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1 **Design and validation of a dual-fluorescence reporter system to**
2 **monitor bacterial gene expression in the gut environment**

3

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8

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13

14 **ABSTRACT**

15 Fluorescence-based reporter systems are valuable tools for studying gene expression dynamics
16 in living cells. However, available strategies to follow gene expression in bacteria within their
17 natural ecosystem that can be typically rich and complex, are scarce. In this work, we designed
18 a plasmid-based tool ensuring both the identification of a strain of interest in complex
19 environments and the monitoring of gene expression through the combination of two distinct
20 fluorescent proteins as reporter genes. The tool was validated in *Escherichia coli* to monitor the
21 expression of *eut* genes involved in the catabolism of ethanolamine. We demonstrated that the
22 constructed reporter strain gradually responds with a bimodal output to increasing ethanolamine
23 concentrations during *in vitro* cultures. The reporter strain was next inoculated to mice and flow
24 cytometry was used to detect the reporter strain among the dense microbiota of intestinal
25 samples and to analyse specifically the expression of *eut* genes. This novel dual-fluorescent
26 reporter system would be helpful to evaluate transcriptional processes in bacteria within
27 complex environment.

28

29

30 **KEY POINTS**

31 . A reporter tool was developed to monitor bacterial gene expression in complex environment

32 . Ethanolamine utilization (*eut*) genes are expressed by commensal *E. coli* in the mouse gut

33 . Expression of *eut* genes follows a bimodal distribution

34

35 **KEYWORDS**

36 gene expression reporter system; single cell; ethanolamine; commensal *E. coli*

37

38

39 INTRODUCTION

40 During the last fifty years, multiple techniques have been developed to monitor gene expression
41 in microorganisms. Among them, transcriptional fusions using fluorescent protein (FP)
42 encoding genes are tools extensively used to record the expression dynamics of genes in living
43 bacterial cells (Rosochacki and Matejczyk 2002; Campbell-Valois and Sansonetti 2014). Since
44 the discovery of green fluorescent protein (GFP), numerous GFP-variants and other FPs with
45 unique spectral properties have been engineered, resulting in a diverse set of colors that could
46 be used separately or combined together. Among their multiple benefits, FPs allow non-
47 destructive spatiotemporal analysis of gene expression at the single-cell level in various
48 conditions. In addition to *in vitro* studies, the monitoring of bacterial gene expression in their
49 natural ecosystem is necessary to integrate the multiple conditions and signals from these
50 complex environments. Numerous works developed strategy to detect specific bacteria in
51 natural environments by immuno-staining with appropriate antibody or by the use of
52 fluorescent tracers (Nielsen et al. 2010; Yang et al. 2017; Peñate-Medina et al. 2019; Packer
53 2021; Yoon et al. 2021). Bacterial detections were also associated with the reporting of
54 physiological functions or gene expression (Burton et al. 2014; Davis et al. 2015; Nuss et al.
55 2016; Li et al. 2021; Striednig et al. 2021). These works were however mainly dedicated to
56 decipher the behaviour of pathogens within host tissues but few were focused on commensals
57 of the gut microbiota which is one of the most complex and dense ecosystem.

58 In this report, we designed a plasmid-based tool ensuring both the identification of a strain of
59 interest in the gut microbiota and the monitoring of gene expression through the combination
60 of two distinct FPs as reporter genes. The gene expression monitoring system includes a genetic
61 amplifier to warrant transcription measurement of weakly expressed genes from their native
62 chromosomal position (Lim and van Oudenaarden 2007). The signal amplification is generated
63 by insertion of the T7 RNA polymerase (T7pol) encoding gene at a locus of interest in the
64 bacterial chromosome. Synthesis of T7pol, which reflects the native expression level of target

65 gene, initiates the transcription of a plasmid-based FP gene controlled by the T7 RNAP-specific
66 promoter. The creation of multiple FP transcripts by each T7pol molecule increases the
67 fluorescence signal and improves the detection of poorly expressed genes (Lim and van
68 Oudenaarden 2007). The FP used to record gene expression in our reporter plasmid is a
69 derivative of GFP obtained by the addition of a specific C-terminal peptide recognized by
70 intracellular tail-specific proteases found in numerous Gram-negative and Gram-positive
71 bacteria (Andersen et al. 1998). This protein extension leads to a destabilized variant of GFP,
72 which is initially a highly stable protein, and allows an accurate monitoring of up- and down-
73 promoter activities for a better study of temporal gene expression. Our designed tool was
74 validated in a gut commensal *Escherichia coli* strain to monitor the expression of *eut* genes
75 involved in the catabolism of ethanolamine (EA). EA is a small organic molecule found in the
76 gastro-intestinal tract of mammals that can be used as nutrients by intestinal bacteria, especially
77 pathogens (Garsin 2010). EA catabolism requires the expression of 17 genes clustered in the
78 *eut* operon and transcription of this unit is mainly controlled by the AraC/XylS-type
79 transcriptional regulatory protein EutR. Activity of this specific regulator is induced by the
80 presence of EA and vitamin B₁₂ and activation of *eut* operon by EutR creates a positive-
81 feedback loop since *eutR* is the last gene of the *eut* operon (Kaval and Garsin 2018). In this
82 work, we first demonstrated that the *eut* reporter *E. coli* strain gradually responds to known EA
83 concentrations during *in vitro* cultures. The strain was next inoculated to mice and digestive
84 sample analysis by flow cytometry allowed to specifically detect the reporter strain among the
85 dense microbiota and to analyse the expression level of *eut* operon in the gut.

86 MATERIAL & METHODS

87

88 *Bacterial strains, plasmids and growth conditions*

89 Strains and plasmids used in this study are listed in Table S1. HS is a human commensal *E. coli*
90 strain isolated from a healthy human available at the Center for Vaccine Development from the
91 University of Maryland School of Medicine (USA). Bacterial strains were cultured in LB with
92 shaking at 37 °C. In some conditions, LB was supplemented with various concentrations of
93 ethanolamine hydrochloride (EA) and/or cyanocobalamin (vitamin B₁₂). When required,
94 antibiotics were used at the following concentrations: chloramphenicol (25 µg.ml⁻¹);
95 streptomycin (Sm) (50 µg.ml⁻¹); gentamicin (15 µg.ml⁻¹); kanamycin (25 µg.ml⁻¹).

96

97 *Construction of reporter plasmids pHL51 and pHL52*

98 The reporter plasmid pHL51 was generated through modifications of pHL32 (Lim and van
99 Oudenaarden 2007). First, the *kan* sequence, obtained by *SacI* and *AatII* digestion, was replaced
100 with a DNA fragment including *aadA* gene (from pMS vector (Thermofisher)) and mTagBFP2
101 (from Addgene plasmid#34632) under the control of a strong constitutive promoter (IGEM
102 database, sequence#BBa_J23119). The DNA fragment was generated by PCR using primers
103 2019_02_F and 2019_02_R (Table S2) and ligated with pHL32 backbone using In-Fusion
104 cloning kit (Takara Bio). *gfp* gene from the resulting plasmid was next modified by inverse
105 PCR using primers pHL50_AAV_F and pHL50_AAV_R in order to obtain the destabilized
106 GFP variant (GFP[AAV]) through addition of the peptide RPAANDENYAAAV at the C-
107 terminal end of GFP (Miller et al. 2000). Sequence of pHL51 was validated by DNA sequencing
108 (Fig. S1). pHL52 was obtained following sequence modifications of pHL51. First, 2 PCR
109 fragments were amplified from pHL51 using primers designed to remove undesired sequences
110 (pHL51_A_fwd_bis and pHL51_A_rev; pHL51_B_fwd and pHL51_A_rev_bis). Following
111 *DpnI* digestion and DNA purification, both fragments were then assembled using the

112 NEBuilder HiFi DNA Assembly kit (New England Biolabs). The sequence of modified region
113 were checked by sequencing.

114

115 ***Strain construction***

116 The *eut* reporter strain was constructed by inserting the coding sequence of the T7 RNA
117 polymerase gene (T7pol) downstream of the *eutR* gene in *E. coli* strain HS. Briefly, a RBS-
118 T7pol-*cam* cassette was amplified from cHNL135 strain with primers 2019_03_F and
119 2019_03_R (Table S2). The 3.5 kb PCR product (Fig. S2) was purified and next introduced
120 into HS (pKD46-Gm) by electroporation following the method of Datsenko and Wanner
121 (Datsenko and Wanner 2000). Correct integration of the T7pol-*cam* cassette was verified by
122 PCR and sequencing. Insertion of *eutR* under the control of the constitutive promoter
123 BBa_J23119 (P_{cst} -*eutR*) was realized at the *attTn7* site as described in (Crepin et al. 2012). The
124 *eutR* gene was amplified from the *E. coli* HS strain using *eutR*_HS_fwd and *eutR*_HS_rev
125 primers and the pGP-Tn7 matrix was amplified from pTn7-Pcst-mTagBFP2 plasmid using
126 pTn7_Pcst_TagBFP2_fwd and pTn7_Pcst_TagBFP2_rev primers (Table S2). Both fragments
127 were assembled using NEBuilder® HiFi DNA Assembly kit (New England Biolabs) to give
128 the pGP-Pcst-*eutR* plasmid. The construct was then transferred by conjugation into the HS *eutR*-
129 T7pol strain containing the thermosensitive vector pSTNSK which allows synthesis of the
130 transposase machinery. Integration of the *Pcst-eutR-Gm* cassette at the *attTn7* site and
131 elimination of pSTNSK was achieved as described in (Crepin et al. 2012) and verified by PCR
132 and sequencing.

133

134 ***Fluorescence quantification***

135 For expression quantification assays, fluorescence was measured with a Spark microplate
136 reader (Tecan). Green fluorescence from GFP was analysed using excitation at 485 ± 20 nm and
137 emission at 530 ± 20 nm and blue fluorescence from mTagBFP2 using excitation at 399 ± 20 nm

138 and emission at 454 ± 20 nm. Cultures were performed directly within the microplate reader at
139 $37\text{ }^{\circ}\text{C}$ with orbital shaking (175 rpm) using black 96-well plates with clear bottoms in which
140 wells were filled with $200\text{ }\mu\text{l}$ of LB supplemented with vitamin B₁₂ (150 nM) and/or EA at
141 indicated concentrations. OD_{600nm} and fluorescence signals were recorded every 20 minutes.
142 Fluorescence quantification was also performed by flow cytometry (Cytoflex, Beckman
143 Coulter). FSC (forward scatter), SSC (side scatter), FITC (488 nm exciter, 525 ± 20 nm emitter)
144 and PB450 (405 nm exciter, 450 ± 22 nm emitter) axes were set to log display. For sample
145 analysis, the following gating strategy was applied: bacterial cells were gated based on a FSC
146 and SSC plot, and then analysed for BFP signal. A new gate covering BFP⁺ cells, which
147 correspond to bacterial cells of our reporter strain was selected and then analysed for GFP
148 signal. At least 10,000 events of BFP⁺ cells were recorded for each sample and data were
149 analysed with CytExpert software v2.2 (Beckman Coulter) or with Floreada tool
150 (<https://floreada.io>).

151

152 ***Real-time microscopy***

153 For microscopy assays, the *eut* reporter strain was grown into M9 minimal medium
154 supplemented with glucose (0.2%), casamino acids (0.4%) (M9 CASA) and streptomycin 50
155 $\mu\text{g}/\text{ml}$ in order to limit medium autofluorescence. Overnight culture of the HS *eutR*-T7pol
156 (pHL51) strain was diluted to an OD_{600nm} of 0.05, grown until an OD_{600nm} of 0.4, and finally
157 diluted in M9 CASA by a factor 4 to make the sample. Agarose-mounted slides are prepared as
158 described by Cayron and Lesterlin (Cayron and Lesterlin 2019). 1% agarose (g/v) were melted
159 into M9 CASA medium. A blue frame (In-Situ Hybridisation Frame, ABGENE) was stucked
160 on a microscope slide, leaving only the opened protecting plastic at the top of the blue frame.
161 $\sim 300\text{ }\mu\text{L}$ of melted agarose were poured into the blue frame and a cover glass was added to
162 remove the exceeding agarose medium and to flatten the surface of the mounted slide. After 30
163 min, the cover glass and the protective plastic were removed, $10\text{ }\mu\text{L}$ of culture were dropped at

164 the surface of the agarose-mounted slide and the slide was tilted gently to spread the sample.
165 After letting the sample dry, a new cover glass was added on the blue frame to perform the
166 observation. For all the samples, the agarose mounted-slides were supplemented with
167 streptomycin 50 µg/ml and when mentioned with Ethanolamine 30 mM and cyanocobalamin
168 150 nM. Microscopy imaging was carried out on a Zeiss Cell Observer Spinning Disk
169 (YOKOGAWA CSU 1X unit and Hamamatsu Orca Flash 4.0 cameras) equipped with
170 CApochromat 40X NA 1.2 Water objective using Zen Blue software for image acquisition.
171 Acquisition were performed using 50% power of laser at 405 nm (BFP) and 25 % power 488
172 nm (GFP) excitation wavelengths. Emissions were filtered at 450 nm (BFP) and 509 nm (GFP)
173 wavelengths. Exposure settings were 100 ms for BFP and 100 ms for GFP. Images were taken
174 every 10 min during 200 minutes. The microscope is equipped with a thermostatic chamber
175 preheated to 37°C before the experiment. Images were visualized and treated with ImageJ/Fiji
176 (<https://fiji.sc/>) (Schindelin et al. 2012) and the Hyper Stack Reg plugin (Sharma 2018) to align
177 time-lapse experiments.

178

179 *Mouse experiments*

180 C3H/HeOuj with specific-pathogen-free (SPF) status were purchased from Charles River
181 laboratories. Five-to six-week-old female mice were housed in cages containing no more than
182 five animals, maintained under a 12 h light/dark cycle at a temperature of 21 ± 2 °C, and fed
183 with standard diet and water ad libitum. Mice were given drinking water supplemented with
184 streptomycin (5 g/L) throughout the experiment, starting from one day prior inoculation with
185 10^7 cells of HS (pHL51) or HS *eutR*-T7pol (pHL51). The drinking water of some groups of
186 mice was also supplemented with 30 mM of ethanolamine hydrochloride and 150 nM of vitamin
187 B₁₂. Five days post-inoculation, mice were euthanized in order to collect cecal contents as well
188 as feces from the rectum. Samples were resuspended in PBS, centrifuged at 1000 rpm for 1 min

189 and supernatants were filtered using a 40- μ m PET membrane before analysis by flow
190 cytometry.

191 RESULTS

192

193 Rationale design and general description of the reporter system

194 Map and sequence of the designed reporter plasmid pHL51 are shown in Figure 1 and Figure
195 S1, respectively. This plasmid originates from pHL32, a low copy vector with a p15A origin of
196 replication, hosting *gfp* gene under the control of the T7pol promoter (Lim and van
197 Oudenaarden 2007). The following modifications have been implemented to yield pHL51.
198 First, gene encoding the blue fluorescent protein mTagBFP2 placed under a constitutive
199 promoter (BBa_J23119) was inserted in opposite orientation to the P_{T7pol} -*gfp* fusion.
200 Consequently, bacterial cells carrying pHL51 permanently produce mTagBFP2 and emit blue
201 fluorescence. Secondly, the *kan* region of pHL32 was replaced by gene *aadA* conferring
202 resistance to spectinomycin/streptomycin. This antibiotic resistance offers the possibility to
203 work *in vivo* with streptomycin-pretreated mice, a common animal model to study colonization
204 and/or infection of the gastro-intestinal tract by *Enterobacteriaceae* (Conway et al. 2004).
205 Finally, the gene *gfp* mut3.1 was modified to insert the peptide RPAANDENYAAAV at the C-
206 terminal end of GFP, leading to the unstable variant GFP[AAV] which allows the studies of
207 transient gene expression in bacteria (Andersen et al. 1998). The functionality of pHL51 was
208 evaluated using the BL21(DE3) *E. coli* strain carrying the T7pol encoding gene under the
209 control of the IPTG inducible promoter P_{lacUV5} . The strain was grown in LB and IPTG 1mM
210 was added at mid-log phase (OD=0.3) to induce the production of T7pol. Four hours after the
211 induction, bacterial cells were analysed by flow cytometry to quantify the levels of BFP and
212 GFP fluorescence (Fig. 2). When compared to the BL21(DE3) strain alone used as a negative
213 control, the BFP signal from BL21(DE3) pHL51 was high and uniform between cells. In
214 addition, the BFP signal was similar between uninduced and IPTG induced conditions. In
215 contrast, GFP fluorescence strongly increased by a factor 60 in IPTG induced culture. This
216 result demonstrates that *E. coli* carrying pHL51 constitutively emits blue fluorescence and

217 expresses GFP in response to the level of T7pol. Next, the stability of GFP[AAV] and
218 mTagBFP2 was assessed using a culture of BL21(DE3) carrying pHL51. The strain was grown
219 in LB until mid-log phase, induced 1 hour with IPTG and then shifted to PBS in order to remove
220 the inducer and to prevent bacterial growth and protein synthesis. BFP and GFP signals were
221 quantified after the shift to estimate the stability of both proteins. The BFP signal stayed stable
222 over the 3-hour experiment. In contrast, the GFP signal decreased overtime and ~40 % of the
223 initial signal was lost after 3 hours (Fig. S3). These data indicate that the GFP[AAV] expressed
224 from pHL51 has a limited half-life as initially reported (Andersen et al. 1998).

225

226 **Use of the reporter system to study the expression of *eut* genes associated with EA** 227 **catabolism in commensal *E. coli***

228 The reporter plasmid pHL51 was next used to monitor the expression of *eut* genes in the *E. coli*
229 commensal strain HS. To this end, we inserted a promoterless T7pol encoding gene
230 immediately downstream *eutR*, which is the last gene of the *eut* operon and this strain was next
231 transformed with pHL51 (Fig. 3A). The strain was cultured in LB supplemented with various
232 concentrations of EA and the co-factor vitamin B₁₂. As expected, the GFP signal gradually
233 increased with EA concentrations (Fig. 3B). Moreover, absence of vitamin B₁₂ impede *eut*
234 expression despite the addition of a high concentration of EA. The different samples were next
235 analysed by flow cytometry to quantify GFP and BFP signals in individual cells (Fig 3C). First,
236 the BFP signal was homogeneous among cells and did not vary between conditions. In contrast,
237 the GFP signal strongly fluctuated in response to EA concentration in the culture medium. Only
238 1 % of cells were GFP⁺ in absence of EA, suggesting that few cells express *eut* genes without
239 supplementation of the inducer. The percentage of GFP⁺ cells increased along with the
240 concentration of EA and reached 43% when EA was added at a final concentration of 30 mM
241 (Fig. 3C). Again, no bacterial cells express the *eut* operon in absence of vitamin B₁₂. It is
242 noteworthy that expression of GFP follows a bimodal distribution with the existence of GFP⁻

243 and GFP⁺ populations. Even at excessive concentrations of EA and/or vitamin B₁₂, bimodal
244 distribution of GFP signal was still observed (Fig. S4), questioning us about a potential artefact
245 associated with the reporting tool pHL51. In particular, the T7 promoter region driving the
246 expression of *gfp* derived initially from the pET-11a vector and thus includes a LacI repressor
247 binding site from the *E. coli lac* operon (Fig. S1). To ensure that potential LacI mediated
248 repression did not interfere with expression and distribution of GFP signal, the LacI operator
249 sequence (as well as a sequence used to add a T7 tag to GFP) was deleted from pHL51 to yield
250 plasmid pHL52 (Fig. S1). The HS *eutR*-T7pol strain carrying either pHL51 or pHL52 were
251 exposed to different concentrations of EA and GFP signals were recorded for comparison. As
252 observed in Fig. S5, GFP patterns obtained from the 2 reporter plasmids were highly similar at
253 a given EA concentration. Moreover, the addition of IPTG, alleviating the repressing activity
254 of LacI, did not alter the GFP expression level in presence of 30 mM EA. These data indicate
255 that the presence of a LacI operator does not influence GFP expression in our experimental
256 conditions and suggest that bimodal expression is a characteristic of the *eut* operon. To go
257 further, we hypothesized that bimodal expression may result from the action of the positive
258 feedback loop generated by the activator EutR since *eutR* is within the *eut* operon. To verify
259 this assumption, we inserted the *eutR* gene under the control of a constitutive promoter ($P_{\text{cst-}}$
260 *eutR*) within the chromosome of the HS *eutR*-T7pol (pHL51) strain, decoupling *eutR* expression
261 from transcription of the *eut* operon. Following culture in presence of 30 mM of EA, more than
262 96 % of cells were GFP⁺, revealing a near-perfect unimodal expression pattern (Fig. 4 and Fig.
263 S6). At lower EA concentrations or without EA supplementation, the percentages of GFP⁺ cells
264 were drastically increased when compared to those recorded with the HS *eutR*-T7pol (pHL51)
265 strain. Altogether, these data strongly suggest that both EutR and EA concentrations in the cell
266 are at play to determine the expression of *eut* genes in individual bacteria, with potential
267 bimodal output at the population level.

268 We also performed time-lapse microscopy to follow the fluorescence signal kinetic in dividing
269 bacteria. The *eut* reporter strain was grown to mid-log phase and few cells were dropped on an
270 agarose pad supplemented or not with EA + vitamin B₁₂. Fluorescence signals were then
271 recorded for 200 minutes under the microscope (Fig. 5, Fig. S7, S8 and S9). Because no EA
272 was added to the initial culture, almost all cells were BFP⁺ and GFP⁻ at the beginning of the
273 time-lapse procedure. However, few cells became GFP⁺ very quickly (~40 min) before shutting
274 down again. Interestingly, a second wave of GFP signal occurred later during the time-lapse
275 (~200 min) only for some cells. As expected, most cells if not all lacked green fluorescence
276 when cells were grown on an agarose pad without EA and vitamin B₁₂ (Fig. S8 and S9). These
277 data confirm the heterogeneous expression of *eut* genes in strain HS and also suggests that *eut*
278 expression is potentially fluctuant in individual cells despite the availability of EA in excess.

279

280 **Monitoring *eut* expression during colonization of the mouse gut**

281 Our reporter system was designed to monitor the expression of a gene of interest in complex
282 environment. Indeed, the constitutive expression of mTagBFP2 should allow the tracking of
283 our strain even in dense ecosystems with high level of diverse microorganisms like within the
284 gastro-intestinal tract. To this end, we orally inoculated streptomycin-treated mice with the
285 strain HS *eutR*-T7pol (pHL51). Streptomycin removes the facultative anaerobes (Maltby et al.
286 2013) and allows gut colonization by the reporter strain which is resistant to streptomycin
287 thanks to *aadA* gene located on pHL51. EA and vitamin B₁₂ were also added to the drinking
288 water of some animals to artificially increase the level of both factors within the gut. Five days
289 post-inoculation, fecal and cecal contents of animals were recovered and analysed by flow
290 cytometry following the gating strategy depicted in figure 6A. A first gate covering events with
291 FSC/SSC parameters usually defined for bacilli was applied to further analyse BFP
292 fluorescence. A small population exhibited a strong BFP signal and this corresponds to our
293 reporter strain constitutively expressing mTagBFP2 (Fig. 6A). These events usually represent

294 less than 0.5 % of the total number of detected events, corroborating with an average of $\sim 1.10^9$
295 CFU of *E. coli* / g feces observed in the streptomycin mouse model (Gardette et al. 2020). These
296 BFP⁺ cells were next gated to analyse green fluorescence in order to report the expression level
297 of *eut* genes. As expected, negligible GFP⁺ events were detected in samples recovered from
298 mice inoculated with HS (pHL51) used as a negative control (Fig. 6B). In contrast, up to 5 %
299 of GFP⁺ cells were detected in the cecal content of mice inoculated with the reporter strain HS
300 *eutR-T7pol* (pHL51). This percentage increased up to 35 % when EA and vitamin B₁₂ were
301 added to the drinking water of animals, demonstrating that more HS cells in the gut express the
302 *eut* operon in response to the supplementation. This was confirmed by a quantitative analysis
303 of the GFP signal with a 6-fold induction of fluorescence in samples recovered from EA
304 supplemented mice (Fig. 6C). We also analysed bacteria recovered from fecal samples to
305 evaluate if *eut* gene expression differs between the upper and the lower part of the large
306 intestine. Whereas the effect of EA supplementation on GFP fluorescence was observed in feces
307 like for the cecal content, the percentage of GFP⁺ cells in feces of untreated mice stayed
308 negligible whereas 5% of cells were GFP⁺ in the cecal content. These data suggest that HS do
309 not encounter enough EA and/or vitamin B₁₂ in feces to express *eut* genes (Fig. 6B and 6C).
310

311 DISCUSSION

312 In this work, we developed a fluorescence reporter system to quantify gene expression level in
313 single cells of a bacterial strain present in its natural complex ecosystem. The dual-fluorescence
314 reporter tool was validated by reporting the expression of *eut* genes involved in the catabolism
315 of EA from a commensal *E. coli* strain present in the gut of experimentally inoculated mice.
316 Our reporter system combines several tools which have been described in the past: *i*) a dual-
317 fluorescence labelling system (Hennessy et al. 2018; Schulte et al. 2021; Lauren et al. 2022) to
318 detect the studied strain through a constitutive expression of *bfp* and to report the expression
319 level of a gene of interest through a transcriptional fusion with *gfp*; *ii*) a signal amplification
320 system based on T7 polymerase (Lim and van Oudenaarden 2007) to enable the detection of
321 poorly expressed genes in their native chromosomal position and *iii*) the use of an unstable
322 variant of GFP (GFP[AAV]) to allow real-time studies of gene regulation *in situ* (Andersen et
323 al. 1998). The use of an unstable variant of GFP in our study has indeed allowed to observe a
324 temporal expression of *eut* genes. In time-lapse microscopy experiment, detection of GFP was
325 recorded in some cells upon transfer to EA-containing agarose pad but the signal quickly
326 disappeared before coming back later in daughter cells. These qualitative data suggest a
327 temporal expression of *eut* genes despite the presence of a high concentration of EA (30mM),
328 but further experiments are required to confirm this possibility. Interestingly, several works
329 highlighted pulsatile expression of genes in *E. coli* under uniform conditions (Patange et al.
330 2018; Kim et al. 2020; Sampaio et al. 2022). Identified genes are involved in various processes
331 including cellular growth, stress response and flagellar biosynthesis, suggesting that pulsing
332 dynamics of gene expression in bacteria under homogeneous conditions might be more
333 common than previously anticipated. Spatio-temporal expression of *eut* genes in the mouse gut
334 is also suggested by our results presented in figure 6. Indeed, the percentage of bacterial cells
335 expressing GFP from fecal samples was systematically lower than the percentage recorded from
336 the cecal sample of the same mouse, for both groups of mice (untreated or EA supplemented

337 mice). These data strongly suggest that EA is present and detected by the reporter strain in the
338 upper part of the large intestine (cecum) and is probably consumed in the colon by the resident
339 microbiota (including the HS reporter strain) and becomes less (or not) available in the lower
340 part of the colon from which the feces were collected. Determination of EA concentration from
341 cecal or fecal contents of animals was performed by NMR spectroscopy to corroborate this
342 statement. Unfortunately, EA was not detected in samples suggesting that the concentration of
343 free untransformed EA in the intestine is below the detection limit even in EA supplemented
344 mice. If the developed reporter tool allowed us to report *eut* gene expression in the mouse gut,
345 the model still presented some limitations. First, mice were treated with an antibiotic to enable
346 gut colonization by the reporter strain and the complexity of the gut microbiota was therefore
347 decreased. Some models of mouse gut colonization have been described to implement a strain
348 of interest in an intact gut microbiota (Lasaro et al. 2014; Payros et al. 2014). It would be
349 interesting to evaluate the relevance of our reporter tool in such models, notably in terms of
350 detection limit by flow cytometry since the concentration of commensal *E. coli* in an intact gut
351 is as low as 10^5 - 10^6 CFU per gram of content. In addition, integration of P_{T7pol}-GFP reporter
352 within the chromosome would be an improvement in order to avoid potential bias such as
353 variation of the plasmid copy number. Another limitation concerns the very low availability of
354 dioxygen in the gut whereas it is essential for maturation of FPs and emission of fluorescence.
355 In our study, BFP and GFP signals recorded from gut samples presented levels similar to those
356 recorded from *in vitro* samples (compare Fig 3C and 6B). Oxygenation of intestinal samples
357 during their preparation for flow cytometry analysis was probably sufficient in our case to
358 enable fluorophore maturation. However, this limitation should be kept in mind for other
359 applications. Finally, the report of gene expression remains a proxy for a function and detailed
360 analysis of EA catabolism would require other complementary approaches.

361 We demonstrated in this study that *eut* genes are expressed by the commensal *E. coli* HS strain
362 in the mouse gut, especially in the cecum. *In vivo* expression of the *eut* operon has been also

363 demonstrated for several gut pathogens such as *Listeria monocytogenes* or *Salmonella enterica*
364 serovar Typhimurium (Toledo-Arana et al. 2009; Anderson et al. 2015). Both pathogens induce
365 a strong inflammation in the gut, contributing to epithelial cell death (Blander 2016) and release
366 of high level of EA in the intestinal lumen. In addition, EA is used both as a nutrient source and
367 as an intestinal signal to control virulence program of pathogens (Toledo-Arana et al. 2009;
368 Garsin 2012; Kendall et al. 2012; Anderson et al. 2015; Rowley et al. 2020). Here, we revealed
369 that commensal *E. coli* express *eut* operon in the mouse gut in absence of an inflammatory
370 context such as during infection, suggesting that EA is catabolized by *E. coli* in the gut under
371 physiological conditions. This corroborates with a recent finding showing that EA utilization is
372 not restricted to intestinal pathogens and occurs in most commensal *E. coli* strains. In addition,
373 this metabolic trait has been demonstrated to be essential for an optimal fitness of commensal
374 *E. coli* in the gastro-intestinal tract (Moreira de Gouveia et al. 2022). Interestingly, *eut*
375 expression in the gut occurs only in a minor fraction of the HS strain population. This bimodal
376 expression pattern was also observed during *in vitro* culture with percentages of both
377 populations varying according to the available EA concentration. Over the last 20 years, many
378 works demonstrate that clonal microbial cells cultured in homogeneous conditions can display
379 significant diversity in their gene expression level, a phenomenon called phenotypic
380 heterogeneity (Avery 2006; Bury-Moné and Sclavi 2017). This variability originates from
381 stochasticity in gene expression but multiples drivers occurring at pre-transcriptional,
382 transcriptional and post-transcriptional levels have been described in the literature (Martin et
383 al. 2022). Particularly, it has been proposed that cellular metabolic pathways in bacteria
384 inherently follow stochastic processes and is a generic source of phenotypic heterogeneity
385 (Kiviet et al. 2014; Takhaveev and Heinemann 2018). Accordingly, many metabolic pathways
386 give rise to distinct single-cell behaviours in *E. coli* (Afroz et al. 2014). Observation of a
387 heterogeneous expression of the *eut* operon in this work add a new example of phenotypic
388 heterogeneity related to metabolic processes in *E. coli*. Functional consequences of metabolic

389 heterogeneity in microorganisms are not fully understood to date but numerous works suggest
390 it offers adaptive benefits to bacteria for persistence in fluctuating environments such as the
391 gastro-intestinal tract, an evolutionary strategy referred as bet hedging (Ackermann 2015). The
392 role of *eut* heterogeneous expression in the fitness of commensal *E. coli* in the gut and/or to the
393 infectious process of pathogenic *E. coli* remains to be investigated.

394 In conclusion, we developed and validated a fluorescence-based tool which enable the
395 monitoring of gene expression at the single cell level in bacteria present in complex
396 environment. This method would facilitate investigations of bacterial gene expression in
397 complex communities and improve our understanding of bacterial behaviours in their natural
398 ecosystem, which are often highly different than those observed in laboratory conditions.

399

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407

408 **STATEMENTS AND DECLARATIONS**

409 *Conflict of Interest:* The authors have no competing interests to declare that are relevant to the
410 content of this article

411 *Author contribution statement:* GJ and ABD conceived and designed research. MIMG, AR,
412 AG, JD and GJ conducted experiments. MIMG and GJ analyzed data. GJ wrote the manuscript.
413 All authors read and approved the manuscript.

414 *Data availability:* All data generated or analyzed during this study are included in this published
415 article

416 *Ethical approval:* All applicable international, national, and/or institutional guidelines for the
417 care and use of animals were followed. The experiments performed herein were reviewed and
418 approved by the Auvergne Committee for Animal Experimentation C2EA and received the
419 agreement number #31333-2021042809157789v2. This article does not contain any studies
420 with human participants performed by any of the authors.

421

422 **FIGURE LEGENDS**

423

424 **Figure 1: Map of the reporter plasmid pHL51.** ORFs are represented by arrows and other
425 elements (promoter, terminator, origin of replication) are represented by rectangles. Indicated
426 enzymes correspond to unique cutters.

427

428 **Figure 2: Functional validation of pHL51 reporter plasmid in *E. coli* BL21(DE3).** The
429 BL21(DE3) strain carrying pHL51 (or without plasmid) were grown in LB supplemented or
430 not with IPTG. Fluorescence emitted from mTagBFP2 and GFP[AAV] were quantified by flow
431 cytometry. Data are shown on histograms with BFP (left panels) or GFP (right panels) signals
432 on the x-axis.

433

434 **Figure 3: Quantification of *eut* gene expression during *in vitro* growth of *E. coli*.** (A)
435 Schematic representation of the EA reporter strain. (B) The HS *eutR*-T7pol (pHL51) strain was
436 grown in LB initially supplemented with vitamin B₁₂ and indicated concentrations of EA. GFP
437 fluorescence and OD_{600nm} were measured every 20 minutes. GFP signals (fluorescence unit)
438 are represented by thick solid lines and growth curves by thin solid lines. Results are presented
439 as mean and standard deviations from three independent experiments (C) Six-hour samples of
440 cultures shown in (B) were analysed by flow cytometry. Data are represented on two-
441 dimensional dot plots with BFP and GFP signals on the y-axis and x-axis, respectively. Gates
442 corresponding to GFP-negative and GFP-positive populations are shown and the percentages
443 of the GFP⁺ cells are indicated at the bottom of each image.

444

445 **Figure 4: Influence of EutR on the bimodal expression of *eut* genes.** The HS *eutR*-T7pol
446 (pHL51) and HS *eutR*-T7pol P_{cst-*eutR*} (pHL51) strains were grown for 6 h in LB supplemented
447 with indicated concentrations of EA and vitamin B₁₂. Bar plots represent the percentage of

448 GFP⁺ cells in the BFP⁺ population quantified by flow cytometry. Mean and standard deviation
449 were obtained from three independent replicates represented by the dots. A 2-way ANOVA
450 with Sidak's multiple comparisons test was performed. ns: non significant, ** P-value<0.01,
451 **** P-value<0.0001.

452

453 **Figure 5: *eut* expression during *in vitro* growth in time-lapse microscopy.** An agarose pad
454 of M9 Glucose supplemented with 150 nM vitamin B₁₂ and 30 mM EA was inoculated with
455 cells of HS *eutR*-T7pol (pHL51) grown in liquid culture. Time-lapse microscopy was
456 performed during 200 minutes on several fields and a representative field is shown here. Images
457 correspond to overlays of BFP, GFP and brightfield acquisitions.

458

459 **Figure 6: Quantification of *eut* gene expression in the gut of mice inoculated with the EA**
460 **reporter strain. (A)** Gating strategy to evaluate *eut* expression in *E. coli* HS sampled from gut
461 contents. Gates are represented by black polygons on the left and middle panels, and gates
462 corresponding to GFP⁻ or GFP⁺ cells are separated by a vertical black line on the right panel.
463 Data are represented on two-dimensional dot plots with indicated parameters on the y-axis and
464 x-axis. Dot plots were generated using all events or from indicated gates. **(B)** Fecal or cecal
465 samples were analysed by flow cytometry following the gating strategy presented in (A). Data
466 are represented on two-dimensional dot plots with BFP and GFP signals on the y-axis and x-
467 axis, respectively. Gates corresponding to GFP-negative and GFP-positive populations are
468 shown and the percentages of the GFP⁺ cells are indicated at the bottom of each image. **(C)**
469 Quantification of GFP signal measured from gut samples of indicated groups of mice. Each dot
470 represents one mouse and means are indicated as a line.

471

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