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Key Role for Efflux in the Preservative Susceptibility and Adaptive Resistance of *Burkholderia cepacia* Complex Bacteria

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Bacteria from the *Burkholderia cepacia* complex (Bcc) are encountered as industrial contaminants, and little is known about the species involved or their mechanisms of preservative resistance. Multilocus sequence typing (MLST) revealed that multiple Bcc species may cause contamination, with *B. lata* ($n = 17$) and *B. cenocepacia* ($n = 11$) dominant within the collection examined. At the strain level, 11 of the 31 industrial sequence types identified had also been recovered from either natural environments or clinical infections. Minimal inhibitory (MIC) and minimum bactericidal (MBC) preservative concentrations varied across 83 selected Bcc strains, with industrial strains demonstrating increased tolerance for dimethylol dimethyl hydantoin (DMDMH). Benzisothiazolinone (BIT), DMDMH, methylisothiazolinone (MIT), a blend of 3:1 methylisothiazolinone-chloromethylisothiazolinone (M-CMIT), methyl paraben (MP), and phenoxyethanol (PH), were all effective anti-Bcc preservatives; benzethonium chloride (BC) and sodium benzoate (SB) were least effective. Since *B. lata* was the dominant industrial Bcc species, the type strain, 383^T (LMG 22485^T), was used to study preservative tolerance. Strain 383 developed stable preservative tolerance for M-CMIT, MIT, BIT, and BC, which resulted in preservative cross-resistance and altered antibiotic susceptibility, motility, and biofilm formation. Transcriptomic analysis of the *B. lata* 383 M-CMIT-adapted strain demonstrated that efflux played a key role in its M-CMIT tolerance and elevated fluoroquinolone resistance. The role of efflux was corroborated using the inhibitor L-Phe-Arg-β-napthylamide, which reduced the MICs of M-CMIT and ciprofloxacin. In summary, intrinsic preservative tolerance and stable adaptive changes, such as enhanced efflux, play a role in the ability of Bcc bacteria to cause industrial contamination.

The *Burkholderia cepacia* complex (Bcc) has recently expanded and is currently comprised of at least 17 genetically distinct species groups (1). Bcc members are ubiquitous in nature and are routinely isolated from a broad range of environments, including soil, plant rhizospheres, and freshwater, where they can have a range of beneficial properties (2). However, as opportunistic pathogens, Bcc bacteria are capable of causing infection in plants, animals, and vulnerable individuals and are commonly associated with respiratory infection in patients with cystic fibrosis (CF) (1, 2). In addition, members of the Bcc have been isolated as contaminants in a variety of industrial processes (3, 4).

Biocides are extensively used in clinical, agricultural, industrial, and domiciliary settings to reduce spoilage and the risk of infection. In industry, biocides are also used as preservative agents incorporated into raw materials and finished products. An inadequate preservative system and/or antimicrobial resistance can result in microbial spoilage; this may cause economic loss and, depending on the contaminating organism(s), could potentially pose a risk to the consumer. Members of the Bcc have been isolated from petroleum products (5), antimicrobial solutions (6), sterile solutions, preserved pharmaceuticals, and preserved cosmetics and toiletries (3). A review of FDA product recalls (1998 to 2006) reported *B. cepacia* as the most frequently isolated Gram-negative contaminant of sterile and nonsterile pharmaceuticals (3). Outbreaks of Bcc infection in vulnerable individuals have resulted from the use of contaminated antimicrobial/preserved industrial products (7–10). *Burkholderia contaminans* (11) was given its systematic species name because of its linkage to a globally distributed strain associated with multiple incidents of contamination and opportunistic infection (12). Overall, Bcc bacteria are now recognized as key risk species in microbial contamination (4).

Bcc bacteria have high innate antimicrobial resistance to both antibiotics and biocides. A recent survey of Bcc bacteria demonstrated that susceptibility to chlorhexidine, cetylpyridinum chloride, triclosan, benzalkonium chloride, and povidone biocides varied across the complex, with species-dependent differences in susceptibility being identified (13). The ability of Bcc bacteria to form biofilms also gives added protection against antibiotics and biocides, with efflux demonstrated as a key mechanism of chlorhexidine resistance in sessile *Burkholderia cenocepacia* cells (14). Adaptation to biocides and preservatives is a recognized phenomenon where resistance increases due to the conditions under which the bacterium is growing (15, 16). The role of adaptive resistance in the emergence of antimicrobial-tolerant microorganisms is perhaps underestimated, as it is often controversially considered to be transient (15). However, studies now indicate the level of increased resistance and the time for which it persists once the inducing stimulus is removed are largely dependent on the dose, time of exposure, and bacterial species (15, 17). Stable adap-

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tive resistance to benzalkonium chloride (18), phenoxyethanol (17), and isothiazolinone biocides (19, 20) has been promoted in *Pseudomonas* spp. via progressive subculture in the presence of sublethal concentrations. *B. cenocepacia* has been shown to adapt to a state of increased antibiotic resistance using multiple mechanisms that are underpinned by stable changes in its global gene expression profile (21). Thus, a major challenge for the manufacturing industry is to ensure that balanced preservation strategies have a sufficient quantity and spectrum of antimicrobials present to prevent microbial growth and not lead to adaptive antimicrobial resistance.

We carried out a novel investigation of Bcc susceptibility to common preservatives used in manufacturing. Since little is known about the distribution of different Bcc species in industrial contamination, a collection of such isolates was characterized using multilocus sequence typing (MLST) (22) to determine their strain diversity and species. Using this information, a genetically diverse panel of Bcc strains was assembled and used to determine the influence of Bcc taxonomy and isolation source on preservative susceptibility. *Burkholderia lata* (11), the most common species identified in our industrial collection, was used as a model species to study adaptive preservative resistance. Progressive subculture of *B. lata* in the presence of sublethal preservative concentrations was carried out, and transcriptomic analysis was used to determine the molecular basis for adaptive resistance to a cosmetic-grade blend of methylisothiazolinone and chloromethylisothiazolinone. A novel role for efflux in the resistance of *B. lata* to these isothiazoline antimicrobials was identified.

MATERIALS AND METHODS

Bacterial collection. A collection of 60 Bcc isolates from a variety of industrial processes (see Table S1 in the supplemental material) was assembled and identified to the species and strain levels by MLST (22). Sequence types and allelic data for the isolates were deposited at the *Burkholderia cepacia* complex Multi Locus Sequence Typing website (http://pubmlst.org/bcc). A panel of 83 Bcc strains used for profiling preservative susceptibility was drawn from the aforementioned industrial collection, the Cardiff University collection (2), and the Belgium Coordinated Collection of Microorganisms (BCCM) (http://bccm.belspo.be/about/lmg.php; see Table S2 in the supplemental material). The collection comprised 15 of the current species groups, five novel Bcc groups defined by MLST, unclassified novel Bcc strains, reference strains from Bcc experimental strain panels (23, 24), type strains, and preservative challenge test organisms (25) (see Table S2 in the supplemental material). The Bcc panel strain isolation sources varied, and the panel included clinical ($n = 41$; CLIN), environmental ($n = 24$; ENV), and industrial ($n = 18$; IND) strains. Ten non-Bcc species were evaluated as a control group, including multidrug-resistant reference strains of *Acinetobacter baumannii* and *Enterococcus faecalis*; clinical isolates of *Pandoraea pnomenusa*, *Pandoraea sputorum*, *Stenotrophomonas maltophilia*, and *Ralstonia pickettii*; and environmental isolates of *Pseudomonas fluorescens*, *Pseudomonas putida*, *Ralstonia mannitolilytica*, and *Burkholderia gladioli* (see Table S3 in the supplemental material). For consistent revival, strains were cultured at 30°C on tryptic soya agar; a defined minimal medium (26) was used for preservative susceptibility and adaptive-resistance testing, as described below.

MIC and MBC determination. Eight commercially available preservative agents frequently used in industrial processes were examined: benzisothiazolinone (BIT), benzethonium chloride (BC), dimethylol dimethyl hydantoin (DMDMH), methylisothiazolinone (MIT), a cosmetic-grade mixture of 3:1 methylisothiazolinone-chloromethylisothiazolinone (M-CMIT), methyl paraben (MP), phenoxyethanol (PH), and sodium benzoate (SB). Standardized agar dilution MIC, broth dilution MIC, and broth dilution minimum bactericidal concentration (MBC) assays were carried out using a modified basal salts medium (BSM) (26; see the supplemental material). The MIC was defined as the lowest concentration of preservative at which there was no visible growth of the test organism. In the context of the study, resistance was defined as an organism's ability to survive "in-use" concentrations (27) as regulated by the cosmetic directive or at levels recommended by the manufacturer. The MBC was determined as the lowest concentration at which growth on the recovery medium ceased (see the supplemental material).

Adaptive preservative resistance. *B. lata* strain 383 (LMG 22485^T), a genome-sequenced strain (http://www.jgi.doe.gov/), was used to evaluate adaptive preservative resistance. To select for preservative-adapted derivatives, approximately 1×10^6 CFU of strain 383 from a fresh overnight BSM broth culture was inoculated onto the surface of BSM agar containing preservative at a concentration up to four times lower than the MIC. The plates were incubated for 24 to 96 h at 30°C. Growth on BSM agar (2 or 3 colonies) with the highest preservative concentration was used to inoculate the next set of training plates, each with a 1.5-fold-increased concentration of preservative. If no growth was apparent after 96 h, the fold increase in the preservative concentration was decreased, and fresh plates were inoculated with growth from the previous training plate. Stepwise training was stopped when subculture onto an increased concentration did not result in growth within 168 h. This resulted in the successful isolation of strains 383-M-CMIT, 383-MIT, 383-BIT, and 383-BC, each named after the preservative to which they had been exposed. Derivatives with increased resistance to PH, MP, and DMDMH were not observed. The stability of the phenotypes was confirmed by 10 culture passages in the absence of preservative. All derivatives were confirmed as *B. lata* strain 383 by random amplification of polymorphic DNA (RAPD) analysis (28). Their preservative MICs in comparison with the parental strain 383 were determined by a standardized broth microdilution assay (see the supplemental material).

Analysis of growth dynamics. The growth dynamics of wild-type *B. lata* strain 383 and its derivatives were examined using an adapted broth dilution assay in a Bioscreen C Microbiological Growth Analyzer (Labsystems, Finland). Overnight broth starter cultures were standardized by optical density (OD) at 600 nm, and triplicate 200-μl cultures in the multiwell plates were inoculated with ~1×10^5 CFU/ml (200 μl). Growth analysis was performed for 72 h at 30°C; turbidity measurements were taken at 5-min intervals using a wide-band filter (450 to 580 nm) after shaking the microplates for 10 s at an intermediate intensity. Experiments were repeated with different starting cultures to obtain two biological replicates. Estimations of the length of the lag phase (in hours) and growth rate (μ) were determined from the mean growth curves generated. The growth rate was calculated as follows: $\mu = (\ln N_t - \ln N_0)/(t - t_0)$, where N_t is the optical density at time t and N_0 is the optical density at time zero (t_0).

Antibiotic susceptibility testing. The antibiotic susceptibility of *B. lata* 383 and its derivatives was determined using Etest strips, as described by the manufacturer (AB Biodisk); two biological replicates were performed for each antibiotic MIC. Eight antibiotics were examined as representative of those active against different cellular targets (29): amikacin, azithromycin, ceftazidime, ciprofloxacin, chloramphenicol, piperacillin, trimethoprim-sulfamethoxazole, and imipenem. An overall antibiotic susceptibility profile score for the wild type and derivatives was calculated by averaging the MICs for the eight antibiotics and was used as a measure of multidrug resistance as described previously (13). A modified broth microdilution assay was used to further evaluate the susceptibilities of the parent strain and derivatives to fluoroquinolone antibiotics. Aqueous solutions of fluoroquinolone antibiotics (10 mg/ml) were added to cation-adjusted Mueller-Hinton (MH) broth to make concentrations ranging from 0 to 80 μg/ml for ciprofloxacin, 0 to 256 μg/ml for levofloxacin, 0 to 1,024 μg/ml for norfloxacin, and 0 to 256 μg/ml for sparfloxacin. Overnight liquid cultures were standardized by optical density, and approximately 1×10^5 CFU of each strain was added to the test and control media in triplicate wells of a 96-well plate. Microplates were incubated with

shaking (150 rpm) at 30°C for 24 h; the MIC was defined as the concentration of preservative at which there was no visible bacterial growth.

Screening for mutations in the topoisomerase genes. The main mechanism of fluoroquinolone resistance in Gram-negative bacteria develops by accumulation of mutations in the quinolone resistance-determining region (QRDR) of the topoisomerase genes (30, 31). The QRDR of the *gyrA* and *gyrB* genes and an extended region (encompassing the QRDR) of the *parC* and *parE* genes were sequenced in the *B. lata* 383 parent strain and derivatives using the primers listed in Table S4 in the supplemental material. The amplification conditions were as follows: DNA was denatured at 96°C for 1 min, followed by 30 cycles of denaturing at 96°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The amplification products were purified, and both strands were sequenced using ABI 3100 and BigDye chemistry (Applied Biosystems). Sequences were analyzed and aligned using the Staden package (32), BioEdit version 7.0.5.3, and ClustalW, as described previously (5).

Efflux inhibition assays. The effect of efflux activity on preservative and antibiotic susceptibility of *B. lata* strain 383 and derivative 383-M-CMIT was studied using the broad-spectrum efflux inhibitor MC-207,110 L-Phe-Arg-β-napthylamide (PAβN) (Sigma-Aldrich, United Kingdom) (33–35). The MIC of PAβN was determined by the modified broth microdilution assay. Appropriate amounts of an aqueous stock solution of PAβN (100 mg/ml) were added to BSM broth to achieve a range of concentrations (0 to 2.048 mg/ml). A checkerboard assay (21) was used to evaluate the effect of PAβN on the MIC of a ciprofloxacin and M-CMIT preservative blend. Concentrations of PAβN ranging from 0 to 0.512 mg/ml were evaluated in combination with ciprofloxacin at 0 to 30 μg/ml and in combination with M-CMIT at a range of 0 to 0.001498%. The MIC was defined as the concentration of ciprofloxacin or preservative (alone and in combination with PAβN) at which there was no visible bacterial growth.

Biofilm and motility assays. A *Burkholderia*-specific biofilm assay (36) was used to determine biofilm biomass production of *B. lata* strain 383. The swarming motilities of the parental *B. lata* strain 383 and derivatives were determined as follows. Molecular-grade agarose (Bioline, United Kingdom) was dissolved in Iso-Sensitest broth (ISO) (Oxoid, United Kingdom) at 2 g/liter and autoclaved. After cooling, 25 ml of the soft agar was poured into triple-vented petri dishes and dried under laminar flow for 20 min. *B. lata* strain 383, adapted derivatives, and *Burkholderia multivorans* ATCC 17616 (a reference Bcc strain with excellent swarming motility) were cultured in ISO broth at 30°C for 18 h and diluted to an OD of 1 at 600 nm. Approximately 5×10^5 CFU was inoculated into the agar at the centers of triplicate soft-agar plates and left to dry for 15 min. The plates were incubated at 30°C, and the diameters of the swarms were measured at 48 h.

Global gene expression. To determine the gene expression alterations responsible for the stable adaptive phenotype of strain 383-M-CMIT, a custom 4,000 by 44,000 microarray for *B. lata* strain 383, designed by Oxford Gene Technology (United Kingdom) and manufactured by Agilent Technologies (Santa Clara, CA; design number 025314), was used. The microarray was composed of 14,132 probes; 14,071 probes were derived from coding genes and intergenic regions in the *B. lata* strain 383 genome, and 61 probes served as internal controls. Each probe was printed three times with a randomized distribution. To determine global gene expression differences, *B. lata* 383 and its preservative-adapted 383-M-CMIT derivative were grown in 250-ml conical flasks with 25 ml of BSM broth using an inoculum of 2×10^8 CFU for each strain. After growth with shaking (150 rpm) at 30°C, cells were harvested at the midlogarithmic growth phase, and total RNA was extracted using the RiboPure Bacteria Kit (Ambion). The experiments were repeated with different starting cultures to obtain three biological replicates. A reference microarray design (21) was used, with Cy5-labeled cDNA derived from the total RNA run against the control channel of Cy3-labeled strain 383 genomic DNA. Washing, scanning, extraction, and analysis of the gene expression data using Genespring GX v7.3.1 (Agilent) were performed as described previously (21). Three biological replicates were compared, and differential gene expression was examined for the 7,749 coding sequences (CDS) and 6,324 intergenic (IG) regions of the *B. lata* genome. Genes significantly altered in expression ($P < 0.05$) were initially identified using a 1.5-fold expression change cutoff, as described previously (21). Bioinformatic analysis of genes and gene cluster data was performed as described previously (21) and by using the *Burkholderia* Genome Database (http://www.burkholderia.com; 37).

Real-time qPCR validation of microarray and efflux gene expression. Quantitative PCR (qPCR) (21) was used to validate the expression of 4 genes observed as upregulated on the microarray analysis (see Table S4 in the supplemental material). Semiquantitative real-time (RT) PCR (38) was used to corroborate the expression of 2 downregulated genes (for genes and primers, see Table S4 in the supplemental material). Relative expression levels of the efflux gene Bcep18194_B1004 were evaluated in the parental *B. lata* strain 383, derivatives of strain 383, and three independent *B. lata* strains isolated from environmental industrial sources: strains BCC1294, BCC1296, and BCC1406. qPCR was performed in triplicate as described previously (21); the primers are listed in Table S4 in the supplemental material.

Amplifications were run on an MJ Research PTC-200 thermal cycler with the option of real-time fluorescence detection (DNA Engine Opticon). Cycling conditions were as follows: after an initial 15-min activation of the modified *Taq* polymerase at 95°C, 50 cycles of 15 s at 95°C, 30 s at 62°C, and 30 s at 72°C were performed. Data were obtained at 72°C, and melting-curve analysis was performed at the end of the PCR to test for specific PCR products. The expression level of *phaC* (Bcep18194_A5090), a gene that had stable expression across all microarray conditions, was used as a reference to correct for differences in the amounts of starting material. This method was applied to the analysis of two total-RNA extractions from independent biological replicates cultured in BSM broth, as described for microarray experiments. A total of 0.1 μg of the extracted RNA was converted to cDNA using an Improm-II Reverse Transcription System (Promega), and 500 ng of cDNA was added to each PCR mixture. A negative control and a genomic-DNA contamination control were included. The efficiency of the PCR was determined by serial dilutions of the cDNA template. Accurate levels of expression were calculated using delta-delta and Pfaffl methods of analysis (39).

Statistical analysis. Significant differences ($P < 0.05$) in the MIC or MBC values of Bcc strains isolated from clinical, environmental, and environmental industrial sources were determined using Kruskal-Wallis and Mann-Whitney (two-tailed test) statistical tests. Only statistically significant gene expression changes ($P < 0.05$) with >1.5-fold alteration were identified, as described previously (21).

Microarray data accession number. The microarray experimental protocols and raw data were submitted to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MEXP-2827.

RESULTS

Bcc species diversity in industry. MLST analysis of a collection of 60 Bcc isolates recovered from industrial sources revealed the following species diversity: *B. lata*, $n = 15$; *B. cenocepacia*, $n = 11$; *Burkholderia vietnamiensis*, $n = 8$; novel Bcc group Kc, $n = 7$; *Burkholderia arboris*, $n = 6$; *Burkholderia stabilis*, $n = 5$; *B. cepacia*, $n = 3$; *B. multivorans*, $n = 2$; *B. contaminans*, $n = 2$; and *Burkholderia ambifaria*, $n = 1$ (see Table S1 in the supplemental material). Only industrial isolates of sequence type 333 (ST333) were encountered at different geographic locations, with the other multiple isolates of single STs identified as duplicate cultures from single incidents. Comparison of the industrial isolates with the MLST database revealed that ST119 isolates had also been independently recovered from the natural environment; representatives of ST50, ST51, ST98, and ST439 had also been cultured from both the

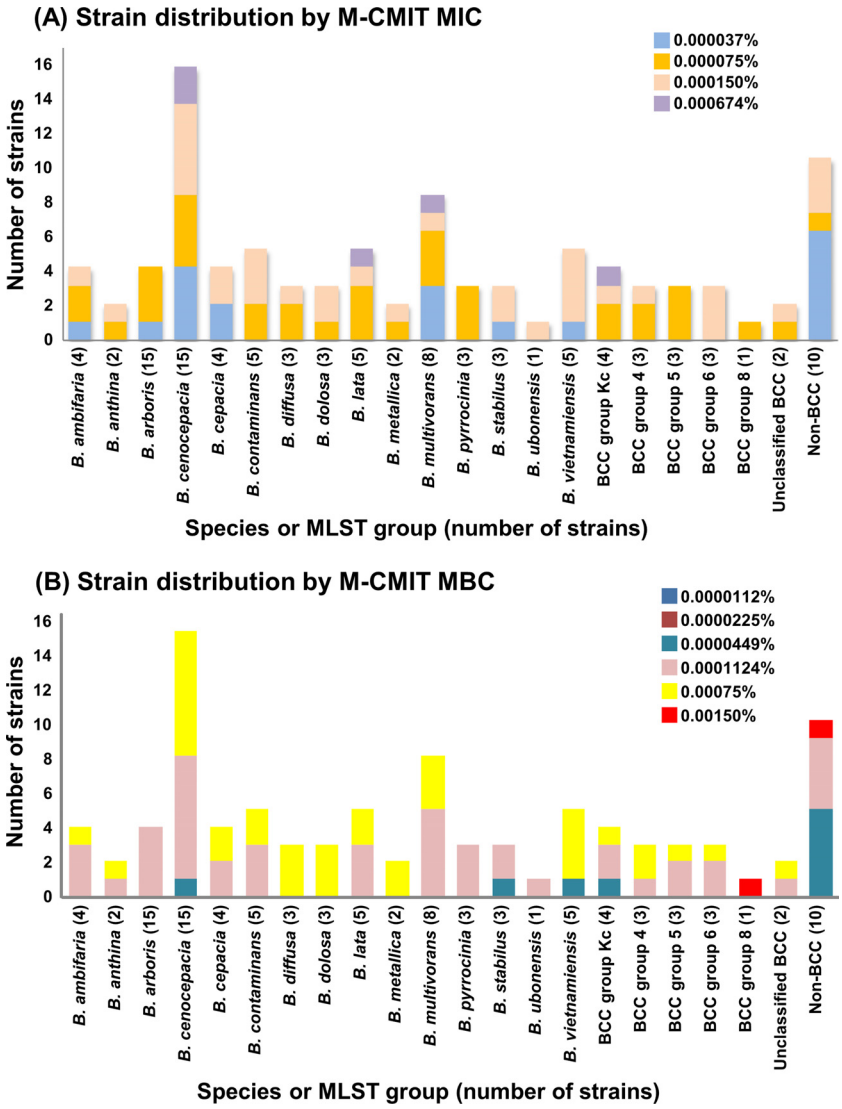

FIG 1 Susceptibility of Bcc species to M-CMIT. (A) MICs. (B) MBCs. The maximum level for use in rinse-off personal-care products in EU-regulated countries is 0.0015% (EU cosmetics directive 76/768/EEC annex VI).

natural environment and clinical infections; and ST3, ST103, ST241, ST250, ST338, and ST340 isolates had also been recovered from clinical infections. A selection of 18 of these industrial isolates spanning six Bcc species groups were included in a strain collection used to evaluate Bcc preservative susceptibility (see Table S2 in the supplemental material).

Bcc preservative susceptibility. The MICs and MBCs of preservative agents used in industrial processes were evaluated for a collection of 83 representative Bcc strains. The mean and range of MICs (see Table S5 in the supplemental material) and MBCs (see Table S6 in the supplemental material) demonstrated that susceptibility varied both between and within species of the Bcc. An example of the distribution of MIC and MBC values for the M-CMIT preservative blend is shown in Fig. 1. For six of the eight preservatives evaluated, strains with MIC and/or MBC values above the maximum level permitted for use in personal-care products were identified (Table 1). The maximum permitted level of sodium benzoate (0.5%) effectively inhibited the growth of 80 Bcc strains but was not high enough to kill the majority of Bcc strains (79/83 survived exposure). Benzethonium chloride failed to inhibit the growth of the majority (77 out of 83) of Bcc strains at maximum permitted levels of ≤0.1%; 14 Bcc strains (predominantly *B. cenocepacia*) showed a high tolerance for benzethonium chloride, with MBCs up to 10 times greater than the maximum permitted level. Isothiazolinone preservatives demonstrated higher efficacy at permitted levels and were bactericidal to 82 out of 83 Bcc strains. Maximum permitted levels of DMDMH (0.3%) and PH (1%) had the greatest anti-Bcc activity, inhibiting and killing all 83 Bcc strains. DMDMH was the only agent to inhibit and kill all 93 test organisms at the maximum permitted level.

The relationship between isolation source and Bcc preservative susceptibility was investigated. DMDMH had significantly higher ($P < 0.0001$) mean MIC and MBC values for Bcc bacteria isolated from industrial sources than for Bcc bacteria from clinical or environmental sources (Fig. 2). Mean MBC values of the remaining preservatives did not significantly differ in relation to Bcc isolation

TABLE 1 Bcc species with individual preservative MIC and MBC values greater than the maximum regulated concentration used in personal-care products

Species or MLST group (no. of strains evaluated)	No. of strains with MICs and MBCs above the regulated preservative concn[a]													
	MIT		M-CMIT[b]		BIT[c]		BC		DMDMH		PH		SB	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. ambifaria (4)							3							2
B. anthina (2)							2							2
B. arboris (4)							4							3
B. cenocepacia (15)							14	9					1	14
B. cepacia (4)							3						1	3
B. contaminans (5)							5	1						5
B. diffusa (3)						1	3							3
B. dolosa (3)		1					3							3
B. lata (5)							4							5
B. metallica (2)							2							2
B. multivorans (8)							8							8
B. pyrrocinia (3)							3							3
B. stabilis (3)							3							2
B. ubonensis (1)							1							1
B. vietnamiensis (5)							1						1	4
BCC Kc (4)							3	2						4
BCC4 (3)							3							3
BCC5 (3)							3							3
BCC6 (3)							3	1						3
BCC8 (1)				1			1							1
unclassified BCC (2)							2							2
non-Bcc (10)		1		1		1	3	1				2		3
Total no. of strains (93)[d]	0	2	0	2	0	2	77	14	0	0	0	2	3	79

[a] The maximum level for use in rinse-off personal-care products according to European Union (EU) cosmetics directive 76/768/EEC annex VI.
[b] A cosmetic-grade commercial blend of methylisothiazolinone and chloromethylisothiazolinone.
[c] Not permitted for use in personal-care products in EU-regulated countries as of 2011; concentrations were evaluated based on the manufacturer's recommended level.
[d] Total, 93 strains: 83 Bcc and 10 non-Bcc.

sources. The mean MICs of phenoxyethanol (0.25%) and methyl paraben (0.1%) for Bcc bacteria isolated from environmental sources were significantly higher ($P < 0.05$) than MICs for Bcc bacteria from other sources. Mean MICs of sodium benzoate for Bcc bacteria from clinical sources (0.23% ± 0.15) were significantly higher ($P < 0.05$) than MICs for Bcc bacteria from other sources.

The preservative susceptibilities of the 83 Bcc bacteria were also compared with those of a control group of 10 non-Bcc species. M-CMIT had significantly higher MIC and MBC values for Bcc bacteria than for the non-Bcc control group. Benzethonium chloride (0.148% ± 0.07%) and sodium benzoate (0.27% ± 0.09%) also had significantly higher mean MICs for the group of Bcc strains than for the non-Bcc species group (mean MICs of 0.049% ± 0.07% and 0.13% ± 0.09%, respectively). Phenoxyethanol had a significantly higher mean MBC (1.3% ± 1.3%) and methyl paraben had a significantly higher mean MIC (0.105% ± 0.114%) for the non-Bcc species group than for the Bcc species group (0.58% ± 0.19% and 0.093% ± 0.02%, respectively).

Adaptive preservative resistance in *B. lata* strain 383. Stable adaptive resistance to M-CMIT, MIT, BIT, and BC preservatives was promoted via progressive subculture on agar containing increasing concentrations of the preservative agent. Adaptive resistance to DMDMH, PH, and MP was not observed. The adaptive resistance remained stable after 10 rounds of progressive subculture in the absence of preservative. The preservative MICs for derivatives 383-M-CMIT, 383-BIT, and 383-BC increased up to 4-fold, while the MIC of MIT for the derivative 383-MIT remained at wild-type levels (Table 2). In the absence of preservative, derivative 383-MIT had a significantly longer generation time (0.33 ± 0.02 h), while that of 383-BC was significantly shorter (0.20 ± 0.01 h) than that of the parental strain (0.23 ± 0.02 h). Derivatives 383-MIT, 383 M-CMIT, and 383-BC also had significantly longer lag phases (7.83 ± 0.44 h, 7.96 ± 0.33 h, and 10.50 ± 0.45 h, respectively) than the wild type (6.25 ± 0.27 h). Growth curve analysis of 383-MIT cultured in the presence of isothiazolinone preservatives demonstrated that the derivative was capable of growth in concentrations 2-fold higher than that of the wild type after an extended lag phase of 35.70 ± 2.95 h. MIC tests, read at 24 h, failed to identify the increase in tolerance for MIT in the *B. lata* 383-MIT derivative. All adapted derivatives except 383-MIT had reduced swarming motility (Table 2). Biofilm biomass formation had significantly increased in derivatives 383-M-CMIT and 383-BC from wild-type levels (Table 2).

The preservative susceptibility profiles of adapted strains varied as follows. M-CMIT- and BIT-induced adaptive resistance in strain 383 conferred cross-resistance between the related compounds: 38-M-CMIT and 383-BIT each demonstrated 4-fold-increased MICs of M-CMIT and BIT compared to parental-strain levels. Adaptive resistance to benzethonium chloride did not confer cross-resistance to isothiazolinone preservatives; however, it

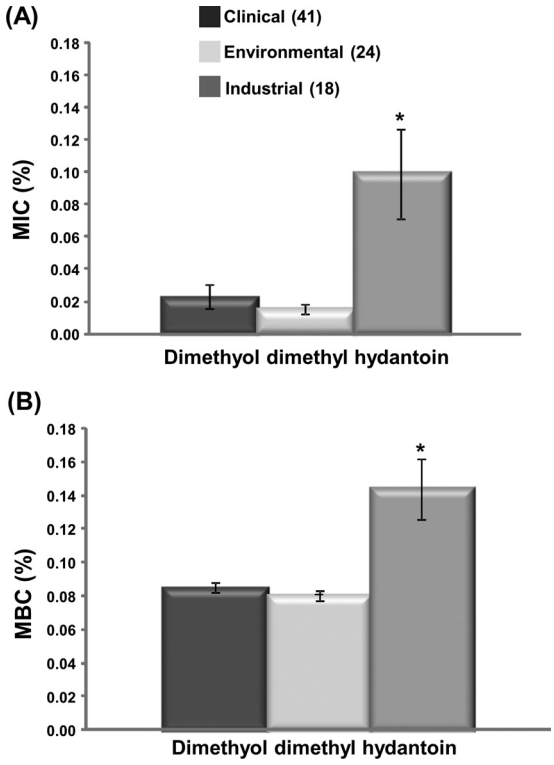

FIG 2 Increased tolerance for dimethyol dimethyl hydantoin in Bcc isolates from industrial sources. The mean minimum inhibitory concentrations (A) and mean minimum bactericidal concentrations (B) of Bcc isolates from industrial sources were significantly higher than the values for Bcc isolates from clinical and environmental sources (*, $P < 0.0001$; two-tailed Mann-Whitney test). In EU-regulated countries, a maximum level of 0.3% DMDMH is regulated for use in rinse-off personal-care products (EU cosmetics directive 76/768/EEC annex VI). The error bars show standard errors.

increased susceptibility to MP and PH (each with a 2-fold decrease in MIC from parental strain levels). Adapted derivatives of *B. lata* 383 had altered antibiotic susceptibility profiles for 5 out of 8 antibiotics tested (Table 3). In particular, derivatives 383-M-CMIT and 383-BIT had increased tolerance for chloramphenicol and a >25-fold-increased MIC of ciprofloxacin (Table 3); the MICs of three additional fluoroquinolones, levofloxacin, norfloxacin, and sparfloxacin, also increased (data not shown). No mutations were found in or surrounding the QRDR genes in the *B.*

TABLE 3 Antibiotic MICs for *B. lata* strain 383 and derivatives

B. lata strain	Mean MIC (μg/ml) value[a]					Antibiotic profile score
	AMK	CIP	CHL	SXT	CAZ	
383 wt[b]	192	1.25	160	0.25	12	73.10
383-MIT	128.0	1.5	128.0	0.25	10	53.55
383-M-CMIT	24	>32	>256	0.315	12	64.86
383-BIT	10.0	>32	>256	0.25	10	61.65
383-BC	64	1.25	72	0.315	12	29.91

[a] AMK, amikacin; CIP, ciprofloxacin; CHL, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; CAZ, ceftazidime. Breakpoints for the BSAC (British Society for Antimicrobial Chemotherapy) disk diffusion assay (version 9.1; March 2010) available for *Pseudomonas* spp. were as follows: AMK,16 μg/ml; CAZ, 8 μg/ml; CIP, 1 μg/ml.
[b] wt, wild type.

lata 383 derivatives to explain their increased fluoroquinolone resistance. The overall antibiotic profile score (Table 3) (13) indicated that all preservative derivatives were less multidrug resistant than the wild-type strain, despite the latter's alterations to specific antibiotics.

Expression analysis of adapted *B. lata* 383-M-CMIT. Transcriptomics were used to identify differentially regulated genes in the preservative-adapted strain *B. lata* 383-M-CMIT because of its striking phenotypic alterations (Tables 2 and 3). Of the 7,749 coding sequences, 126 genes were significantly upregulated (see Table S7 in the supplemental material) and 90 downregulated (see Table S8 in the supplemental material) in the 383-M-CMIT derivative. Microarray performance was robust and reproducible, as had been observed with other *Burkholderia* custom arrays using the same platform (14, 21, 38, 40), with 4 upregulated genes validated by qPCR (see Table S4 in the supplemental material) and 2 downregulated genes validated by semiquantitative RT-PCR (data not shown). Of the 216 differentially expressed genes, 81, 103, and 32 were located on the first, second, and third chromosomal replicons of *B. lata*, respectively. The genes were associated with 19 clusters of orthologous groups (COG) categories; 89 were poorly characterized, 80 were involved in metabolism, 31 were involved in cellular processes and signaling, and 16 were involved in information storage and processing (see Table S7 in the supplemental material). Of the 90 downregulated genes, the largest change (6.3-fold) was associated with a gene encoding chorismate mutase (B0668) (see Table S8 in the supplemental material). This had 73% homology to the salicylate biosynthesis protein PchB in *P.*

TABLE 2 Preservative susceptibility profiles, swarming motilities, and biofilm biomass production of *B. lata* 383 derivatives with adaptive resistance

B. lata strain	Preservative susceptibility[a]				Swarming motility (diam [mm]) (SD)	Biofilm production[e] (mean OD at 570 nm) (SD)
	Mean MIC (%)[b]	Increase in MIC (fold)[c]	Highest tolerated concn (%)[d]	Increase in concn (fold)[c]		
Wild type					50.0 (0)	0.192 (0.025)
383-M-CMIT	0.00018725	4	0.0003745	8	27.5 (2.1)	0.163 (0.019)[f]
383-MIT	0.0012125	0	0.0012125	2	50.5 (0.7)	0.202 (0.02)
383-BIT	0.0025	4	0.0025	2	27.5 (0.7)	0.192 (0.021)
383-BC	0.025	2	0.025	4	26.0 (2.8)	0.446 (0.112)[f]

[a] Preservative agent to which the *B. lata* derivative developed adaptive resistance through stepwise training.
[b] Broth dilution assay.
[c] From wild-type levels.
[d] Highest concentration of preservative at which growth was observed (Bioscreen MBG analyser).
[e] Biomass production (crystal violet assay).
[f] Significant difference from wild type ($P < 0.0001$; Mann-Whitney two-tailed test).

aeuriginosa PAO1, involved in the biosynthesis of the siderophore pyochelin (41).

Three classes of predicted efflux pumps (42) also had altered expression levels in 383-M-CMIT: the ATP-binding cassette (ABC) superfamily (genes A3512_3517 and B1768), major facilitator superfamily (MFS) (genes B1327 and A4968), and resistance-nodulation-division (RND) superfamily (genes B1004 to B1006; see Table S7 in the supplemental material). The largest change in expression was associated with the coregulated RND efflux pump gene cluster, B1004 to B1006, located on the second replicon (see Table S7 in the supplemental material). All components of the tripartite system were significantly upregulated; the membrane fusion protein (Bcep18194_B1004 gene) was upregulated 25-fold, and the transport efflux protein and outer membrane protein were upregulated 9.3- and 10-fold, respectively. Additional microarray analysis demonstrated that expression of this system was not induced by sublethal concentrations of M-CMIT (0.00001498%) in the wild type (data not shown).

RND efflux pump gene expression and inhibition. qPCR was used to determine the expression of the RND efflux membrane fusion protein gene B1004 in all isothiazolinone preservative-adapted *B. lata* 383 derivatives and in three genetically distinct *B. lata* strains isolated from industrial sources. In comparison to the wild type, transcription was significantly higher in derivatives 383-M-CMIT (116.8 ± 62.4) (values indicate fold change) and 383-BIT (81.0 ± 20.2), whereas 383-MIT had only a 1.6-fold (±1.5) increase from wild-type levels. Three independent *B. lata* strains from industrial sources all had higher levels of transcription than the wild-type *B. lata* 383 and derivative 383-MIT (BCC1296, 5.7 ± 1; BCC1406, 3.3 ± 2.4), with the process contaminant BCC1294 having the largest expression change of the efflux-encoding gene (236.8 ± 184.4). (The values represent fold change [mean of biological duplicates] in comparison to the transcription level in the wild-type *B. lata* strain 383.) To corroborate a phenotypic role for efflux in *B. lata* isothiazolinone resistance, the ability of the efflux inhibitor PAβN (33) to alter preservative susceptibility was examined. A concentration of 0.512 mg/ml PAβN reduced the MICs of M-CMIT for *B. lata* strain 383 to 0.0007% (3-fold) and for the adapted derivative 383-M-CMIT to 0.00625% (4-fold). A corresponding reduction in the fluoroquinolone MIC was also observed at this PAβN concentration, with the ciprofloxacin MIC reduced by 2-fold (0.7 ± 0.3 μg/ml) and 6-fold (1.88 μg/ml) for the parental strain and the 383-M-CMIT derivative, respectively.

DISCUSSION

The Gram-negative species "*B. cepacia*" is becoming recognized as one of the most problematic bacterial species for causing industrial contamination (4). "*B. cepacia*" bacteria belong to a taxonomic complex of closely related species, and this complicates their study in relation to microbial contamination. Our research has led to a much clearer understanding of which Bcc species are prevalent in industrial contamination; the preservative agents to which they are susceptible; and, for the first time, which preservatives may develop adaptive resistance and the molecular pathways behind this preservative tolerance, where efflux was a key player.

Which Bcc species are associated with industrial contamination? Incidents of Bcc species contamination are often generically reported as caused by "*B. cepacia*" (3, 4); they may often be detected in house by manufacturers and not publically reported. Using MLST (22), *B. lata* and *B. cenocepacia* were identified as the two most common industrial contaminant species in our collection (see Table S1 in the supplemental material). *B. lata* belongs to the taxon K subdivision within the Bcc and was defined at the same time as another species, *B. contaminans* (11). *B. lata* causes less than 3% of all Bcc CF infections reported in the United States and has not yet been associated with major outbreaks of infection (1). In contrast, *B. cenocepacia* is one of the most problematic Bcc species, causing 31% of CF infections in the United States and being linked to multiple outbreaks and epidemics in CF and non-CF patients (1). While *B. cenocepacia* is known to be more tolerant of biocides and antibiotics (13), the industrial prevalence of *B. lata* and *B. cenocepacia* could not be explained by their preservative susceptibility profiles. No particular Bcc species specifically had a high preservative tolerance; however, we did observe that Bcc bacteria with elevated tolerance for the formaldehyde-releasing agent DMDMH were more prevalent in industrial settings (Fig. 2). Bcc species potentially possess multiple pathways for formaldehyde detoxification (43); however, a preliminary investigation of whether Bcc bacteria could grow on DMDMH demonstrated that they were incapable of using it as a sole carbon source (data not shown).

Which commonly used preservatives are most effective against the Bcc? Bcc bacteria are innately resistant to antimicrobials (2), but in relation to preservatives, resistance pathways are poorly characterized. BIT, DMDMH, MIT, M-CMIT, MP, and PH all possessed excellent activity against Bcc species, and none of the 83 strains examined survived exposure to levels of these agents currently in use (Table 1). BC and SB were not effective agents on their own (Table 1). DMDMH and PH were the most bactericidal agents, killing all Bcc strains examined at their maximum concentrations in use, however, the remaining six individual preservative agents did not achieve this 100% efficacy. Preservative formulations vary greatly, depending on what is permissible or effective in an industry and product. Single preservative agents are not generally used in industry, and our data support this practice, as Bcc strains that could escape inhibition or killing by individual preservatives at the maximum regulated levels (with the exception of DMDMH) were encountered.

Bcc bacteria can adapt to higher levels of preservative tolerance. Adaptive resistance may also play a role in the emergence of resistant organisms. The ability of Bcc bacteria to increase their antibiotic resistance is well known, with stable gene expression changes and cross-resistance to other antibiotics seen after selection for spontaneous resistance (21). We successfully isolated *B. lata* 383 derivatives with elevated tolerance for MIT, C-MIT, BIT, and BC but could not achieve this for MP, PH, and DMDMH. The lack of success in promoting DMDMH tolerance failed to correlate with our industrial phenotypic screening data (Fig. 2). Whether the lack of adaptive resistance seen reflects their efficacy or was due to selection on agar as opposed to broth-based exposures warrants further study. There are conflicting reports of stable (20) and unstable (19) isothiazolone resistance seen in adapted *P. aeruginosa*. Our data clearly demonstrated that isothiazolone-adapted *B. lata* derivatives stably maintain their preservative tolerance in the absence of these biocides.

As seen with spontaneous antibiotic resistance in *B. cenocepacia* (21), exposure of *B. lata* to single preservative agents led to stable cross-resistance to other preservatives, especially with regard to tolerance within the isothiazoline family. This emphasizes the importance of formulating preservatives appropriately to

avoid leaving particular agents at sub-MIC levels, which can promote tolerance. No overall increase in antibiotic multidrug resistance was seen in the preservative-adapted Bcc derivatives; however, one of the most striking cross-resistances observed was the association of an elevated fluoroquinolone MIC with the isothiazolone-adapted derivatives 383-BIT and 383-M-CMIT (Table 3). This was not due to an accumulation of mutations in the topoisomerase genes, as noted for fluoroquinolones (30). Elevated chlorhexidine resistance has been observed in CF outbreak strains of *B. multivorans* and *B. cenocepacia*, which were multidrug resistant (13). Linkages between antibiotic and biocide resistances have now been observed for several bacterial species (44). Whitehead et al. (45) demonstrated that *Salmonella enterica* mutants surviving a single exposure to in-use concentrations of Superkill (a mixture of aldehydes and quaternary ammonium compounds) and Trigene (a halogenated tertiary amine) acquired both elevated antibiotic (nalidixic acid, chloramphenicol, tetracycline, and ciprofloxacin) and triclosan resistance.

Efflux is a key mechanism of adaptive preservative resistance in *B. lata*. Transcriptomic analysis of the isothiazolinone-adapted *B. lata* 383-M-CMIT derivative revealed that the factors necessary for preservative adaptation were generally inherently encoded traits of *B. lata*. Multiple transport-associated genes had altered expression in 383-M-CMIT (see Tables S7 and S8 in the supplemental material), with the greatest change from wild-type transcription associated with the RND efflux pump genes B1004 to B1006. In *B. cenocepacia* J2315, the orthologous efflux pump, the RND-9 system (genes BCAM1945 to BCAM1947), is implicated in ethidium bromide, beta-lactam, aminoglycoside, and fluoroquinolone efflux, and gene deletion results in reduced motility and increased biofilm formation (40). This phenotype from *B. cenocepacia* corroborates that of the preservative-adapted *B. lata* 383-M-CMIT and 383-BIT derivatives. The phylogenetic distribution of this RND efflux pump within the available Bcc genomes shows that orthologous systems are limited to *B. cenocepacia*, *Burkholeria dolosa*, *B. multivorans*, and *B. lata* (40).

Overexpression of the B1004-to-B1006 RND operon in the adapted *B. lata* derivative remained stable in the absence of isothiazolones, suggesting mutation in either the local repressor gene (B1003, a MerR homologue [40]), a global regulatory gene, or the promoter region had occurred in 383-M-CMIT and 383-BIT. Induction of the RND operon did not occur when the 383 wild type was exposed to sublethal concentrations of M-CMIT, suggesting that the preservative substrate may not be directly involved in regulation of the efflux system. However, because the susceptibility of both derivatives and the wild-type increased in the presence of an RND efflux inhibitor, the role of efflux in tolerance for M-CMIT and BIT preservatives was confirmed. All isothiazolinones are thiol-interactive biocides; however, chloromethylisothiazolinone and benzisothiazolinone are thought to have highly similar modes of action (46), perhaps inducing the same RND efflux-mediated adaptive resistance in *B. lata*. Outer membrane protein alterations have been associated with the onset of resistance to M-CMIT in Bcc bacteria (47) and resistance to CMIT, MIT, MIT/CMIT and BIT in *Pseudomonas* spp. (19, 20).

Conclusions. Understanding the diversity of contaminating Bcc species and their mechanisms of preservative resistance will enable manufacturers to target these organisms more effectively. We have identified *B. lata* as a commonly encountered industrial species and a suitable model organism for the study of preservative resistance. *B. lata* can evolve stable adaptive resistance to preservatives, conferring cross-resistance to structurally related and unrelated antimicrobial agents, including certain antibiotics. The potential for adaptive resistance should perhaps be considered in parallel with the ability of preservative agents to inhibit growth and/or kill spoilage organisms. Targeting efflux-mediated adaptive resistance with inhibitors may potentiate preservative activity and help reduce the emergence of such resistant organisms. Preservative challenge test organisms should be expanded in light of our findings to include genetically diverse Bcc bacteria and preservative-tolerant "in-house" strains recovered from preserved products. Finally, in order to increase the depth of our understanding in this poorly reported area, all Bcc bacteria from formal product recalls should be accurately identified to the species level, using available molecular assays (22, 48).

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