Engineered bacteria detect tumor DNA *in vivo*.

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Summary
In vitro nucleic acid analysis has become a valuable diagnostic tool. However, *in vitro* measurements have many disadvantages when compared to *in vivo* techniques. Synthetic bacterial biosensors have been engineered to sense many target signals *in vivo*, but no biosensor exists to detect specific DNA sequences. Here, we engineered naturally competent *Acinetobacter baylyi* bacteria to detect engineered donor DNA inserted into the genomes of colorectal cancer (CRC) cells and organoids. The DNA biosensor concept was developed *in vitro* and then validated *in vivo* with sensor bacteria delivered orally or rectally to mice that had been injected with orthotopic donor CRC organoids. Horizontal gene transfer occurred from the donor tumor to the sensor bacteria *in vivo*, conferring antibiotic resistance to the sensor bacteria and allowing their detection in stool. The sensor bacteria differentiated mice with and without CRC. Life detecting life has many implications for future diagnosis, prevention, and treatment of disease. This approach may also be useful in any application that requires the detection of mutations or organisms within environments that are difficult to sample.

Main text
In vitro DNA analysis helps detect and manage important human diseases, including cancer and infection[1]. However, *in vitro* sensing by definition removes samples from their environment, which may introduce artifacts[2]. In addition, many DNA diagnostics cannot achieve clinically relevant sequence resolution, and more advanced sequencing remains too expensive for routine use[3].

Synthetic bacteria present promising chassis for *in vivo* diagnostic devices[4]. Engineered bacterial sensors report on a variety of signals, including gut inflammation[5], intestinal bleeding[6], quorum sensing pathogens[7] and hypoxic tumors[8]. Bacteria can easily access the entire gastrointestinal tract via oral administration[9], and they can produce outputs that can be noninvasively measured in stool[5] or urine[8]. The use of cellular memory, e.g. bistable switches[5,10] or genomic rearrangements[11], allows bacteria to store information over time. However, biosensors have not yet been engineered to detect specific DNA sequences or mutations.

Some bacteria are "naturally competent" and can sample extracellular DNA directly from their environment[12]. Natural competence promotes horizontal gene transfer (HGT), the exchange of genetic material between organisms outside vertical, "parent to offspring" transmission[13]. HGT is common between microbes[14] and has been described from microbes into animals and plants[15]. However, gene transfer in the opposite direction, from animals and plants into microbes, is poorly characterized. *Acinetobacter baylyi* is a highly competent and well-studied bacterium[16] that is largely non-pathogenic in healthy humans[17] and can colonize the murine gastrointestinal tract[18]. This combination of traits made *A. baylyi* a suitable candidate to test whether engineered bacteria could detect CRC-promoting DNA mutations *in vivo*.

The human gastrointestinal tract presents unique opportunities for DNA-based diagnostics. Colorectal cancer (CRC) is the second most frequent cause of cancer death in the US[19], and precancerous polyps, from which CRC develops, occur in most adults >50 years old[20]. Genetic predisposition, ageing, diet, and the microbiome are all implicated in colorectal carcinogenesis[20,21]. These factors ultimately promote cancer development through predictable genetic events that progressively subvert normal epithelial homeostasis[22]. However, DNA-based, *in vitro* fecal and serum assays are not yet sufficiently sensitive to reliably detect DNA mutation within precancerous colorectal polyps, which are necessary to diagnose and remove in order to prevent CRC[23,24].

Here we present a proof-of-principle, non-invasive, bacterial sensor for CRC *in vivo* (Fig. 1). Our strategy non-invasively delivers bacterial biosensors to the gastrointestinal tract, where they sample and genomically integrate target tumor DNA *in situ*. The work presented here uses engineered tumor cells and, now having demonstrated feasibility, future biosensors could be modified to detect non-engineered target

DNA using more advanced genetic circuits. This technology, while studied here for its utility in detecting CRC, may have important applications in many other clinical, and non-clinical, settings.

Sensor bacteria can detect human cancer DNA

To test the hypothesis that bacteria could detect human tumor DNA, we generated transgenic "donor" human cancer cells and "sensor" bacteria (Fig. 2a). The donor cassette comprised a kanamycin resistance gene and GFP (*kan^R-GFP*) flanked by 1 kb homology arms from human *KRAS* (Fig. 2b-c and Extended Data Fig. 1). *KRAS* is an important oncogene in human cancer and the *KRASG12D* mutation is present in advanced adenomas and in 13% of CRC[25]. We stably transduced this donor cassette into both RKO and LS174T human CRC cell lines using a lentiviral vector. To construct the sensor bacteria, we inserted a complementary landing pad with *KRAS* homology arms into a neutral genomic site of *A. baylyi* cells. We tested both a "large insert" design (2 kb), with a different resistance marker between the *KRAS* arms to be replaced by the donor cassette (Fig. 2b, Extended Data Fig. 2a), and a "small insert" design (8 bp), with the same *kan^R-GFP* cassette as in the tumor donor DNA but interrupted by 2 stop codons in *kan^R* (Fig. 1 & 2c, Extended Data Fig. 2b). The biosensor output was growth on kanamycin plates, measured as colony-forming units (CFUs).

We tested both designs using various donor DNA sources, both in liquid culture and on solid agar (Fig. 2a). The "large insert" biosensors detected donor DNA from purified plasmids and genomic DNA both in liquid (Fig. 2d) and on agar (Fig. 2e). On agar, they also detected raw, unpurified lysate, albeit at just above the limit of detection (Fig. 2e). Consistent with previous results[26], the "small insert" design improved detection efficiency roughly 10-fold, reliably detecting donor plasmid, purified genomic DNA, and raw lysate both in liquid and on agar (Fig. 2f-g, Extended Data Supplemental Movie). Regardless of the source of DNA or the biosensor design, detection on solid agar was approximately 10-fold more efficient than in liquid culture. Importantly, detection of donor DNA from raw lysate demonstrated that the biosensors do not require *in vitro* DNA purification[27].

Sensor bacteria can discriminate wild-type from mutant *KRAS* DNA

KRASG12D is a common oncogene in CRC and in solid tumors generally[28]. To test whether sensor bacteria could discriminate between wild-type and mutant *KRAS (KRASG12D)*, which differ by a single G > A transition, we utilized *A. baylyi's* endogenous Type I-F CRISPR-Cas system[29]. We stably transduced an RKO cell line with the *kan^R-GFP* donor cassette flanked by wild-type *KRAS* (RKO-*KRAS*), and a second line with *KRASG12D* flanking sequences (RKO-*KRASG12D*). Next, we designed 3 CRISPR spacers targeting the wild-type *KRAS* sequence at the location of the *KRASG12D* mutation, using the *A. baylyi* protospacer-adjacent motif (PAM) of 5'-CC-protospacer-3' (Fig. 2h). We inserted these as single-spacer arrays into a neutral locus in the "large insert" *A. baylyi* sensor genome.

The sensor bacteria, if effective, should reject wild-type *KRAS* through CRISPR-mediated DNA cleavage. Conversely, the *KRASG12D* sequence should alter the target sequence and evade DNA cleavage. Two of the three spacers blocked transformation by both wild-type and mutant DNA (Fig. 2i-j). However, spacer 2, for which the *KRASG12D* mutation eliminated the PAM site, selectively permitted HGT only with *KRASG12D* donor DNA (Fig. 2E-F). Thus, sensor *A. baylyi* can be engineered to detect hotspot mutations in the *KRAS* gene with single-base specificity.

Sensor bacteria can integrate cancer DNA in organoid culture

Ex vivo organoid culture faithfully reflects endogenous tumor biology[30]. We therefore evaluated our sensor and donor constructs in organoid culture (Fig. 3a). We previously used CRISPR/Cas9 genome engineering to generate compound *Braf^{V600E}; Tgfbr2$^{\Delta/\Delta}$; Rnf43$^{\Delta/\Delta}$; Znrf3$^{\Delta/\Delta}$; p16Ink4a$^{\Delta/\Delta}$* (BTRZI) mouse organoids that recapitulate serrated CRC when injected into the mouse colon[31].

We transduced BTRZI organoids with the human *KRAS*-flanked donor DNA construct (*KRAS-kan*^{R}) to generate donor CRC organoids, and incubated their lysate with "small inert" *A. baylyi* biosensors. As with the CRC cell lines, the sensor *A. baylyi* incorporated DNA from donor organoid lysate, but not from control lysates from the parental organoids (Fig. 3b, Extended Data Fig. 3a). Next, we co-cultured GFP-expressing sensor *A. baylyi* with BTRZI parental or BTRZI-*KRAS-kan*^{R} donor organoids for 24 hours on Matrigel. The GFP-expressing sensor bacteria surrounded the organoids (Fig. 3c and Extended Data Fig. 3b). Following co-culture with donor, but not parental, organoids, the *A. baylyi* sensor bacteria underwent HGT, as evidenced by acquired kanamycin resistance (Fig. 3d). HGT-induced antibiotic resistance was confirmed by Sanger sequencing of individual colonies (Extended Data Fig. 3c). Note that these experiments did not test specificity for mutant *KRAS*, but whether organoid-to-bacteria HGT would occur in organoid co-culture.

Sensor bacteria can detect tumor DNA *in vivo*

Given that cancer to bacterial HGT occurred *in vitro*, both in cell lines and in organoid co-culture, we sought to test this system *in vivo*. *A. baylyi* previously survived transit through the mouse gastrointestinal tract in germ-free animals[18]. To confirm this finding and to optimize our own experimental protocol, we used *mCherry*-expressing, kanamycin resistant, *A. baylyi*. One week after antibiotic gut decontamination, we administered 10^{10} *A. baylyi* either by single oral gavage or rectal enema. Mice administered *A. baylyi*, by either route, maintained gastrointestinal colonization for at least one week as measured by stool CFU assays and fluorescence (Extended Data Fig. 4). Next, we confirmed that our BTRZI, orthotopic CRC model would release tumoral DNA into the fecal stream. In this mouse model of CRC, engineered CRC organoids were injected orthotopically, by mouse colonoscopy, into the mouse colon to form colonic tumors, as previously described[31]. Using digital droplet PCR, we measured *Braf* mutant tumor DNA in stools collected from tumor-bearing and control mice. The BRTZI model reliably released tumor DNA into the colonic lumen (Extended Data Fig. 5).

Having confirmed that sensor bacteria would colonize the mouse gastrointestinal tract and that DNA is released from the tumor, we designed and conducted our orthotopic CRC experiment (Fig. 4a). At week -4, NSG mice were either injected colonoscopically, or not, with BRTZI-*KRAS-kan*^{R} organoids. At week -1, mice underwent a gut decontamination regimen. A single dose of 10^{10} "small insert" *A. baylyi* sensor bacteria, with additional constitutive resistance to chloramphenicol, was administered by oral gavage or enema to tumor-bearing and non-tumor-bearing mice. We included control mice with and without tumors, that were administered PBS rather than sensor bacteria (Fig. 4a). All study groups were housed in separate cages. At day 3 after sensor bacteria delivery, mice were administered 2 days of low dose kanamycin in their drinking water before having their stools collected at day 5. HGT was measured by stool culture on chloramphenicol or kanamycin agar plates, with CFU presented as the mean CFU per 2-4 stools collected for each mouse.

Following sensor bacteria delivery, either by oral (Fig. 4b) or rectal (Fig. 4c) delivery, the kan-resistant CFUs were significantly higher in the tumor-bearing compared to the non-tumor mice. The sensor bacteria perfectly discriminated tumor from non-tumor bearing mice (Fig. 4d). The mean stool CFU was the same regardless of tumor size at the time of stool collection (Fig. 4e). HGT-mediated antibiotic resistance was confirmed by Sanger sequencing of individual colonies (Extended Data Fig. 6). Finally, to ensure that HGT was occurring within the colorectal lumen *in vivo*, rather than on the agar plates *ex vivo*, the collected stool was pre-treated with DNase, which did not cause any reduction in CFUs (Extended Data Fig. 7).

Discussion

In this study, naturally competent *A. baylyi* were engineered to sense tumoral donor DNA *in vivo*. The donor-sensor system was optimized *in vitro* and then validated *in vivo* using an orthotopic mouse model of CRC. Furthermore, *in vitro*, we engineered a CRISPR-based technique to provide specificity for the mutant

KRASG12D vs. wild-type *KRAS*. The sensor bacteria described here demonstrate that a living biosensor can detect tumor DNA shed from CRC *in vivo*, with no sample preparation or processing.

For the gut in particular, sampling *in vivo* may offer important detection advantages. The gastrointestinal tract contains significant DNase activity[32], which limits the lifetime of free DNA in both rodents and humans[33,34]. In previous attempts to use *A. baylyi* as DNA biosensors, gastrointestinal contents from rats and mice inhibited transformation of *A. baylyi* by purified DNA, possibly due to that DNase activity[35]. Degradation may also explain why fecal samples fail to accurately capture microbiome diversity[2], and why DNA testing is less sensitive for proximal precancerous lesions[23]. However, living biosensors located *in situ* could capture and preserve DNA shortly after its release, before degradation by local DNase. In addition, biosensors could amplify target DNA *in vivo* through HGT-induced fitness, intercellular quorum sensing or intracellular genetic memory switches[10,11]. Perhaps most exciting, however, is that unlike *in vitro* diagnostics, detection of target DNA by a living biosensor could potentially be coupled to direct and genotype-complementary nanobodies, peptides, or other small molecules for the treatment of cancer or infection[36,37].

Further developments are still required to translate this proof-of-concept into a clinical application. *A. baylyi* may not be the ideal chassis species for use in humans, either because of its performance as a biosensor in the human colorectum or due to safety concerns in some immunosuppressed patients[17]. This system also required engineered tumor DNA to select for sensor bacteria that had undergone HGT. However, this could be addressed so that HGT leads to an excision of repressive elements within the sensor bacteria rather than the acquisition of engineered DNA from the tumor. The single base specificity, as demonstrated here *in vitro* for *KRASG12D*, may not be practical for all genetic loci of interest; here it relied on *KRASG12D* overlapping the CC PAM. This occurs on average every 8 bp, but other CRISPR-Cas systems with different PAMs can be used to expand coverage. Furthermore, future readouts should be optimized to avoid the need for antibiotic selection and could include colorimetric or fluorescent outputs that may prove easier to implement in the field. Finally, biocontainment concerns would need to be re-examined as this technology advances.

In summary, here we present the proof-of-concept that bacterial sensors can detect and report on specific sequences of DNA *in vivo*. This suggests sensor bacteria could be developed as cellular analytical laboratories, for use in the body, the factory, the crop, or the sewer. Their greatest value will be for applications in which sampling is difficult, continuous surveillance is desirable, diagnostic resources are scarce, and/or a response would be best delivered to the target organism at the time and place of its detection.

Online content

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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Figure legends

Figure 1. Engineered bacteria to detect tumor DNA. Engineered *A. baylyi* bacteria are delivered orally or rectally in an orthotopic mouse model of CRC. The naturally competent *A. baylyi* take up tumor DNA shed into the colonic lumen. The tumor donor DNA is engineered with a kan^R cassette flanked by *KRAS* homology arms (HA). The sensor bacteria are engineered with a defective kan^R cassette, due to two mutations, 8 bp apart that induce two TAA stop codons. Horizontal gene transfer is promoted by the homology arms. Sensor bacteria that undergo HGT, from tumor DNA, acquire kanamycin resistance and are quantified in stool by colony forming unit analysis on kanamycin selection plates.

Figure 2: Sensing *KRASG12D* DNA *in vitro*. a-c) Donor DNA consisting of plasmid, purified cancer cell genomic DNA, or raw lysate (top) recombines into biosensor *A. baylyi* cells (bottom), transferring either a large, 2 kb insert (**b**), or a small, 8 bp insert to repair 2 stop codons (**c**), in both cases conferring kanamycin resistance. **d-g**) *A. baylyi* biosensors were incubated with plasmid DNA, purified RKO-*KRAS* or LS174T-*KRAS* genomic DNA, or raw RKO-*KRAS* lysate, all containing the donor cassette, or purified RKO or LS174T genomic DNA as controls. Biosensor cells included either "large insert" (**b,d,e**) or "small insert" (**c,f,g**) designs, and transformations were performed in liquid culture (**d,f)** or on solid agar surfaces (**e,g**). Two-sample t-tests compared data to combined RKO and LS174T genomic DNA controls for the same conditions. **h**) CRISPR spacers targeting the *KRAS* G12D mutation (boxed), using the underlined PAMs. **i,j**) Fraction of total biosensor cells expressing the indicated CRISPR spacers that were transformed by plasmid donor DNA with wild type (**i**) or mutant G12D (**j**) *KRAS*. Statistics were obtained using two-sample t-tests. Data points below detection are shown along the x-axis.

Figure 3: Detection of donor DNA from BRTZI-*KRAS-kan^R* organoids.
Schema depicting *in vitro* co-culture of *A. baylyi* sensor bacteria with BRTZI-*KRAS-kan^R* (CRC donor) organoid lysates or viable organoids to assess HGT repair of kanamycin resistance gene (kan^R). **b**. Recombination with DNA from crude lysates enables growth of *A. baylyi* sensor on kanamycin plates with transformation efficiency of 1.4×10^{-5}. **c**. Representative images of GFP-tagged *A. baylyi* sensor surrounding parental BRTZI (control) and BRTZI-*KRAS-kan^R* donor organoids at 24h. Scale bar 100µm **d**. Co-culture of established CRC BRTZI-*KRAS-kan^R* donor organoids with *A. baylyi* sensor enables growth of *A. baylyi* sensor on kanamycin plates with transformation efficiency of 3.8×10^{-7}. In **b, d,** n = 5 independent experiments each with 5 technical replicates, one sample t-test on transformed data was used for statistical analysis with *P* values as indicated.

Figure 4 Horizontal gene transfer detected in stool from mice bearing BRTZI-*KRAS-kan^R* tumors after oral or rectal dosing of *A. baylyi* sensor bacteria.
a, Schema depicting *in vivo* HGT experiments: generation of BRTZI-*KRAS-kan^R* (CRC donor) tumors in mice, administration of PBS control or sensor *A. baylyi* and stool collection. Scale bars 200µm. **b,** oral or **c,** rectal delivery of *A. baylyi* sensor to mice bearing CRC donor tumors results in kanamycin resistant *A. baylyi* sensor in stool via HGT. Average CFU per stool from 2-4 stools per mouse grown on Kanamycin selection plates is shown, n=3-8 mice/group. **d**. ROC curve analysis of HGT CFU following oral gavage. **e**. HGT CFU rate in stool was not affected by donor tumor size in recipient mice, as determined by colonoscopic scoring (S small, M medium, L large). In **b,c,e**, one-way ANOVA on $\log_{10}$ transformed data was used for statistical analysis, with *P* values shown in the corresponding panels.

Extended Data Movie 1:
A. baylyi biosensors taking up plasmid donor DNA.
A. baylyi were grown overnight, washed into fresh LB, mixed with saturating pLenti-KRAS donor DNA, and sandwiched between an agar pad and a glass bottom dish. Images were taken every 10 minutes. GFP fluorescence indicates that the cells have taken up and genomically integrated the donor DNA cassette.

Extended Data DNA Files:
DNA cassettes and surrounding regions corresponding to the “large insert” and “small insert” designs for *A. baylyi*, and the plasmid donor DNA, as shown in Extended Data 1,2, in Genbank format.

Methods and Materials

Bacterial cell culture and cloning to generate biosensors

Acinetobacter baylyi ADP1 was obtained from the American Type Culture Collection (ATCC #33305) and propagated in standard LB media at 30 or 37 °C. *KRAS* homology arms were inserted into a neutral genetic locus denoted *Ntrl1*, replacing the gene remnant ACIAD2826. For the "large insert" design, a spectinomycin resistance gene was placed between the *KRAS* homology arms. For the "small insert" design, two stop codons were placed near the beginning of the kan^R gene of the donor cassette, and the broken cassette was inserted into *A. baylyi*. CRISPR arrays were inserted into a neutral locus used previously, replacing ACIAD2186, 2187 and part of 2185. Ectopic CRISPR arrays were driven by a promoter region that included 684 bp from upstream of the first repeat of the endogenous, 90-spacer array.

In vitro biosensor transformation experiments

A. baylyi were grown overnight in LB at 30 °C. Cells were then washed, resuspended in an equal volume of fresh LB, and mixed with donor DNA. For transformation in liquid, 50 µl cells were mixed with 250 ng donor DNA and incubated in a shaker at 30 °C for 2 hours or overnight. For transformation on agar, 2 µl cells were mixed with >50 ng donor DNA, spotted onto LB plates containing 2% wt/vol agar, and incubated at 30 °C overnight. Spots were cut out the next day and resuspended in 500 µl phosphate buffered saline solution (PBS). To count transformants, cells were 10-fold serially diluted 5 times, and 2 µl spots were deposited onto selective (30 ng/ml kanamycin) and non-selective 2% agar plates, with 3 measurement replicates at each dilution level. Larger volumes of undiluted samples were also spread onto agar plates to increase detection sensitivity (25 µl for liquid culture, 100 µl for resuspended agar spots). Colonies were counted at the lowest countable dilution level after overnight growth at 30 °C, and measurement replicates were averaged. Raw, unpurified lysate was produced by growing donor RKO cells in a culture dish until confluence, trypsinizing and harvesting cells, pelleting them in a 15 ml tube, resuspending them in 50 µl PBS, and placing the tube in a –20 °C freezer overnight to disrupt cell membranes.

In vitro statistics

Hypothesis testing was performed using 2-sample t-tests in Matlab after taking base 10 logarithms, since serial dilutions produce log-scale data. Where data points were below the limit of detection, they were replaced by the limit of detection as the most conservative way to include them in log-scale analysis. Comparisons between large vs small inserts or liquid vs solid agar culture were performed using paired t-tests, where data were matched for donor DNA and either culture type (liquid vs agar) or insert size, respectively.

Creation of BTRZI CRC donor organoids

BTRZI ($Braf^{V600E}$;$Tgfbr2^{\Delta/\Delta}$;$Rnf43^{\Delta/\Delta}$ /$Znf43^{\Delta/\Delta}$;p16 $Ink4a^{\Delta/\Delta}$) organoids were generated using CRISPR-Cas9 engineering (Lannagan et al, 2019 Gut) and grown in 50 µl domes of GFR-Matrigel (Corning,; 356231) in organoid media: Advanced Dulbecco's modified Eagle medium/F12 (Life Technologies) supplemented with 1x gentamicin/antimycotic/antibiotic (Life Technologies), 10mM HEPES (Gibco), 2 mM GlutaMAX (Gibco), 1x B27 (Life Technologies; 12504-044), 1x N2 (Life Technologies; 17502048), 50 ng/ml mouse recombinant EGF (Peprotech; 315-09), 10 ng/ml human recombinant TGF-β1 (Peprotech; 100-21). Following each split, organoids were cultured in 10 µM Y-27632 (MedChemExpress; HY-10583), 3 µM iPSC (Calbiochem; 420220), 3 µM GSK-3 inhibitor (XVI, Calbiochem; 361559) for the first 3 days.

To create BTRZI CRC donor organoids, lentiviral expression plasmid pD2119-FLuc2 KRasG12D donor was co-transfected with viral packaging vectors, psPAX2 (Addgene; plasmid; 12260) and MD2G (Addgene; plasmid; 12259), into HEK293T cells. At 48 and 72 h after transfection, viral supernatants were harvested, filtered through a 0.45-µm filter, and concentrated using Amicon Ultra Centrifugal Filters (Merck Millipore; UFC910024). Concentrated lentivirus particles were used for transduction. The viral supernatant generated was used to transduce BTRZI organoids by spinoculation. Briefly, organoids were dissociated to single cells using TrypLE. $1x10^5$ single cells were mixed with 250 µl organoid media; 10 µM Y-27632; 250 µl concentrated viral supernatant and 4 µg/ml polybrene (Sigma,; H9268) in a 48 well tray before centrifugation at 600 xg for 90 minutes at 32 °C. Meanwhile, 120 µl 50:50 ADMEM:Matrigel mixture was added to a cold 24-well tray before centrifugation of this bottom matrigel layer for 40 minutes at 200xg at room temperature, followed by solidifying the Matrigel by incubating at 37 °C for 30 minutes. After spinoculation, cells were scraped from the well and plated on top of the Matrigel monolayer with organoid media. The following day, the media was removed and the upper layer of Matrigel was set over the organoids by adding 120 µl 50:50 ADMEM:Matrigel and allowing to set for 30 minutes before adding organoid media. 48 hours after transduction, BTRZI donor organoids were selected with 8 µg/ml puromycin for 1 week, then maintained in organoid media with 4 µg/ml puromycin.

Organoid lysate mixed with *A. baylyi* sensor bacteria

BTRZI (parental) and BTRZI donor organoids were grown for 5 days in 50 ml Matrigel domes. Organoids were dissociated to single cells with TrypLE, counted and $6x10^5$ single cells were collected in PBS and snap frozen. The CFU equivalence of exponentially growing *A. baylyi* sensor culture at OD_{600} 0.35 was ascertained by serial dilution of 3 independent cultures with 5 technical replicates plated on 10 µg/ml Chloramphenicol LB agar plate to be 2.4 x 10^8 CFU per ml. *A. baylyi* sensor was grown in liquid culture with 10 µg/ml Chloramphenicol to OD_{600} 0.35 before mixing with organoid lysate at a 1:1 ratio and grow overnight on LB agar plates at 30 °C. All bacteria was scraped into 200 µl LB/20% glycerol before spotting 5x 5 µl spots onto kanamycin and chloramphenicol plates and grown overnight at 37 °C. Colonies were counted and the dilution factor was accounted for to calculate CFU per ml. Rate of HGT was calculated by dividing the CFU per ml of transformants (Kanamycin plates) by the CFU per of total *A. baylyi* (chloramphenicol plates) for 5 independent experiments.

Coculture organoids with *A. baylyi* sensor bacteria

For co-culture experiments, 24-well trays were coated with Matrigel monolayers. Briefly, 200 µl 50:50 ADMEM:Matrigel mixture was added to a cold 24-well tray and centrifuged for 40 minutes at 200xg at room temperature, followed by a 30 minute incubation at 37 °C to solidify matrigel. BTRZI (parental) and BTRZI donor organoids were dissociated into small clusters using TrypLE and grown for 5 days on a Matrigel monolayer in organoid media without antibiotics before 50 µl OD_{600} 0.35 *A. baylyi* sensor was added to each well. After 24 hours, organoids were photographed then collected and grown overnight on LB agar plates at 30 °C. All bacteria was scraped into 200 µl LB/20% glycerol before spotting 5x 5 µl spots onto kanamycin and chloramphenicol plates and grown overnight at 37 °C. Colonies were counted and the dilution factor was accounted for to calculate CFU per ml. Rate of HGT was calculated by dividing the CFU per ml of transformants (kanamycin plates) by the CFU per ml of total *A. baylyi* (chloramphenicol plates) for 5 independent experiments.

***A. baylyi* colonisation trial**

This study was approved by the SAHMRI Animal Ethics committee (SAM20.036). NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice (male and female, 10-13 weeks old) were obtained from

the SAHMRI Bioresources facility and housed under pathogen-free conditions. NSG mice were administered with antibiotics (2.7mM Ampicillin, Sigma; A1066 and 0.55mM Neomycin, Sigma; N1876) in drinking water a week prior to oral gavage/enema. *A. baylyi*-mCherry/KanR was grown in liquid culture with 50 µg/ml kanamycin to OD_{600} 0.3. *A. baylyi* was washed with PBS before 3 mice received 10^{10} *A. baylyi* via oral gavage, 3 mice received 10^{10} *A. baylyi* via enema and 2 control mice received PBS (1x enema and 1x oral gavage). Oral gavage was administered using a 20G curved feeding needle at a volume of 200 µl per mouse. Enema was performed as per previous publication. Briefly, mice were anaesthetised with isofluorane and colon flushed with 1 ml of room temperature sterile PBS to clear the colon cavity of any remaining stool. A P200 pipette tip coated with warm water was then inserted parallel into the lumen to deliver 50 mL of bacteria into the colon over the course of 30 seconds. After infusion, the anal verge was sealed with Vetbond Tissue Adhesive (3M; 1469SB) to prevent luminal contents from being immediately excreted. Animals were maintained on anaesthesia for 5 minutes, and then allowed to recover on heat mat and anal canal inspected 6 hours after the procedure to make sure that the adhesive has been degraded. Stool was collected for 2 weeks in 250 µl PBS/20% glycerol, vortexed and stored at -80 °C. Stool slurry (50 µl) was plated onto a LB agar plate and grown overnight at 37 ºC. All bacteria was scraped into 200 µl LB/20% glycerol. 5x 5µl serial dilutions were spotted onto kanamycin plates. Colonies were counted and dilutions were factored to calculate CFU *A. baylyi* per stool.

Horizontal gene transfer *in vivo*

BTRZI donor organoids were isolated from Matrigel and dissociated into small clusters using TrypLE. The cell clusters (equivalent to ~150 organoids per injection) were washed three times with cold PBS containing 10 µM Y-27632 and then resuspended in 20 µl 10% GFR matrigel 1:1000 india ink, 10 µM Y-27632 in PBS and orthotopically injected into the mucosa of the proximal and distal colon of anaesthetised 10-13 week old NSG mice (150 organoids per injection), as previously described (Lannagan et al, 2019 Gut). Briefly, a customised needle (Hamilton Inc. part number 7803-05, removable needle, 33 gauge, 12 inches long, point 4, 12 degree bevel) was used. In each mouse up to 2 injections of 20µl were performed. CRC donor tumor growth was monitored by colonoscopy for 4 weeks and the videos were viewed offline using QuickTime Player for analysis. Colonoscopy was performed using a Karl Storz Image 1 Camera System comprised of: Image1 HDTV HUB CCU; Cold Light Fountain LED Nova 150 light source; Full HD Image1 3 Chip H3-Z Camera Head; Hopkins Telescope, 1.9mm, 0 degrees. A sealed luer lock was placed on the working channel of the telescope sheath to ensure minimal air leakage (Coherent Scientific, # 14034-40). Tumor growth of the largest tumor visualised was scored as previously described using the Becker Scale (Rex et al, 2012 Am J Gastroenterol). Mice were administered antibiotics (2.7mM Ampicillin, Sigma; A1066 and 0.55mM Neomycin, Sigma; N1876) in drinking water a week prior to oral gavage/enema. *A. baylyi* sensor was grown in liquid culture with 10 µg/ml Chloramphenicol to OD_{600} 0.3. *A. baylyi* sensor was washed with PBS before 13 mice received 10^{10} *A. baylyi* sensor via oral gavage (7 mice without tumors and 6 mice with CRC donor tumors), 7 mice received 10^{10} *A. baylyi* sensor via enema (3 mice without tumors and 4 mice with CRC donor tumors). Three days after *A. baylyi* administration, mice received 10 mg/L kanamycin in their drinking water, except 2 mice from the oral gavage *A. baylyi* sensor, CRC donor tumor cohort, 5 mice from the oral gavage *A. baylyi* sensor, no tumor cohort and 2 mice from the enema *A. baylyi* sensor, no tumor cohort . Stool was collected 5 days after *A. baylyi* administration into 250 µl PBS/20% glycerol, vortexed and stored at -80 °C. Stool slurry (50 µl) was plated onto a LB agar plate and grown overnight at 37 °C. All bacteria was scraped into 200 µl LB/20% glycerol. 5x 5µl serial dilutions were spotted onto chloramphenicol and

kanamycin plates. Colonies were counted and dilutions were factored to calculate CFU *A. baylyi* per stool.

Sequencing gDNA from bacterial colonies grown on kanamycin plates
A. baylyi transformants were individually picked from kanamycin plates and grown in liquid culture LB supplemented with 25 µg/ml Kanamycin. gDNA was extracted using purelink genomic DNA minikit (Invitrogen; K182001). Genomic regions of interest were amplified using Primestar Max DNA polymerase (Takara, # R045A) and primers HGTpcrF: CAAAATCGGCTCCGTCGATACTA ; HGTpcrR: TAGCATCACCTTCACCCTC; Kan seqF: AAAGATACGGAAGGAATGTCTCC; Kan seqR: CGGCCGTCTAAGCTATTCGT. Sanger sequencing was conducted by AGRF using the same primers.

DNase treatment of stool
Stool slurry (25 µl) was mixed with 2.5 µl 10x DNase 1 buffer with or without 1 µl DNase 1 (2.7 U/µl) using RNase-free DNase 1 kit (Qiagen,; 79254). Samples were incubated at 37 °C for 30 minutes then the mixture was plated onto LB agar plates and grown overnight at 37 °C. A control to assess DNase 1 activity was set up simultaneously with 25 µl stool (from mouse with no tumor); 1 µl 100 ng/µl KRasG12D donor plasmid DNA (2 ng/ul final concentration); 2.5 µl 10x DNase 1 buffer with or without 1 µl DNase 1 (2.7 U/µl), which was incubated at 37 °C for 30 minutes. Following DNase 1 treatment, controls were mixed with 25 µl of *A. baylyi* sensor liquid culture (OD_{600} 0.35) and incubated at 37 °C 2 hrs before the mixture was plated onto LB agar plates and grown overnight at 37 °C. All bacteria was scraped into 200 µl LB/20% glycerol. 5x 5µl serial dilutions were spotted onto kanamycin plates. Colonies were counted and dilutions were factored to calculate CFU *A. baylyi* per stool.

Supplementary Materials & Methods

ddPCR detection of CRC mutant DNA in stool DNA samples
DNA was isolated from 2-8 stools combined (up to 220mg dry stool weight) per mouse using QIAamp FAST DNA Stool mini kit (Qiagen; 51604) as per manufacturer's instructions, except that a Qiagen Tissue Lyser was used for stool homogenisation. To prepare stool DNA (sDNA) for droplet digital PCR (ddPCR), sDNA was ethanol precipitated with NaAc pH 5.5 to reduce DNA volume, then sheared by sonication (Covaris, M220). PCR reactions containing 1.5 mg sDNA/combined stool sample, Kapa Probe mix (Sigma-Aldrich) with 10mM each Braf primer, 5mM each Braf assay (wild type-HEX/VIC and mutant V600E-FAM) were prepared with droplet stabiliser (RainDrop/Biorad), and subjected to droplet production (RainDance Source), PCR amplification (RainDance Sensor) and analysis (RainDrop Analyst II) as per the manufacturer's instructions (RainDrop/Biorad).

Braf F 5'- AAATAGGTGACTTTGGTCTAGC
Braf R 5'- AGATCCAGACAACTGTTCAAA
Braf wt probe 5'- HEX-CAGTGAAAT/ZEN/CTCGGTGGAGTGGGT/3lABkFQ
Braf V600E probe 5'- FAM-CACAGAGAA/ZEN/ATCTAGATGGAGTGGGTCCC/3lABkFQ
PCR program- 95°C 10 min; 92°C 15 sec, 65°C 1 min x45 cycles; 98°C 10 min; 12°C 15 min; ramp speed 0.5°C/sec.

Live imaging of *A. baylyi* DNA biosensing

A. baylyi cells were transformed with a modified "small insert" sensor design, with the GFP gene interrupted by adjacent stop codons instead of the kanamycin resistance gene. These cells always grow on kanamycin, but they only express GFP upon recombination with the donor DNA cassette. These sensors were grown overnight, washed into fresh LB, and mixed at equal volumes with plasmid donor DNA. An agar pad was prepared by pouring molten LB with 2% wt/vol agar into a 35 mm glass-bottom dish, allowing it to cool, and scooping out the solidified agar. 0.5 µl of cell-DNA mixture was spotted on the pad and allowed to dry. The pad was then replaced into the dish, sandwiching the cells between the agar and the glass. The cells were imaged every 10 minutes on a Nikon TE microscope, in both brightfield and GFP channels.

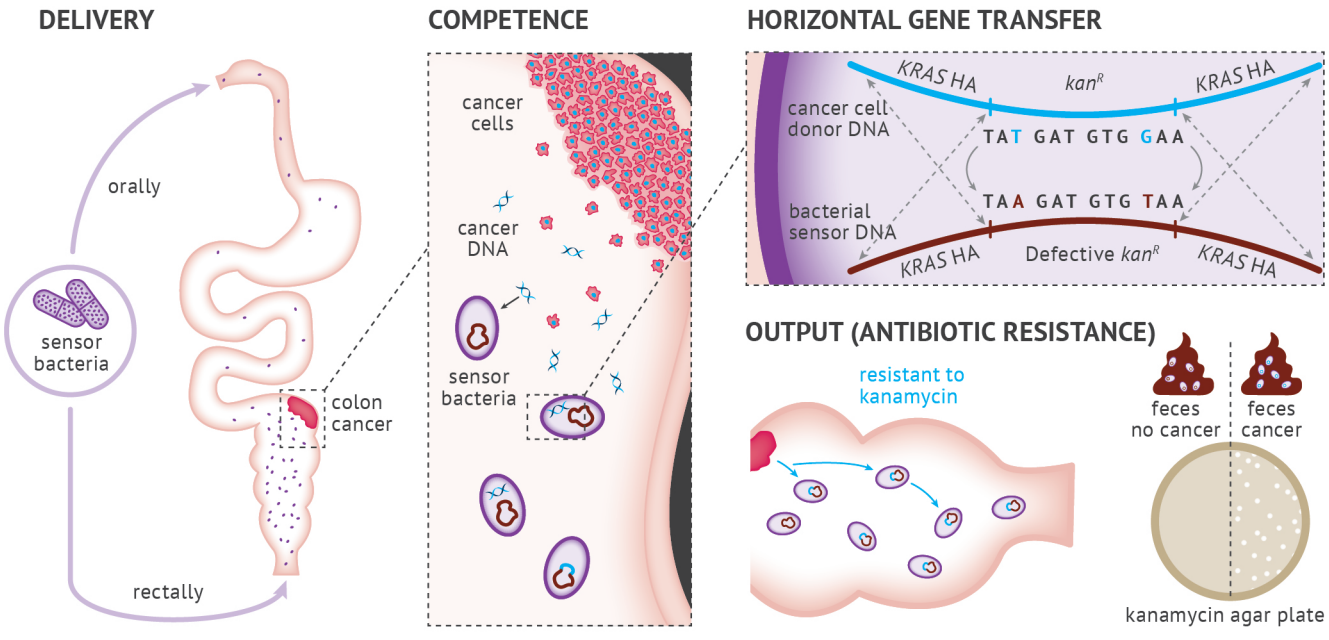

Figure 1. Engineered bacteria to detect tumor DNA. Engineered *A. baylyi* bacteria are delivered orally or rectally in an orthotopic mouse model of CRC. The naturally competent *A. baylyi* take up tumor DNA shed into the colonic lumen. The tumor donor DNA is engineered with a *kan*^{R} cassette flanked by *KRAS* homology arms (HA). The sensor bacteria are engineered with a defective *kan*^{R} cassette, due to two mutations, 8 bp apart that induce two TAA stop codons. Horizontal gene transfer is promoted by the homology arms. Sensor bacteria that undergo HGT, from tumor DNA, acquire kanamycin resistance and are quantified in stool by colony forming unit analysis on kanamycin selection plates.

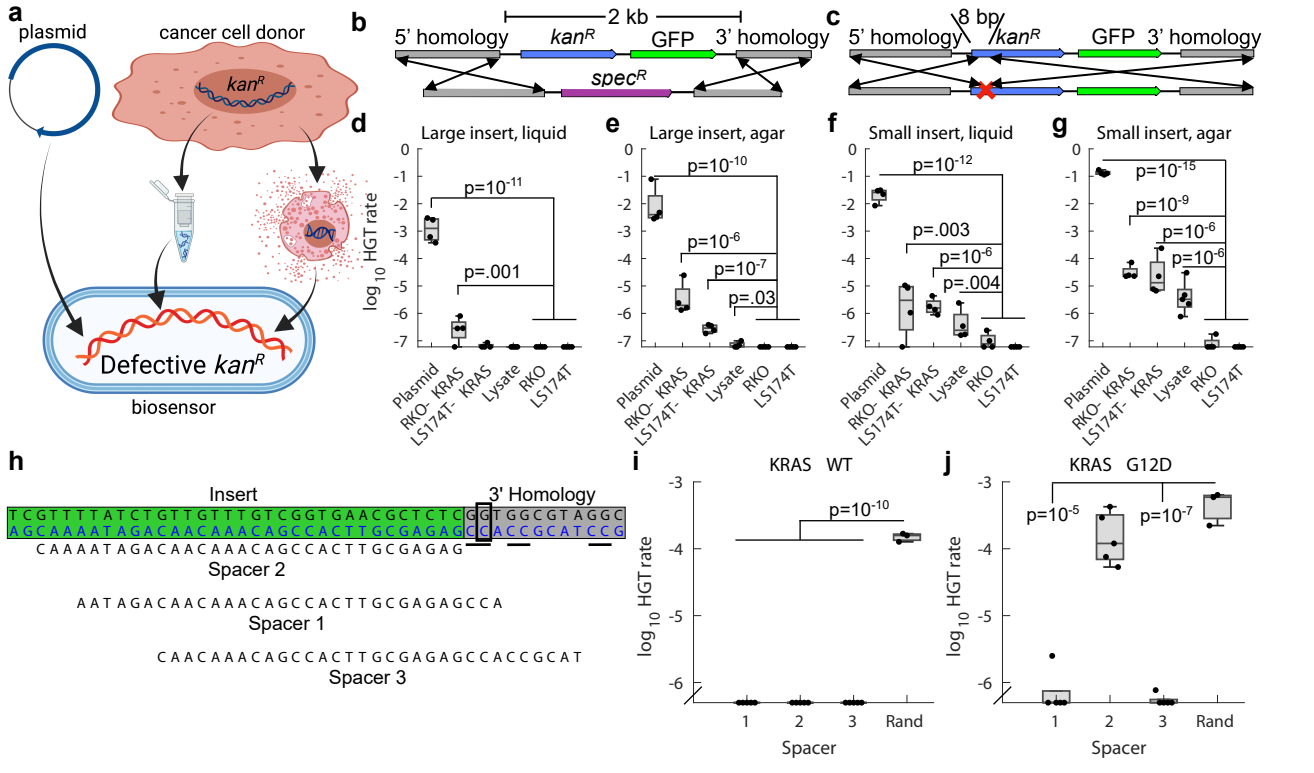

Figure 2: Sensing *KRASG12D* DNA *in vitro*. a-c , Donor DNA consisting of plasmid, purified cancer cell genomic DNA, or raw lysate (top) recombines into biosensor *A. baylyi* cells (bottom), transferring either a large, 2 kb insert (**b**) or a small, 8 bp insert to repair 2 stop codons (**c**) in both cases conferring kanamycin resistance. **d-g**, *A. baylyi* biosensors were incubated with plasmid DNA, purified RKO-*KRAS* or LS174T-*KRAS* genomic DNA, or raw RKO-*KRAS* lysate, all containing the donor cassette, or purified RKO or LS174T genomic DNA as controls. Biosensor cells included either "large insert" (**b,d,e**) or "small insert" (**c,f,g**) designs, and transformations were performed in liquid culture (**d,f**) or on solid agar surfaces (**e,g**). Two-sample t-tests compared data to combined RKO and LS174T genomic DNA controls for the same conditions. **h**, CRISPR spacers targeting the *KRASG12D* mutation (boxed), using the underlined PAMs. **i,j**, Fraction of total biosensor cells expressing the indicated CRISPR spacers that were transformed by plasmid donor DNA with wild type (**i**) or mutant G12D (**j**) *KRAS*. Statistics were obtained using two-sample t-tests. Data points below detection are shown along the x-axis.

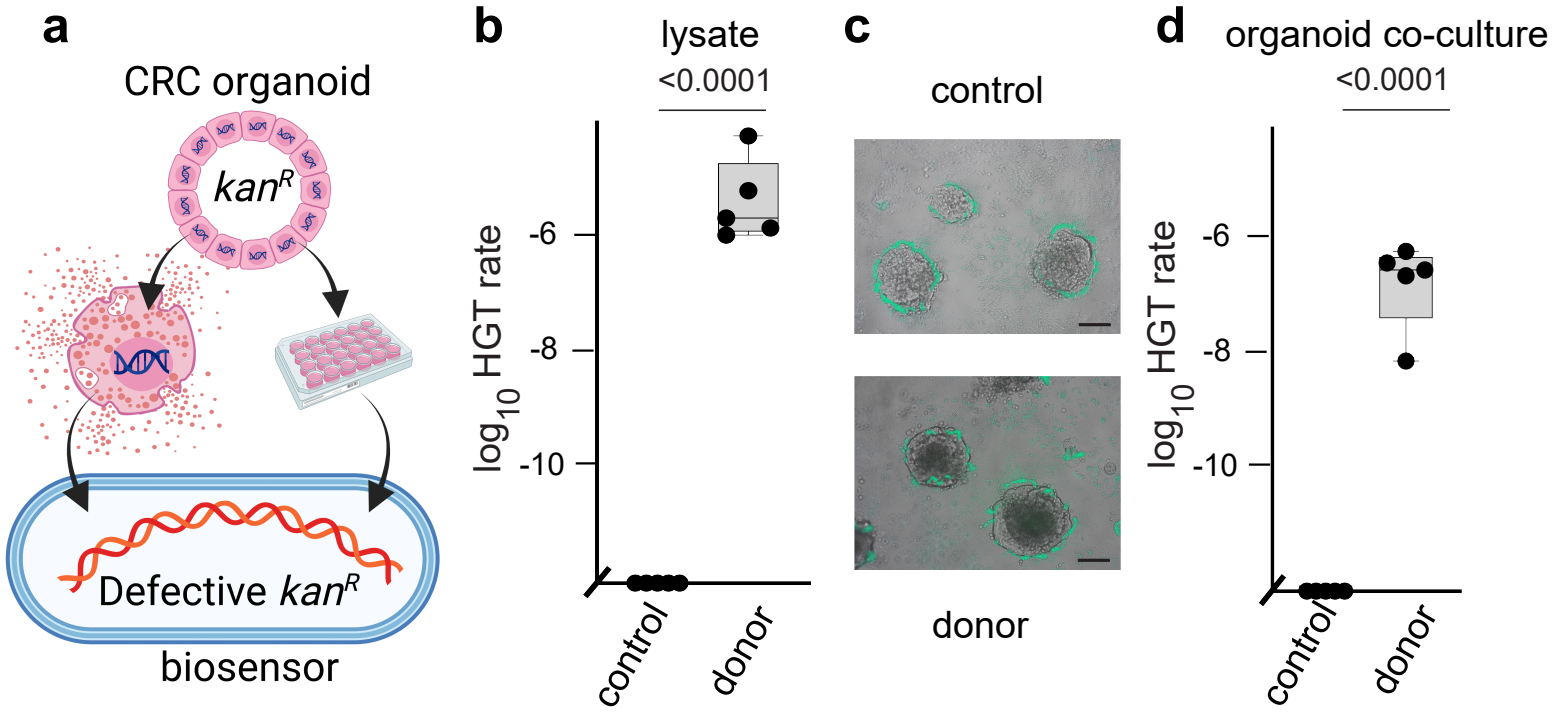

Figure 3: Detection of donor DNA from BTRZI-*KRAS-kan*^{R} organoids.
a, Schema depicting *in vitro* co-culture of *A.baylyi* sensor bacteria with BTRZI-*KRAS-kan*^{R} (CRC donor) organoid lysates or viable organoids to assess HGT repair of kanamycin resistance gene (*kan*^{R}).
b, Recombination with DNA from crude lysates enables growth of *A.baylyi* sensor on kanamycin plates with transformation efficiency of 1.4×10^{-5}. **c**, Representative images of GFP-tagged *A.baylyi* sensor surrounding parental BTRZI (control) and BTRZI-*KRAS-kan*^{R} donor organoids at 24h. Scale bar 100µm. **d**, Co-culture of established CRC BTRZI-*KRAS-kan*^{R} donor organoids with *A.baylyi* sensor enables growth of *A.baylyi* sensor on kanamycin plates with transformation efficiency of 3.8×10^{-7}. In **b**, **d**, n = 5 independent experiments each with 5 technical replicates, one sample t-test on transformed data was used for statistical analysis with P values as indicated.

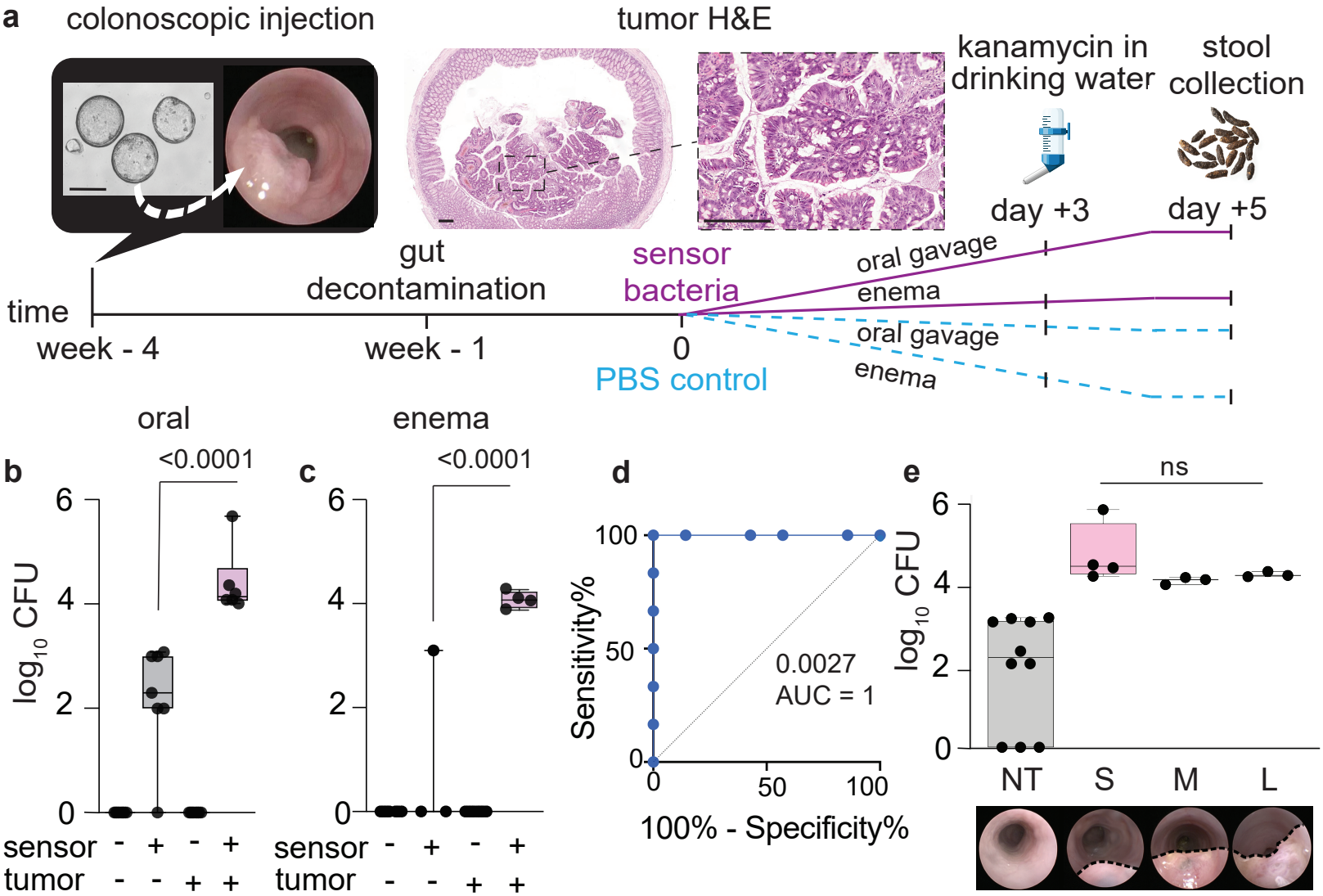

Figure 4. Horizontal gene transfer detected in stool from mice bearing BTRZI-*KRAS-kan*^{R} tumors after oral or rectal dosing of *A. baylyi* sensor bacteria.
a Schema depicting *in vivo* HGT experiments: generation of BTRZI-*KRAS-kan*^{R} (CRC donor) tumors in mice, administration of PBS control or sensor *A. baylyi* and stool collection. Scale bars 200µm. **b**, oral or **c**, rectal delivery of *A. baylyi* sensor to mice bearing CRC donor tumors results in kanamycin resistant *A. baylyi* sensor in stool via HGT. Average CFU per stool from 2-4 stools per mouse grown on Kanamycin selection plates is shown, n=3-8 mice/group. **d**, ROC curve analysis of HGT CFU following oral gavage.**e**, HGT CFU rate in stool was not affected by donor tumor size in recipient mice, as determined by colonoscopic scoring (S small, M medium, L large). In **b**,**c**,**e**, one-way Anova with Tukey's post-hoc on $\log_{10}$ transformed data was used for statistical analysis with P values shown in the corresponding panels.