CANCER DETECTION

Engineered bacteria detect tumor DNA

Robert M. Cooper[1]†, Josephine A. Wright[2]†, Jia Q. Ng[3], Jarrad M. Goyne[2], Nobumi Suzuki[2,3], Young K. Lee[3], Mari Ichinose[2,3], Georgette Radford[3], Feargal J. Ryan[2,4], Shalni Kumar[5], Elaine M. Thomas[3], Laura Vrbanac[3], Rob Knight[6,7,8,9], Susan L. Woods[2,3]*, Daniel L. Worthley[2,10]*, Jeff Hasty[1,5,6,9]*

Synthetic biology has developed sophisticated cellular biosensors to detect and respond to human disease. However, biosensors have not yet been engineered to detect specific extracellular DNA sequences and mutations. Here, we engineered naturally competent *Acinetobacter baylyi* to detect donor DNA from the genomes of colorectal cancer (CRC) cells, organoids, and tumors. We characterized the functionality of the biosensors in vitro with coculture assays and then validated them in vivo with sensor bacteria delivered to mice harboring colorectal tumors. We observed horizontal gene transfer from the tumor to the sensor bacteria in our mouse model of CRC. This cellular assay for targeted, CRISPR-discriminated horizontal gene transfer (CATCH) enables the biodetection of specific cell-free DNA.

Bacterial engineering has allowed the development of living cell diagnostics and therapeutics (*1*–*3*), including microbes that respond to gut inflammation (*4*), intestinal bleeding (*5*), pathogens (*6*), and hypoxic tumors (*7*). Bacteria can access the entire gastrointestinal tract to produce outputs measured in stool (*4*) or urine (*7*). Cellular memory, such as bistable switches (*4*, *8*, *9*) or genomic rearrangements (*10*), enables bacteria to store information over time. Some bacteria are naturally competent for transformation and can sample extracellular DNA directly from their environment (*11*). Natural competence is one mechanism of horizontal gene transfer (HGT), the exchange of genetic material between organisms outside vertical, "parent to offspring" transmission (*12*). HGT is common between microbes (*12*). It may also occur from microbes into animals and plants (*13*) and, in the opposite direction, from eukaryotes to prokaryotes (*14*). The forward engineering of bacteria to detect and respond to mammalian DNA through HGT, however, has not been explored.

Acinetobacter baylyi is a highly competent and well-studied bacterium (*15*, *16*) that is largely nonpathogenic in healthy humans (*17*), can colonize the murine gastrointestinal tract (*18*), and acquires unpurified, environmental DNA from lysed cells (*19*). Our cellular assay for targeted, CRISPR-discriminated horizontal gene transfer (CATCH) strategy delivers bacterial biosensors to sample and genomically integrate target DNA (Fig. 1). To demonstrate this concept, we used the biosensor to detect engineered tumor cells. We then developed genetic circuits to detect natural, nonengineered tumor DNA sequences, discriminating oncogenic mutations at the single-base level. Because the target sequence and output gene are modular, our approach can be generalized to detect arbitrary DNA sequences and respond in a programmable manner.

[1]Synthetic Biology Institute, University of California, San Diego, La Jolla, CA 92093, USA. [2]Precision Cancer Medicine Theme, South Australia Health and Medical Research Institute, Adelaide, SA 5000, Australia. [3]Adelaide Medical School, University of Adelaide, Adelaide, SA 5000, Australia. [4]Flinders Health and Medical Research Institute, Flinders University, Bedford Park, SA 5042, Australia. [5]Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093, USA. [6]Molecular Biology Section, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093, USA. [7]Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093, USA. [8]Department of Computer Science and Engineering, University of California, San Diego, La Jolla, CA 92093, USA. [9]Center for Microbiome Innovation, University of California, San Diego, La Jolla, CA 92093, USA. [10]Colonoscopy Clinic, Brisbane, QLD 4000, Australia.
*Corresponding author. Email: susan.woods@adelaide.edu.au (S.L.W.); dan@colonoscopyclinic.com.au (D.L.W.); hasty@ucsd.edu (J.H.)
†These authors contributed equally to this work.

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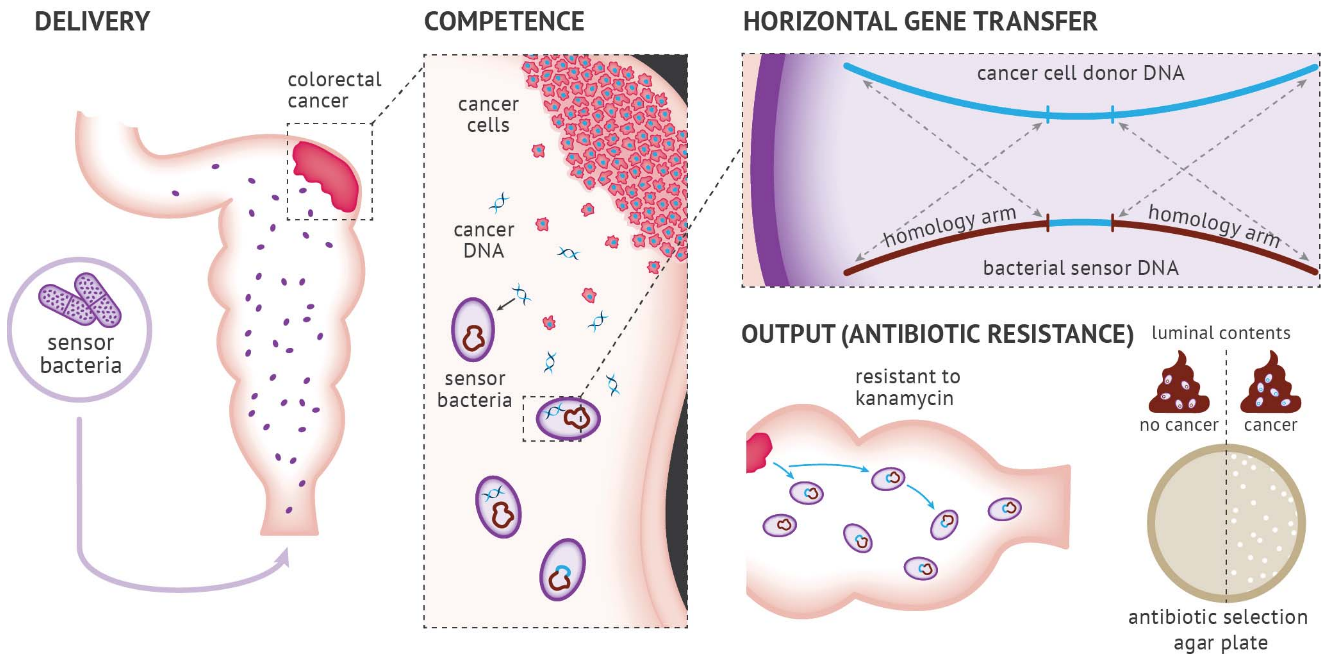

Fig. 1. Engineered bacteria to detect tumor DNA. Engineered *A. baylyi* bacteria are delivered rectally in an orthotopic mouse model of CRC. The naturally competent *A. baylyi* take up tumor DNA shed into the colorectal lumen. The tumor donor DNA is engineered with a *kanR* cassette flanked by *KRAS* homology arms. The sensor bacteria are engineered with matching *KRAS* homology arms that promote homologous recombination. Sensor bacteria that undergo HGT from tumor DNA acquire kanamycin resistance and are quantified from luminal contents by serial dilution on antibiotic selection plates.

Results

Engineering cancer cell lines, organoids, and sensor bacteria

To test the hypothesis that bacteria could detect specific mammalian DNA, we generated transgenic donor human cancer cells with a kanamycin resistance gene (*kanR*) inside *KRAS* homology arms (Figs. 1 and 2, A to C, and figs. S1 and S2). *KRAS* is an important oncogene in human cancer, and a driver mutation in *KRAS* often accompanies the progression of simple into advanced colorectal adenomas (*20*). Our technology is currently confined to the detection of specific sequences and thus for cancer detection is limited to hotspot mutations, such as *KRASG12D*.

We stably transduced this donor cassette into three conventional human colorectal cancer (CRC) cell lines with differing background genetic alterations [RKO is microsatellite instability high (MSI-H), *BRAFV600E*; LS174T is MSI-H, *KRASG12D*; SW620 is microsatellite stable (MSS), *KRASG12V*] and two human CRC organoid lines (RAH057T is MSS, *KRASG12D*; RAH038T is MSI-H, *BRAFV600E*) using a lentiviral vector. To construct the sensor bacteria, we inserted a complementary landing pad with *KRAS* homology arms into *A. baylyi*. We tested both a "large insert" design, where 2 kb of donor cassette must transfer (Fig. 2, A and B; fig. S2A; and data file S1), and a "small insert" design, where only 8 base pairs (bp) must transfer to repair two stop codons (Fig. 2C, fig. S2B, and supplementary materials and methods) (*21*). The initial biosensor output was growth on kanamycin plates (Fig. 2 and fig S2).

Detection of cell-free DNA from cancer cell lines

We tested these 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). As expected (*22*), the "small insert" design improved detection efficiency ~10-fold, reliably detecting even raw lysate (Fig. 2, F and G, and movie S1). Across conditions, detection on solid agar was more efficient than in liquid culture. Notably, these experiments confirmed that the biosensors did not require DNA purification (*19*).

Mutations in codon 12 of *KRAS* occur in 27% of CRC tumors (*23*), accounting for 72% of all CRC *KRAS* mutations (*24*), and are common in solid tumors generally (*25*). To test whether sensor bacteria could discriminate between wild-type and mutant *KRAS* (*KRASG12D*), we used *A. baylyi*'s endogenous type I-F CRISPR-Cas system (*26*). We stably transduced a donor RKO cell line with the *kanR-GFP* (green fluorescent protein) donor cassette flanked by wild-type *KRAS*, and a second line with *KRASG12D* flanking sequences. Next, we designed three CRISPR spacers targeting the wild-type *KRAS* sequence at the location of the *KRASG12D* mutation, using the *A. baylyi* protospacer-adjacent motif (PAM) (Fig. 2H). We inserted these as single-spacer arrays into a neutral locus in the "large insert" *A. baylyi* sensor genome.

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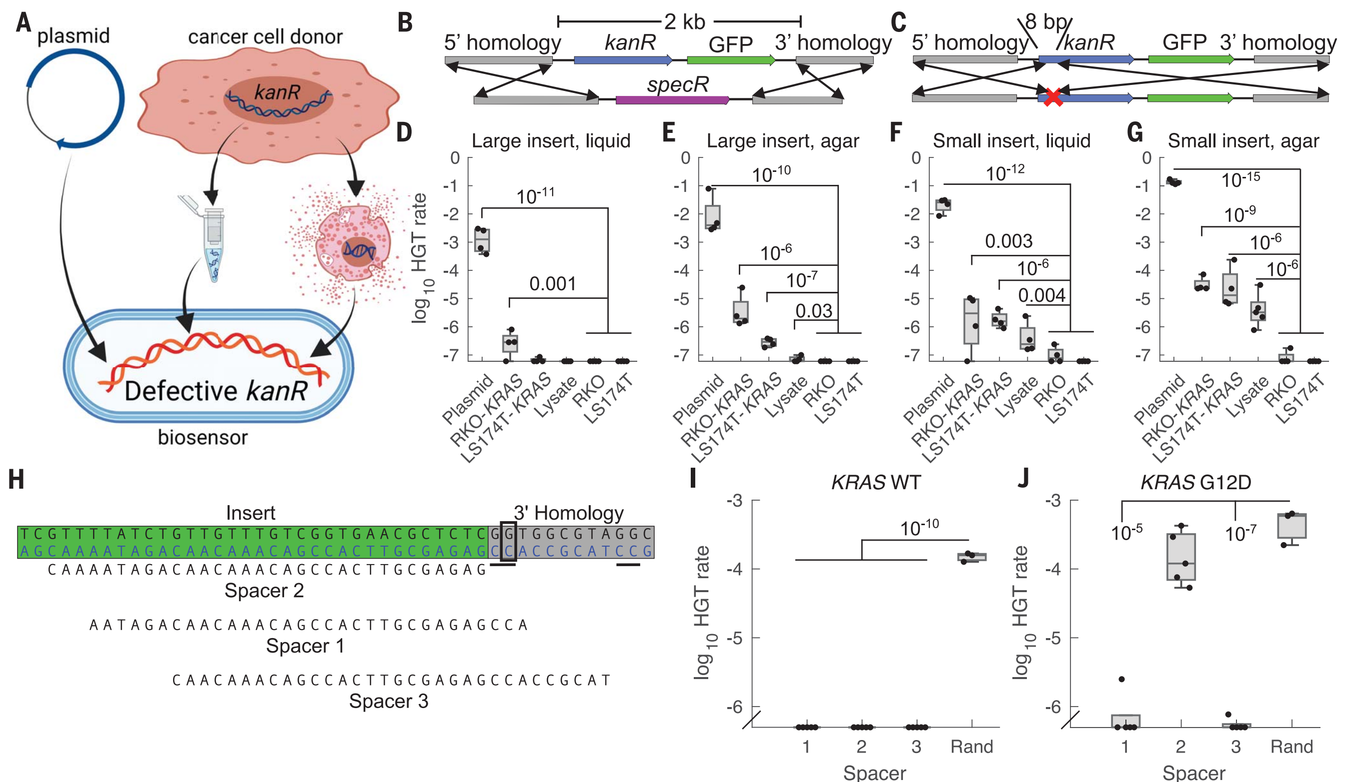

Fig. 2. Sensing *KRASG12D* DNA in vitro. (**A**) Donor DNA was derived from plasmid, purified cancer cell genomic DNA, or raw lysate (top) that recombined into biosensor *A. baylyi* cells (bottom). Horizontal gene transfer included either a large, 2-kb insert (**B**) or a small, 8-bp insert to repair two stop codons (**C**), in both cases conferring kanamycin resistance. (**D** to **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), and (E)] or "small insert" [(C), (F), and (G)] designs, and transformations were performed in liquid culture [(D) and (F)] or on solid agar surfaces [(E) and (G)]. Two sample *t* tests compared data to RKO and LS174T genomic DNA controls under the same conditions. (**H**) CRISPR spacers targeting the *KRAS* G12D mutation (boxed), using the underlined PAMs. (**I** and **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 by using two sample, one-sided *t* tests, with *P* values displayed on the figures. Data points below detection are shown along the *x* axis, at the limit of detection.

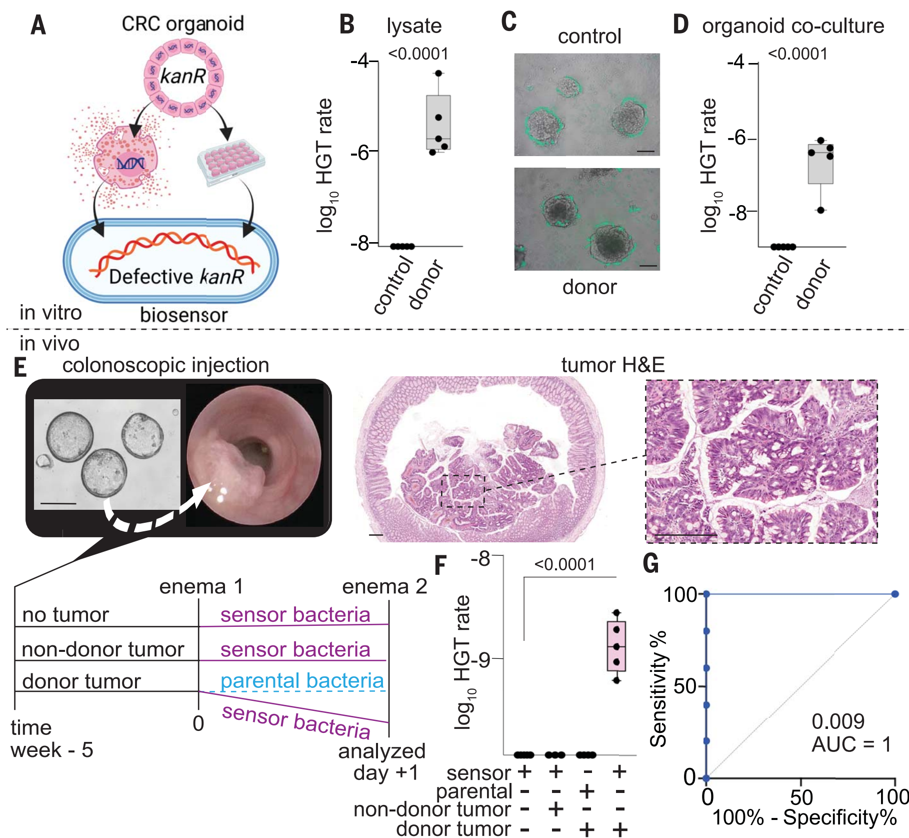

Fig. 3. Detection of donor DNA from BTRZI-*KRAS-kanR* organoids in both an in vitro and an in vivo model of colorectal cancer. (**A**) Schema depicting in vitro coculture of *A. baylyi* sensor bacteria with BTRZI-*KRAS-kanR* (CRC donor) organoid lysates or viable organoids to assess HGT repair of kanamycin resistance gene (*kanR*). (**B**) Recombination with DNA from crude lysates enables growth of *A. baylyi* sensor on kanamycin. (**C**) Representative images of GFP-tagged *A. baylyi* biosensor surrounding parental BTRZI (control) and BTRZI-*KRAS-kanR* donor organoids at 24 hours. Scale bar, 100 μm. (**D**) Coculture of established CRC BTRZI-*KRAS-kanR* donor organoids with *A. baylyi* sensor enables growth of *A. baylyi* sensor on kanamycin. In (B) and (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. (**E**) Schema depicting in vivo HGT experiments: generation of BTRZI-*KRAS-kanR* (CRC donor) tumors in mice through colonoscopic injection, with tumor pathology validated by hematoxylin and eosin (H&E) histology, administration of biosensors, and analysis of luminal contents. Scale bars, 200 μm. (**F**) Rectal delivery of *A. baylyi* biosensor to mice bearing CRC donor tumors results in kanamycin-resistant *A. baylyi* biosensor in luminal contents through HGT with transformation efficiency of 1.5×10^{-9} (limit of detection 1.25×10^{-10}). HGT rate calculated from colony-forming units (CFU) on kanamycin–chloramphenicol–vancomycin (transformants) and chloramphenicol–vancomycin (total *A. baylyi*) selection plates, n = 3 to 5 mice per group. One-way analysis of variance with Tukey's post-hoc on $\log_{10}$-transformed data was used for statistical analysis. (**G**) ROC (receiver operating characteristic) curve analysis of HGT CFU following enema; area under the curve = 1, P = 0.009.

The sensor bacteria should reject wild-type *KRAS* through CRISPR-mediated DNA degradation but allow integration of the *KRASG12D* sequence. Two of the three spacers blocked transformation by both wild-type and mutant DNA (Fig. 2, I and J). However, spacer 2, for which the *KRASG12D* mutation eliminated the PAM site, selectively permitted only *KRASG12D* donor DNA (Fig. 2, I and J). The other common mutations in codon 12 of *KRAS* all eliminate this PAM as well (*23*). Thus, sensor *A. baylyi* can be engineered to detect a mutational hotspot in the *KRAS* gene with single-base specificity.

Detection of cell-free DNA from tumorigenic organoid lines

Next, we 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 (*27*). We transduced BTRZI organoids with the donor DNA construct to generate donor CRC organoids and incubated their lysate with the more efficient "small insert" *A. baylyi* biosensors. Using quantitative polymerase chain reaction (qPCR), we confirmed that the BTRZI organoids that we generated contained only two copies of the target donor DNA (fig. S3). 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 and figs. S4 and S5A). Next, we cocultured GFP-expressing sensor *A. baylyi* with parental or donor organoids for 24 hours on Matrigel. The GFP-expressing sensor bacteria enveloped the organoids (Fig. 3C). After coculture with donor, but not parental, organoids, the *A. baylyi* sensor bacteria acquired donor DNA through HGT (Fig. 3D and fig. S5, B and C). Finally, we estimated the detection limit of our biosensor for target DNA in stool. To achieve this, we added increasing amounts of donor plasmid to a defined mixture of biosensor and stool (5×10^7 biosensor mixed with 0.017 g per 100 μl of stool slurry). The detection limit was 3 pg of plasmid or 2.7×10^5 copies of target DNA, for a given incubation volume and time (fig. S6).

Detection of cell-free tumor DNA in an orthotopic mouse model of colorectal cancer

Given that cancer-to-bacterial HGT occurred in vitro and in the presence of stool, we sought to test the CATCH system in vivo. We first confirmed that our BTRZI, orthotopic CRC model released tumoral DNA into the colorectal lumen. Engineered CRC organoids were injected orthotopically, by mouse colonoscopy, into the mouse colon to form colonic tumors, as previously described (*27*). Using digital droplet PCR, we measured *Braf* mutant tumor DNA in stools collected from tumor-bearing and control mice. The BTRZI model reliably released tumor DNA into the colorectal lumen (fig. S7).

We next conducted an orthotopic CRC experiment (Fig. 3E). Immunodeficient NSG mice were injected with donor or nondonor organoids, or neither. At week 5, once the tumors had grown into the lumen, sensor (or parental) *A. baylyi* bacteria were delivered twice through rectal enema. The mice were subsequently euthanized and the colorectum harvested with the luminal effluent plated for analysis. Serial dilutions were then plated on agar with different antibiotic combinations (Fig. 3F).

HGT from tumors to biosensors was only detected in donor tumor-bearing mice that were administered sensor bacteria. There was no HGT detected in any control group (Fig. 3F). The resistant colonies were confirmed to be the engineered biosensor strain by antibiotic resistance, green fluorescence, 16*S* sequencing, and HGT-mediated *kanR* repair of

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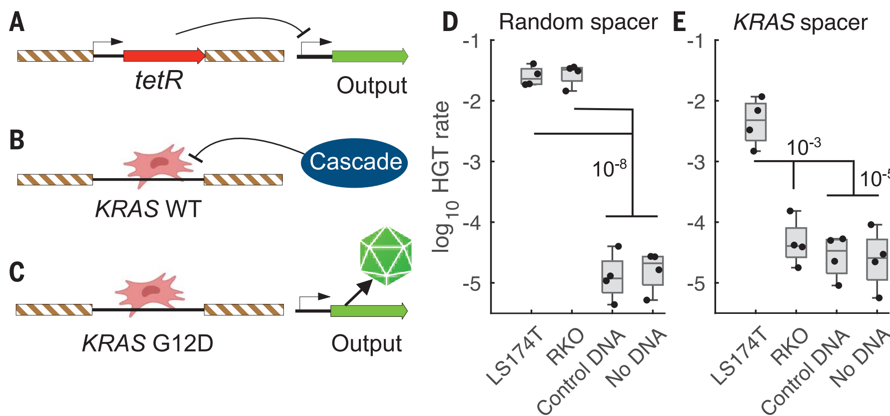

Fig. 4. Detection of nonengineered DNA. (**A**) *tetR* located between the homology arms on the *A. baylyi* genome represses expression of the output gene. (**B**) Target DNA with wild-type *KRAS* sequence is recognized and degraded by the type I-F CRISPR-Cas effector complex, Cascade. (**C**) Target DNA with the *KRASG12D* mutation avoids degradation, replaces *tetR* in the biosensor genome, and relieves repression of the output gene. (**D** and **E**) Fraction of biosensors with either a random CRISPR spacer (D) or a spacer targeting wild-type *KRAS* (E) that detected donor DNA. Statistics were obtained in two sample *t* tests and are displayed on the figure.

individual colonies (fig. S8). Thus, CATCH discriminated mice with and without CRC in our experimental model (Fig. 3G).

Detection of nonengineered DNA

Finally, we designed living biosensors to detect and analyze nonengineered cancer DNA. The *tetR* repressor gene was inserted between the *KRAS* homology arms in the biosensor, and in a second locus, we placed an output gene under control of the P_LtetO-1 promoter (*28*) (Fig. 4A). Here, the output gene was kanamycin resistance for ease of measurement, but the output gene is arbitrary and exchangeable.

In this design, expression of the output gene is constitutively repressed (Fig. 4A). Upon recombination with the *KRAS* target DNA, the repressor *tetR* is deleted from the genome. If the incoming *KRAS* sequence is wild type at the G12 locus, Cascade, the type I-F CRISPR-Cas effector complex, detects and degrades it (Fig. 4B). However, if the G12 locus is mutated, the PAM site and therefore CRISPR-Cas targeting are eliminated, and expression from the output gene turns on (Fig. 4C).

We tested this natural DNA sensor design in vitro using PCR products from LS174T and RKO genomes as donor DNA. Natural DNA biosensors with a random CRISPR spacer detected DNA sequences from both cell lines (Fig. 4D), and biosensors with the *KRAS* spacer accurately detected only DNA sequence from LS174T cells, which contain the *KRASG12D* mutation (Fig. 4E), demonstrating biosensor detection and discrimination of natural target DNA.

Discussion

The sensor bacteria described here demonstrate that a living biosensor can detect specific mammalian DNA shed from CRC in vivo in the gut, with no sample preparation or processing. Engineered donor cassettes are not required for CATCH biosensors to detect, discriminate, and report on target sequences, although the final natural DNA biosensors will need an improved signal-to-background ratio to reliably detect sequences within whole genomic DNA. The homology arms and CRISPR spacers are modular, so this strategy could be readily adapted to detect and analyze arbitrary target sequences of interest.

Our technology is not yet ready for clinical application. This approach requires further development to ensure that future versions, at least those designed for gastrointestinal use, may be delivered orally and achieve sufficient luminal density to allow reliable detection by noninvasive sampling such as in stool or blood. As the technology advances toward clinical care, we will also need to more critically evaluate the performance of CATCH compared to other relevant disease-specific tests such as, in this case, colonoscopy and in vitro nucleic acid assays (*29*, *30*). Further bioengineering is required to limit the risk of biosensors escaping circuit-mediated cell death and to improve the efficiency of natural DNA detection. As our technology progresses, careful analysis is essential to ensure patient safety, to minimize the risk of spreading antibiotic resistance, and to satisfy biocontainment concerns. These necessary next steps are being actively pursued and are important as CATCH is applied to additional preclinical models and before it is trialed in humans.

In vitro DNA analysis helps detect and manage important human diseases, including cancer and infection (*31*). However, in vitro sensing requires potentially invasive removal of samples, and many DNA diagnostics do not achieve clinically relevant sequence resolution, with more advanced techniques remaining too expensive for routine use in all settings (*32*). Direct sampling of the gut in vivo may offer important advantages. The gastrointestinal tract contains marked deoxyribonuclease (DNase) activity (*33*), which limits the lifetime of free DNA in both rodents and humans (*18*, *34*, *35*) and may thus reduce the information content of downstream fecal samples. Bacterial biosensors located in situ could capture and preserve DNA shortly after its release before degradation by local DNases. Perhaps the most exciting aspect of CATCH, however, is that unlike in vitro diagnostics, once target DNA is captured, it could be coupled to direct and genotype-complementary delivery of nanobodies, peptides, or other small molecules for the treatment of cancer or infection (*36*, *37*). CATCH allows for the cellular detection of cell-free DNA and thus may prove useful in future synthetic biology applications, wherever, and whenever, DNA detection and analysis are important.

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Correspondence and requests for materials should be addressed to J.H. (hasty@ucsd.edu), D.W. (dan@colonoscopyclinic.com.au), or S.W. (susan.woods@adelaide.edu.au).
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SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.adf3974
Materials and Methods
Figs. S1 to S8
References (*38*–*41*)
MDAR Reproducibility Checklist
Movie S1
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