Mapping the efficacy and mode of action of ethylzingerone [4-(3-ethoxy-4-hydroxyphenyl) butan-2-one] as an active agent against *Burkholderia* bacteria.

Laura Rushton,[a#] Ahmad Khodr,[b]* Florence Menard-Szczebara,[b*] Jean-Yves Maillard,[c] Sylvie Cupferman,[b]and Eshwar Mahenthiralingam [a#]

*: contributed equally

[a] Cardiff School of Biosciences, Cardiff University, Cardiff, UK

[b] International Microbiology Department, L'Oréal Research and Innovation, Chevilly-Larue, France

[c] Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK

Running heading: *Burkholderia* are susceptible to ethylzingerone

[#]**Corresponding author**: Laura Rushton, RushtonL3@cardiff.ac.uk

(ORCID: 0000-0002-4643-6489)

[#]**Co-correspondence:** Eshwar Mahenthiralingam, MahenthiralingamE@cardiff.ac.uk

(ORCID: 0000-0001-9014-3790)

ABSTRACT

Burkholderia cepacia complex (Bcc) bacteria are intrinsically antimicrobial resistant opportunistic pathogens and key risk species in the contamination of non-food industrial products. New agents and formulations to prevent growth of *Burkholderia* in home care (cleaning agents) and personal care (cosmetics and toiletries) products are required. We characterised how ethylzingerone [4-(3-ethoxy-4-hydroxyphenyl) butan-2-one)] (HEPB) acts as a preservative with activity against *Burkholderia* species encountered in industry. *Burkholderia* (n = 58) and non-*Burkholderia* (n = 7) bacteria were screened for susceptibility to HEPB, and its mode of action/resistance determined for a model *B. vietnamiensis* strain using transposon mutagenesis, transcriptomics and genome resequencing analysis. The susceptibility of *Burkholderia spp.* to HEPB (MIC = 0.45 ±0.11 % w/v; MBC = 0.90 ±0.3 % w/v) was characterised, with limited inter-/intra-species differences. HEPB (1% w/v) was rapidly bactericidal producing a 6-Log reduction in viability within 4 hours. Spontaneous resistance to HEPB did not develop, but transient phenotypes with altered growth characteristics and susceptibility to antibiotics were identified after prolonged exposure to sub-lethal HEPB concentrations. Transposon mutagenesis and RNA-sequencing analysis identified multiple genetic pathways associated with HEPB exposure, including stress response mechanisms, altered permeability, regulation of intracellular pH, damage/repair of intracellular components and alteration/repair of lipopolysaccharides. Key pathways included the stringent response, homeostasis of intracellular pH by the *kdp* operon, protection against electrophiles by KefC, and repair of oxidised proteins by methionine sulfoxide reductase enzymes. In summary, we show that HEPB has potent, targeted efficacy against *Burkholderia* bacteria without promoting wider stable antimicrobial resistance. The mode of action of HEPB against *Burkholderia* is multifactorial but killing by intracellular oxidation is a key mechanism of this promising agent.

Importance. *Burkholderia* bacteria are opportunistic pathogens that can overcome preservatives used in the manufacture of non-sterile industrial products, and occasionally cause contamination. Consequently, new preservatives to prevent the growth of key risk *Burkholderia cepacia* complex bacteria in non-food industrial products are urgently required. Here we show that ethylzingerone is active against these problematic bacteria, killing them via a multifactorial mode of action which involves intracellular oxidation.

INTRODUCTION

Antimicrobials are used extensively in healthcare, domiciliary, agricultural and industrial settings to inhibit proliferation of spoilage organisms and kill potential pathogens. In industry, combinations of antimicrobials are incorporated at low levels into raw materials and finished products as preservative agents to protect against microbial contamination. Failure of these preservative systems can result in significant economic loss and, depending on the contaminant, may pose a risk to consumer health. A diverse range of yeasts, moulds and bacteria are encountered as contaminants of industrial products. Gram-negative bacteria are commonly encountered in the pharmaceutical, cosmetics and personal care industries, with a predominance of *Pseudomonas spp.* and *Burkholderia cepacia* reported in product recalls (1). As it is difficult to differentiate species by conventional phenotypic or biochemical tests, *Burkholderia* contaminants are routinely recorded as '*B. cepacia'* in these incident reports. This obscures the diversity of *Burkholderia* species actually encountered in the industrial environment. Multiple *Burkholderia* species have been isolated from non-food industrial products, with a predominance of species from the *Burkholderia cepacia* complex (Bcc) (2). The Bcc comprises over 20 closely related, but genetically distinct, species within the diverse genus *Burkholderia*. As highly adaptable environmental bacteria, the Bcc have been studied for their potential biotechnological applications in plant promotion, bioremediation, and biological control of plant pests (3). In parallel to their beneficial properties, however,

68 members of the Bcc have been extensively studied as opportunistic pathogens capable of
69 causing infection in multiple hosts, such as chronic respiratory infection in people with cystic
70 fibrosis (CF) (4). As industrial contaminants, Bcc bacteria have been isolated from petroleum
71 products (5), antimicrobial solutions (6), pharmaceuticals, and preserved cosmetics and
72 toiletries (1, 7). Outbreaks of Bcc infection in vulnerable individuals, although rare, have
73 resulted from the use of contaminated industrial products (6, 8) and these bacteria have
74 gained recognition as key risk species in microbial contamination (1, 9).

75 The ability of Bcc bacteria to survive as contaminants is in part due to high innate
76 antimicrobial resistance, and their metabolic flexibility and adaptability (3). A recent survey
77 characterising the susceptibility of the Bcc to key groups of preservatives used in industry
78 revealed inter-/intra-species differences in susceptibility, and highlighted that the permitted
79 levels of sodium benzoate and benzethonium chloride were ineffective in the control of Bcc
80 contamination (2). The study also demonstrated that *Burkholderia* bacteria can develop stable
81 adaptations to biocides via repeated exposure to sub-lethal concentrations of preservatives as
82 priming agents (2). Adaptation to key preservatives resulted in derivatives with decreased
83 preservative susceptibility and altered antibiotic susceptibility profiles, that persisted in the
84 absence of the priming biocide (2). Transcriptomic analysis of *B. lata*, a commonly
85 encountered industrial contaminant, revealed that efflux by a resistance-nodulation-division
86 (RND) system played a key role in adaptation to isothiazolinone agents. In addition, Bcc
87 strains isolated from industrial sources demonstrated increased tolerance to a formaldehyde
88 releasing agent. Therefore, the selection and emergence of antimicrobial tolerant Bcc bacteria
89 in industry is of concern (2).

90 There is an unmet need for preservatives that, when used at low levels, are efficacious against
91 intrinsically resistant *Burkholderia* bacteria. Ethylzingerone, which is also referred to as
92 'Hydroxyethoxyphenyl Butanone' (HEPB), is a novel cosmetic ingredient recently regulated

by the European Union (EU) as a preservative in rinse-off, oral care, and leave-on cosmetic products at levels ≤ 0.7% w/v (10-12). This phenolic derivative is structurally similar to Zingerone [4-(4-hydroxy-3-methoxyphenyl) butan-2-one], which is derived from bioactive molecules (such as gingerols) found in the root of the ginger plant (*Zinger officinale).* Zingerone is well characterised for its anti-inflammatory activity (13). In a recent investigation, zingerone was shown to have activity against Gram-negative *Pseudomonas spp.* (14). Our working hypothesis, that HEPB also had antimicrobial activity against bacteria of this phyla, including *Burkholderia* species, warranted further investigation. We carried out an exploration of HEPB susceptibility, utilising a diverse panel of Bcc bacteria, other *Burkholderia* species, and reference non-*Burkholderia* bacteria. *B. vietnamiensis* strain G4 (LMG 22486), a key species seen in industrial contamination (2), was used as a model strain to study adaptation to HEPB and the genetic basis for tolerance to the preservative. We defined stable adaptation as a non-transient change in phenotype, specifically antimicrobial susceptibility, that persisted in the absence of the priming preservative agent. Transposon mutagenesis and transcriptomic analysis of *B. vietnamiensis* strain G4 identified key genes and pathways involved in HEPB susceptibility, and revealed its multifactorial mode of action against *Burkholderia* bacteria.

111 **RESULTS**

112 ***Burkholderia* susceptibility to HEPB.** The minimum inhibitory concentrations (MIC) and
113 minimum bactericidal concentrations (MBC) of HEPB for 58 *Burkholderia* strains (Table
114 S1), representative of species commonly encountered as industrial contaminants, and 7
115 reference non-*Burkholderia* (Table S2) were evaluated. Data for individual strains is
116 available in the supplemental Tables S3 and S4. The 58 *Burkholderia* strains belonged to 22
117 species, with 54 strains belonging to 20 species of the Bcc. In this experimental system,
118 HEPB solubility prevented the evaluation of test concentrations above 2% w/v. Nearly a
119 quarter of the *Burkholderia* strains (24%, n = 14) were killed by 0.7% w/v HEPB
120 (supplemental Table S3), which is the maximum concentration permitted for use in rinse-off,
121 oral care and leave on cosmetic products by EU Regulation No 1223/2009 Annex V (10). The
122 mean MICs and MBCs of HEPB demonstrated little variation for *Burkholderia* strains (Table
123 1). The majority of the strains had a median MIC of 0.5 % w/v (48 strains; 83%), and 44
124 strains (76%) had a median MBC of 1% w/v (Table S3). The largest variation in
125 susceptibility within a species group was a four-fold difference in MICs for three *B.*
126 *ambifaria* strains (ranging from 0.125% to 0.5% w/v, Table S3).

127 The MICs and MBCs of the non-*Burkholderia* panel were more variable (see supplemental
128 Table S4). *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* strains
129 were not killed by 2% (w/v) HEPB, the limit of the agent's solubility, and therefore MICs
130 and MBCs could not be determined. *Paraburkholderia* strains (Table S2) had increased
131 susceptibility to HEPB compared with *Burkholderia* strains, as shown in Table 1.

132 The preservative susceptibility of 39 *Burkholderia* strains utilised in this study was published
133 previously by Rushton *et al.* (2) as supplemental data. Metadata analysis shows that in
134 comparison to other established preservatives, the effectiveness of industrially relevant levels

of HEPB against *Burkholderia* bacteria is similar to that of methyl paraben or phenoxyethanol (supplemental Fig.S1). Inhibitory concentrations of HEPB for the 39 *Burkholderia* strains ranged from 1.4 to 5.6-fold lower than the EU maximum permitted level of 0.7% w/v; while inhibitory concentrations of methyl paraben or phenoxyethanol were 4 to 8-fold lower than maximum regulated levels of 0.4% w/v and 1% v/v respectively.

B. vietnamiensis strain G4 was an ideal model strain to evaluate HEPB mode of action and resistance as it possessed an intermediate susceptibility to HEPB, with an MIC of 0.25% w/v and MBC of 0.5% w/v. Time-kill curves of *B. vietnamiensis* strain G4 exposed to HEPB demonstrated the rapid bactericidal activity of HEPB, with a 6-Log reduction in viability occurring within an hour of exposure to 1% w/v HEPB. In contrast, *P. aeruginosa* ATCC 19249 did not show a reduction in viability at this concentration of HEPB (Fig. 1). Confocal microscopy of *B. vietnamiensis* strain G4 exposed to HEPB concentrations varying from 0.125% to 1% w/v for 18 hours revealed cell death increased in a dose-dependent manor to the agent (Supplemental Fig. S3). At 1 % w/v HEPB, only fragments of non-viable bacterial cells, as determined by total viable count (TVC), were observed by a live/dead stain (Fig.S3).

Spontaneous resistance and adaptation of *B. vietnamiensis* strain G4 to HEPB. The propensity of *Burkholderia* to develop resistance to HEPB was investigated using *B. vietnamiensis* strain G4. The frequency of spontaneous resistance to HEPB was calculated as zero: as none of the ~$1x10^6$ CFU plated onto agar containing 2x MIC (0.5 % w/v) of HEPB grew as resistant colonies. Adaption to HEPB was developed via the progressive sub-culture of the parental strain G4 on agar with sub-inhibitory HEPB concentrations. Stable adaptation was defined as a change in HEPB susceptibility and phenotype, that persisted in the absence of HEPB. The resulting three HEPB-adapted derivatives of *B. vietnamiensis* G4 (named T2s, T2L and T3) did not demonstrate a large decrease in susceptibility to HEPB: all three had an MIC of 0.375% w/v (1.5-fold higher than the parental G4 strain). Derivatives T2s and T3 also

had altered colony morphology with smaller discrete colonies than that of the parental strain (Fig. 2A). Such changes in morphology and susceptibility were not observed after the equivalent serial passage of the parental strain in culture media without HEPB. In the absence of HEPB, adapted derivatives demonstrated a slower rate of growth than that of the parental strain. The HEPB-adapted derivative T3 achieved a significantly lower final optical density than the parental strain when cultured for 48 hours in the absence of HEPB (mean Log_{10} OD 0.260±0.011 and 0.336 ±0.023 respectively, $p<0.05$) (Fig. 2B). In culture with 0.375% w/v HEPB, adapted derivative T2s achieved a significantly higher final OD than that of the parental strain (mean Log_{10} OD 0.078 ± 0.01 and 0.026 ± 0.011 respectively, $p = 0.05$), whereas its final OD in the absence of HEPB was similar to that of the parent (Fig. 2B). HEPB-adapted derivatives had varied degrees of altered susceptibility to eight antibiotics that were representative of agents active against different cellular targets (Table 2). To evaluate the stability of HEPB-adaptation, and antibiotic susceptibility, derivatives were sub-cultured repeatedly in the absence of HEPB. After serial passage, susceptibility to ceftazidime, imipenem, piperacillin and ciprofloxacin reverted to wild-type levels; sensitivity to amikacin and azithromycin were also closer to that of the parental strain. Increased tolerance to chloramphenicol in HEPB-adapted derivatives persisted after serial passage in the absence of HEPB).

Whole-genome re-sequencing, and comparison to the parental *B. vietnamiensis* G4 genome (8.4 Mb in size), revealed limited (n = 9) nucleotide polymorphisms in coding sequences (CDS) across the genomes of the HEPB-adapted derivatives. Three missense variants with putative function where common to all three derivatives: His102Tyr in a putative voltage-gated CIC-type chloride channel (gene Bcep1808_1681); Gln1527Arg in a putative modification methylase (gene Bcep1808_7553); and Lys21Glu in a putative purine nucleoside phosphoramidase (gene Bcep1808_0414). Derivatives T2s and T3 shared the

185 missense variant Arg222Cys in a putative murein hydrolase activator (gene Bcep1808_1748).
186 Derivative T3 had the missense variant Cys266Gly in a DNA-dependent RNA polymerase
187 (gene Bcep1808_0356). The remaining nucleotide polymorphisms were synonymous variants
188 that did not change the encoded amino acid.

189 **Mapping *B. vietnamiensis* strain G4 genes associated with HEPB susceptibility by**
190 **transposon mutagenesis.** A single mutant bank of 3984 derivatives of *B. vietnamiensis* strain
191 G4 was created using the transposon mini-Tn5-*luxCDABE*-km (15). The initial phenotypic
192 screen by agar dilution assay identified 1229 mutants (30.8% of the bank) with increased or
193 decreased HEPB susceptibility. The 1229 mutants were re-screened by broth dilution assay,
194 and a sub-set of 46 mutants with increased/decreased HEPB susceptibility were selected for
195 detailed growth analysis in the presence/absence of the preservative. Growth curve analysis
196 revealed that 18 of the 46 mutants reached a lower final mean OD (Log_{10} adjusted OD ≤ 0.25)
197 than that of the wild-type strain G4 (Log_{10} adjusted OD ≥ 0.35) under control conditions.
198 Consequently, these 18 mutants were excluded from further characterisation.

199 The genetic context was determined for 28 mutants that displayed a change in HEPB
200 susceptibility, but maintained wild-type levels of growth under control conditions (Table 3).
201 Transposon insertion had occurred at random, covering the three main genomic replicons and
202 five plasmids of strain G4. *B. vietnamiensis* strain G4 genes associated with HEPB
203 susceptibility were involved in a range of putative functions, the predominant COG
204 categories were transcription (n = 5), unknown function (n = 4), and signal transduction (n =
205 3) (Table 3). Mutation of genes involved in the regulation of intracellular pH, response to
206 external chemical stimuli or adverse conditions, and the response to oxidative stress increased
207 HEPB susceptibility. A 4-fold decrease in HEPB MIC (0.0625% w/v by agar dilution assay)
208 was associated with mutation of a *spoT* homolog (Bcep1808_0918, Mutant 22:E11). This
209 gene putatively encodes a bi-functional (p)ppGpp synthase/hydrolase, a mediator of the

stringent response, which coordinates a variety of cellular activities in response to change/adverse conditions (16). Also associated with the response to extracellular stimuli, the mutation of gene Bcep1808_3929 (Mutant 35:G5) resulted in increased HEPB susceptibility. This putative sensor hybrid histidine kinase has a predicted protein-protein interaction with an osmolality response regulator homologous to *ompR* (17).

Mutation of two genes involved in the regulation of intracellular pH increased HEPB susceptibility: gene Bcep1808_2370 (Mutant 19:H7) homologous to *kdpD*, a member of a two-component regulatory system of the *kdp* operon, which encodes a high affinity potassium (K^+) transporter; and a homolog of *kefC* (Bcep1808_2890; Mutant 29:B7), a potassium/sodium antiporter efflux system that confers protection against externally derived electrophiles (18). Mutation of a third gene, with a putative involvement in intracellular homeostasis and polyamine-mediated protection against oxidative stress, also resulted in increased HEPB susceptibility. Gene Bcep1808_3088 (Mutant 5:D5), homologous to *puuB*, is a putative gamma-glutamylputrescine oxidoreductase involved in putrescine catabolism - a precursor of the polyamines spermidine and spermine (19). A large increase in HEPB susceptibility (confirmed by growth curve analysis) was associated with the mutation of a putative *msrA1* gene homolog (Bcep1808_5515; Mutant 27:G3). Methionine sulfoxide reductase (Msr) enzymes are involved in the repair of proteins inactivated by oxidation (20). Interruption of key genes associated with transcription, and DNA replication or repair, also changed HEPB susceptibility (Table 3). A large increase in HEPB susceptibility (4-fold reduction in MIC) resulted via the mutation of gene Bcep1808_3794 (Mutant 20:G12). This homolog of *noc* that putatively encodes a *parB*-family protein, has involvement in nucleoid occlusion, and is recognised as a transcriptional regulator of stress response genes in *P. aeruginosa* (21).

Mapping *B. vietnamiensis* global gene expression in the presence of HEPB. RNA-sequencing analysis was used to identify differential gene expression of *B. vietnamiensis* strain G4 in response to sub-inhibitory concentrations of HEPB. Growth curve analysis revealed that strain G4 was able to grow in the presence of 0.2 x, 0.5 x, and 0.75 x MIC of HEPB, but with significantly ($p < 0.05$) altered growth kinetics in comparison to control conditions (Fig. S2). The mean final optical density (OD) of the considered test concentrations were impaired by 22%, 55% and 68% respectively (MIC reduced mean final OD by 82%) in comparison to that of control at 24 h (Fig. S2). A test concentration of 0.5 x MIC (0.125% w/v HEPB), sampled at 8 h, was utilised for gene expression analysis as the cultures had significantly altered growth but consistently reached a density that yielded high quality RNA from live cells (determined by viable count) and a growth rate equivalent to that of the control condition cultured in parallel.

Paired-end sequence reads (ranging from 1.82E+06 to 3.16E+06 in total, for the control and test conditions) were aligned to the reference *B. vietnamiensis* strain G4 genome. A mean of 94.35% (range 96% to 93%) of the sequence reads were found to map to coding sequences. Differential gene expression occurred in response to HEPB at sub-inhibitory concentrations: 21.94% of the *B. vietnamiensis* strain G4 genome had significantly altered expression, including 189 up-regulated genes (Table S5) and 70 down-regulation genes (Table S6) with a significant Log2-fold change ≥1.5. In total, 18 operons were significantly upregulated and eight downregulated with a Log2-fold change ≥1.5 ($p<0.05$). This included the upregulation of two RND-efflux systems, with up to +1.89-fold changes in expression (Table 53); and three operons involving transposable elements with homology to IS30, TniB, and Tn3 family proteins (present on the plasmid and the chromosome), with up to +3.05-fold changes in expression. Additionally, many integrases distributed along the genome of strain G4 were

upregulated (Table S5). Expression of two copies of H-NS, a global regulator and a xenogeneic silencer (22-24), was also upregulated.

The largest significant change in expression (+ 4.26-fold) was associated with gene Bcep1808_2705, which putatively encodes a sorbitol dehydrogenase. Network analysis of KEGG pathways indicated that the operon was putatively involved in fatty acid biosynthesis (17). All three genes within the operon were significantly upregulated ≥ +2.85-fold in response to HEPB. In connection, genes encoding a sorbitol-binding extracellular binding protein (Bcep1808_2709), and the operon Bcep1808_2705 to 2708, which encodes an ABC-transporter system, were significantly upregulated 3.32 to 4.06-fold in response to HEPB (Table S5). 25 of the genes upregulated by ≥2-fold were located on plasmid pBVIE02. The majority of these genes (with putative function) were involved in transcription, replication, recombination and repair: including a *parB* homolog and several transposable elements.

Genes significantly downregulated ≥1.5-fold in response to HEPB were located on chromosome 1 (n = 29), chromosome 2 (n=23), chromosome 3 (n = 7), and plasmids pBVIE01 (n=10) and pBVIE03 (n = 1). These genes were predominantly of unknown function (n = 19) or had a putative role in intracellular trafficking and secretion (n = 10), amino acid transport (n = 8) or energy production (n = 8) (Table S6). This included a -2.64 to -1.67-fold change in an operon of genes encoding a putative type II secretion system (Bcep1808_1487 to 1491, chromosome 1) involved in protein secretion. Genes involved in the high-affinity binding and transport of the aliphatic branched chain amino acids leucine/Isoleucine/valine were down-regulated ≤ -2.84-fold (Bcep1808_6683/6691 and 3370 respectively). Two porins were significantly down-regulated by -2.08 and -2.28-fold (genes Becp1808_4025 and 4974 respectively).

DISCUSSION

282 Manufacturers strive to formulate robust preservative systems with a wide antimicrobial
283 spectrum, that prevent microbial growth and will not lead to the development of resistant
284 microorganisms. Although there are currently over 100 chemical substances regulated as
285 primary synthetic/natural preservatives for use in personal care products, toiletries, and
286 cosmetics, under EU Commission Regulation no. 1223/2009 Annex V (25), this challenge to
287 manufacturers is compounded by tighter regulations on preservative limits of use, and by
288 consumer pressure for milder preservation in personal care and cosmetic products. As a
289 result, the most commonly used preservative agents fall into just 11 groups by chemical
290 composition (26). In recent years, safety concerns over estrogenic activity and sensitization
291 have led to a significant reduction in formulations containing parabens and isothiazolinone
292 preservatives, both highly efficacious agents against Bcc bacteria (2). Currently, the industry
293 suffers from a considerable lack of less-toxic preservatives with a potent antimicrobial
294 activity against these key contaminants.

295 ***HEPB is efficacious against key risk Burkholderia cepacia complex bacteria.*** HEPB
296 demonstrated good antimicrobial activity against a diverse panel of 58 *Burkholderia* strains
297 representing species commonly encountered as contaminants. In contrast to previous
298 antibiotic, biocide and preservative susceptibility surveys (27, 2), there were little or no inter-
299 /intra-species differences in HEPB susceptibility, with the majority of strains inhibited by the
300 industrially relevant level of 0.5% w/v. Greater inter-/intra-species differences in HEPB
301 susceptibility may well become apparent in a larger strain collection. However, this finding
302 suggests that the antimicrobial activity of HEPB exploits a vulnerability shared by
303 *Burkholderia* species. The majority of *Burkholderia* strains evaluated were inhibited, and
304 24% were killed, by the maximum permitted level of HEPB for use in EU cosmetics and
305 toiletries. The targeted antimicrobial potency against *Burkholderia* species was in stark
306 contrast to the HEPB susceptibility of the non-*Burkholderiales* bacteria evaluated. In this

study, and a recent *in-vitro* investigation by Wesgate *et al*. (28), industrially relevant levels of HEPB were sub-inhibitory for the same *P. aeruginosa*, *E. coli* and *S. aureus* reference strains. Even at 2% w/v, HEPB was non-lethal for these bacteria. However, it is noteworthy that the HEPB susceptibility of other genera has not yet been systematically surveyed utilising taxonomically diverse test strain panels. This study also investigated the inherent activity of HEPB in an aqueous solution with the co-solvent DMSO. In application, HEPB will be deployed in product formulations containing surfactants, sequestrants, and other compounds that can interact with cellular targets and may affect, even potentiate, its antimicrobial potency (29).

B. vietnamiensis adaptation to HEPB is transient and does not confer high-level HEPB resistance. Concerns regarding the potential link between widespread biocide use and the emergence of antimicrobial resistant microorganisms, have been voiced for many years. In support of recent opinions from scientific committees (30), the EU now requires that biocidal product manufacturers provide information on the development of resistance to their products in target organisms (31). The challenge to manufacturers of biocides, and preserved non-food products, is to predict resistance developing in formulation using low-cost high throughput techniques that reflect in-use conditions (32). Bcc bacteria have been shown to increase their antibiotic resistance after selection for spontaneous resistance (33), and increase tolerance to isothiazolinone and benzethonium chloride preservatives via adaptive resistance (2). In both studies, stable gene expression changes and cross-resistance to other antimicrobials were shown to persist in the absence of selective pressure/priming agents. However, changes in antibiotic susceptibility were not clinically relevant. The propensity for Bcc bacteria to develop resistance to HEPB was therefore carefully considered, as both spontaneous resistance and adaptation could contribute to the emergence of resistant organisms.

331 Spontaneous resistance, which occurs naturally in culture, did not develop to HEPB, even at a
332 concentration below the in-use recommendation (0.5% w/v). This may have been the result of
333 a multifactorial mode of action, and/or the absence of mutation(s) accumulating in a specific
334 cellular target or resistance mechanism. Unlike the previously observed stable adaptation of
335 *Burkholderia spp.* to isothiazolinone and benzethonium chloride preservatives (2), adaptation
336 to sub-lethal concentrations of HEPB was transient. The susceptibility of HEPB-adapted *B.*
337 *vietnamiensis* G4 derivatives to HEPB, and seven of the eight antibiotics evaluated, reverted
338 to wild-type levels in the absence of the priming agent. The mechanism(s) responsible for the
339 elevated tolerance to chloramphenicol in HEPB-adapted derivatives, that persisted even in the
340 absence of HEPB, was not elucidated. However, the contribution of efflux pump activity to
341 chloramphenicol resistance in *Burkholderia cepacia* complex species is well documented
342 (34). Wesgate *et al.*(28) also reported HEPB to have a low propensity to induce phenotypic
343 resistance in other genera; short-term exposure to HEPB (24 hours, in a tryptone and sodium
344 chloride suspension) did not change the antimicrobial susceptibility profile of *P. aeruginosa*
345 or *S. aureus* strains, although *E. coli* became susceptible to gentamycin. The propensity of
346 formulated HEPB to induce resistance in these genera was not investigated. Studies suggest
347 that the frequency, and extent of decreases in susceptibility to preservatives, can be
348 significantly lower when the biocides are incorporated into product formulation (29, 32, 35).

349 Genomic analysis of the HEPB-adapted derivatives revealed few DNA polymorphisms
350 resulted from the prolonged exposure of *B. vietnamiensis* to sub-inhibitory levels of HEPB.
351 This finding supports the opinion of the SCCS that, based on bacterial reversion mutation
352 tests in *Salmonella spp.*, HEPB was not a potential mutagen (10). The low rate of mutation in
353 HEPB-adapted *B. vietnamiensis* derivatives suggests that the transient change in phenotype,
354 and antimicrobial susceptibility, were a result of gene expression changes and/or the
355 epigenetic regulation of gene expression at a transcriptomic level (36). Reversible changes to

antimicrobial susceptibility have been attributed to transient phenotypic adaptations, such as the induction of bacterial stress responses (37), or non-specific reductions in the cellular permeability (38), that revert to pre-exposure levels once the antimicrobial is removed (29). DNA methylation has been shown to epigenetically regulate gene expression in *B. cenocepacia* (39) playing an important role in biofilm formation and motility. The role of DNA methylation in the preservative resistance of *Burkholderia spp.* remains to be determined. The 'methylome' of *B. vietnamiensis* strain G4 was not characterised in this study, but results suggest that key genetic pathways associated with HEPB may be regulated by DNA methylation. The mutation of gene Bcep1808_0037 (mutant 14:C2, Table 3), which encodes a highly conserved type III methyltransferase orthologous to *B. cenocepacia* J2315 gene BCAL3494 (39), resulted in a 2-fold decrease in MIC for HEPB.

HEPB mode of action against Bcc bacteria is multifactorial. A large global gene expression effort was required for *B. vietnamiensis* strain G4 to grow in the presence of HEPB at half the MIC. Key genetic pathways, identified by transposon mutagenesis and transcriptomic analysis, suggested that HEPB had a multifactorial mode of action, and did not target a specific cellular target. The highest significantly upregulated gene pathways in response to HEPB were associated with fatty acid biosynthesis pathways (17) involved in fructose and mannose metabolism. Lipids are major targets during oxidative stress (40); therefore, the effect of HEPB on the outer membrane and lipopolysaccharides of *Burkholderia* bacteria warrants further investigation.

Multiple key gene pathways associated with HEPB were involved in stress responses, and survival of adverse conditions. This included *parB* gene homologs, shown to regulate stress response in *Pseudomonas* (21), and *spoT*, a mediator of the stringent response shown to influence expression of numerous genes with an effect on bacterial cell physiology that impacts antimicrobial susceptibility (41). Mutation of *relA* or *spoT* in *P. aeruginosa*, have

been shown to increase susceptibility to antimicrobials that cause oxidative stress (16). Transposable elements within *Burkholderia* have also been shown to have increased activity in response to oxidative stress (42). The role of transposable elements in HEPB susceptibility, remains to be elucidated. The activity of these elements may also influence the regulation of genes and modulate the organisms' stress response(s), an epigenetic effect that has been observed in plant cells (43).

Key gene pathways involved in the repair of proteins damaged by oxidation, and repair of damaged DNA, were associated with HEPB. This suggested that HEPB damages, and potentially kills cells, via oxidation. Based on its chemistry, the carbonyl group is the likely, albeit weak, electrophilic centre of the molecule that would require activation of the oxygen for reactivity. Not considered to be an oxidant, the oxidative damage caused by HEPB may result from a reactive metabolite or by an indirect mechanism that produces endogenous reactive oxygen species (ROS) (44). Methionine sulfoxide reductase enzymes (including MsrA) have been shown to be important for resistance against oxidative stress in a range of bacteria (45), and are key participants in maintaining the homeostasis of the cytoplasm and envelope of bacteria. Mutation of the *msrA* gene in *B. vietnamiensis* resulted in increased HEPB susceptibility. This suggests that HEPB causes the oxidation of methionine (Met) residues in *B. vietnamiensis*, and that the full repair of these proteins requires the action of both msrA and msrB enzymes. Isothiazolinone preservatives, shown to be highly efficacious against Bcc bacteria (2), also target amino acids particularly vulnerable to oxidation. These reactive electrophilic biocides oxidate the thiol (-SH) functional group of cysteine residues of cytoplasmic and membrane bound enzymes, and damage DNA at higher concentrations (46) .

A key role for the *kdp* operon, encoding a high-affinity K^+ transporter, and kefC potassium channels, suggested that the regulation of intracellular pH was important for the survival of *B. vietnamiensis* exposed to HEPB. kefC efflux systems have been shown to protect *E.coli*

against intracellular damage caused by externally derived electrophiles, via potassium efflux and the rapid acidification of the cytoplasm (47). Other key genes identified suggested that altered cellular permeability and defence mechanisms were potentially required to reduce uptake/intracellular concentrations of HEPB and aid survival. There was a reduction in the expression of porins and increased expression of efflux systems, including those of the RND-family. However, these RND-systems were not homologs of those previously associated with adaptive-resistance to isothiazolinone preservatives (2).

CONCLUSIONS

In a study of susceptibility and genetic analysis, we have demonstrated that HEPB is active against *Burkholderia* species encountered as industrial contaminants, and has low risk of promoting its own or other antimicrobial resistance in these Gram-negative bacteria. Key genetic pathways associated with HEPB susceptibility were involved in bacterial stress responses and damage/repair mechanisms. These indicated that the agent is multifactorial in nature causing oxidative stress and damage to intracellular components. Overall, this study supports the use of HEPB as an efficacious preservative against *Burkholderia* bacteria, recognised as antimicrobial resistant and objectionable industrial contaminants.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A panel of 58 *Burkholderia* and 7 non-*Burkholderia* strains used for profiling HEPB susceptibility was drawn from the Cardiff University Collection (3), including reference strains from the Belgium Coordinated Collection of Microorganisms (BCCM) (http://bccm.belspo.be/about/lmg.php; see Table S1 and S2). The collection comprised 20 of the current *Burkholderia cepacia* complex (Bcc)

429 species groups, reference strains from the Bcc experimental strain panel (48), and 39 strains
430 previously profiled for preservative susceptibility by Rushton *et al.* (2). Two non-Bcc
431 species, *B. gladioli* and *B. plantarii*, were also included. The *Burkholderia* panel strains were
432 originally isolated from clinical (*n = 27*), environmental (*n = 23*), and industrial (*n = 8*)
433 sources. Seven non-*Burkholderia* species were evaluated as a control group, including two
434 members of the closely related *Paraburkholderia* clade (4), antibiotic and biocide testing
435 reference strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and a reference
436 strain of *Escherichia coli.* For consistent revival strains were cultured at 30˚C on tryptone
437 soya media (TSB/TSA; Oxoid Ltd, UK). Strains were stored in TSB containing 8% v/v
438 dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) at -80˚C.

439 **Preservative susceptibility testing.** The minimum inhibitory concentration (MIC) and
440 minimum bactericidal concentration (MBC) of HEPB (L'Oréal, France) were determined by
441 standardised agar dilution and broth dilution assays as described by Rushton *et al.* (2), using
442 TSA/TSB. Bacterial culture was performed at 30˚C to be representative of industrial product
443 manufacture and storage. Preservative stock solutions (50% w/v) were prepared in DMSO
444 (Sigma-Aldrich, UK) and the required volume then added to the growth medium to achieve
445 the desired test concentration (range 0.03125 – 2% w/v HEPB). Test media was used on the
446 day of preparation. The final concentration of DMSO in the presence of the bacteria was non-
447 toxic (not exceeding 4% v/v) and was included as a control condition in assays to negate its
448 effect on growth. The MIC was defined as the lowest concentration of preservative at which
449 there was an 80% reduction in liquid culture OD (at 630 nm), or no visible growth of the test
450 organisms on an agar medium (TSA). The bactericidal activity of HEPB up to 2% w/v, above
451 the maximum concentration for intended use (0.7% w/v) in rinse-off, oral care and leave-on
452 cosmetic products, was examined. The MBC was determined as the lowest concentration to
453 elicit a 99% rate of kill, at which growth on recovery medium (TSA) ceased. Preservatives

were inactivated prior to the recovery and enumeration of surviving test organisms by dilution in a 1.5% v/v Tween 80, 3 % lecithin neutralising solution as described by Rushton *et al.*(2). The efficiency and toxicity of the neutralising solution were evaluated prior to experimentation as described by Lear *et al* .2006 (49). Three biological replicates, each with three technical replicates, were obtained.

***Burkholderia* resistance to HEPB.** *B. vietnamiensis* strain G4 (LMG 22486) was used to evaluate the propensity of *Burkholderia* to develop resistance to HEPB. To enumerate spontaneous resistance occurring within a culture, 1 x 10^6 CFU of strain G4 from a fresh overnight (18 h) TSB culture was inoculated onto the surface of replicate TSA plates containing HEPB 2-fold higher than the MIC, mutants with decreased susceptibility were enumerated after 24 hours of culture.

A stepwise training assay was performed to select for HEPB-adapted derivatives. Approximately 1 x 10^6 CFU of an 18 h TSB culture of *B. vietnamiensis* strain G4 were inoculated onto the surface of TSA plates containing HEPB concentrations up to eight-fold lower than that of the MIC as described by Rushton *et al.*(2). After 24 hours culture at 30 °C, growth from the starting TSA-HEPB plates were sub-cultured onto TSA containing a 2-fold increase in HEPB concentration. The serial passage of *B. vietnamiensis* strain G4 on TSA with a gradual increase in HEPB concentrations (to above that of the MIC), was repeated until growth ceased. HEPB-adapted *B. vietnamiensis* strain G4 derivatives were stored at – 80 °C as described above.

Stability of the HEPB-adapted phenotype was then evaluated after five passages on TSA plates without HEPB. HEPB-Adapted derivatives were confirmed as *B. vietnamiensis* strain G4 by Random Amplified Polymorphic DNA (RAPD) analysis (50).

Analysis of growth dynamics. The growth dynamics of the wild-type *B. vietnamiensis* strain G4, HEPB-adapted derivatives of strain G4, and *P. aeruginosa* (ATCC 19429), were evaluated using a broth dilution assay in a Bioscreen C Microbiological Growth Analyser (Labsystems, Finland). Starting cultures were standardized by OD as described by Rushton *et al.* (2) and quadruplicate 200 µl cultures in the multi-well plate inoculated with 1 x 10^6 CFU. Turbidity readings were taken using a wideband-filter (450 to 580 nm) every 15 minutes after shaking the microplates for 10 seconds at medium amplitude. Experiments were repeated to obtain three biological replicates. The mean OD of the uninoculated media were subtracted from the test wells and the data transformed by Log_{10}+1 to obtain a 'Log_{10} adjusted OD' for growth curve analysis. Growth rate (µ) and length of lag-phase (hours) were determined from the mean growth curves generated using the GroFit package in R software (51).

Antibiotic susceptibility assay. Phenotypic changes in HEPB-adapted *B. vietnamiensis* strain G4 derivatives were characterised by antibiotic susceptibility profiling using ETest strips (BioMerieux, UK) according to manufacturers' guidelines. Eight antibiotics with varying modes of action were examined: amikacin (AK), azithromycin (AZ), ceftazidime (TZ), chloramphenicol (CHL), ciprofloxacin (CI) imipenem, piperacillin (PP) and trimethoprim-sulfamethoxazole (SXT).

Construction of transposon mutants of *B. vietnamiensis* strain G4. *Escherichia coli* S17-1 λpir donor strain carrying pUTmini-Tn5-*luxCDABE*-Km was grown on Luria Butani (LB) media containing 20 µg ml^{-1} kanamycin (KAN) (Sigma) at 37˚C. The mini-Tn5-*luxCDABE* transposon was delivered into the recipient *B. vietnamiensis* strain by conjugal mating with the *E. coli* donor as described by Lewenza *et al.*(15) with the following modifications. Fresh overnight cultures of recipient and donor were concentrated by centrifugation at 1600 *x g* for 10 minutes and re-suspended in LB broth containing 10 mM $MgSO_4$**.** Donor and recipient were mixed at a ratio of 1:1 on a nitrocellulose filter (0.2 µm pore size) and incubated on LB

agar containing 10 mM $MgSO_4$ for 24 hours at 37˚C. To select *B. vietnamiensis* transconjugants, the mixture was diluted in TSB and inoculated onto TSA agar containing 30 µg ml^{-1} KAN and 240 units/ml polymyxin B (PMB;Sigma) (PMB was added to counter-select against the *E.coli* donor). Transconjugants were picked into 96-well plates containing 200 µl TSB and cultured for 24 hours on an orbital shaker at 30˚C. DMSO was added to achieve a final concentration of 8% v/v and plates were stored at -80˚C.

Transposon-insertion mapping. Mutant genomic DNA was extracted from overnight cultures using the Maxwell®16 instrument (Promega, Southampton, UK) and a Maxwell® 16 Tissue DNA purification kit, according to the manufacturers' instruction. Genomic DNA flanking the site of transposon insertion was amplified by nested PCR with primers 1-4 (Table S7 in supplemental materials) as described by O'Sullivan *et al.* (52). The PCR product was sequenced using Primer 3 (Table S7. Eurofins Genomics, Germany) and interrupted genes were located on the annotated reference *B. vietnamiensis* strain G4 genome (GenBank accession: GCA_000016205.1).

Bioinformatic analysis was carried out using a virtual machine, hosted by the Cloud Infrastructure for Microbial Bioinformatics (CLIMB) (53). Coding sequence features were extracted (https://github.com/bac-genomics-scripts/cds_extractor) and a basic local alignment search tool (BLAST) algorithm was used to compare nucleotide queries. Additional analysis was performed using the *Burkholderia* Genome Database (54). Twenty mutants from across the mutant bank were selected for validation of single random chromosomal integration of the transposon and sufficient coverage of the multi-replicon G4 chromosome. Mutants were confirmed as *B. vietnamiensis* strain G4 by RAPD analysis (50).

HEPB susceptibility screening of G4 mutants. *B. vietnamiensis* mutants with altered HEPB susceptibility were identified by agar/broth dilution MIC assays and growth curves analysis as described above. To confirm the phenotype, mutants with altered HEPB susceptibility (to

527 that of the wild-type) identified by agar dilution assay in a 96-well plate format were re-
528 screened by broth dilution assay with TSB containing HEPB at the MIC and 0.5 x MIC.
529 Mutants with altered susceptibility were selected based on an additional 20% reduction in OD
530 (600 nm) to that of wild-type growth reduction, at both test concentrations. To confirm
531 altered HEPB susceptibility and evaluate fitness, growth curve analysis of the selected
532 mutants was performed in TSB and TSB containing HEPB at half the MIC as described
533 above. Alteration to HEPB susceptibility was defined as equivalent wild-type growth
534 dynamics under control conditions (TSB without HEPB) in conjunction with a lower final
535 OD and/or significantly altered growth rate and longer lag-phase. The genetic context of
536 mutants of interest with altered HEPB susceptibility was determined as described above.

537 **HEPB time-kill assay.** *B. vietnamiensis* strain G4 and *P. aeruginosa* ATCC 19429 were
538 prepared and cultured (as three biological replicates) as described for the susceptibility testing
539 above. Bactericidal activity of HEPB against approximately 1 x 10^6 CFU was assessed in
540 TSB using 2-fold dilutions of HEPB from 2% w/v to 0.125% w/v (2 x to 0.5 x MIC for strain
541 G4). 100 µl of culture were collected 0, 1, 4, 24 and 168 h post-inoculation, diluted in
542 neutralising solution to inactivate the HEPB, and viable cells were enumerated on TSA using
543 a drop count method.

544 **Gene expression analysis.** RNA-sequence analysis was utilised to determine differential
545 gene expression in response to HEPB, as described by Green *et al*. (55) with the following
546 modifications. To determine a suitable time-point for RNA extraction, growth curve analysis
547 was performed on *B. vietnamiensis* strain G4 cultured in TSB (control) and TSB containing
548 HEPB at 0.75 x and 0.5 x MIC (test condition) for 24 hours as described above. A suitable
549 test concentration and time-point were chosen, at which the test and control conditions were
550 at an equivalent growth phase, and the cell numbers would yield sufficient RNA for analysis
551 (Fig S2). Four biological replicates of *B. vietnamiensis* G4 culture (each with four technical

552 replicates) under test and control conditions were harvested. Cells were rapidly cooled using
553 liquid nitrogen and total RNA (toRNA) extracted using the RiboPure RNA Purification
554 Bacteria Kit (Ambion) according to manufacturers' guidelines. This included a DNase 1
555 treatment step to deplete trace amounts of genomic DNA from the toRNA. RNA was
556 quantified using a Qubit fluorometer system and a broad-range RNA kit (Invitrogen). The
557 quality of RNA was determined using a Bioanalyzer with RNA 6000 Nano kit (Agilent
558 Technologies, Ltd), according to manufacturers' recommendations. Total RNA with a high
559 Integrity Number (≥ 8) and a ratio of 23S:16S rRNA ≥ 1.5 was concentrated by precipitation
560 to ≥ 100 ng/ul, and messenger RNA (mRNA) was enriched using the MICROBExpress
561 bacterial mRNA enrichment kit (Ambion). Complementary DNA (cDNA) library
562 preparations (using the Illumina TruSeq Stranded mRNA kit) and sequencing (on an Illumina
563 NextSeq 500) were conducted by the Genomics Research Hub at Cardiff School of
564 Biosciences: https://www.cardiff.ac.uk/biosciences/research/technology-research-
565 hubs/genomics-research. Bioinformatic analysis was carried out as described by Green *et*
566 *al*.(55) with the following modifications. Quality control and adaptor trimming of the paired-
567 end sequencing data were conducted using Trim Galore software v0.4.4 (56). As an
568 additional enrichment step for mRNA sequence data, Artemis software (v16.0) (57) was used
569 to obtain the 16S to 23S rRNA gene region sequence data from the complete *B. vietnamiensis*
570 strain G4 genome sequence (obtained from NCBI). The RNA-seq reads were first aligned to
571 the 16S:23S rRNA gene region via a Burrows-Wheeler Aligner transformation (BWA) with
572 the BWA-MEM algorithm (v0.7.13-r1126)(58). The aligned sequence reads were removed
573 from the data set using the SAM Tools toolkit (v1.3)(59). The rRNA-clean sequence reads
574 were then aligned to the complete *B. vietnamiensis* strain G4 genome sequence via a BWA
575 using the BWA-MEM algorithm. Sequence Alignment/Map (SAM) files of the aligned
576 sequence reads were then sorted into BAM files using the SAM Tools toolkit. The aligned

577 reads that mapped to annotated gene features of the *B. vietnamiensis* strain G4 genome were
578 counted using the Python programme HTSeq-count (v0.6.0) (60). Differential gene
579 expression was determined using the R Bioconductor programme DESeq2 (v1.14.1) (61) and
580 was defined as exhibiting a log2-fold change of >1.5 as previously described (2, 33, 55).

581 **Identifying genomic alterations in HEPB-adapted *B. vietnamiensis* G4.** Genomic DNA of
582 the wild-type and HEPB-adapted derivatives ("T2s", "T2L" and "T3") was extracted using
583 the Maxwell®16 Tissue DNA purification kit according to manufacturers' guidelines. DNA
584 library preparations and sequencing on an Illumina NextSeq 500, were carried out in
585 cooperation with the Genomics Research Hub at Cardiff School of Biosciences. The resulting
586 reads were trimmed using Trim Galore software v0.0.4 and assembled using Unicycler
587 (v0.4.7)(62). Annotation of the assembled draft genomes was conducted using Prokka (v1.12)
588 (63). DNA Polymorphisms between the sequence reads of the wild-type genome and the
589 HEPB-adapted derivatives genomes were identified using Snippy (v4.1.0) (64) with default
590 parameters of a minimum base quality of 20, minimum read coverage of 10X, and 90% read
591 concordance at each locus.

592 **Statistical analysis.** All experiments were performed as 3 biological replicates except for
593 RNA-sequencing which was performed in quadruplicate for each condition. Significant
594 differences ($p < 0.05$) in the mean MIC or MBC values for test groups were determined using
595 a Two-sampled T-test for equal or unequal variances as appropriate. Significant differences
596 ($p < 0.05$) in gene expression changes with ≥ 1.5-fold alteration were determined as described
597 above.

598 **Accession numbers.** RNA sequence reads are available in the ArrayExpress database
599 (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7906. The whole-
600 genome sequence reads of *B. vietnamiensis* strain G4 (wild-type) and HEPB-adapted

derivatives “T2s”, “T2L” and “T3” are available at European Nucleotide Archive (accession numbers ERS4125347, ERS4125348, ERS4125349, ERS4125347, ERS4125350 respectively). Gene numbers and nomenclature correlate to those shown on www.burkholderia.com for the *B.vietnamiensis* G4 genome.

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AUTHOR CONTRIBUTIONS

We describe author contributions to the paper using the CRedit taxonomy.

Conceptualisation: E.M, JY.M and F.M.S; *Data Curation:* L.R. and E.M.; *Formal analysis:* L.R.; *Funding Acquisition:* E.M., JY.M and F.M.S; *Investigation:* L.R.; *Methodology:* L.R., E.M., J.Y.M, F.M.S, and A.K.; *Project Administration:* L.R. and E.M.; *Resources:* F.M.S., A.K., and E.M.; *Software:* L.R.; *Supervision:* E.M.; *Validation:* L.R.; *Visualisation:* L.R.; *Writing-Original Draft:* L.R and E.M.; *Writing-Review & Editing:* L.R., E.M., J.Y.M, A.K, S.C and F.M.S.

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TRANSPARENCY DECLARATIONS

L.R., J-Y.M. and E.M. do not declare any conflicts of interest. F.M.S., A.K. and S.C. are employees of L'Oréal (Paris, France).

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TABLE 1 The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of HEPB for *Burkholderia* and *Paraburkholderia* strains.

Grouped Strains	HEPB MIC (% w/v)		HEPB MBC (% w/v)	
	Median	Mean (± STDEV)	Median	Mean (± STDEV)
Burkholderia *(n = 58)*	0.5	0.45 ± *0.12*	1	0.90 ± *0.30*
BCC *(n = 54)*	0.5	0.46 ± *0.11*	1	0.91 ± *0.29*
Paraburkholderia *(n = 2)*	0.125	0.125 *0*	0.5	0.625 ± *0.25*

BCC, Burkholderia cepacia complex; n, number of strains. Median and mean, values derived from three biological replicate experiments. MIC and MBC data for individual strains is available in supplemental Table S3.

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839 **TABLE 2. Antibiotic susceptibility profile of HEPB-adapted *B. vietnamiensis* strain G4**
840 **derivatives.**

	Mean MIC (µg/ml)							
***B. vietnamiensis* strain G4**	**AMK**	**AZM**	**CIP**	**CHL**	**IPM**	**PIP**	**SXT**	**CAZ**
Parent	1	4	0.19	12	0.125	0.5	0.125	0.5
Derivative T2s	1	4	0.19	32	0.125	0.5	0.19	0.38
Derivative T2L	1	3	0.19	12	0.125	0.5	0.19	0.5
Derivative T3	0.25	1	0.64	24	0.16	0.25	0.125	0.25

841 Abbreviations: AMK, Amikacin; AZM, Azithromycin; CIP, ciprofloxacin; CHL,
842 chloramphenicol; IMP, imipenem; PIP, piperacillin; Trimethoprim-sulfamethoxazole; CAZ,
843 Ceftazidime. The table shows the antibiotic susceptibility profile of HEPB-adapted
844 derivatives before serial passage in the absence of HEPB.

845 **TABLE 3. Transposon interrupted genes of *B. vietnamiensis* strain G4 exhibiting altered susceptibility to HEPB and wild-type**
846 **growth in control conditions.**

Mutant ID	G4 DNA flanking transposon insertion site (20 bp)	Gene ID	Replicon	Name and Putative Function of Mutated Gene [1]	COG Category [2]
9:G3	ACCCATCACCATGCCCACA	4698	chr2	*ilvD*, Dihydroxy-acid dehydratase	Amino acid transport and metabolism
44:B9	CGTTCCGGCGCGGCGCTGCC	6500	chr3	Cellulose synthase domain-containing protein	Carbohydrate transport and metabolism
42:E3	AAGTAAGACAGGTCACGAAC	1471	chr1	RND efflux system outer membrane lipoprotein	Cell wall/membrane/envelope biogenesis
14:C2	AGCAGTTCATCGCGCTGGC	0037	chr1	Type III restriction enzyme, res subunit	Defence mechanisms
9:D8	ATCGCTGACCACCCGCGCT	0174	chr1	Hypothetical protein (putative restriction endonuclease)	Defence mechanisms
5:D5	CGGCTAGGCGGCCAGATCT	3088	chr1	*puuB*, Gamma-glutamylputrescine oxidoreductase	Energy production and conversion
43:B11	AGGAGAAAGGCCCCGTCATC	3033	chr1	2-oxoacid ferredoxin oxidoreductase	Energy production and conversion
6:F3	AGGCGGCCAGATCTGATCA	7566	pBVIE04	Hypothetical protein	Function unknown
9:D7	CCCCCCCGTACTAGTCGAC	3807	chr2	Amine oxidase	Function unknown
19:E4	CGCTGGCGGCCAGATCTGA	1415	chr1	Hypothetical protein	Function unknown
44:E6	GTCAACGCGTGGCCAAATCG	6803	pBVI01	Hypothetical protein	Function unknown
29:B7 [3]	CGGCTAGGCGGCCAGATCT	2890	chr1	*kefC*, Glutathione-regulated potassium-efflux system protein	Inorganic ion transport and metabolism
27:G3	CCCCCGCCGTACTAGTCGA	5515	chr3	*MsrA*, Peptide methionine sulfoxide reductase	Posttranslational modification, protein turnover, chaperones
19:D4	CTGCTCCGGCACGACGTCCA	0426	chr1	DNA primase TraC	Replication, recombination and repair
42:B11	ATAATAGTCAAGGCGTGGCC	6093	chr3	Transposase Tn3 family protein	Replication, recombination and repair
7:G7	TGAGTTTAATGTCTTCGCT	6805	pBVIE01	Putative signal transduction protein	Signal transduction mechanisms
19:H7	CCGACGCGCGCCGGCAGCG	2370	chr1	*kdpD*,Sensor protein	Signal transduction mechanisms
35:G5	CGTGACCAGGTGCTCGCGA	3929	chr2	Integral membrane sensor hybrid histidine kinase	Signal transduction mechanisms
8:D10	GGCAGGCCAGATCTGATCA	7553	pBVIE04	Helicase domain-containing protein	Transcription
6:F4	CGTGACGACCGAGTCGAAG	3650	chr2	Chromosome replication initiation inhibitor protein	Transcription
43:G1	CGCTCACTGCCGGCCGGCAA	4632	chr2	LysR family transcriptional regulator	Transcription
20:G12 [3]	TACGACCAGTCTGCGAATCG	3794	chr2	*noc*, Nucleoid occlusion (*parB*-like) protein	Transcription
22:E11 [3]	TCAGCTAGGCGGCCAGATCT	0918	chr1	*spoT*, Bifunctional (p)ppGpp synthase/hydrolase	Transcription, signal transduction mechanisms

[1] Putative function of poorly characterised proteins based on predicted protein-protein interactions networks in the STRING database(17)

[2] COG, Cluster of orthologous groups category identified using the EggNOG database (65).

[3] Mutant demonstrated a 4-fold decrease in MIC (0.0625% w/v) of HEPB by agar dilution assay. All other mutants demonstrated a 2-fold decrease in MIC of HEPB by agar dilution assay.

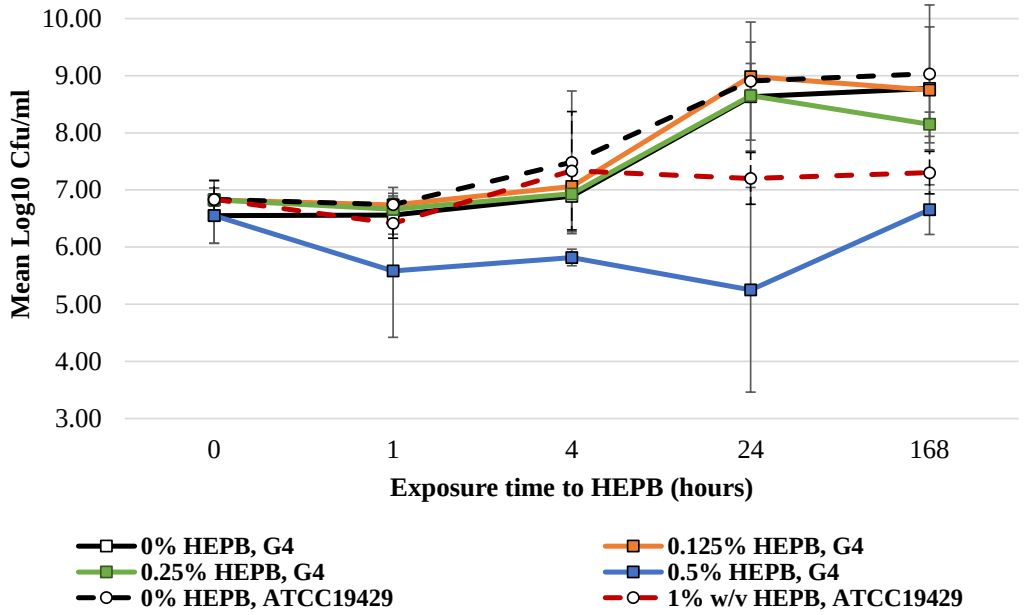

FIG. 1. **Time-kill curves of *B. vietnamiensis* strain G4 and *P. aeruginosa* ATCC19429 cultured in TSB with HEPB at concentrations below in-use levels.** *B. vietnamiensis* strain G4 viability decreased to undetectable levels after exposure to 1% w/v HEPB for 1 hour (not shown). *P. aeruginosa* ATCC19429 is indicated by dash lines. Cultures were sampled over 1 week and neutralised before enumeration of viable cells. Each symbol indicated the means ± STDEV for three biological replicates. The lower detection threshold was 10^3 CFU/ml. A final concentration of 2% v/v DMSO control did not reduce cell viability.

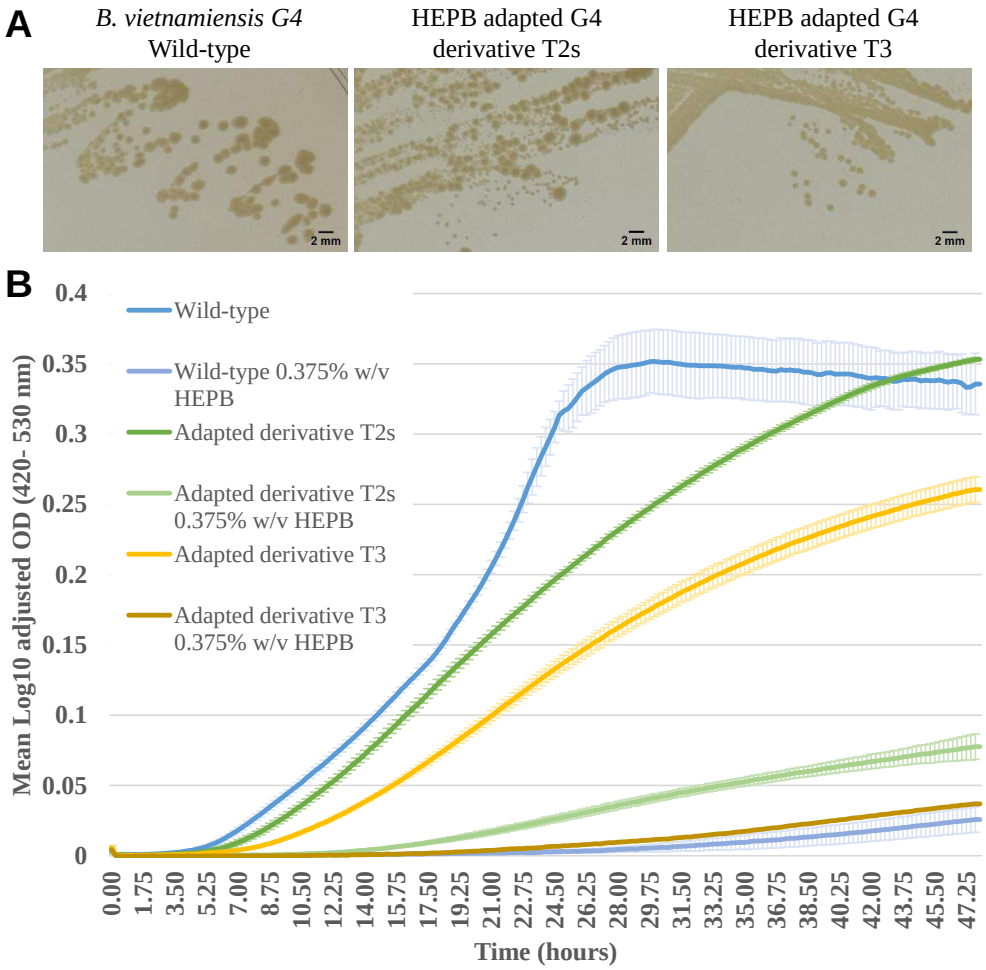

FIG 2. Colony morphology and growth curve of *B. vietnamiensis* strain G4 wild-type and HEPB-adapted derivatives T2s and T3. (A) Colony morphology of wild-type (parental) strain and HEPB adapted derivates cultured on TSA without HEPB for 24 hours. HEPB-adapted derivatives form smaller discrete round colonies than the wild-type. Scale bar represents 2 mm. **(B)** Growth of the wild-type and HEPB adapted derivatives in TSB without HEPB and 0.375% w/v HEPB. Each symbol indicated the means ± STDEV for two biological replicates. Growth of T2s is similar to the wild-type in the absence of HEPB. HEPB-adapted derivatives reached

higher final OD than the wild-type in the presence of HEPB. HEPB-adapted derivative T2L was not shown, as its colony morphology and growth in the absence/presence of HEPB was similar to that of the wild-type.