

The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms

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A miniaturized, disposable microbial culture chip has been fabricated by microengineering a highly porous ceramic sheet with up to one million growth compartments. This versatile culture format, with discrete compartments as small as $7 \times 7 \mu\text{m}$, allowed the growth of segregated microbial samples at an unprecedented density. The chip has been used for four complementary applications in microbiology. (i) As a fast viable counting system that showed a dynamic range of over 10,000, a low degree of bias, and a high culturing efficiency. (ii) In high-throughput screening, with the recovery of 1 fluorescent microcolony in 10,000. (iii) In screening for an enzyme-based, non-dominant phenotype by the targeted recovery of *Escherichia coli* transformed with the plasmid pUC18, based on expression of the *lacZ* reporter gene without antibiotic-resistance selection. The ease of rapid, successive changes in the environment of the organisms on the chip, needed for detection of β -galactosidase activity, highlights an advantageous feature that was also used to screen a metagenomic library for the same activity. (iv) In high-throughput screening of >200,000 isolates from Rhine water based on metabolism of a fluorogenic organophosphate compound, resulting in the recovery of 22 microcolonies with the desired phenotype. These isolates were predicted, on the basis of rRNA sequence, to include six new species. These four applications suggest that the potential for such simple, readily manufactured chips to impact microbial culture is extensive and may facilitate the full automation and multiplexing of microbial culturing, screening, counting, and selection.

microdish | microcolony | cellular assay | nanoporous aluminum oxide

The cultivation of bacteria and fungi has hardly changed in centuries. Culture on gel surfaces, such as agar in a Petri dish, and the liquid growth media used in most microbiology laboratories would be recognized by Robert Koch, who first described the growth of bacteria into a colony in the 19th century (1). Post-genomic sciences (2, 3), the need for industrial strain improvement, and the high level of threat posed by pathogens demand versatile, automatable microbial culture formats that can be widely used (4, 5). Recent microbial cultivation technologies include highly subdivided multiwell plates (6) and capillaries (7), as well as methods of sorting encapsulated microcolonies (8). These systems require expensive and sophisticated hardware and are often specialized in application. Most parallel, miniaturized culture methods are based on growth in liquid media and are variants on the multiwell plate (6). Multiwell formats have problems of oxygen transfer by shaking, the accumulation of waste products, and poor image resolution, and if miniaturized too far suffer from rapid evaporation. Additionally, the throughput is still rather low for genuine high-throughput screening (HTS). Alternatively, in situations in which microorganisms must be detected or assayed (such as routine quality control or diagnostics), low cost is needed, and only limited laboratory facilities are available. Further miniaturization, and therefore multiplexing, of culture formats is feasible because the cells are generally

only 1–10 μm in size. The challenge is how to do this in a way that will be widely used.

Centuries of experience have shown that agar-supported growth of microorganisms in the Petri dish is robust and reliable, and this is reflected in the near ubiquity of this method in microbiology. Weaknesses of the agar-containing Petri dish lie in a poor facility for automation, the requirement for user skill, limited speed to result, the generation of waste (including the enrichment of pathogenic or genetically modified organisms), a short shelf life, and limited storage time of organisms *in situ* after culture. Additionally, agar does not suit the growth of all microbial species. Microbial culture on flexible porous membranes, on filters, or in chambers provides valuable alternatives, permitting manipulations such as staining *in situ* and changes in the nutrient environment of the cells, and culturing otherwise intractable species (9, 10). However, these culture devices have never been subdivided to the point of creating sufficient growth compartments for genuine HTS. In part, this may be because flexible membranes are hard to engineer at the micrometer scale and, possibly, the perception that micro-engineered mechanical systems (MEMS) devices cannot compete on cost with a cheap plastic disposable. Porous aluminum oxide (PAO) has been recently identified as a microbial culture support (11–13). This material is exceptionally porous (40% by volume) compared with other surfaces, inert, and stable with nano-range pores (20–200 nm diameter) and retains microorganisms on the rigid planar surface while allowing nutrients to pass. Culture on PAO to date (11) has been limited to hundreds of growth areas, which is inadequate for HTS. Hence, we developed the concept to provide far more advanced culture formats fabricated by MEMS techniques. Micro-engineering of PAO is facilitated by the great stability, rigidity, and inertness to temperature, wetting, or solvents of the starting material. A range of disposable, surface-culture, microbial growth chips, or “micro-Petri dishes,” were therefore created by using a MEMS approach to engineer growth compartments on top of PAO, which acts as the surface on which an exceptional number of microbial samples can be grown, assayed, and recovered.

Results

Chip Manufacture, Specifications, and Physical Properties. Reactive ion etching (RIE), a process of etching by using plasma directed by

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Abbreviations: FDG, fluorescein di- β -D-galactopyranoside; FDP, fluorescein 3,6-diphosphate; HTS, high-throughput screening; L-agar, Luria agar; PAO, porous aluminum oxide; RIE, reactive ion etching.

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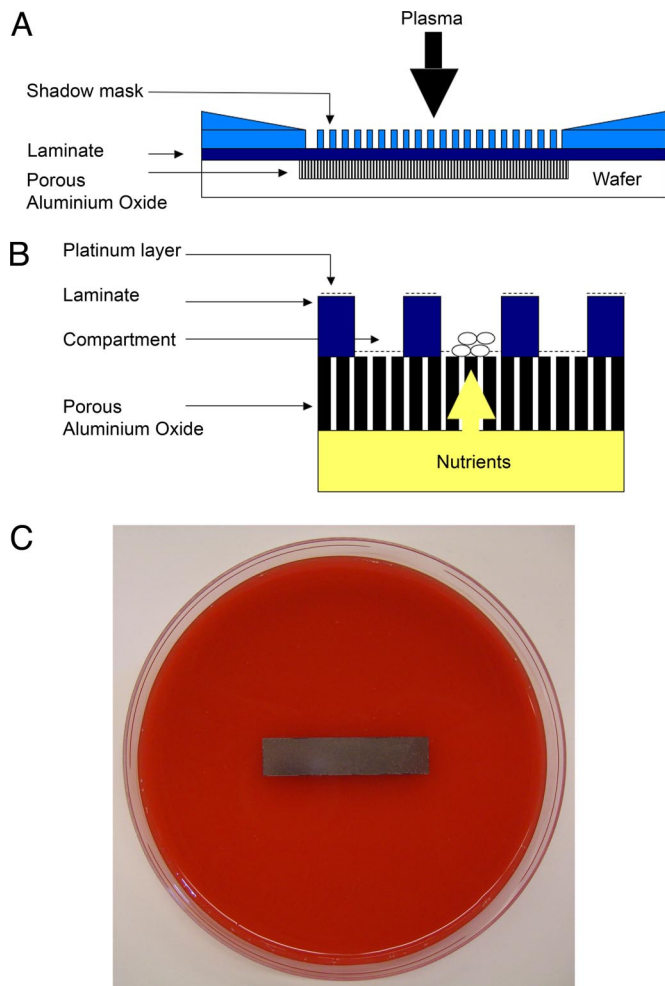


Fig. 1. Culture chip use and manufacture. (A) Cross-section of set-up for RIE of a 36×8 -mm strip of aluminum oxide (not to scale). (B) Cross-sectional diagram of a small part of a chip, illustrating microbial growth in the central compartment (7 – $150 \mu\text{m}$ wide) and supply of nutrients from beneath the $60\text{-}\mu\text{m}$ thickness of the aluminum oxide and $10\text{-}\mu\text{m}$ -high walls. (C) Photograph of a platinum-coated chip placed on sheep's blood agar in a standard Petri dish.

a shadow mask (14), was used to selectively remove an acrylic polymer laminated on the surface of the PAO to create areas open for growth (Fig. 1). The compartments formed (Fig. 2) were precisely defined by the photo mask, with coefficient of variation values of $<4\%$ for the X and Y dimensions of the compartments and accuracies of $<2 \mu\text{m}$ across the complete chip. Some erosion of the walls occurred during etching, leading to narrowing toward the top (e.g., Fig. 2C), but breaches into adjacent compartments were rare, being found in $<0.5\%$ of cases ($n = 6,840$). SEM of the compartment surface suggested that the porous material forming the compartment base was undamaged and not blocked by debris, and that the walls were effectively bonded to the PAO (Fig. 2C). The completed chips were permeable to water or ethanol drawn through from the upper surface by using a vacuum manifold. The PAO was strengthened by the acrylic laminate, forming a more robust composite material. Optimization of the etching time proved critical to the manufacturing process. Too little etching time resulted in incomplete removal of the laminate, and over-etching resulted in deposition of material onto the PAO from the mask. This back-sputtering and the resultant erosion of the mask during etching was minimized by a coating of aluminum oxide on the mask. The full range of chip formats created is listed in supporting information (SI) Table 1. The maximum density of growth areas was $>340,000$

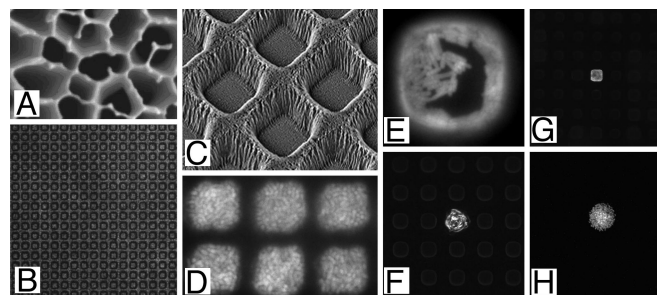


Fig. 2. Images of materials, growth compartments, and microbial culture on chips. (A) SEM of aluminum oxide showing pores on average 200 nm diameter. (B) Transmission light microscopy of hundreds of $20 \times 20\text{-}\mu\text{m}$ compartments viewed from above. (C) SEM of $7 \times 7\text{-}\mu\text{m}$ compartments from above at a 30° angle. (D) Culture of *L. plantarum* in six compartments of the same dimensions as C stained with a fluorogenic dye (Syto 9) after growth and imaged from above. (E) Detection of β -galactosidase activity using the fluorogenic substrate FDG, from *E. coli* containing plasmid pUC18 grown in a $20 \times 20\text{-}\mu\text{m}$ compartment. (F) As in E with one plasmid-containing microcolony, viewed at lower magnification. (G) View of $20 \times 20\text{-}\mu\text{m}$ format chip with one area supporting a GFP-expressing strain of *E. coli* in a background of nonfluorescent cells. (H) Previously uncultivated oligotrophic bacterium related to *Dechloromonas* sp. labeled by FDP metabolism and grown in a $20 \times 20\text{-}\mu\text{m}$ compartment supplied by Rhine water, before recovery and identification by PCR.

per cm^2 , permitting growth formats to be created with millions of culture areas. The smallest compartment size made was $7 \times 7 \mu\text{m}$ at the base, which gave 1 million discrete growth areas on an $8 \times 36 \text{ mm}$ chip. This density allows 40 million compartments to be placed on the footprint of a standard multiwell plate. The most commonly used compartment size ($8 \times 36 \text{ mm}$ chip, $20 \times 20 \mu\text{m}$ growth areas, $20\text{-}\mu\text{m}$ -wide walls) had 180,000 compartments per chip. This configuration was used for experiments, unless noted otherwise. Assuming exponential growth, each compartment in the standard format allows ≈ 5 – 10 divisions of a typical rod-shaped bacterium from a single viable cell as the inoculum to a detectable monolayer of hundreds of cells or filling the compartment (thousands of bacterial cells). This gives sufficient time and biomass for culture-based screening operations to be performed.

The Ordyl 314 (acrylic) material forming the grid showed a degree of autofluorescence that partially interfered with the imaging of cells by fluorescence microscopy. This autofluorescence (515 – 730 nm) was detectable with the 10 - to 400 -ms exposures typically required for imaging fluorescent cells by CCD camera. To counter this, a 20-nm -thick layer of platinum was applied by sputtering over the whole chip (Fig. 1B). This was sufficient to mask autofluorescence >20 -fold (515 – 730 nm). The platinum did not block the pores of the aluminum oxide; when measured by SEM, the decrease in average pore area due to the platinum sputtering was negligible, measuring the face of the PAO shown in Fig. 2A ($P > 0.5$ by Student's t test; $n = 500$ for both samples). The platinum-sputtered format was therefore used for all experiments.

Growth of Test Microorganisms on Chips. The chips were tested for the growth of microorganisms. For comparison, a description of the growth of the same organisms on unmodified PAO is included in SI Methods and SI Fig. 5. Pretreatment with ethanol (20 min with $95\% \text{ vol/vol}$) was sufficient to sterilize the chips for most applications. The chips were stored individually in sterile tubes. Chips were deployed to agar plates directly from the tubes by tapping the tube until one end of the chip contacted the agar, then withdrawing the tube so that the base of the chip contacted the agar. Capillary action rapidly wetted the chip, which was now ready for inoculation. Chips were moved between media or staining slides by using a piece of sterile Parafilm. Effective growth and segregation of all three test organisms (*Lactobacillus plantarum* WCFS1, *Escherichia coli* XL2

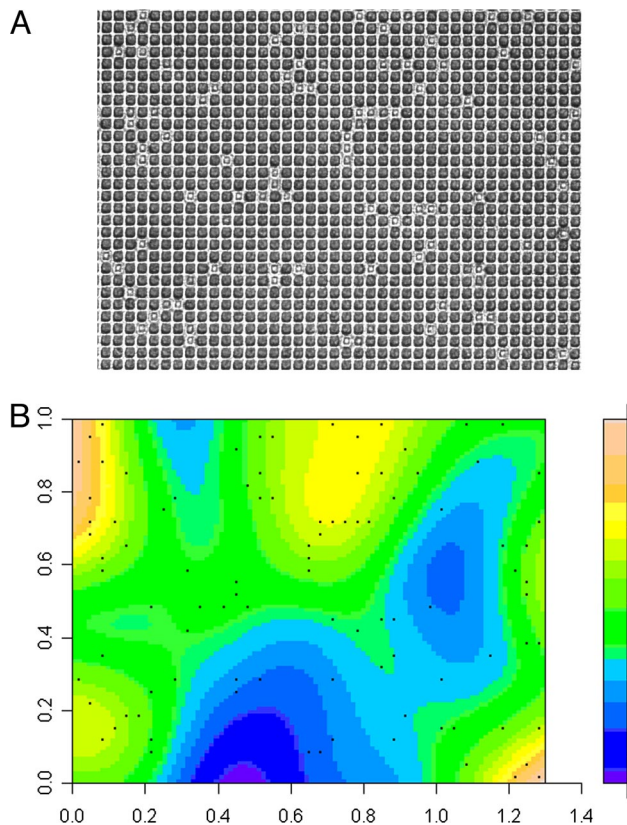


Fig. 3. Example of bias and distribution of culture on chips. (A) Original image (inverted transmission light microscopy image; lighter areas show growth of *L. plantarum*) of a single field of view of $1,170 \times 20 \times 20\text{-}\mu\text{m}$ compartments. (B) Heat-density map of local colony density, computed by using a Gaussian smoothing kernel used, to construct statistical models of chip performance and assess bias.

Blue, and *Candida albicans* JBZ32) was seen in compartments of inoculated chips with culture areas from $150 \times 150 \mu\text{m}$ down to $7 \times 7 \mu\text{m}$ (Figs. 2 and 3A). Spread plating $20 \times 20\text{-}\mu\text{m}$ -compartment chips with >10 colony-forming units (cfu) per compartment indicated that $>99.95\%$ of compartments could support growth. Culturability of *L. plantarum*, *C. albicans*, and *E. coli* was 49%, 96%, and 97%, respectively, comparing culture on the chip with growth on unmodified PAO.

Inoculation with *L. plantarum* was used to assess biases in culturability across the chips (Fig. 3). A statistical model was used to assess whether inoculation in one compartment lead to an unusually high probability of growth in an adjacent compartment (described in detail in *SI Methods*). A plot of the Poisson probability distribution function of the local density of compartment occupancy with an expected density equal to the global density showed no indications of extreme deviations. In addition, a χ^2 goodness-of-fit test on 5×5 quadrats gave no indications that the occupation of compartments deviated from a uniform Poisson point process: in a series of 12 samples with three different dilutions of bacteria, the lowest *P* value was 0.37. Taken together, this suggests that the manufacture process created culture areas that were highly consistent and that biases—e.g., due to differences in the etching process across the chips or between chips, or because of differences in inoculation—were minimal.

A test for local bias, in particular for the detection of an influence of the occupation of a compartment on the occupation of compartments in the immediate neighborhood, indicated only significant deviations from a random model at only the highest inoculation densities ($P < 0.05$ above 10% occupancy). This was due to

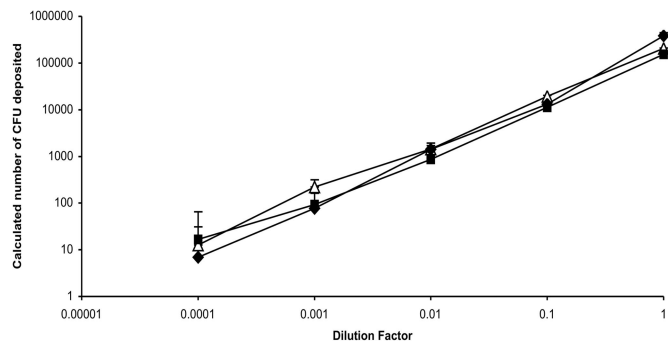


Fig. 4. Culture and viable counting of microorganisms on chips. Dilution series of microorganisms plated on a series of $20 \times 20\text{-}\mu\text{m}$ format chips. The number of deposited cfu per chip was calculated from the fraction of compartments supporting growth, as scored by microscopy, with the numbers corrected for inoculation of compartments with multiple cfu. Open triangles, *E. coli*; filled squares, *C. albicans*; filled diamonds, *L. plantarum*. Error bars show SD with lower limits not shown at lowest dilution factor because of log₁₀ transformation of the data.

limited spill-over of bacteria from one compartment to the other, which may happen at high densities and when the colony volume exceeds the compartment volume. Microscopic examination of chips suggested that there was no association of local bias with incomplete wall manufacture. With overnight growth of *E. coli*, greater invasion of orthogonally adjacent compartments was observed, increasing this bias (data not shown). Modifying the compartment size had the expected effect, altering overgrowth by an amount consistent with the time to fill the new volume, a few hours difference for most organisms. The culturability of the organisms in the test panel was not greatly affected by compartment size (data not shown). All experiments described were conducted under conditions in which spill-over was not a significant bias.

A dilution series of *L. plantarum* was used to assess the ability of the chip to act as a counter for cfu. By scoring growth as present or absent in a total of 150,000 compartments (a total area of $<3 \text{ cm}^2$), a linear relationship was obtained between the number of cfu in the inoculum and the percentage of compartments supporting growth over four orders of magnitude (Figs. 3A and 4). This was also found to be the case for *E. coli* and *C. albicans* (Fig. 4). These data show that viable counts can be made on these chips for very different organisms. For comparison, a conventional viable count would require multiple Petri dishes and a 10-fold dilution series of the microbial culture, with only plates containing $\approx 30\text{--}300$ colonies acceptable for counting. Culture on the chips required 0.5 ml of nutrient agar; assuming duplicate plating, the miniaturization factor for the chip is approximately three orders of magnitude.

Screening and Recovery of a Variant on the Basis of Fluorescence. To demonstrate a simple screening application, a green fluorescent protein (GFP)-expressing, ampicillin-resistant strain of *E. coli* (15) was diluted 1:10,000 into cells of the same strain carrying pUC18 to confer ampicillin resistance but not expressing GFP. This mixture was used to inoculate chips by spread plating and incubated on Luria agar (L-agar) for 5 h at 37°C. Inoculation at a cell density supporting growth in 8.1% of compartments across six chips (a total of 1,080,000 compartments with 87,480 supporting growth) yielded five instances where GFP-expressing cells were detected by fluorescence microscopy after systematic scanning of the chips (e.g., Fig. 2G). By manual use of a sterile toothpick, targeted by microscopy, GFP-expressing strains could be recovered, and any contaminating nonfluorescent clones were removed by subculture on agar. Contamination with nontargeted cells, when it occurred ($< 50\%$ of cases in GFP experiments), had two causes: (i) occupancy of a

compartment by two clones, as seen by segregation of fluorescence in a compartment, and (ii) imprecise recovery caused by scraping the toothpick against an adjacent compartment in addition to the target, which could be deduced by damage to the platinum coat and the disturbance of microorganisms. However, these problems were not a significant obstacle to screening. A simple purification by restreaking on a recovery plate was sufficient to purify an isolate and was not always necessary. These data suggest that screening operations and targeted strain recovery can be conducted effectively with only minimal secondary purification required to achieve a purified isolate with the desired properties or phenotype.

Screening for *E. coli* Transformants on the Basis of β -Galactosidase Activity. A screening was performed to recover *E. coli* transformed with plasmid pUC18 without using a growth-based selection marker (such as an antibiotic-resistance gene) by means of a plasmid-dependent enzyme activity; i.e., β -galactosidase expression (16). The plasmid (10 ng) was transformed into chemically competent cells of *E. coli* strain OmniMax (Invitrogen) according to the manufacturer's protocol. An aliquot of the transformation mixture was used for conventional plating, resulting in 1.1×10^9 ampicillin-resistant transformants per μg pUC18 DNA with 1 in 2,000 cells in the original mixture gaining ampicillin resistance. Other aliquots were analyzed on the chips for α -complementation and the production of a functional β -galactosidase. This was done by using the fluorogenic substrate fluorescein di- β -D-galactopyranoside (FDG) with a competitive inhibitor of β -galactosidase to regulate the rate of FDG hydrolysis (17, 18). The cells were loaded with the fluorogenic substrate by osmotic shock, by moving chips from growth medium to a hypertonic medium to a hypotonic medium containing FDG then to a recovery medium. Osmotic shock is a standard method of loading FDG (18), and this demonstrates an important advantage of the chip: effectively a series of changes in cell environment were made for a large number of samples. By screening six chips (a total of 113,000 microcolonies) for the most highly fluorescent microcolonies (e.g., Fig. 2 E and F), using customized GridFinder software and an automated XY table to locate and center "hits" (see *SI Methods*) for recovery, seven transformants were recovered successfully in 12 attempts. This was confirmed by restriction digestion of the isolated plasmids after further culture and DNA purification. No successful recoveries were made with the same number of recovery attempts of the most fluorescent microcolonies in control experiments using cells transformed with a noncomplementing plasmid (pUC18 containing an insert in the polylinker) or in mock transformations. Additionally, a metagenomic fosmid library of DNA isolated from human ileum effluent constructed in *E. coli* (E. Zoetendal, C. Booijnck, M. Kleerebezem, and W.M.d.V., unpublished work) was screened by the same procedure. This resulted in the detection and isolation of four β -galactosidase-producing microcolonies from 12,000 clones (data not shown).

When exchanging medium with the bulk phase, a distance of only 60 μm had to be traversed from the base of the chips to the cells on the upper surface. Therefore, the rapid exchange of salt and FDG, required for the osmotic shock experiments as described above, was effective. Control experiments were performed by using fluorescein to follow the kinetics of small-molecule exchange between the chips and the bulk medium (method described in *SI Methods*). In loading the chip with small molecules from beneath, an influx of fluorescein equivalent to >90% of the concentration of the medium below was achieved in <10 s. In terms of efflux from a chip loaded with fluorescein, dilution of more than two orders of magnitude occurred in <25 s.

Screening Oligotrophic Bacteria on the Basis of Organic Phosphate Metabolism. A screen of microbial samples isolated from a low-nutrient environment (the Dutch region of the Rhine River at Wageningen, The Netherlands) was undertaken on the chips. The

Rhine water that was the source of the inoculum was also filter-sterilized and used as the growth medium; this was only supplemented with 0.3% (wt/vol) Gelrite (Merck) as a minimal gelling agent. The total cell count by microscopy was 8.7×10^5 cells per ml of Rhine water. After 6 days of incubation under aerobic conditions at 15°C, the viable count on Rhine water Gelrite was 4.4×10^3 cfu/ml (0.5% culturability). By counting microcolonies on chips placed on the same medium, the viable count was 6.9×10^4 cfu/ml, a >10-fold increase in culturability. These data are consistent with viable counts of oligotrophs obtained from similar environments (19). Higher viable counts are known to be observed when assessing microcolonies grown on porous supports than visible colonies on agar (20). Chips ($n = 25$, average compartment occupancy of 4.6%) were screened after culture for 6 days (207,000 microcolonies). Selection of microcolonies for recovery was on the basis of uptake of fluorescein 3,6-diphosphate (FDP) and rapid conversion to fluorescein (21) (Fig. 2H). The criteria for recovery were the conversion of FDP and microcolony labeling >100-fold more intense than the mean, and a cell morphology likely to be prokaryotic. Less than 1 in 2,000 cfu fit these criteria. The fluorescence of FDP-converting cells was sufficiently intense that it was clear from imaging the toothpick during recovery that the intended sample was being picked up. Hundreds of cells were recovered from each target strain. These were identified after DNA extraction by PCR amplification, sequencing of the variable regions of DNA encoding 16S rRNA, and comparative analysis with the RDP II ribosomal database (22, 23). Of 23 recovered strains, 22 amplified and gave good quality sequence. This suggests that recovery was from a single bacterial strain. A more detailed phylogenetic analysis of the recovered strains is available in *SI Fig. 6*. Of the previously cultured organisms, several were from recently identified groups known to be involved in organic phosphate degradation, including *Paucibacter toxinivorans* and *Malikia granulosa* (23–26). Of the six previously uncultured organisms, the nearest relatives were *P. toxinivorans*, and members of the genera *Dechloromonas*, *Sphaerotilus*, *Aeromonas*, and *Sphingomonas*. At least one newly cultured isolate (Rhine 6) may be a member of an uncultured genus. The remainder of strains were identified to the genus level and included *Aeromonas*, *Acidovorax*, *Klebsiella*, and *Rhodobacter* spp. Attempts to perform a similar screening on PAO without the compartment structure failed because of cross-contamination by microorganisms capable of spreading across the surface over the 6- to 9-day incubation period. Taken together, these data suggest that HTS for a phenotype, followed by recovery, from a complex microbial population was possible on the chips.

Discussion

A microengineering method has been used to create a series of simple but effective microbial growth chips based around a nanoporous support. The manufacturing method was reliable; compartments on the micrometer scale could be constructed with precise dimensions that were consistent and homogeneous with respect to microbial growth. Almost every compartment appeared to be available for culture, showing that selective removal of the laminate without degrading the functional properties of the PAO beneath was efficient. An advantage of the RIE method is that the spacing of the compartments directed by the shadow mask is extremely precise, which is an important feature when other tools (e.g., printing, inoculation, or recovery systems) need to be aligned with the growth chip.

Culture and containment of three very different microorganisms was demonstrated. The culturability of these organisms on the chips was similar to that on unmodified PAO. If necessary, improved containment was possible by increasing wall height. However, given that microcolonies of hundreds of cells can be contained in the standard format (*SI Table 1*), the current height was sufficient for most detection and assay methods. Manual recovery, or with a basic micromanipulator, of variant cells was possible at a greater dilution

GFP Screening and Strain Recovery Experiment. *E. coli* XL2 Blue was transformed with the plasmid pEGFP1 (Clontech), expressing of GFP (15, 16). As a control, the same strain was transformed with the plasmid pUC18. The GFP-expressing strain was diluted 1:10,000 with the nonfluorescent strain, spread plated on six chips, and incubated for 5 h on L-agar plates with 200 $\mu\text{g}/\text{ml}$ ampicillin at 37°C. This dilution for spread plating was made with the aim that 10% of the compartments on each chip should support growth, so that in most cases only a single cfu would be inoculated per growth area. After incubation, the chips were scanned by microscopy (11) to visualize compartments supporting the growth of GFP-expressing cells. The percentage of compartments supporting growth was scored, imaging 1,170 compartments per field of view for the 20 \times 20- μm configuration. Recovery of GFP-expressing cells was accomplished by hand, using a sharp, sterile toothpick (tip <60 μm in diameter) targeted by microscopy. The result of each recovery attempt was streaked on an L-agar plate with ampicillin.

Screening for Transformation Events. Transformation and recovery of a plasmid was performed on the chips by direct screening of β -galactosidase activity, without antibiotic selection, to identify plasmid-containing clones. OmniMax-competent cells (Invitrogen) were transformed with 10 ng of plasmid pUC18 (16). As controls, cells were transformed with pUC18 containing an insert in the polylinker to inactivate α -complementation. Inoculation was by spread plating dilutions from transformation mixes directly onto chips. Incubation of chips was on L-agar plates with 100 μM IPTG and 0.5 mM D-glucose for 5 h at 37°C. An on-chip osmotic shock was used to load cells with the fluorogenic β -galactosidase substrate FDG (Sigma) (17). A 1-mm layer of L-agar with 0.3 M NaCl was poured on a sterile microscope slide and used to transfer the chips to subject the microcolonies on each chip to a hypertonic environment. After 10 min, the chips were transferred to a second set of slides coated with 1/5 strength Luria broth gelled with 1.5% agar with 100 μM FDG and 20–100 μM phenylethyl-D-thiogalactopyranoside (18). After 10 min, chips were transferred to a third set of slides covered with L-agar for cell recovery and FDG hydrolysis (15 min at 37°C). More than 110,000 microcolonies were screened by

fluorescence microscopy in each experiment. Recovery of plasmid-containing clones was performed in a fashion similar to the GFP screening experiment but using L-agar plates containing X-gal and IPTG at standard concentrations (16) without antibiotics. Ten recoveries were made per experiment, targeting the most fluorescent microcolonies from six chips. Plasmid recovery was confirmed by liquid culture of blue colonies in 1.5 ml of Luria broth in the absence of antibiotics with plasmid preparation and restriction digestion (16).

Screening for Oligotrophs Metabolizing Organophosphate Dye. Samples of water from the Rhine River (The Netherlands) were plated on plates of filter-sterilized Rhine water solidified with 0.3% (wt/vol) Gelrite (Merck) directly or on chips placed on this medium. Incubation of samples was at 15°C for 6–9 days under aerobic conditions. Additionally, filter counts were made with the Sybr II Gold strain to determine the number of microorganisms in the samples (20). After incubation, chips were transferred to slides with low-melting-point agarose containing the fluorogenic substrate FDP at 100 μM (21). After incubation for 20 min at 15°C, the chips were transferred to a second slide without dye and imaged. Recovery of selected samples was by toothpick with the criteria for selection being unusually strong FDP conversion to fluorescein (>100-fold greater than the mean for microcolonies grown from this source) and observation of cellular morphology, selecting for strains likely to be prokaryotic. Bacterial cells were harvested by using a toothpick. A few hundred cells were resuspended in 20 μl of InstaGene matrix (Bio-Rad), and the bacterial DNA was extracted. The partial DNA sequence of 16S rRNA genes of the isolates was determined after PCR amplification (22). Stain identification and phylogenetic analysis used the Ribosomal Database Project II, Release 9.52 (23).

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