Loss of Function in Escherichia coli exposed to Environmentally Relevant Concentrations of Benzalkonium Chloride

FORBES, Sarah <http://orcid.org/0000-0002-8361-6390>, MORGAN, Nicola, HUMPHREYS, Gavin J. <http://orcid.org/0000-0002-9138-0859>, AMÉZQUITA, Alejandro, MISTRY, Hitesh and MCBAIN, Andrew J. <http://orcid.org/0000-0002-5255-5425>

Loss of Function in *Escherichia coli* exposed to Environmentally Relevant Concentrations of Benzalkonium Chloride

Sarah Forbes[1], Nicola Morgan[2], Gavin J Humphreys[2], Alejandro Amézquita[3], Hitesh Mistry and Andrew J McBain[2*]

[1]Biomolecular Sciences Research Centre, Sheffield Hallam University, Sheffield. [2]Division of Pharmacy and Optometry, School of Health Sciences, Faculty of Biology, Medicine and Health, Stopford Building, The University of Manchester, Manchester, UK [3]Unilever, Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedfordshire, MK44 1LQ, UK.

Running Title: *Escherichia coli* adaptation to residual concentrations of benzalkonium chloride

Key Words: Biocide, Bacteria, benzalkonium chloride.

*For correspondence: Andrew J McBain, Division of Pharmacy and Optometry, Faculty of Biology, Medicine and Health, The University of Manchester, Oxford Road, Manchester M13 9PT, UK. Tel: 44 161 275 2360; Email: andrew.mcbain@manchester.ac.uk

ABSTRACT

Assessing the risk of resistance associated with biocide exposure commonly involves exposing microorganisms to biocides at concentrations close to the MIC. With the aim of representing exposure to environmental biocide residues, *Escherichia coli* MG1655 was grown for 20 passages in the presence or absence of benzalkonium chloride (BAC) at 100 ng/L and 1000 ng/L (0.0002% and 0.002% of the MIC respectively). BAC susceptibility, planktonic growth rates, motility and biofilm-formation were assessed, and differentially expressed genes determined via RNA-sequencing. Planktonic growth rate and biofilm-formation were significantly reduced ($p<0.001$) following BAC adaptation, whilst BAC minimum bactericidal concentration increased two-fold. Transcriptomic analysis identified 289 upregulated and 391 downregulated genes after long-term BAC adaptation when compared to the respective control organism passaged in BAC-free-media. When the BAC-adapted bacterium was grown in biocide-free medium, 1052 genes were upregulated and 753 were down regulated. Repeated passage solely in biocide-free medium resulted in 460 upregulated and 476 downregulated genes compared to unexposed bacteria. Long-term exposure to environmentally relevant BAC concentrations increased the expression of genes associated with efflux and reduced gene expression associated with outer-membrane porins, motility and chemotaxis. This was manifested phenotypically through loss-of-function (motility). Repeated passage in a BAC-free-environment resulted in the up-regulation of multiple respiration-associated genes, which was reflected by increased growth rate. In summary, repeated exposure of *E. coli* to BAC residues resulted in significant alterations in global gene expression that were associated with minor decreases in biocide susceptibility, reductions in growth-rate and biofilm-formation, and loss of motility.

IMPORTANCE. Exposure to very low concentrations of biocide in the environment is a poorly understood risk factor for antimicrobial resistance. Repeated exposure to trace levels of the biocide BAC resulted in loss of function (motility) and a general reduction in bacterial fitness, but relatively minor decreases in susceptibility. These changes were accompanied by widespread changes in the *E. coli* transcriptome. This demonstrates the importance of including phenotypic characterisation in studies designed to assess the risks of biocide exposure.

INTRODUCTION

Benzalkonium chloride (BAC) is a broad-spectrum quaternary ammonium biocide (1), which has a variety of applications in antisepsis, disinfection or preservation in industrial (2-4), medical (5) (6-8) and domestic (9-11) fields. Interest in the biological mechanisms that underpin the antibacterial mode of action of BAC has been longstanding (12). There is concern that biocides could select for reduced susceptibility in bacteria (13-15), which may occur through the

increased expression of efflux pumps (16-17), changes in cell permeability (18) or modification of cell surface electrostatic charge (17) (19). The potential for this to occur has been demonstrated through *in vitro* studies (20) but less so in environmental surveys (as previously reviewed (21)).

Regardless of the application, microorganisms are likely to be exposed to effective concentrations of biocides at the point of primary application and to lower concentrations through secondary exposure. This may result in a microorganism being exposed to concentrations of biocides that are substantially above the minimum bactericidal concentration, or anywhere on a continuum between this and zero, as previously reviewed (22) (13) (21) (23).

Biocide residues are commonly detected in aquatic environments with concentrations in the region of 100 ng/L reported in wastewater effluents (24) that will be further diluted as effluents are dissipated into the riverine and marine environments (25). Such biocide concentrations are several hundred times lower than MICs and could therefore be assumed to exert minimal selective pressure on exposed microbial populations.

The fact that environmentally relevant concentrations of biocides are likely to be considerably below those required to inhibit microbial growth has significance for risk assessment where *in vitro* testing has an important role (26-27). In such *in vitro* testing microorganisms are generally exposed to the biocides at sub-lethal concentrations. Concentrations used at the point of primary deployment are however often considerably higher than minimum bactericidal concentrations (14) (28).

We have previously assessed the effect of formulation (29-30), growth in taxonomically diverse biofilm communities (31), and potential impairment of fitness in biocide-adapted bacteria (32-33) in a series of studies intended to develop and apply methods for assessing the risk of biocide resistance based more closely on real-life conditions.

The current investigation considers biocide concentration as the variable of interest. We exposed *E. coli* to concentrations of BAC representing environmental residues in treated effluent, riverine and other environments (24). Antimicrobial susceptibility and phenotypic variables of relevance to environmental fitness and persistence were assessed. With the aim of correlating phenotypic changes with omics data, the transcriptomes of bacteria exhibiting loss of function following exposure to 100 ng/L BAC were profiled using RNA sequencing (RNA-seq).

RESULTS

E. coli was adapted to BAC at 100 ng/L and 1000 ng/L over 10 and 20 passages. Susceptibility (MIC and MBC) and planktonic and biofilm growth dynamics were assessed before and after adaptation and compared to the respective bacterium passaged in BAC-free medium. Alterations in the transcriptome were assessed using RNA-seq for BAC-adapted and non-adapted bacteria, following immediate growth in BAC-containing or BAC-free medium to determine the stability of any transcriptomic changes once the biocide was removed.

Effects of BAC exposure on antimicrobial susceptibility. No change in susceptibility (MIC or MBC) to BAC was observed after 10 passages in the presence of BAC at either concentration. The MBC increased two-fold (the minimum detectable change) for both exposure concentrations after 20 passages, whilst the MIC remained unchanged.

Alterations in planktonic growth and biofilm formation after BAC exposure. Repeated passage in biocide-free medium led to reduced lag phase, increased growth rate and decreased maximum culture density ($p<0.001$). Following adaptation (10 and 20 passages) with 100 ng/L BAC, growth rates did not change but maximum culture densities were significantly decreased. Adaptation to 1000 ng/L of BAC (10 and 20 passages) also resulted in significantly decreased maximum culture densities but did not alter growth rates relative to the control.

However, lag phase increased following 10 passages with BAC at 1000 ng/L, which was not the case following 20 passages.

Repeated passage of bacteria solely in biocide-free medium resulted in significantly decreased biofilm formation when compared to unexposed parent bacterium ($p<0.05$). Adaptation to BAC at 100 or 1000 ng/L resulted in significantly greater reductions in biofilm formation ($p<0.05$) (Figure 2). Biofilm formation was significantly ($p<0.05$) lower following 10 passages in the presence of BAC at 1000 ng/l compared to the equivalent number of passages at 100 ng/L although there was no significant difference in biofilm formation between treatment concentrations following 20 passages.

Effect of BAC adaptation and exposure on the transcriptome. Transcriptome analysis by RNA-seq was used to compare the effects of passaging bacteria 20 times in the presence of 100 ng/L of BAC (B (P20)) to a control organism, passaged 20 times in a BAC free environment (C (P20)). Bacteria were grown in the presence (PR) or absence of BAC prior to RNA extraction to determine the stability of any transcriptional adaptations towards the biocide once it was removed from the growth medium. The effects of immediate BAC exposure on the unexposed parent bacterium (C (P0)) and the effect of repeatedly passaging the parent bacterium in BAC-free growth media (C (P20)) were also evaluated. Differential expression analysis was conducted in the following pairwise comparisons; C (P0) v C (P20), C P20) v B (P20), C P20)-PR v B (P20)-PR and C (P0) v C P0)-PR. BAC exposed bacteria were therefore compared to their respective control, with regards to number of passages performed. Transcription profiles for the untreated/unpassaged control bacterium C0 when grown in the immediate presence of 100 ng/L of BAC (C (P0)-PR) showed little variation, with a single gene being upregulated and two downregulated. In contrast for C (P0) v C (P20), representing continuous growth in BAC-free medium, 460 genes were upregulated and 476 were down regulated. For B (P20) (20 passages in 100 ng/ L of BAC) v C (P20) (20-passage control)

grown in the absence of BAC prior to RNA extraction, 1052 genes were upregulated and 753 were down regulated. When the same bacteria were maintained in the presence of BAC prior to extraction, 289 DE genes were upregulated and 391 were down regulated (Supplementary material). Figure 3, a PCA plot of log2 count data from all libraries for the second and third components indicates that C P0), C (P20) and B (P20) are clearly separated reflecting distinct differences in gene expression profiles. Figure 3 indicates that growing bacteria in the presence of 100 ng/L of BAC had a lesser effect on gene expression than the effects of repeated BAC exposure over a longer period.

Growing the unexposed *E. coli* parent strain (C (P0) in the presence of BAC (C (P0)-PR) resulted in a minor -1.12 log fold-decrease in *grxA* encoding glutaredoxin-1 which catalyzes the reduction of protein disulfides. A -0.9 log fold change in in *rsbA* and a 0.9 log fold-change in *mgtA* were also observed, which are involved in ribose transport and peptidoglycan biosynthesis, respectively. Data in Tables 2-5 show data fot differentially expressed genes associated with electron transport, cell permeability and efflux, motility and chemotaxis data for other genes are in Supplementary material. When repeatedly passaged in a BAC-free environment (C (P20)) an increase in the transcription of genes involved in respiration and generalised protein synthesis occurred compared to the unexposed parent strain (C (P0); Table 2). In terms of respiration, there was a substantial increase (log 3.4 to 5-fold-change) in transcription of the cytochrome o oxidase complex encoded by cyoABCDE. Similarly, there was an increase (log 2.7 to 4.2-fold) in formate dehydrogenase O (FDH-O) a heterotrimeric complex encoded by *fdoG* (α-subunit), *fdoH* (β-subunit) and *fdoI* (γ-subunit). A log 1.8 to 3.3-fold-increase in genes *nuoA* to *nuoN* encoding an NADH:ubiquinone oxidoreductase was also observed after repeated passage in binary culture in a BAC free environment. When passaged in the presence of BAC (B (20)), increases in these respiratory enzymes were not observed, with transcriptional reductions evident in *cyo*, *fdo* and *nuo* genes in comparison to the

respective passage control C20 (Table 2). Whilst repeated passage in the absence of BAC lead to an overall increase in the transcription of 50S Rpl and 30 S Rps ribosomal proteins (Supplementary Table 1), passaging in the presence of BAC (B20) caused a reduction in the transcription of these proteins relative to the BAC-free control (C (P20)). A significant upregulation in proteins associated with cellular efflux occurred after BAC exposure including MdtEF and MdtNOP, in addition to multi-drug resistance genes MdtG, MdtL, MdtM and MdtQ (Table 3). An increase in genes associated with the predicted Yhi efflux system (*yhiD, yhiJ, yhiM*, *yhiL* and *yhiS,*) was also observed (Table 3). A reduction in transcription in a number of omp genes associated with outer-membrane permeability occurred when *E. coli* was passaged in the presence of BAC.

The transcription of multiple genes associated with motility and chemotaxis in *E. coli* was reduced following BAC adaptation, including the transcription factor *flhDC* a master regulator of a multi-tiered transcriptional network including genes responsible for flagella regulation, biosynthesis and assembly (*fli* and *flg*) in addition to the flagella motor complex proteins MotA and MotB. The down-regulation of these motility-associated genes was further enhanced in the presence of BAC prior to RNA extraction (Table 4). This reduction in motility was further confirmed using a triphenyltetrazolium chloride-based motility assay (Figure 4). Several genes associated with chemotaxis were downregulated after BAC exposure, including some within the Che family, which are associated with the transmission of sensory signals from chemoreceptors to the flagella motors (Table 5). Additionally, methyl-accepting chemoreceptors Tsr, Tar and signal transducer Tap were downregulated. The transcription of these chemotaxis associated proteins were further downregulated when bacteria were grown in the presence of BAC prior to RNA extraction (Table 5).

DISCUSSION

Used appropriately, biocides offer an important means of reducing the transmission and growth of microorganisms (34-36). They have the potential, through disinfection and antisepsis, to reduce the incidence of infections and the burden on antibiotics (37) (35) and to protect consumers through the preservation of liquid formulations (38).

Decisions on what constitutes appropriate biocide use and optimisation of current practices must be based on evidence. In this context, assessing the risks of resistance associated with biocide use relies mainly on data generated from environmental surveys or in the laboratory through the exposure of microorganisms to biocides. The former approach offers the possibility of determining susceptibility in exposed environments but directly linking susceptibility in exposed microbial populations to biocide exposure has been challenging (15) (39). Laboratory studies on the other hand enable the effects of biocide exposure to be determined, but are by definition artificial (40) since many factors associated with the biocide and the microorganisms in the laboratory do not necessarily closely reproduce the conditions of environmental exposure. These include the fact that *in vitro* studies most commonly involve the exposure of single-species, planktonic cultures of bacteria to biocides delivered in simple aqueous solution, at concentrations that have been selected to be sublethal, but are relatively close to MICs (20). There is therefore the potential to increase the realism in such studies and this has been done to a limited extent by introducing complex communities (28) and formulation (26) (29).

Concentration is a critical variable in determining the outcome of the exposure of a microorganism to an antimicrobial compound, since exposure to concentrations greater than the susceptibility of the exposed microorganism will lead to complete inhibition or inactivation, whilst exposure to various sub-lethal concentrations is more likely to be selective. It is necessary to use sub-lethal concentrations, that are mostly considerably lower than those used at the point of biocide application in experimental risk assessment since inactivated cultures

cannot adapt. However, concentrations utilized in many reports are relatively close to the MIC and as such best reflect a limited subset of environmental scenarios. In real-world use, biocides are variously eluted into aquatic environments through wastewater disposal systems (41) which is particularly relevant in risk assessment due to the high bacterial densities and taxonomic diversity in sewage treatment and aquatic environments, and the presence of biocides on a continuum between hundredths of the MIC to zero.

E. coli may be exposed in the environment to biocide concentration many times lower than would be required to inhibit growth (42) with poorly understood implications. In the current investigation therefore, the bacterium was exposed in the fluid-phase to environmentally relevant residual concentrations of the commonly used quaternary ammonium compound BAC. Biocide-free cultures were also assessed to control for general adaptation occurring during repeated growth on normal medium. Changes occurred following 20 passages with or without BAC. Susceptibility decreased two-fold after exposure to BAC residues (MBC) but not following growth in biocide-free conditions. Compared to bacteria grown in biocide-free conditions, which increased in planktonic growth rate and maximum culture density after adaption, BAC adapted bacteria were significantly less productive in terms of maximum culture density when grown in normal medium.

In previous reports, large changes in susceptibility have been reported following repeated or long-term exposure to relatively high concentrations of BAC (for example, 3 mg/L initial MIC to 90mg/L in *E. coli* (43) and for *Pseudomonas aeruginosa*, 50mg/L to 1,600 mg/L) (44-45). These susceptibility changes have been associated in various bacterial species, with reductions in cell permeability (46), increased expression of broad range efflux systems (47) and the induction of general stress response (48) although it is likely that several adaptation mechanisms will be involved during adaptation (43). Importantly however, in many previous

studies adapted bacteria were generated by exposure to BAC concentrations of up to 200 mg/L (17) (47) (49) over 1000 times higher than in the current study.

Repeated exposure BAC concentrations representing environmental residues resulted in considerably smaller reductions in susceptibility than the majority of previous reports. The MBC of the unexposed bacterium was 50 µg/ml and no change occurred after 10 passages with 100 or 1000 ng/L BAC. By 20 passages only a two-fold decrease in susceptibility occurred. Significant changes were however observed in biofilm formation in adapted bacteria. Whilst planktonic passage in biocide-free conditions reduced biofilm accumulation, BAC adapted organisms were further suppressed in biofilm formation and following 20 passages with BAC residues, adapted bacteria became non-motile. Thus, exposure to concentrations of BAC representing environmental residues was associated with reduction in function (growth rate and biofilm formation) and loss of function (motility), whilst by comparison, passage in normal medium increased planktonic fitness.

With respect to RNAseq data, passage in biocide-free medium alone (C (P20)) resulted in 460 upregulated and 476 downregulated genes compared to the unpassaged parent bacterium (C P0)). Long-term exposure to environmentally relevant BAC concentrations (B (P20)) increased transcription of efflux proteins and led to a reduction in outer-membrane porins and genes associated with motility and chemotaxis, which was manifested phenotypically through loss-of-function (motility). Repeated passage of control cultures in a BAC-free-environment resulted in the up-regulation of multiple respiration-associated proteins, which was reflected by increases in planktonic growth rates. Thus, repeated exposure of *E. coli* to BAC residues resulted in significant alterations in global gene expression that were associated with marginal decreases in biocide susceptibility, reductions in growth-rate and biofilm-formation, and loss of function for motility.

The minor decreases in susceptibility observed in BAC-adapted bacteria are potentially attributable to the down-regulation of outer membrane proteins and upregulation of proposed efflux systems, as observed in the transcriptomic data. This agrees with previous reports documenting the adaptive BAC insusceptibility in *E. coli*, where microarray analysis indicated the involvement of active efflux (43). In contrast to previous reports however, significant upregulation of the acrAB efflux system or the outer membrane protein TolC was not observed and there was no significant increase in the transcription of stress response systems, such as those belonging to the soxRS regulon, or the multiple antibiotic resistance operon MarA (50-51). An increase in transcription of genes associated with the Mdt and Yhi efflux systems was however observed which could further explain the minor changes in BAC susceptibility.

Increases in the transcription of genes associated with cellular respiration and protein synthesis were observed following adaptation to BAC-free environment. Since the bacteria are actively growing, this observed increase in cellular activity may represent adaption to the conditions of rapid growth on laboratory media. This increase did not occur following adaptation to BAC, possibly due to the inhibitory effect of BAC on respiratory processes or the associated metabolic burden as evidenced by data presented in Figure 1 associated with damage to the cell membrane and potentially impaired function of associated cell membrane bound proteins impacting electron transport (52).

The effect of adaptation to BAC on motility and chemotaxis in was particularly notable since it resulted in loss of motility. In terms of mechanisms responsible, flagella and motility-associated proteins were down regulated in response to BAC exposure. Flagella are widely documented virulence factors in *E. coli* and have been previously suggested to play a key role in biofilm formation (53-54) . Thus, a decrease in flagella expression, in addition to the down-regulation in the transcription of various chemotaxis related proteins, could account for the decrease in biofilm formation observed after BAC exposure (Figure 2).

In summary, concentrations of BAC representing environmental residues induced minor changes in antimicrobial susceptibility in *E. coli* that were associated with a decrease in outer membrane proteins and up-regulation of efflux systems. Cellular respiration associated proteins were transcriptionally down regulated, and planktonic growth rates reduced following BAC adaptation. BAC adaptation also resulted in a significant decrease in motility and biofilm formation, possibly due to a decrease in the transcription of proteins involved in flagella synthesis, function and chemotaxis in the bacterial cell. The combination of phenotypic and transcriptomic analysis presents the opportunity to consider the two distinct approaches in the context of risk assessment. Omics and molecular genetics are increasingly applied in this field (17, 55) and in some cases have indicated potential changes of concern associated with biocide exposure; for example virulence potential (56) and transfer of genes associated with resistance (57). Determining the biological or real-word significance of such data without phenotypic corroboration can be problematic, and this is of concern where the objective is diligent risk assessment to inform product composition. Thus, more work is required before genotype-phenotype relationships can be effectively integrated in to the process of risk assessment. Similarly, genome-wide association studies, applied with the aim of predicting antibiotic resistance (58) may be less successful when applied to biocide studies due to the complexity of modes of action and resistance mechanisms.

Data generated in the current investigation suggest that despite the differences in gene expression profiles, endpoints of relevance for decision-making (i.e. reduced biocide susceptibility) remained practically unchanged. Had the study generated only transcriptomic data, the message of relevance to risk assessment would probably be different (e.g. overexpression of efflux pumps). This investigation therefore highlights the importance of utilising phenotypic analysis to supplement transcriptome profiling when assessing functional consequence and determining risk assessment of biocide usage in bacteria.

METHODS

Bacteria, bacteriological media and antimicrobial agents. *E. coli* MG1655 was selected a well-characterized example of this bacterium, for which whole genome sequence data are available. The bacterium, and growth media were purchased from Oxoid (Basingstoke, United Kingdom). The bacterial growth medium was sterilized at 121°C and 15 lb/in^2 for 15 min prior to use. *E. coli* was grown on Tryptone Soy Agar and Tryptone Soy Broth. BAC (≥95.0%) was purchased from Sigma-Aldrich (Dorset, United Kingdom) and was prepared at 1 mg/ml in deionised water and filter sterilised (0.2 µM pore size) prior to use.

Determination of MICs and MBCs. MICs were determined by microdilution according to EUCAST methods (https://onlinelibrary.wiley.com/doi/pdf/10.1046/j.1469-0691.2003.00790.x) using TSB as growth medium. BAC concentrations ranged between 0.8 and 800 µg/ml. Growth was determined through turbidity (OD_{600}) compared to uninoculated wells (negative control) using a microtiter plate reader (PowerWave XS, BioTek, Bedfordshire, United Kingdom).

Minimum bactericidal concentrations were determined as described previously (14, 59). Briefly, aliquots (10 µl) from wells exhibiting no turbidity were transferred to sterile TSA prior to 4 days of incubation at 37°C to determine the MBC. The MBC was defined as the lowest concentration of biocide at which no growth occurred after 4 days of incubation.

Planktonic growth rate. Overnight cultures of *E. coli* were diluted to an OD_{600} of 0.8 then further diluted 1:100 in TSB in the presence and absence of 100 or 1000 ng/L BAC. Bacteria were incubated in 96 well microtiter plates at 37ºC for 24 h and the OD_{600} was determined every hour spectrophotometrically.

Crystal violet biofilm formation assay. Overnight cultures of *E. coli* were diluted to an OD_{600} of 0.8 then further diluted 1:100 in TSB. 150 µl of diluted bacterial inoculum was delivered to each test well of a 96-well microtiter plate prior to incubation for 48 h at 37 °C and 20 rpm to promote biofilm growth. Wells were washed twice with 250 µl of sterile PBS before addition of 200 µl of 0.5% (w/v) crystal violet solution. Plates were incubated for 30 min at room temperature and the wells were subsequently washed twice with 250 µl of PBS and left to dry at room temperature for 1 h. Attached crystal violet was solubilised in 250 µl of 95% ethanol and plates were agitated at room temperature at 20 rpm for 1 h. After solubilisation, biofilm growth was viewed as change in OD_{600} relative to a sterile negative control. Biofilm bound crystal violet was quantified and average values were calculated using data from two separate experiments each with three technical replicates. Statistical significance was determined using an ANOVA with Post-Hoc Tukey analysis where $p<0.05$ was deemed significant.

Motility assay. Single colonies of *E. coli* were stab inoculated into 10 ml of Remel™ Motility Test Medium (Thermo Scientific, UK) containing 2,3,5-triphenyltetrazolium chloride. Tubes were incubated for 48 h at 37°C, growth was determined visually as the presence of a red pigment at the line of inoculation. Three biological replicates were done for each test bacterium.

Repeated exposure to low levels of BAC. Conical flasks containing 50 ml of TSB were set-up containing 100 or 1000 ng/ L of BAC. These concentrations were selected to represent residual concentrations that have been reported in natural aquatic environments and in wastewater (24-25). Overnight cultures of bacteria were diluted to 0.8 OD_{600} then further diluted 1:100 into the BAC containing growth medium. Flasks were incubated at 37°C and 100 rpm aerobically for 48 h. Bacteria were passaged 10 or 20 times in the presence of the biocide whilst using a consistent inoculation density for each passage (OD_{600} 0.008; 3 - 5 x 10^7 cfu/ml). Cultures were plated between all passages to check for purity and viability. The progenitor

(control) strain that has not been passaged or exposed to BAC was designated "C (P0)". For passaged bacteria "B" refers to the BAC concentration and "P" to the number of passages. Thus, "B100 (P20)" was grown on the presence of BAC at 100ng/L for 20 passages.

RNA extraction. RNA was extracted from 30 ml of a mid-log phase culture of the previously generated adapted bacteria of *E. coli* (OD_{600}=0.2). Bacteria were grown in the presence or absence of 100 ng/L of BAC prior to extraction to determine the stability of any induced changes in gene expression once the BAC selective pressure was removed. Transcription was stopped using 3.6 ml of 5 % phenol in ethanol prior to the addition of 10 ml RNAprotect™ bacteria reagent (Qiagen, UK). Bacteria were pelleted at 4000 rpm for 15 mins. Bacterial pellets were resuspended in 1 ml of 0.5 % Tween 80 prior to re-pelleting at 4000 rpm for 15 mins. Pellets were resuspended in 200 µl of RNase free water and added to 750 µl of extraction buffer (0.5 M sodium acetate, 0.5 % SDS, pH 4). Resuspensions were transferred to 1.5 ml lock top tubes containing sterile glass beads (sigma) and vortexed 10 x for 1 min with 10 min cooling on ice in between vortexing. Liquid was removed from tubes and transferred to a sterile 1.5ml microcentrifuge tube containing 500 µl of phenol on ice before centrifugation for 15 min at 13,000 rpm. The aqueous layer was removed from each tube and transferred to 1 ml of 100 % ethanol on ice containing 100 µl 3 M sodium acetate. RNA was then precipitated at -20ºC for 30 min before centrifugation at 13,000 rpm for 15 min. Supernatant was subsequently removed and the pellet was washed in 1 ml of 80 % ethanol and air dried before dissolving in 50 µl RNAse free water. 5 µl of RNA was added to 5 µl of 2 x RNA gel loading dye (ThermoFisher Scientific, UK) and heated at 65ºC for 10 min before being run at 45 V on a 1 % agarose gel for 45 min to check RNA integrity. RNA was DNAse treated (DNAse1) for 30 min at 37ºC, quantified on a NanoDrop Microvolume Spectrophotometer (ThermoFisher, UK) prior to being sent to the Centre for Genomic Research at University of Liverpool for RNA-sequencing and analysis.

388 **RNA sequencing.** Samples were prepared for RNA sequencing using the Epicentre
389 ScriptSeq Complete Bacterial kit with an input of 2000ng of RNA for ribosomal depletion.
390 Ribosomal depleted samples were run on an Agilent Bioanalyzer to check for successful
391 depletion of the ribosomal RNA. All purified depleted RNA was used in ScriptSeq v2 Library
392 preparation according to manufacturer's instructions. Libraries were purified using AxyPrep
393 Mag beads. Each library was quantified using Qubit and the size distribution assessed using the
394 Agilent 2100 Bioanalyser. The 18 amplified libraries were multiplexed as a single pool. The
395 pooled sample was further purified with AxyPrep Mag beads to remove small amount of
396 adaptor in a few of the individual libraries. The template DNA was denatured according to the
397 protocol described in the Illumina cBot User guide and loaded at 13 pM concentration. The
398 sequencing was carried out on one lane of an Illumina HiSeq2500 at 2x125 bp paired-end
399 sequencing with v4 chemistry. RNA sequencing and initial data analysis was done at The
400 Centre for Genomic Research, University of Liverpool.

401 **Data processing and sequence alignment.** Base calling and de-multiplexing of
402 indexed reads was done using by CASAVA version 1.8.2 (Illumina). The raw fastq files were
403 trimmed to remove Illumina adapter sequences using Cutadapt version 1.2.1 (60). The reads
404 were further trimmed to remove low quality bases using Sickle version 1.200, with a minimum
405 window quality score of 20. Reads were aligned to the genome sequences using Bowtie2
406 version 2.2.5 (61) (62). The option for read mate orientation was set as --fr and the other
407 options were default.

408 **Differential expression analysis.** Gene expression was calculated from read alignment
409 files using htseq-count (Simon et al., 2014). The count numbers were also converted into
410 FPKM values. The count numbers per gene were put into differential expression analysis. The
411 main processes of the analysis include data variation assessment, data modelling, model fitting,
412 testing and DE genes detecting. All the DGE (Differential Gene Expression) analyses were

413 performed in R (version 3.2.2) environment using the edgeR (Robinson et al, 2010) package.
414 With respect to the assessment of variation in the count data, the variation between samples
415 within a sample group (within-group variation) is usually smaller than that between samples
416 from different sample groups (overall variation) because the former consists of technical and
417 biological variation only, while the latter also contains variation because of other factors. When
418 the factor effect is the dominant term of variation, the sample groups can be clearly separated
419 by using assessment tools. If the factor effect is weak compared to the technical and biological
420 variation within sample groups will be difficult to be discriminated from the data. An
421 assessment reveals that the within-group variation was strong for C0, C0P, B20 and B20P four
422 sample groups. In each of them, one sample is not correlated closely with the other two samples
423 of the same group. Whilst the C (P20) and C (P20)-PR two groups had obviously higher
424 within-group correlation (see correlation heatmap Figure 2). The pairwise scatter plots of $\log_{10}$
425 count data of the samples for each group give the same view as the correlation heatmap. Two
426 example figures are presented here: Figure 3 is the pairwise scatter plot for the C0 group, and
427 Figure 4 is for the C20 group. Figure 5 is the PCA plot of log10 count data from all libraries,
428 the second and third components are plotted. The plot shows that the C (P0), C (P20) and B
429 (P20) can be clearly separated; indicating the differences between them caused notable
430 differences in gene expression. Comparatively, the impact of growing cells in the presence of
431 BAC or not is relative weaker. Therefore, it is expected that more genes will be identified as
432 DE from C (P0) vs C (P20) or C (P20) vs B (P20) or C (P20)-PR vs B (P20)-PR than from C
433 (P0) vs C (P0)-PR.

434 **Statistical analyses.** Data from crystal violet biofilm formation assays were analyzed
435 using a one-way ANOVA (analysis of variance) and post-hoc Tukey analysis.

To compare growth curves a non-linear regression analysis using the logistic model was used. As follows:

$$Y(t) = \frac{L}{(1 + e^{-k(t_{50} - t)})}.$$

The above growth law was used to analyse the following 3-way comparisons: C0 v C10 v C20, ii C0 v B100 (P10) v B100 (P20); iii) C0 v B1000 (P10) v B1000 (P20); iv) C10 v B100 (P10) v B1000 (P10) and v) C20 v B100 (P20) v B1000 (P20). For each comparison the following step-wise analysis procedure was used to assess experimental condition effects. The logistic model was fitted to all three conditions without accounting for the experimental condition. Subsequently parameter values were varied according to experimental condition type, therefore three L, k and t50 values representing each condition were estimated, and assessed whether this improved model fit using the F-test. To arrive at the final model, if the 95 percent confidence interval, generated using the profile likelihood, for the same parameter across the three conditions overlapped, then that parameter was grouped across conditions. Subsequently an assessment of the effect that had on model fit was conducted using the F-test and the new model accepted with a p-value threshold of 0.05. The resulting differences between treatments via model parameters were reported with 95 percent confidence intervals (CI). A P-value from the F-test was reported together with quantitative details, maximal growth and time to reach 50% of maximal growth, on how the growth curves differed.

ACKNOWLEDGMENTS

A.A. is an employee of Unilever. The rest of us have no conflicts of interest to declare.

S.F., A.A., and A.J.M. conceived and designed the experiments. S.F. and N.C. per- formed the experiments. S.F., N.C., G.H., A.A., and A.J.M. analyzed the data. H.M. performed the statistical modeling. S.F. and A.J.M. wrote the paper. This project was funded by Unilever's Safety and Environmental Assurance Centre (to AJM).

REFERENCES

1. **Furi L, Ciusa ML, Knight D, Di Lorenzo V, Tocci N, Cirasola D, Aragones L, Coelho JR, Freitas AT, Marchi E, Moce L, Visa P, Northwood JB, Viti C, Borghi E, Orefici G, Consortium tB, Morrissey I, Oggioni MR.** 2013. Evaluation of Reduced Susceptibility to Quaternary Ammonium Compounds and Bisbiguanides in Clinical Isolates and Laboratory-Generated Mutants of *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 57:3488-3497.
2. **Alzubeidi YS, Udompijitkul P, Talukdar PK, Sarker MR.** 2018. Inactivation of *Clostridium perfringens* spores adhered onto stainless steel surface by agents used in a clean-in-place procedure. Int J Food Microbiol 277:26-33.
3. **Condell O, Iversen C, Cooney S, Power KA, Walsh C, Burgess C, Fanning S.** 2012. Efficacy of biocides used in the modern food industry to control Salmonella-links between biocide tolerance and resistance to clinically relevant antimicrobial compounds. Applied and Environmental Microbiology:AEM. 07534-11.
4. **Jia R, Li Y, Al-Mahamedh HH, Gu T.** 2017. Enhanced Biocide Treatments with D-amino Acid Mixtures against a Biofilm Consortium from a Water Cooling Tower. Front Microbiol 8:1538.
5. **Uppal S, Bazzi A, Reynolds RK, Harris J, Pearlman MD, Campbell DA, Morgan DM.** 2017. Chlorhexidine-Alcohol Compared With Povidone-Iodine for Preoperative Topical Antisepsis for Abdominal Hysterectomy. Obstet Gynecol 130:319-327.
6. **Barbolt TA.** 2002. Chemistry and safety of triclosan, and its use as an antimicrobial coating on Coated VICRYL* Plus Antibacterial Suture (coated polyglactin 910 suture with triclosan). Surg Infect (Larchmt) 3 Suppl 1:S45-53.
7. **Maki DG, Stolz SM, Wheeler S, Mermel LA.** 1997. Prevention of Central Venous Catheter-Related Bloodstream Infection by Use of an Antiseptic-Impregnated Catheter. Annals of Internal Medicine 127:257-266.
8. **Gaonkar TAP, Sampath LABA, Modak SMP.** 2003. Evaluation of the Antimicrobial Efficacy of Urinary Catheters Impregnated with Antiseptics in an In Vitro Urinary Tract Model. Infection Control and Hospital Epidemiology 24:506-513.
9. **Bloomfield SF.** 2002. Significance of biocide usage and antimicrobial resistance in domiciliary environments. Symp Ser Soc Appl Microbiol:144S-157S.
10. **Vander Heyde Y, Nguyet AN, Detaevenier MR, Massart DL, Plaizier-Vercammen J.** 2002. Simultaneous determination of ketoconazole and formaldehyde in a shampoo: liquid chromatography method development and validation. J Chromatogr A 958:191-201.
11. **Koehler DA, Strevett KA, Papelis C, Kibbey TCG.** 2017. The impact of antibacterial handsoap constituents on the dynamics of triclosan dissolution from dry sand. Chemosphere 186:251-256.
12. **Klimek JW, Bailey JH.** 1956. Factors influencing the rate of killing of *Escherichia coli* exposed to benzalkonium chloride. Appl Microbiol 4:53-9.
13. **McBain AJ, Gilbert P.** 2001. Biocide tolerance and the harbingers of doom. International Biodeterioration & Biodegradation 47:55-61.
14. **Moore LE, Ledder RG, Gilbert P, McBain AJ.** 2008. In vitro study of the effect of cationic biocides on bacterial population dynamics and susceptibility. Appl Environ Microbiol 74:4825-34.
15. **Marshall BM, Robleto E, Dumont T, Levy SB.** 2012. The frequency of antibiotic-resistant bacteria in homes differing in their use of surface antibacterial agents. Curr Microbiol 65:407-15.

16. **Chuanchuen R, Karkhoff-Schweizer RR, Schweizer HP.** 2003. High-level triclosan resistance in *Pseudomonas aeruginosa* is solely a result of efflux. Am J Infect Control 31:124-7.
17. **Kim M, Hatt JK, Weigand MR, Krishnan R, Pavlostathis SG, Konstantinidis KT.** 2018. Genomic and Transcriptomic Insights into How Bacteria Withstand High Concentrations of Benzalkonium Chloride Biocides. Appl Environ Microbiol 84.
18. **Jennings MC, Forman ME, Duggan SM, Minbiole KPC, Wuest WM.** 2017. Efflux Pumps Might Not Be the Major Drivers of QAC Resistance in Methicillin-Resistant Staphylococcus aureus. Chembiochem 18:1573-1577.
19. **Nagai K, Murata T, Ohta S, Zenda H, Ohnishi M, Hayashi T. 2**003. Two different mechanisms are involved in the extremely high-level benzalkonium chloride resistance of a Pseudomonas fluorescens strain. Microbiology and immunology 47:709-715.
20. **Mc Cay PH, Ocampo-Sosa AA, Fleming GT.** 2010. Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of *Pseudomonas aeruginosa* grown in continuous culture. Microbiology 156:30-8.
21. **Gilbert P, McBain AJ.** 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin Microbiol Rev 16:189-208.
22. **Gilbert P, Allison DG, McBain AJ.** 2002. Biofilms in vitro and in vivo: do singular mechanisms imply cross-resistance? J Appl Microbiol 92 Suppl:98S-110S.
23. **Russell AD.** 2003. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. Lancet Infect Dis 3:794-803.
24. **Clara M, Scharf S, Scheffknecht C, Gans O.** 2007. Occurrence of selected surfactants in untreated and treated sewage. Water Res 41:4339-48.
25. **Grillitsch B, Gans O, Kreuzinger N, Scharf S, Uhl M, Fuerhacker M. 2**006. Environmental risk assessment for quaternary ammonium compounds: a case study from Austria. Water Sci Technol 54:111-8.
26. **Knapp L, Amezquita A, McClure P, Stewart S, Maillard JY.** 2015. Development of a protocol for predicting bacterial resistance to microbicides. Appl Environ Microbiol 81:2652-9.
27. **Knapp L, Rushton L, Stapleton H, Sass A, Stewart S, Amezquita A, McClure P, Mahenthiralingam E, Maillard JY.** 2013. The effect of cationic microbicide exposure against *Burkholderia cepacia* complex (BCC); the use of *Burkholderia lata* strain 383 as a model bacterium. J Appl Microbiol 115:1117-26.
28. **McBain AJ, Ledder RG, Moore LE, Catrenich CE, Gilbert P.** 2004. Effects of quaternary-ammonium-based formulations on bacterial community dynamics and antimicrobial susceptibility. Appl Environ Microbiol 70:3449-56.
29. **Cowley NL, Forbes S, Amezquita A, McClure P, Humphreys GJ, McBain AJ.** 2015. Effects of Formulation on Microbicide Potency and Mitigation of the Development of Bacterial Insusceptibility. Appl Environ Microbiol 81:7330-8.
30. **Forbes S, Knight CG, Cowley NL, Amezquita A, McClure P, Humphreys G, McBain AJ.** 2016. Variable Effects of Exposure to Formulated Microbicides on Antibiotic Susceptibility in Firmicutes and Proteobacteria. Appl Environ Microbiol 82:3591-3598.
31. **Forbes S, Cowley N, Humphreys G, Mistry H, Amezquita A, McBain AJ.** 2017. Formulation of Biocides Increases Antimicrobial Potency and Mitigates the Enrichment of Nonsusceptible Bacteria in Multispecies Biofilms. Appl Environ Microbiol 83.
32. **Latimer J, Forbes S, McBain AJ.** 2012. Attenuated virulence and biofilm formation in *Staphylococcus aureus* following sublethal exposure to triclosan. Antimicrob Agents Chemother 56:3092-100.

33. **Forbes S, Latimer J, Bazaid A, McBain AJ.** 2015. Altered Competitive Fitness, Antimicrobial Susceptibility, and Cellular Morphology in a Triclosan-Induced Small-Colony Variant of *Staphylococcus aureus*. Antimicrob Agents Chemother 59:4809-16.
34. **Koski TA, Stuart LS, Ortenzio LF.** 1966. Comparison of chlorine, bromine, iodine as disinfectants for swimming pool water. Appl Microbiol 14:276-9.
35. **Harnoss JC, Assadian O, Kramer A, Probst P, Muller-Lantzsch C, Scheerer L, Bruckner T, Diener MK, Buchler MW, Ulrich AB.** 2018. Comparison of chlorhexidine-isopropanol with isopropanol skin antisepsis for prevention of surgical-site infection after abdominal surgery. Br J Surg 105:893-899.
36. **Garcia MT, Pelaz C.** 2008. Effectiveness of disinfectants used in cooling towers against *Legionella pneumophila*. Chemotherapy 54:107-16.
37. **Lai NM, Chaiyakunapruk N, Lai NA, O'Riordan E, Pau WS, Saint S.** 2016. Catheter impregnation, coating or bonding for reducing central venous catheter-related infections in adults. Cochrane Database Syst Rev 3:CD007878.
38. **Bloomfield SF.** 2007. Importance of disinfection as a means of prevention in our changing world hygiene and the home. GMS Krankenhhyg Interdiszip 2:Doc25.
39. **Aiello AE, Marshall B, Levy SB, Della-Latta P, Larson E.** 2004. Relationship between triclosan and susceptibilities of bacteria isolated from hands in the community. Antimicrob Agents Chemother 48:2973-9.
40. **McBain AJ, Ledder RG, Sreenivasan P, Gilbert P.** 2004. Selection for high-level resistance by chronic triclosan exposure is not universal. J Antimicrob Chemother 53:772-7.
41. **Kümmerer K, Eitel A, Braun U, Hubner P, Daschner F, Mascart G, Milandri M, Reinthaler F, Verhoef J. 1**997. Analysis of benzalkonium chloride in the effluent from European hospitals by solid-phase extraction and high-performance liquid chromatography with post-column ion-pairing and fluorescence detection. Journal of chromatography A 774:281-286.
42. **Reinthaler F, Posch J, Feierl G, Wüst G, Haas D, Ruckenbauer G, Mascher F, Marth E.** 2003. Antibiotic resistance of *E. coli* in sewage and sludge. Water Research 37:1685-1690.
43. **Bore E, Hebraud M, Chafsey I, Chambon C, Skjaeret C, Moen B, Moretro T, Langsrud O, Rudi K, Langsrud S.** 2007. Adapted tolerance to benzalkonium chloride in Escherichia coli K-12 studied by transcriptome and proteome analyses. Microbiology 153:935-46.
44. **Martinez-Carballo E, Sitka A, Gonzalez-Barreiro C, Kreuzinger N, Furhacker M, Scharf S, Gans O.** 2007. Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part I. Application to surface, waste and indirect discharge water samples in Austria. Environ Pollut 145:489-96.
45. **Martinez-Carballo E, Gonzalez-Barreiro C, Sitka A, Kreuzinger N, Scharf S, Gans O.** 2007. Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part II. Application to sediment and sludge samples in Austria. Environ Pollut 146:543-7.
46. **Mechin L, Dubois-Brissonnet F, Heyd B, Leveau JY.** 1999. Adaptation of Pseudomonas aeruginosa ATCC 15442 to didecyldimethylammonium bromide induces changes in membrane fatty acid composition and in resistance of cells. J Appl Microbiol 86:859-66.
47. **Heir E, Sundheim G, Holck AL.** 1999. The qacG gene on plasmid pST94 confers resistance to quaternary ammonium compounds in staphylococci isolated from the food industry. J Appl Microbiol 86:378-88.

48. **Ceragioli M, Mols M, Moezelaar R, Ghelardi E, Senesi S, Abee T.** 2010. Comparative transcriptomic and phenotypic analysis of the responses of *Bacillus cereus* to various disinfectant treatments. Appl Environ Microbiol 76:3352-60.
49. **Langsrud S, Sundheim G, Holck AL.** 2004. Cross-resistance to antibiotics of Escherichia coli adapted to benzalkonium chloride or exposed to stress-inducers. J Appl Microbiol 96:201-8.
50. **Bore E, Hebraud M, Chafsey I, Chambon C, Skjæret C, Moen B, Møretrø T, Langsrud Ø, Rudi K, Langsrud S.** 2007. Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses. Microbiology 153:935-946.
51. **Moen B, Rudi K, Bore E, Langsrud S.** 2012. Subminimal inhibitory concentrations of the disinfectant benzalkonium chloride select for a tolerant subpopulation of *Escherichia coli* with inheritable characteristics. International journal of molecular sciences 13:4101-4123.
52. **Gilbert P, Moore LE.** 2005. Cationic antiseptics: diversity of action under a common epithet. J Appl Microbiol 99:703-15.
53. **Girón JA, Torres AG, Freer E, Kaper JB.** 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Molecular microbiology 44:361-379.
54. **Pratt LA, Kolter R.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Molecular microbiology 30:285-293.
55. **Farkas A, Craciunas C, Chiriac C, Szekeres E, Coman C, Butiuc-Keul A.** 2016. Exploring the Role of Coliform Bacteria in Class 1 Integron Carriage and Biofilm Formation During Drinking Water Treatment. Microb Ecol 72:773-782.
56. **Chin CY, Tipton KA, Farokhyfar M, Burd EM, Weiss DS, Rather PN.** 2018. A high-frequency phenotypic switch links bacterial virulence and environmental survival in *Acinetobacter baumannii*. Nat Microbiol 3:563-569.
57. **Gillings MR, Gaze WH, Pruden A, Smalla K, Tiedje JM, Zhu YG.** 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J 9:1269-79.
58. **Earle SG, Wu CH, Charlesworth J, Stoesser N, Gordon NC, Walker TM, Spencer CCA, Iqbal Z, Clifton DA, Hopkins KL, Woodford N, Smith EG, Ismail N, Llewelyn MJ, Peto TE, Crook DW, McVean G, Walker AS, Wilson DJ.** 2016. Identifying lineage effects when controlling for population structure improves power in bacterial association studies. Nat Microbiol 1:16041.
59. **Forbes S, Latimer J, Bazaid A, McBain AJ.** 2015. Altered Competitive Fitness, Antimicrobial Susceptibility, and Cellular Morphology in a Triclosan-Induced Small-Colony Variant of *Staphylococcus aureus*. Antimicrobial Agents and chemotherapy 59:4809-4816.
60. **Martin M.** 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet journal 17:pp. 10-12.
61. **Langmead B, Salzberg SL.** 2012. Fast gapped-read alignment with Bowtie 2. Nature methods 9:357-359.
62. **Blattner FR, Plunkett G, 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277:1453-62.

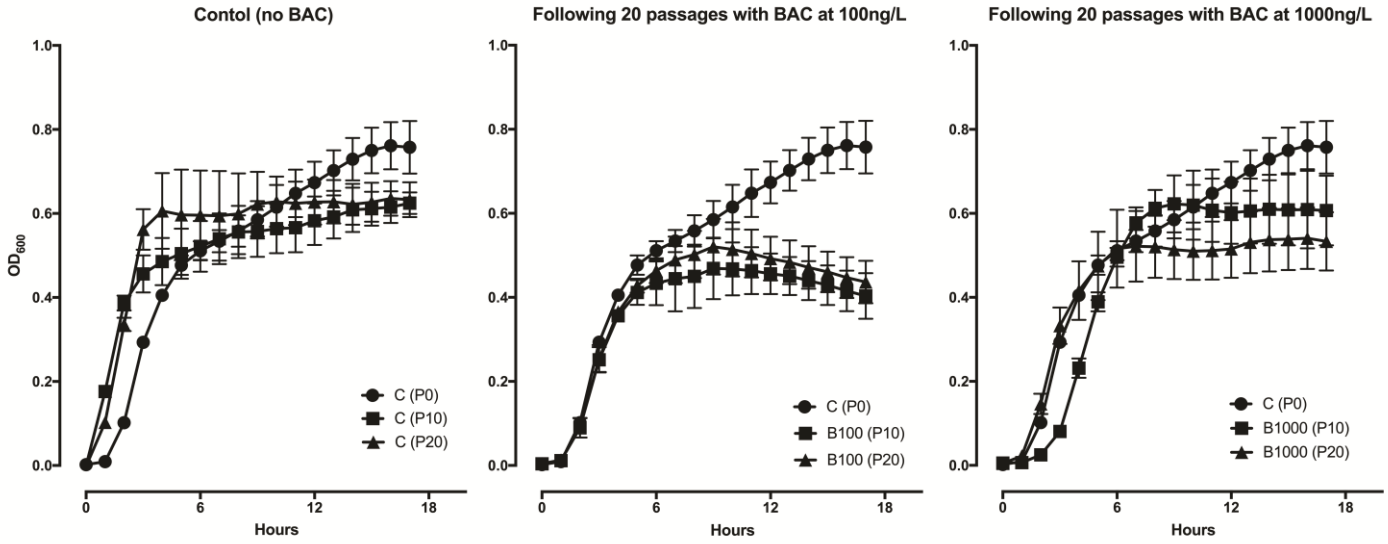
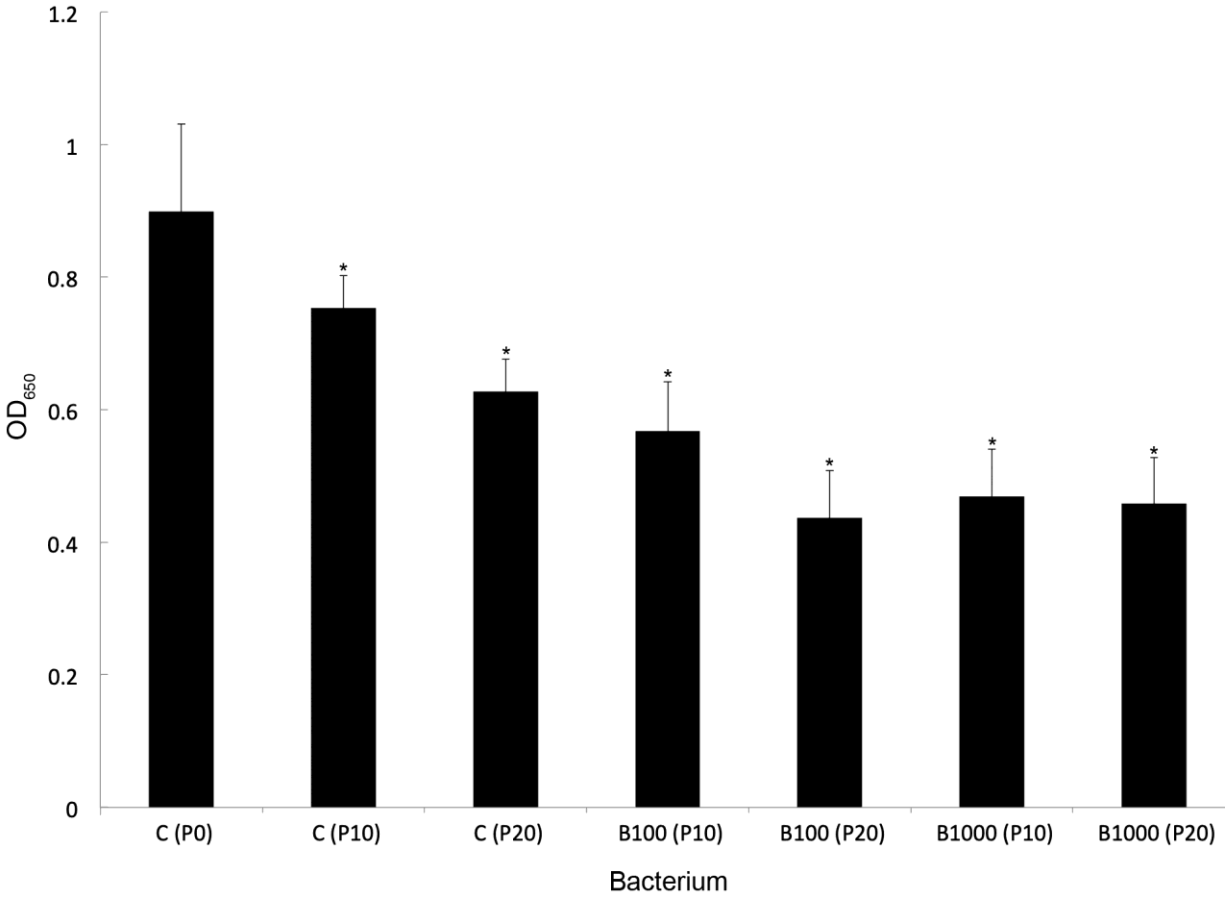


FIG 1 *E. coli* planktonic growth after 10 or 20 passages in the absence or presence of BAC. *E. coli* was passaged 10 (P 10) and 20 (P20) times in binary culture in the presence of 100 ng/L (B100) or 1000 ng/L (B1000) of BAC prior to determination of planktonic growth. Control cultures prior to passage (C P0) or passaged in a BAC-free environment over 10 and 20 passages were included (C (P10) and C (P20) respectively). Data represent two separate experiments each with three technical replicates Error bars are representative of standard deviations.



687

688 **FIG 2** Crystal violet assay indicating biofilm formation (OD_{600}) before (control; C (P0)) and after 10 or
689 20 passages in the absence (C (P10) and C (P20) or the presence of 100 ng/L of BAC (B100 P10/20) or
690 1000 ng/L of BAC (B1000 P10/20). Error bars represent standard deviations. Data represent two
691 separate experiments each with three technical replicates. Biofilm formation was significantly ($p<0.05$)
692 reduced following exposure to residual concentrations of BAC (C (P10)) and (C (P20)). This was the
693 case for exposure 100 ng/L and 1000ng/L BAC after 10 and 20 passages. Exposure to BAC at 1000
694 ng/L for 10 passages resulted in significantly lower biofilm formation than 10 passages with 100 ng/L of
695 BAC. Following 20 passage exposures differences for 100 and 1000ng/L BAC were not statistically
696 significant.

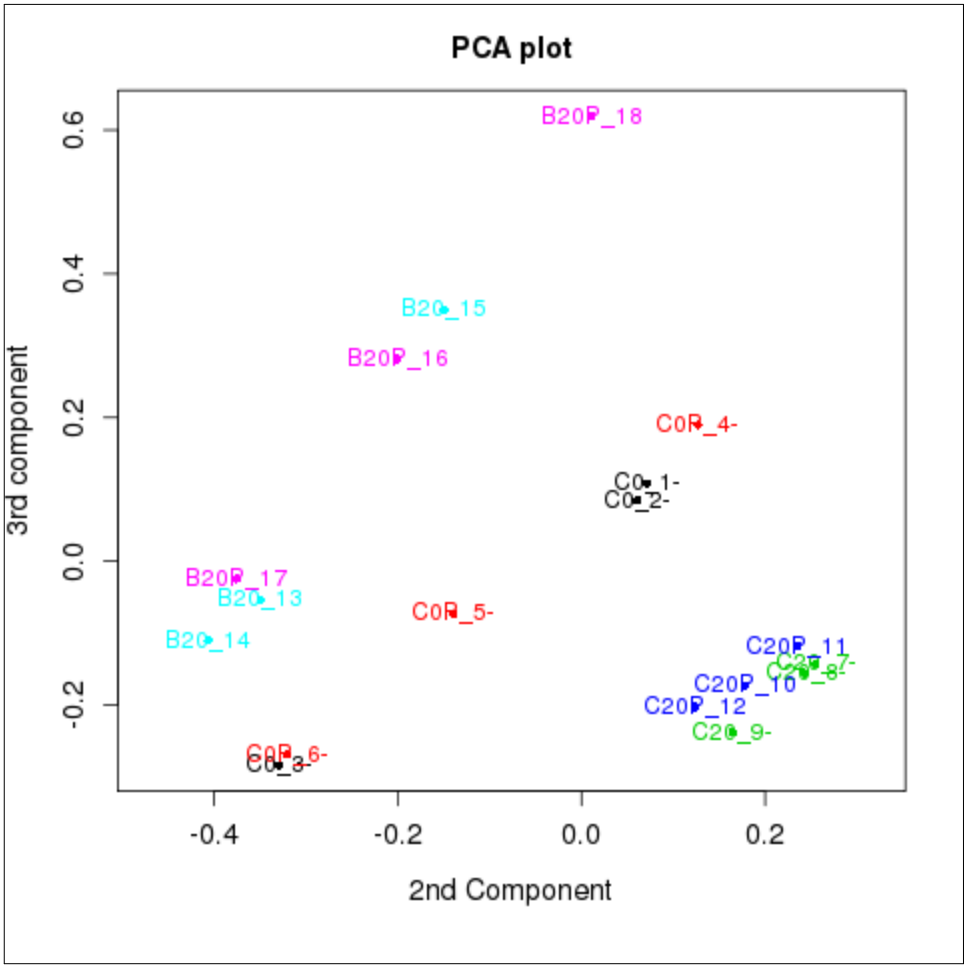


FIG 3 PCA plot indicating variation in DE genes for *E. coli* when comparing an unpassaged control bacterium C0, a bacterium passaged 20 times in a BAC-free environment (C20) and a bacterium passaged 20 times in the presence of 100 ng/L BAC (B 20). C0 (black), C20 (green) and B20 (cyan) were grown in biocide free-media prior to RNA extraction whilst C0P (red), C20P (dark blue) and B20P (pink) indicate bacteria that were grown in the presence of BAC immediately prior to RNA extraction. This allowed us to determine whether any changes in the transcriptome were maintained when BAC was removed from the growth medium. Data are derived from three biological replicates for each bacterium.

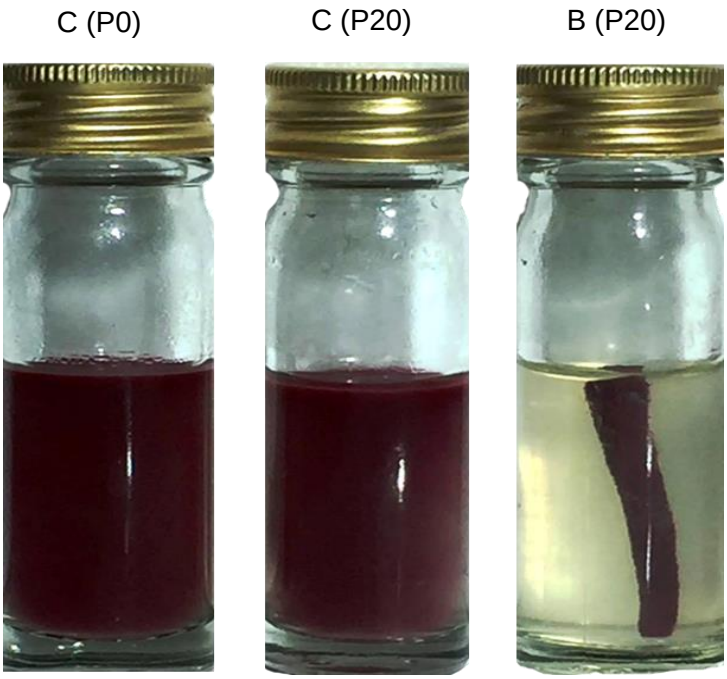


FIG 4 Motility before (C (P0)) and after 20 passages in binary culture in the absence (C (P20)) or presence (B (P20)) of 100 ng/L BAC.

TABLE 1 *E. coli* MICs and MBCs before (P0) and after 10 (P10) and 20 (P20) passages in residual concentrations of BAC

	MIC (µg/ml)			MBC (µg/ml)		
	P0	P10	P20	P0	P10	P20
Control	50	50	50	50	50	50
BAC 100	50	50	50	50	50	**100**
BAC 1000	50	50	50	50	50	**100**

Data show the mean minimum inhibitory concentrations and minimum bactericidal concentrations of bacteria before and after BAC exposure and represent samples taken from two separate experiments each with three technical replicates (values did not vary between replicates). Control refers to the unpassaged bacterium. P, passage. Bold text indicates ≥2-fold changes when comparing P0 to P10 or P20.

836 **TABLE 2** Differentially expressed genes associated with electron transport

Gene	Gene product	logFC C20 *v* C0	logFC B20 v C20	logFC B20P *v* C20P
cyoA	Cytochrome o oxidase subunit a	4.96	-3.90	-3.81
cyoB	Cytochrome o oxidase subunit b	4.27	-3.54	-2.68
cyoC	Cytochrome o oxidase subunit c	4.53	-3.87	-2.82
cyoD	Cytochrome o oxidase subunit d	3.50	-2.68	-3.81
cyoE	Cytochrome o oxidase subunit e	3.42	-3.46	-3.41
fdoH	Formate dehydrogenase-O iron-sulfur subunit	3.81	-2.39	-2.12
fdoI	Formate dehydrogenase cytochrome b556 subunit	2.72	-3.44	-3.09
fdoG	Formate dehydrogenase-O major subunit	4.18	-3.69	-3.55
nuoA	NADH-quinone oxidoreductase subunit A	2.36	-2.49	-1.89
nuoB	NADH-quinone oxidoreductase subunit B	3.25	-2.92	-2.21
nuoC	NADH-quinone oxidoreductase subunit C	3.08	-2.68	-2.30
nuoE	NADH-quinone oxidoreductase subunit E	3.21	-2.51	-2.26
nuoF	NADH-quinone oxidoreductase subunit F	3.16	-2.69	-2.09
nuoG	NADH-quinone oxidoreductase subunit G	3.20	-3.13	-2.56
nuoH	NADH-quinone oxidoreductase subunit H	2.72	-2.81	-2.21
nuoI	NADH-quinone oxidoreductase subunit I	2.70	-2.61	-1.92
nuoJ	NADH-quinone oxidoreductase subunit J	2.24	-2.23	-1.90
nuoK	NADH-quinone oxidoreductase subunit K	2.27	Ns	-2.25
nuoL	NADH-quinone oxidoreductase subunit L	2.05	Ns	-1.71
nuoM	NADH-quinone oxidoreductase subunit M	2.66	-1.91	-2.20
nuoN	NADH-quinone oxidoreductase subunit N	1.80	-2.21	-1.44

837
838 Differentially expressed genes in in *Escherichia coli* after repeated passage in a BAC free (C20 to C0)
839 and BAC at 100ng/L (B20 to C20 and B20P to C20P). Data represent three biological repeats. *v*, versus.
840
841
842
843
844
845
846
847
848
849
850
851

852 **TABLE 3** Differentially expressed genes associated with outer membrane permeability and efflux

Gene	Gene product	logFC C20 *v* C0	logFC B20 *v* C20	logFC B20P *v* C20P
tolC	Outer membrane protein	1.14	-1.22	ns
ompA	Outer membrane protein A	ns	-1.35	ns
ompC	Outer membrane protein C	ns	-1.85	ns
ompF	Outer membrane protein F	ns	-2.18	-1.70
ompG	Outer membrane protein G	ns	3.02	ns
ompL	Outer membrane protein L	ns	3.40	ns
ompN	Outer membrane protein N	ns	3.13	ns
ompT	Outer membrane protein T	ns	-1.62	-1.38
ompR	Outer membrane protein R	0.93	-0.89	-0.86
ompX	Outer membrane protein X	ns	-1.88	ns
ompW	Outer membrane protein W	ns	-2.19	-2.29
acrA	Multidrug efflux pump subunit	1.44	-1.04	ns
acrD	Aminoglycoside efflux pump	na	0.92	0.84
mdtE	Multidrug resistance protein	2.46	4.13	3.24
mdtF	Multidrug resistance protein	1.32	1.92	0.97
mdtJ	Multidrug resistance protein	1.50	ns	ns
mdtG	Multidrug resistance protein	ns	1.63	ns
mdtL	Multidrug resistance protein	ns	2.13	ns
mdtM	Multidrug resistance protein	ns	2.05	ns
mdtN	Multidrug resistance protein	3.16	4.40	ns
mdtO	Multidrug resistance protein	2.55	3.99	2.78
mdtP	Multidrug resistance protein	2.51	3.66	2.46
mdtQ	Multidrug resistance protein	2.58	3.51	ns
yhiD	Putative magnesium transporter	2.48	3.24	2.72
yhiJ	Putative uncharacterised protein	3.13	3.88	2.93
yhiM	Inner membrane protein	2.38	3.39	2.37
yhiL	Putative uncharacterised protein	3.56	4.37	3.83
yhiS	Putative uncharacterised protein	3.90	4.88	3.95

853 [a] See footnote to Table 2. ns, no significant change.

854

855

856

857

858

859

860

861

862

863

864

865 **TABLE 4** Differentially expressed genes associated with motility

Gene	Gene product	logFC C20 *v* C0	logFC B20 *v* C20	logFC B20P *v* C20P
fliA	RNA polymerase sigma factor	ns	-6.07	-7.16
fliC	Flagellin	3.46	-6.69	-6.60
fliD	Flagellar hook-associated protein 2	2.00	-3.41	-4.53
fliE	Flagellar hook-basal body complex protein	ns	-4.42	-5.66
fliF	Flagellar M-ring protein	ns	-6.46	-7.63
fliG	Flagellar motor switch protein	ns	-5.67	-6.99
fliH	Flagellar assembly protein	1.12	-6.20	-7.29
fliI	Flagellum-specific ATP synthase	ns	-5.86	-7.16
fliJ	Flagellar protein	1.22	-5.16	-5.64
fliK	Flagellar hook-length control protein	ns	-4.06	-5.06
fliL	Flagellar protein	ns	-6.97	-7.84
fliM	Flagellar motor switch protein	ns	-5.64	-6.22
fliN	Flagellar motor switch protein	ns	-6.38	-6.72
fliO	Flagellar protein	ns	-5.74	-5.97
fliP	Flagellar biosynthetic protein	ns	-4.47	-5.89
fliQ	Flagellar biosynthetic protein	ns	-4.38	-5.02
fliS	Flagellar secretion chaperone	2.30	-4.72	-5.76
fliT	Flagellar protein	ns	-3.05	-3.46
fliZ	Regulator of sigma S factor	ns	-3.79	-4.48
flgA	Flagella basal body P-ring formation protein	ns	-5.71	-5.78
flgB	Flagellar basal body rod protein	2.39	-6.99	-8.56
flgC	Flagellar basal-body rod protein	2.33	-7.86	-7.74
flgD	Basal-body rod modification protein	2.17	-6.72	0.00
flgE	Flagellar hook protein	2.48	-5.96	-6.82
flgF	Flagellar basal-body rod protein	2.26	-6.89	-7.17
flgG	Flagellar basal-body rod protein	2.36	-5.64	-6.33
flgH	Flagellar L-ring protein	1.98	-5.20	-6.03
flgI	Flagellar P-ring protein	1.77	-5.64	-6.22
flgJ	Peptidoglycan hydrolase	1.20	-5.55	-5.93
flgK	Flagellar hook-associated protein 1	2.11	-5.85	0.00
flgL	Flagellar hook-associated protein 3	1.75	-5.69	-6.05
flgN	Flagella synthesis protein	1.60	-5.76	-6.50
flhA	Flagellar biosynthesis protein	ns	-4.45	-4.76
flhB	Flagellar biosynthetic protein	ns	-5.38	-6.17
flhC	Flagellar transcriptional regulator	ns	-5.37	-5.99
flhD	Flagellar transcriptional regulator	ns	-2.55	-2.51
flhE	Flagellar protein	ns	-4.65	-5.08
motA	Motility protein A	ns	-7.13	-7.83
motB	Motility protein B	ns	-5.27	-6.11

866 [a] See footnote to Tables 2 and 3.

867

868

869

870

871 **TABLE 5** Differentially expressed genes associated with chemotaxis

Gene	Gene product	logFC C20 *v* C0	logFC B20 *v* C20	logFC B20P *v* C20P
tsr	Methyl-accepting chemotaxis protein	2.57	-6.34	-7.05
tar	Methyl-accepting chemotaxis protein	1.69	-6.46	-5.57
tap	Methionine import ATP-binding protein	1.98	-4.94	-6.09
cheR	Chemotaxis protein methyltransferase	ns	-3.52	-4.67
cheZ	Protein phosphatase	ns	-5.77	-5.96
cheA	Chemotaxis protein	ns	-5.20	-6.11
cheR	Chemotaxis protein methyltransferase	ns	-3.52	-4.67
cheB	Protein-glutamate methylesterase	ns	-6.01	-6.41
cheY	Chemotaxis protein	ns	-6.19	-6.94
cheW	Chemotaxis protein	ns	-6.54	-6.97

872 [a] See footnote to Tables 2 and 3.

873

874

875

876

877

878

879

880

881

882

883

884

885

886

887

888

889