Detecting changes in the Caenorhabditis elegans intestinal environment using an engineered bacterial biosensor: Supplementary Information

Jack W. Rutter∗1, Tanel Ozdemir∗1, Evgeny Galimov3, Leonor M. Quintaneiro2, Luca Rosa1, Geraint Thomas1, Filipe Cabreiro2,3, and Chris P Barnes1,4

1Department of Cell and Developmental Biology, University College London, London, UK
2Institute of Structural and Molecular Biology, University College London and Birkbeck College, London, UK
3MRC London Institute of Medical Sciences, London, UK
4Department of Genetics, Evolution and Environment, University College London, London, UK

Materials and Methods

Plasmids and Cloning Methods

Ratiometric sensor assay systems were designed with a constitutive mCherry reporter plasmid and a separate GFP reporter plasmid (plasmid maps can be seen in Figure S1). The GFP reporter plasmids were constructed from the promoterless OG241_GFP plasmid (Figure S1A), containing an upstream multiple cloning site, a dasher GFP reporter gene, a pUC high copy origin-of-replication and a kanamycin resistance cassette (Oxford Genetics, UK). As a positive control, the strong constitutive OXB19 promoter (Oxford Genetics, UK) was placed upstream of GFP to derive OXB19_GFP. In addition to these constructs, an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible GFP sensor (pLac_GFP) was created. Briefly, this was created by PCR cloning the region containing the LacI protein, tetR promoter and trc promoter from pKDL071 [1] and adding SpeI and EcoRI flanks. This region was then cloned into the MCS of OG241_GFP with the appropriate restriction enzymes. The p47_mCherry construct was created from pSEVA471, a 3120bp plasmid based on the pSEVA format [2]. This involved using PacI and SpeI to clone out the constitutively expressed mCherry fragment from pTn7-M-pEM7-mCherry and ligate into the empty multiple cloning site (MCS) of pSEVA471 to derive a low copy SC101 plasmid with streptomycin resistance and strong constitutive mCherry expression under the pEM7 promoter. EcN was then simultaneously transformed with p47_mCherry and the respective GFP plasmid via heat-shock methods to give the strains listed below.

Table S1: Bacterial strains used within this work.

| Strain designation     | Host     | Plasmids                      | Source               |
|------------------------|----------|-------------------------------|----------------------|
| EcN_OG241_mCherry      | E. coli | OG241_GFP, pUC_KanR, p47 M7_mCherry, SC101_StrpR | Oxford Genetics, UK  |
| EcN_OXB19_GFP_mCherry  | E. coli | OXB19_GFP, pUC_KanR, p47 M7_mCherry, SC101_StrpR | This work            |
| EcN_pLac_GFP_mCherry   | E. coli | pLac_GFP, pUC_KanR, p47 M7_mCherry, SC101_StrpR | This work            |
Media and Strains

Lysogeny broth (LB) media and agar were used during propagation and cloning of bacteria. Assays were carried out in LB or M9 minimal media supplemented with 1 mM thiamine hydrochloride, 0.4% glycerol, 0.2% casamino acids, 2 mM MgSO$_4$ and 0.1 mM CaCl$_2$. When antibiotic selection was applied, kanamycin was used at 25 µg/mL and streptomycin was used at 50 µg/mL. All DNA manipulations were performed in *E. coli* DH5α (NEB). All sequences were confirmed via Sanger sequencing (Source Bioscience, UK). All final induction assays were performed in *E. coli* Nissle 1917 (EcN) (Prof. Ian Henderson from the University of Birmingham, UK). Chemically competent EcN was made using standard protocols.

Briefly, this involved diluting an overnight EcN LB culture 1:100 into 50 ml of fresh LB media and grown at 37°C in a shaking incubator to an OD$_{600}$ of 0.25 to 0.3. The culture was then chilled on ice for 15 minutes and then centrifuged for 10 minutes at 5000 g and 4°C. The medium was then discarded and the cell pellet resuspended in 30 ml of cold 0.1 M CaCl$_2$ before being kept on ice for a further 30 minutes. This suspension was then centrifuged for 10 minutes at 5000 g and 4°C. The supernatant was once again removed and the cell pellet was resuspended in 3 ml of cold 0.1 M CaCl$_2$ solution with 15% glycerol. The final cell suspension was then aliquoted and flash frozen before being stored at -80°C.

Induction Assays in Liquid Culture

All induction assay cultures were grown for 3 hours at 37°C (350 rpm shaking), before being induced and then transferred to 20°C (no shaking) or kept in the same conditions (37°C, 350 rpm shaking), at a volume of 500 µL in a sterile autoclaved 96-well deep square well (2.2 mL) polypropylene plate (BRAND®, Sigma-Aldrich, UK) sealed with Breathe-Easy sealing membranes (Sigma-Aldrich, UK). Induction was calculated through flow cytometry or plate-reader analysis. IPTG was used to induce the pLac_GFP plasmid. Concentration range induction assays were performed by diluting triplicate overnight cultures 1:500 into LB or supplemented M9 media, incubating for 3 hours (37°C) unless stated otherwise and inducing with the appropriate inducer concentration. The cultures were then allowed to incubate for 18 hours (at desired shaker speed and temperature) before flow cytometry analysis, as described in the main manuscript methods section.

Plate Reader Assays

Plate reader assays were performed in a Tecan Spark® plate reader (Tecan, Switzerland). Overnight cultures of uninduced bacteria were diluted 1:1000 into sterile LB media and incubated for 3 hours at 37°C. The cultures were then induced with the desired IPTG concentration and 200 µL of each culture transferred to a clear bottom 96-well plate (Greiner, Germany). Plates were then incubated at the desired shaking speed and temperature. Every effort was made to achieve a consistent 20°C temperature for the plate reader timecourse (Figure 1D); however, due to technical limitations of the plate reader a consistent incubation temperature could not be achieved. For the ‘cold’ timecourse a final temperature of 22.48±0.126°C (mean ± standard error) was achieved. Values for GFP (excitation: 488/20 nm, emission: 530/20 nm, gain: 70) and mCherry (excitation: 531/20 nm, emission: 620/20 nm, gain: 90) were recorded every 20 minutes over 20 hours.

At present, the calibration and MEF conversion applied to flow cytometry data is not applicable to plate reader data. Therefore, the data from plate reader experiments is presented as arbitrary units (Figure 1D).

Confocal Microscopy

For confocal microscopy a nematode strain, GK70 (unc-119 (ed3) III; dklIs37[Pact-5-GFP-pgp-1;unc-119(+)]) [3], in which the intestinal apical membrane is labelled with GFP was used. L1 nematodes
were transferred to EcN-NGM plates and grown until the L4 stage. The L4 nematodes were then
transferred to EcN-NGM plates supplemented with 20 µM FuDR and aged to day 5.

Day 5 old worms were paralyzed with levamisole, then washed from plates using M9 worm buffer
plus 0.1% Triton X-100, then rinsed with M9 worm buffer and surface bleached for 10 min at 4°C in a
1:1,000 bleach solution in M9 to remove any live bacteria from their surface. Worms were then imaged
using a Leica TCS SP5 microscope. Auto-fluorescent gut granules were excited with a 405 nm laser
(emitted light in the range 417-472 nm). GFP was excited with a 488 nm laser (emission between
498-508 nm), mCherry was excited with a 561 nm laser (emission between 569-661 nm).

Automated Biosensor Induction Quantification

The image analysis pipeline was developed using Matlab version R2016b and implemented on a 2015
MacBook Pro (8GB RAM). In brief, brightfield images were converted to black and white and seg-
mented to extract the nematode body from the surrounding agar (removing any variation introduced
by the agar plate background). The extracted nematode body was then applied as a mask to the GFP
and mCherry images. For images which contained segments of a worm already fully present within
another image, this area was manually excluded (prior to placing within the automated pipeline) to
ensure that every worm was only considered within a single image and to prevent oversampling. A
threshold was also placed on object size (when extracting the nematode body masks), to ensure that
small fragments of agar or eggs were not included in the worm mask (thereby removing any potential
for variation caused by objects outside of the nematode bodies). The colonised area of the mCherry
intestine was then extracted (through threshold based on mCherry brightness) and the colonised area
applied as a mask to the GFP image. GFP autofluorescence was accounted for by subtracting the
background GFP signal of the nematode body (from the colonised area). This adjustment was added
to account for some of the autofluorescence of the nematode illustrated by the blue areas within Figure
S4. Uncolonised images were discarded using an arbitrary mCherry threshold. Finally, the average
pixel intensity of the GFP (green channel) and mCherry (red channel) for each image were collected
in order to calculate the biosensor ratio. Where appropriate, the Mann-Whitney-Wilcoxon test was
used to demonstrate statistical significance. All plots were prepared using custom scripts based on
the ‘ggplot2’ package in R.

Additional in vitro characterisation of EcN strains.

Figure S2 shows in vitro characterisation of the two control strains. From this data we can see
that the ratios achieved by EcN_OXB19_GFP_mCherry (positive control) were higher than those
from EcN_OG241_mCherry (negative control) and that both remained largely unaffected by IPTG
concentration.

Figure S3 shows the parameters fitted during Hill Function modelling on a typical biosensor in-
duction curve.

Additional in vivo characterisation of pLac biosensor.

Figure S4 contains confocal microscopy images of a nematode colonised with the EcN_pLac_GFP_mCherry
biosensor strain (an accompanying z-stack of the colonised nematode can be seen within the supple-
mentary materials). From these images it can be clearly seen that the mCherry expression biosensor
(red) is clearly contained within the fluorescently labeled intestinal membrane (green). These images
provide strong evidence that the bacterial strains are contained solely within the C. elegans digestive
tract.

Figure S5 shows the number of images which were discarded (deemed ‘uncolonised’ as below
mCherry threshold) during image analysis, for each of the bacterial strains. It can be seen that only
17.23% of the total images were discarded. From this data no clear trends could be seen between percentage of ‘colonised’ images and either the strain, imaging timepoint or inducer concentration. This supports a hypothesis that the colonisation period may have a greater effect on the percentage of ‘colonised’ images.

Figure S6 shows characterisation of both the EcN_OG241_mCherry and EcN_OXB19_GFP_mCherry control strains, in vivo within the C. elegans digestive tract. From this characterisation it can be seen that the GFP:mCherry ratios for both control strains remain relatively constant, both in time and +/- the IPTG inducer.

Figure S7 contains the results of the original attempt to induce the pLac strain in vivo, after transferring to unseeded inducer plates. From the results it can be seen that there was no significant change in the GFP:mCherry ratio on transferring worms from seeded to unseeded plates, for a period of 8 hours without any inducer present. However, the addition of IPTG produced a significant increase in the GFP:mCherry ratio.

Figure S8 contains additional, higher magnification, images of a nematode colonised with the OXB19 control strain.

References

[1] Kevin D. Litcofsky, Raffi B. Afeyan, Russell J. Krom, Ahmad S. Khalil, and James J. Collins. Iterative plug-and-play methodology for constructing and modifying synthetic gene networks. Nat. Methods, 9(11):1077–1080, November 2012.

[2] Esteban Martinez-Garcia, Tomas Aparicio, Angel Goni-Moreno, Sofia Fraile, and Victor de-Lorenzo. SEVA 2.0: an update of the Standard European Vector Architecture for de-/re-construction of bacterial functionalities. Nucleic Acids Res., 43(Database issue):D1183–D1189, January 2015.

[3] Keiko Saegusa, Miyuki Sato, Katsuya Sato, Junko Nakajima-Shimada, Akihiro Harada, and Ken Sato. Caenorhabditis elegans chaperonin cct/tric is required for actin and tubulin biogenesis and microvillus formation in intestinal epithelial cells. Molecular biology of the cell, 25(20):3095–3104, 2014.
Figure S1: Maps of plasmids used within this work. (A) OG241, promoterless GFP. (B) OXB19, constitutive GFP. (C) pLac, IPTG inducible GFP. (D) p47_mCherry, constitutive mCherry.
Figure S2: *In vitro* characterisation of the EcN control strains (EcN_OG241_mCherry and EcN_OXB19_GFP_mCherry). (A) 20°C, (B) 37°C, overnight induction assays. From left to right: density plot of GFP induction, median GFP fluorescence, density plot of mCherry fluorescence, median mCherry fluorescence and GFP:mCherry ratios over all IPTG inducer concentrations. The characterisation presented here was completed in LB media. Flow cytometry data with 10,000 events (n=3).
Figure S3: A typical biosensor induction response curve, highlighting the parameters estimated during Hill function fitting.

Figure S4: Confocal microscopy of a *C. elegans* nematode, colonised with the EcN_pLac_GFP_mCherry biosensor. Pseudocolours: green= GFP-PGP-1 labelled *C. elegans* intestinal apical membrane, red= EcN_pLac_GFP_mCherry within the intestinal lumen constitutively expression mCherry, blue= autofluorescent granules within the *C. elegans* gut. (A) without DIC and (B) with DIC. (scale bar = 20µm).
Figure S5: (A) Example image of a nematode which did not show mCherry fluorescence above the mCherry threshold and was therefore excluded during automated image analysis. Percentage of images which were kept (‘colonised’) or excluded (‘uncolonised’) during automated image analysis for each strain; for (B) all images, (C) images split by strain, (D) images split by timepoint, (E) images split by inducer concentration.
Figure S6: Characterisation of the EcN_OG241_mCherry (top) and EcN_OXB19_GFP_mCherry (bottom) control strains within the *C. elegans* digestive extract. Both with and without exposure to IPTG. *(n ≥ 4 images).*
Figure S7: Preliminary characterisation of the pLac sensor within *C. elegans*. Images were collected at 0 hours and then 8 hours after transferring to inducer plates, supplemented with 0 or 1mM IPTG. The ratios of colonised nematodes are presented. (n ≥ 15 images, p-values: n.s.>0.05, ***<0.001).

Figure S8: Images of colonised *C. elegans* nematode. Representative 7 day old *C. elegans* worm grown on EcN_OXB19_GFP, constitutively expressing GFP. GFP shows localisation of the strain from the pharynx onwards and throughout the intestines. (A) x120 magnification, (B) x200 magnification.