

Biocide susceptibility of the *Burkholderia cepacia* complex

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Received 22 September 2008; returned 30 October 2008; revised 9 December 2008; accepted 15 December 2008

Objectives: The *Burkholderia cepacia* complex (Bcc) species are important opportunistic pathogens with intrinsic antibiotic resistance. They are also well known as contaminants of disinfectants, yet their biocide susceptibility has not been studied in detail. We investigated Bcc biocide susceptibility and correlated it to their taxonomy, antibiotic susceptibility and ability to form biofilms.

Methods: Genetically distinct Bcc strains belonging to 12 of the defined species were examined. Biocide susceptibility was assessed by (i) broth dilution MIC assays, (ii) agar growth-based MBC screens and (iii) suspension tests. Antibiotic MIC was determined by Etest[®] strips, and the ability to form biofilms was examined in a 96-well plate assay.

Results: Biocide susceptibility varied across the Bcc complex with high MIC recorded for chlorhexidine (>100 mg/L), cetylpyridinium chloride (>200 mg/L), triclosan (>500 mg/L), benzalkonium chloride (>400 mg/L) and povidone (>50000 mg/L). Species-dependent differences were apparent only for cetylpyridinium chloride. There was no correlation between biocide susceptibility and (i) antibiotic susceptibility or (ii) the ability to form biofilms. Biocide MBC was considerably higher than the MIC (chlorhexidine, 6-fold greater; cetylpyridinium chloride, 20-fold greater). Cystic fibrosis outbreak strains (*Burkholderia multivorans* Glasgow strain and *Burkholderia cenocepacia* ET12) possessed elevated chlorhexidine resistance, and Bcc bacteria were also shown to remain viable in current commercial biocide formulations.

Conclusions: Bcc bacteria are resistant to a wide range of biocides and further representatives of this group should be included as reference strains in the development of new anti-infectives and commercial formulations.

Keywords: antibiotics, resistance and susceptibility, minimal inhibitory concentration

Introduction

The *Burkholderia cepacia* complex (Bcc) species are problematic Gram-negative opportunistic pathogens, and people with cystic fibrosis (CF) are particularly prone to chronic Bcc lung infection.¹ The Bcc consists of multiple closely related species, all of which have been isolated from clinical infections.^{1,2} Until very recently, the following 10 species had been formally designated as members of the complex:³ *B. cepacia*, *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia dolosa*, *Burkholderia ambifaria*, *Burkholderia anthina*, *Burkholderia pyrrocinia* and *Burkholderia ubonensis*. Two recent studies have extended the complex to 17 species by the addition of *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov., *Burkholderia metallica*

sp. nov.,⁴ *Burkholderia lata* and *Burkholderia contaminans*.⁵ Of these 17 species, *B. cenocepacia* and *B. multivorans* are most associated with CF infection, which together account for ~85% to 95% of all Bcc infections in this disease.¹ The epidemiology of Bcc infection outside CF has not been extensively studied; however, the recent studies of Reik *et al.*⁶ have shown *B. cenocepacia* (25.6%), *B. cepacia* (18.9%) and *B. multivorans* (15.6%) to be the most common non-CF infections seen in the USA.

An important feature of Bcc bacteria is their very high innate antimicrobial resistance to both antibiotics and biocides.^{1,2} Bcc species are intrinsically resistant to many antibiotics such as aminoglycosides and polymyxin B and often require combination therapy to suppress infection in CF.⁷ The antibiotics polymyxin, gentamicin and vancomycin are used at high concentrations in *B. cepacia* Selective Agar, a highly effective

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medium for their isolation from CF sputum.⁸ Nzula *et al.*⁹ compared the antibiotic susceptibility of six Bcc species and concluded that it was highly variable except for innate polymyxin resistance and not linked to the taxonomic status of the isolates examined. Efflux, the secretion of chromosomal β -lactamases and the impermeability of the outer envelope of Bcc bacteria have been implicated in antibiotic resistance.¹

In contrast, the molecular basis for biocide resistance in Bcc bacteria has been poorly studied despite these organisms being linked with many instances of contamination in disinfectants and other anti-infective solutions.¹⁰ Pharmaceutical formulations containing benzalkonium chloride,¹¹ cetylpyridinium chloride¹² and chlorhexidine^{13,14} have become contaminated with Bcc bacteria and led to large outbreaks of infection. Even during commercial manufacture, disinfectants such as povidone-iodine may become contaminated with Bcc bacteria.¹⁵ A review of commercial recalls in the USA from 1998 to 2006 demonstrated that Bcc bacteria were the most commonly isolated Gram-negative bacteria in both sterile and non-sterile products.¹⁰ Multiple instances of a single Bcc strain causing either contamination or clinical infection at geographically distinct sites have been identified using multilocus sequence typing (MLST);^{16–18} these data suggest that in our burgeoning world economy, the potential for global distribution of contaminating bacteria in anti-infective agents may exist.

The aim of this study was to provide an up-to-date survey of the biocide susceptibility profiles of a representative panel of Bcc species that accounts for taxonomy and genetic diversity. To ensure that a wide genetic diversity of Bcc bacteria were sampled, isolates were also selected on the basis of their MLST

type.¹⁹ Biocide susceptibility was investigated using a standardized broth dilution method to determine MICs; this was also correlated to the MBC for resistant and susceptible strains. A possible link between biocide resistance and multidrug resistance has been proposed,²⁰ and this hypothesis was also explored by correlating biocide and antibiotic resistance profiles of the Bcc isolates tested.

Material and methods

Bacterial collection

A panel of 101 genetically distinct Bcc strains were selected from the Cardiff University collection³ and the Belgium Coordinated Collection of Microorganisms (BCCM; <http://bccm.belspo.be/about/lmg.php>). The selected strains encompassed reference strains from the defined Bcc strain panels.^{21,22} Additional *B. multivorans* strains from the Glasgow outbreak²³ were kindly provided by Dr Alan Brown and Prof. John R.W. Govan (Edinburgh University, UK). MLST was used to type and select strains spanning the genetic diversity of the Bcc.¹⁹ The strains spanned 11 of the most prevalent Bcc species (Table 1) and also two strains from the Bcc novel Group K (a distinct MLST cluster from the former Group K isolates re-named as the new species *B. lata* and *B. contaminans*); the source of the strains varied and included clinical (56), non-clinical (9) and environmental strains (36). Initially all strains were screened against chlorhexidine (ICN Biomedicals) and cetylpyridinium chloride (ICN Biomedicals) as representatives of agents that could be easily screened in a broth dilution assay. From this screen, a subcollection of 38 strains was selected that spanned the range of

Table 1. Susceptibility of Bcc and other species to chlorhexidine and cetylpyridinium chloride

Species (number of strains tested)	Chlorhexidine MIC (mg/L)		Cetylpyridinium chloride MIC (mg/L)	
	mean minimum	mean maximum	mean minimum	mean maximum
<i>B. cepacia</i> (10)	57	65	170 ^a	175 ^b
<i>B. multivorans</i> (30)	36.6	46	77.7	101.3
<i>B. cenocepacia</i> (31)	47.4	54.8	175.5 ^c	184.5 ^c
<i>B. stabilis</i> (3)	40	46.7	146.7	166.7
<i>B. vietnamiensis</i> (5)	52	58	90	106
<i>B. dolosa</i> (3)	26.7	36.7	66.7	86.7
<i>B. ambifaria</i> (6)	33.3	41.7	98.3	116.7
<i>B. anthina</i> (2)	10	20	95	125
<i>B. pyrrocinia</i> (2)	15	25	50	70
<i>B. lata</i> (4)	15	25	183.3 ^d	200 ^d
<i>B. contaminans</i> (3)	13.3	23.3	200 ^e	200 ^f
Novel Bcc Group K (2)	50	60	200 ^g	200 ^g
Mean Bcc (101)	33.03	41.9	129.4	144.3

^aAt the mean minimum cetylpyridinium chloride MIC, *B. cepacia* was significantly more resistant than *B. multivorans*, *B. dolosa*, *B. ambifaria* and *B. pyrrocinia* ($P < 0.05$).

^bAt the mean maximum cetylpyridinium chloride MIC, *B. cepacia* was significantly more resistant than *B. multivorans*, *B. dolosa* and *B. pyrrocinia* ($P < 0.05$).

^cAt the mean minimum and mean maximum cetylpyridinium chloride MICs, *B. cenocepacia* was significantly more resistant than *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina* and *B. pyrrocinia* ($P < 0.05$).

^dAt the mean minimum and mean maximum cetylpyridinium chloride MICs, *B. lata* was significantly more resistant than *B. multivorans* ($P < 0.05$).

^eAt the mean minimum cetylpyridinium chloride MIC, *B. contaminans* was significantly more resistant than *B. multivorans* and *B. ambifaria* ($P < 0.05$).

^fAt the mean maximum cetylpyridinium chloride MIC, *B. contaminans* was significantly more resistant than *B. multivorans* ($P < 0.05$).

^gAt the mean minimum and mean maximum cetylpyridinium chloride MICs, Bcc novel Group K was significantly more resistant than *B. multivorans* ($P < 0.05$).

chlorhexidine and cetylpyridinium chloride susceptibility observed: 22 strains with low MICs and 16 with high MICs were selected to provide a spectrum of Bcc biocide resistance.

The subcollection was then used (i) to examine the susceptibility to three additional biocides povidone (Sigma Aldrich), benzalkonium chloride (Sigma Aldrich) and triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol; Fluka, Biochemika) and (ii) to determine the MBC values of chlorhexidine and cetylpyridinium chloride. In addition, 10 non-Bcc species were also screened for chlorhexidine and cetylpyridinium chloride susceptibility as control species (which, except for *Escherichia coli*, can infect the CF lung): *Staphylococcus aureus* [both methicillin-resistant (MRSA, two isolates) and -sensitive (MSSA, four isolates)] and *Pseudomonas aeruginosa* (24 isolates), as well as one control isolate for *Burkholderia gladioli*, *Stenotrophomonas maltophilia*, *Ralstonia mannitolytica*, *Pseudomonas stutzeri*, *Ralstonia picketti*, *Pseudomonas putida*, *Achromobacter xylosoxidans* and *E. coli*. All strains were grown in Tryptone soya broth (TSB) or agar (TSA; Oxoid) and incubated for 24–72 h at 37°C.

Broth dilution MIC method

A broth dilution assay adapted to examine biocide susceptibility of Bcc bacteria was carried out as follows. Aqueous solutions (sterilized by filtration) of chlorhexidine (10 mg/mL) and cetylpyridinium chloride (5 mg/mL) were used to make up stock concentrations of 100 and 1000 mg/L biocides. These solutions were then added to TSB to make up biocide concentrations ranging from 0 to 100 mg/L for chlorhexidine and 0 to 200 mg/L for cetylpyridinium chloride. We found that growth of bacteria in a liquid medium when compared with that on slopes of solid agar produced consistent numbers of viable cells for the Bcc strains studied. Therefore, each Bcc strain was grown overnight (18 h) in 3 mL of TSB at 37°C (in a 14 mL tube, shaken horizontally at 200 rpm); the culture was diluted to an optical density (OD) of 1 (630 nm) corresponding to a viable count of $\sim 1 \times 10^8$ cfu/mL. Approximately 1×10^5 cfu of each test Bcc strain were then added to 1 mL of each biocide concentration, and 200 μ L of this was added to three replicate wells within a 96-well plate. The microplates were incubated with shaking (200 rpm) at 37°C for 24 h and the OD was read using a Dynex Technologies MRX[®] microplate absorbance reader with Revelation application. An endpoint reading at 630 nm was taken with 10 s of shaking beforehand.

B. multivorans LMG 13010 was included in all assays as a control strain as preliminary experiments with this strain exhibited a reproducible effect. Addition of biocides to the growth media resulted in changes in OD. Therefore, the MIC value was designated as the concentration of biocides that resulted in an 80% knockdown of culture OD in comparison with the control wild-type growth with no added biocide. Viable counts performed on the control strain after running the broth dilution MIC assay demonstrated the presence of 9.7×10^5 cfu/mL at a chlorhexidine concentration of 20 mg/L, while no viable cells were detected above this concentration. Using the 80% knockdown in culture OD, the MIC for the control strain was found to be 30–40 mg/L, which corresponded well to the absence of viable cells in concentrations of chlorhexidine above 20 mg/L. The results were subjected to statistical analysis in order to determine levels of susceptibility.

Agar dilution MIC method

To screen the subset of 38 strains against the three additional biocides povidone, benzalkonium chloride and triclosan, an agar

dilution method was used. The method allowed for higher concentrations of biocides to be examined when the broth dilution assay was prevented due to the biocides altering the OD reading. Stock solutions of the three biocides were made up as follows: 10000 mg/L of triclosan in DMSO (this solvent did not inhibit the growth of Bcc bacteria at the concentrations used); 200000 mg/L of povidone in water and 10000 mg/L of benzalkonium chloride in water. After autoclaving, TSA was cooled to 50°C and the appropriate amount of each biocide was added to 40 mL of agar for concentration ranges of 0–500 mg/L for triclosan, 0–400 mg/L for benzalkonium chloride and 0–50000 mg/L for povidone. The bacterial strains were cultured and diluted to 1×10^8 cfu/mL as described above and 200 μ L of this suspension was then added to a 96-well plate in order to create a master plate containing eight replicates of each test strain. The strain suspensions from the master plate were then replicated onto the biocide-containing agar plates using a 96-well replicator and left to grow for 48 h at 37°C. The MIC was designated as the concentration of biocides of which no visible growth was apparent.

MBC

Bacterial cultures were grown as described above and diluted to $\sim 10^6$ cfu/mL. Chlorhexidine or cetylpyridinium chloride was added to diluted bacteria up to a maximum of 5000 mg/L; 200 μ L of this suspension was placed into 96-well plates and incubated at 37°C with shaking for 24 h. After incubation, 20 μ L of the bacterial suspension was placed into 100 μ L of neutralizer solution (0.75% azolectin and 5% Tween)²⁴ and left in contact for 5 min. The neutralizer solution was previously screened for efficiency of biocide termination and lack of toxicity towards the test bacteria (data not shown). After neutralization, the culture was replicated onto TSA and incubated for 24–48 h at 37°C; the MBC value was observed as the concentration at which growth ceased. Given the known starting viable count of bacteria, the dilution made in the neutralizer and the volume transferred by the replica plater (1.5 μ L), in the absence of bacterial growth, the MBC assay was capable of detecting a 99.995% rate of bacterial kill.

Antibiotic MIC testing: Etest[®] strips

The susceptibility of Bcc strains to 10 antibiotics was determined using Antibiotic Etest[®] strips as described by the manufacturer (AB Biodisk). The strains (60 tested) were selected on the basis of their resistance to chlorhexidine and cetylpyridinium chloride. The 10 antibiotics examined were amikacin, azithromycin, ciprofloxacin, ceftazidime, chloramphenicol, imipenem, meropenem, piperacillin, tobramycin and trimethoprim/sulfamethoxazole; all these antibiotics may be used to treat microbial infection in individuals with CF. The selection of antibiotics used to treat Bcc infection in CF is dependent on multiple factors including strain antibiogram, patient tolerance and clinic practice. Double or triple antibiotic combination therapy is recommended with meropenem/high-dose tobramycin combined with ceftazidime, trimethoprim/sulfamethoxazole, chloramphenicol or amikacin.⁷ The bacterial strains tested were revived on agar plates, and fresh colony growth resuspended in Iso-Sensitest broth (Oxoid) to an OD of 0.5 at 630 nm. This culture was then swabbed onto Iso-Sensitest agar plates and left to dry for 15 min. Etest[®] strips were then placed on top of the lawn of bacteria and left at 37°C overnight. The MIC was read from the strips and the resistance/susceptibility determined using the manufacturer's guidelines. Spontaneous resistance of each Bcc strain was measured by counting the number of colonies that grew within the initial zone of

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clearing after 48 h of incubation; spontaneous resistance was defined by the appearance of one or more colonies after 48 h of growth.

Suspension Tests

Two commercial skin cleanser biocide formulations were assessed in a standardized suspension test assay:²⁵ HibiSCRUB™ (containing an alcoholic solution of 4% chlorhexidine gluconate; Regent Medical Ltd) and Cuticura™ Waterless Hand Sanitizer (containing an unspecified amount of triclosan; Cuticura Labs Corp., NY, USA). Bacterial cultures were grown as described above and diluted to $\sim 10^8$ cfu/mL, and 1 mL of this suspension was added to 9 mL of commercial biocide. After exposure for 5, 10, 20 and 60 min, 100 μ L of this suspension was removed and placed into 900 μ L of a neutralizer solution (0.75% azolectin and 5% Tween 80) for 5 min. A control experiment containing water instead of biocides was also carried out at the same time. To enumerate viable bacteria, serial dilutions and viable counts were conducted.

Biofilm assays

An adapted biofilm assay was used to determine the biofilm production for Bcc strains.²⁶ Plates (96-well) were coated with 1 mg/well of porcine mucin (Sigma Aldrich, M1778; 200 μ L of a 5000 mg/L solution was placed in each well) and left at 37°C overnight. Porcine mucin was used to aid the adherence of bacterial cells to the wells of the plate as mucin is a protein commonly found in the lungs. Plates were washed three times with PBS and 0.1% Tween 20 and then stored at 4°C until use. Bacterial cultures were grown in nutrient broth as described above and diluted to $\sim 10^6$ cfu/mL; 200 μ L of each strain was then placed into 96-well plates. Eight replicates of each strain were assessed with the control row containing only broth. The plates were incubated at 37°C statically for 72 h. After incubation, the plates were washed three times with PBS to remove planktonic growth. The remaining biofilm was fixed with methanol for 15 min. Once methanol was removed and plates were dried, biofilms were stained with 1% Crystal Violet for 5 min. The stain was removed by washing with water and plates were dried. Biofilm thickness was measured by adding 33% glacial acetic acid and taking an OD reading at 570 nm using an automated plate reader (see above).

Statistical analysis

Due to the multifactorial mechanisms of biocide action, the MIC values obtained in replicate experiments spanned a range; therefore the results were split into a mean minimum value and a mean maximum value. All the results were analysed using the statistical software Minitab V.14. Kruskal–Wallis and Mann–Whitney statistical tests were carried out on the datasets and significant differences were determined ($P < 0.05$ for Kruskal–Wallis and $P < 0.05$ for Mann–Whitney). An antibiotic profile score was also calculated as a measure of overall antibiotic resistance within a Bcc species as follows: (i) for a given antibiotic, the mean MIC for all the strains in the species tested was calculated and (ii) the mean for all 10 of the antibiotics tested was then calculated to produce an overall score value for the Bcc species. Mann–Whitney tests were applied to determine the significance of the species' mean antibiotic profile score ($P < 0.05$); however, the significance of the score was not determined for Bcc species represented by less than two strains. The antibiotic profile score did not correlate to clinically defined break points for resistance; it just served as a measure of the overall multiple drug resistance observed in each Bcc species.

Results

Chlorhexidine and cetylpyridinium chloride susceptibility

MICs of chlorhexidine and cetylpyridinium chloride were initially determined for 101 strains of the Bcc; after replicate analysis, the mean minimum and mean maximum MIC values for each species were calculated (Table 1). For chlorhexidine, a variable range of growth inhibition was observed around the Bcc species mean (33–42 mg/L), from 10 mg/L (*B. lata*) to 65 mg/L (*B. cepacia*; Table 1). Although there were no significant differences observed between species, strains of *B. cepacia*, *B. vietnamiensis* and *B. cenocepacia* possessed the highest chlorhexidine MIC values observed (Table 1). Approximately 3-fold larger amounts of cetylpyridinium chloride were required to inhibit the growth of Bcc compared with chlorhexidine (mean MIC range, 129–144 mg/L; Table 1). In addition, species differences were apparent, with *B. cepacia*, *B. cenocepacia*, *B. lata*, *B. contaminans* and novel Bcc Group K strains being significantly less susceptible to cetylpyridinium chloride than other members of the Bcc (Table 1; $P < 0.05\%$).

The collection of isolates examined contained nine examples of Bcc strains that were involved in outbreaks involving multiple CF patients; interestingly, two of these epidemic strains demonstrated very high levels of chlorhexidine resistance. The *B. cenocepacia* ET12 epidemic strain J2315 and two additional strains that were single-locus variants (part of the same epidemic cable pilus and *B. cepacia* Epidemic Strain Marker-positive lineage) of J2315 had high MIC values for chlorhexidine that were > 100 mg/L (one of these, LMG 18827, is shown in Table 2). Of the remaining 28 *B. cenocepacia* strains tested, only one other *recA* Group III-A outbreak strain (MU1; Table 2) and one non-epidemic III-B group strain (LMG 18832; Table 2) possessed a chlorhexidine MIC in excess of 100 mg/L. Other epidemic strains within *B. cenocepacia* III-B such as the Philadelphia–District Columbia²⁷ and Mid-Western strains²⁸ were prevented from growing by < 60 mg/L chlorhexidine.

The *B. multivorans* isolate LMG 16660, a strain that caused a major outbreak among CF patients in Glasgow,²³ possessed a very high chlorhexidine MIC value (90–100 mg/L; Table 2) compared with the other *B. multivorans* strains. To determine whether this was a feature of the outbreak isolates, 19 additional Glasgow strains (from 17 CF patients involved in the outbreak) were examined and their chlorhexidine MICs were found to range from 44 to 100 mg/L. These minimum and maximum chlorhexidine MICs were significantly higher than the respective mean levels observed for all the other *B. multivorans* strains examined (22–33 mg/L; $P < 0.05$). However, in contrast to the elevated chlorhexidine MIC, the Glasgow outbreak strains were significantly more susceptible to cetylpyridinium chloride (76.5–89.5 mg/L; $P < 0.05$) than the other *B. multivorans* strains (105–127 mg/L; $P < 0.05$).

The Bcc strains were also compared with a control group of non-Bcc species commonly encountered in CF infection. With chlorhexidine, there were no significant differences between the MIC values for Bcc compared with non-Bcc species, although 6 of 10 species tested were prevented from growth by < 20 mg/L chlorhexidine. *R. pickettii*, *R. mannitolytica*, *S. aureus* and *P. aeruginosa* required more than 20 mg/L chlorhexidine to inhibit growth. Cetylpyridinium chloride resistance was significantly elevated in several Bcc species compared with

Table 2. Biocide susceptibility and MBCs for selected Bcc strains

Species and strain name	Chlorhexidine		Cetylpyridinium chloride		Triclosan MIC ^a (mg/L)	Benzalkonium chloride MIC ^a (mg/L)
	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)		
<i>B. cepacia</i> (7)						
LMG 17997	100	100	70–100	2000	400	200
ATCC 49709	100	100	>200	500	300	50
IST431	90–100	—	>200	3000	400–450	200
LMG 18821	90–100	300	>200	2000	>500	100
AVC1717	70–80	300	>200	>5000	500	200
ATCC 17759	20–30	100	>200	3000	>500	100
Bcc0176	10–20	50	30–40	50	500	100
<i>B. multivorans</i> (5)						
LMG 16660	90–100	300	>200	>5000	>500	200–250
ATCC 17616	10–20	300	50–150	1000	50	150
C1582	100	300	>200	1000	450–500	150
C1607	100	500	200	>5000	500	250
LMG 18825	20–30	100	50–100	50	50	100
<i>B. cenocepacia</i> (12)						
J2315 ^b	>100	100	>200	>5000	>500	350
X100	90–100	500	70–100	>5000	>500	350
LMG 18827 ^b	>100	—	>200	—	500	150
MU1	>100	1000	>200	>5000	10	350
WH1	10–20	100	150–180	4000	450–500	200
LMG 16654	80–90	300	>200	>5000	>500	50
DN	100	500	>200	2000	450–500	50
K56-2 ^b	90–100	300	>200	>5000	>500	150
PC002	100	300	>200	>5000	>500	>400
ATCC 25609	50–60	300	>200	>5000	400	350
LMG 18832	>100	1000	>200	>5000	>500	350
PC523	10–20	100	150–190	3000	500	200
<i>B. stabilis</i> (2)						
ATCC 35254	10–20	100	200	>5000	500	200
LMG 14294	100	100	180–200	>5000	400	200
<i>B. vietnamiensis</i> (4)						
LMG 16232	100	100	180–200	4000	450–500	100
G4	10–20	300	30–50	500	50	50
J1738	20–30	100	10–30	50	50	50
LMG 10929	100	100	30–50	300	100	50
<i>B. dolosa</i> (2)						
LMG 18943	20–30	300	50–70	2000	450–500	200
AU0090	40–50	100	100–120	5000	450–500	100
<i>B. ambifaria</i> (3)						
MC40-6	20–30	50	100–120	2000	50	50
ATCC 53267	100	—	180–200	5000	>500	50
LMG 19467	20–30	50	70–100	1000	>500	150
<i>B. anthina</i> (2)						
LMG 16670	10–20	50	70–100	500	150–200	50
LMG 20980	10–20	50	120–150	2000	150	50–100
<i>B. pyrrocinia</i> (1)						
LMG 14191	10–20	50	50–70	2000	50	50
Mean maximum value (mg/L)	74	229	155	2998	374	164

^aThe MIC of triclosan and benzalkonium chloride was determined on agar media.^bIsolates representative of the *B. cenocepacia* ET12 epidemic CF strain.

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other CF colonizers, with *B. cepacia*, *B. cenocepacia* and Bcc novel Group K being significantly less susceptible ($P < 0.05$). Overall, *P. aeruginosa* was the only CF pathogen that demonstrated chlorhexidine MICs (33–42 mg/L) and cetylpyridinium chloride MICs (200 mg/L) equivalent to those found in Bcc bacteria.

MBC

After screening 101 strains from the Bcc for growth inhibition, 38 strains spanning the observed MIC ranges for chlorhexidine and cetylpyridinium chloride were selected for MBC analysis (Table 2). The mean Bcc chlorhexidine MBC was 229 mg/L (Table 2), ~6-fold greater than the mean MIC for this biocide. The endpoint difference between the MIC and MBC assays demonstrated that the MIC concentration should not be taken as a measure of biocide efficacy, since for all but two of the strains examined it was below the bactericidal concentration. The majority of strains tested (35 out of 38) required no more than 300 mg/L for a bactericidal outcome. One *B. multivorans* and two *B. cenocepacia* strains had MBCs of 500 mg/L; two *B. cenocepacia* strains needed 1000 mg/L of chlorhexidine to achieve killing, 10-fold above their respective MIC concentration (Table 2). In contrast, 27 of the 38 Bcc strains required over 20-fold more cetylpyridinium chloride than the MIC for a bactericidal outcome, with a mean cetylpyridinium chloride MBC of 2998 mg/L (Table 2). In addition, 13 Bcc strains (eight of which were *B. cenocepacia*) were still viable after a 24 h exposure to 5000 mg/L of cetylpyridinium chloride.

Triclosan, povidone and benzalkonium chloride susceptibility

The same subgroup of Bcc strains selected for MBC analysis was tested for their growth susceptibility to triclosan, povidone and benzalkonium chloride (Table 2). All Bcc strains tested possessed povidone MICs in excess of 50000 mg/L, except for

one *B. pyrrocinia* strain (MIC 2000 mg/L). The mean maximum MIC observed for triclosan was 374 mg/L with 11 Bcc strains having MICs > 500 mg/L. The mean maximum MIC of benzalkonium chloride was 164 mg/L, similar to that of the other quaternary ammonium compound tested, cetylpyridinium chloride (155 mg/L for the subgroup of strains tested). Both *B. cenocepacia* J2315 and K56-2 from the ET12 epidemic lineage (which possessed high chlorhexidine and cetylpyridinium chloride MICs; Table 1) had high MICs of triclosan (> 500 mg/L), povidone [> 50000 mg/L (5%)] and benzalkonium chloride (350 mg/L).

Antibiotic susceptibilities

Antibiotic susceptibility to 10 antibiotics commonly used in CF was determined for 60 Bcc strains (Table 3). Clinically defined resistance or susceptibility to individual antibiotics varied widely for the Bcc strains tested. However, when the total antibiotic MIC score was examined, *B. cepacia*, *B. multivorans*, *B. cenocepacia* and *B. dolosa* possessed the greatest levels of multiple drug resistance (Table 3). *B. dolosa*, in particular, possessed very high levels of antibiotic resistance (Table 3); however, this did not correlate to elevated biocide resistance. For example, the *B. dolosa* strain LMG 18943, a strain associated with an outbreak in a hospital in Boston,²⁹ had a chlorhexidine MIC value of 20–30 mg/L and a cetylpyridinium chloride MIC value of 50–70 mg/L, but was resistant to all 10 antibiotics tested.

Overall, the Bcc strains examined were most susceptible to meropenem, imipenem, piperacillin and trimethoprim/sulfamethoxazole; *B. cenocepacia* strains such as J2315 were, however, innately resistant to this antibiotic combination. Spontaneous resistant colonies appearing within the zones of clearing on the Etest[®] strips occurred most frequently with the β -lactam antibiotics (meropenem, imipenem and piperacillin). In addition, *B. multivorans* and *B. cenocepacia* strains (41% and 33%, respectively, of the strains tested) produced the most

Table 3. Mean MIC (mg/L) values for Bcc species to 10 antibiotics

Organism (number of strains tested)	AMK	AZM	CAZ	CHL	CIP	IPM	MEM	PIP	TOB	SXT	Antibiotic profile score
<i>B. cepacia</i> (5)	109.6	125.3	5.98	13.3	5.2	17.6	0.9	13.1	88.23	0.5	37.9 ^a
<i>B. multivorans</i> (24)	81.6	110.4	52.9	101.6	1.976	25.8	2.2	21.4	30	0.4	42.8 ^b
<i>B. cenocepacia</i> (14)	111.5	81.2	2.6	12.2	1	35.6	3.1	23.4	37.5	9.1	31.7 ^c
<i>B. stabilis</i> (2)	8	32.7	1.6	22	16.3	3	1.5	3	4.5	2	9.5 ^d
<i>B. vietnamiensis</i> (4)	6.3	13.5	2.5	8	0.2	0.4	0.5	1.25	4.6	0.3	3.8
<i>B. dolosa</i> (4)	224	256	66.7	192.7	10.3	26.2	17.5	82.3	118	12	100.6 ^e
<i>B. ambifaria</i> (4)	9.8	23.6	1.2	3.25	0.1	1.1	0.2	1.1	6.6	0.1	4.7
<i>B. anthina</i> (1)	16	32	1.5	4	0.19	2	0.38	1	8	0.19	6.5 ^d
<i>B. pyrrocinia</i> (1)	0.094	24	0.75	1.5	0.003	0.19	0.002	0.25	2	0.002	2.9 ^d
<i>B. contaminans</i> (1)	1	16	1.5	8	0.25	24	0.75	1	0.38	0.125	5.3 ^d

AMK, amikacin; AZM, azithromycin; CIP, ciprofloxacin; CAZ, ceftazidime; CHL, chloramphenicol; IPM, imipenem; MEM, meropenem; PIP, piperacillin; TOB, tobramycin; SXT, trimethoprim/sulfamethoxazole.

^a*B. cepacia* was significantly more resistant than *B. vietnamiensis* and *B. ambifaria* ($P < 0.05$).

^b*B. multivorans* was significantly more resistant than *B. ambifaria* ($P < 0.05$).

^c*B. cenocepacia* was significantly more resistant than *B. vietnamiensis* and *B. ambifaria* ($P < 0.05$).

^d*B. stabilis*, *B. anthina*, *B. pyrrocinia* and *B. contaminans* were not statistically significant owing to small sample size.

^e*B. dolosa* was significantly more resistant than *B. stabilis*, *B. vietnamiensis* and *B. ambifaria* ($P < 0.05$).

spontaneous resistance to the β -lactams. All other Bcc species tested demonstrated low rates of this phenomenon with *B. vietnamiensis* (at 11% of strains tested) being the next highest observed.

Susceptibility to commercial biocide formulations

To determine whether commercial biocide formulation was bactericidal for Bcc strains that possessed high MIC and MBC to specific disinfectants, suspension tests were performed. Two biocides were evaluated: Hibiscrub, a chlorhexidine-based disinfectant used in clinical settings for pre-operative hand disinfection and skin antiseptics, and Cuticura, a hand gel sold for both home and clinical use. Four Bcc strains with high resistance to chlorhexidine (90–100 mg/L) were tested: *B. cepacia* LMG 18821, *B. multivorans* LMG 16660, *B. cenocepacia* J2315 and Bcc novel Group K strain 24. *B. multivorans* ATCC 17616 was used as a susceptible control isolate. Five minutes of exposure to the commercial biocide formulation was sufficient to achieve killing of three Bcc strains (strain LMG 16660, Group K strain 24 and ATCC 17616). However, *B. cepacia* LMG 18821 remained viable after 1 h of exposure to Cuticura (only a 1 log reduction in viable count was observed); the strain was killed within 5 min in Hibiscrub. *B. cenocepacia* J2315 remained viable in Hibiscrub after 1 h of exposure; however, it was more susceptible to Cuticura with complete killing observed after 20 min.

Biofilm production

Bacteria within biofilms are less susceptible to killing by antimicrobial agents than planktonic cells.³⁰ To determine whether there was a correlation between biofilm formation and biocide resistance in a planktonic state, 11 Bcc strains were screened for biofilm production. Six strains were selected with high (>80 mg/L) and five with low (<30 mg/L) resistance to chlorhexidine (Figure 1). No correlation between chlorhexidine MIC and the ability to form a biofilm was observed. For example, *B. multivorans* strain ATCC 17616, which possessed a

low chlorhexidine MIC (10–20 mg/L) and low MICs to the other biocide screens (Table 1), produced the most biofilms. In contrast, *B. cenocepacia* J2315, which was representative of a highly transmissible lineage associated with major clinical infections and possessed high biocide (Table 2) and antibiotic resistance (see above), was a poor biofilm former.

Discussion

Bcc bacteria are known to possess high levels of antimicrobial resistance; however, a survey of their biocide resistance that accounts for the recent changes in their taxonomy has not been performed. Our analysis of the biocides chlorhexidine, cetylpyridinium chloride, triclosan and benzalkonium chloride has demonstrated that susceptibility to these agents varies considerably across a panel of genetically distinct Bcc strains. Species-dependent differences were apparent only for cetylpyridinium chloride. No specific correlations between biocide susceptibility, antibiotic susceptibility and the ability to form biofilms were seen; however, certain CF outbreak strains possessed very high chlorhexidine resistance. The ability of biocide-resistant Bcc strains to survive exposure to two widely used commercial skin washing biocides was also demonstrated, suggesting that there is still room to improve disinfectant formulations to ensure they kill this group of bacteria.

While the correlation of antibiotic susceptibility to six genovars has been reported,⁹ the influence of Bcc taxonomy on biocide susceptibility has not been examined. The MIC for Bcc strains was investigated in great depth for chlorhexidine and cetylpyridinium chloride, with *B. cepacia*, *B. cenocepacia*, *B. lata*, *B. contaminans* and Bcc novel Group K being significantly less sensitive to cetylpyridinium chloride than other species. Previous work correlating clinical antibiotic resistance to Bcc taxonomy did not find any significant trends,⁹ however, we found that if an overall antibiotic resistance score was calculated based on the MIC value, then *B. cepacia*, *B. multivorans*, *B. cenocepacia* and *B. dolosa* were significantly more resistant. Interestingly, *B. cenocepacia*, which overall possessed both high biocide (Tables 1 and 2) and antibiotic resistance (Table 3), was also the most prevalent Bcc species encountered in a recent survey of both CF and other clinical infections.⁶

Correlations between antibiotic resistance and biocide resistance were not obvious among the Bcc bacteria. Certain epidemic Bcc strains such as those of the ET12 group (e.g. J2315, Table 2) possessed the highest levels of resistance to both classes of antimicrobial agents. The Glasgow outbreak *B. multivorans* strain²³ possessed chlorhexidine MICs that were nearly double that of other *B. multivorans* strains (Table 2), yet had similar levels of antibiotic resistance within this species group. Other strains, such as the epidemic *B. dolosa* LMG 18943,²⁹ possessed low chlorhexidine and cetylpyridinium chloride MICs (Table 2) but were resistant to all 10 antibiotics tested. There was also a lack of correlation between planktonic biocide resistance and the ability to form biofilms (Figure 1) for Bcc bacteria. This suggests that despite the advantages biofilm growth confers in terms of survival under antimicrobial stress,³⁰ not all Bcc bacteria have adopted this phenotype, but instead have other enhanced resistance mechanisms that function during planktonic growth. However, strains such as *B. cenocepacia* C4455 and Bcc novel Group K 24 (Figure 1), which have both high intrinsic biocide

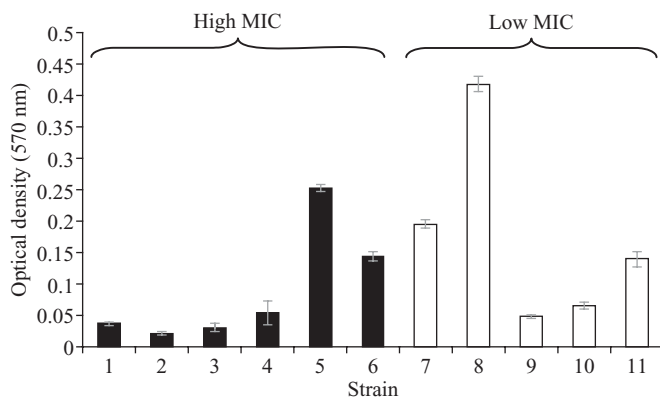


Figure 1. Biofilm production by selected Bcc strains. Six strains with high (black bars) and five with low (open bars) chlorhexidine MICs were tested for biofilm formation. The biofilm biomass readout is shown on the vertical axis. Strains are as follows: 1, *B. cepacia* LMG 18821; 2, *B. multivorans* LMG 16660; 3, *B. cenocepacia* LMG 18832; 4, *B. cenocepacia* J2315; 5, *B. cenocepacia* C4455; 6, *B. cepacia* novel Group K 24; 7, *B. multivorans* LMG 13010; 8, *B. multivorans* ATCC 17616; 9, *B. dolosa* LMG 18943; 10, *B. ambifaria* AMMD; 11, *Burkholderia vietnamiensis* G4.

B. cepacia complex biocide susceptibility

and a good ability to form biofilms, have the potential to be particularly problematic in contamination or outbreak scenarios. The lipopolysaccharide (LPS) structure of Bcc bacteria is very unusual and also varies between species.³¹ Given the important role this part of the Bcc cell envelope plays in its resistance to cationic antimicrobial agents, it would be interesting in future studies to compare the LPS structures of strains that are resistant or susceptible to membrane-active biocides such as chlorhexidine.

Maintaining product sterility is a problem faced by many manufacturing industries and a recent survey of the pharmaceutical industry has shown that Bcc bacteria are one of the leading causes of pharmaceutical product recalls.¹⁰ Our analysis of MBC data showed that Bcc bacteria may need more than 25 times more biocides over the MIC value to achieve killing. The concentrations of biocides used in commercial products are dependent on the biocide and type of product application. Chlorhexidine, cetylpyridinium chloride and triclosan are used in a variety of commercial products in concentrations ranging from 0.1% to 4% (1000–40000 mg/L).³² At the lowest level, this is insufficient to kill Bcc bacteria. For example, two *B. cenocepacia* strains had chlorhexidine MBCs of 1000 mg/L and 31 Bcc strains had cetylpyridinium chloride MBCs in excess of 1000 mg/L (Table 2). Dilute solutions of chlorhexidine appear particularly prone to contamination with Bcc bacteria, and outbreaks associated with mouthwash (0.2%)³³ or blood-product disinfectant solutions (0.5% and lower concentrations)¹³ have been reported. The MIC of five disinfectants used for organ irrigation in CF lung-transplant procedures was evaluated by Perry *et al.*³⁴ Taurolin and Noxyflex were found to be more inhibitory towards the 19 Bcc isolates tested than chlorhexidine, triclosan and povidone, and were specifically recommended for use where these bacteria may be present.³⁴

The efficacy of skin disinfection products and procedures may be tested according to standard European guidelines using bacterial species such as *E. coli*, *P. aeruginosa*, *S. aureus* and *Enterococcus hirae*.²⁵ Preservative challenge test guidelines include the *B. cepacia* Type strain ATCC 25416, among the recommended test strains.³⁵ However, although this strain possessed the maximal cetylpyridinium chloride MIC (>200 mg/L), its chlorhexidine MIC was 20–30 mg/L and was not among the most resistant encountered in our survey. Three well-defined strains stand out in our analysis as being resistant to multiple biocides (Table 2): (i) *B. cenocepacia* LMG 18832 (ATCC 17765), a urinary tract infection isolate;²² (ii) *B. cenocepacia* J2315 (LMG 16656), an epidemic CF isolate,²² and (iii) *B. multivorans* LMG 16660 (C1576), a CF isolate from the Glasgow outbreak.²³ All the latter strains were included in a historical reference panel of Bcc strains²² and are available from recognized strain collections. Given the major role Bcc organisms may play in the contamination of such products,¹⁰ future testing should also include these bacteria.

In summary, the characterization of Bcc as a group of problematic opportunistic pathogens has advanced considerably in the last decade.³ The ability to genetically define strains^{17,19} and systematically identify new species within the group^{4,5} has improved considerably. We have shown that as a closely related group of Gram-negative pathogens, Bcc bacteria possess very high resistance to biocides and commercial formulations of these agents. This understanding, together with the potential use of the biocide-resistant reference strains we have defined, can be used to develop new anti-infectives capable of killing Bcc

bacteria and preventing their occurrence in disease² and contamination of commercial products.¹⁰

Acknowledgements

We thank Alan Brown and John R.W. Govan (Edinburgh University) for providing additional Bcc strains and Gisli Einarsson (Queens University, Belfast) for information on the biofilm assay.

Funding

This work was funded via the UK Cystic Fibrosis Microbiology Consortium with grants from the CF Trust (Grant No. MbC003) and Big Lottery. MLST analysis was funded by grants from the Wellcome Trust (Grant No. 72853) and CF Trust (PJ535).

Transparency declarations

None to declare.

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