

Gene-targeted microfluidic cultivation validated by isolation of a gut bacterium listed in Human Microbiome Project's Most Wanted taxa

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This paper describes a microfluidics-based workflow for genetically targeted isolation and cultivation of microorganisms from complex clinical samples. Data sets from high-throughput sequencing suggest the existence of previously unidentified bacterial taxa and functional genes with high biomedical importance. Obtaining isolates of these targets, preferably in pure cultures, is crucial for advancing understanding of microbial genetics and physiology and enabling physical access to microbes for further applications. However, the majority of microbes have not been cultured, due in part to the difficulties of both identifying proper growth conditions and characterizing and isolating each species. We describe a method that enables genetically targeted cultivation of microorganisms through a combination of microfluidics and on- and off-chip assays. This method involves (i) identification of cultivation conditions for microbes using growth substrates available only in small quantities as well as the correction of sampling bias using a “chip wash” technique; and (ii) performing on-chip genetic assays while also preserving live bacterial cells for subsequent scale-up cultivation of desired microbes, by applying recently developed technology to create arrays of individually addressable replica microbial cultures. We validated this targeted approach by cultivating a bacterium, here referred to as isolate microfluidicus 1, from a human cecal biopsy. Isolate microfluidicus 1 is, to our knowledge, the first successful example of targeted cultivation of a microorganism from the high-priority group of the Human Microbiome Project's “Most Wanted” list, and, to our knowledge, the first cultured representative of a previously unidentified genus of the *Ruminococcaceae* family.

microscale | anaerobe | aerobic | cultivate | metagenome

This paper describes an integrated microfluidic workflow for genetically targeted cultivation and isolation of microorganisms. Microbes play critical functional roles in diverse environments ranging from soil and oceans to the human gut. The emergence of culture-independent techniques has provided insights into microbial ecology by revealing genetic signatures of uncultured microbial taxa (1–5). It also suggests that certain microbes may impact host phenotypes such as obesity, inflammation, and gastrointestinal integrity (6, 7). This explosion of sequencing data has presented new challenges and opportunities for microbial cultivation, which is critical for allowing direct access to microorganisms to test hypotheses experimentally, and is crucial for proper taxonomic classification, functional annotation of metagenomic sequences, and use of such microbes for environmental remediation, energy applications, and formulation of probiotics. However, a direct approach that cultivates, in a targeted fashion, microbes carrying genes of interest identified in metagenomic data sets remains mostly unexplored. As a result, for example, a list of the “Most Wanted” taxa that are urgently in need of cultivation has been issued by the Human Microbiome Project (HMP) from the National Institutes of Health. These microorganisms are highly prevalent and abundant

in the human microbiome but poorly represented in cultured collections (2).

Most microbes do not grow using traditional cultivation methods and hence are referred to as “unculturable” (8–10). Although these microbes could be grown in their natural habitats (9), where effects such as cross-feeding (11) and microbe–host interactions (12, 13) are present, some biological samples, such as clinical biopsies, are often limited in quantity. This makes it challenging to set up cultivation experiments in large scale with these native media, but creates opportunities for miniaturized methods. Further, miniaturized methods that use compartmentalization can eliminate competition among species. Cultivation methods that use miniaturization and compartmentalization, including gel microdroplets (14), miniaturized Petri dishes (15), and microfluidics (16–19), have become increasingly promising as a basis for targeted microbial cultivation and isolation platforms, as they can limit the consumption of precious samples and also control the microenvironment around cells (20). We envisioned implementing targeted cultivation with microfluidics by focusing on two goals. The first goal is to efficiently identify cultivation

Significance

Obtaining cultures of microbes is essential for developing knowledge of bacterial genetics and physiology, but many microbes with potential biomedical significance identified from metagenomic studies have not yet been cultured due to the difficulty of identifying growth conditions, isolation, and characterization. We developed a microfluidics-based, genetically targeted approach to address these challenges. This approach corrects sampling bias from differential bacterial growth kinetics, enables the use of growth stimulants available only in small quantities, and allows targeted isolation and cultivation of a previously uncultured microbe from the human cecum that belongs to the high-priority group of the Human Microbiome Project's “Most Wanted” list. This workflow could be leveraged to isolate novel microbes and focus cultivation efforts on biomedically important targets.

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Data deposition: The genome sequences reported in this paper have been deposited in the Joint Genome Institute's Integrated Microbial Genomes database, <https://img.jgi.doe.gov/cgi-bin/w/main.cgi> (accession no. 254555870). The 16S rRNA gene sequences of isolate microfluidicus 1 reported in this paper have been deposited in the GenBank database (accession nos. KJ875866 and KJ875867).

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controlled by positive pressure using a pipettor. This process of injection–collection is repeated three times. Immiscible oil is then injected to further displace the remaining aqueous phase. We used a red dye experiment to visualize the device operation described above (Fig. 2*B*), which allowed us to observe that the droplets remained intact during purging when gas was introduced into the channels. In addition, in the chip wash step, the solutions from the channel and the wells were merged and could be visualized by the originally colorless solutions from the channel turning red. The removal of red dye can be observed in Fig. 2*B*, *vii* as the solution in the channel turned back to colorless. To quantify the recovery efficiency of this method, a solution with a fluorescent dye was injected into the device and subsequently collected and quantified using a fluorospectrometer. We determined a recovery rate of 96% when comparing the fluorescence signal from the chip wash solution with the starting stock solution normalized to the same volume. A recovery rate of 83% was observed when *Escherichia coli* cells labeled with red fluorescent protein were used to quantify the recovery efficiency of bacterial cells.

Validating the Chip Wash Method with a Two-Species Model Community.

Having validated the device's operation, we next tested the functionality of the chip wash method using a model community from the human gut microbiome (Fig. 3). First, we tested whether chip wash can detect microbial growth on SlipChip. We cultivated a mixture of *Clostridium scindens* and *Enterococcus faecalis* at a 5:1 ratio on the chip or agar plates. The genomic DNA of the starting inoculum and chip wash solution were extracted and quantified by quantitative PCR (qPCR). Cultivation on the chip followed by chip wash resulted in an ~1,000-fold increase of DNA for each strain compared with DNA from the starting inoculum used as a nongrowth control (Fig. 3*E*), showing that chip wash can be used to detect microbial growth.

Second, we hypothesized that chip wash would detect, without bias, the growth of bacteria that grow at different rates but with similar carrying capacity, for the following reason. For the interest of detection, the optimal time for sampling is the late exponential phase or early stationary phase of the target to maximize the yield of biomass. A single cell growing on a plate starts at a density of ~10 cfu mL⁻¹ assuming the inoculation density is 300 cfu with 30 mL of medium, whereas a single cell growing in a 6-nL well starts at a density of ~1.7 × 10⁵ cfu mL⁻¹. Typical carrying capacity of the media we used for gut anaerobes is ~10⁹ cfu mL⁻¹; therefore, on the device the carrying capacity can be reached more rapidly, and for a larger range of growth

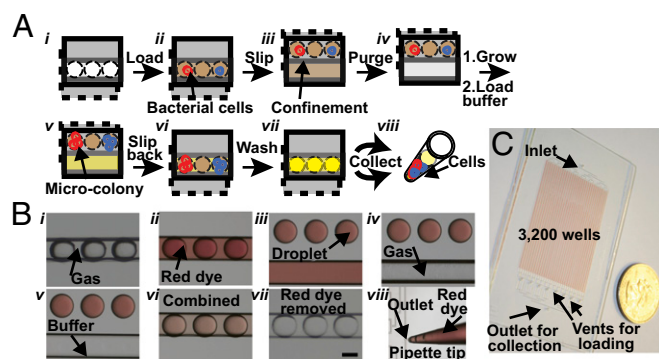


Fig. 2. Design and operation of the chip wash device. (A) Schematic drawings of the chip wash method illustrating device design for handling microbial cells. (B) Representative photographs showing device operation as visualized with red dye. See text for details. Scale bar in *i*–*vii*, 200 μm . (C) Photograph of 3,200 droplets generated and stored on the chip for chip wash, shown next to a US quarter.

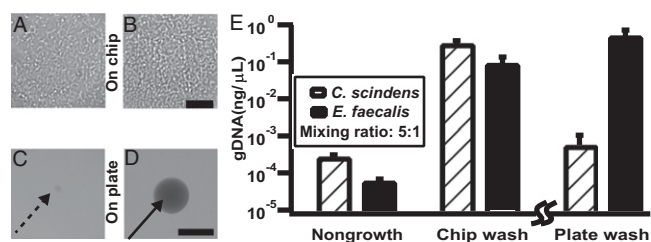


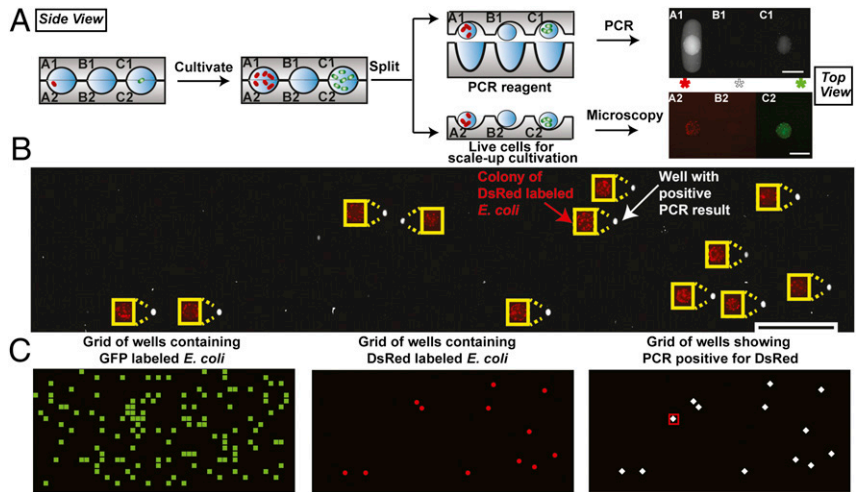
Fig. 3. Validation of the chip wash method with a model community of *C. scindens* and *E. faecalis*. Samples were collected on day 1. (A and B) Representative optical microscopy of *C. scindens* (A) and *E. faecalis* (B) grown on SlipChip. (C and D) Representative photographs of *C. scindens* (C) and *E. faecalis* (D) grown on an agar plate. (E) Graph showing genomic DNA of *C. scindens* and *E. faecalis* recovered from nongrowth negative control, chip wash, and plate wash solutions. The nongrowth control and the chip wash experiments were performed using an identical procedure and can be directly compared. Because the plate wash experiment requires a different protocol, only the relative values can be compared (emphasized by the break in the axis). Error bars indicate SD ($n = 3$). Scale bar, 30 μm for A and B and 1 mm for C and D.

rates, than on a plate. To test this hypothesis, we confirmed that under this particular cultivation condition, *E. faecalis* grew faster than *C. scindens* on agar plates, as observed from the difference in colony size on day 1 (Fig. 3*C* and *D*). The cultivation medium has a similar carrying capacity for the two strains (*SI Appendix*). Consistent with the prediction, the two strains grew on the chip to a comparable density on day 1 (Fig. 3*A* and *B*). As shown by the quantity of genomic DNA recovered from the two strains, sampling on day 1 by plate wash resulted in an ~1,000-fold bias toward rapidly growing bacteria, whereas the chip wash method effectively corrected this bias, as the genomic DNA was comparable for each strain (Fig. 3*E*). This chip wash method provides an efficient way to detect slowly growing bacteria and is complementary to the plate wash method (21). Because we have shown that SlipChip is compatible with solutions used in membrane protein crystallization (28), we expect that SlipChip would be compatible with testing a wide range of growth media with different viscosities and surface tensions.

Using Splitting to Preserve Cultivar and Perform Genetic Assays.

We next tested whether genetic assays could be used to identify and characterize microbes on the chip. We used a replica-SlipChip described in an accompanying paper (22) to split the microcolonies into two halves so that PCR could be performed with one of these halves and live microbes could be preserved on the other. To unambiguously establish the mapping from genotype to phenotype, we used *E. coli* cells expressing DsRed or GFP genes to ensure the genotype could be characterized by PCR, and the phenotype could be monitored by fluorescence microscopy (Fig. 4). We tested if this on-chip PCR approach could reliably distinguish the DsRed-labeled *E. coli* from the GFP-labeled *E. coli*. A mixture of *E. coli* cells labeled with GFP and DsRed proteins was loaded onto the chip, at final densities of 2×10^4 cfu mL⁻¹ and 2×10^3 cfu mL⁻¹, respectively. We assume that the cells are distributed in wells randomly and therefore that their distribution is governed by the Poisson statistics. We used a motile strain of *E. coli* to ensure uniform distribution of bacterial cells in both wells within 3 h of incubation. Individual cells were compartmentalized and cultivated, and then the chip was split into two daughter halves, each carrying a copy of the microcolonies (Fig. 4*A*). One chip was mixed with PCR reagents containing primers targeting the plasmid of DsRed and the other was imaged with a fluorescence microscope to check for the presence or absence of fluorescent proteins. We observed 125 wells that contained colonies with GFP *E. coli* and 12 wells

Fig. 4. Cultivating pure microcolonies from a mixture and using PCR to identify specific microcolonies. Schematics show side views, whereas photographs show top views. (A) Schematic illustrating the cultivation of single cells from a mixture of *E. coli* expressing GFP and DsRed genes, as well as a method for splitting individual colonies. PCR was used to identify the *E. coli* expressing DsRed gene on one half of the split chip. The PCR reagents wells have an ellipsoidal cross-section from top view. An increase in fluorescence intensity indicated a positive result for PCR, and thus, the presence of the DsRed gene. Fluorescence microscopy identified wells that contained microbes expressing red and green fluorescent proteins, matching corresponding results in PCR. (B) To test the accuracy of the PCR assay, results from microscopy imaging (red), indicating *E. coli* colonies expressing DsRed gene, and PCR assay (white) were montaged with an offset to allow visualization without overlap. (C) Plot of a 20 × 50-well grid was used to represent the position of each well on the same device. Elements corresponding to wells were colored to highlight the presence of *E. coli* GFP colonies (green squares), *E. coli* DsRed colonies (red dots), and PCR positive results for DsRed (white diamonds). A red square in the third plot denotes a false positive result from PCR. The different shapes of markers used in C do not represent the shapes of wells. Scale bar, 200 μm for A, 2 mm for B. A 200-μm-wide yellow rectangle was used as scale bar for images showing DsRed expressing *E. coli* colonies in B. Note: schematics are not to scale; dimensions are provided in *SI Appendix*.



containing DsRed *E. coli*. The wells showing PCR-positive matched the corresponding wells containing DsRed *E. coli* (Fig. 4 B and C); in contrast, blank wells that contained no bacteria and wells containing GFP-labeled *E. coli* were PCR-negative. We also noticed one well that showed increased fluorescence intensity in the PCR result, but no bacterial colony was detected in the other copy, which indicates that the well may have contained nongrowing cells or cell-free DNA from the solution. Many microbes residing in the human gut are not motile and might also adhere to surfaces. Therefore, we wanted to verify that this method would work with such organisms or whether active mixing inside SlipChip wells (25) would be required.

Identifying Cultivation Conditions for One of the Most Wanted Microbial Targets. To test this workflow, we focused on isolating microbes from the human gut that belong to the high-priority groups of the Most Wanted list. The genus *Oscillibacter* is frequently observed in the Most Wanted list (2) and other sequencing data sets (29–31), but no human-associated member of this genus has been cultivated yet. To cultivate this genus, we collected samples from the human cecum using a brushing technique to obtain mucosa-associated microbes of high biomedical interest that may directly interact with the host (Fig. 5A). To identify microbial targets in the cultivar, we used 16S rRNA gene high-throughput sequencing with the V4 variable region (32) as a first screening. Reads were clustered to operational taxonomic units (OTUs) de novo with mothur software (33). First, the sample was cultured on agar plates in M2GSC medium (see *SI Appendix* for ingredients) and examined by plate wash. No OTU from the cultivar was classified as *Oscillibacter*. Next, miniaturization enabled by microfluidics allowed us to test if we could culture this genus by supplementing the medium with washing fluid from the sampling site in the human cecum. We obtained washing fluid by a lavage technique, autoclaved it, and spiked into the cultivation medium, referred to in this paper as M2LC (see *SI Appendix* for ingredients). The same amount of inoculum as plated on M2GSC agar was cultivated on SlipChip with M2LC medium and then chip wash was performed as described above. High-throughput sequencing of the V4 region of the 16S rRNA gene showed the successful cultivation of *Oscillibacter* on the chip (*SI Appendix*). We performed high-throughput sequencing of the V1V3 region of the 16S rRNA gene to test if the *Oscillibacter* recovered from chip wash

belonged to the Most Wanted list. We were able to assign the reads classified as *Oscillibacter* to OTU_158_V1V3 (OTU158 for short, with an estimated ~0.7% relative abundance in stool samples in the HMP dataset) from the list (*SI Appendix*) using a custom script (provided in *SI Appendix*) based on usearch software (34). PCR with OTU158-specific primers (OTU158P) confirmed that the target OTU indeed could be found in the cultivar (*SI Appendix*). OTU158P allowed us to validate results of the 16S survey conclusively by qPCR (Fig. 5B). We quantified the genomic DNA of OTU158 using OTU158P and total bacterial genomic DNA using 16S rRNA V4 universal primers. We observed genomic DNA of OTU158 from the chip wash experiment

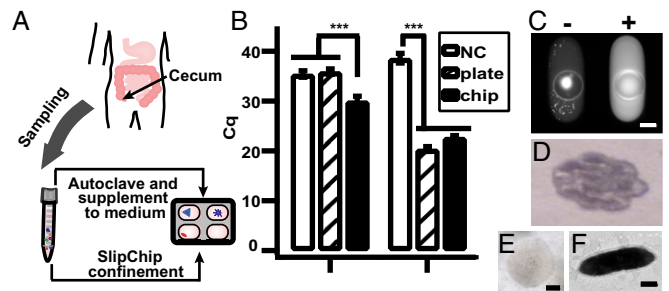


Fig. 5. Targeted isolation of isolate microfluidicus 1 from SlipChip. (A) Illustration showing that mucosal biopsies obtained from the human cecum were used for stochastic confinement as well as supplemented into the medium to stimulate growth of microbes. (B) Identifying the cultivation condition of the microbial target OTU158 using qPCR. (Left) Graph showing that the use of target-specific primers revealed that the target was found in the chip wash solution (M2LC) but not in the blank negative control (NC) or the plate wash solution (M2GSC). (Right) Graph showing that the use of universal primers of 16S rRNA gene showed that both chip wash and plate wash solutions contained bacterial genomic DNA. A lower Cq value indicates higher concentration of DNA. Error bars indicate SD ($n = 3$). (C) Fluorescence microscopy photograph of on-chip colony PCR after the chip was split, showing a positive well (Right) for OTU158. A PCR negative well is shown on the left, as indicated by the low fluorescence intensity of the solution. The bright spot was presumably from cell material stained with SYBR Green. (D) Photograph of the first round of scaled-up culture of OTU158. (E) Microphotograph of a single colony of isolate microfluidicus 1. (F) Transmission electron microscopy image of a single OTU158 cell. Scale bar, 200 μm for C and E, and 0.5 μm for F.

but not in the blank negative control or the plate wash experiment, whereas both plate wash and chip wash solutions had similar quantities of bacterial DNA that were higher than that of the blank negative control. Chip wash with M2GSC medium did not recover OTU158 (*SI Appendix*). We concluded that the M2LC medium with the washing fluid is an optimal condition to cultivate OTU158.

Isolating OTU158 Using Replica-SlipChip. We further tested isolation and scale-up of microcolonies by cultivating the sample on the replica-SlipChip (22) with the M2LC medium containing the washing fluid from the sampling site. PCR was carried out with primers OTU158P targeting OTU158. We observed two positive wells (one is shown in Fig. 5C) from a single device with ~500 microbial colonies (a negative PCR well is shown in Fig. 5C, *Left*). We scaled up the cultivar from one of the positive wells on an agar plate using the M2GSC medium. The intact scale-up culture after 3 d of incubation is shown in Fig. 5D. The culture contained multiple colonies, as shown in the picture, due to the presence of multiple cfus transferred from the same well of the chip. Although we did not observe the target from plate wash and chip wash experiments in the same medium, the cells could be scaled up on an agar plate with M2GSC medium. It is possible that the target grew in M2GSC medium but was outcompeted by rapidly growing strains in both plate wash and chip wash experiments, or that the target was in a dormant state until it was primed by washing fluid from the sampling site (35). Alternatively, the scaled-up colonies may represent a subpopulation of cells that can be cultivated with M2GSC, and the microcolony grown on the chip offered enough cells to cultivate these rare cells. We expect this observation can be understood as similar isolates are obtained using this method and as improved analytics are developed for quantitatively understanding the unculturable state of cells from environmental samples (10). Next, we performed colony PCR on this isolate with both species-specific

and universal primers in bulk, and confirmed by Sanger sequencing that it was indeed the desired target. Although we observed that this was an almost pure culture (with some minor heterogeneity observed from chromatogram, shown in *SI Appendix*), we streaked the plates five times for purification to obtain single colonies (Fig. 5D) of target cells. This isolate, hereafter referred to in this paper as isolate microfluidicus 1, could then be routinely grown in bulk liquid culture to obtain enough biomass to initiate in vivo studies and whole genome sequencing. For example, the draft genome of this isolate was sequenced and assembled into 83 contigs comprising 3.4 Mbp sequences. We observed rod-shaped cells (Fig. 5F and *SI Appendix*) and two 16S rRNA gene types of 99.4% sequence identity to one another, each with 99% identical to OTU_158_V1V3 and OTU_896_V1V3 from the Most Wanted list (*Table S1*). Both OTUs are from the high-priority group classified as *Oscillibacter*, but their relative abundances differ by 20-fold in stool samples surveyed by the HMP (2). Although sequence heterogeneity among multiple 16S rRNA genes on the same genome is not uncommon (36), these two sequence types could either have been derived from a single strain or indicated the presence of two closely related strains. Therefore, we designed two oligonucleotide probes able to distinguish between the two sequence types and used them in FISH experiments (37, 38). All FISH-positive cells bound both sequence type-specific FISH probes (Clostr183-I and Clostr183-II, Fig. 6A), as well as the general probe mix EUB338I-III (*SI Appendix*), which specifically detects most members of the bacteria (39, 40). Together, these results demonstrate the presence of a single *Ruminococcaceae* species in the culture.

Improved Taxonomic Assignment of the Isolate. Short reads from 16S rRNA high-throughput sequencing may not be sufficient for assignment of taxonomy if the organisms are poorly represented in culture collections. Based on both 16S rRNA V4 and V1V3 high-throughput sequencing, the target was classified as *Oscillibacter* (see *SI Appendix* for Ribosomal Database Project classification). However, the pure culture suggests that isolate microfluidicus 1 is a member of a previously unidentified genus. The closest described relative for which a 16S rRNA sequence is available is *Oscillibacter valericigenes*, isolated from a Japanese clam (*Corbicula*) (41), which exhibits a sequence identity of 93.0% to the isolate of isolate microfluidicus 1. Phylogenetic analyses of the 16S rRNA of isolate microfluidicus 1 confirmed the unique positioning of this microbe within the *Ruminococcaceae* (42, 43) (Fig. 6B). These observations suggest that this highly sought (*Table S2*) bacterium may represent, to our knowledge, the first discovered species of an uncultured genus.

Materials and Methods

Sample Collection. Brush and luminal cecum samples were collected from a healthy volunteer. Samples were transferred into an anaerobic chamber immediately after collection and homogenized in grants buffered saline solution (GBSS) supplemented with 5% DMSO by vortexing for 5 min. Aliquots of the samples were flash frozen with liquid nitrogen and preserved at -80°C . Work with clinical samples for this project is approved by the Institutional Review Boards at California Institute of Technology and The University of Chicago, and by the Institutional Biosafety Committee.

SlipChip Cultivation. The brush sample was serially diluted in GBSS buffer and then suspended in M2LC medium. This bacterial suspension was then loaded onto SlipChip designed for chip wash and incubated for 3 d.

Chip Wash. The cultivar was collected into an Eppendorf pipette tip by flowing 90 μL PBS buffer three times and then 90 μL tetradecane into the SlipChip. The solution was then transferred into an Eppendorf tube. DNA was extracted using a QiaAmp kit following the manufacturer's protocol and then used to prepare the library for high-throughput sequencing and PCR.

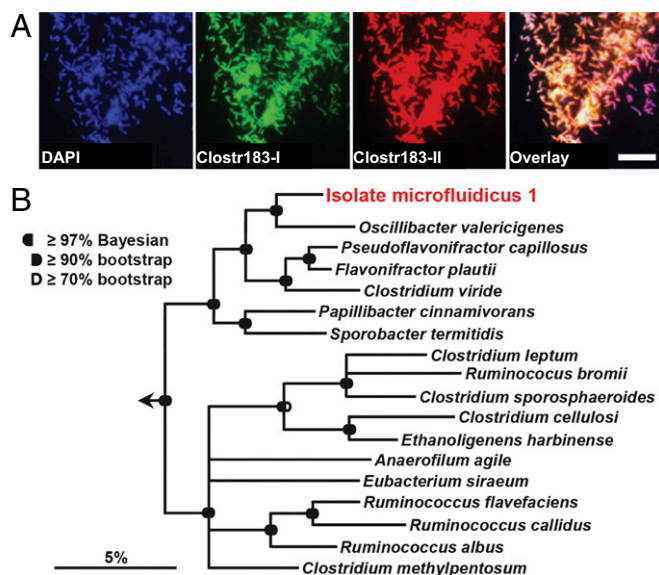


Fig. 6. Phylogenetic affiliation of isolate microfluidicus 1 and validation of the purity of the culture by FISH. (A) Fluorescence images showing that both 16S rRNA types obtained from the culture are expressed within the same cells, demonstrating the presence of a single *Ruminococcaceae* species within the culture. Clostr183-I and Clostr183-II indicate FISH probes, each specific to a different sequence type. (Scale bar, 10 μm .) (B) 16S rRNA-based consensus tree demonstrating the positioning of isolate microfluidicus 1 within the *Ruminococcaceae* (Clostridia cluster IV). Please see *SI Appendix* for details.

Isolation of isolate Microfluidicus 1. We used the replica-SlipChip to cultivate and split the microcolonies. One copy was used for colony PCR to identify the wells containing OTU158. The microcolony from the other copy was transferred on an M2GSC agar plate for scale-up culture.

Conclusions

In this paper, we describe an integrated microfluidic workflow for genetically targeted isolation of microbes, and validate it by successful isolation and cultivation of isolate microfluidicus 1 from the HMP's Most Wanted list. To our knowledge, this is the first example of targeted isolation of a high-priority member from the list, and is the first successful targeted cultivation from a complex biological sample of a previously uncultured taxon defined only by short reads from high-throughput sequencing of the 16S rRNA gene. We believe this genetically targeted workflow can become a general method beyond the isolate described in this paper, as in our preliminary experiments, an additional high-priority and three medium-priority organisms on the Most Wanted list have been isolated. We envision that the microfluidics-based workflow described in

this paper will be useful for conclusively testing hypotheses generated from culture-independent studies by providing pure cultures of biomedically and environmentally significant microorganisms.

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- Schloss PD, Handelsman J (2005) Metagenomics for studying unculturable microorganisms: Cutting the Gordian knot. *Genome Biol* 6(8):229.
- Fodor AA, et al. (2012) The "most wanted" taxa from the human microbiome for whole genome sequencing. *PLoS ONE* 7(7):e41294.
- Kennedy J, et al. (2011) Functional metagenomic strategies for the discovery of novel enzymes and biosurfactants with biotechnological applications from marine ecosystems. *J Appl Microbiol* 111(4):787–799.
- Rooks DJ, McDonald JE, McCarthy AJ (2012) Metagenomic approaches to the discovery of cellulases. *Methods Enzymol* 510:375–394.
- Reddy BVB, et al. (2012) Natural product biosynthetic gene diversity in geographically distinct soil microbiomes. *Appl Environ Microbiol* 78(10):3744–3752.
- Ridaura VK, et al. (2013) Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 341(6150):1241214.
- Frank DN, et al. (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA* 104(34):13780–13785.
- Staley JT, Konopka A (1985) Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu Rev Microbiol* 39(1):321–346.
- Kaeberlein T, Lewis K, Epstein SS (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* 296(5570):1127–1129.
- Stewart EJ (2012) Growing unculturable bacteria. *J Bacteriol* 194(16):4151–4160.
- D'Onofrio A, et al. (2010) Siderophores from neighboring organisms promote the growth of uncultured bacteria. *Chem Biol* 17(3):254–264.
- Giel JL, Sorg JA, Sonenshein AL, Zhu J (2010) Metabolism of bile salts in mice influences spore germination in *Clostridium difficile*. *PLoS ONE* 5(1):e8740.
- Singh S, et al. (2013) Cell extract-containing medium for culture of intracellular fastidious bacteria. *J Clin Microbiol* 51(8):2599–2607.
- Zengler K, et al. (2002) Cultivating the uncultured. *Proc Natl Acad Sci USA* 99(24):15681–15686.
- Ingham CJ, et al. (2007) The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. *Proc Natl Acad Sci USA* 104(46):18217–18222.
- Eun Y-J, Utada AS, Copeland MF, Takeuchi S, Weibel DB (2011) Encapsulating bacteria in agarose microparticles using microfluidics for high-throughput cell analysis and isolation. *ACS Chem Biol* 6(3):260–266.
- Martin K, et al. (2003) Generation of larger numbers of separated microbial populations by cultivation in segmented-flow microdevices. *Lab Chip* 3(3):202–207.
- Park J, Kerner A, Burns MA, Lin XN (2011) Microdroplet-enabled highly parallel cultivation of microbial communities. *PLoS ONE* 6(2):e17019.
- Liu W, Kim HJ, Lucchetta EM, Du W, Ismagilov RF (2009) Isolation, incubation, and parallel functional testing and identification by FISH of rare microbial single-copy cells from multi-species mixtures using the combination of chemistride and stochastic confinement. *Lab Chip* 9(15):2153–2162.
- Vincent ME, Liu W, Haney EB, Ismagilov RF (2010) Microfluidic stochastic confinement enhances analysis of rare cells by isolating cells and creating high density environments for control of diffusible signals. *Chem Soc Rev* 39(3):974–984.
- Stevenson BS, Eichorst SA, Wertz JT, Schmidt TM, Breznak JA (2004) New strategies for cultivation and detection of previously uncultured microbes. *Appl Environ Microbiol* 70(8):4748–4755.
- Ma L, et al. (2014) Individually addressable arrays of replica microbial cultures enabled by splitting slipchips. *Integr Biol*, 10.1039/C4IB00109E.
- Du W, Li L, Nichols KP, Ismagilov RF (2009) SlipChip. *Lab Chip* 9(16):2286–2292.
- Shen F, et al. (2010) Nanoliter multiplex PCR arrays on a SlipChip. *Anal Chem* 82(11):4606–4612.
- Liu W, Chen D, Du W, Nichols KP, Ismagilov RF (2010) SlipChip for immunoassays in nanoliter volumes. *Anal Chem* 82(8):3276–3282.
- Begolo S, Shen F, Ismagilov RF (2013) A microfluidic device for dry sample preservation in remote settings. *Lab Chip* 13(22):4331–4342.
- Fuerstman MJ, et al. (2007) The pressure drop along rectangular microchannels containing bubbles. *Lab Chip* 7(11):1479–1489.
- Li L, Du W, Ismagilov R (2010) User-loaded SlipChip for equipment-free multiplexed nanoliter-scale experiments. *J Am Chem Soc* 132(1):106–111.
- Claesson MJ, et al. (2012) Gut microbiota composition correlates with diet and health in the elderly. *Nature* 488(7410):178–184.
- Raman M, et al. (2013) Fecal microbiome and volatile organic compound metabolome in obese humans with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 11(7):868–875, e1–e3.
- Lam YY, et al. (2012) Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice. *PLoS ONE* 7(3):e34233.
- Caporaso JG, et al. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* 108(Suppl 1):4516–4522.
- Schloss PD, et al. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75(23):7537–7541.
- Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26(19):2460–2461.
- Lewis K (2007) Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* 5(1):48–56.
- Klappenbach JA, Saxman PR, Cole JR, Schmidt TM (2001) Rndb: The ribosomal RNA operon copy number database. *Nucleic Acids Res* 29(1):181–184.
- Amann R, Fuchs BM (2008) Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. *Nat Rev Microbiol* 6(5):339–348.
- Stoecker K, Daims H, Wagner M (2005) Fluorescence in situ hybridization for the detection of prokaryotes. *Molecular Microbial Ecology*, eds Osborn AM, Smith CJ (Bios Advanced Methods, Abingdon, UK), pp 213–239.
- Amann RI, et al. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 56(6):1919–1925.
- Daims H, Brühl A, Amann R, Schleifer KH, Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* 22(3):434–444.
- Iino T, Mori K, Tanaka K, Suzuki K, Harayama S (2007) *Oscillibacter valericigenes* gen. nov., sp. nov., a valerate-producing anaerobic bacterium isolated from the alimentary canal of a Japanese corbicula clam. *Int J Syst Evol Microbiol* 57(Pt 8):1840–1845.
- Collins MD, et al. (1994) The phylogeny of the genus *Clostridium*: Proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* 44(4):812–826.
- Yutin N, Galperin MY (2013) A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. *Environ Microbiol* 15(10):2631–2641.