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**).

72 Using this system, we constructed a DNA sensor strain to detect the presence of *E. coli*
73 (EC sensor) by introducing homology to the *E. coli* MG1655 *xdhABC* operon, encoding genes for
74 purine catabolism[27]. To characterize the DNA sensor, the EC sensor was exposed to purified *E.*
75 *coli* genomic DNA (gDNA) in media supplemented with 50 mM xylose to induce natural
76 competence (**Figure 1B**). The transformation frequency of the EC sensor was quantified by
77 antibiotic selection in liquid or solid media in the presence of IPTG. We investigated the
78 transformation frequency of the EC sensor as a function of homology length to the target DNA
79 sequence by constructing a set of EC sensor strains that varied in length of homology to the target
80 DNA sequence. The transformation frequencies of EC sensors with greater than 1 kbp homology
81 on the 3' and 5' ends of the kill switch were higher in the presence of *E. coli* gDNA than in the
82 absence of gDNA (**Figure 1C**). In addition, transformation frequency increased with the homology
83 length (**Figure 1C and Figure S2B**).
84 A moderate number of transformants was observed in the absence of gDNA and the
85 transformation frequency in this condition did not vary with the EC sensor homology length. These
86 transformants consisted of both GFP OFF and ON colonies due to mutations in either *txpA* and/or
87 *lacI* (**Figure S3**). The GFP ON colonies contained mutations in *txpA*, suggesting that these
88 mutations reduced the growth inhibitory activity of TxpA. The GFP OFF colonies contained
89 mutations in *lacI* near the ligand-binding site, suggesting that they may reduce binding affinity of
90 IPTG to LacI, which in turn could reduce the expression of TxpA and GFP[28,29]. Using the 2.5 kbp
91 EC sensor that maximized the DNA sensor transformation frequency, we found that the
92 transformation frequency increased and saturated by 10 hr (**Figure 1D and S2A**).
93
94 **Building living DNA sensors to sense major pathogens**
95 Since the target DNA sequences are programmable and modular, we constructed DNA sensors
96 to detect the opportunistic intestinal pathogens *Salmonella typhimurium*[30] (*S. typhimurium*) or
97 *Clostridioides difficile*[31] (*C. difficile*) or skin pathogen *Staphylococcus aureus*[32] (*S. aureus*). We
98 introduced 2.5 kbp sequences homologous to the pathogenicity island *sipBCDA* in *S. typhimurium*
99 (ST sensor), the heme biosynthesis pathway *hemEH* in *S. aureus* (SA sensor), or phenylalanyl-
100 tRNA synthetase *pheST* in *C. difficile* (CD sensor)[33–35]. The target sequences used to construct
101 each pathogen DNA sensor were either linked to virulence activities of the pathogen or critical for
102 fitness.
103 The SA sensor transformation frequency was approximately one order of magnitude
104 higher than the other sensor strains, suggesting that the identity of the target sequence in addition
105 to the homology length determines the transformation frequency for each DNA sensor (**Figure**

106 **2A**). To test the specificity of the sensors, we performed time-series measurements of absorbance
107 at 600 nm (OD600) and GFP for each sensor in the presence of individual gDNA (100 ng mL^{-1})
108 from each donor species (**Figure S4**). To quantify the specificity of each sensor strain for the
109 gDNA with homology, we analyzed GFP expression at 12 hr using a fluorescence plate reader
110 (**Figure 2B**). Our results showed significantly higher GFP expression at 12 hr in the presence of
111 the gDNA with homology to the given DNA sensor strain than in the absence of gDNA (p-
112 value<0.01). By contrast, the GFP signal in the presence of gDNA without homology to the given
113 DNA sensor strain was not significantly different from the negative control lacking gDNA except
114 for the SA sensor in the presence of *S. typhimurium* gDNA (p-value=0.0415). These data indicate
115 that each DNA sensor strain has high specificity to the correct target DNA sequence.
116 We evaluated the sensitivity of each sensor strain to individual gDNA with homology by
117 quantifying the temporal response of each DNA sensor strain to a range of gDNA concentrations
118 (**Figure S5**). The GFP response time decreased with gDNA concentration for all sensor strains
119 and the steady-state GFP expression increased with gDNA concentration for the EC, ST and SA
120 sensor strains (**Figure 2C-2F**). We determined the sensitivity of each DNA sensor strain to the
121 target sequence by evaluating the statistical significance of the difference in GFP expression at
122 12 hr with the negative control lacking gDNA (**Figure 2G-2J**). Due to the variation in the temporal
123 response of each sensor strain to its target sequence, we analyzed the GFP expression in the
124 saturated fluorescence regime at 12 hr for each sensor strain. Our results showed that the EC
125 and ST sensors displayed the highest sensitivity of 1 ng mL^{-1} gDNA, whereas the sensitivities of
126 the SA and CD sensors were lower (4 ng/mL and 62.5 ng mL^{-1}, respectively). The observed
127 sensitivities of the EC and ST sensors to the target sequence are sufficient for detection of DNA
128 release in cell culture or cell extracts (**Figure 2K and S6**)[36–38].
129 We compared sensitivities of Cell-Sens to qPCR using primers that anneal to each target
130 sequence. Our results showed that qPCR was approximately one order of magnitude more
131 sensitive than the EC and ST sensors with the highest sensitivity to the target sequence. In sum,
132 each sensor displayed high sensitivity to the target DNA sequence except for the CD sensor which
133 displayed moderate sensitivity. Growth of the non-transformed sub-population and GFP ON
134 escape mutants in liquid culture contributed to the GFP background signal (**Figure S4 and S5**),
135 which in turn may reduce the sensitivity and specificity of Cell-Sens.
136
137 **Cell-Sens can perform multiplexed detection of bacterial genomic DNA**
138 To determine whether the sensor strains could detect gDNA from multiple donor species
139 simultaneously, we used three orthogonal fluorescent reporters including the EC-GFP (EC-G),

140 ST-RFP (ST-R) and SA-BFP (SA-B) sensor strains. We introduced all combinations of gDNA (100
141 ng mL^{-1}) from the pathogen donor species into a mixed culture containing EC-G, ST-R and SA-B.
142 The mixed culture was transferred onto agar plates and the fluorescent imaging was used to
143 determine the number of RFP, BFP and GFP colonies in each condition (**Figure 3A, 3B, and S7**).
144 We found that the mixed DNA sensor culture could accurately detect the presence and absence
145 of each donor species gDNA across all conditions (**Figure 3C-E**). In sum, a mixture of DNA sensor
146 strains each individually labeled with a unique fluorescent reporter enabled accurate detection of
147 all possible combinations of donor species gDNA derived from diverse bacterial pathogens.
148

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

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

162 The fluorescence intensity of each DNA sensor strain co-cultured with individual donor
163 species that did not have homology to the given DNA sensor strain was similar to the negative
164 control lacking gDNA (**Figure 4B-4E)**. In addition, the fluorescence intensity of the EC and CD
165 sensors co-cultured with each corresponding donor species with homology displayed similar
166 fluorescence levels to the positive control (PC) supplemented with purified gDNA containing the
167 target sequence (100 ng mL^{-1}), suggesting that the transformation was efficient in co-culture
168 (**Figure 4B and 4E)**. Time-series eDNA measurements using qPCR demonstrated that each
169 donor species released eDNA into the environment (**Figure S9A**). The eDNA concentration
170 released by each donor species in co-culture with the individual DNA sensor strains ranged
171 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
172 the concentration of purified gDNA used to characterize the DNA sensor strains (**Figure S9A**).
173 Therefore, the SA sensor was able to detect a lower concentration of gDNA in co-culture with *S.*

174 *aureus* than in monoculture supplemented with *S. aureus* gDNA, indicating that the presence of
175 certain donor species can enhance the sensitivity of Cell-Sens (**Figure 2I and 4D**)[40]. To determine
176 if the DNA sensing mechanism occurred via natural competence, we introduced DNase I into the
177 co-culture containing each donor species and the corresponding DNA sensor strain. The
178 presence of DNase I reduced the transformation frequency to a similar background fluorescence
179 level of each DNA sensor as the negative control lacking gDNA (**Figure S9B**). In sum, our results
180 demonstrate that Cell-Sens can accurately detect the presence of diverse species in a microbial
181 community.
182

183 **DISCUSSION**

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

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

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

208 controlled by an orthogonal transcriptional repressor to directly couple the expression of GFP to
209 homologous recombination with the target DNA sequence. To achieve autonomous sensing
210 without inducer induction, the kill switch may be coupled to a quorum-sensing systems such as
211 *agrBDCA* so that non-transformed cells will be eliminated after the population reaches a threshold
212 density allowing only the transformed sub-population to survive[46]. Due to the transformation
213 efficiency and doubling time of the cells, the total experimental time for Cell-Sens to perform
214 detection of a target DNA sequence was approximately one day. To speed up the DNA detection
215 workflow, flow cytometry or fluorescence microscopy could be used to quantify fluorescent cells
216 within hours following transformation before selection[47]. Future work will investigate the
217 generalizability of the DNA sensor to sensing other organisms beyond bacteria, including viruses,
218 fungi or cancer cells[48].
219

220 **MATERIALS AND METHODS**

221 *Plasmid and strain construction*

222 All sensor strains were derived from *B. subtilis* PY79. The pAX01-comK plasmid was used to
223 introduce P_{xyl}-*comK* at the *lacA* locus in *B. subtilis* PY79 to enhance the transformation efficiency
224 in Luria broth (LB)[25,43]. Reporter plasmids (pOSV00170, pOSV00455 and pOSV00456) were
225 constructed to introduce $P_{hyperspank}$-*gfp*, $P_{hyperspank}$-*rfp*, or $P_{hyperspank}$-*bfp* at the *ycgO* locus[21,49] (**Table**
226 **S1**). The kill switch plasmid pOSV00157 was composed of *lacI*, $P_{hyperspank}$-*txpA*-*ratA*, flanked by
227 two target sequences, and was introduced at the *amyE* locus. The toxin-antitoxin system *txpA*-
228 *ratA* was amplified from purified gDNA of *B. subtilis* 168 by PCR and cloned onto the *amyE*
229 integration plasmid creating pOSV00157. The target sequences *xdhABC* (Location: 3001505-
230 3004004 and 3004005-3006504) were PCR amplified from the purified gDNA of *E. coli* MG1655
231 (NCBI Reference Sequence: NC_000913.3), *sipBCDA* (Location: 3025979-3028478 and
232 3028479-3030978) from *Salmonella enterica* serovar Typhimurium LT2 ATCC 700720 (NCBI
233 Reference Sequence: NC_003197.2), *hemEH* (Location: 553-2770 and 2864-5638) from *S.*
234 *aureus* DSM 2569 (GenBank: LHUS02000002.1), and *pheST* (Location: 770923-773144 and
235 773157-775686) from *C. difficile* DSM 27147 (GenBank: FN545816.1). These sequences were
236 used to construct a set of plasmids (pOSV00169, pOSV00205, pOSV00206, pOSV00207,
237 pOS00208, pOSV00292, pOSV00459 and pOSV00475) using restriction enzymes BamHI-HF
238 (New England Biolabs) and EcoRI-HF (New England Biolabs) or Golden Gate Assembly Mix (New
239 England Biolabs). A nucleotide BLAST search (megablast with default setting) was used to
240 determine if the target sequences were unique to each of the species used in our study.

241 Plasmids were cloned in *E. coli* DH5α and transformed into *B. subtilis* using MC medium[50].
242 Plasmids were extracted using Plasmid Miniprep Kit (Qiagen). MC medium is composed of 10.7
243 g/L potassium phosphate dibasic (Chem-Impex International), 5.2 g/L potassium phosphate
244 monobasic (MilliporeSigma), 20 g/L glucose (MilliporeSigma), 0.88 g/L sodium citrate dihydrate
245 (MilliporeSigma), 0.022 g/L ferric ammonium citrate (MilliporeSigma), 1 g/L Oxoid casein
246 hydrolysate (Thermo Fisher Scientific), 2.2 g/L potassium L-glutamate (MilliporeSigma), and 20
247 mM magnesium sulfate (MilliporeSigma). *B. subtilis*, *E. coli*, *S. typhimurium*, and *S. aureus* were
248 cultured in Lennox LB medium (MilliporeSigma) for gDNA extraction. *C. difficile* was cultured in
249 YBHI medium in an anaerobic chamber (Coy Laboratory). YBHI medium is Brain-Heart Infusion
250 Medium (Acumedia Lab) supplemented with 0.5% Bacto Yeast Extract (Thermo Fisher Scientific),
251 1 mg/mL D-Cellobiose (MilliporeSigma), 1 mg/mL D-maltose (MilliporeSigma), and 0.5 mg/mL L-
252 cysteine (MilliporeSigma). The gDNA from each species was extracted using DNeasy Blood &
253 Tissue Kit (Qiagen). For *S. aureus* gDNA extraction used for PCR target sequence or
254 transformation experiments, 0.1 mg/mL Lysostaphin (MilliporeSigma) was added in the
255 pretreatment step. For building the DNA sensor strains, a double crossover and thus stable
256 integration of each plasmid introduced into *B. subtilis* PY79 was confirmed by the replacement of
257 a different antibiotic resistance gene at the integration locus. gDNA of modified *B. subtilis* was
258 then extracted and transformed into another modified *B. subtilis* to introduce multiple
259 modifications into the genome. Bacterial strains are listed in **Table S2**. DNA sequences of genetic
260 parts are listed in **Table S3**.
261
262 *gDNA detection experiments*
263 The DNA sensor strains were inoculated from a -80 °C glycerol stock into LB with 100 µg/mL
264 spectinomycin and incubated at 37 °C with shaking (250 rpm) for 14 hours. The OD600 was
265 measured by NanoDrop One (Thermo Fisher Scientific) and then the DNA sensor strains then
266 diluted to OD600 of 0.1 in 1 mL LB in 14 mL Falcon™ Round-Bottom Tube (Thermo Fisher
267 Scientific) supplemented with 50 mM xylose (Thermo Fisher Scientific) and 100 µg/mL
268 spectinomycin (Dot Scientific). Xylose was added to induce the competence and spectinomycin
269 was used to prevent the contamination of other bacteria. Purified gDNA from each donor species
270 was quantified by a Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific) and added to the
271 cultures with known concentration. The positive control in all experiments was 100 ng/mL gDNA
272 from a given donor species. The DNA sensor strains cultures were incubated at 37°C with shaking
273 (250 rpm) for 10 hours and then transferred to liquid LB with a 1:20 dilution or 20 µL was plated
274 on an LB plate for CFU counting. The liquid or solid LB media was supplemented with 2 mM IPTG

275 (Bioline), 5 μg/mL chloramphenicol (MilliporeSigma), and MLS (1 μg/mL erythromycin from
276 Sigma-Aldrich and 25 μg/mL lincomycin from Thermo Fisher Scientific). In liquid culture, OD600
277 and fluorescence were measured using a SPARK Multimode Microplate Reader (TECAN). In
278 these experiments, 100 uL of the 1:20 diluted cultures of transformed DNA sensors were
279 transferred to 96-well black and clear-bottom CELLSTAR® format sterile cell culture microplates
280 (Greiner Bio-One). Plates were sealed with Breathe-Easy Adhesive Microplate Seals (Thermo
281 Fisher Scientific) and incubated in the microplate reader at 37 °C with shaking for time-series OD
282 and GFP measurements. On agar plate, CFU of transformed *B. subtilis* and total *B. subtilis* were
283 counted for both fluorescent and non-fluorescent colonies. To determine the CFU of transformed
284 *B. subtilis*, 20 μL of cell culture was plated on LB agar plate with 2 mM IPTG, 5 μg/mL
285 chloramphenicol and MLS. To determine the CFU of total *B. subtilis*, cell culture was diluted in
286 phosphate-buffered saline (PBS) (Dot Scientific) and plated on LB agar plate with 5 μg/mL
287 chloramphenicol and MLS. Colonies were imaged using an Azure Imaging System 300 (Azure
288 Biosystems) using Epi Blue LED Light Imaging with 50 millisecond exposure time to determine
289 the fluorescence expression. Transformation frequency was defined as the ratio of fluorescent or
290 non-fluorescent transformed *B. subtilis* to the total number of *B. subtilis* in **Figure 1C and 1D**.
291 Transformation frequency was defined as the ratio of fluorescent transformed *B. subtilis* to the
292 total number of *B. subtilis* in **Figure 2A and S9B**.
293
294 *Multiplexed gDNA detection experiment*
295 The DNA sensor strains EC-G, ST-R, and SA-B sensors were diluted to an OD600 of 0.1, 0.1,
296 and 0.01, respectively, in 1 mL LB supplemented with 50 mM xylose to induce expression of *comK*
297 (Thermo Fisher Scientific) and 100 μg/mL spectinomycin (Dot Scientific). Different combinations
298 of 100 ng/mL purified gDNA from each donor species were introduced into a mixed culture
299 containing different DNA sensor strains (EC-G, ST-R and SA-B). The DNA sensor strain mixtures
300 were incubated at 37 °C with shaking (250 rpm) for 10 hours and 5 μL of each culture was plated
301 on 6-well plates (Thermo Fisher Scientific). The 6-well plates were large enough to visualize
302 approximately a hundred colonies in each well and could be imaged by the Nikon Ti II Microscope.
303 In these plates, each well contained solidified LB agar supplemented with 2 mM IPTG, 5 μg/mL
304 chloramphenicol, and MLS. These plates were incubated at 37 °C overnight.
305 On the next day, each well was imaged using a Nikon Eclipse Ti2-E Microscope.
306 Brightfield images were collected at 4X magnification using the built-in transilluminator of the
307 microscope. The epifluorescence light source was the SOLA-III Light Engine (Lumencor) and
308 standard band filter cubes (Nikon) including DAPI (Excitation: 375/28, Emission: 460/50), FITC

309 (Excitation: 480/30, Emission: 535/40) and TRITC (Excitation: 540/25, Emission: 605/55) filter
310 cubes were used to image BFP, GFP, and RFP, respectively. The exposure times for BFP, GFP,
311 RFP were 500 ms, 9 ms, and 50 ms, respectively. Since the DAPI filter was not optimized for BFP,
312 a strong BFP signal was observed from the LB agar. To reduce the autofluorescence from LB
313 agar, images were processed as follows: complete images of each LB agar well were generated
314 from multipoint images using the stitching function in the Nikon NIS Elements software. Once the
315 full images were assembled, each of the four channels were mapped to unity by the minimum
316 and maximum pixel values of all images for that channel using ImageJ. Fluorescence channels
317 were further processed to remove background signal from agar fluorescence by masking out all
318 signals not associated with a colony. Masks for this step were generated from the DIC images
319 using the pixel classification tool in Ilastik[51]. Any remaining noise in the fluorescence channels
320 was reduced by clipping each image by the average noise value for that channel.
321
322 *Co-culture experiments*
323 *E. coli*, *S. typhimurium*, and *S. aureus* and the corresponding DNA sensor strains were each
324 inoculated from a -80 °C glycerol stock into LB media and incubated at 37 °C with shaking (250
325 rpm) overnight. *C. difficile* was cultured in YBHI medium in an anaerobic chamber (Coy
326 Laboratory). On the next day, the cultures were diluted to an OD600 of 0.1 each in 100 µL LB
327 supplemented with 50 mM xylose and 100 µg/mL spectinomycin into 96-well plates at 37 °C with
328 shaking (250 rpm). Overnight cultures of target bacteria were also diluted in PBS (Dot Scientific)
329 and plated onto an LB or YBHI agar plate to determine the initial CFU of target bacteria in the co-
330 culture, which was 2.54 x 10^6 cells per mL, 1.19 x 10^7 cells per mL, 1.22 x 10^7 cells per mL, and
331 1.05 x 10^6 cells per mL for *E. coli*, *S. typhimurium*, *S. aureus*, and *C. difficile*, respectively.
332 Following 10 hours of incubation, cell culture were diluted into PBS with 1:10 dilution and 10 uL
333 diluted cell culture were spotted on a large LB agar plate (140 mm diameter and 20 mm height
334 petri dish, Thermo Fisher Scientific). The LB agar was supplemented with 2 mM IPTG, 5 µg/mL
335 chloramphenicol, and MLS and incubated overnight at 37 °C. On the next day, each plate was
336 imaged with an Azure Imaging System 300 (Azure Biosystems) using the Epi Blue LED Light
337 Imaging with 50 millisecond exposure time. The pixel fluorescence intensity of the colony spots
338 was quantified by ImageJ[52].
339 To determine if the detection of target bacteria was via transformation, 1 unit/mL DNase I
340 (Thermo Fisher Scientific) was added to the 1 mL of each co-culture and CFU of both transformed
341 *B. subtilis* and total *B. subtilis* were counted to calculate the transformation frequency change. To
342 quantify the extracellular DNA (eDNA), a given DNA sensor strain and donor species were diluted

343 into OD0.1 each in 3 mL LB containing 50 mM xylose and 100 μg/mL spectinomycin and
344 incubated at 37 °C with shaking (250 rpm) for 10 h. Because the sensor also contained the same
345 DNA sequence targeted by the qPCR primers, to quantify the eDNA released from only the target
346 bacteria but not the sensor, a sensor strain (msOSV00409) without homologous sequence was
347 used in the co-culture instead. At every two hours, supernatants were collected and filtered with
348 0.2 μm Whatman Puradisc Polyethersulfone Syringe Filter (GE Healthcare) to remove the cells,
349 and stored in the -20 °C freezer for qPCR measurements.
350
351 *qPCR measurements*
352 To determine the detection limit of qPCR, purified gDNA from *E. coli*, *S. typhimurium*, *S. aureus*,
353 or *C. difficile* was extracted using DNeasy Blood & Tissue Kit (Qiagen) and quantified by Quant-
354 iT dsDNA Assay Kit (Thermo Fisher Scientific). The purified gDNA samples were serially diluted
355 using a 1:10 dilution from 10 μg/mL to 10^{-5} μg/mL and mixed with 2X SsoAdvanced Universal
356 SYBR® Green Supermix (Bio-Rad) and 500 nM of each primer (**Table S4**). Primers were designed
357 and synthetized by Integrated DNA Technologies to target the sequences used to construct each
358 DNA sensor strain. The qPCR reactions were performed on the CFX Connect Real-Time PCR
359 Detection System (Bio-Rad). The gDNA concentration produced at least 95% positive replicates
360 (no missing Ct values) is defined as the detection limit[53]. To quantify the rate of eDNA release of
361 *E. coli*, *S. typhimurium*, *S. aureus*, or *C. difficile* in each co-culture, gDNA standards previously
362 quantified by a Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific) were included in the qPCR
363 run and the gDNA concentration of the supernatant collected at different time points was inferred
364 from the standard curve based on a titration of purified gDNA.
365
366 **DATA AVAILABILITY**
367 All data presented in this manuscript will be available from Zenodo. Plasmids and strains are
368 available upon request.
369
370 **CODE AVAILABILITY**
371 Code for data analysis will be available from Github. The microscopic images of multiplexed
372 detection were processed by ImageJ and ilastik to reduce the background fluorescence from the
373 agar using the basic features such as segmentation and pixel classification and no code was
374 generated in this process.
375
376 **ACKNOWLEDGEMENTS**

We would like to thank Dr. Scott Coyle and Zhejing Xu for providing access to key equipment used in this study. This work was supported by the Defense Advanced Research Projects Agency (DARPA) Grant under grant number HR0011-19-2-0002, Multi University Research Initiative (MURI) under grant number W911NF-19-1-0269, National Institutes of Health under grant number R35GM124774, National Institute of Biomedical Imaging and Bioengineering under grant number R01EB030340.

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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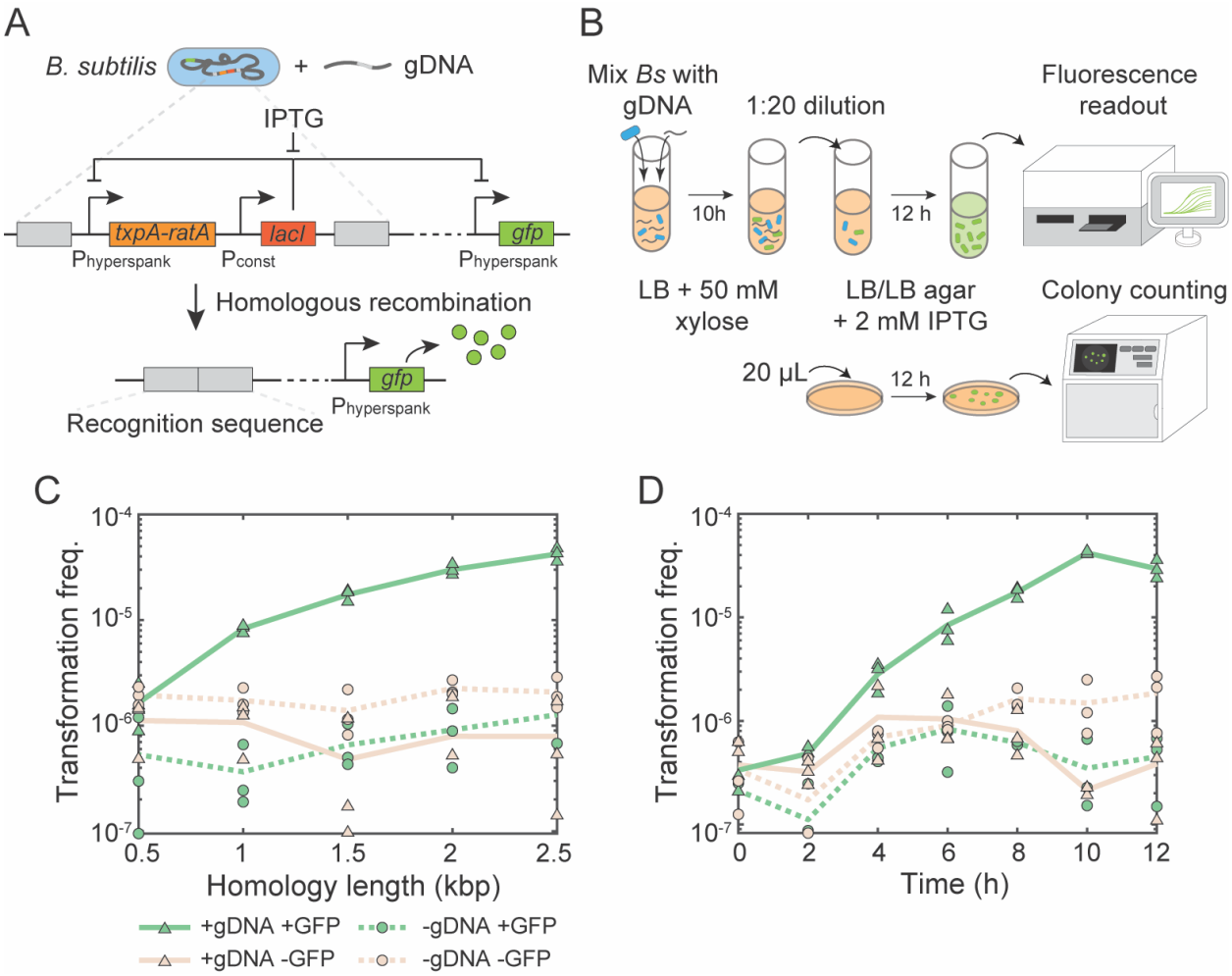

Figure 1. Construction and characterization of a cell-based DNA sensor (Cell-Sens). (A) Schematic of a synthetic genetic circuit in *Bacillus subtilis* that can specifically recognize programmable target DNA sequences. The gene expression of the toxin-antitoxin system *txpA-ratA* and a fluorescent reporter *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

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

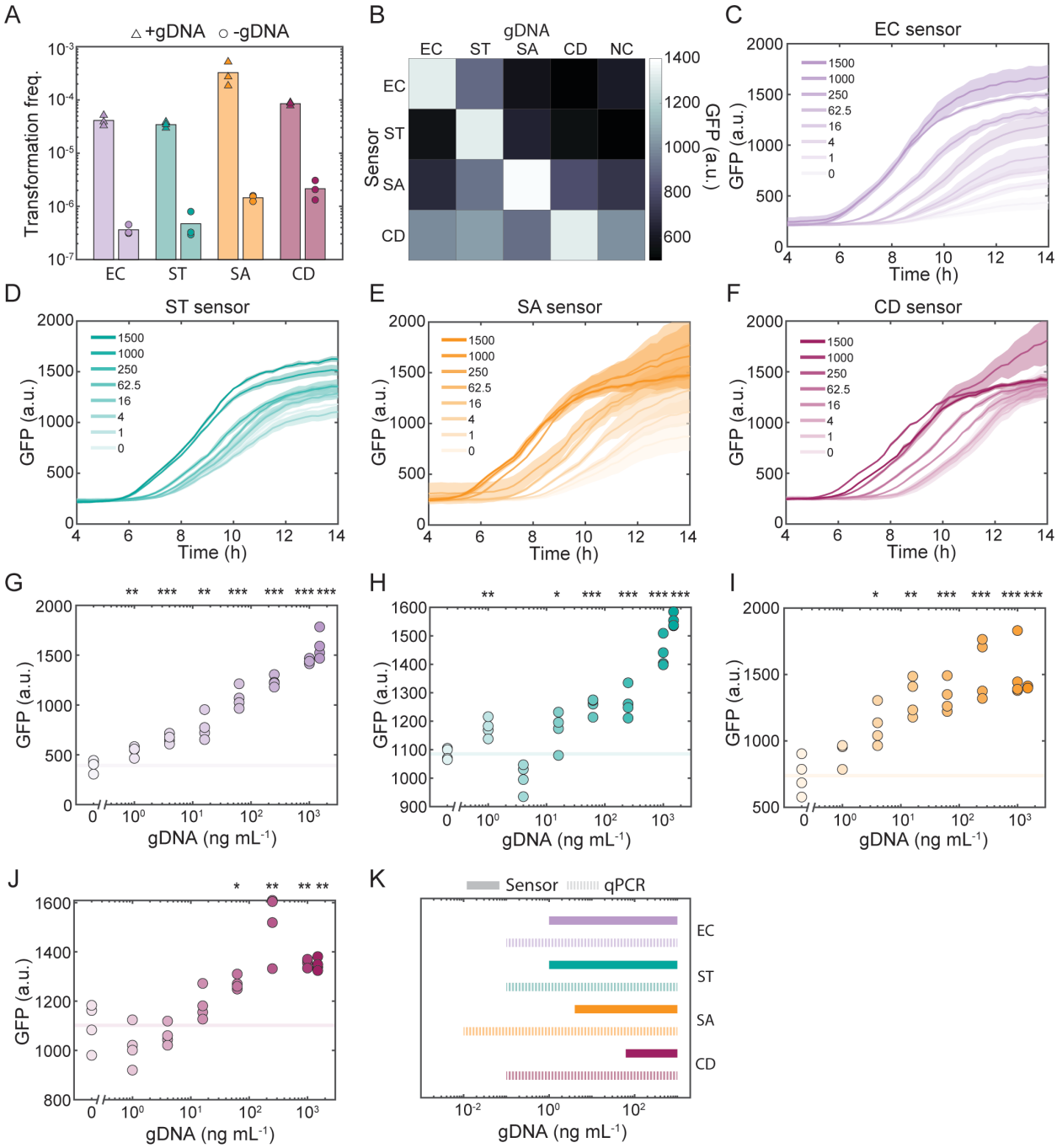

Figure 2. Cell-Sens can detect diverse bacterial species including major human pathogens. (A) Transformation frequencies of DNA sensor strains that can detect *E. coli* (EC sensor), *S. typhimurium* (ST sensor), *S. aureus* (SA sensor), or *C. difficile* (CD sensor) in the presence of 100 ng/mL purified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

546 gDNA of a given donor species or no gDNA representing the negative control (NC). DNA sensor
547 strains were cultured in LB media supplemented with 2 mM IPTG, 5 µg/mL chloramphenicol and
548 MLS (1 µg/mL erythromycin and 25 µg/mL lincomycin) for 12 h. Data are the average of four
549 biological replicates. GFP fold changes were 2.6, 2.6, 2.1, and 1.5 for the EC, ST, SA, or CD
550 sensor in the presence of *E. coli*, *S. typhimurium*, *S. aureus* or *C. difficile* purified gDNA,
551 respectively compared to NC with *p*-values of 8.5×10^{-3}, 4.5×10^{-5}, 1.3×10^{-4}, and 3.5×10^{-4},
552 respectively based on an unpaired *t*-test. Expression of GFP as a function of time for the **(C)** EC
553 sensor, **(D)** ST sensor, **(E)** SA sensor, or **(F)** CD sensor transformed with different concentrations
554 of target bacterial gDNA. Lines represent the mean of biological replicates (n=4). Shaded region
555 denotes 1 s.d. from the mean. Expression of GFP as a function of gDNA concentration at 12 hr
556 for the **(G)** EC sensor, **(H)** ST sensor, **(I)** SA sensor, and **(J)** CD sensor. Unpaired *t*-test was
557 performed on GFP fluorescence in the presence and absence of gDNA where *, **, or *** denotes
558 *p*-values < 0.05, 0.01 or 0.001, respectively. Horizontal line denotes the mean GFP fluorescence
559 in the absence of gDNA (n=4). **(K)** Horizontal bar plot denoting the ability to detect gDNA for each
560 DNA sensor strain based on data in **(G)**-**(J)** or based on quantitative real-time PCR. Solid bars
561 indicate gDNA concentrations where the given DNA sensor strain displayed higher GFP
562 fluorescence than the no gDNA condition based on the unpaired t-test in **(G)**-**(J)**. For the qPCR
563 data, dashed bars represent gDNA concentration conditions where Ct values were determined in
564 each of the three technical replicates.

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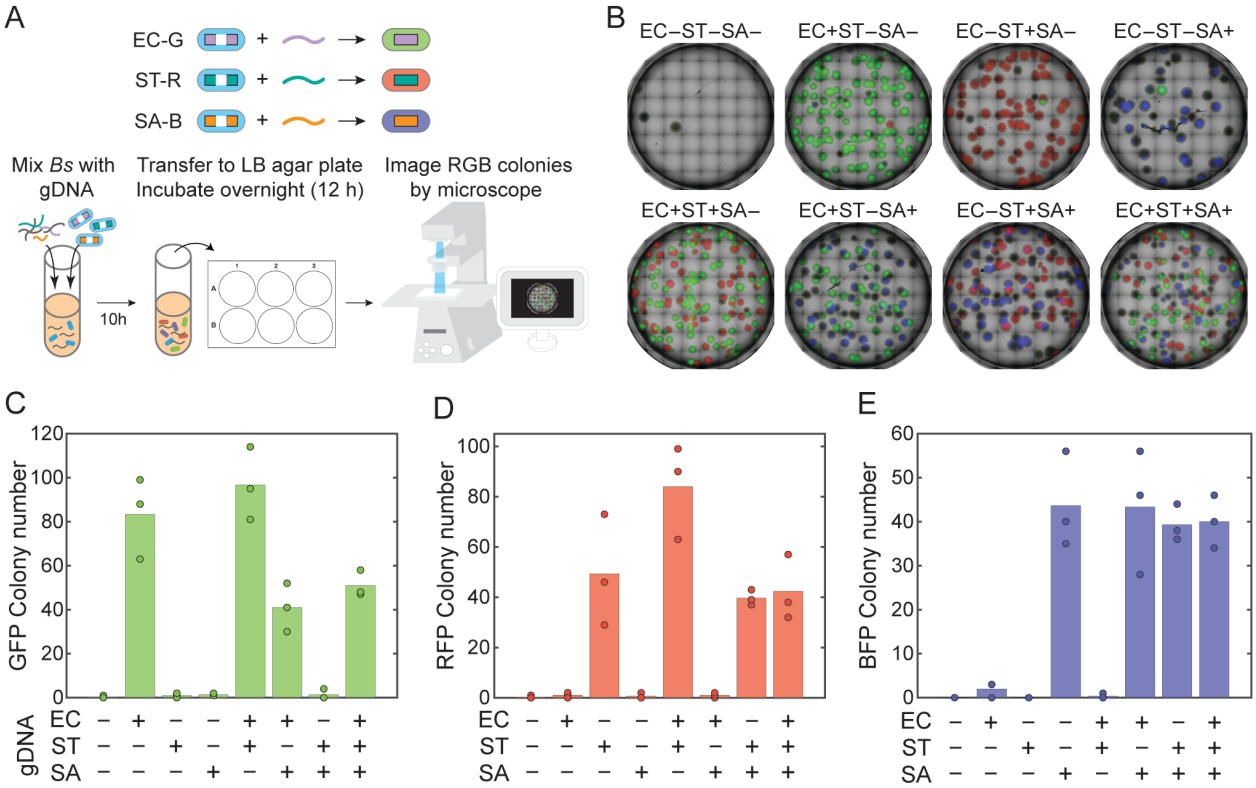

Figure 3. Cell-Sens can perform multiplexed detection of diverse bacterial genomic DNA. (A) Schematic of the experimental design for multiplexed detection of genomic DNA (gDNA) of *E. coli*, *S. typhimurium*, and *S. aureus*. The *E. coli*, *S. typhimurium*, and *S. aureus* DNA sensor strains were labeled 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).

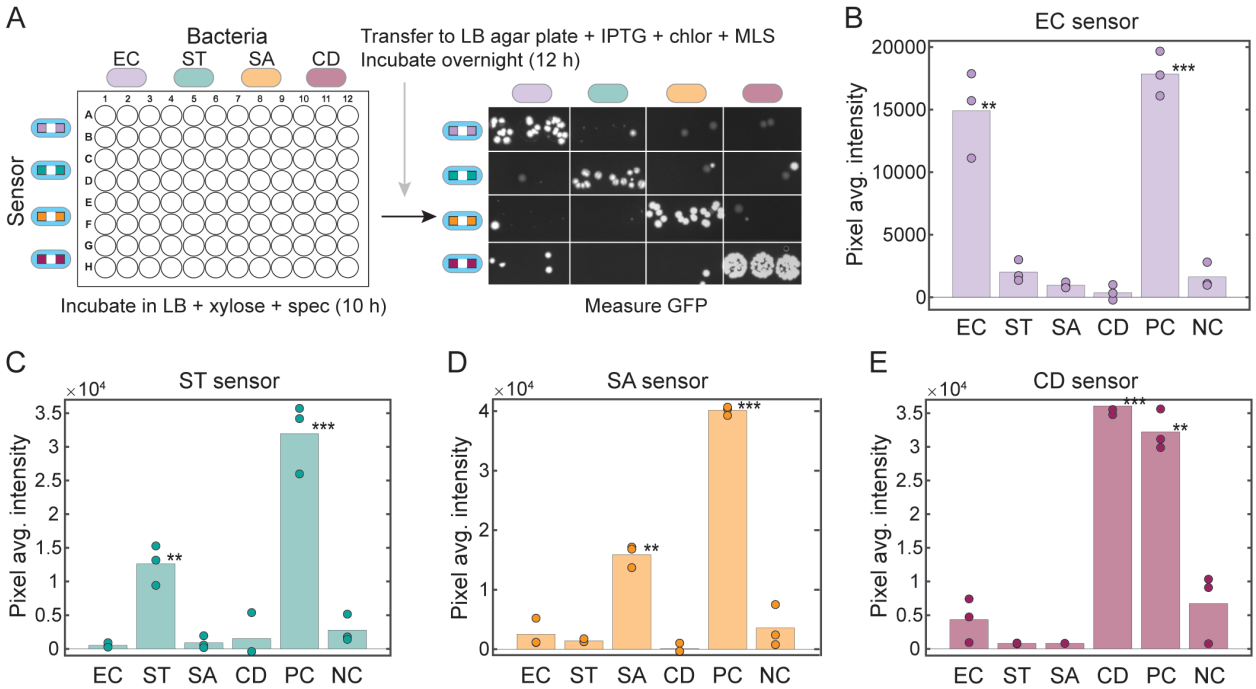

Figure 4. Cell-Sens can detect diverse bacteria in co-culture. **(A)** Schematic of the experimental design for co-culturing each DNA sensor strain with a given donor species in 96-well plates with an initial OD600 of 0.1 for each species. The pairwise communities were co-cultured 10 hours in LB containing 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.