

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

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

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31

32 **ABSTRACT**

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

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

61

62 **INTRODUCTION**

63 Benzalkonium chloride (BAC) is a broad-spectrum quaternary ammonium biocide (1), which has a
64 variety of applications in antiseptics, disinfection or preservation in industrial (2-4), medical (5)
65 (6-8) and domestic (9-11) fields. Interest in the biological mechanisms that underpin the
66 antibacterial mode of action of BAC has been longstanding (12). There is concern that biocides
67 could select for reduced susceptibility in bacteria (13-15), which may occur through the

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

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

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

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

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

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

99 **RESULTS**

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

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

110 **Alterations in planktonic growth and biofilm formation after BAC exposure.**
111 Repeated passage in biocide-free medium led to reduced lag phase, increased growth rate and
112 decreased maximum culture density ($p < 0.001$). Following adaptation (10 and 20 passages) with
113 100 ng/L BAC, growth rates did not change but maximum culture densities were significantly
114 decreased. Adaptation to 1000 ng/L of BAC (10 and 20 passages) also resulted in significantly
115 decreased maximum culture densities but did not alter growth rates relative to the control.

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

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

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

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

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

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

176

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

190

191 **DISCUSSION**

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

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

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

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

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

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

240 studies adapted bacteria were generated by exposure to BAC concentrations of up to 200 mg/L
241 (17) (47) (49) over 1000 times higher than in the current study.

242 Repeated exposure BAC concentrations representing environmental residues resulted in
243 considerably smaller reductions in susceptibility than the majority of previous reports. The
244 MBC of the unexposed bacterium was 50 µg/ml and no change occurred after 10 passages with
245 100 or 1000 ng/L BAC. By 20 passages only a two-fold decrease in susceptibility occurred.
246 Significant changes were however observed in biofilm formation in adapted bacteria. Whilst
247 planktonic passage in biocide-free conditions reduced biofilm accumulation, BAC adapted
248 organisms were further suppressed in biofilm formation and following 20 passages with BAC
249 residues, adapted bacteria became non-motile. Thus, exposure to concentrations of BAC
250 representing environmental residues was associated with reduction in function (growth rate and
251 biofilm formation) and loss of function (motility), whilst by comparison, passage in normal
252 medium increased planktonic fitness.

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

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

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

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

289 In summary, concentrations of BAC representing environmental residues induced minor
290 changes in antimicrobial susceptibility in *E. coli* that were associated with a decrease in outer
291 membrane proteins and up-regulation of efflux systems. Cellular respiration associated proteins
292 were transcriptionally down regulated, and planktonic growth rates reduced following BAC
293 adaptation. BAC adaptation also resulted in a significant decrease in motility and biofilm
294 formation, possibly due to a decrease in the transcription of proteins involved in flagella
295 synthesis, function and chemotaxis in the bacterial cell. The combination of phenotypic and
296 transcriptomic analysis presents the opportunity to consider the two distinct approaches in the
297 context of risk assessment. Omics and molecular genetics are increasingly applied in this field
298 (17, 55) and in some cases have indicated potential changes of concern associated with biocide
299 exposure; for example virulence potential (56) and transfer of genes associated with resistance
300 (57). Determining the biological or real-world significance of such data without phenotypic
301 corroboration can be problematic, and this is of concern where the objective is diligent risk
302 assessment to inform product composition. Thus, more work is required before genotype-
303 phenotype relationships can be effectively integrated in to the process of risk assessment.
304 Similarly, genome-wide association studies, applied with the aim of predicting antibiotic
305 resistance (58) may be less successful when applied to biocide studies due to the complexity of
306 modes of action and resistance mechanisms.

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

314

315

316 **METHODS**

317 **Bacteria, bacteriological media and antimicrobial agents.** *E. coli* MG1655 was
318 selected a well-characterized example of this bacterium, for which whole genome sequence
319 data are available. The bacterium, and growth media were purchased from Oxoid (Basingstoke,
320 United Kingdom). The bacterial growth medium was sterilized at 121°C and 15 lb/in² for 15
321 min prior to use. *E. coli* was grown on Tryptone Soy Agar and Tryptone Soy Broth. BAC
322 ($\geq 95.0\%$) was purchased from Sigma-Aldrich (Dorset, United Kingdom) and was prepared at 1
323 mg/ml in deionised water and filter sterilised (0.2 μ M pore size) prior to use.

324 **Determination of MICs and MBCs.** MICs were determined by microdilution
325 according to EUCAST methods ([https://onlinelibrary.wiley.com/doi/pdf/10.1046-j.1469-0691.2003.00790.x](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
326 and 800 μ g/ml. Growth was determined through turbidity (OD₆₀₀) compared to uninoculated
327 wells (negative control) using a microtiter plate reader (PowerWave XS, BioTek, Bedfordshire,
328 United Kingdom).

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

334 **Planktonic growth rate.** Overnight cultures of *E. coli* were diluted to an OD₆₀₀ of 0.8
335 then further diluted 1:100 in TSB in the presence and absence of 100 or 1000 ng/L BAC.
336 Bacteria were incubated in 96 well microtiter plates at 37°C for 24 h and the OD₆₀₀ was
337 determined every hour spectrophotometrically.

338 **Crystal violet biofilm formation assay.** Overnight cultures of *E. coli* were diluted to
339 an OD₆₀₀ of 0.8 then further diluted 1:100 in TSB. 150 µl of diluted bacterial inoculum was
340 delivered to each test well of a 96-well microtiter plate prior to incubation for 48 h at 37 °C and
341 20 rpm to promote biofilm growth. Wells were washed twice with 250 µl of sterile PBS before
342 addition of 200 µl of 0.5% (w/v) crystal violet solution. Plates were incubated for 30 min at
343 room temperature and the wells were subsequently washed twice with 250 µl of PBS and left to
344 dry at room temperature for 1 h. Attached crystal violet was solubilised in 250 µl of 95%
345 ethanol and plates were agitated at room temperature at 20 rpm for 1 h. After solubilisation,
346 biofilm growth was viewed as change in OD₆₀₀ relative to a sterile negative control. Biofilm
347 bound crystal violet was quantified and average values were calculated using data from two
348 separate experiments each with three technical replicates. Statistical significance was
349 determined using an ANOVA with Post-Hoc Tukey analysis where p<0.05 was deemed
350 significant.

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

355 **Repeated exposure to low levels of BAC.** Conical flasks containing 50 ml of TSB
356 were set-up containing 100 or 1000 ng/ L of BAC. These concentrations were selected to
357 represent residual concentrations that have been reported in natural aquatic environments and in
358 wastewater (24-25). Overnight cultures of bacteria were diluted to 0.8 OD₆₀₀ then further
359 diluted 1:100 into the BAC containing growth medium. Flasks were incubated at 37°C and 100
360 rpm aerobically for 48 h. Bacteria were passaged 10 or 20 times in the presence of the biocide
361 whilst using a consistent inoculation density for each passage (OD₆₀₀ 0.008; 3 - 5 x 10⁷ cfu/ml).
362 Cultures were plated between all passages to check for purity and viability. The progenitor

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

366 **RNA extraction.** RNA was extracted from 30 ml of a mid-log phase culture of the
367 previously generated adapted bacteria of *E. coli* (OD₆₀₀=0.2). Bacteria were grown in the
368 presence or absence of 100 ng/L of BAC prior to extraction to determine the stability of any
369 induced changes in gene expression once the BAC selective pressure was removed.
370 Transcription was stopped using 3.6 ml of 5 % phenol in ethanol prior to the addition of 10 ml
371 RNeasy Protect™ bacteria reagent (Qiagen, UK). Bacteria were pelleted at 4000 rpm for 15 mins.
372 Bacterial pellets were resuspended in 1 ml of 0.5 % Tween 80 prior to re-pelleting at 4000 rpm
373 for 15 mins. Pellets were resuspended in 200 µl of RNase free water and added to 750 µl of
374 extraction buffer (0.5 M sodium acetate, 0.5 % SDS, pH 4). Resuspensions were transferred to
375 1.5 ml lock top tubes containing sterile glass beads (sigma) and vortexed 10 x for 1 min with 10
376 min cooling on ice in between vortexing. Liquid was removed from tubes and transferred to a
377 sterile 1.5ml microcentrifuge tube containing 500 µl of phenol on ice before centrifugation for
378 15 min at 13,000 rpm. The aqueous layer was removed from each tube and transferred to 1 ml
379 of 100 % ethanol on ice containing 100 µl 3 M sodium acetate. RNA was then precipitated at -
380 20°C for 30 min before centrifugation at 13,000 rpm for 15 min. Supernatant was subsequently
381 removed and the pellet was washed in 1 ml of 80 % ethanol and air dried before dissolving in
382 50 µl RNase free water. 5 µl of RNA was added to 5 µl of 2 x RNA gel loading dye
383 (ThermoFisher Scientific, UK) and heated at 65°C for 10 min before being run at 45 V on a
384 1 % agarose gel for 45 min to check RNA integrity. RNA was DNase treated (DNase1) for 30
385 min at 37°C, quantified on a NanoDrop Microvolume Spectrophotometer (ThermoFisher, UK)
386 prior to being sent to the Centre for Genomic Research at University of Liverpool for RNA-
387 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 \log_{10} 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.

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

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

438
439 The above growth law was used to analyse the following 3-way comparisons: C0 v C10 v C20, ii C0 v
440 B100 (P10) v B100 (P20); iii) C0 v B1000 (P10) v B1000 (P20); iv) C10 v B100 (P10) v B1000 (P10)
441 and v) C20 v B100 (P20) v B1000 (P20). For each comparison the following step-wise analysis
442 procedure was used to assess experimental condition effects. The logistic model was fitted to all three
443 conditions without accounting for the experimental condition. Subsequently parameter values were
444 varied according to experimental condition type, therefore three L, k and t50 values representing each
445 condition were estimated, and assessed whether this improved model fit using the F-test. To arrive at the
446 final model, if the 95 percent confidence interval, generated using the profile likelihood, for the same
447 parameter across the three conditions overlapped, then that parameter was grouped across conditions.
448 Subsequently an assessment of the effect that had on model fit was conducted using the F-test and the
449 new model accepted with a p-value threshold of 0.05. The resulting differences between treatments via
450 model parameters were reported with 95 percent confidence intervals (CI). A P-value from the F-test
451 was reported together with quantitative details, maximal growth and time to reach 50% of
452 maximal growth, on how the growth curves differed.

453

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456 S.F., A.A., and A.J.M. conceived and designed the experiments. S.F. and N.C. per- formed the
457 experiments. S.F., N.C., G.H., A.A., and A.J.M. analyzed the data. H.M. performed the
458 statistical modeling. S.F. and A.J.M. wrote the paper. This project was funded by Unilever's
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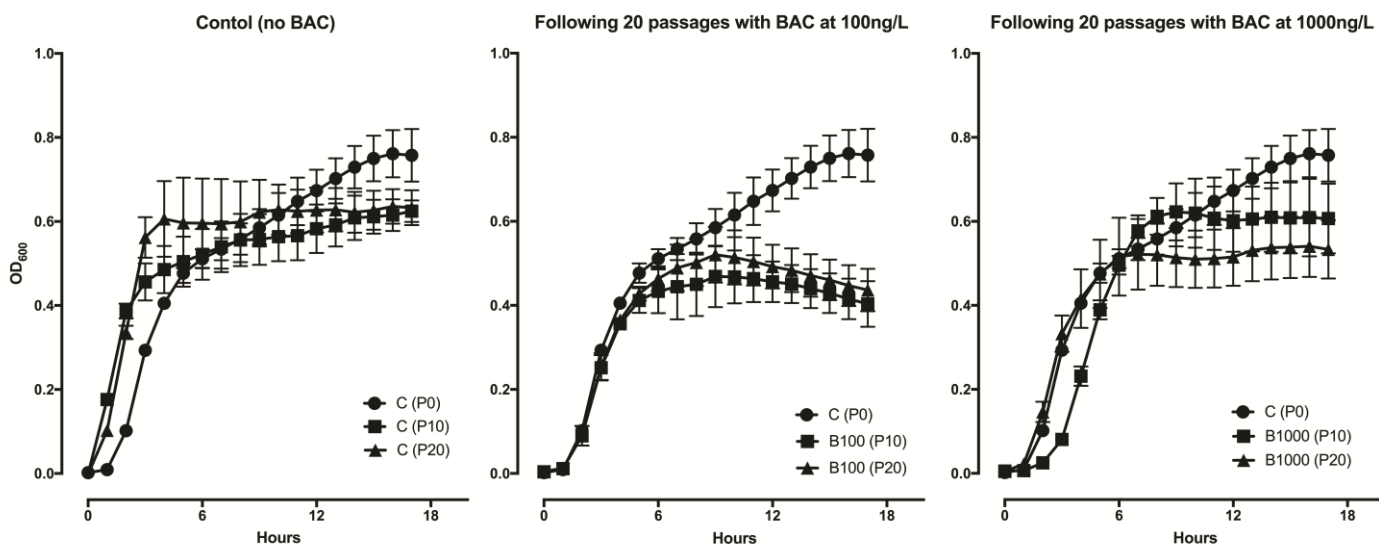
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660 **FIG 1** *E. coli* planktonic growth after 10 or 20 passages in the absence or presence of BAC. *E. coli* was
661 passaged 10 (P 10) and 20 (P20) times in binary culture in the presence of 100 ng/L (B100) or 1000
662 ng/L (B1000) of BAC prior to determination of planktonic growth. Control cultures prior to passage (C
663 P0) or passaged in a BAC-free environment over 10 and 20 passages were included (C (P10) and C
664 (P20) respectively). Data represent two separate experiments each with three technical replicates Error
665 bars are representative of standard deviations.

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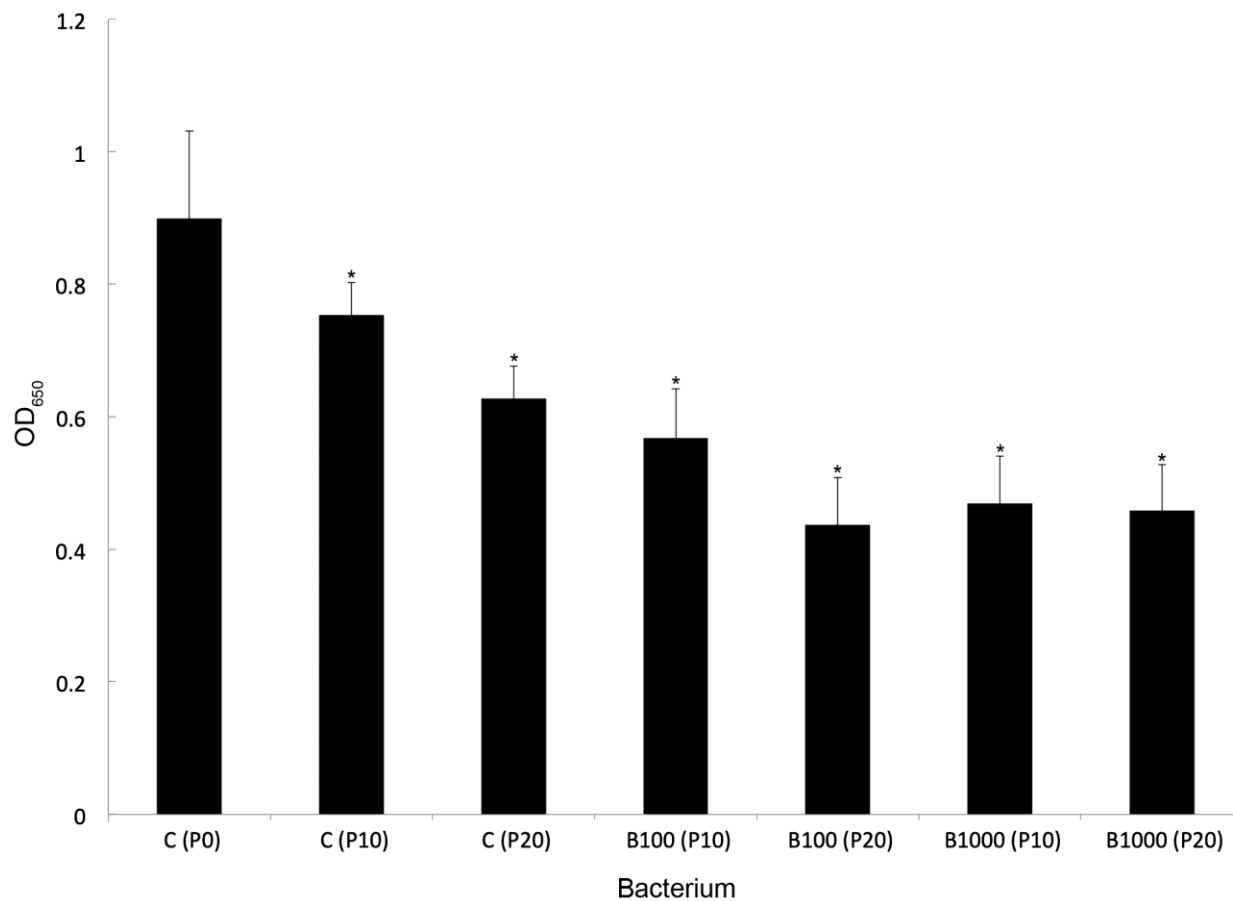
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688 **FIG 2** Crystal violet assay indicating biofilm formation (OD₆₀₀) before (control; C (P0)) and after 10 or
 689 20 passages in the absence (C (P10) and C (P20) or the presence of 100 ng/L of BAC (B100 P10/20) or
 690 1000 ng/L of BAC (B1000 P10/20). 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.

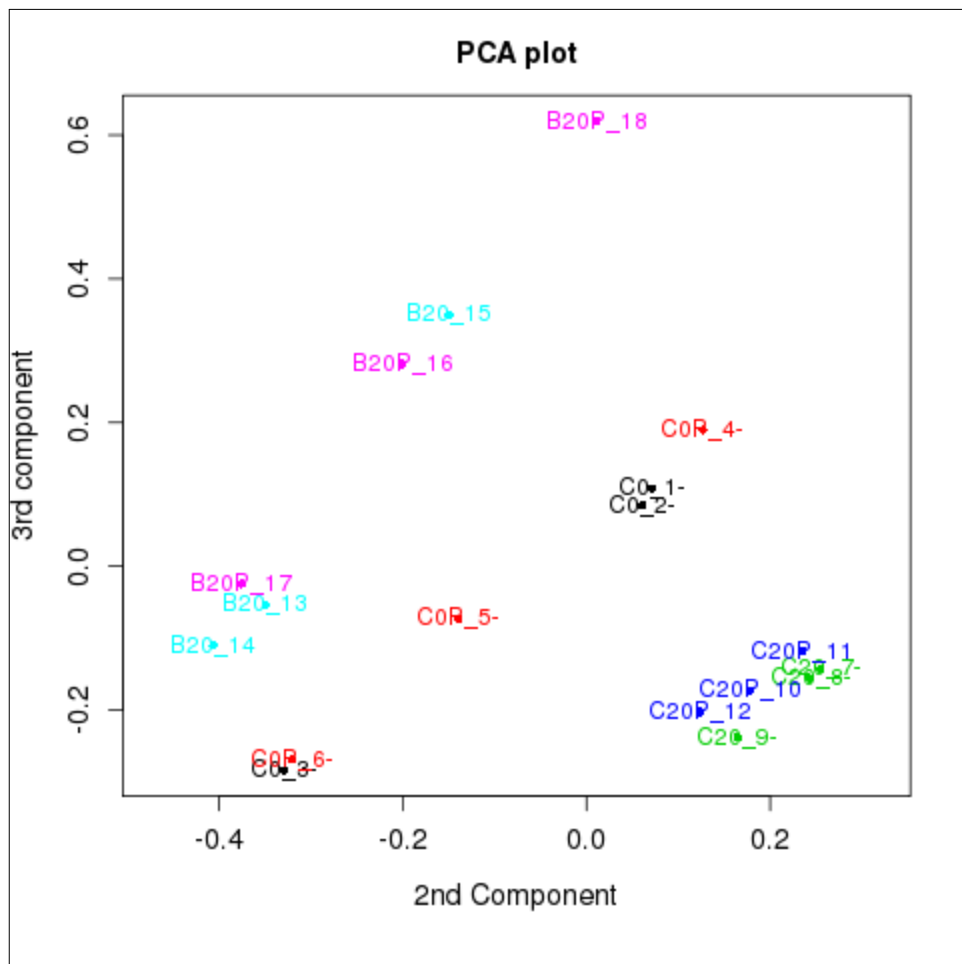
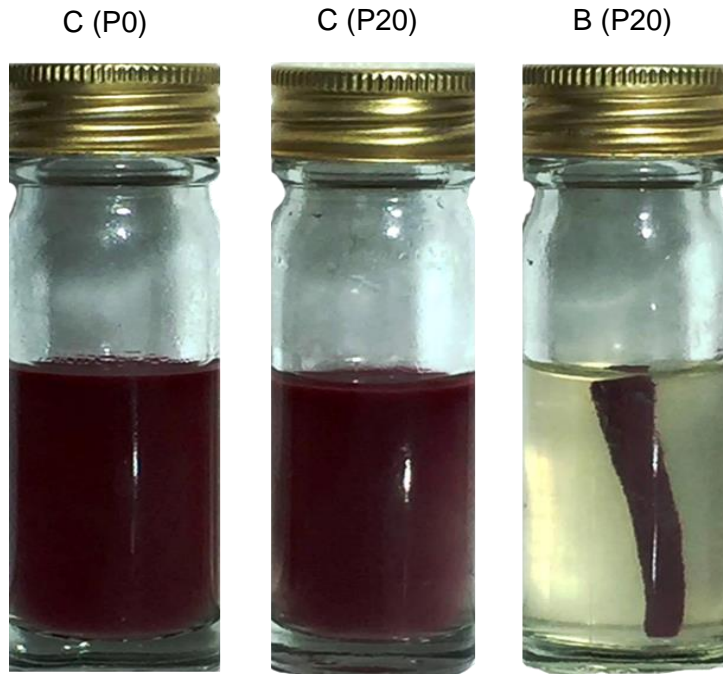


FIG 3 PCA plot indicating variation in DE genes for *E. coli* when comparing an unpassed control bacterium C0, a bacterium passaged 20 times in a BAC-free environment (C20) and a bacterium passaged 20 times in the presence of 100 ng/L BAC (B 20). C0 (black), C20 (green) and B20 (cyan) were grown in biocide 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.

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

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805 **TABLE 1** *E. coli* MICs and MBCs before (P0) and after 10 (P10)
 806 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
BAC 100	50	50	50	50	50	100
BAC 1000	50	50	50	50	50	100

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

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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.
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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.

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

Gene	Gene product	logFC	logFC	logFC
		C20 v C0	B20 v C20	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.

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

Gene	Gene product	logFC	logFC	logFC
		C20 v C0	B20 v C20	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.

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