Electronic Supporting Information

Synthetic Cellular Communication-Based Screening for Strains with Improved 3-Hydroxypropionic Acid Secretion

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Supplementary Methods

Method S1. Development of a genetic sensor for the sensitive detection of 3-hydroxypropionic acid (3-HP)

A fresh colony of *E. coli* W strain harboring the pCDF-116C4sgfp plasmid[1] (WSEN3, Table S1) on an LB agar plate was inoculated in 3 mL of the LB medium. Following overnight cultivation, the cell culture was refreshed by diluting into 3 mL of fresh medium at 0.05 OD_{600} (optical density at 600 nm). When the OD_{600} reached 1, the culture was further diluted into 20 mL of the medium at 0.05 OD_{600}. Specifically for this main culture, 0.1 g/L 3-HP was included. After 6 h of incubation, the top 0.01% of most high-fluorescence cells out of the total library of approximately 10^7 cells were sorted using Bio-Rad Laboratories FACS. The cells obtained were inoculated into 3 mL of the medium and cultured overnight. The culture was used as a seed culture for another round of sorting. A total of three rounds of sorting were conducted. Finally, sorted cells were spread on an LB agar plate and their plasmids were sequenced and characterized. The screened cells had an identical mutation (-69delA from the coding sequence of *sgfp*) on the promoter region of the 3-HP sensor plasmid.

Method S2. Fabrication of the microfluidic chips

Droplet generation and droplet sorting chips were fabricated from polydimethylsiloxane (PDMS) chips using standard soft lithography[2] and multilayer soft lithography[3], respectively. A single layer of photoresist (SU8 3025, Microchem, Westborough, MA, USA) was spin-coated onto a silicon wafer for constructing molds for the droplet generation chip (20 μm height) or valve layer (50 μm height) of the droplet sorting chip. Then, the coated photoresists on the wafers were photo-polymerized through the channel-patterned masks. The mold for the fluidic layer of the droplet sorting chip was fabricated from double layers (10 μm and 20 μm height) of photoresists (SU8 3005, 3025, Microchem). The PDMS mixture, at a 10:1 ratio of elastomer and curing agent, was poured onto the valve layer mold and incubated in a 65℃ oven for 1 h. A partially cured PDMS slab was peeled off from the mold and a hole was punched at the end of the valve. The PDMS mixture at a 20:1 ratio was spin-coated onto the fluidic layer mold with 40 μm height and partially cured for 40 min. The PDMS slab of the valve was aligned on the fluidic layer and placed in an oven at 65℃ for overnight incubation. For the droplet generation chip, PDMS mixture at a 10:1 ratio was poured onto the corresponding mold and incubated at 65℃ overnight. Fully cured PDMS slabs were peeled off from each mold and holes were introduced to create inlets and outlets. Then, the slabs were bonded with PDMS spin-coated glass slides as the bottom substrate using plasma treatment. The assembled chips were incubated overnight in a 65℃ oven to restore the hydrophobic nature of the PDMS surface.

Method S3. Screening confirmation by specific amplification of an SC1-specific genetic region

To monitor the enrichment, two different plasmids, pMD20 and pMD20-2, were introduced into the SC2 and SC1 strains, respectively. Specifically, the pMD20-2 plasmid contained an additional 1 kb non-coding sequence compared to the pMD20 plasmid. When a set of the universal primers (AmpR_F and AmpR_B, Table S2) was used, a common region that existed in both plasmids was amplified. The extra_PCR_F and extra_PCR_B primers are specifically bound to the additional sequence of pMD20-2 in SC1. Therefore, this region could be amplified only when SC1 cells were present (Figure 1D). Separately generated co-culture droplets (SC1-RC2 and SC2-RC2) were incubated at 37℃ for 9 h. To validate the chip-based device sorting capability of the co-culture system, droplets were mixed at a 9:1 ratio of SC1-RC2 to SC2-RC2 droplets. Mixed droplets were aliquoted and labeled 'Before'. The mixed droplets were re-injected into the sorting device and only the top 1% of most fluorescent droplets were harvested and labeled 'After'. Both samples were placed in test tubes filled with 3 mL modified glycerol M9 medium and grown for 9 h at 37℃. Single colonies of SC1 and SC2 were inoculated into 3 mL of the medium, grown for 9 h at 37℃, and labeled 'SC1' and 'SC2'. All samples were denatured by incubating at 98°C for 20 min using a thermal cycler. The PCR was performed with 0.2 μM of each primer, 1 μL of a sample, 2X Takara EmeraldAmp GT PCR Master Mix (Kusatsu, Japan), and water (a reaction volume of 10 μL). Amplification was performed with a program: 2 min at 98°C followed by 30 cycles of 10 sec at 98°C, 30 sec at 56°C, and 40 sec at 72°C followed by a 5 min as a final extension step.

Method S4. Genomic DNA library preparation

Extracted *E. coli* W genomic DNA was fragmented via sonication (300 watts, 2 s x 2 rounds) using a Sonics^{R} Vibra cell^{TM} VCX-750 (Newtown, CT, USA). Fragmented DNA of 3-5 kb was obtained by DNA gel purification using a GeneAll^{R} Expin^{TM} Gel SV kit (GeneAll Biotechnology). Then, both ends of the fragments were repaired and 5'-phosphorylated using a NEBNext^{R} End Repair Module kit (NEB). The resulting blunt-ended and phosphorylated fragments were further ligated with the vector fragment amplified from pMD20 (pMB1-derived origin, high copy) using pMD20_F and pMD20_B. The ligated products were transformed into ElectroMAX^{TM} DH10B cells (NEB) for the high efficiency of transformation. Subsequently, plasmids were extracted and reintroduced into the SC3 strain harboring the 3-HP-producing plasmid.

Method S5. Plasmid cloning

The pACYC_3HP plasmid was constructed by subcloning a gene expression cassette of *kgsadh* from the pUC_K plasmid into the pACYC_B4 plasmid containing gene expression cassettes of *dhaB1*, *dhaB2*, *dhaB3*, and *gdrAB*. The gene expression cassette was amplified using the KGSADH_F and KGSADH_B primers. Both the pACYC_B4 plasmid and the amplified fragment were digested using the Bsu36I enzyme and ligated with each other.

To construct the pCDF_*tetA* plasmid, the *tetA* gene was amplified from the pBR322 plasmid using the tetA_F and tetA_B primers. Then, the pCDF plasmid and the amplified fragment were digested using the KpnI and SacI enzymes and ligated with each other. To construct the pMD20_extra plasmid, a vector fragment was amplified from the pMD20 plasmid using the pMD20_F and pMD20_B primers. An insert fragment harboring an extra 1 kb sequence was amplified from the pET_mCherry plasmid using 5'-phosphorylated extra_F and extra_B primers. The two amplified fragments were blunt-end ligated. To construct the pMD20_*gltP*, pMD20_*yjcO*, pMD20_*sgrST*, pMD20_*sgrST-setA*, and pMD20_*leuD* plasmids, a vector fragment was amplified from the pMD20 plasmid using the pMD20_SacI and pMD20_NcoI primers. Gene fragments were amplified using the *E. coli* W genomic DNA as a template and the gltP_F, gltP_B, yjcO_F, yjcO_B, sgrST_F, sgrST_B, setA_B, leuD_F, and leuD_B primers. The vector fragment and gene fragments were digested using the SacI and NcoI enzymes and ligated to yield their respective plasmids. To construct the pUC_K2 plasmid, the pUC_K plasmid lacking the ampicillin resistance gene, the pUC_K plasmid was amplified using 5'-phosphorylated pUCK2_F and pUCK2_B primers. Subsequently, the resulting fragment was blunt-end ligated. To construct the pUC_kan plasmid, the pUC_K2 plasmid was amplified using 5'-phosphorylated pUCkan_F and pMD20_B* primers. Subsequently, the resulting fragment was blunt-end ligated. To construct the pMD20_mCherry-FKF plasmid, fragments were amplified from pET-mCherry and pMD20-FKF(f72) using 5'-phosphorylated

mCherry_F and mCherry_B, and FRT_F and FRT_B. Two fragments were blunt-end ligated. Homology site1-mCherry-FRT-kan^{R}-FRT-Homology site2 was amplified from pMD20_mCherry-FKF using mCFKF_H1_F and mCFKF_H2_B to insert mCherry gene into the genome.

Supplementary Figures

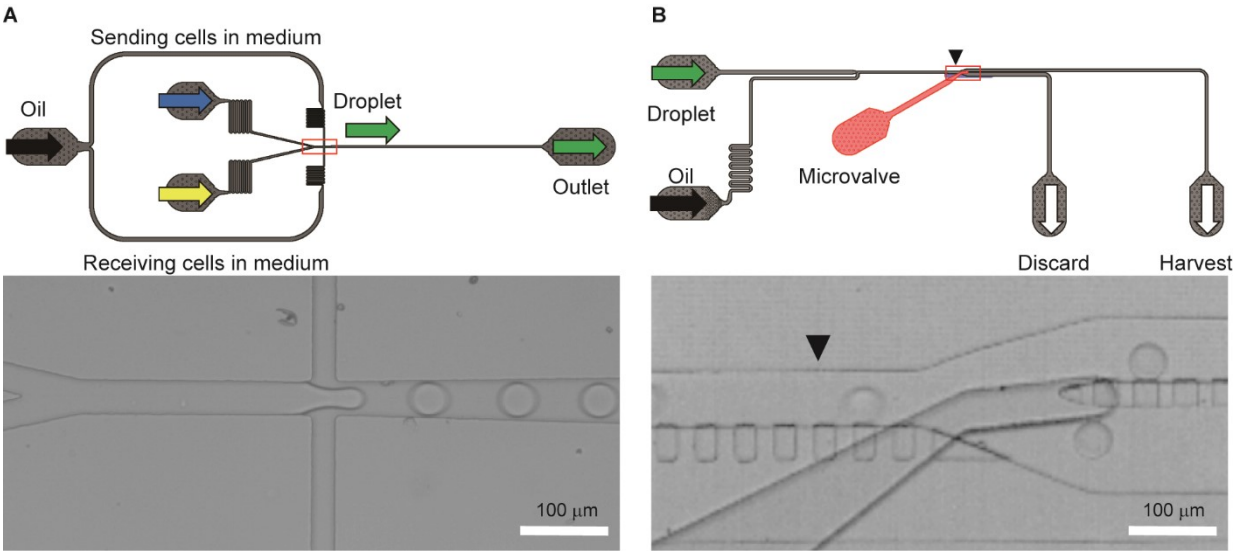

Figure S1. Schematics of the developed microfluidic platform. (A) Schematic image of coculture droplet generation chip. The flow directions of oil, sending cells, receiving cells, and droplets are represented by black, blue, yellow, and green arrows, respectively. The inset figure is the microscope image of the flow-focusing junction (red box in A) in which the droplet is generated. (B) Schematic images of sorting chip. The flow of oil and droplets are indicated by black and green arrows, respectively. The inset figure shows the sorting junction. The region irradiated with the laser is indicated by a black triangle. All scale bars indicate 100 μm.

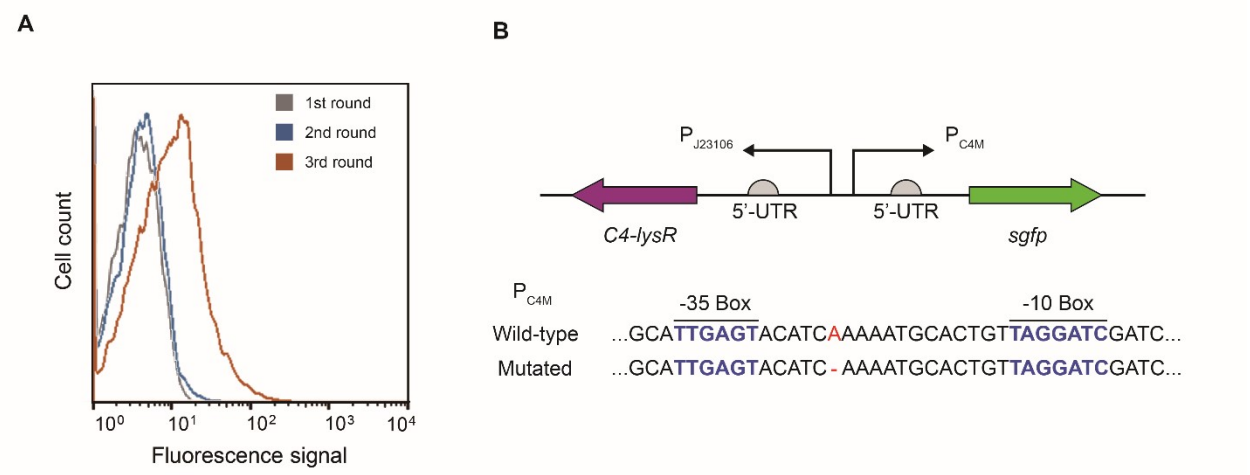

Figure S2. Screening of a mutant sensor for the sensitive detection of 3-HP. (A) To obtain a mutated sensor with higher sensitivity, the WSEN3 strain (Supplementary Table 1) was cultivated in the M9 minimal medium with 0.1 g/L 3-HP for 6 h, and the top 0.01% fraction showing high fluorescence was enriched using a conventional fluorescence-activated cell sorter (FACS). The *x*-axis indicates fluorescence intensities in an arbitrary unit and the *y*-axis indicates cell numbers. (B) The mutated sensor has a single A deletion in the promoter region of *sgfp*. The -35 and -10 boxes were predicted using the PromoterHunter tool from phiSITE (http://www.phisite.org)[4]

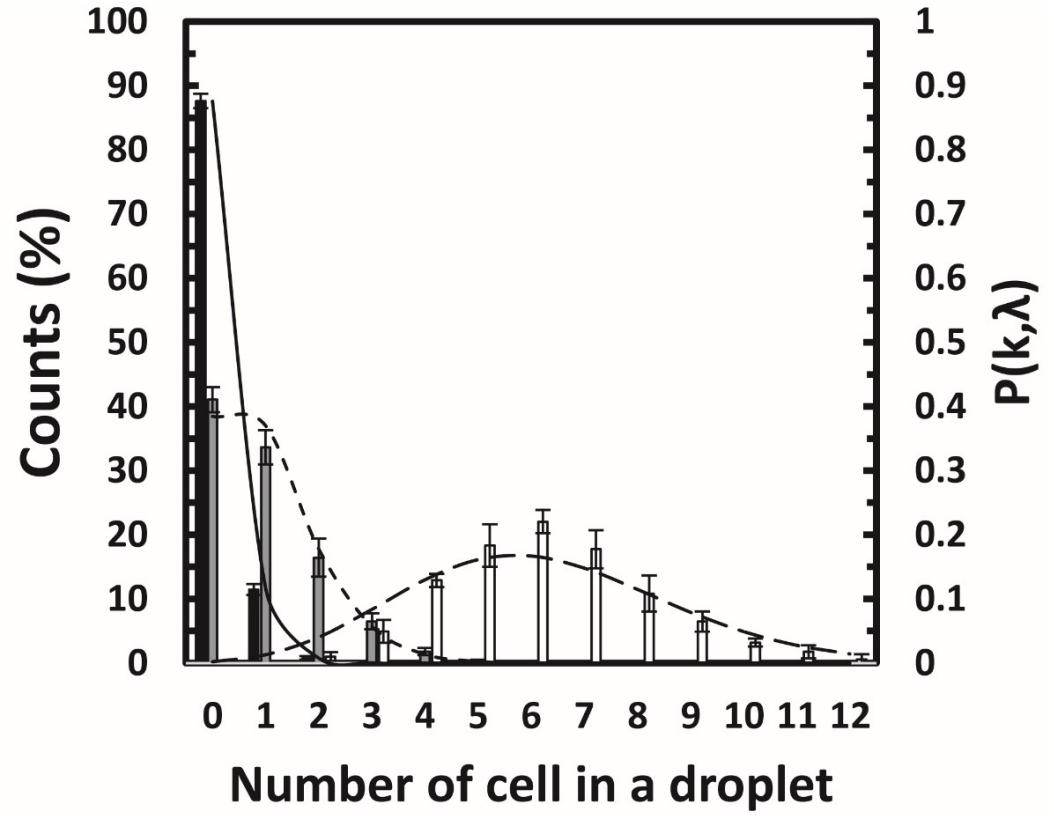

Figure S3. Distribution of encapsulated cell numbers depending on the injected cell concentrations. Bar graphs show the fraction of each encapsulated cell number and lines indicate the Poisson distribution. The number of cells in each microdroplet is counted to estimate the ratio of single-cell encapsulating microdroplets ($P_{(k,\lambda)} = \lambda^k exp(-\lambda)/k!$ where $P_{(k,\lambda)}$ is the probability of a droplet containing k number of cells, with λ being the mean number of cells per droplet). The RC2 strain (Supplementary Table 1) cultivated with 0.1 g/L 3-HP for *gfp* expression was injected into one of the inlets while the medium without cells was injected into the other inlet. The throughput for generating droplets was 3000 Hz. The numbers of fluorescent cells in droplets were counted using a microscope. Although there appears to be an inconsistency between OD_{600} and Lambda values because OD loses linearity at high cell densities, we confirmed that high reproducibility in three independent replicates with actual cell counts under the microscope.

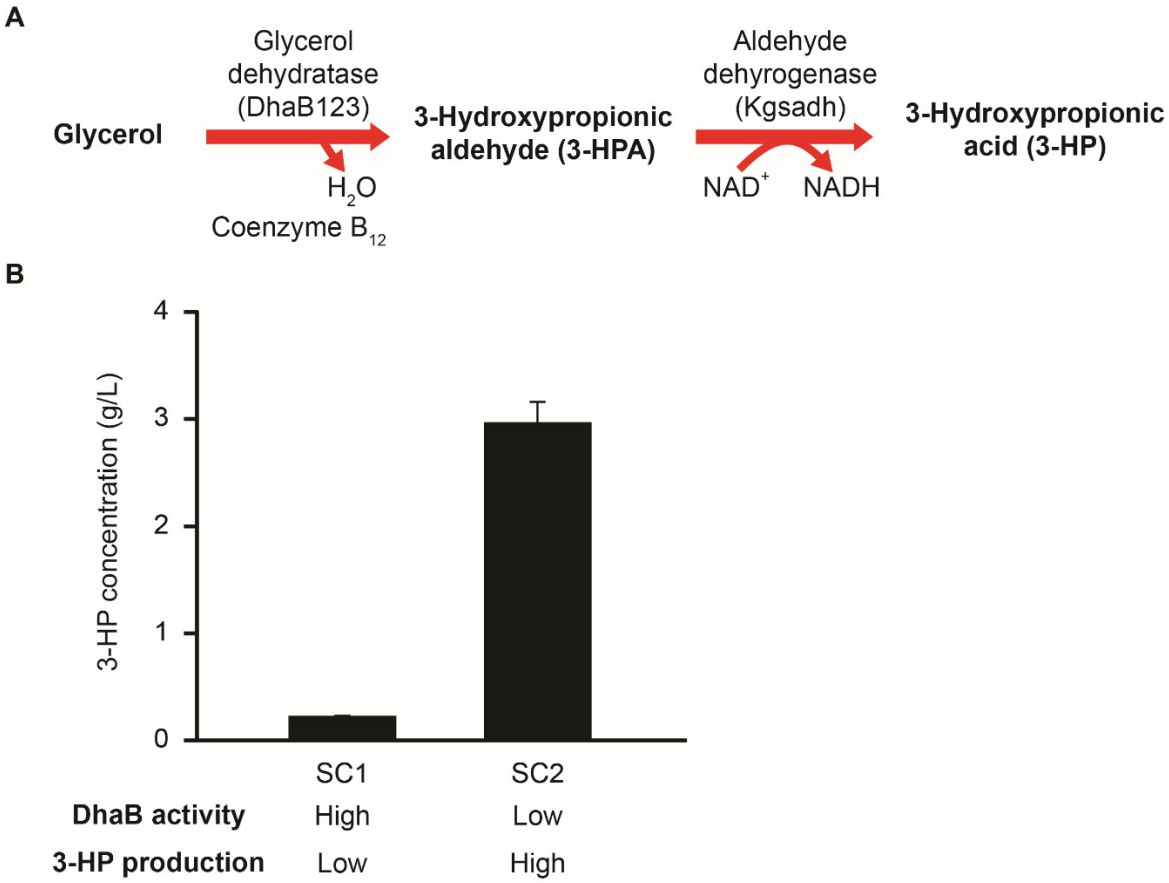

Figure S4. A schematic diagram of the 3-HP production pathway from glycerol. (A) A schematic diagram of the 3-HP biosynthetic pathway. Glycerol is converted into 3-hydroxypropionic aldehyde (3-HPA) by glycerol dehydratase consisting of three subunits (DhaB1, DhaB2, and DhaB3) from *Klebsiella pneumoniae*. 3-HPA is further oxidized into 3-HP by aldehyde dehydrogenase (Kgsadh) from *Azospirillum brasilense*. (B) 3-HP production of the SC1 and SC2 strains after being cultured for 24 h with 100 mM glycerol. The two strains have different activities of glycerol dehydratase. Error bars indicate the actual two data points of the biological duplicates (n = 2).

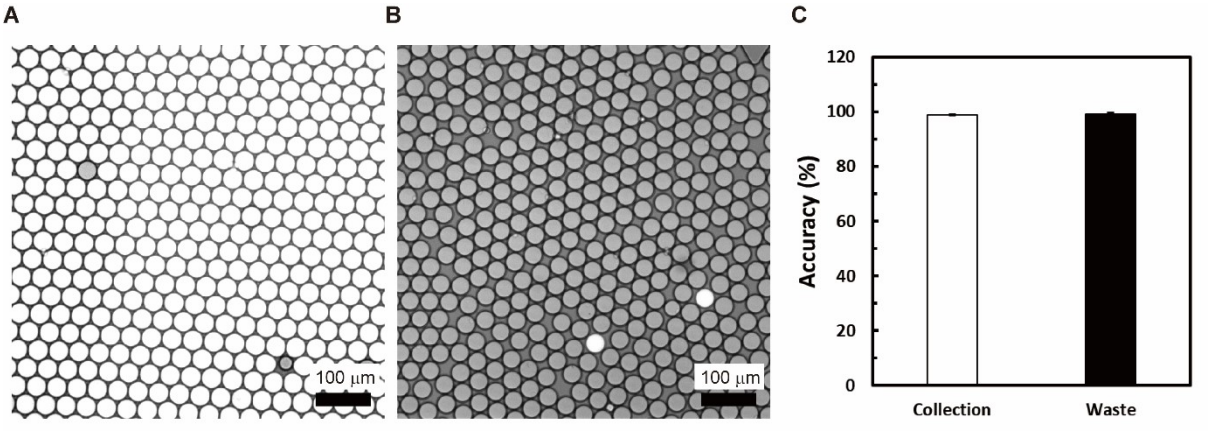

Figure S5. Validation of sorting accuracy. (A-B) Images of droplets from (A) collection and (B) waste tubes after sorting on a chip. To validate the sorting accuracy, two different droplets (bright and dark) containing 1 mM and 0.5 mM fluorescein sodium salt, respectively, were used. The dark droplets additionally contained 25% v/v green dye to distinguish the two droplets when monitored by a microscope. For testing, the two different droplets were mixed at a ratio of 1:1, and the resulting mixture was injected into the sorting chip. The sorting accuracy in the collection and waste outlets was 98.94 ± 0.18 and 99.08 ± 0.37, respectively (triplicates, $n > 800$ droplets).

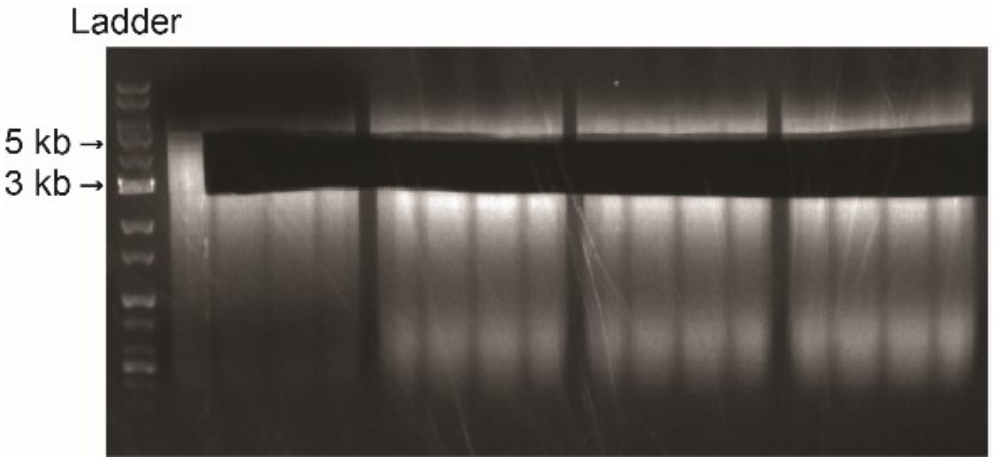

Figure S6. Genomic DNA fragments for constructing the library. To generate a library, *E. coli* genomic DNA was extracted and fragmented via sonication. To improve the cloning efficiency, the exposure to UV light was avoided by selectively exposing a ladder and a partial sample lane. After obtaining the desired gel fragment containing 3-5 kb DNA, the rest of the gel was monitored under UV for confirmation. A total of 10^7 library cells were obtained.

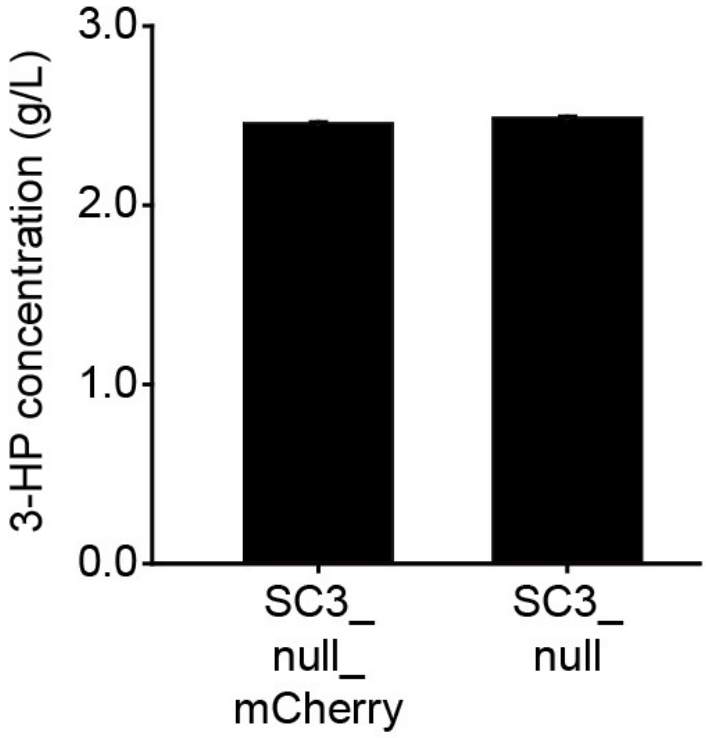

Figure S7. Comparison of 3-HP production by SC3_null_mCherry and SC3_null. SC3_null_mCherry was generated by integrating a gene expression cassette for the constitutive expression of *mCherry* in the intergenic region of *yjiP* and *yjiR* of the SC3_null strain genome. The expression of *mCherry* did not affect the 3-HP production (SC3_null_mCherry: 2.46 g/L, SC3_null: 2.49 g/L). Error bars indicate standard deviation (n = 3).

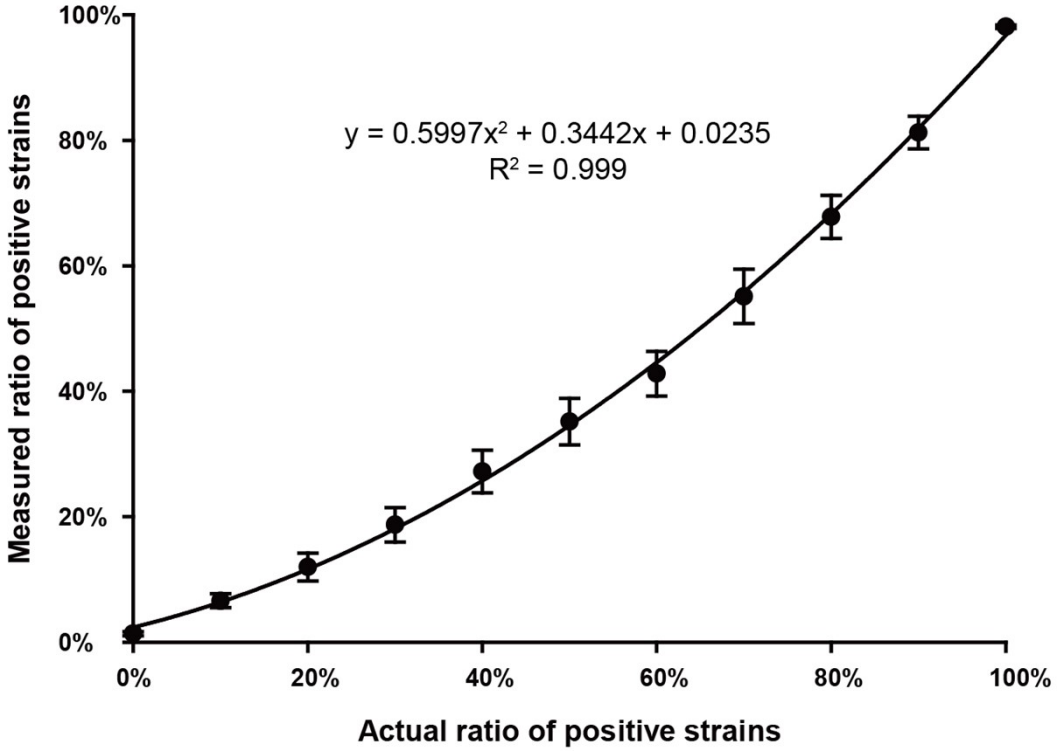

Figure S8. Standard curve to determine the fractions of each strain in a mixture. The SC3_null_mCherry and the two positive strains (SC3_fragment1 and SC3_fragment2) were individually grown and they were mixed at various ratios (0-100%). At least 100,000 cells in a mixture were analyzed by using a Cytoflex. The fluorescence threshold was determined at which signals of the strains were most well distinguished. The obtained data was fitted to a quadratic equation. Error bars indicate the actual two data points (n = 2).

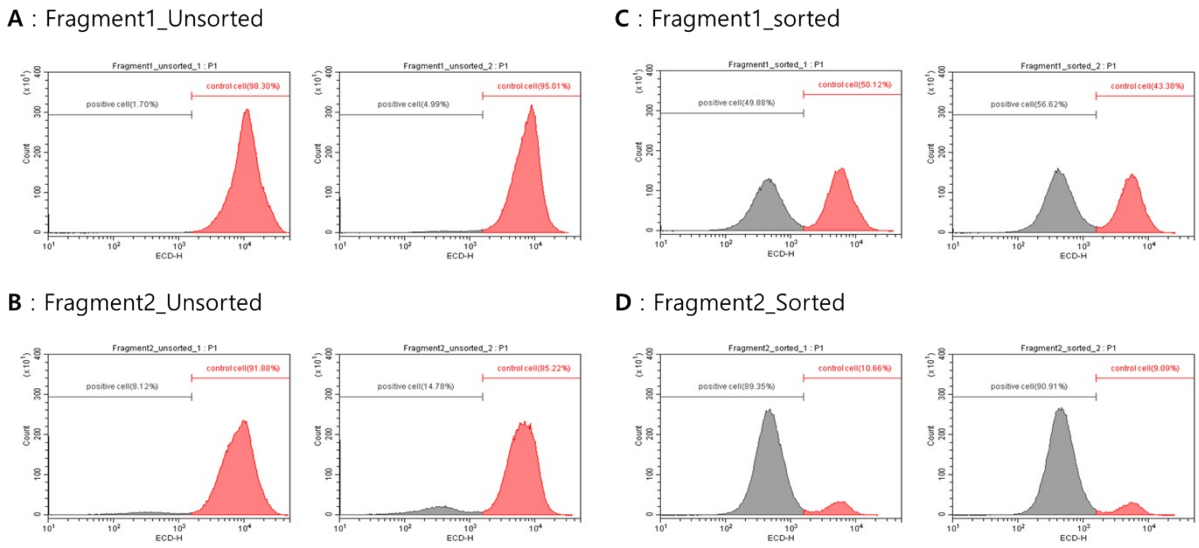

Figure S9. Flow cytometry assay of unsorted and sorted cell mixtures. (A-D) Fluorescence distribution of (A and B) unsorted mixtures and (C and D) sorted mixtures. (A and C) The initial mixture contains the SC3_null_mCherry and SC3_fragment1 strains at a 9:1 ratio (B and D). The initial mixture contains the SC3_null_mCherry and SC3_fragment2 strains at a 9:1 ratio. The unsorted and sorted mixtures were incubated for 12 h and the fluorescence of cells was analyzed by using a Cytoflex. This analysis was performed in technical duplicates. *x*- and *y*-axis indicate fluorescence intensity and cell numbers, respectively.

Supplementary Tables

Table S1. Strains and plasmids used in this study.

Name	Description	Antibiotic resistance	Source
Strains			
E. coli W	An acid tolerant and fast-growing *E. coli* strain, ATCC 9637	None	The American Type Culture Collection (ATCC)
E. coli Mach1-T1R	A cloning host	None	Invitrogen
WSEN3	*E. coli* W / pCDF-116C4sgfp	Sm	[1]
SC1	*E. coli* W / pCDF_tetA / pMD20-2 / pACYC_B1 / pUC_K2 /	Cm, Amp, Kan, Sm, Tet	This study
SC2	*E. coli* W / pCDF_tetA / pMD20 / pACYC_B4 / pUC_K2	Cm, Amp, Kan, Sm, Tet	This study
SC3	*E. coli* W / pACYC_3HP / pCDF_tetA	Cm, Sm, Tet	This study
SC3_null	SC3 / pMD20	Cm, Sm, Tet, Amp	This study
SC3_null_mCherry	*E. coli* W_J23100-mCherry-FRT-kan^R-FRT / pACYC_3HP / pCDF_tetA / pMD20	Cm, Sm, Tet, Amp, Kan	This study
SC3_fragment1	SC3 / pMD20_fragment1	Cm, Sm, Tet, Amp	This study
SC3_fragment2	SC3 / pMD20_fragment2	Cm, Sm, Tet, Amp	This study
SC3_*gltP*	SC3 / pMD20_*gltP*	Cm, Sm, Tet, Amp	This study
SC3_*yjcO*	SC3 / pMD20_*yjcO*	Cm, Sm, Tet, Amp	This study
SC3_*sgrST*	SC3 / pMD20_*sgrST*	Cm, Sm, Tet, Amp	This study
SC3_*sgrST-setA*	SC3 / pMD20_*sgrST-setA*	Cm, Sm, Tet, Amp	This study
SC3_*leuD*	SC3 / pMD20_*leuD*	Cm, Sm, Tet, Amp	This study
RC1	*E. coli* W / pMD20 / pACYC / pUC_kan / pCDF-116C4sgfp	Cm, Sm, Amp, Kan	This study
RC2	*E. coli* W / pMD20 / pACYC / pUC_kan / pCDF_116C4sgfp(-69delA)	Cm, Sm, Amp, Kan	This study
RC3	*E. coli* W / pMD20 / pACYC / pCDF_116C4sgfp(-69delA)	Cm, Sm, Amp	This study
RC4_null	*E. coli* W / pCDF-116C4sgfp / pMD20	Sm, Amp	This study

RC4_*leuD*	*E. coli* W / pCDF-116C4sgfp / pMD20_*leuD*	Sm, Amp	This study
RC4_*sgrST*	*E. coli* W / pCDF-116C4sgfp / pMD20_*sgrST*	Sm, Amp	This study
RC4_*sgrST-setA*	*E. coli* W / pCDF-116C4sgfp / pMD20_*sgrST-setA*	Sm, Amp	This study
RC4_*yjcO*	*E. coli* W / pCDF-116C4sgfp / pMD20_*yjcO*	Sm, Amp	This study

Plasmids

pCDF	CloDF13 ori	Sm	Novagen
pMD20	pMB1-derived ori	Amp	Takara
pMD20-2	pMB1-derived ori, containing extra marker sequence	Amp	This study
pACYC	p15A ori	Cm	Novagen
pBR322	pMB1 ori	Amp, Tet	Takara
pCDF-116C4*sgfp*	pCDF-P_{J23116}-synUTR$_{C4lysR}$-C4lysR-T-P_{C4M}-synUTR*sgfp-sgfp*	Sm	[1]
pACYC_B1	p15A ori, P_{tac}-SynUTR1$_{dhaB1}$-*dhaB1*-P_{tac}-SynUTR$_{dhaB2}$-*dhaB2-dhaB3-gdrA-gdrB*	Cm	[5]
pACYC_B4	p15A ori, P_{tac}-SynUTR4$_{dhaB1}$-*dhaB1*-P_{tac}-SynUTR$_{dhaB2}$-*dhaB2-dhaB3-gdrA-gdrB*	Cm	[5]
pUC_K	pMB1-derived ori, P_{tac}-SynUTR$_{kgsadh}$-*kgsadh*	Amp, Kan	[5]
pUC_K2	pUC_K lacking the AmpR gene	Kan	This study
pCDF_116C4*sgfp*(-69delA)	A mutated pCDF-116C4*sgfp* plasmid	Sm	This study
pACYC_3HP	p15A ori, P_{tac}-SynUTR4$_{dhaB1}$-*dhaB1*-P_{tac}-SynUTR$_{dhaB2}$-*dhaB2-dhaB3-gdrA-gdrB*-P_{tac}-SynUTR$_{kgsadh}$-*kgsadh*	Cm	This study
pCDF_*tetA*	pCDF containing the *tetA* gene	Sm, Tet	This study
pMD20_fragment1	pMD20 containing the *sgrR-sgrS-setA-leuD-leuC* region of *E. coli* W	Amp	This study
pMD20_fragment2	pMD20 containing the *gltP-yjcO-fdhF* region of *E. coli* W	Amp	This study
pMD20_*gltP*	pMD20 containing the *gltP* gene of *E. coli* W	Amp	This study
pMD20_*yjcO*	pMD20 containing the *yjcO* gene of *E. coli* W	Amp	This study
pMD20_*sgrST*	pMD20 containing the *sgrS and sgrT* genes of *E. coli* W	Amp	This study
pMD20_*sgrST-setA*	pMD20 containing the *sgrS*, *sgrT*, and *setA* genes of *E. coli* W	Amp	This study
pMD20_*leuD*	pMD20 containing the *leuD* genes of *E. coli* W	Amp	This study
pUC_kan	pMB1-derived ori, kanR gene	Kan	This study
pET-mCherry	A source of the extra marker sequence	Amp	[6]

	used in the pMD20-2 plasmid and a source of the mCherry sequence used in the pMD20_mCherry-FKF		
pKD46	Red recombinase expression vector	Amp	[7]
pCP20	FLP expression vector	Amp, Cm	[7]
pMD20-FKF(f72)	A source of the FRT-kan^R-FRT sequence	Amp	[8]
pMD20_mCherry-FKF	pMD20-FKF(f72) containing mCherry-FRT-kan^R-FRT	Amp	This study

[a]Abbreviations: Amp, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Km, kanamycin; Tet, tetracycline.

Table S2. Oligonucleotide sequences used in this study.

Name	Sequence (5′−3′)[a]
AmpR_F	CCGCATACACTATTCTCAGAATGACTTGG
AmpR_B	ACTCCCCGTCGTGTAGATAACTACG
extra_PCR_F	GGTCACAGCTTGTCTGTAAGC
extra_PCR_B	ATGAACTCGCCATCCTGC
KGSADH_F	ACACCACCTCAGGGAGTTAGCTCACTCGAGCTCTTGAC
KGSADH_B	GATCGCCCTGAGGAAAAAAGGGCGATCTTGCGACCGCCCTTTTTTTCAGACGGCCATCACCGTC
tetA_F	GAGCTCCACGAGGCCCTTTCGTCTTCA
tetA_B	ACACCAGGTACCCGCATTCACAGTTCTCCGCAAG
pMD20_F	CGTCGTGACTGGGAAAACCC
pMD20_B	GGTTTGCGTATTGGGCGCTCTTCC
pMD20_B*	*GGTTTGCGTATTGGGCGCTCTTCC
extra_F	*GCTGGCTTAACTATGCGGCTTGACAATTAATCATCGGCTGTATAATGTGTGGAGAAATTCAGTGGAAGGAGGAAGAAATGGTTTCCAAGGGCGAGG
extra_B	*GAGCTCTTATTTGTACAGCTCATCCATGCCA
pMD20_SacI	ATCTGATGATGAGCTCCGTCGTGACTGGGAAAACCC
pMD20_NcoI	TACATTACTGCCATGGGGTTTGCGTATTGGGCGCTCTTCC
gltP_F	AGTCCAGTACCCATGGCACCAATCAGTATTATTGCAG
gltP_B	AGGTACCATTGAGCTCGGTTTGATACCGGGTGTG
yjcO_F	AGTCCAGTACCCATGGCATTTTGCCGTCAACGTCGTGG
yjcO_B	AGGTACCATTGAGCTCGGTTCGACGCTGTATCAAAGTATTGCC
sgrST_F	CAATGCAGTCCCATGGGTTGTTGCAGACGAGCAGATG
sgrST_B	TACCATGTCAGAGCTCCATTGCCGATAGCCATCAAACAG
setA_B	AGTTACGTAGGAGCTCCACGACGACGCCATTGC
leuD_F	GTACATCAGTCCATGGCGAAGTGGCTATCGACAAAGTG
leuD_B	AATGGCATGTGAGCTCGCTACCACCTTATTTACTAACAGTATTTCTAC
pUCK2_F	*CAGGACCACTTCTGCGCTC
pUCK2_B	*CCCGAAAAGTGCCACCTG
pUCkan_F	*TGGACAGCAAGCGAACCG
tet promoter	TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCAC
Terminator (BBa_B1002)	CGCAAAAAACCCCGCTTCGGCGGGGTTTTTTCGC
mCherry_F	ACTGTACATGGATTTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCGTGTGGAGAAATTCAGTGGAAGGAGG
mCherry_B	AATCTCATGACCAAAATCCCTTAACGTGAG
FRT_F	GCATGACCGGCGCGATGC
FRT_B	GCTCAGCGGATCTCATGCGC
mCFKF_H1_F	TCTATTGAATTTGCGCGGTTTGTAGGCCGGATAAGGCGTTCACGCCGCATAACTGTTGGGAAGGGCGATCG
mCFKF_H2_B	ATCGCCACGTTCCAGCATGGCTTAGGCAAAGTGTGCTTCGTTCATACCGGCTCAGCGGATCTCATGCGC

[a]Underlined sequences are restriction enzyme recognition sites.

[b]* indicates 5’ phosphorylation.

Supplementary Video

Video S1. The workflow of the synthetic cellular communication-based screening.

Sending and receiving cell suspensions are mixed and encapsulated to generate a co-culture droplet. Co-culture droplets are sorted based on the fluorescence of receiving cells according to the secretion phenotype of a sending cell.

Supplementary references

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