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

Maria Ines Moreira de Gouveia, Audrey Reuter, Annie Garrivier, Julien Daniel, Annick Bernalier Donadille, Grégory Jubelin

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

Maria Ines Moreira de Gouveia[1#], Audrey Reuter[1#], Annie Garrivier[1], Julien Daniel[1], Annick Bernalier-Donadille[1], Gregory Jubelin[1]*

[1] *Université Clermont Auvergne, INRAE, MEDIS UMR454, F-63000 Clermont-Ferrand, France*

[#] These authors contributed equally to this work

* corresponding author: Gregory Jubelin, INRAE Auvergne Rhône-Alpes, UMR454 MEDIS, site de Theix, 63122 Saint-Genès-Champanelle, France. Email: gregory.jubelin@inrae.fr. ORCID : 0000-0002-9902-0663

ABSTRACT

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

KEY POINTS

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

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

. Expression of *eut* genes follows a bimodal distribution

KEYWORDS

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

INTRODUCTION

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

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

gene, initiates the transcription of a plasmid-based FP gene controlled by the T7 RNAP-specific promoter. The creation of multiple FP transcripts by each T7pol molecule increases the fluorescence signal and improves the detection of poorly expressed genes (Lim and van Oudenaarden 2007). The FP used to record gene expression in our reporter plasmid is a derivative of GFP obtained by the addition of a specific C-terminal peptide recognized by intracellular tail-specific proteases found in numerous Gram-negative and Gram-positive bacteria (Andersen et al. 1998). This protein extension leads to a destabilized variant of GFP, which is initially a highly stable protein, and allows an accurate monitoring of up- and down-promoter activities for a better study of temporal gene expression. Our designed tool was validated in a gut commensal *Escherichia coli* strain to monitor the expression of *eut* genes involved in the catabolism of ethanolamine (EA). EA is a small organic molecule found in the gastro-intestinal tract of mammals that can be used as nutrients by intestinal bacteria, especially pathogens (Garsin 2010). EA catabolism requires the expression of 17 genes clustered in the *eut* operon and transcription of this unit is mainly controlled by the AraC/XylS-type transcriptional regulatory protein EutR. Activity of this specific regulator is induced by the presence of EA and vitamin B_{12} and activation of *eut* operon by EutR creates a positive-feedback loop since *eutR* is the last gene of the *eut* operon (Kaval and Garsin 2018). In this work, we first demonstrated that the *eut* reporter *E. coli* strain gradually responds to known EA concentrations during *in vitro* cultures. The strain was next inoculated to mice and digestive sample analysis by flow cytometry allowed to specifically detect the reporter strain among the dense microbiota and to analyse the expression level of *eut* operon in the gut.

MATERIAL & METHODS

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table S1. HS is a human commensal *E. coli* strain isolated from a healthy human available at the Center for Vaccine Development from the University of Maryland School of Medicine (USA). Bacterial strains were cultured in LB with shaking at 37 ºC. In some conditions, LB was supplemented with various concentrations of ethanolamine hydrochloride (EA) and/or cyanocobalamin (vitamin B_{12}). When required, antibiotics were used at the following concentrations: chloramphenicol (25 $\mu g.ml^{-1}$); streptomycin (Sm) (50 $\mu g.ml^{-1}$); gentamicin (15 $\mu g.ml^{-1}$); kanamycin (25 $\mu g.ml^{-1}$).

Construction of reporter plasmids pHL51 and pHL52

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

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

Strain construction

The *eut* reporter strain was constructed by inserting the coding sequence of the T7 RNA polymerase gene (T7pol) downstream of the *eutR* gene in *E. coli* strain HS. Briefly, a RBS-T7pol-*cam* cassette was amplified from cHNL135 strain with primers 2019_03_F and 2019_03_R (Table S2). The 3.5 kb PCR product (Fig. S2) was purified and next introduced into HS (pKD46-Gm) by electroporation following the method of Datsenko and Wanner (Datsenko and Wanner 2000). Correct integration of the T7pol-*cam* cassette was verified by PCR and sequencing. Insertion of *eutR* under the control of the constitutive promoter BBa_J23119 (P_{cst}-*eutR*) was realized at the *att*Tn*7* site as described in (Crepin et al. 2012). The *eutR* gene was amplified from the *E. coli* HS strain using eutR_HS_fwd and eutR_HS_rev primers and the pGP-Tn7 matrix was amplified from pTn7-Pcst-mTagBFP2 plasmid using pTn7_Pcst_TagBFP2_fwd and pTn7_Pcst_TagBFP2_rev primers (Table S2). Both fragments were assembled using NEBuilder® HiFi DNA Assembly kit (New England Biolabs) to give the pGP-P*cst-eutR* plasmid. The construct was then transferred by conjugation into the HS *eutR*-T7pol strain containing the thermosensitive vector pSTNSK which allows synthesis of the transposase machinery. Integration of the *Pcst-eutR-Gm* cassette at the *att*Tn7 site and elimination of pSTNSK was achieved as described in (Crepin et al. 2012) and verified by PCR and sequencing.

Fluorescence quantification

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

and emission at 454±20 nm. Cultures were performed directly within the microplate reader at 37 °C with orbital shaking (175 rpm) using black 96-well plates with clear bottoms in which wells were filled with 200 µl of LB supplemented with vitamin B_{12} (150 nM) and/or EA at indicated concentrations. OD_{600nm} and fluorescence signals were recorded every 20 minutes. Fluorescence quantification was also performed by flow cytometry (Cytoflex, Beckman Coulter). FSC (forward scatter), SSC (side scatter), FITC (488 nm exciter, 525±20 nm emitter) and PB450 (405 nm exciter, 450±22 nm emitter) axes were set to log display. For sample analysis, the following gating strategy was applied: bacterial cells were gated based on a FSC and SSC plot, and then analysed for BFP signal. A new gate covering BFP^+ cells, which correspond to bacterial cells of our reporter strain was selected and then analysed for GFP signal. At least 10,000 events of BFP^+ cells were recorded for each sample and data were analysed with CytExpert software v2.2 (Beckman Coulter) or with Floreada tool (https://floreada.io).

Real-time microscopy

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

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

Mouse experiments

C3H/HeOuJ with specific-pathogen-free (SPF) status were purchased from Charles River laboratories. Five-to six-week-old female mice were housed in cages containing no more than five animals, maintained under a 12 h light/dark cycle at a temperature of 21 ± 2 °C, and fed with standard diet and water ad libitum. Mice were given drinking water supplemented with streptomycin (5 g/L) throughout the experiment, starting from one day prior inoculation with 10^7 cells of HS (pHL51) or HS *eutR*-T7pol (pHL51). The drinking water of some groups of mice was also supplemented with 30 mM of ethanolamine hydrochloride and 150 nM of vitamin B_{12}. Five days post-inoculation, mice were euthanized in order to collect cecal contents as well 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-µm PET membrane before analysis by flow
190 cytometry.

RESULTS

Rationale design and general description of the reporter system

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

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

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

The reporter plasmid pHL51 was next used to monitor the expression of *eut* genes in the *E. coli* commensal strain HS. To this end, we inserted a promoterless T7pol encoding gene immediately downstream *eutR*, which is the last gene of the *eut* operon and this strain was next transformed with pHL51 (Fig. 3A). The strain was cultured in LB supplemented with various concentrations of EA and the co-factor vitamin B_{12}. As expected, the GFP signal gradually increased with EA concentrations (Fig. 3B). Moreover, absence of vitamin B_{12} impede *eut* expression despite the addition of a high concentration of EA. The different samples were next analysed by flow cytometry to quantify GFP and BFP signals in individual cells (Fig 3C). First, the BFP signal was homogeneous among cells and did not vary between conditions. In contrast, the GFP signal strongly fluctuated in response to EA concentration in the culture medium. Only 1 % of cells were GFP^+ in absence of EA, suggesting that few cells express *eut* genes without supplementation of the inducer. The percentage of GFP^+ cells increased along with the concentration of EA and reached 43% when EA was added at a final concentration of 30 mM (Fig. 3C). Again, no bacterial cells express the *eut* operon in absence of vitamin B_{12}. It is noteworthy that expression of GFP follows a bimodal distribution with the existence of GFP^-

and GFP$^+$ populations. Even at excessive concentrations of EA and/or vitamin B_{12}, bimodal distribution of GFP signal was still observed (Fig. S4), questioning us about a potential artefact associated with the reporting tool pHL51. In particular, the T7 promoter region driving the expression of *gfp* derived initially from the pET-11a vector and thus includes a LacI repressor binding site from the *E. coli lac* operon (Fig. S1). To ensure that potential LacI mediated repression did not interfere with expression and distribution of GFP signal, the LacI operator sequence (as well as a sequence used to add a T7 tag to GFP) was deleted from pHL51 to yield plasmid pHL52 (Fig. S1). The HS *eutR*-T7pol strain carrying either pHL51 or pHL52 were exposed to different concentrations of EA and GFP signals were recorded for comparison. As observed in Fig. S5, GFP patterns obtained from the 2 reporter plasmids were highly similar at a given EA concentration. Moreover, the addition of IPTG, alleviating the repressing activity of LacI, did not alter the GFP expression level in presence of 30 mM EA. These data indicate that the presence of a LacI operator does not influence GFP expression in our experimental conditions and suggest that bimodal expression is a characteristic of the *eut* operon. To go further, we hypothesized that bimodal expression may result from the action of the positive feedback loop generated by the activator EutR since *eutR* is within the *eut* operon. To verify this assumption, we inserted the *eutR* gene under the control of a constitutive promoter (P_{cst}-*eutR*) within the chromosome of the HS *eutR*-T7pol (pHL51) strain, decoupling *eutR* expression from transcription of the *eut* operon. Following culture in presence of 30 mM of EA, more than 96 % of cells were GFP$^+$, revealing a near-perfect unimodal expression pattern (Fig. 4 and Fig. S6). At lower EA concentrations or without EA supplementation, the percentages of GFP$^+$ cells were drastically increased when compared to those recorded with the HS *eutR*-T7pol (pHL51) strain. Altogether, these data strongly suggest that both EutR and EA concentrations in the cell are at play to determine the expression of *eut* genes in individual bacteria, with potential bimodal output at the population level.

We also performed time-lapse microscopy to follow the fluorescence signal kinetic in dividing bacteria. The *eut* reporter strain was grown to mid-log phase and few cells were dropped on an agarose pad supplemented or not with EA + vitamin B_{12}. Fluorescence signals were then recorded for 200 minutes under the microscope (Fig. 5, Fig. S7, S8 and S9). Because no EA was added to the initial culture, almost all cells were BFP^+ and GFP^- at the beginning of the time-lapse procedure. However, few cells became GFP^+ very quickly (~40 min) before shutting down again. Interestingly, a second wave of GFP signal occurred later during the time-lapse (~200 min) only for some cells. As expected, most cells if not all lacked green fluorescence when cells were grown on an agarose pad without EA and vitamin B_{12} (Fig. S8 and S9). These data confirm the heterogeneous expression of *eut* genes in strain HS and also suggests that *eut* expression is potentially fluctuant in individual cells despite the availability of EA in excess.

Monitoring *eut* expression during colonization of the mouse gut

Our reporter system was designed to monitor the expression of a gene of interest in complex environment. Indeed, the constitutive expression of mTagBFP2 should allow the tracking of our strain even in dense ecosystems with high level of diverse microorganisms like within the gastro-intestinal tract. To this end, we orally inoculated streptomycin-treated mice with the strain HS *eutR*-T7pol (pHL51). Streptomycin removes the facultative anaerobes (Maltby et al. 2013) and allows gut colonization by the reporter strain which is resistant to streptomycin thanks to *aadA* gene located on pHL51. EA and vitamin B_{12} were also added to the drinking water of some animals to artificially increase the level of both factors within the gut. Five days post-inoculation, fecal and cecal contents of animals were recovered and analysed by flow cytometry following the gating strategy depicted in figure 6A. A first gate covering events with FSC/SSC parameters usually defined for bacilli was applied to further analyse BFP fluorescence. A small population exhibited a strong BFP signal and this corresponds to our 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 ~ 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 B_{12} 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 B_{12} in feces to express *eut* genes (Fig. 6B and 6C).
310

DISCUSSION

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

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

We demonstrated in this study that *eut* genes are expressed by the commensal *E. coli* HS strain 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 persistance 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

ACKNOWLEDGMENTS

We thank the staff of CLIC (Clermont Confocal Imaging) facility and especially Caroline VACHIAS for the excellent assistance in time-lapse microscopy experiments. This work was supported by fundings from the National Research Institute for Agriculture, Food and Environment (INRAE). Maria Ines Moreira de Gouveia was a PhD Research Fellow funded by the French ministry of Education and Research. Audrey Reuter was a Postdoctoral Research Fellow granted by the "Microbiology and Food Chain" Division of INRAE.

STATEMENTS AND DECLARATIONS

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

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

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

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

FIGURE LEGENDS

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

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

Figure 3: Quantification of *eut* gene expression during *in vitro* growth of *E. coli*. (A) Schematic representation of the EA reporter strain. **(B)** The HS *eutR*-T7pol (pHL51) strain was grown in LB initially supplemented with vitamin B_{12} and indicated concentrations of EA. GFP fluorescence and OD_{600nm} were measured every 20 minutes. GFP signals (fluorescence unit) are represented by thick solid lines and growth curves by thin solid lines. Results are presented as mean and standard deviations from three independent experiments **(C)** Six-hour samples of cultures shown in (B) were analysed by flow cytometry. Data are represented on two-dimensional dot plots with BFP and GFP signals on the y-axis and x-axis, respectively. Gates corresponding to GFP-negative and GFP-positive populations are shown and the percentages of the GFP^+ cells are indicated at the bottom of each image.

Figure 4: Influence of EutR on the bimodal expression of *eut* genes. The HS *eutR*-T7pol (pHL51) and HS *eutR*-T7pol P_{cst}-*eutR* (pHL51) strains were grown for 6 h in LB supplemented with indicated concentrations of EA and vitamin B_{12}. 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 B_{12} 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

REFERENCES

Ackermann M (2015) A functional perspective on phenotypic heterogeneity in microorganisms. Nat. Rev. Microbiol. 13

Afroz T, Biliouris K, Kaznessis Y, Beisel CL (2014) Bacterial sugar utilization gives rise to distinct single-cell behaviours. Mol Microbiol 93. https://doi.org/10.1111/mmi.12695

Andersen JB, Sternberg C, Poulsen LK, Bjørn SP, Givskov M, Molin S (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl Environ Microbiol 64. https://doi.org/10.1128/aem.64.6.2240-2246.1998

Anderson CJ, Clark DE, Adli M, Kendall MM (2015) Ethanolamine signaling promotes *Salmonella* niche recognition and adaptation during infection. PLoS Pathog 11. https://doi.org/10.1371/journal.ppat.1005278

Avery S V. (2006) Microbial cell individuality and the underlying sources of heterogeneity. Nat. Rev. Microbiol. 4

Blander JM (2016) Death in the intestinal epithelium—basic biology and implications for inflammatory bowel disease. FEBS J.

Burton NA, Schürmann N, Casse O, Steeb AK, Claudi B, Zankl J, Schmidt A, Bumann D (2014) Disparate impact of oxidative host defenses determines the fate of *Salmonella* during systemic infection in mice. Cell Host Microbe 15. https://doi.org/10.1016/j.chom.2013.12.006

Bury-Moné S, Sclavi B (2017) Stochasticity of gene expression as a motor of epigenetics in bacteria: from individual to collective behaviors. Res. Microbiol. 168:503–514

Campbell-Valois FX, Sansonetti PJ (2014) Tracking bacterial pathogens with genetically-encoded reporters. FEBS Lett. 588

Cayron J, Lesterlin C (2019) Multi-scale analysis of bacterial growth under stress treatments. J Vis Exp 2019. https://doi.org/10.3791/60576

Conway T, Krogfelt KA, Cohen PS (2004) The life of commensal *Escherichia coli* in the

498 mammalian intestine. EcoSal Plus 1. https://doi.org/10.1128/ecosalplus.8.3.1.2
499 Crepin S, Harel J, Dozois CM (2012) Chromosomal complementation using Tn7 transposon
500 vectors in *enterobacteriaceae*. Appl Environ Microbiol 78.
501 https://doi.org/10.1128/AEM.00986-12
502 Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in E*scherichia*
503 *coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645.
504 https://doi.org/10.1073/pnas.120163297
505 Davis KM, Mohammadi S, Isberg RR (2015) Community behavior and spatial regulation
506 within a bacterial microcolony in deep tissue sites serves to protect against host Attack.
507 Cell Host Microbe 17. https://doi.org/10.1016/j.chom.2014.11.008
508 Gardette M, Daniel J, Loukiadis E, Jubelin G (2020) Role of the nitric oxide reductase NorVW
509 in the survival and virulence of enterohaemorrhagic *Escherichia coli* during infection.
510 Pathogens 9:1–14. https://doi.org/10.3390/pathogens9090683
511 Garsin DA (2010) Ethanolamine utilization in bacterial pathogens: Roles and regulation. Nat.
512 Rev. Microbiol. 8
513 Garsin DA (2012) Ethanolamine: A signal to commence a host-associated lifestyle? MBio 3
514 Hennessy RC, Christiansen L, Olsson S, Stougaard P (2018) A broad-host range dual-
515 fluorescence reporter system for gene expression analysis in Gram-negative bacteria. J
516 Microbiol Methods 144. https://doi.org/10.1016/j.mimet.2017.11.024
517 Kaval KG, Garsin DA (2018) Ethanolamine utilization in bacteria. MBio 9.
518 https://doi.org/10.1128/mBio.00066-18
519 Kendall MM, Gruber CC, Parker CT, Sperandio V (2012) Ethanolamine controls expression of
520 genes encoding components involved in interkingdom signaling and virulence in
521 enterohemorrhagic *Escherichia coli* O157:H7. MBio 3.
522 https://doi.org/10.1128/mBio.00050-12
523 Kim JM, Garcia-Alcala M, Balleza E, Cluzel P (2020) Stochastic transcriptional pulses

orchestrate flagellar biosynthesis in *Escherichia coli*. Sci Adv 6. https://doi.org/10.1126/sciadv.aax0947

Kiviet DJ, Nghe P, Walker N, Boulineau S, Sunderlikova V, Tans SJ (2014) Stochasticity of metabolism and growth at the single-cell level. Nature 514. https://doi.org/10.1038/nature13582

Lasaro M, Liu Z, Bishar R, Kelly K, Chattopadhyay S, Paul S, Sokurenko E, Zhu J, Goulian M (2014) *Escherichia coli* isolate for studying colonization of the mouse intestine and its application to two-component signaling knockouts. J Bacteriol 196. https://doi.org/10.1128/JB.01296-13

Lauren DM, Shailab S, W. RJ, Pola K, Maria K, Carolina AF, Aimee S (2022) Development of a dual-fluorescent-reporter system in *Clostridioides difficile* reveals a division of labor between virulence and transmission Gene Expression. mSphere 7:e00132-22. https://doi.org/10.1128/msphere.00132-22

Li J, Claudi B, Fanous J, Chicherova N, Cianfanelli FR, Campbell RAA, Bumann D (2021) Tissue compartmentalization enables *Salmonella* persistence during chemotherapy. Proc Natl Acad Sci U S A 118. https://doi.org/10.1073/pnas.2113951118

Lim HN, van Oudenaarden A (2007) A multistep epigenetic switch enables the stable inheritance of DNA methylation states. Nat Genet 39:269–275. https://doi.org/10.1038/ng1956

Maltby R, Leatham-Jensen MP, Gibson T, Cohen PS, Conway T (2013) Nutritional basis for colonization resistance by human commensal *Escherichia coli* strains HS and Nissle 1917 against *E. coli* O157:H7 in the mouse intestine. PLoS One 8. https://doi.org/10.1371/journal.pone.0053957

Martin C Saint, Jubelin G, Darsonval M, Leroy S, Leneveu-Jenvrin C, Hmidene G, Omhover L, Stahl V, Guillier L, Briandet R, Desvaux M, Dubois-Brissonnet F (2022) Genetic, physiological, and cellular heterogeneities of bacterial pathogens in food matrices:

Consequences for food safety. Compr Rev food Sci food Saf. https://doi.org/10.1111/1541-4337.13020

Miller WG, Leveau JHJ, Lindow SE (2000) Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol Plant-Microbe Interact 13. https://doi.org/10.1094/MPMI.2000.13.11.1243

Moreira de Gouveia MI, Daniel J, Garrivier A, Bernalier-Donadille A, Jubelin G (2022) Diversity of ethanolamine utilization by human commensal *Escherichia coli*. Res Microbiol 103989. https://doi.org/10.1016/j.resmic.2022.103989

Nielsen AT, Dolganov NA, Rasmussen T, Otto G, Miller MC, Felt SA, Torreilles S, Schoolnik GK (2010) A bistable switch and anatomical site control *Vibrio cholerae* virulence gene expression in the intestine. PLoS Pathog 6. https://doi.org/10.1371/journal.ppat.1001102

Nuss AM, Schuster F, Roselius L, Klein J, Bücker R, Herbst K, Heroven AK, Pisano F, Wittmann C, Münch R, Müller J, Jahn D, Dersch P (2016) A precise temperature-responsive bistable switch controlling *Yersinia* virulence. PLoS Pathog 12. https://doi.org/10.1371/journal.ppat.1006091

Packer D (2021) The history of the antibody as a tool. Acta Histochem. 123

Patange O, Schwall C, Jones M, Villava C, Griffith DA, Phillips A, Locke JCW (2018) *Escherichia coli* can survive stress by noisy growth modulation. Nat Commun 9. https://doi.org/10.1038/s41467-018-07702-z

Payros D, Secher T, Boury M, Brehin C, Ménard S, Salvadorcartier C, Cuevas-Ramos G, Watrin C, Marcq I, Nougayrède JP, Dubois D, Bedu A, Garnier F, Clermont O, Denamur E, Plaisancié P, Theodorou V, Fioramonti J, Olier M, Oswald E (2014) Maternally acquired genotoxic *Escherichia coli* alters offspring's intestinal homeostasis. Gut Microbes 5. https://doi.org/10.4161/gmic.28932

Peñate-Medina O, Tower RJ, Peñate-Medina T, Will O, Saris PEJ, Suojanen J, Sorsa T, Huuskonen L, Hiippala K, Satokari R, Glüer CC, de Vos WM, Reunanen J (2019)

576 Universal membrane-labeling combined with expression of Katushka far-red fluorescent
577 protein enables non-invasive dynamic and longitudinal quantitative 3D dual-color
578 fluorescent imaging of multiple bacterial strains in mouse intestine. BMC Microbiol 19.
579 https://doi.org/10.1186/s12866-019-1538-z
580 Rosochacki SJ, Matejczyk M (2002) Green fluorescent protein as a molecular marker in
581 microbiology. Acta Microbiol. Pol. 51
582 Rowley CA, Sauder AB, Kendall MM (2020) The ethanolamine-sensing transcription factor
583 EutR promotes virulence and transmission during *Citrobacter rodentium* intestinal
584 infection. Infect Immun 88. https://doi.org/10.1128/IAI.00137-20
585 Sampaio NMV, Blassick CM, Andreani V, Lugagne JB, Dunlop MJ (2022) Dynamic gene
586 expression and growth underlie cell-to-cell heterogeneity in *Escherichia coli* stress
587 response. Proc Natl Acad Sci U S A 119. https://doi.org/10.1073/pnas.2115032119
588 Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S,
589 Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K,
590 Tomancak P, Cardona A (2012) Fiji: An open-source platform for biological-image
591 analysis. Nat. Methods 9
592 Schulte M, Olschewski K, Hensel M (2021) Fluorescent protein-based reporters reveal stress
593 response of intracellular *Salmonella enterica* at level of single bacterial cells. Cell
594 Microbiol 23. https://doi.org/10.1111/cmi.13293
595 Sharma V (2018) ImageJ plugin HyperStackReg V5.6
596 Striednig B, Lanner U, Niggli S, Katic A, Vormittag S, Brülisauer S, Hochstrasser R, Kaech A,
597 Welin A, Flieger A, Ziegler U, Schmidt A, Hilbi H, Personnic N (2021) Quorum sensing
598 governs a transmissive *Legionella* subpopulation at the pathogen vacuole periphery .
599 EMBO Rep 22. https://doi.org/10.15252/embr.202152972
600 Takhaveev V, Heinemann M (2018) Metabolic heterogeneity in clonal microbial populations.
601 Curr. Opin. Microbiol. 45

602 Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Balestrino D, Loh E,
603 Gripenland J, Tiensuu T, Vaitkevicius K, Barthelemy M, Vergassola M, Nahori MA,
604 Soubigou G, Régnault B, Coppée JY, Lecuit M, Johansson J, Cossart P (2009) The *Listeria*
605 transcriptional landscape from saprophytism to virulence. Nature 459.
606 https://doi.org/10.1038/nature08080
607 Yang C, Ren C, Zhou J, Liu J, Zhang Y, Huang F, Ding D, Xu B, Liu J (2017) Dual fluorescent-
608 and isotopic-labelled self-assembling vancomycin for *in vivo* imaging of bacterial
609 infections. Angew Chemie - Int Ed 56:2356–2360.
610 https://doi.org/10.1002/anie.201610926
611 Yoon SA, Park SY, Cha Y, Gopala L, Lee MH (2021) Strategies of detecting bacteria using
612 fluorescence-based dyes. Front. Chem. 9
613