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Engineered bacteria function in the mammalian gut as long term live diagnostics of inflammation

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Abstract

Bacteria can be engineered to function as diagnostics or therapeutics in the mammalian gut but commercial translation of these technologies has been hindered by the susceptibility of synthetic genetic circuits to mutation and unpredictable function during extended gut colonization. Here we report stable, engineered bacterial strains that maintain their function for 6 months in the mouse gut. We engineered a commensal murine *Escherichia coli* strain to detect tetrathionate, which is produced during inflammation. Using our engineered diagnostic strain, which retains memory of exposure in the gut for analysis by fecal testing, we detected tetrathionate in both infection-induced and genetic mouse models of inflammation over 6 months. The synthetic genetic circuits in the engineered strain were genetically stable and functioned as intended over time. The robust,

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Author Contributions

D.T.R., T.W.G., M.B., S.J.K., G.K.G., J.C.W. and P.A.S. designed experiments. D.T.R. and M.J.N. performed and analyzed *in vitro* characterization. D.T.R. performed and analyzed all mouse experiments. T.W.G. performed and analyzed tetrathionate mass spectrometry. M.B. performed and analyzed whole genome sequencing. S.J.K. and J.W.K. generated strains and collected preliminary data for the study. R.T.B. performed all histology scoring. D.T.R. and P.A.S. wrote the manuscript.

Competing Financial Interests Statement

The authors declare no competing financial interests.

Accession Codes

PAS638 sequencing reads are available at NCBI SRA (BioSample accession numbers SAMN06671873 - SAMN06671878) linking to BioProject PRJNA380756.

Data Availability

Sequence reads are available at NCBI SRA (BioSample accession numbers SAMN06671873 - SAMN06671878) linking to BioProject PRJNA380756. *E. coli* NGF-1 assemblies for chromosome and plasmids are available with NCBI references NZ_CP016007.01, NZ_CP016008.01 and NZ_CP016009.01.

The conversion of thiosulfate, which is produced from hydrogen sulfide, into tetrathionate in the mouse intestine has been reported during *S. typhimurium*¹⁴ and *Yersinia enterocolitica*¹⁵ induced inflammation (Supplementary Fig. 1). Tetrathionate is detected in these bacteria by the TtrR/TtrS two-component system¹⁶ and can be used as a terminal electron acceptor for anaerobic respiration providing a growth advantage to these pathogens during inflammation^{14, 15, 17, 18}. A range of other bacteria, including pathogens, may also be able to grow preferentially using tetrathionate¹⁹. Increased production of tetrathionate in toll-like receptor-1 deficient mice¹⁵ and an enrichment of tetrathionate utilization genes in the

microbiota of a $Tbet^{-/-}$ $Rag^{-/-}$ mouse ulcerative colitis (TRUC) model²⁰ point to the potential of tetrathionate as a general marker of inflammation. However, non-invasive detection of tetrathionate is not possible, which has limited further investigation.

We engineered a commensal mouse *E. coli* strain, NGF-1 to detect tetrathionate (Fig. 1a.). Briefly we constructed a trigger element using *ttrR/S* genes and the P_{ttrBCA} promoter from *S. typhimurium* to drive Cro expression and inserted it into the genome of strain PAS637, already containing our previously published memory circuit (see Online Methods)¹², thereby creating strain PAS638 (Fig. 1a; Supplementary Table 1). All synthetic constructs were integrated into the chromosome of *E. coli* strain, NGF-1 to reduce burden and allow long-term circuit retention without selection. Engineered strain PAS638 exhibits memory upon tetrathionate exposure, driving Cro and β -galactosidase (β -gal) expression from the synthetic memory element and maintaining their expression in the absence of tetrathionate (Fig. 1c). We induced memory during anaerobic growth in liquid culture by adding tetrathionate, which should activate expression through P_{ttrBCA} in the trigger element and switch the memory element from the off (CI^+/Cro and β -gal⁻) to on (Cro and β -gal⁺/ CI^-) state. We quantified the response of the circuit by plating tetrathionate activated cultures onto solid media containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (x-gal), which turns blue in the presence of β -gal. Plating in the absence of tetrathionate and under aerobic conditions allowed optimal maturation of the x-gal indicator plates along with ensuring only colonies retaining a record of tetrathionate exposure were counted. (Fig. 1b). PAS638 bacteria were effectively induced by tetrathionate (EC50:0.38–0.85 μ M 95% CI) whereas the control strain, PAS637 (Supplementary Table 1), which does not contain the P_{ttrBCA} -cro trigger, showed no switching even at saturating concentrations of 2mM tetrathionate (Fig. 1c).

Having validated that PAS638 could record tetrathionate exposure *in vitro*, we next tested the specificity of PAS638 for tetrathionate *in vivo* using a murine *S. typhimurium*-induced colitis model²¹ (Fig. 1d). The presence of inflammation, specifically ROS, causes elevated levels of tetrathionate, which are rapidly reduced to thiosulfate by wild-type *S. typhimurium* strains but not by strains deficient in their tetrathionate reduction-capacity¹⁴. We pre-treated C57Bl/6 mice with streptomycin to reduce colonization resistance for *S. typhimurium* in the colon, and introduced PAS638, PAS638 with *S. typhimurium*, or PAS638 with a *S. typhimurium ttrR* variant which is unable to express tetrathionate reductase by oral gavage (Fig. 1d; Supplementary Table 1)¹⁸. Fecal samples were collected at days 2–5 post-administration. PAS638 did not switch on β -gal expression in control and *S. typhimurium* infected mice (Fig. 1e). However, when PAS638 was co-administered with *S. typhimurium ttrR* switching was increased, with 4/7 mice showing a higher proportion of memory-on colonies than any samples from control mice on days 4–5 post-infection (Fig. 1e, Supplementary Fig. 2a). Colony forming unit (CFU) counts for PAS638 *E. coli* (Supplementary Fig. 2b) and *S. typhimurium* variants (Supplementary Fig. 2c) were generally consistent between groups and showed no correlation with switching. We hypothesized that the mice in which PAS638 was switched on should also show signs of more acute inflammation.

We independently validated the presence of inflammation in the colitis model using quantification of the lipocalin-2 (LCN-2) biomarker (Supplementary Fig. 2d)²² and blinded histopathology scoring of cecum and colon sections (Supplementary Fig. 2e–f). Taken together these techniques identified inflammation in all *S. typhimurium* and *S. typhimurium ttrR* infected mice¹⁴. Of note, PAS638 + *S. typhimurium ttrR* mice in which memory-on state colonies were detected had significantly higher cumulative LCN-2 values at day 4 post-infection than those in which PAS638 remained off (Fig. 1f). These results show that our engineered memory strain specifically senses tetrathionate and that tetrathionate sensing corresponds to a more acute inflammatory response *in vivo*.

Next we set out to use PAS638 to further probe the pathways required for tetrathionate production. The Cytochrome B-245 Beta Chain gene (*cybb*; also known as *gp91phox* and *Nox2*), which encodes a subunit of the phagocyte NADPH-oxidase complex, has previously been implicated in the generation of tetrathionate¹⁴. We tested the response of PAS638 to *S. typhimurium ttrR* infection of *cybb*-deficient (*cybb*^{-/-}) mice (Fig. 2a). Unexpectedly, PAS638 showed increased switching specifically in *S. typhimurium ttrR* infected mice (Fig. 2b). *S. typhimurium ttrR*-infected *cybb*^{-/-} mice also had more acute weight loss and increased fecal LCN-2 levels compared with *S. typhimurium ttrR*-infected C57Bl/6 controls (Supplementary Fig. 3a–b). Uninfected *cybb*^{-/-} mice also produced increased LCN-2 compared with uninfected C57Bl/6 suggesting a propensity to inflammation in this mouse strain (Supplementary Fig 3b). These results suggest that tetrathionate is produced during *S. typhimurium* infection, even in the absence of *Cybb*.

Mass spectrometry confirmed these results, directly detecting tetrathionate in cecum extracts from *S. typhimurium ttrR*-infected C57Bl/6 and all *cybb*^{-/-} mice, with infected mice showing significantly higher tetrathionate levels than uninfected ones (Fig. 2c). We detect ~1 nanomole of tetrathionate from each inflamed mouse (Fig. 2c), translating to low micromolar concentrations if diluted across the approximate volume of the cecum (assuming 0.1–0.5cm³ cecum volume); this concentration is in the order of the *in vitro* EC50 of our sensor (Fig. 1c). Together these data show that the threshold of PAS638 sensing *in vivo* is in the order of 1 micromolar, and show that phagocyte NADPH-oxidase- independent tetrathionate production can be detected in the gut by our engineered *E. coli* strain. In contrast to previous reports we identified tetrathionate in *cybb*^{-/-} mice using the *S. typhimurium ttrR* colitis model, both non-invasively by PAS638 response and by mass spectrometry detection¹⁴. These findings could foreseeably derive from sample measurement or microbiota differences between the studies.

Increased ROS levels are also associated with IBD in humans²³ and may be present in patients even during subclinical inflammation. Interleukin-10 deficient mice (IL10^{-/-}) have features of human IBD, including the effect of microbiota composition on disease progression²⁴. To test whether PAS638 could detect subclinical inflammation in this model we colonized ~6 month-old, retired-breeder IL10^{-/-} mice with PAS638 *E. coli* (Fig. 2d). These mice do not have acute colitis because they have been raised in gnotobiotic and barrier SPF conditions. The engineered bacteria (3 measurements over 2-weeks) detected tetrathionate in 8/9 IL10^{-/-} mice, but not in separately-reared retired C57Bl/6 breeder controls (Fig. 2e). Measurement of fecal LCN-2 showed that this marker of inflammation

was temporarily increased in IL10^{-/-} mice immediately following administration but reduced after a week (Supplementary Fig. 3c). Switching levels in IL10^{-/-} mice were therefore measured >1-week after bacterial administration, which means that the inflammation sensed was not caused by the process of administration. To confirm that PAS638 did not cause inflammation, we administered PAS638 by oral gavage without antibiotic pre-treatment into IL10^{-/-} mice. PAS638 successfully colonized (Fig. 2f) and detected tetrathionate in 2/10 mice (2 measurements over 9 days) (Supplementary Fig. 3d). LCN-2 levels were not raised in contrast to streptomycin treated IL10^{-/-} mice (Supplementary Fig. 3e), suggesting that streptomycin rather than PAS638 caused the previously observed post-administration LCN-2 increase. This means that PAS638 can detect elevated tetrathionate in a physiologically relevant subclinical inflammatory environment.

To evaluate PAS638 in different mouse backgrounds we administered the bacteria by oral gavage to Balb/c, C57Bl/6 or 129X1/SvJ mice, after overnight provision of streptomycin in their drinking water to assist in colonization, and measured the response over 5–8 days (3 measurements) (Fig. 2g). PAS638 did not respond in Balb/c or C57Bl/6 mice; surprisingly, however, bacteria from a subset (~20–40%) of 129X1/SvJ mice showed elevated switching (Fig. 2h). PAS638 gavage in the absence of streptomycin pre-treatment, while producing successful colonization (Supplementary Fig. 3f), also showed similar switching results (Supplementary Fig. 3g). Interestingly, histology did not reveal consistent signs of inflammation (Supplementary Fig. 3h). However, the presence of memory-on state PAS638 bacteria correlated with the onset of elevated LCN-2 levels in 5 mice that were tested daily for 7 days (Fig. 2i), despite absolute LCN-2 levels being orders of magnitude lower than those measured during *Salmonella* infection (Supplementary Fig. 2d). While a definitive cause for the inflammation remained unclear, the 129X1/SvJ strain has documented defects in macrophage recruitment to sites of inflammation^{25, 26}. Detection of tetrathionate in the presence of fluctuating and low-level inflammation that remained below histological detection further demonstrates the ability of PAS638 to detect a subclinical inflammatory environment.

Finally, we tested whether PAS638 could be used for long-term monitoring of tetrathionate. We used the PAS638-inducing environment of the 129X1/SvJ mouse to continually induce memory, thus allowing the fitness costs associated with both on- and off- states to be assessed. Five mice were colonized with PAS638 by oral gavage, after overnight provision of streptomycin in their drinking water, and maintained for 200 days, which corresponds to >1600 bacterial generations.²⁷ We used intermittent fecal testing to analyze for the presence and function of PAS638 (Fig. 3a). Throughout this experiment PAS638 remained colonized at detectable levels without continued antibiotic selection (Fig. 3b) and colonies in the memory-on state were detected at each timepoint (Fig. 3c). Remarkably, colonies that were in the memory-off state in fecal samples at day 159 and day 200 retained the ability to respond to tetrathionate *ex vivo* (Fig. 3d). Similarly, colonies that were in the memory-on state in fecal samples retained an ability to turn off memory under repeat streaking on agar plates (Fig. 3d). Together these results indicate that PAS638 had not acquired mutations to prevent its correct function.

PAS638 can colonize the mouse gut and retain the ability to respond to inflammation *in vivo* for at least 6-months. Colonies that retained *ex vivo* function were pooled from either one or three mice in a repeat long-term experiment, after 200 days colonization (Supplementary Fig. 4a). Bacteria from these colonies were administered to C57Bl/6 mice in the presence or absence of *S. typhimurium ttrR* (Fig. 3e). Blinded scoring of histopathology, weight loss, and LCN-2 concentrations all indicated successful infection (Supplementary Fig. 4b–d). The post-colonization PAS638 showed elevated switching specifically in *S. typhimurium ttrR* co-infected mice (Fig. 3f), confirming the ability of the engineered bacteria to function *in vivo* after extended growth in the gut.

Whole genome sequencing further demonstrates circuit stability. To analyze whether mutations had accrued in PAS638 during colonization, at day 159 PAS638 colonies from five mice (Fig. 3a) were pooled at the individual mouse level and these were compared to the ancestral PAS638 by whole genome sequencing. The sequencing depth that was achieved (average chromosomal fold-coverage of 80–152) can identify mutations that occur in more than ~10% of the population (see Online Methods). The only unique mutation identified from the 5 mouse samples was a repeat expansion in an intergenic region of the *E. coli* NGF-1 colicin-like plasmid, which was detected in 89% and 92% of reads from two mice (Fig. 3d). No mutations or rearrangements were detected on the chromosome, including the synthetic gene elements, or the NGF-1 *Citrobacter rodentium*-like plasmid (see Online Methods). Further, junction prediction indicated that the synthetic element remained in the same genomic location in all samples. The detected mutation, (A)₉→₁₀ in an intergenic region, is not expected to affect the function of the bacteria in any way. Detection of a single mutant is consistent with the number expected from wild-type *E. coli* over the approximate 1200 generations in the gut^{28, 29}. The two mice carrying bacteria with the detected mutations were housed together, while other mice were separated at day 105 (Fig. 3a). Given this mutation spread and fixed amongst the cohoused mice, we are confident that other beneficial mutations would have been detected at the sequencing depth achieved. Together these findings indicate that these synthetic circuits place sufficiently low burden on the bacteria carrying them to allow robust function over >6-months in the complex gut environment.

These long-term experiments were facilitated by the use of *E. coli* NGF-1, which has high colonization capacity (Fig. 3b)¹². Further, colonization by this strain does not require pre-administration of antibiotics which means that the models are more physiologically relevant. Our results confirm the association of increased tetrathionate concentrations with inflammation both in the presence and absence of pathogen infection. We detected low-level inflammation in uninfected IL10^{-/-} and 129X1/SvJ mice. Further, in at least one case the PAS638 response provided more consistent results over a multiple day period than LCN-2 measurement (Fig. 2i) due to the memory capacity of the engineered bacterial strain. In summary, these results show that synthetic bacterial devices can colonize the complex host mammalian gut and be used to monitor and understand disease.

Online Methods

Strain construction

Details of strains constructed for this study are provided (Supplementary Table 1). All synthetic constructs were integrated chromosomally in the mouse commensal *Escherichia coli* NGF-1 strain¹² or *Salmonella typhimurium* 14028s.

The lambda derived CI/Cro memory element was originally inserted between the *mhpR* and *lacZ* loci of *E. coli* TB10³⁰ and transferred to *E. coli* NGF-1 by P1 *vir* transduction³¹ as previously described¹². The tetrathionate responsive trigger circuit, consisting of *ttrR*, *ttrS* genes and the P_{ttrBCA} promoter was amplified directly from *S. typhimurium* LT2. Overlap PCR was used to append the *cro* gene downstream of P_{ttrBCA} and add ~36bp flanks for insertion into the *araB-araC* locus of *E. coli* TB10. The primer sets used were as follows: set 1: FW: ttatggataaaaatgctatggcatagcaaagtgtga, REV:

accagaatacgaacaacgtatgagccatgatgtgacggaagatcacttcgcagaataaat; set 2: FW

atttattctgcgaagtatcttccgtcacatcatggctcatagctgttctgtattctggt, REV:

ataatcttccagggtatgcgtgttccatattgactcccgccacattgccacaataga; and set 3: FW

tcattgttggaatgtggacgggagtcgaatggaacaacgcataaccctgaagattat, REV:

acggcagaaaagtccacattgattattgcacggcggtatgctgtgtttttgttactcgggaaggg. The locus was ultimately transferred to *E. coli* NGF-1 by P1 *vir* transduction.

S. typhimurium strains were derived from *S. typhimurium* 14028s. To confer tetracycline resistance to all *S. typhimurium* strains a zhj-1401::Tn10 construct from *S. typhimurium* LT2 SA2700 (Salmonella Genetic Stock Center) transferred to both *S. typhimurium* strains used in this study by P22 transduction³². To prevent tetrathionate reduction capacity, a *ttrR* knock-out TT22470¹⁸ construct was transferred to *S. typhimurium* by P22 transduction.

In vitro induction with tetrathionate

Tetrathionate-based memory was induced under *in vitro* conditions in either liquid culture or during growth on plates. For liquid culture induction, strains were backdiluted 1:1000 from overnight culture in SOC broth into pre-reduced anaerobic SOC broth and grown at 37°C in an anaerobic chamber (Coy Lab Products) using 7% H₂/ 20% CO₂/63% N₂ culture gas. After 1h, potassium tetrathionate (Sigma) dissolved in pre-reduced SOC media was added to cultures and induction was undertaken at 37°C in the anaerobic chamber for 4h. Memory was assayed by plating and growth in aerobic conditions of serial dilutions of the bacteria on luria-broth (LB) + 300µg/mL streptomycin sulfate (Sigma) + 60µg/mL 5-Bromo-4-chloro-3-indolyl b-D-galactopyranoside (x-gal) (Santa Cruz Biotechnology) agar plates. Switching levels were estimated through counting greater than 250 blue and white colonies unless otherwise noted. *In vitro* testing on solid plates involved plating on LB + 300µg/mL streptomycin sulfate (Sigma) + 60µg/mL x-gal (Santa Cruz Biosciences) + 10 mM sodium tetrathionate (Sigma) agar. Growth was maintained in anaerobic conditions for approximately 8–12 hours using the Gaspak system (BD Biosciences), followed by further growth in aerobic conditions to allow development of memory and the x-gal reporter.

***In vivo* testing of tetrathionate memory**

The Harvard Medical School Animal Care and Use Committee approved all animal study protocols.

General analysis—For most experiments, female, Balb/c (Charles River), C57Bl/6 (Charles River/ Jackson laboratories), 129X1/SvJ (Jackson laboratories), B6.129P2-*Il10^{tm1Cgn}/J* (Jackson laboratories) or B6.129S-*Cybb^{tm1Din}/J* (Jackson laboratories) mice of 8–12 weeks, including ~2 weeks acclimatization to our mouse facility, were administered PAS638 *E. coli* NGF-1 bacteria ($1 \times 10^{7-8}$ CFU/ mouse) by oral gavage. Except where specifically stated this occurred following pre-treatment with USP-grade streptomycin sulfate (Gold Biotechnology; 0.5g/L in drinking water or 20mg per mouse by oral gavage). Bacteria were pelleted and resuspended at a dilution of ~1/10 from overnight culture in sterile PBS prior to gavage of 100uL/ mouse.

Fecal samples were collected from mice under study and homogenized at 50 or 100 mg/mL in sterile PBS by vortexing in 1.5mL eppendorf tubes for ~5 minutes. Large debris was pelleted from homogenized feces by centrifugation at 200rpm (4g) for 20min in a benchtop centrifuge. Bacteria were cultured on agar plates following serial dilution of the resulting supernatant. Enumeration by colony counting and analysis of switching by comparison of blue (*lac*⁺, ON) and white (*lac*⁻, OFF) colony counts was achieved by plating on LB + 60µg/mL x-gal (Santa Cruz Biosciences) agar with 300µg/mL streptomycin sulfate (Sigma), 34 µg/mL chloramphenicol (Sigma) or a combination of both drugs. All mice were pre-screened for the presence of resistant colonies in feces pre bacterial administration. At least 250 colonies were counted per sample and where this was not possible results were excluded from switching quantification.

LCN-2 quantification was undertaken using the Mouse lipocalin-2/NGAL DuoSet ELISA kit (R&D Systems), using the manufacturer recommended protocol. Samples for ELISA were prepared as previously described²², briefly, involving homogenization of 100mg/mL fecal pellets in PBS + 0.1% Tween20 by vortexing for 20 minutes at 4°C, followed by spinning at 12,000g for 10 minutes at 4°C. Clear supernatant was diluted at least 10 fold for quantification by ELISA. ELISA results were obtained on a Victor³V plate reader (Perkin Elmer) with 450/8nm and 540/8nm absorbance filters. For analysis, absorbance corrected values were interpolated from a sigmoidal four parameter logistic standard curve using Prism 6 for Mac OS X software (GraphPad).

At the conclusion of the experiments, where required, mice were sacrificed and dissected, their bowel removed and fixed whole in Bouin's fixative (Sigma). Fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Scoring of severity of inflammation was undertaken on single longitudinal sections of whole gut in a blinded fashion by a trained rodent histologist (RTB).

At least 4 mice were used per experimental group, to which mice were assigned randomly where relevant. This sample size was estimated as a minimum based on its power to detect whether PAS638 had an overall detection capacity (a factor of both bacterial sensitivity and variability of mouse models) of ~47% with 95% confidence. These calculations treated the

occurrence of above-threshold tetrathionate as a dichotomous variable given PAS638's low false positive rate *in vitro* and in pilot *in vivo* experiments. For inflammation models determined to have higher variability or lower penetrance, larger sample sizes were used accordingly.

Streptomycin treated Salmonella colitis model—The streptomycin-treated Salmonella colitis model was undertaken in C57Bl/6 (Charles River or Jackson laboratories) and B6.129S-*Cybb^{tm1Din}* (Jackson laboratories) mice as previously described²¹. Briefly, mice were administered with 20mg USP grade streptomycin sulfate (Gold Biotechnology) by oral gavage following 4h nil per os (NPO). 24 hours later, again following 4h NPO, mice were administered bacterial strains, resuspended from overnight culture in phosphate buffered saline (Gibco), by oral gavage. Bacteria were administered at $\sim 1 \times 10^7$ *E. coli* and $\sim 1 \times 10^8$ *S. typhimurium* per mouse as previously described²¹.

Selective plating for enumeration of *S. typhimurium* strains was achieved on M9 + 0.4% glucose + 30µg/mL tetracycline (Sigma) agar. Enumeration and analysis of switching was achieved using plating on LB + 300µg/mL streptomycin sulfate (Sigma) + 60µg/mL x-gal (Santa Cruz Biosciences) agar.

IL10 knockout model—For initial experiments, male retired breeder mice (~6 months age) from an IL10^{-/-} background³³ were transferred to barrier SPF conditions, following growth under gnotobiotic conditions. The mice were colonized with a complex set of human commensal microbes, Gnotocomplex 2.0 (Supplementary Table 2), which is an expanded version of the Gnotocomplex designed to capture additional functionality and phylogenetic diversity³⁴. Control mice were male retired breeder mice (typically 6–8 months age) from a C57Bl/6 background raised in SPF conditions (Charles river). Mice were administered PAS638 bacteria ($\sim 3 \times 10^7$ per mouse) by oral gavage following USP streptomycin sulfate treatment (Gold Biotechnology; 0.5g/L in drinking water). In mice where colonization was lower than 2.5×10^4 CFU/g following gavage, streptomycin was re-administered within the first week following bacterial administration for up to 48h to assist robust colonization.

Analysis of fecal samples was undertaken as described above. To avoid adverse influence of streptomycin and bacterial administration in IL10^{-/-} mice, which are sensitive to aberrant inflammatory responses (as evident from LCN-2 quantification in week 1 post administration (Fig. 4b)), blue-white colony screening was not undertaken in the week following administration. Results were generated from 3-4 fecal samples taken over the subsequent 2 weeks for control C57Bl/6 mice and ~1 month for IL10^{-/-} mice.

IL10^{-/-} studies were also undertaken in B6.129P2-*Il10^{tm1Cgn}/J* with administration of PAS638 occurring without streptomycin pre treatment.

Tetrathionate quantification by mass spectrometry

Tetrathionate was quantified from extracts prepared from whole mouse cecum. Following dissection, the cecum was cut open and cecal contents were carefully removed from the underlying tissue and mucous layer. The mucosa was scraped with a blunt spatula and this was added to 500µL of water, which was additionally used to wash the cecal tissue by

pipetting vigorously over the surface approx. 10 times. The remaining cecal tissue was then added to the same tube, and tissue and debris was removed by centrifugation at 10,000 g for 10 minutes. Supernatants were then filter sterilized using 13mm 0.20 μ m, Hydrophilic, PTFE syringe filters (Millex-LG) and frozen at -80°C prior to quantification.

HPLC-MS/MS analysis of standards and extracts was carried out using an Agilent 1260 Infinity HPLC system equipped with an Agilent Eclipse Plus C18 column (100×4.6 mm, particle size 3.5 mm, flow rate: 0.5 mL/min, solvent A: 10 mM ammonium acetate in dd.H₂O, solvent B: acetonitrile, injection volume: 5 mL) connected to an Agilent 6530 Accurate-Mass Q-TOF instrument. The following gradient was used (time/min, %B): 0, 0; 1, 0; 2, 95; 6, 95; 7, 0, 9, 0. The mass spectrometer was operated in negative mode and the autosampler was kept at 4°C . MS-fragmentation experiments were carried out using collision induced dissociation (CID) fragmentation with nitrogen as the collision gas at 10 eV. By comparing fragmentation patterns of standards and samples, tetrathionate could be unequivocally identified.

The following fragments could be detected: 224.8660 ([S₄O₆H]⁻, parent ion), 144.9078 ([S₃O₃H]⁻) and 80.9652 ([SO₃H]⁻) (Supplementary Fig. 5).

To quantitate tetrathionate levels in extracts, a standard curve was recorded using freshly prepared Sodium Tetrathionate (Sigma) standards in distilled water (0.0001, 0.001, 0.01, 0.1 and 1 mM). Samples were kept on ice or at 4°C . After HPLC-MS/MS analysis, EIC peaks (224.84-224.88 Da) were automatically integrated using the MassHunter Workstation Software (version: B.07.00). A log-log plot of peak area versus tetrathionate concentration was used to generate a linear fit.

Whole Genome Sequencing and analysis

Bacterial samples were selected by plating from feces, as described above, on LB + 300 μ g/mL streptomycin sulfate (Sigma) + 60 μ g/mL x-gal (Santa Cruz Biosciences) agar. Colonies were scraped from plates, resuspended and prepared for sequencing as a pool using a small-volume modification of the Illumina Nextera XP kit as described previously³⁵. Sequencing was performed on the Illumina Miseq platform, using paired end 50-bp reads. Raw sequence reads are available with links to NCBI BioProject accession number PRJNA380756.

Alignment and base calling of the ancestral sample was done via BreSeq³⁶ using default options, aligning to *E.coli* NGF-1 (NCBI references NZ_CP016007.01, NZ_CP016008.01 and NZ_CP016009.01) and *E. coli* MG1655 (NCBI reference NC_000913.3) for sequences flanking engineered elements, which were cloned in *E. coli* MG1655 and transferred to the strain by P1 transduction. Breseq's gdttools package was used to modify the ancestral genbank file to eliminate mutations in the ancestral strain. We then ran breseq at high sensitivity (settings-consensus-minimum-coverage-each-strand 3 –consensus-frequency-cutoff 0.1) to call mutations in the passaged strains. Genomic coverage is as reported by breseq. As the number of reads at any given point is high, the chance of not seeing a mutation that occurs at x% of the population in at least k reads can be computed directly via

Poisson approximation, i.e. $1 - \sum_{k=0}^{k-1} \frac{10^k e^{-10}}{k!}$, so for the case of a mutation in 10% of the population, at 100× coverage, there is a 99% chance it will appear in at least three independent reads.

Statistical analysis

All statistical analyses were undertaken in Prism 6 or 7 for Mac OS X (GraphPad). Details of individual tests and resulting p-values are included in figure legends and text where appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Engineering a tetrathionate responsive memory device in *E. coli* NGF-1 a)
A bacterial memory device, PAS638, was constructed in mouse commensal *E. coli* NGF-1. *S. typhimurium ttrR/S* and P_{ttrBCA} drive Cro ‘trigger’ expression to switch a phage lambda-based memory circuit¹². In the presence of tetrathionate, TtrS becomes phosphorylated, in turn phosphorylating TtrR, which activates expression through P_{ttrBCA} in anaerobic conditions. Cro protein expression switches memory ON, accompanied by *lacZ* reporter expression. b) Testing of *in vitro* memory showed. c) a dose-response curve of PAS638 (EC₅₀: 0.38–0.85 μM 95%CI) but no response of the triggerless control PAS637 strain.

Graph shows individual values from 6 replicate colonies from 2 separate experiments, non-linear fit $\pm$ SEM. d) PAS638 and *S. typhimurium* 14028s (*S. tm*) bacteria were administered by oral gavage to mice one day after streptomycin treatment, and fecal samples were analyzed d2–5 post administration (green stars) e) showing specific response of PAS638 on day 4 and/or 5 (see also Supplementary Fig. 2a) when co-infected with *S. typhimurium ttrR* bacteria (n=7) but not control (n=7) or *S. typhimurium* wt bacteria (n=6). f) Cumulative LCN-2 levels in mice administered PAS638+ *S. typhimurium ttrR* were higher in mice with PAS638 response (green lines) than in those without (black lines). Graph shows plots for individual mice (dotted) and ON or OFF averages (solid lines). ** t-ratio(5) = 4.3, p=0.03 using multiple t-tests with Holm-Sidak multiple comparisons test and each timepoint analyzed individually without assuming a consistent SD. d2 (t-ratio(5) = 1.2, p = 0.3), d3 (t-ratio(5) = 2.5, p = 0.1) and d5 (t-ratio(5) = 2.3, p = 0.1) were not significantly different.

Figure 2. Tetrathionate-sensing of inflammation in vivo

a) PAS638 and *S. typhimurium* *trr* bacteria were administered to *cybb*^{-/-} and C57Bl/6 control mice (n= 5 per group) by oral gavage a day following streptomycin pre-treatment. b) Elevated PAS638 switching in the presence of *S. typhimurium* *trr* indicated the presence of tetrathionate in both strains. Graph shows max. percent over 5 day experiment, means are marked. *p=0.01 and ** p = 0.03 using Kruskal-Wallis test with Dunn's multiple comparison correction. A comparison between the two *S. tm* *trr* samples was also done, p >0.99. c) Mass spectrometry from cecal extracts confirmed that tetrathionate was raised in

cybb^{-/-} and C57Bl/6 mice co-infected with *S. typhimurium ttrR*, along with intermediate levels in cybb^{-/-} uninfected controls. Graph shows total tetrathionate detected per whole cecum; means are marked. *p<0.0001, **p0.0008, ***p=0.002 and **** p=0.0009; F(3,15) = 42.92 using one-way ANOVA with Tukey's multiple comparison correction. Comparison of both infected groups (p=0.996) and C57Bl/6 control with cybb^{-/-} infected mice (p<0.0001) were performed but not shown. d) PAS638 was administered to retired breeder IL10^{-/-} mice raised in gnotobiotic and barrier SPF conditions and retired breeder C57Bl/6 control mice raised in SPF conditions. e) Indicator plating of fecal samples from C57Bl/6 and IL10^{-/-} mice (> 1 week post administration) showed elevated tetrathionate response in IL10^{-/-} mice. Blue values: average of 3–4 measurements from individual mice. Grey values: single measurement from individual mice. Means are marked ** p=0.009 between groups (including only blue values) using a two-tailed Mann-Whitney test. f) PAS638 stably colonized IL10^{-/-} mice (n=10) even without streptomycin pre-treatment. Graph shows CFU counts for individual mice (dotted lines) and average of all mice (solid) g) Balb/c, C57Bl/6 and 129X1/SvJ mice were colonized with PAS638 and the bacterial memory state was analyzed (green star = fecal sample collected). h) Balb/c and C57Bl/6 mice showed no switching, while a subset of 129X1/SvJ mice showed elevated response. Graph shows averages from at >3 days for individual mice and means. i) PAS638 levels broadly correlated with increased LCN-2 levels in five 129X1/SvJ mice. Timepoints in which bacteria were more highly switched into the memory-on state (memory values >1% highlighted green) tended to correspond with higher LCN-2 levels (values >20ng/g highlighted red).

Figure 3. PAS638 does not accrue mutations in synthetic elements over long periods

a) 129X1/SvJ mice were colonized with PAS638 for a 200-day period, >1600 bacterial generations. Mice were housed together for days 1–105, and then separated into two cages.

b) PAS638 titers remained detectable following establishment in the streptomycin pre treated gut, without further antibiotic selection.

c) Function was evident by switching at timepoints throughout the 200-day experiment.

d) *Ex vivo* functional tests and next-generation sequencing analysis of PAS638 colonies following 159 and 200 days colonization confirmed that synthetic circuits did not mutate in any way over this period. Only one mutational

insertion in the Colicin-like plasmid was identified in two cohoused mice, m1 and m2. e) *In vivo* function was further confirmed through administration of PAS638 and *S. typhimurium ttrR* to C57Bl/6 mice (n=5 per group), using PAS638 colonies isolated after colonization of three separate 129X1/SvJ mice for 200 days. Pooled colonies from a single mouse (94 colonies), or all three mice (280 colonies) were tested. f) Infection specific PAS638 switching response confirmed retained functionality *in vivo*. Graph shows max. switching percent measured from d3–5 post-infection; means are marked. ** p=0.008 using a two-tailed Mann-Whitney test of each experiment separately.

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