 | K272E, Y239D | CfxA3 | AAL79549 |
| UK CF | 32 | 2 | 12 | K272E | CfxA2 | ADD23513 |
| UK CF | 64 | 4 | 6 | K272E | CfxA2 | ADD23513 |
| UK CF | 64 | 3 | 12 | K272E | CfxA2 | ADD23513 |
| UK CF | 48 | >256 | 64 | K272E, Y239D | CfxA3 | AAL79549 |
| UK CF | 96 | >256 | 0.064 | K272E, Y239C | CfxA5 | AAV37206 |
| UK invasive | >256 | >256 | 3 | K272E, Y239D | CfxA3 | AAL79549 |
| UK invasive | >256 | 4 | 3 | K272E | CfxA2 | ADD23513 |
| UK invasive | >256 | >256 | 3 | K272E, Y239D | CfxA3 | AAL79549 |
| UK invasive | 64 | 1.5 | 0.125 | K272E | CfxA2 | ADD23513 |
| UK invasive | 48 | 3 | 0.064 | K272E, Y239D | CfxA3 | AAL79549 |
| UK invasive | >256 | >256 | 0.25 | K272E | CfxA2 | ADD23513 |
| UK healthy control | 192 | 32 | 0.25 | K272E | CfxA2 | ADD23513 |
| UK healthy control | 3 | 1.5 | 0.25 | K272E | CfxA2 | ADD23513 |
| UK healthy control | 4 | 0.25 | 0.032 | K272E | CfxA2 | ADD23513 |
| UK healthy control | 2 | 16 | 0.023 | K272E | CfxA2 | ADD23513 |
| UK healthy control | >256 | >256 | 3 | K272E, Y239D | CfxA3 | AAL79549 |
| Type strain | 1.5 | 3 | 0.064 | K272E | CfxA2 | ADD23513 |

NA, not applicable; AAL79549, *Capnocytophaga ochracea*; ADD23513, *Prevotella intermedia*; AAV37206, *Parabacteroides distasonis*.

The species-specific linker genes were present in the majority of *Prevotella* spp. isolates tested (83/87, 95%) by PCR. The remaining isolates (*P. melaninogenica*) were either negative by PCR for one ($n=2$) or both ($n=2$) of the predicted linker genes. The transcription levels of the species-specific efflux pumps were analysed in 25 clinical isolates and *P. melaninogenica* ATCC 25845 by qRT-PCR. Twenty-one of 25 (84%) isolates and the type strain expressed the expected linker genes (Figure S2, available as Supplementary data at *JAC* Online). For the remaining four isolates either no expression was detected (*P. melaninogenica*, $n=1$; and *P. histicola*, $n=2$) or one of two expected pumps was expressed ($n=1$; Figure S2). The MICs of seven antibiotics in the presence or absence of an EPI was also investigated. A statistically significant decrease in the MICs of tetracycline ($P=0.001$, Wilcoxon signed rank test) was detected in the presence of the EPI, with no statistically significant differences apparent for the remaining antibiotics (Figure 3). A further analysis of the tetracycline MIC data revealed that 17/25 (68%) *Prevotella* isolates were negative by PCR for *tetQ*. A significant decrease in MICs was detected among the *tetQ*-negative isolates in the presence of the EPI ($P=0.025$, Wilcoxon signed rank test). In contrast, no statistical difference in the MIC of tetracycline was detected in the presence or absence of an EPI for the *tetQ*-positive isolates ($n=8$).

## Discussion

*Prevotella* spp. have been detected in the airway microbiota in diseases characterized by chronic infection, including CF, COPD and bronchiectasis.[4,6,8] Although their role in infection and inflammation in these conditions has not been clearly defined, this obligate anaerobe may be a future target for antimicrobial treatment. *In vitro* antimicrobial susceptibility testing may be used clinically to guide the treatment of individual anaerobic isolates. Unfortunately, *in vitro* susceptibility testing of *Prevotella* spp. can be difficult to perform given the fastidious nature of this genus. A genotypic prediction of resistance may be an attractive alternative to susceptibility testing with some antibiotics and has proved useful to detect single antibiotic resistance gene targets associated with resistance, e.g. *mecA* associated with methicillin resistance in *Staphylococcus aureus*.[20] Before genotypic prediction can be used, it is important to understand the mechanisms of resistance that are commonly associated with reduced susceptibility in the target bacterium. Therefore, in this study we investigated, in clinical *Prevotella* isolates, putative mechanisms of resistance to antibiotics used in the treatment of chronic lung infections. We also explored the relationship between the presence of specific antibiotic resistance genes, amino acid mutations within the CfxA-type β-lactamase and the expression of efflux pumps and *in vitro* antimicrobial susceptibility and determined whether the genotype could predict phenotypic resistance.

β-Lactamase production by bacteria is a key mechanism of resistance to β-lactam antibiotics, and the presence of a *cfxA*-type gene in the *Prevotella* isolates in this study was associated with high MICs of amoxicillin, as has previously been reported for *Prevotella* isolates from dental infection.[21] In the current study, isolates harbouring the *cfxA*-type gene also had

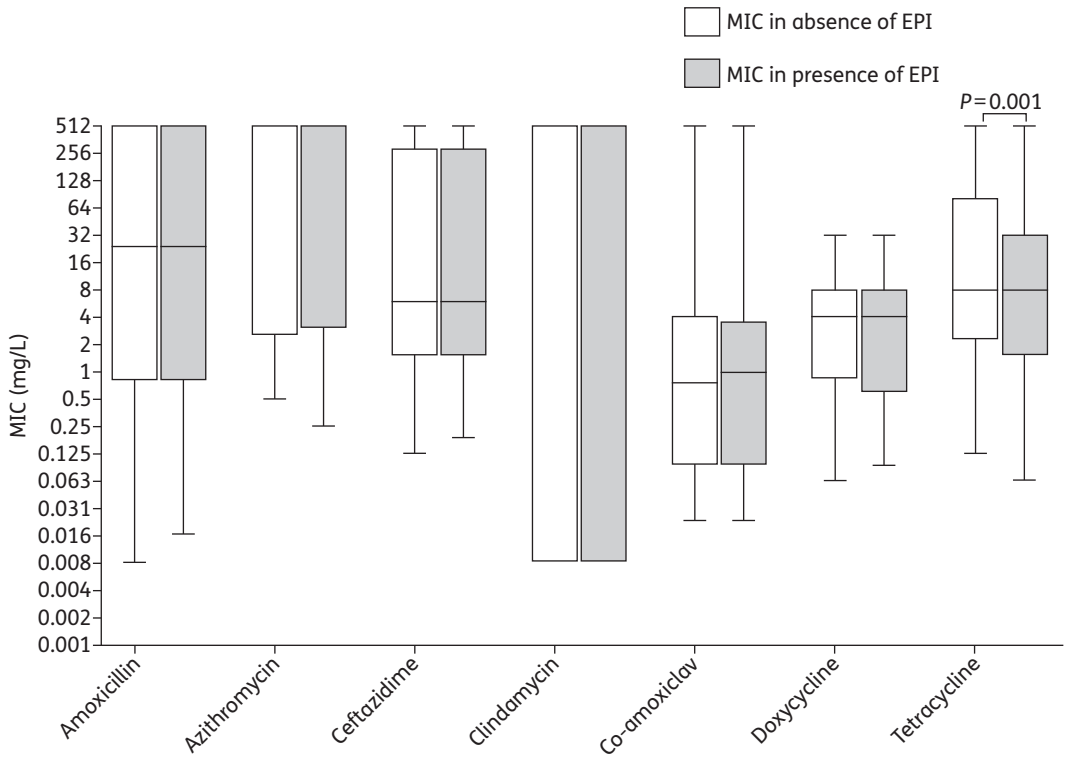


**Figure 3.** Antibiotic susceptibility (MICs) of *Prevotella* isolates ($n=25$) in the absence or presence of the EPI Phe-Arg β-naphthylamide dihydrochloride. In the box and whisker plots, the top and bottom boundaries of each box indicate the 75th and 25th quartile values, respectively, with the line inside the box representing the median (50th quartile). The ends of the whiskers indicate the range. Any isolates recorded as having an MIC greater than the maximum concentration (>256 mg/L) or lower than the minimum concentration (<0.016 mg/L) on the Etest® strip are shown as double the maximum concentration or half the lower concentration, respectively.

higher MICs of ceftazidime and co-amoxiclav compared with isolates negative for the gene. This is in contrast to the findings of a previous study, which found that all *Prevotella* isolates positive for the β-lactamase-encoding gene *cfxA2* ($n=62$) were amoxicillin resistant, but susceptible to ceftazidime and co-amoxiclav.[11] Giraud-Morin *et al.*[11] proposed that substrate specificity may be extended by mutations within the *cfxA*-type gene as it is recognized that mutations in other β-lactamase-encoding genes (e.g. TEM-type β-lactamases) widen substrate specificity.[13] In the present study, when the CfxA-type amino acid sequence was analysed, the K272E substitution, common to CfxA2, was detected in all 20 isolates tested. However, additional substitutions at amino acid residue 239 were also found, which are characteristic of other CfxA2-like sequences (CfxA3 and CfxA5) suggesting that *Prevotella* isolates from a range of sources produce variants of the CfxA2 β-lactamase. Furthermore, the data indicate that a Y239D substitution within the CfxA2-type protein sequence is associated with significantly higher MICs of ceftazidime. Therefore, the presence of this substitution may reduce susceptibility to ceftazidime.

Resistance to MLS antibiotics in *Prevotella* spp. may be due to an *ermF*-encoded methylase, which alters the binding site of these antibiotics. Isolates positive for *ermF* were associated with higher MICs of azithromycin and clindamycin. In agreement with this, Xie *et al.*[22] detected 13/19 (68%) clindamycin- and/or roxithromycin-resistant *Prevotella* isolates from dental abscesses to be positive for *ermF*. Furthermore, the risk of UK CF isolates harbouring *ermF* was much higher compared with those collected from other patient groups. This may reflect the fact that the UK CF isolates were cultured from adult CF patients, where long-term azithromycin is prescribed in a high percentage of patients compared with the paediatric US CF isolates or UK invasive and healthy control isolates, for whom the use of azithromycin is less likely. The hypothesis of higher resistance in *Prevotella* being linked to azithromycin use appears to be confirmed by the association between a current patient prescription of azithromycin and the presence of *ermF* among the *Prevotella* isolates. Ideally, we would like to investigate the prevalence of *ermF* in *Prevotella* isolates from young patients with CF from the UK in future studies. However, as bronchoscopy is not performed in young children in the UK as part of routine clinical practice, obtaining clinically relevant respiratory samples is challenging. The expression of *ermF* in these isolates could also be evaluated between CF patients treated with or without chronic azithromycin. However, this would also be difficult to do as most CF patients are prescribed long-term azithromycin therapy.

Between 14% and 30% of *Prevotella* isolates in each group were also positive for *tetQ*, which has been associated with resistance to tetracyclines, with *tetQ*-positive isolates having statistically higher MICs of both doxycycline and tetracycline. A previous study also reported that ~25% of *Prevotella* isolates cultured from clinical specimens and the resident microbiota of healthy subjects were positive for *tetQ*.[12] Similarly,

Arzese *et al.*[12] investigated *tetQ* and susceptibility to tetracycline, and reported that all *tetQ*-positive isolates ($n=14$) were resistant to tetracycline.

The results of this study indicate that if one of the target resistance genes is detected in clinical *Prevotella* isolates, it is more likely that these isolates will have higher MICs of the associated antibiotics. However, susceptible isolates with low MICs were also positive for the target resistance gene; this suggests that the choice of antibiotic treatment cannot be based solely on the presence of the gene. This was particularly evident for co-amoxiclav, where only 23% of isolates positive for the *cfxA*-type gene were resistant or intermediately resistant to co-amoxiclav. There were no amino acid mutations identified within the CfxA-type β-lactamase that were associated with reduced susceptibility to this antibiotic. These data suggest that the *cfxA*-gene itself may not be a major mechanism of resistance to co-amoxiclav. However, the determination of co-amoxiclav susceptibility post-complementation of a susceptible *Prevotella* strain with mutated *cfxA* genes would be required to support this hypothesis.

We also investigated whether the expression of efflux pumps could contribute to antibiotic resistance in *Prevotella* spp. The constitutive expression or overexpression of efflux pumps by bacteria is an important putative resistance mechanism as it may contribute to the reduced susceptibility to structurally dissimilar antibiotics. This study is the first to report the type of efflux pump in the *Prevotella* genome and whether these efflux pumps were expressed and potentially contributing to antibiotic resistance in five different species. Although detected in higher numbers in other bacterial genomes,[14,15] a maximum of two RND-type efflux pumps were detected within five *Prevotella* genomes, which were homologous to efflux pumps found in *P. aeruginosa* PAO1 and *B. fragilis* ATCC 25285. In *P. aeruginosa*, it is recognized that MexAB-OprM and MexXY-OprM efflux systems are constitutively active.[14] In this study, 22/25 (88%) clinical *Prevotella* isolates tested demonstrated pump expression. It is also known that efflux pumps expressed by *P. aeruginosa* contribute to resistance to a range of antibiotics including β-lactams and tetracycline.[14] In our study, the presence of an EPI resulted in a statistically significant decrease in the MICs of tetracycline, suggesting that this antibiotic is a potential substrate of *Prevotella* efflux pumps, especially in isolates negative for *tetQ*. Further work includes comparing the expression levels of efflux pumps in a large number of isolates susceptible, intermediately resistant and resistant to tetracycline across all the different species.

A number of *Prevotella* isolates in this study also had high MICs of an antibiotic, but lacked a *cfxA*-type gene, *ermF* or *tetQ*. Although it is feasible that these isolates harbour a novel resistance mechanism, it is also possible that the primers used in this study did not recognize the target antibiotic resistance genes, which could be due to mutations within the target sequence. Furthermore, we cannot rule out that *Prevotella* isolates may harbour more than one mechanism of resistance for the same antibiotics.

There are a number of limitations to this study. Single bacterial colonies from each clinical sample were isolated and those identified as *Prevotella* spp. were subsequently used in this study. We cannot discount that other genotypes of *Prevotella* spp., which may have a different susceptibility profile from that detected, were present in the same sample. Furthermore, the microenvironment in chronic lung infections may induce different metabolic conditions and affect *in vivo* susceptibility compared with our *in vitro* results; however, the current approach to testing susceptibility and detecting resistance genes mirrors techniques used frequently in clinical practice.

In summary, this study has shown that *Prevotella* isolated from chronic pulmonary infection, invasive infections and the healthy respiratory tract harbour a common pool of resistance genes and possess RND-type efflux pumps, which potentially contribute to a reduced susceptibility to tetracycline. The presence of a *cfxA*-type gene, *ermF* or *tetQ* was associated with significantly higher MICs of β-lactam, MLS and tetracycline antibiotics, respectively, and an amino acid substitution within the CfxA-type β-lactamase was associated with higher MICs of ceftazidime. The data suggest that the presence of these genes increases the likelihood of resistance, but absence does not imply susceptibility. There was also an association between current patient prescriptions of azithromycin and the presence of *ermF* among the CF *Prevotella* isolates, suggesting that antibiotic pressure may have contributed to the spread of *ermF* among these isolates.

## Acknowledgements

We thank Mr Gerry McGrillen (School of Pharmacy, Queen's University Belfast, Belfast, UK) for technical assistance and Dr Valerie Hall (Anaerobe Reference Laboratory, Cardiff, UK) for kindly providing the UK invasive isolates.

## Funding

This work was supported by grants from the Health and Social Care Research and Development, Public Health Agency, Northern Ireland, the Medical Research Council and the United States National Institutes of Health (grant numbers HL092964, HL084934 and 5R01 HL092964-04) through a US-Ireland Partnership Grant. M. M. T. was supported by a Health and Social Care Research and Development, Public Health Agency, Northern Ireland, funded UK National Institute for Health Research Career Scientist Award. L. J. S. and K. A. G. were supported by Department of Employment and Learning, Northern Ireland, Studentships.

## Transparency declarations

None to declare.

### *Author contributions*

L. J. S., B. S., M. M. T., D. F. G., T. S. and J. S. E. conceived and designed research. L. J. S., B. S., K. A. G., S. J. M., L. M. and J. H. performed research. L. J. S., B. S. and M. M. T. analysed data. L. J. S., B. S., M. M. T., M. S. M. and J. S. E. wrote the paper. M. C. W., D. F. G. and T. S. reviewed and revised the paper.

## Supplementary data

Tables S1 to S3, Figure S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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