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Burkholderia Cepacia Complex in Personal Care Products: Molecular Epidemiology and Susceptibility to Preservatives

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Synopsis

Many outbreaks of *Burkholderia cepacia* complex (Bcc) infections are associated with contaminations in personal care products (PCPs). This study aimed to analyze a collection of Bcc isolates in PCPs and assess the susceptibility of preservatives, including dimethoxy dimethyl hydantoin (DMDMH), methylisothiazolinone–chloromethylisothiazolinone (MIT/cMIT), and methyl 4-hydroxybenzoate (MH). The Bcc isolates collected during the 3-year (2015–2017) study period were further examined by biochemical identification system, phylogenetic analysis based on *recA* nucleotide sequences, and multilocus sequence typing analysis. Preservatives susceptibility testing of Bcc bacteria were evaluated by minimum inhibitory concentration and minimum bactericidal concentration. A total of seven distinct sequence types (STs) were identified, which belonged to four different Bcc species: *Burkholderia cenocepacia* (ST621, ST258, and novel ST), *Burkholderia lata* (ST339 and ST336), *Burkholderia contaminans* (ST482), *Burkholderia cepacia* (ST922). For DMDMH and MH, the maximum permitted concentrations according to the safety specification of cosmetics (0.6% and 0.4%) were able to inhibit or kill all Bcc strains, but 40% of Bcc isolates could survive at higher than maximum permitted concentrations of MIT/cMIT (of a mixture in the ratio 3:1 of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one). The PCPs contamination of Bcc strains should be given more attention by manufacturers because of its diversity in molecular epidemiology and its low susceptibility to preservatives such as MIT/cMIT.

INTRODUCTION

Although personal care products (PCPs) are an indispensable part of everyone's daily grooming routine, the water and available nutrients they contain make them susceptible to microbial growth. Microbial contamination in PCPs, including toiletries and cosmetics, is very common and has been of great concern to the manufacturers of such products for many years. Most often, microorganisms are the cause of organoleptic alterations, such

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as offensive odors and changes in viscosity and color. Moreover, contaminating microorganisms may be pathogenic, exposing users to possible skin irritation, allergic contact dermatitis, and infection, especially in the eyes, mouth, or open wounds (1–5).

The Gram-negative species *Burkholderia cepacia* complex (Bcc) is emerging as one of the most intractable bacterial species to cause industrial contamination (6). Bcc organisms remain significant pathogens in patients with cystic fibrosis (CF) (7,8). Many nosocomial infections originate from the use of PCPs contaminated with Bcc. Outbreaks of *Burkholderia* infections due to contaminated mouthwash have been reported in hospitalized individuals (9–11). An outbreak of *B. cepacia* in an intensive care unit was caused by intrinsically contaminated moisturizing milk (12). An outbreak of *B. cepacia* complex was associated with contaminated liquid soap for hospital use (13). A hospital-wide outbreak of *Burkholderia contaminans* was caused by moist prefabricated washcloths (14).

Currently, the Bcc consists of at least 24 phenotypically similar but genotypically distinct Gram-negative bacteria (Table I) (15–30). According to a review of Food and Drug Administration (FDA) records, *B. cepacia* was the most common reason for recalls (34%), followed by *Pseudomonas* spp. (31). To explore the sources of potential contamination, it is first and foremost necessary to understand the species distribution characteristics. Until now, few studies have investigated the molecular epidemiology of the Bcc in PCPs. Identification of the Bcc at the species level in routine microbiology laboratories using manual and/or automated commercial systems is problematic because of the homogeneity of biochemical test results obtained from some species of Bcc, making them difficult to identify using phenotypic methods (32). Genetic methods such as 16S rRNA and *recA* gene sequence analysis have proven useful for Bcc species identification, but 16S rRNA gene sequencing is not sufficiently discriminatory to resolve all the species as the gene is >98% identical for members of the Bcc, and *recA* based species specific polymerase chain reaction (PCR) can identify most Bcc strains more accurately than 16S rRNA gene sequencing (7), although it cannot be used as a means to differentiate Bcc strains recovered from different sources, such as clinical infections versus those that occur in natural environments (33). Multilocus sequence typing (MLST) has been used for the molecular epidemiological study of Bcc since 2005 and improved in 2009; it is a globally accepted method that provides resolution at a species level higher than other methods (34,35). To date, approximately 1,722 sequence types (STs) of the Bcc have been identified worldwide, and the number of new cases identified by MLST increases each year. The ability of MLST to differentiate the existing Bcc species is greater than the analysis of the *recA* gene alone and also shows an excellent correlation to the multiple polyphasic taxonomic methods used to fully characterize these bacteria (7). Therefore, the *recA* gene and MLST are used in combination to more accurately identify species and molecular epidemiological types. Second, the sensitivity of different Bcc species to preservatives must be investigated because Bcc bacteria exhibit high levels of innate antimicrobial resistance to both antibiotics and biocides. Recently, research on the antimicrobial efficiency of preservatives in cosmetic products has received much attention. A survey of Bcc bacteria demonstrated that susceptibility to chlorhexidine, cetylpyridinium chloride, triclosan, benzalkonium chloride, and povidone biocides varied across the complex, with species-dependent differences in susceptibility being identified (36). Studies also indicated that the level of increased resistance is largely dependent on the dose, time of exposure, and bacterial species (37).

In this study, we collected 25 Bcc strains from the finished product of PCPs and determined strain diversity and species using MLST and *recA* gene sequence analysis. We also

Table I
The 24 Currently Established Species within the Bcc

Bcc species	Type strain	Source	Year identified and/or named	Country/region	Reference
B. catarinensis	DSM 103188T (BR 10601T)	Native grassland soil	2017	Southern Brazilian	(14)
B. alpine	PO-04-17-38^T = DSM 28031^T = LMG 28138^T	Volcanic soils	2017	Pico de Orizaba (Mexico)	(15)
B. puraquae	CAMPA 1040^T (=LMG 29660^T = DSM 103137^T)	Hospital and soils	2017	Argentina	(16)
B. paludis	MSh1^T (=DSM 100703^T = MCCC1K01245^T)	Surface peat	2016	Southeast Pahang, Malaysia	(17)
B. stagnalis	LMG28156^T (=CCUG65686^T)	Soil and water	2015	Australia	(18)
B. territorii	LMG 28158^T (=CCUG65687^T)	Soil and water sampling	2015	Territory of Australia	(18)
B. pseudomultivorans	LMG 26883^T (=CCUG 62895^T)	CF sputum	2013	USA	(19)
B. latens	FIRENZE 3^T = LMG 24064^T = CCUG 54555^T	CF sputum	2008	Italy	(20)
B. diffusa	AU1075^T = LMG 24065^T 5CCUG 54558^T	CF sputum	2008	USA	(20)
B. arboris	ES0263A^T = LMG 24066^T = CCUG 54561^T	Morris Arboretum	2008	Philadelphia (PA, USA)	(20)
B. seminalis	AU0475^T = LMG 24067^T = CCUG 54564^T	CF sputum	2008	USA	(20)
B. metallica	AU0553^T = LMG 24068^T = CCUG 54567^T	CF sputum	2008	USA	(20)
B. ubonensis	LMG 20358^T	Soil	2008	Thailand	(20)
B. dolosa	LMG 18943^T (=CCUG 47727^T)	Environment and CF sputum	2004	USA	(21)
B. cenocepacia	LMG 16656 (=NCTC 13227, ATCC BAA-245, CCM 4899)	CF sputum	2003	Edinburgh (UK)	(22)
B. anthina	LMG 20980 (=CCUG 46047)	Rhizosphere of a house-plant	2002	Nashville (TN, USA)	(23)
B. pyrrocinia	LMG 14095	Soil	2002	USA	(23)
B. contaminans	J2956^T (=LMG 23361^T = CCUG 55526^T)	Milk of a sheep with mastitis	2001	Spain	(24)
B. ambifaria	LMG 19182^T (=CCUG 44356^T)	Pea plants	2001	Wisconsin (USA)	(25)
B. stabilis	LMG 14294	CF sputum	2000	Leuven (Belgium)	(26)
B. multivorans	LMG 13010	CF sputum	1997	Brussels (Belgium)	(27)
B. lata	383^T (=ATCC 17760^T = LMG 22485^T = CCUG 55525^T)	Forest soil	1966	Trinidad	(24)
B. vietnamiensis	TVV75 (=LMG 10929)	Rice rhizosphere soil	1995	Vietnam	(28)
B. cepacia	Burkholder (=717-ICPB 25, =ATCC 25416, =NCTC 10743)	Rot of onion bulbs	1950	New York State (USA)	(29)

carried out investigation of Bcc susceptibility to the common preservatives used in PCPs. The aim of this study was to reveal insights into the diversity of Bcc isolates from PCPs and the susceptibility to different preservatives commonly used.

MATERIALS AND METHODS

BACTERIAL STRAINS

A total of 25 *B. cepacia* complex isolates were obtained from contaminated PCPs, including creams, shampoos, lotions, and conditioners, and stored for a period of 2 years (2015–2017) (Table II). *B. cepacia* ATCC 25416 was used as a reference strain in the study. All strains were grown in Tryptone soya agar (TSA; Oxoid™; Thermo Fisher Scientific, Waltham, MA) and incubated for 24–48 h at 37°C (±1°C). Strains were stored at −80°C (±1°C) with 20% glycerol until used. All Bcc samples were prepared in duplicate.

ANTIMICROBIALS

2-methyl-4-isothiazolin-3-one (MIT), consisting of a 3:1 (volume:volume) ratio of 5-chloro-2-methyl-4-isothiazolin-3-one (cMIT) and MIT at a final concentration of 14% (m/vol) total active ingredient, was obtained from Guangdong Demei Biology Technology Co., Ltd (Guangzhou, China). Dimethoxy dimethyl hydantoin (DMDMH; 55%, m/vol) and methyl 4-hydroxybenzoate (MH) were purchased from Sigma-Aldrich (St. Louis, MO).

ISOLATION AND IDENTIFICATION OF STRAINS

One gram of recovered PCPs was introduced onto the lecithin tween-80 nutrient agar plates (Guagdong Huankai Microbial Sci. & Tech. Co., Ltd., Guangzhou, China), the samples were streaked using a sterile loop, and the plates incubated for 18–24 h at 37°C. Gram staining and microscopic observation were performed on all isolated strains. Strains were identified by the API 20NE system (API-bioMérieux, La Balme les Grottes, France) according to the manufacturer's directions. Genomic DNA was isolated using a genomic DNA extraction kit from TaKaRa (Tokyo, Japan) according to the manufacturer's instructions for bacterial cells. PCR was performed using primer pairs for BCR1 (5′-TGACCGCCGAGAAGAGCAA-3′) and BCR2 (5′-CTCTTCTTCGTCCATCGCCTC-3′) in a Mastercycler® 5330 (Eppendorf AG, Hamburg, Germany) and run for 35 thermal cycles of 94°C (4 min), 58°C (30 s), and 72°C (30 s). A 10-min elongation step was included in the final cycle (38). The reaction products were separated and detected on an ABI PRISM Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA). Sequencing was performed by Huada Gene Technology Co. Ltd (Shenzhen, China).

The resulting sequences were assessed for similarities among known sequences using the Basic Local Alignment Tool at the National Center for Biotechnology Information (NCBI, NIH, Bethesda, MD). Multiple nucleotide sequence alignments of the *recA* gene were constructed using CLUSTAL X (Dublin, Ireland). Phylogenetic and molecular evolutionary

Table II
Bcc Strain Isolation Details along with Allelic Profiles and Sequence Type Identified

Strain ID	Year and month	Source	Microbial content (cfu g^{-1})	Type of preservative[a]	Preservative residual (mg kg^{-1})	*atpD*	*gltB*	*gyrB*	*recA*	*lepA*	*phaC*	*trpB*	ST
BC-01	2015.3	Shampoos	1.2×10^4	MIT/CMIT; PH	Not detected	15	11	481	14	11	6	147	621
BC-02	2015.3	Hair conditioners	2.0×10^6	DMDMH; MH; PH	Not detected	15	11	481	14	11	6	147	621
BC-03	2015.7	Shampoos	1.3×10^5	MH; PH	Not detected	361	325	640	382	431	329	330	922
BC-04	2015.7	Shampoos	1.7×10^5	MIT/CMIT	MIT:10.5	15	64	506	14	11	6	147	novel
BC-05	2016.1	Raw materials	1.2×10^6	/	/	361	325	640	382	431	329	330	922
BC-06	2016.1	Raw materials	3.6×10^4	/	/	361	325	640	382	431	329	330	922
BC-07	2016.1	Raw materials	4.8×10^5	/	/	361	325	640	382	431	329	330	922
BC-08	2016.1	Raw materials	2.5×10^5	/	/	15	11	481	14	11	6	147	621
BC-09	2016.7	Shampoos	2.9×10^5	MIT/CMIT; BP	MIT:13.3	15	11	481	14	11	6	147	621
BC-10	2016.11	Body lotion	3.1×10^5	MIT/CMIT	MIT:15.6	15	11	481	14	11	6	147	621
BC-11	2016.11	Body lotion	4.5×10^6	MIT/CMIT	MIT:14.8	15	11	481	14	11	6	147	621
BC-12	2016.11	Shower gel	2.3×10^5	MIT/CMIT	MIT:13.9	15	11	481	14	11	6	147	621
BC-13	2016.11	Shower gel	1.5×10^6	MIT/CMIT	MIT:10.8	15	11	481	14	11	6	147	621
BC-14	2016.11	Shower gel	2.3×10^6	MIT/CMIT	MIT:11.9	15	11	481	14	11	6	147	621
BC-15	2016.11	Shower gel	1.6×10^5	MIT/CMIT	MIT:14.5	15	11	481	14	11	6	147	621
BC-16	2017.1	Toner	2.3×10^4	/	/	184	224	301	200	219	53	210	339
BC-17	2017.1	Cream	1.5×10^5	MH; PH	Not detected	184	224	301	200	219	53	210	339
BC-18	2017.3	Cream	5.4×10^5	MH; PH	Not detected	15	11	481	14	11	6	147	621
BC-19	2017.3	Raw material	3.4×10^6	/	/	15	11	481	14	11	6	147	621
BC-20	2017.3	Production water	3.8×10^5	/	/	182	390	291	146	214	162	217	336
BC-21	2017.3	Production water	4.3×10^3	/	/	182	390	291	146	214	162	217	336
BC-22	2017.3	Bubble water	2.6×10^4	MH	Not detected	182	390	291	146	214	162	217	336
BC-23	2017.3	Bubble water	1.5×10^5	MH	Not detected	182	390	291	146	214	162	217	336
BC-24	2017.5	Baby lotion	5.3×10^4	/	/	151	192	254	152	158	173	151	482
BC-25	2017.5	Baby lotion	1.2×10^5	/	/	15	11	236	14	11	6	79	258

[a]The name of the preservative comes from the product packaging.
PH: propyl *p*-hydroxybenzoate; /: preservative free.

analyses were conducted using genetic-distance–based neighbor-joining algorithms within MEGA version 4.0 software package (Tokyo, Japan).

MLST LOCI AMPLIFICATION AND SEQUENCING

MLST was performed by sequencing the following housekeeping genes: ATP synthase beta chain (*atpD*), glutamate synthase large subunit (*gltB*), DNA gyrase subunit B (*gyrB*), recombinase A (*recA*), GTP-binding protein (*lepA*), acetoacetyl-CoA reductase (*phaC*), and tryptophan synthase subunit B (*trpB*) according to the previously published method available at www.pubmlst.org/Bcc. Primer sequences used for the MLST locus amplification are listed in Table III.

Genomic DNA was isolated using the genomic DNA extraction kit (TaKaRa) mentioned previously, according to the manufacturer's instructions for bacterial cells. Amplification of MLST loci was performed in 50 µl PCR volumes containing 2× EasyTaq® PCR SuperMix (TransGen Biotech, Beijing, China), 0.4 µM primers, DNA templates, and double distilled water. Amplification was performed with the Eppendorf Mastercycler® 5,330 using the following cycling conditions: initial denaturation for 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 30 s at an annealing temperature of 58°C, and 2 min at 72°C, followed by a final extension step of 5 min at 72°C. The reaction products were separated and detected on an ABI PRISM Genetic Analyzer 3100 (Applied Biosystems). The sequences from both strands of a given locus of the same isolate were aligned, trimmed to the desired length, and edited using the SeqMan II program from the Lasergene software package. The specific ST of the analyzed strains was determined using the *B. cepacia* complex multilocus sequence typing website (http://pubmlst.org/Bcc/) developed by Keith Jolley at the University of Oxford (England, United Kingdom) (39).

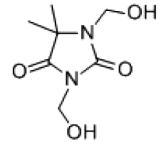
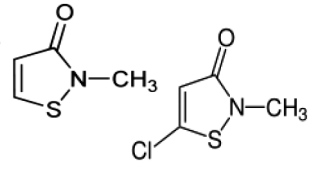
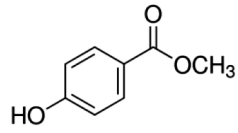
ANTIMICROBIAL SUSCEPTIBILITY TESTING

Determination of the MIC. Minimum inhibitory concentrations (MICs) were determined on a panel of 25 strains illustrating the diverse origins of the collection, broth microdilution using 96-well microplate. First, preservative mother solution was prepared according to the concentrations from Table IV and diluted 10 times, and then a double dilution up to six different dilutions (Table IV) was made. For each bacterial isolate to be tested, 100 µl

Table III
List of Primers Used in the MLST Locus Amplification

Gene name	Size (bp) of fragment analyzed	Locus primer (5′→3′)	Locus primer (5′→3′)
atpD	443	GTTCATCTGGCCGTACAC	AACTGACGCTCGAAGTCC
gltB	400	CTTCTTCTTCGTCGCCGA	TTGCCGACGTAGTCGTTG
gyrB	454	ATCGTGATGACCGAGCTG	CGTTGTAGCTGTCGTTCC
recA	393	TGACCGCCGAGAAGAGCAA	GACCGAGTCGATGACGAT
lepA	397	GGCATCAAGGAACTGACG	CTGCGGCATGTACAGGTT
phaC	385	AGACGGCTTCAAGGTGGT	ACACGGTGTTGACCGTCA
trpB	301	CTGGGTCACGAACATGGA	CCGAATGCGTCTCGATGA

Table IV
Compounds Used in this Study

Compounds	Range tested	Mother solution	Chemical formula	Chemical structures
Dimethoxy dimethyl hydantoin (DMDMH 0.6%)[a]	0.0125–0.4%	4.0%	$C_7H_{12}N_2O_4$	
Methylisothiazolinone and chloromethylisothiazolinone (MIT/cMIT 0.0015%)[a]	1.5625×10^{-4}–0.05%	0.5%	C_4H_5NOS C_4H_4NClOS	
Methyl 4-hydroxybenzoate (MH 0.4%)[a]	0.0125–0.4%	4.0%	$C_8H_8O_3$	

[a]Maximum permitted concentrations.

of each preservative dilution was added into the respective well, and 200 µl of broth was pipetted into the sterility control well (G well in 96-microplate) and 100 µl into the growth control well (H well in 96-microplate). Second, the bacterial isolates were streaked onto MH agar plates and incubated for 18–24 h at 37°C. For each isolate, three to five colonies were transferred into the MH broth, and the bacterial suspension was adjusted to 1×10^8 cfu ml^{-1} (the OD_{600} is about 0.1) by vortexing and diluted by a factor of 1:100. The test and control wells (growth control) were inoculated with 100 µl of the bacterial suspension. This results in the final desired inoculum of 5×10^5 cfu ml^{-1}. A 10-µl sample from the growth control tube was removed immediately after inoculation and pipetted into a sterile tube holding 990 µl of sterile saline. The sample was mixed well by vortexing. A further dilution of this suspension (1:10) was made by pipetting 100 µl into 900 µl of sterile saline. Then, 100 µl of each of the two dilutions was plated onto two agar plates. The agar plates were incubated at 37°C for 24 h. The purpose of this step was to ensure the accuracy of the bacterial inoculation amount. Finally, the 96-well plates were placed in a multifunctional microplate reader (TECAN Spark, Shanghai, China) for incubation for 24–30 h. Growth curves were obtained by measuring OD_{600} at 37°C every hour. The MIC was defined as the lowest concentration of an antimicrobial at which no bacterial growth was observed visually on the well plate and without change in OD_{600} compared with the negative control from the growth curves. These tests were repeated three times.

Determination of the MBCs. Minimum bactericidal concentrations (MBCs) were determined after the MIC test. Bacterial suspensions in well plates were resuspended at $1/10^e$ and $1/100^e$ in neutralizing solution (Fischer Scientific Bioblock, Shanghai, China), which was commonly used in cosmetics laboratories to check the presence of surviving bacteria by inhibiting preservative activity. In detail, a 10-µl sample was removed from MIC test wells and introduced into a sterile tube holding 90 µl ($1/10^e$) and 990 µl ($1/100^e$) of neutralizing solution, then 100 µl and 1 ml of each of the two dilutions were plated onto two agar plates

and incubated at 37°C for 24 h (40). The MBC was estimated as the lowest concentration of preservative with the absence of colony in the case of agar plates.

RESULTS

BCC SPECIES ISOLATION AND IDENTIFICATION

We collected 25 Bcc strains from 2015 to 2017, isolated from shampoos, hair conditioners, raw materials, body lotion, shower gel, toner, cream, production water, and bubble water. The number of bacteria exceeds 10^3 cfu ml^{-1}, of which 18 Bcc strains are greater than 10^5 cfu ml^{-1}. The types of preservatives were MIT/cMIT, DMDMH, and MH. Except for the two PCPs (body lotion and shower gel) collected in July and November 2016, other types of cosmetics can be detected with residual MIT, the content of which is between 10.8 and 15.6 mg kg^{-1} (Table II). All of the spoilage strains were Gram-negative, non–spore-forming rods, and four species were identified by *recA* nucleotide sequence analysis, including *Burkholderia cenocepacia*, *B. contaminans*, *Burkholderia lata*, and *B. cepacia*, with the phylogenetic tree of Bcc based on the *recA* gene sequence (Figure 1).

BCC SPECIES DIVERSITY IN CONTAMINATED PCPS

RecA gene sequence and MLST analysis of a collection of 25 Bcc isolates recovered from PCPs revealed the following species diversity: *B. cenocepacia* (ST621, ST258, and nST), *B. lata* (ST339 and ST336), *B. contaminans* (ST482), and *B. cepacia* (ST922). A total of seven distinct STs were identified belonging to four different Bcc species, including one novel ST. The new ST type, BC-04, was identified as *B. cenocepacia* by *recA* gene, the evolutionary tree established by *recA* gene showed that it was not in the same branch as other *B. cenocepacia*, and the MLST results also showed the *gltB* and *gyrB* were 64 and 506, which were different among the seven housekeeping genes. These all indicate that BC-04 might be the new ST type of *B. cenocepacia* (Figure 1). The proportion of 48% of stains we detected is *B. cenocepacia* ST621, the dominant STs were ST621 among all ST types, followed by ST339 (16%) and ST336 (16%).

The colony morphology of Bcc species grown on agar was different according to STs (Figure 2). Different ST types in the same Bcc show different colony morphologies. It was worth noting that the colonies of *Burkholderia cepacia* (ST922) showed a purple pigment spreading from the center to the edge (add 5 µl of the bacterial solution that was stored in a glycerol storage tube to the TSA plate and incubate at 37°C for 5 days); this phenomenon might become a specific phenotype of ST922.

SUSCEPTIBILITY OF BCC STRAINS ISOLATED FROM CONTAMINATED PCPS

MIC and MBC methods allow the rapid evidence of bacterial susceptibility in response to biocides contact and action in general, and to preservatives in particular. In this study, the methods were used to evaluate the susceptibility of preservatives agents (DMDMH, MIT/cMIT, and MH) for a collection of 25 Bcc strains and *B. cepacia* ATCC 25416. The mean of MICs and MBCs demonstrated susceptibility varied within Bcc species (Table V). Our

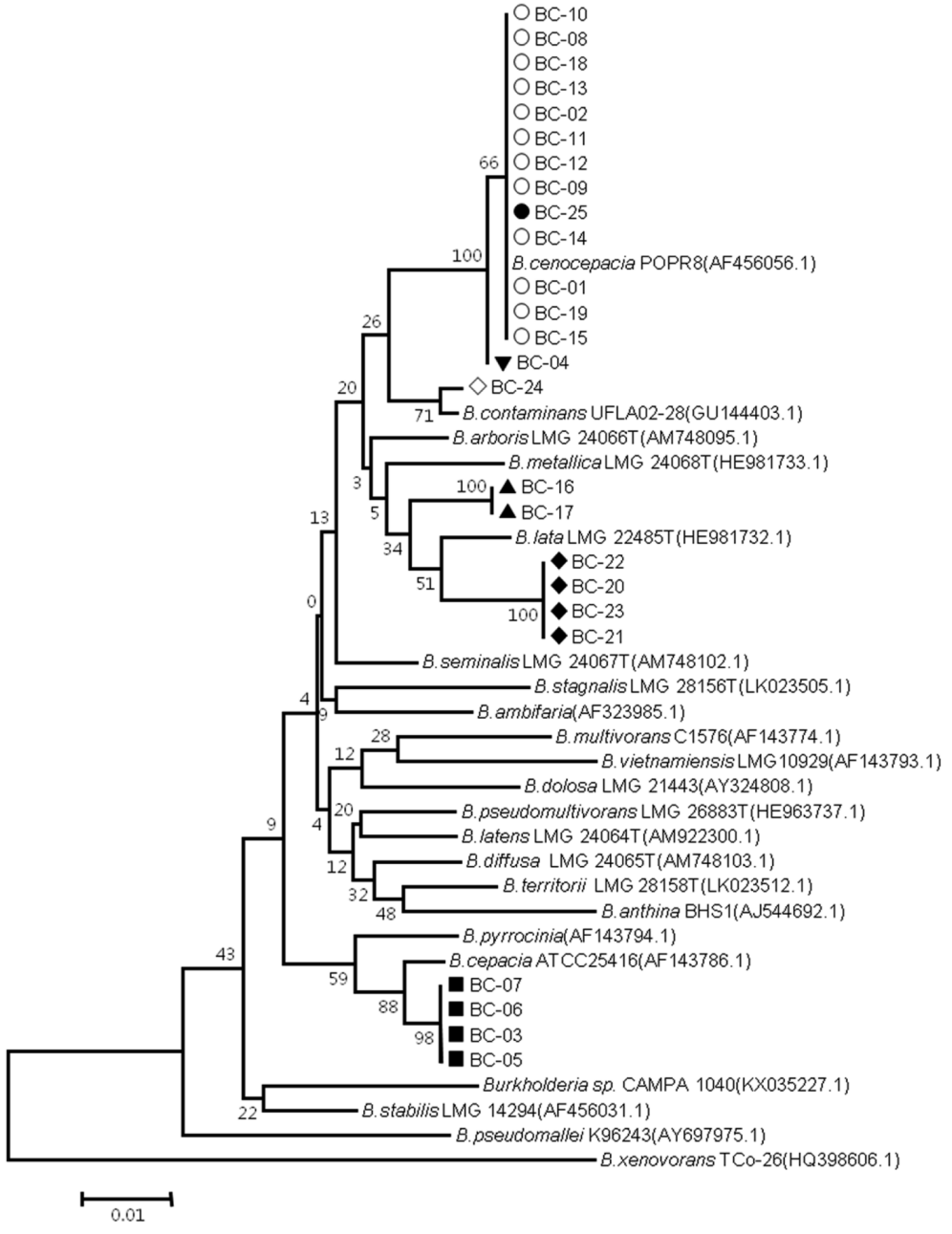


Figure 1. Phylogenetic tree of the *B. cepacia* complex based on the complete *recA* gene sequence. Bootstrap values are shown on each horizontal limb of the tree. The tree was rooted with the *B. xenovorans* strain TCo-26 *recA* gene as a representative member of a species outside the Bcc group. Different symbols represent different ST types: ○: ST621; ▼: novel ST; ■: ST922; ◆: ST336; ◇: ST482; ▲: ST339; ●: ST258.

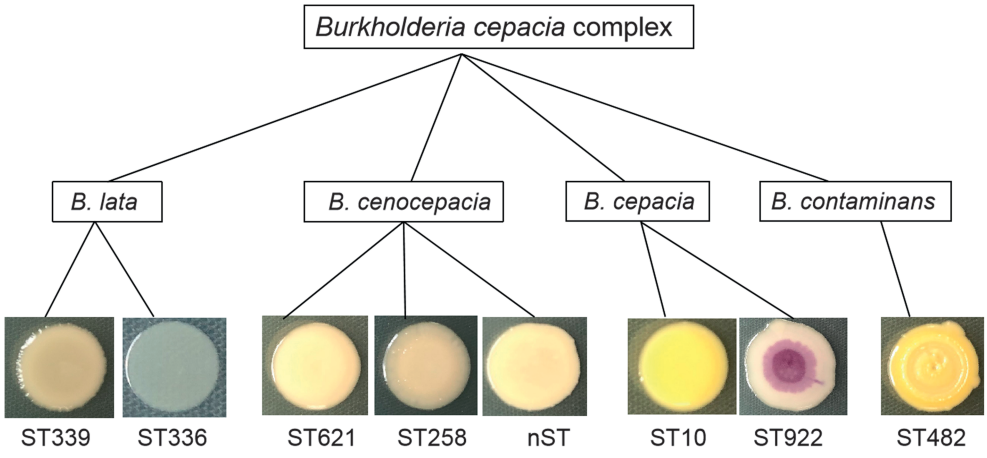


Figure 2. Morphological differences of Bcc bacteria grown on TSA.

protocol involved performing MIC/MBC testing in 2-fold dilutions, and the MIC and MBC values were highly reproducible across three separate experiments, as indicated by low standard deviations.

Growth curves were also performed in this study to determine the susceptibility of all Bcc in the presence of three preservatives (DMDMH, MIT/cMIT, and MH). Part of the growth curves of four different types of Bcc and the standard strain are shown in Figure 3. When the concentration of MIT/cMIT was 0.00015625% and 0.003125%, the *B. cepacia* ATCC 25416 could grow well; except for 0.005%, other low concentrations of MIT/cMIT could not inhibit the growth of BC12 (*B. cenocepacia*); the MIT/cMIT MIC to BC12 was 8-fold greater than it to *B. cepacia* ATCC 25416. For BC05 (*B. cepacia*), the MIT/cMIT MIC was 0.0025%. For BC16 (*B. lata*) and BC04 (*B. contaminans*), the OD_{600} values of the wells were unchanged, except for the positive control wells after 16 h of incubation. When cultured to 24 h, MIT concentration of 0.0003125% and 0.00015625% could not inhibit the growth of BC16, and 0.00015625% MIT could not prevent the reproduction of BC24.

Overall, the MBC values for three preservatives were higher than or equal to those of MIC for all strains, and MBC dilutions at $1/100^{e}$ were higher than or equal to that dilution at $1/10^{e}$. For DMDMH and MH, the maximum permitted concentrations according to the safety specification of cosmetics (0.6% and 0.4%) were able to inhibit or kill all strains. Regarding the MIT/cMIT, the MIC values for 10 strains (BC09-13, BC18, and BC20-23), belonging to *B. cenocepacia* (ST621) and *B. lata* (ST336), were higher than the maximum permitted concentrations (0.0015%); the MBC values even reached 0.005% or 0.01% (Table V); and these Bcc strains were mainly isolated from cosmetics containing the preservatives of MIT/cMIT.

DISCUSSION

Bcc members consist of multiple closely related species that are ubiquitous in nature. Phenotypic variation can occur in all Bcc species, even within sequential clinical isolates of the same strain (41). There have also been reports of morphological differences based

Table V
MIC and MBC Values of Three Common Preservatives for Bcc

			DMDMH (%) (0.6%)[a]			MIT/CMIT (%) (0.0015%)[a]			MH (%) (0.4%)[a]		
				MBC			MBC (e^{-4})			MBC	
Strain ID	Species	STs	MIC	1/10[e]	1/100[e]	MIC (e^{-4})	1/10[e]	1/100[e]	MIC	1/10[e]	1/100[e]
BC-00	*B. cepacia* ATCC 25416	ST10	0.05	0.1	0.2	3.125	6.25	6.25	0.025	0.05	0.05
BC-01	*B. cenocepacia*	ST621	0.2	0.2	0.4	6.25	12.5	12.5	0.025	0.05	0.05
BC-02	*B. cenocepacia*	ST621	0.2	0.2	0.4	6.25	6.25	12.5	0.025	0.05	0.05
BC-04	*B. cenocepacia*	nST	0.05	0.1	0.1	6.25	6.25	12.5	0.025	0.05	0.05
BC-08	*B. cenocepacia*	ST621	0.2	0.2	0.2	12.5	25	25	0.05	0.1	0.1
BC-09	*B. cenocepacia*	ST621	0.2	0.2	0.4	50	50	50	0.05	0.1	0.1
BC-10	*B. cenocepacia*	ST621	0.1	0.2	0.4	25	50	50	0.05	0.1	0.1
BC-11	*B. cenocepacia*	ST621	0.1	0.4	0.4	25	50	100	0.05	0.1	0.1
BC-12	*B. cenocepacia*	ST621	0.1	0.2	0.2	50	50	50	0.05	0.1	0.1
BC-13	*B. cenocepacia*	ST621	0.1	0.2	0.2	50	50	100	0.05	0.1	0.1
BC-14	*B. cenocepacia*	ST621	0.2	0.2	0.4	12.5	25	50	0.05	0.1	0.1
BC-15	*B. cenocepacia*	ST621	0.2	0.2	0.4	6.25	12.5	12.5	0.05	0.1	0.1
BC-18	*B. cenocepacia*	ST621	0.2	0.2	0.2	25	25	25	0.1	0.1	0.1
BC-19	*B. cenocepacia*	ST621	0.2	0.2	0.2	12.5	12.5	25	0.05	0.05	0.05
BC-25	*B. cenocepacia*	ST258	0.025	0.05	0.1	3.125	3.125	6.25	0.05	0.05	0.1
BC-03	*B. cepacia*	ST922	0.1	0.1	0.2	3.125	6.25	6.25	0.05	0.05	0.1
BC-05	*B. cepacia*	ST922	0.2	0.2	0.2	12.5	25	25	0.05	0.05	0.1
BC-06	*B. cepacia*	ST922	0.2	0.2	0.2	12.5	25	25	0.05	0.05	0.05
BC-07	*B. cepacia*	ST922	0.2	0.2	0.2	12.5	25	25	0.05	0.05	0.05
BC-16	*B. lata*	ST339	0.2	0.2	0.2	6.25	6.25	12.5	0.1	0.1	0.1
BC-17	*B. lata*	ST339	0.1	0.1	0.1	6.25	6.25	6.25	0.05	0.1	0.1
BC-20	*B. lata*	ST336	0.1	0.4	0.4	25	50	50	0.25	0.1	0.1
BC-21	*B. lata*	ST336	0.1	0.4	0.4	25	50	50	0.25	0.1	0.1
BC-22	*B. lata*	ST336	0.05	0.2	0.2	25	50	100	0.1	0.2	0.2
BC-23	*B. lata*	ST336	0.2	0.2	0.2	50	50	50	0.1	0.2	0.2
BC-24	*B. contaminans*	ST482	0.1	0.2	0.2	3.125	3.125	6.25	0.05	0.1	0.2

[a]The maximum level for use in rinse-off personal-care products according to the Safety and Technical Standards for Cosmetics (2015 edition).

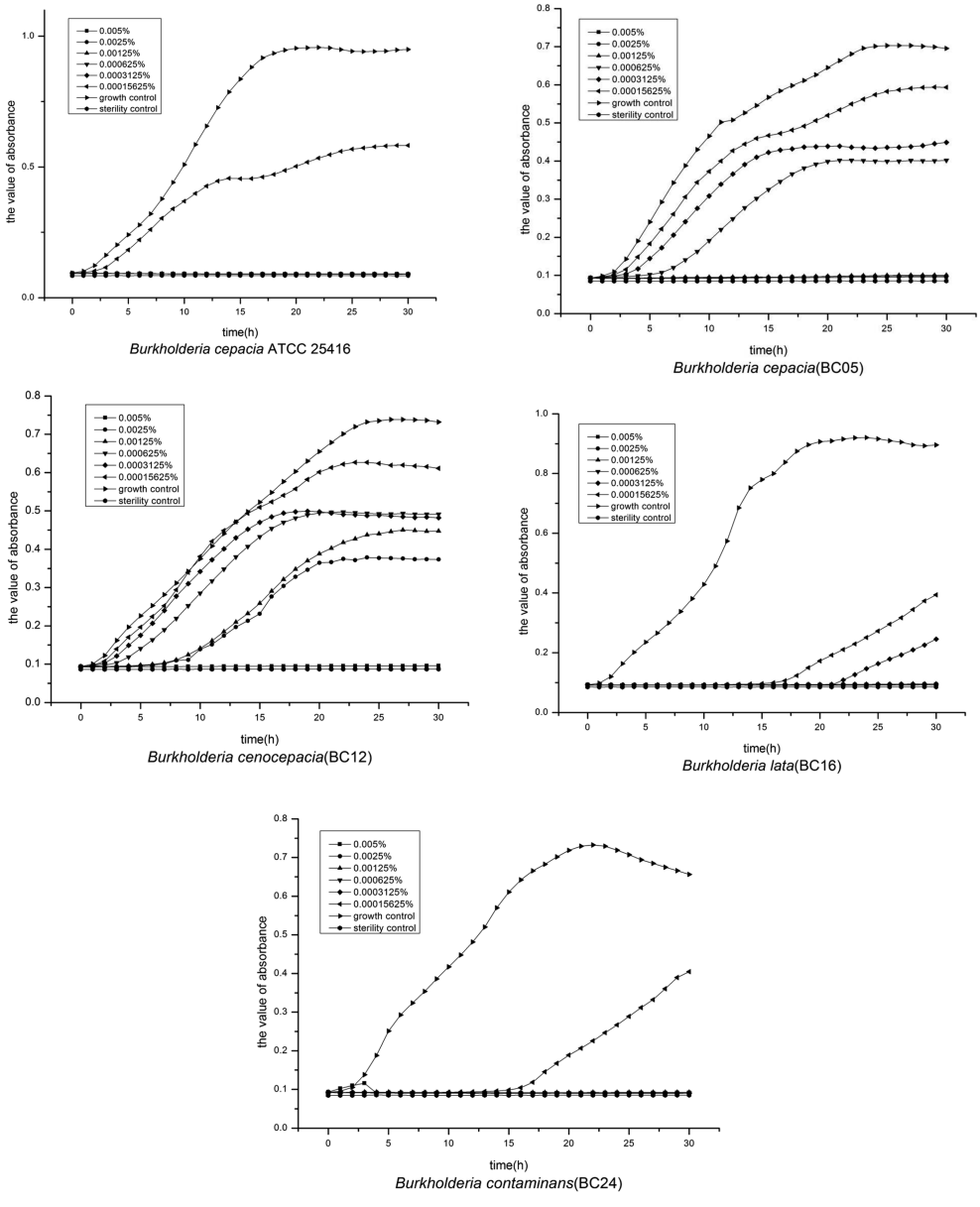


Figure 3. The growth curves of four types of Bcc strains and the standard strain in the presence of different concentrations of MIT/cMIT.

on different nitrogen sources (7). This phenotypic variability makes it difficult to correctly identify strains in diagnostic microbiology laboratories (42). This variation also occurred in the present study, seven ST-types Bcc colonies showed different forms on TSA, and novel ST type was confirmed because *gltB* and *gyrB* were different among the seven housekeeping genes. The emergence of new strains or ST types may be due to the adaptive changes of the strains to the oligotrophic environment, which may indicate an increase in the number of resistant strains or an enhancement ability of strain resistance.

In the present study, most of the contaminated bacterial count (72%) is greater than 10^5 cfu ml^{-1}; it was a huge amount in PCPs, and most contaminating bacteria showed reduced susceptibility in products where MIT/cMIT is present, which is consistent with previous studies (43), indicating that the MIT/cMIT in these products cannot inhibit the growth of Bcc for several reasons: (i) intrinsic and acquired resistance in Bcc bacteria, including efflux pump, outer membrane permeability barrier, and alterations in drug targets (44); (ii) Bcc bacteria has an adaptive mechanism for oligotrophic environments that can use substances in the environment as a source of nutrients for metabolism; and (iii) preservative compatibility with formulations: formulation water activity, pH range, and partition coefficients of preservatives (e.g., oil-in-water and water-in-oil emulsions). The work of the resistance mechanism will be performed in the next part of our study.

B. cenocepacia is considered one of the most frequent isolates in our collection. *B. cenocepacia* ST621 was also identified in 42 Bcc isolates, which were obtained from tertiary referral hospitals (45). The fact that these STs from PCPs were also isolated from patients suggested that conservation of intrinsic determinants necessary to thrive in PCPs may confer an ability to colonize susceptible humans. Similar reports showed that 21.5% of the clinical isolates were indistinguishable by MLST in isolates from environmental sources (46). Therefore, more detailed MLST research is needed to study the pathogenic members of Bcc in nonclinical settings, and Bcc from clinical sources will be collected for further research.

In this study, a total of 25 Bcc isolates were obtained from contaminated PCPs. *B. cenocepacia* was the most common contaminating microorganism, followed by *B. lata* and *B. cepacia*. The most common molecular type was ST621. However, Bcc is susceptible to genetic mutations that lead to the emergence of new types of mutations. Therefore, continuous monitoring and prevention of Bcc stains is extremely necessary, and the low susceptibility of Bcc to MIT/cMIT is one of the important reasons for its contamination of PCPs. This characteristic of Bcc should be taken seriously by manufacturers to avoid more nosocomial infection due to the use of Bcc-contaminated PCPs.

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REFERENCES

(1) M. Saeb-Lima, G. V. Solis-Arreola, and A. Fernandez-Flores, Mycobacterial infection after cosmetic procedure with botulinum toxin A, *J. Clin. Diagn. Res.*, 9, WD01–WD02 (2015).
(2) K. L. Warburton and M. Wilkinson, Contact allergy to methylisothiazolinone: has there been any change? Experience of a UK centre, *Contact Dermatitis*, 72, 398–400 (2015).
(3) R. Campana, C. Scesa, V. Patrone, E. Vittoria, and W. Baffone, Microbiological study of cosmetic products during their use by consumers: health risk and efficacy of preservative systems, *Lett. Appl. Microbiol.*, 43, 301–306 (2006).
(4) A. S. B. Tan, M. Tuysuz, and G. Otuk, Investigation of preservative efficacy and microbiological content of some cosmetics found on the market. *Pak. J. Pharm. Sci.*, 26, 153–157 (2013).

(5) M. D. Lundov, L. Moesby, C. Zachariae, and J. D. Johansen, Contamination versus preservation of cosmetics: a review on legislation, usage, infections, and contact allergy, *Contact Dermatitis*, **60**, 70–78 (2009).
(6) L. Torbeck, D. Raccasi, D. Guilfoyle, R. L. Friedman, and D. Hussong, *Burkholderia cepacia*: this decision is overdue, *PDA J. Pharm. Sci. Technol.*, **65**, 535–543 (2011).
(7) E. Mahenthiralingam, A. Baldwin, and C. G. Dowson, *Burkholderia cepacia* complex bacteria: opportunistic pathogens with important natural biology, *J. Appl. Microbiol.*, **104**, 1539–1551 (2008).
(8) R. Reik, T. Spilker, and J. J. Lipuma, Distribution of *Burkholderia cepacia* complex species among isolates recovered from persons with or without cystic fibrosis, *J. Clin. Microbiol.*, **43**, 2926–2928 (2005).
(9) P. K. Kutty, B. Moody, J. S. Gullion, M. Zervos, M. Ajluni, R. Washburn, R. Sanderson, M. Kainer, T. Powell, C. Clarke, R. J. Powell, N. Pascoe, A. Shams, J. LiPuma, B. Jensen, J. Noble-Wang, M. Arduino, and L. McDonald, Multistate outbreak of *Burkholderia cenocepacia* colonization and infection associated with the use of intrinsically contaminated alcohol-free mouthwash, *Chest*, **132**, 1825–1831 (2007).
(10) L. Matrician, G. Ange, S. Burns, W. L. Fanning, C. Kioski, G. D. Cage, and K. K. Komatsu, Outbreak of nosocomial *Burkholderia cepacia* infection and colonization associated with intrinsically contaminated mouthwash. *Infect. Control Hosp. Epidemiol.*, **21**, 739–741 (2000).
(11) J. Molina-Cabrillana, M. Bolaños-Rivero, E. E. Alvarez-León, A. M. M. Sánchez, M. Sánchez-Palacios, D. Alvarez, and J. A. Sáez-Nieto, Intrinsically contaminated alcohol-free mouthwash implicated in a nosocomial outbreak of *Burkholderia cepacia* colonization and infection. *Infect. Control Hosp. Epidemiol.*, **27**, 1281–1282 (2006).
(12) F. Álvarez-Lerma, E. Maull, R. Terradas, C. Segura, I. Planells, P. Coll, H. Knobel, and A. Vázquez, Moisturizing body milk as a reservoir of *Burkholderia cepacia*: outbreak of nosocomial infection in a multidisciplinary intensive care unit, *Crit. Care*, **12**, R10 (2008).
(13) K. Süer, M. Güvenir, B. Otlu, and E. Tunç, Outbreak of *Burkholderia cepacia* complex associated with contaminated liquid soap for hospital use: a case study, *Afr. J. Microbiol. Res.*, **10**, 791–795 (2016).
(14) M. Martin, B. Christiansen, G. Caspari, M. Hogardt, A. J. Von Thomsen, E. Ott, and F. Mattner, Hospital-wide outbreak of *Burkholderia contaminans* caused by prefabricated moist washcloths, *J. Hosp. Infect.*, **77**, 267–270 (2011).
(15) E. Bach, F. H. Sant'Anna, J. F. Magrich dos Passos, E. Balsanelli, V. A. de Baura, F. O. Pedrosa, E. M. de Souza, and L. M. P. Passaglia, Detection of misidentifications of species from the *Burkholderia cepacia* complex and description of a new member, the soil bacterium *Burkholderia catarinensis* sp. nov, *Pathog. Dis.*, **75**, ftx076 (2017).
(16) C. F. Weber and G. M. King, Volcanic soils as sources of novel CO-Oxidizing *Paraburkholderia* and *Burkholderia: Paraburkholderia hiiakae* sp. nov., *Paraburkholderia metrosideri* sp. nov., *Paraburkholderia paradisi* sp. nov., *Paraburkholderia peleae* sp. nov., and *Burkholderia alpina* sp. nov.a member of the *Burkholderia cepacia* complex, *Front. Microbiol.*, **8**, 207 (2017).
(17) P. Martina, M. Leguizamon, C. I. Prieto, S. A. Sousa, P. Montanaro, W. O. Draghi, M. Stämmler, M. Bettiol, C. C. C. R. de Carvalho, J. Palau, C. Figoli, F. Alvarez, S. Benetti, S. Lejona, C. Vescina, J. Ferreras, P. Lasch, A. Lagares, A. Zorreguieta, J. H. Leitão, O. M. Yantorno, and A. Bosch, *Burkholderia puraquae* sp.nov., a novel species of the *Burkholderia cepacia* complex isolated from hospital settings and agricultural soils, *Int. J. Syst. Evol. Microbiol.*, **68**, 14–20 (2017).
(18) K. S. Ong, Y. K Aw, L. H. Lee, C. M. Yule, Y. L. Cheow, and S. M. Lee, *Burkholderia paludis* sp. nov., an antibiotic-siderophore producing novel *Burkholderia cepacia* complex species, isolated from Malaysian tropical peat swamp soil, *Front. Microbiol.*, **7**, 2046 (2016).
(19) B. De Smet, M. Mayo, C. Peeters, J. E. Zlosnik, T. Spilker, T. J. Hird, J. John, L. Puma, T. J. Kidd, M. Kaestli, J. L. Ginther, D. M. Wagner, P. Keim, S. C. Bell, J. A. Jacobs, B. J. Currie, and P. Vandamme, *Burkholderia stagnalis* sp. nov. and *Burkholderia territorii* sp. nov., two novel *Burkholderia cepacia* complex species from environmental and human sources, *Int. J. Syst. Evol. Microbiol.*, **65**, 2265–2271 (2015).
(20) C. Peeters, J. E. Zlosnik, T. Spilker, T. J. Hird, J. J. LiPuma, and P. Vandamme, *Burkholderia pseudomultivorans*sp.nov., a novel *Burkholderia cepacia* complex species from human respiratory samples and the rhizosphere, *Syst. Appl. Microbiol.*, **36**, 483–489 (2013).
(21) E. Vanlaere, J. J. LiPuma, A. Baldwin, D. Henry, E. De Brandt, E. Mahenthiralingam, D. Speert, C. Dowson, and P. Vandamme, *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica s*p. nov., novel species within the *Burkholderia cepacia* complex, *Int. J. Syst. Evol. Microbiol.*, **58**, 1580–1590 (2008).

(22) K. Vermis, T. Coenye, J. J. LiPuma, E. Mahenthiralingam, H. J. Nelis, and P. Vandamme, Proposal to accommodate *Burkholderia cepacia* genomovar VI as *Burkholderia dolosa* sp. nov. *Int. J. Syst. Evol. Microbiol.*, **54**, 689–691 (2004).
(23) P. Vandamme, B. Holmes, T. Coenye, J. Goris, E. Mahenthiralingam, J. J. LiPuma, and J. R. Govan, *Burkholderia cenocepacia* sp. nov.-a new twist to an old story, *Res. Microbiol.*, **154**, 91–96 (2003).
(24) P. Vandamme, D. Henry, T. Coenye, S. Nzula, M. Vancanneyt, J. J. LiPuma, D. P. Speert, J. R. W. Govan, and E. Mahenthiralingam, *Burkholderia anthina* sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia cepacia* complex bacteria, may confound results of new molecular diagnostic tools, *FEMS Immunol. Med. Microbiol.*, **33**, 143–149 (2002).
(25) E. Vanlaere, A. Baldwin, D. Gevers, D. Henry, E. De Brandt, J. J. LiPuma, E. Mahenthiralingam, D. P. Speert, C. Dowson, P. Vandamme, and K. Taxon, A complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. *Int. J. Syst. Evol. Microbiol.*, **59**, 102–111 (2009).
(26) T. Coenye, E. Mahenthiralingam, D. Henry, J. J. LiPuma, S. Laevens, M. Gillis, D. P. Speert, and P. Vandamme, *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates, *Int. J. Syst. Evol. Microbiol.*, **51**, 1481–1490 (2001).
(27) P. Vandamme, E. Mahenthiralingam, B. Holmes, T. Coenye, B. Hoste, P. De Vos, D. Henry, and D. P. Speert, Identification and population structure of *Burkholderia stabilis* sp. nov.(formerly *Burkholderia cepacia* Genomovar IV), *J. Clin. Microbiol.*, **38**, 1042–1047 (2000).
(28) P. Vandamme, B. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauwers, M. Gillis, K. Kersters, and J. R. W. Govan, Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov, *Int. J. Syst. Evol. Microbiol.*, **47**, 1188–1200 (1997).
(29) M. Gillis, T. Van, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kersters, T. Heulin, and M. P. Fernandez, Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N2-fixing isolates from rice in Vietnam, *Int. J. Syst. Evol. Microbiol.*, **45**, 274–289 (1995).
(30) W. H. Burkholder, Sour skin, a bacterial rot of onion bulbs, *Phytopathology*, **40**, 115–117 (1950).
(31) S. Sutton and L. Jimenez, Microbiology-A review of reported recalls involving microbiological control 2004–2011 with emphasis on FDA considerations of "objectionable organisms", *Am. Pharmaceut. Rev.*, **15**, 42 (2012).
(32) Y. C. Chien, C. H. Liao, W. H. Sheng, J. Y. Chien, Y. T. Huang, C. J. Yu, and P. R. Hsueh, Clinical characteristics of bacteraemia caused by *Burkholderia cepacia* complex species and antimicrobial susceptibility of the isolates in a medical centre in Taiwan. *Int. J. Antimicrob. Agents*, **51**, 357–364 (2018).
(33) G. W. Payne, P. Vandamme, S. H. Morgan, J. J. Lipuma, T. Coenye, Weightman A. J., T. Hefin-Jones, and E. Mahenthiralingam, Development of a recA gene-based identification approach for the entire burkholderia genus, *Appl. Environ. Microbiol.*, **71**, 3917 (2005).
(34) A. Baldwin, E. Mahenthiralingam, K. M. Thickett, D. Honeybourne, M. C. Maiden, J. R. Govan, D. P. Speert, J. J. LiPuma, P. Vandamme, and C. G. Dowson, Multilocus sequence typing scheme that provides both species and strain differentiation for the *Burkholderia cepacia* complex, *J. Clin. Microbiol.*, **43**, 4665–4673 (2005).
(35) T. Spilker, A. Baldwin, A. Bumford, C. G. Dowson, E. Mahenthiralingam, and J. J. Lipuma, Expanded multilocus sequence typing for *Burkholderia* species, *J. Clin. Microbiol.*, **47**, 2607–2610 (2009).
(36) H. Rose, A. Baldwin, C. G. Dowson, and E. Mahenthiralingam, Biocide susceptibility of the *Burkholderia cepacia* complex, *J. Antimicrob. Chemother.*, **63**, 502–510 (2009).
(37) L. Fernández, E. B. M. Breidenstein, and R. E. W. Hancock, Creeping baselines and adaptive resistance to antibiotics, *Drug Resist. Updates*, **14**, 1–21 (2011).
(38) E. Mahenthiralingam, J. Bischof, S. K. Byrne, C. Radomski, J. E. Davies, Y. Av-Gay, and P. Vandamme, DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis, Burkholderia multivorans, Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III, *J. Clin. Microbiol.*, **38**, 3165–3173 (2000).
(39) K. A. Jolley and M. C. Maiden, BIGSdb: scalable analysis of bacterial genome variation at the population level, *BMC Bioinf.*, **11**, 595 (2010).
(40) M. Périamé, J. M. Pagès, and A. Davin-Regli, *Enterobacter gergoviae* adaptation to preservatives commonly used in cosmetic industry, *Int. J. Cosmet. Sci.*, **36**(4), 386–395 (2014).
(41) G. Y. Larsen, T. L. Stull, and J. L. Burns, Marked phenotypic variability in *Pseudomonas cepacia* isolated from a patient with cystic fibrosis, *J. Clin. Microbiol.*, **31**, 788–792 (1993).

(42) T. Coenye, P. Vandamme, J. R. Govan, and J. J. LiPuma, Taxonomy and identification of the *Burkholderia cepacia* complex, *J. Clin. Microbiol.*, **39**, 3427–3436 (2001).
(43) G. Zhou, Q. Shi, Y. Ouyang, and Y. B. Chen, Involvement of outer membrane proteins and peroxide-sensor genes in *Burkholderia cepacia* resistance to isothiazolone, *World J. Microbiol. Biotechnol.*, **30**, 1251–1260 (2014).
(44) K. A. Rhodes and H. P. Schweizer, Antibiotic resistance in *Burkholderia* species, *Drug Resist. Updates*, **28**, 82–90 (2016).
(45) V. Gautam, P. P. Patil, S. Kumar, S. Midha, M. Kaur, S. Kaur, M. Singh, S. Mali, J. Shastri, A. Arora, P. Ray, and P. B. Patil, Multilocus sequence analysis reveals high genetic diversity in clinical isolates of *Burkholderia cepacia* complex from India, *Sci. Rep.*, **6**, 35769 (2016).
(46) A. Balwin, A. Mahenthiralingam, P. Drevinek, P. Vandamme, J. Govan, and D. Waine, Environmental *Burkholderia cepacia* complex isolates in human infections, *Emerg. Infect. Dis.*, **13**, 458–461 (2007).