Programming bacteria to sense environmental DNA for multiplexed pathogen detection

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ABSTRACT

DNA is a universal and programmable signal of living organisms. Here we developed Cell-based DNA sensors (Cell-Sens) by engineering the naturally competent bacterium *Bacillus subtilis* (*B. subtilis*) to detect specific DNA sequences in the environment. The DNA sensor strains can identify diverse bacterial species including major human pathogens with high specificity and sensitivity. Multiplexed detection of combinations of different species genomic DNA was achieved by coupling the sensing mechanism to orthogonal fluorescent reporters. We demonstrate that the DNA sensors can detect presence of species in a microbial community without requiring DNA purification of the donor species. In sum, the modularity of the living cell-based DNA sensing mechanism and simple measurement procedure could enable programmable DNA sensing for broad applications.

INTRODUCTION

Chemical and electrical signaling in microbial communities play key roles in biofilm development, activation of virulence pathways and symbioses with multicellular organisms[1,2]. These signals can be exploited to control the collective growth or gene expression of the population or mediate interactions between constituent community members[3,4]. For example, circuits have been designed in engineered organisms to sense specific signals produced by pathogens for selective inhibition of growth[5,6]. However, there are limited well-characterized and orthogonal chemical signals systems for building communication networks between strains due to signal crosstalk[7,8]. In addition, there are challenges to engineering these chemical signals for inter-species communication[4,9]. Therefore, new versatile mechanisms are needed for sensing diverse species in microbial communities.

Towards this goal, sensing of bacterial pathogens is a critical and unsolved challenge as new pathogens can emerge[10]. Current methods for pathogen detection include quantitative polymerase chain reaction (qPCR), immunology-based testing, selective culturing, and Next-Generation Sequencing (NGS)[11,12]. Further, diagnostic tools based on CRISPR-Cas nucleases have also been developed[13,14]. While qPCR is sensitive to the concentration of the target sequence, this method requires specialized equipment and trained personnel which may limit its broad deployment. Immunological detection methods have lower sensitivity and specificity than PCR-based techniques but have a faster turnaround time. Due to these limitations, new cost-effective, sensitive, generalizable, and easy to implement pathogen detection methods are needed.

DNA is a universal and programmable biological signal that selectively distinguish among distinct organisms[15]. For example, mammalian immune system can detect the presence of bacterial DNA and execute protective molecular programs[16,17]. We develop a living cell-based sensor (Cell-Sens) by exploiting the natural competence ability of *B. subtilis* to detect unique DNA

sequences present in the environment. The uptake and integration of the programmable target DNA sequence onto the *B. subtilis* genome enables growth of the DNA sensor by eliminating a kill switch via homologous recombination (**Figure 1A**). We demonstrate that Cell-Sens can detect diverse bacteria including major human pathogens (*Escherichia coli* or *E. coli*, *Salmonella typhimurium* or *S. typhimurium*, *Staphylococcus aureus* or *S. aureus*, and *Clostridium difficile* or *C. difficile*)[18–20]. Cell-Sens can detect the presence of donor species in a microbial community, thus obviating the need for purification of DNA from the target species. In addition, we show that Cell-Sens can perform multiplexed detection of purified genomic DNA (gDNA) from multiple species. In sum, Cell-Sens is a low-cost and versatile method for the selective detection of bacteria present in the environment.

RESULTS

Construction of a living DNA sensor strain

To build a living DNA sensor strain, we exploited the natural competence ability of the well characterized soil bacterium *B. subtilis*[21]. The natural competence ability of *B. subtilis* enables the uptake of environmental DNA and integration of specific sequences with sufficient homology into the genome via homologous recombination[22]. The recombination efficiency of homologous sequences depends stringently on the sequence length and percent identity, which can be exploited to build a highly specific DNA sensor[23,24].

To control the ability to enter the natural competence state and enhance transformation efficiency, we introduced a xylose-regulated (P_{xylA}) master regulator of competence *comK* onto the *B. subtilis* genome[25]. To select for the transformed sub-population, we designed a circuit for negative growth selection consisting of a kill switch that is eliminated by homologous recombination with an target DNA sequence. To this end, we constructed a kill switch composed of the Isopropyl β-D-1-thiogalactopyranoside (IPTG) regulated toxin-antitoxin system *txpA-ratA*[26] flanked on the 3' and 5' ends by homology to the target DNA sequence (**Figure S1A**). In the presence of IPTG, *txpA* inhibits cell growth by inducing cell lysis. TxpA is predicted to have activity on the membrane and block cell wall synthesis but the exact mechanism of growth inhibition is unknown. Homologous recombination with the target DNA sequence eliminates the kill switch and the LacI transcriptional repressor. The LacI repressor regulates both the expression of *txpA-ratA* and GFP integrated at a different location in the *B. subtilis* genome (**Figure S1B and S1C**). In the presence of IPTG, the transformed sub-population that has eliminated *txpA-ratA* is able to grow exponentially and amplifies the GFP fluorescence signal (**Figure 1A and S2A**).

Using this system, we constructed a DNA sensor strain to detect the presence of *E. coli* (EC sensor) by introducing homology to the *E. coli* MG1655 *xdhABC* operon, encoding genes for purine catabolism[27]. To characterize the DNA sensor, the EC sensor was exposed to purified *E. coli* genomic DNA (gDNA) in media supplemented with 50 mM xylose to induce natural competence (**Figure 1B**). The transformation frequency of the EC sensor was quantified by antibiotic selection in liquid or solid media in the presence of IPTG. We investigated the transformation frequency of the EC sensor as a function of homology length to the target DNA sequence by constructing a set of EC sensor strains that varied in length of homology to the target DNA sequence. The transformation frequencies of EC sensors with greater than 1 kbp homology on the 3' and 5' ends of the kill switch were higher in the presence of *E. coli* gDNA than in the absence of gDNA (**Figure 1C**). In addition, transformation frequency increased with the homology length (**Figure 1C and Figure S2B**).

A moderate number of transformants was observed in the absence of gDNA and the transformation frequency in this condition did not vary with the EC sensor homology length. These transformants consisted of both GFP OFF and ON colonies due to mutations in either *txpA* and/or *lacI* (**Figure S3**). The GFP ON colonies contained mutations in *txpA*, suggesting that these mutations reduced the growth inhibitory activity of TxpA. The GFP OFF colonies contained mutations in *lacI* near the ligand-binding site, suggesting that they may reduce binding affinity of IPTG to LacI, which in turn could reduce the expression of TxpA and GFP[28,29]. Using the 2.5 kbp EC sensor that maximized the DNA sensor transformation frequency, we found that the transformation frequency increased and saturated by 10 hr (**Figure 1D and S2A**).

Building living DNA sensors to sense major pathogens

Since the target DNA sequences are programmable and modular, we constructed DNA sensors to detect the opportunistic intestinal pathogens *Salmonella typhimurium*[30] (*S. typhimurium*) or *Clostridioides difficile*[31] (*C. difficile*) or skin pathogen *Staphylococcus aureus*[32] (*S. aureus*). We introduced 2.5 kbp sequences homologous to the pathogenicity island *sipBCDA* in *S. typhimurium* (ST sensor), the heme biosynthesis pathway *hemEH* in *S. aureus* (SA sensor), or phenylalanyl-tRNA synthetase *pheST* in *C. difficile* (CD sensor)[33–35]. The target sequences used to construct each pathogen DNA sensor were either linked to virulence activities of the pathogen or critical for fitness.

The SA sensor transformation frequency was approximately one order of magnitude higher than the other sensor strains, suggesting that the identity of the target sequence in addition to the homology length determines the transformation frequency for each DNA sensor (**Figure**

2A). To test the specificity of the sensors, we performed time-series measurements of absorbance at 600 nm (OD600) and GFP for each sensor in the presence of individual gDNA (100 ng mL^{-1}) from each donor species (**Figure S4**). To quantify the specificity of each sensor strain for the gDNA with homology, we analyzed GFP expression at 12 hr using a fluorescence plate reader (**Figure 2B**). Our results showed significantly higher GFP expression at 12 hr in the presence of the gDNA with homology to the given DNA sensor strain than in the absence of gDNA (p-value<0.01). By contrast, the GFP signal in the presence of gDNA without homology to the given DNA sensor strain was not significantly different from the negative control lacking gDNA except for the SA sensor in the presence of *S. typhimurium* gDNA (p-value=0.0415). These data indicate that each DNA sensor strain has high specificity to the correct target DNA sequence.

We evaluated the sensitivity of each sensor strain to individual gDNA with homology by quantifying the temporal response of each DNA sensor strain to a range of gDNA concentrations (**Figure S5**). The GFP response time decreased with gDNA concentration for all sensor strains and the steady-state GFP expression increased with gDNA concentration for the EC, ST and SA sensor strains (**Figure 2C-2F**). We determined the sensitivity of each DNA sensor strain to the target sequence by evaluating the statistical significance of the difference in GFP expression at 12 hr with the negative control lacking gDNA (**Figure 2G-2J**). Due to the variation in the temporal response of each sensor strain to its target sequence, we analyzed the GFP expression in the saturated fluorescence regime at 12 hr for each sensor strain. Our results showed that the EC and ST sensors displayed the highest sensitivity of 1 ng mL^{-1} gDNA, whereas the sensitivities of the SA and CD sensors were lower (4 ng/mL and 62.5 ng mL^{-1}, respectively). The observed sensitivities of the EC and ST sensors to the target sequence are sufficient for detection of DNA release in cell culture or cell extracts (**Figure 2K and S6**)[36–38].

We compared sensitivities of Cell-Sens to qPCR using primers that anneal to each target sequence. Our results showed that qPCR was approximately one order of magnitude more sensitive than the EC and ST sensors with the highest sensitivity to the target sequence. In sum, each sensor displayed high sensitivity to the target DNA sequence except for the CD sensor which displayed moderate sensitivity. Growth of the non-transformed sub-population and GFP ON escape mutants in liquid culture contributed to the GFP background signal (**Figure S4 and S5**), which in turn may reduce the sensitivity and specificity of Cell-Sens.

Cell-Sens can perform multiplexed detection of bacterial genomic DNA

To determine whether the sensor strains could detect gDNA from multiple donor species simultaneously, we used three orthogonal fluorescent reporters including the EC-GFP (EC-G),

ST-RFP (ST-R) and SA-BFP (SA-B) sensor strains. We introduced all combinations of gDNA (100 ng mL^{-1}) from the pathogen donor species into a mixed culture containing EC-G, ST-R and SA-B. The mixed culture was transferred onto agar plates and the fluorescent imaging was used to determine the number of RFP, BFP and GFP colonies in each condition (**Figure 3A, 3B, and S7**). We found that the mixed DNA sensor culture could accurately detect the presence and absence of each donor species gDNA across all conditions (**Figure 3C-E**). In sum, a mixture of DNA sensor strains each individually labeled with a unique fluorescent reporter enabled accurate detection of all possible combinations of donor species gDNA derived from diverse bacterial pathogens.

Cell-Sens can detect diverse donor species in co-culture

Specific bacterial species have been shown to release extracellular DNA (eDNA) in response to certain environmental stimuli but the mechanisms leading to DNA release and prevalence of DNA release in microbial communities are unknown[39]. To determine if the DNA sensors could detect the presence of the donor cells without performing a DNA extraction, we performed high-throughput co-culture experiments by combining each DNA sensor strain with its corresponding donor species into a single culture. The cultures contained the antibiotic spectinomycin which specifically inhibited the growth of the donor species and not the DNA sensor strain to reduce ecological competition (**Figure 4A**). Following 10 hr, the transformed DNA sensor sub-populations were selected on agar plates containing antibiotics that inhibit growth of the donor species and IPTG. By quantifying the fluorescence intensities of the colonies, we demonstrated that all four DNA sensors can detect the presence each donor species in co-culture without requiring gDNA purification (**Figure 4B-4E and S8**).

The fluorescence intensity of each DNA sensor strain co-cultured with individual donor species that did not have homology to the given DNA sensor strain was similar to the negative control lacking gDNA (**Figure 4B-4E)**. In addition, the fluorescence intensity of the EC and CD sensors co-cultured with each corresponding donor species with homology displayed similar fluorescence levels to the positive control (PC) supplemented with purified gDNA containing the target sequence (100 ng mL^{-1}), suggesting that the transformation was efficient in co-culture (**Figure 4B and 4E)**. Time-series eDNA measurements using qPCR demonstrated that each donor species released eDNA into the environment (**Figure S9A**). The eDNA concentration released by each donor species in co-culture with the individual DNA sensor strains ranged between 0.5 ng mL^{-1} for *S. aureus* to 42 ng mL^{-1} for *C. difficile* after 10 hr and was thus lower than the concentration of purified gDNA used to characterize the DNA sensor strains (**Figure S9A**). Therefore, the SA sensor was able to detect a lower concentration of gDNA in co-culture with *S.*

aureus than in monoculture supplemented with *S. aureus* gDNA, indicating that the presence of certain donor species can enhance the sensitivity of Cell-Sens (**Figure 2I and 4D**)[40]. To determine if the DNA sensing mechanism occurred via natural competence, we introduced DNase I into the co-culture containing each donor species and the corresponding DNA sensor strain. The presence of DNase I reduced the transformation frequency to a similar background fluorescence level of each DNA sensor as the negative control lacking gDNA (**Figure S9B**). In sum, our results demonstrate that Cell-Sens can accurately detect the presence of diverse species in a microbial community.

DISCUSSION

By exploiting the natural competence ability of *B. subtilis*, we developed a cell-based DNA sensor to selectively detect programmable DNA sequences in the environment. We demonstrated that the DNA sensors achieved high sensitivity to the concentration of target DNA and specificity for diverse bacteria including major human pathogens. Cell-Sens requires a simple procedure to detect a given target sequence. First, purified DNA or donor cells are introduced into a culture containing the DNA sensor strain(s), incubated for a period and then readouts include colony forming units (CFU) counting or fluorescence measurements. This workflow could be parallelized using high-throughput automation techniques to simultaneously process many samples[41]. In addition, the design of the DNA sensor is modular since any target sequence of interest can be used.

Since homologous recombination has stringent requirements for target sequence length and percent identity, Cell-Sens could potentially achieve higher specificity than standard techniques such as PCR due to off-target primer annealing[42]. In addition, new DNA sensor strains can be constructed and tested within week timescales for emerging pathogens. Finally, the unique properties of *B. subtilis* enables detection of donor cells without DNA purification. Since DNA released in co-culture may be more easily taken up and/or recombined onto the recipient genome[43], the sensitivity of Cell-Sens may be enhanced in a microbial community containing the target bacterial species than purified DNA.

The performance of the DNA sensor mechanism could be further optimized for real-world applications. To reduce the background signal, proteins promoting mutagenesis such as *mfd* can be deleted to reduce the number of escaped mutants once *txpA* is overexpressed[44]. Combining different selection mechanisms such as toxin overexpression and essential protein degradation through proteolysis tagging could also improve the strength of negative selection of the non-transformed sub-population[45]. In addition, GFP could be expressed from a unique promoter

controlled by an orthogonal transcriptional repressor to directly couple the expression of GFP to homologous recombination with the target DNA sequence. To achieve autonomous sensing without inducer induction, the kill switch may be coupled to a quorum-sensing systems such as *agrBDCA* so that non-transformed cells will be eliminated after the population reaches a threshold density allowing only the transformed sub-population to survive[46]. Due to the transformation efficiency and doubling time of the cells, the total experimental time for Cell-Sens to perform detection of a target DNA sequence was approximately one day. To speed up the DNA detection workflow, flow cytometry or fluorescence microscopy could be used to quantify fluorescent cells within hours following transformation before selection[47]. Future work will investigate the generalizability of the DNA sensor to sensing other organisms beyond bacteria, including viruses, fungi or cancer cells[48].

MATERIALS AND METHODS

Plasmid and strain construction

All sensor strains were derived from *B. subtilis* PY79. The pAX01-comK plasmid was used to introduce P_{xyl}-*comK* at the *lacA* locus in *B. subtilis* PY79 to enhance the transformation efficiency in Luria broth (LB)[25,43]. Reporter plasmids (pOSV00170, pOSV00455 and pOSV00456) were constructed to introduce $P_{hyperspank}$-*gfp*, $P_{hyperspank}$-*rfp*, or $P_{hyperspank}$-*bfp* at the *ycgO* locus[21,49] (**Table S1**). The kill switch plasmid pOSV00157 was composed of *lacI*, $P_{hyperspank}$-*txpA*-*ratA*, flanked by two target sequences, and was introduced at the *amyE* locus. The toxin-antitoxin system *txpA*-*ratA* was amplified from purified gDNA of *B. subtilis* 168 by PCR and cloned onto the *amyE* integration plasmid creating pOSV00157. The target sequences *xdhABC* (Location: 3001505-3004004 and 3004005-3006504) were PCR amplified from the purified gDNA of *E. coli* MG1655 (NCBI Reference Sequence: NC_000913.3), *sipBCDA* (Location: 3025979-3028478 and 3028479-3030978) from *Salmonella enterica* serovar Typhimurium LT2 ATCC 700720 (NCBI Reference Sequence: NC_003197.2), *hemEH* (Location: 553-2770 and 2864-5638) from *S. aureus* DSM 2569 (GenBank: LHUS02000002.1), and *pheST* (Location: 770923-773144 and 773157-775686) from *C. difficile* DSM 27147 (GenBank: FN545816.1). These sequences were used to construct a set of plasmids (pOSV00169, pOSV00205, pOSV00206, pOSV00207, pOS00208, pOSV00292, pOSV00459 and pOSV00475) using restriction enzymes BamHI-HF (New England Biolabs) and EcoRI-HF (New England Biolabs) or Golden Gate Assembly Mix (New England Biolabs). A nucleotide BLAST search (megablast with default setting) was used to determine if the target sequences were unique to each of the species used in our study.

Plasmids were cloned in *E. coli* DH5α and transformed into *B. subtilis* using MC medium[50]. Plasmids were extracted using Plasmid Miniprep Kit (Qiagen). MC medium is composed of 10.7 g/L potassium phosphate dibasic (Chem-Impex International), 5.2 g/L potassium phosphate monobasic (MilliporeSigma), 20 g/L glucose (MilliporeSigma), 0.88 g/L sodium citrate dihydrate (MilliporeSigma), 0.022 g/L ferric ammonium citrate (MilliporeSigma), 1 g/L Oxoid casein hydrolysate (Thermo Fisher Scientific), 2.2 g/L potassium L-glutamate (MilliporeSigma), and 20 mM magnesium sulfate (MilliporeSigma). *B. subtilis*, *E. coli*, *S. typhimurium*, and *S. aureus* were cultured in Lennox LB medium (MilliporeSigma) for gDNA extraction. *C. difficile* was cultured in YBHI medium in an anaerobic chamber (Coy Laboratory). YBHI medium is Brain-Heart Infusion Medium (Acumedia Lab) supplemented with 0.5% Bacto Yeast Extract (Thermo Fisher Scientific), 1 mg/mL D-Cellobiose (MilliporeSigma), 1 mg/mL D-maltose (MilliporeSigma), and 0.5 mg/mL L-cysteine (MilliporeSigma). The gDNA from each species was extracted using DNeasy Blood & Tissue Kit (Qiagen). For *S. aureus* gDNA extraction used for PCR target sequence or transformation experiments, 0.1 mg/mL Lysostaphin (MilliporeSigma) was added in the pretreatment step. For building the DNA sensor strains, a double crossover and thus stable integration of each plasmid introduced into *B. subtilis* PY79 was confirmed by the replacement of a different antibiotic resistance gene at the integration locus. gDNA of modified *B. subtilis* was then extracted and transformed into another modified *B. subtilis* to introduce multiple modifications into the genome. Bacterial strains are listed in **Table S2**. DNA sequences of genetic parts are listed in **Table S3**.

gDNA detection experiments

The DNA sensor strains were inoculated from a -80 °C glycerol stock into LB with 100 µg/mL spectinomycin and incubated at 37 °C with shaking (250 rpm) for 14 hours. The OD600 was measured by NanoDrop One (Thermo Fisher Scientific) and then the DNA sensor strains then diluted to OD600 of 0.1 in 1 mL LB in 14 mL Falcon™ Round-Bottom Tube (Thermo Fisher Scientific) supplemented with 50 mM xylose (Thermo Fisher Scientific) and 100 µg/mL spectinomycin (Dot Scientific). Xylose was added to induce the competence and spectinomycin was used to prevent the contamination of other bacteria. Purified gDNA from each donor species was quantified by a Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific) and added to the cultures with known concentration. The positive control in all experiments was 100 ng/mL gDNA from a given donor species. The DNA sensor strains cultures were incubated at 37°C with shaking (250 rpm) for 10 hours and then transferred to liquid LB with a 1:20 dilution or 20 µL was plated on an LB plate for CFU counting. The liquid or solid LB media was supplemented with 2 mM IPTG

(Bioline), 5 μg/mL chloramphenicol (MilliporeSigma), and MLS (1 μg/mL erythromycin from Sigma-Aldrich and 25 μg/mL lincomycin from Thermo Fisher Scientific). In liquid culture, OD600 and fluorescence were measured using a SPARK Multimode Microplate Reader (TECAN). In these experiments, 100 uL of the 1:20 diluted cultures of transformed DNA sensors were transferred to 96-well black and clear-bottom CELLSTAR® format sterile cell culture microplates (Greiner Bio-One). Plates were sealed with Breathe-Easy Adhesive Microplate Seals (Thermo Fisher Scientific) and incubated in the microplate reader at 37 °C with shaking for time-series OD and GFP measurements. On agar plate, CFU of transformed *B. subtilis* and total *B. subtilis* were counted for both fluorescent and non-fluorescent colonies. To determine the CFU of transformed *B. subtilis*, 20 μL of cell culture was plated on LB agar plate with 2 mM IPTG, 5 μg/mL chloramphenicol and MLS. To determine the CFU of total *B. subtilis*, cell culture was diluted in phosphate-buffered saline (PBS) (Dot Scientific) and plated on LB agar plate with 5 μg/mL chloramphenicol and MLS. Colonies were imaged using an Azure Imaging System 300 (Azure Biosystems) using Epi Blue LED Light Imaging with 50 millisecond exposure time to determine the fluorescence expression. Transformation frequency was defined as the ratio of fluorescent or non-fluorescent transformed *B. subtilis* to the total number of *B. subtilis* in **Figure 1C and 1D**. Transformation frequency was defined as the ratio of fluorescent transformed *B. subtilis* to the total number of *B. subtilis* in **Figure 2A and S9B**.

Multiplexed gDNA detection experiment

The DNA sensor strains EC-G, ST-R, and SA-B sensors were diluted to an OD600 of 0.1, 0.1, and 0.01, respectively, in 1 mL LB supplemented with 50 mM xylose to induce expression of *comK* (Thermo Fisher Scientific) and 100 μg/mL spectinomycin (Dot Scientific). Different combinations of 100 ng/mL purified gDNA from each donor species were introduced into a mixed culture containing different DNA sensor strains (EC-G, ST-R and SA-B). The DNA sensor strain mixtures were incubated at 37 °C with shaking (250 rpm) for 10 hours and 5 μL of each culture was plated on 6-well plates (Thermo Fisher Scientific). The 6-well plates were large enough to visualize approximately a hundred colonies in each well and could be imaged by the Nikon Ti II Microscope. In these plates, each well contained solidified LB agar supplemented with 2 mM IPTG, 5 μg/mL chloramphenicol, and MLS. These plates were incubated at 37 °C overnight.

On the next day, each well was imaged using a Nikon Eclipse Ti2-E Microscope. Brightfield images were collected at 4X magnification using the built-in transilluminator of the microscope. The epifluorescence light source was the SOLA-III Light Engine (Lumencor) and standard band filter cubes (Nikon) including DAPI (Excitation: 375/28, Emission: 460/50), FITC

(Excitation: 480/30, Emission: 535/40) and TRITC (Excitation: 540/25, Emission: 605/55) filter cubes were used to image BFP, GFP, and RFP, respectively. The exposure times for BFP, GFP, RFP were 500 ms, 9 ms, and 50 ms, respectively. Since the DAPI filter was not optimized for BFP, a strong BFP signal was observed from the LB agar. To reduce the autofluorescence from LB agar, images were processed as follows: complete images of each LB agar well were generated from multipoint images using the stitching function in the Nikon NIS Elements software. Once the full images were assembled, each of the four channels were mapped to unity by the minimum and maximum pixel values of all images for that channel using ImageJ. Fluorescence channels were further processed to remove background signal from agar fluorescence by masking out all signals not associated with a colony. Masks for this step were generated from the DIC images using the pixel classification tool in Ilastik[51]. Any remaining noise in the fluorescence channels was reduced by clipping each image by the average noise value for that channel.

Co-culture experiments

E. coli, *S. typhimurium*, and *S. aureus* and the corresponding DNA sensor strains were each inoculated from a -80 °C glycerol stock into LB media and incubated at 37 °C with shaking (250 rpm) overnight. *C. difficile* was cultured in YBHI medium in an anaerobic chamber (Coy Laboratory). On the next day, the cultures were diluted to an OD600 of 0.1 each in 100 µL LB supplemented with 50 mM xylose and 100 µg/mL spectinomycin into 96-well plates at 37 °C with shaking (250 rpm). Overnight cultures of target bacteria were also diluted in PBS (Dot Scientific) and plated onto an LB or YBHI agar plate to determine the initial CFU of target bacteria in the co-culture, which was 2.54×10^6 cells per mL, 1.19×10^7 cells per mL, 1.22×10^7 cells per mL, and 1.05×10^6 cells per mL for *E. coli*, *S. typhimurium*, *S. aureus*, and *C. difficile*, respectively. Following 10 hours of incubation, cell culture were diluted into PBS with 1:10 dilution and 10 uL diluted cell culture were spotted on a large LB agar plate (140 mm diameter and 20 mm height petri dish, Thermo Fisher Scientific). The LB agar was supplemented with 2 mM IPTG, 5 µg/mL chloramphenicol, and MLS and incubated overnight at 37 °C. On the next day, each plate was imaged with an Azure Imaging System 300 (Azure Biosystems) using the Epi Blue LED Light Imaging with 50 millisecond exposure time. The pixel fluorescence intensity of the colony spots was quantified by ImageJ[52].

To determine if the detection of target bacteria was via transformation, 1 unit/mL DNase I (Thermo Fisher Scientific) was added to the 1 mL of each co-culture and CFU of both transformed *B. subtilis* and total *B. subtilis* were counted to calculate the transformation frequency change. To quantify the extracellular DNA (eDNA), a given DNA sensor strain and donor species were diluted

into OD0.1 each in 3 mL LB containing 50 mM xylose and 100 μg/mL spectinomycin and incubated at 37 °C with shaking (250 rpm) for 10 h. Because the sensor also contained the same DNA sequence targeted by the qPCR primers, to quantify the eDNA released from only the target bacteria but not the sensor, a sensor strain (msOSV00409) without homologous sequence was used in the co-culture instead. At every two hours, supernatants were collected and filtered with 0.2 μm Whatman Puradisc Polyethersulfone Syringe Filter (GE Healthcare) to remove the cells, and stored in the -20 °C freezer for qPCR measurements.

qPCR measurements

To determine the detection limit of qPCR, purified gDNA from *E. coli*, *S. typhimurium*, *S. aureus*, or *C. difficile* was extracted using DNeasy Blood & Tissue Kit (Qiagen) and quantified by Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific). The purified gDNA samples were serially diluted using a 1:10 dilution from 10 μg/mL to 10^{-5} μg/mL and mixed with 2X SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) and 500 nM of each primer (**Table S4**). Primers were designed and synthetized by Integrated DNA Technologies to target the sequences used to construct each DNA sensor strain. The qPCR reactions were performed on the CFX Connect Real-Time PCR Detection System (Bio-Rad). The gDNA concentration produced at least 95% positive replicates (no missing Ct values) is defined as the detection limit[53]. To quantify the rate of eDNA release of *E. coli*, *S. typhimurium*, *S. aureus*, or *C. difficile* in each co-culture, gDNA standards previously quantified by a Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific) were included in the qPCR run and the gDNA concentration of the supernatant collected at different time points was inferred from the standard curve based on a titration of purified gDNA.

DATA AVAILABILITY

All data presented in this manuscript will be available from Zenodo. Plasmids and strains are available upon request.

CODE AVAILABILITY

Code for data analysis will be available from Github. The microscopic images of multiplexed detection were processed by ImageJ and ilastik to reduce the background fluorescence from the agar using the basic features such as segmentation and pixel classification and no code was generated in this process.

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AUTHOR CONTRIBUTIONS

Y.Y.C. and O.S.V. conceived the research. Y.Y.C., and Z.C. performed the experiments. Y.Y.C., Z.C., and X.C. constructed the sensors. T.D.R. developed custom code for data analysis. T.G.F. assisted with the strain construction. Y.Y.C. and O.S.V. wrote the manuscript. O.S.V., B.M.B., T.G.F., and Z.C. analyzed the data. All authors provided feedback on the manuscript. O.S.V. and B.M.B. secured the funding.

COMPETING INTERESTS

O.S.V., Y.Y.C. and Z.C. are inventors on a provisional patent application filed by the Wisconsin Alumni Research Foundation (WARF) with the US Patent and Trademark Office, which describes and claims concepts disclosed herein (Application No. 63/290,442 Filing Date: 12/16/2021). All other authors declare no conflict of interest.

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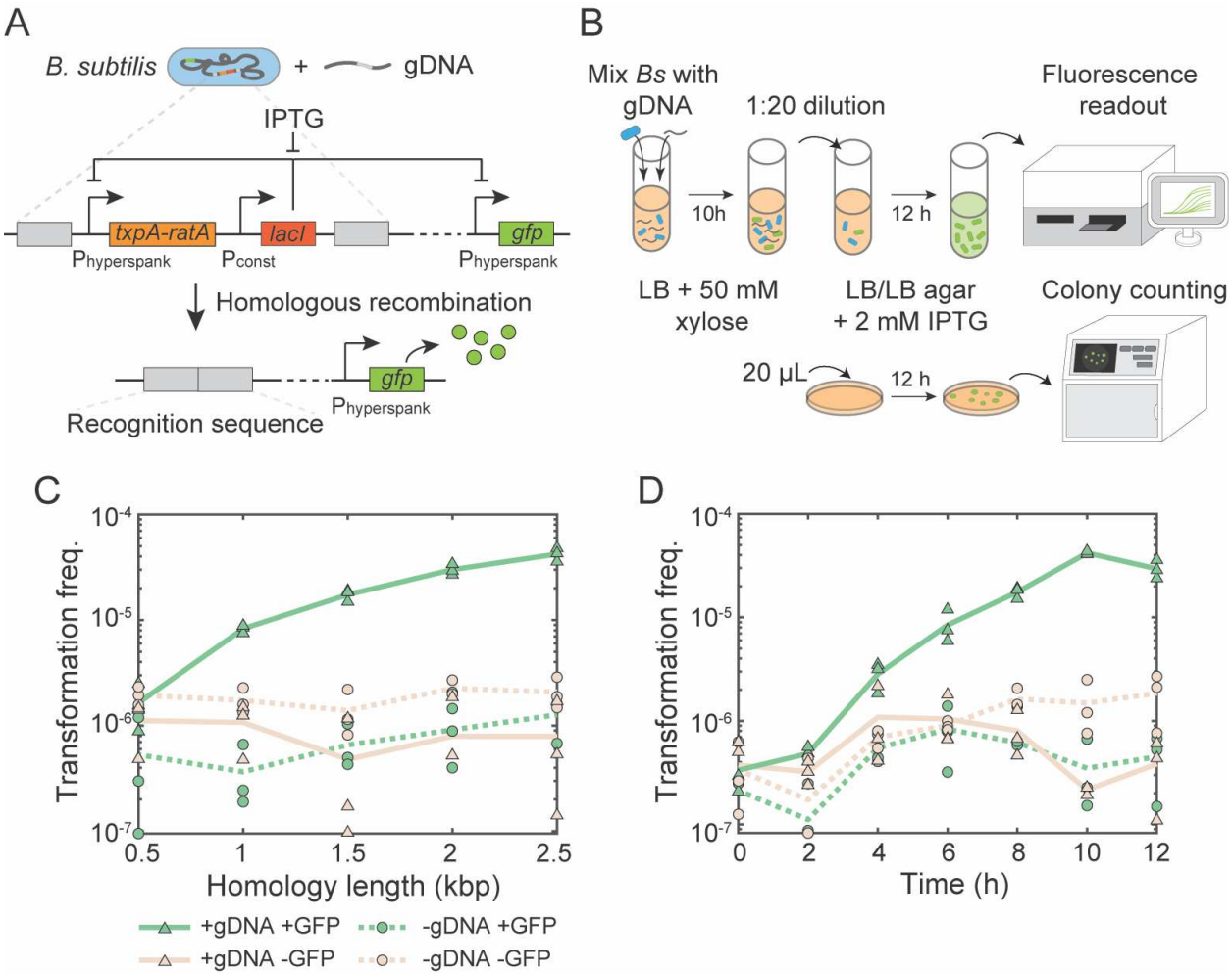

Figure 1. Construction and characterization of a cell-based DNA sensor (Cell-Sens). (A) Schematic of a synthetic genetic circuit in *Bacillus subtilis* that can specifically recognize programmable target DNA sequences. The gene expression of the toxin-antitoxin system *txpA-ratA* and a fluorescent reporter *gfp* integrated onto the genome in a different position are regulated by the IPTG-inducible $P_{hyperspank}$ promoter. In the presence of the target DNA sequence, DNA uptake and homologous recombination via natural competence can eliminate the toxin and the LacI repressor. Consequently, growth of the GFP expressing transformed sub-population is permitted in the presence of IPTG and the target DNA sequence. **(B)** Schematic of the purified DNA detection procedure for Cell-Sens. The DNA sensor is inoculated into a liquid culture containing the target DNA sequence. Xylose controls the competence state of *B. subtilis* and enhances transformation efficiency by inducing the expression of the master regulator of competence *comK*. The fluorescence output of the DNA sensor can be quantified in liquid culture or solid media supplemented with IPTG, which induces expression of the kill switch and GFP and thus selects for the transformed sub-population. **(C)** Line plot of homology length versus transformation frequency for the *E. coli* DNA sensor strain targeting sequences in *xdhABC* in the presence (solid lines) and absence (dashed lines) of 100 ng/mL purified *E. coli* genomic DNA. Kill switch escape mutants that can grow in the presence of IPTG and absence of gDNA either express GFP (triangles) or are not fluorescent (circles). Data points represent biological replicates and lines are the average of three biological replicates. **(D)** Line plot of time versus transformation

536 frequency for the *E. coli* DNA sensor strain. Data points represent biological replicates and lines
537 are the average of three biological replicates.

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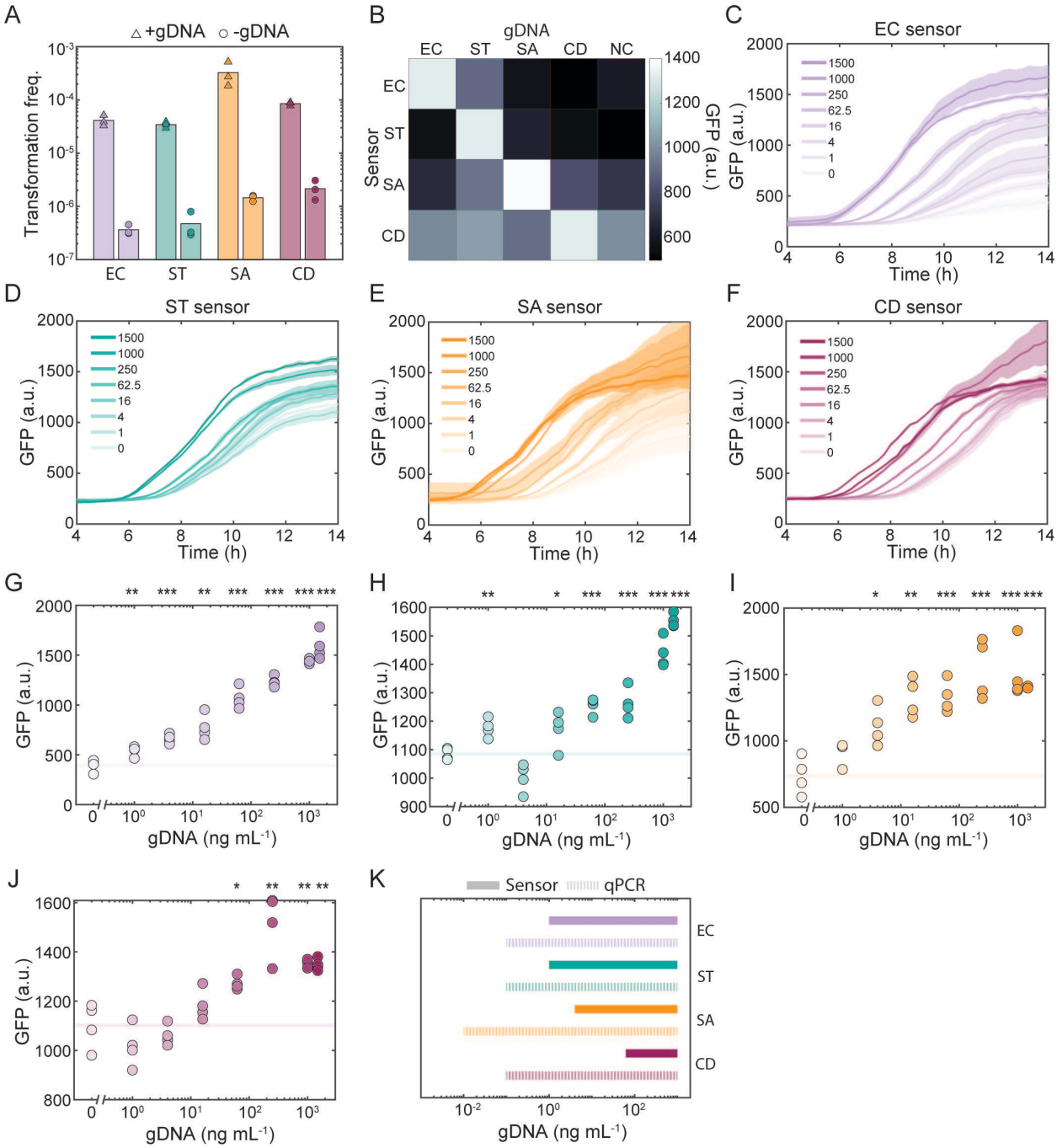

539
540 **Figure 2. Cell-Sens can detect diverse bacterial species including major human pathogens.**
541 **(A)** Transformation frequencies of DNA sensor strains that can detect *E. coli* (EC sensor), *S.*
542 *typhimurium* (ST sensor), *S. aureus* (SA sensor), or *C. difficile* (CD sensor) in the presence of
543 100 ng/mL purified gDNA from each target bacteria (triangles) or no gDNA (circles) at 10 hr. Bar
544 height represents the mean of biological replicates. Data points represent biological replicates (n
545 = 3). **(B)** GFP expression of each DNA sensor strain at 12 hr in response to 100 ng/mL purified

gDNA of a given donor species or no gDNA representing the negative control (NC). DNA sensor strains were cultured in LB media supplemented with 2 mM IPTG, 5 µg/mL chloramphenicol and MLS (1 µg/mL erythromycin and 25 µg/mL lincomycin) for 12 h. Data are the average of four biological replicates. GFP fold changes were 2.6, 2.6, 2.1, and 1.5 for the EC, ST, SA, or CD sensor in the presence of *E. coli*, *S. typhimurium*, *S. aureus* or *C. difficile* purified gDNA, respectively compared to NC with *p*-values of $8.5x10^{-3}$, $4.5x10^{-5}$, $1.3x10^{-4}$, and $3.5x10^{-4}$, respectively based on an unpaired *t*-test. Expression of GFP as a function of time for the **(C)** EC sensor, **(D)** ST sensor, **(E)** SA sensor, or **(F)** CD sensor transformed with different concentrations of target bacterial gDNA. Lines represent the mean of biological replicates (n=4). Shaded region denotes 1 s.d. from the mean. Expression of GFP as a function of gDNA concentration at 12 hr for the **(G)** EC sensor, **(H)** ST sensor, **(I)** SA sensor, and **(J)** CD sensor. Unpaired *t*-test was performed on GFP fluorescence in the presence and absence of gDNA where *, **, or *** denotes *p*-values < 0.05, 0.01 or 0.001, respectively. Horizontal line denotes the mean GFP fluorescence in the absence of gDNA (n=4). **(K)** Horizontal bar plot denoting the ability to detect gDNA for each DNA sensor strain based on data in **(G)**-**(J)** or based on quantitative real-time PCR. Solid bars indicate gDNA concentrations where the given DNA sensor strain displayed higher GFP fluorescence than the no gDNA condition based on the unpaired t-test in **(G)**-**(J)**. For the qPCR data, dashed bars represent gDNA concentration conditions where Ct values were determined in each of the three technical replicates.

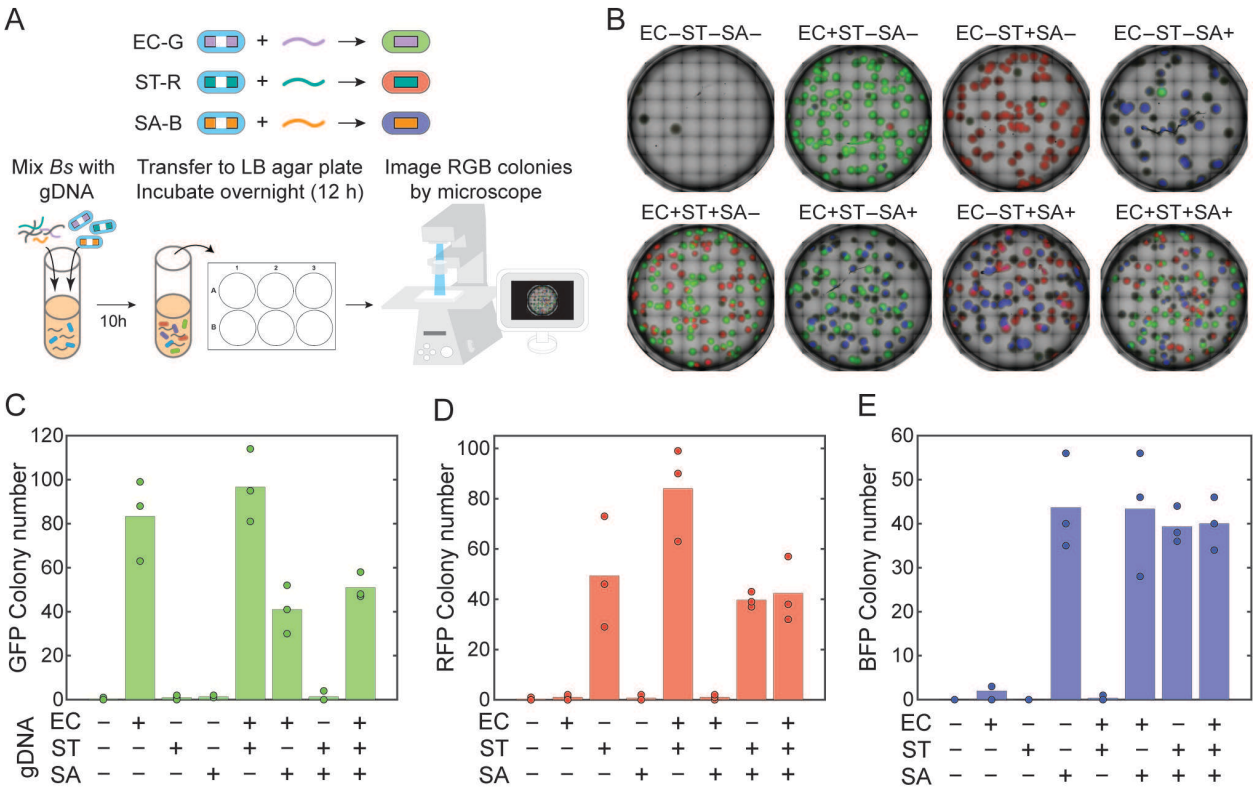

Figure 3. Cell-Sens can perform multiplexed detection of diverse bacterial genomic DNA. **(A)** Schematic of the experimental design for multiplexed detection of genomic DNA (gDNA) of *E. coli*, *S. typhimurium*, and *S. aureus*. The *E. coli*, *S. typhimurium*, and *S. aureus* DNA sensor strains were labeled GFP (EC-G), RFP (ST-R) and BFP (SA-B), respectively. EC-G, ST-R, and SA-B sensors were combined into a mixed culture and exposed to all combinations of gDNA from each species (100 ng/mL each). The mixed culture of DNA sensor strains supplemented with combinations of species' gDNA was incubated for 10 hours and then plated onto 6-well LB agar plates containing 2 mM IPTG, 5 µg/mL chloramphenicol and MLS (1 µg/mL erythromycin and 25 µg/mL lincomycin). Fluorescent imaging was used to quantify the number of GFP, RFP and BFP expressing *B. subtilis* colonies in each condition. **(B)** Fluorescent microscopy images of GFP, RFP and BFP in the presence of different combinations of gDNA from *E. coli*, *S. typhimurium*, and/or *S. aureus*. Bar plots of the number of colonies expressing each fluorescent reporter in each condition for the **(C)** EC-G sensor, **(D)** ST-R sensor, and **(E)** SA-B sensor. Bar height represents the mean of biological replicates (n = 3). Data points denote biological replicates (n=3).

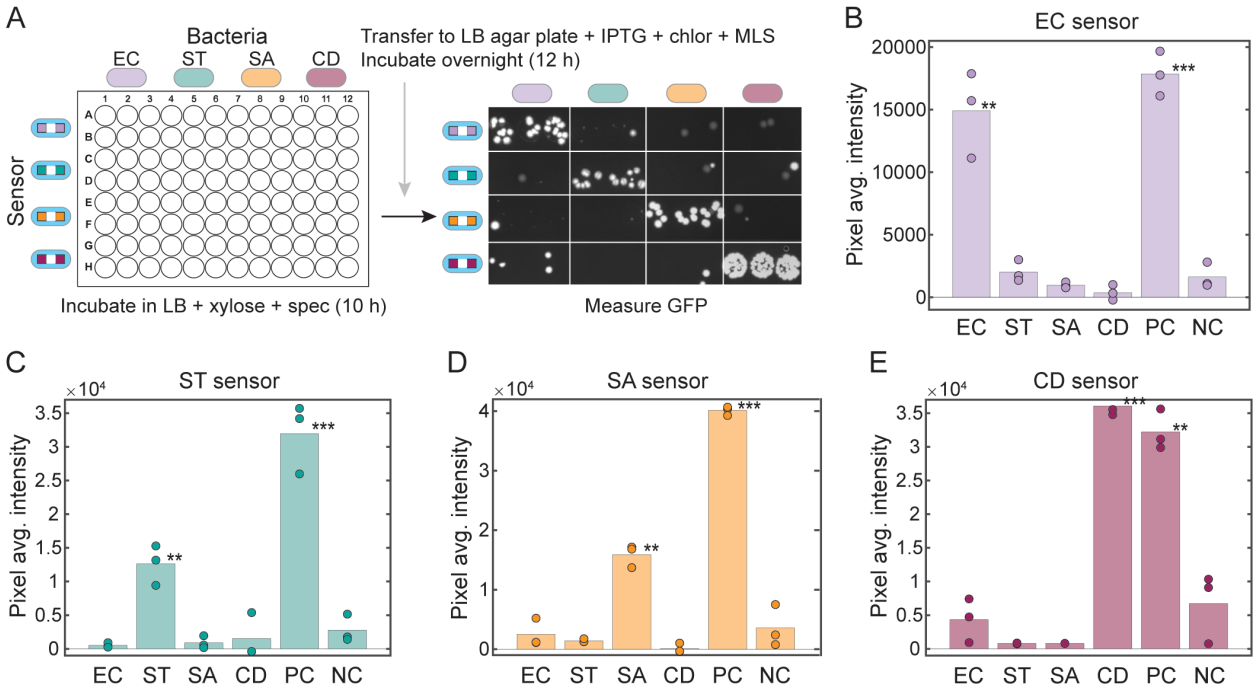

Figure 4. Cell-Sens can detect diverse bacteria in co-culture. **(A)** Schematic of the experimental design for co-culturing each DNA sensor strain with a given donor species in 96-well plates with an initial OD600 of 0.1 for each species. The pairwise communities were co-cultured 10 hours in LB containing xylose (50 mM) and spectinomycin (100 µg/mL) and then plated onto LB plates containing 2 mM IPTG, 5 µg/mL chloramphenicol and MLS (1 µg/mL erythromycin and 25 µg/mL lincomycin). Antibiotics inhibit the growth of the donor species. Fluorescence microscopy image (right) of *B. subtilis* DNA sensor strain colonies on the agar plate for each co-culture condition with three biological replicates (n=3). Pixel average fluorescence intensity of colonies on agar plates were analyzed for the **(B)** EC sensor, **(C)** ST sensor, **(D)** SA sensor, or **(E)** CD sensor. A positive control of 100 ng/mL target bacterial gDNA was included (PC) and no gDNA representing the negative control (NC). Unpaired *t*-test was performed in the presence and absence (NC) of the donor species where ** and *** denote *p*-values < 0.01 or 0.001, respectively.