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Growth and Multiplexed Analysis of Microorganisms on a Subdivided, Highly Porous, Inorganic Chip Manufactured from Anopore

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A highly porous inorganic material (Anopore) was shown to be an effective support for culturing and imaging a wide range of microorganisms. An inert barrier grid was printed on the rigid surface of Anopore to create a "living chip" of 336 miniaturized compartments (200/cm^2) with broad applications in microbial culture.

There is a pressing need in microbiology for the development of automated, miniaturized, and multiplexed growth formats that improve on the petri dish and the microtiter plate (1, 3). One approach to microbial culture has been to use flexible organic membranes as a support, permitting growth and imaging in situ (2, 10). We suggest in this work that an inorganic and rigid porous surface may be a better choice for many applications. Anopore is an inert ceramic, an aluminum oxide that is formed in sheets by a high-pressure and etching technique (4, 6, 8), creating a uniquely porous planar material; up to 50% of the volume is pores. Anopore has been identified as a superior surface for the imaging of microorganisms compared to flexible organic membranes (8, 9), in part due to its flatness and limited autofluorescence. The motivation for this work was to test the suitability of Anopore in the creation of versatile new growth formats or "living chips."

Anopore strips (8 by 36 mm; 60 μm thick; 0.2-μm-diameter pores; 3×10^9 pores cm^{-2}) were a gift from PamGene International ('s-Hertogenbosch, The Netherlands). A latex solution (masking fluid 052; Royal Talens, The Netherlands) was applied to one surface in 1-mm-thick lines using a mapping pen and allowed to polymerize at room temperature for 20 min. The strips were then washed with distilled water and twice with 96% (vol/vol) ethanol and air dried. The grid formed a surface barrier 0.5 mm wide and 0.4 mm high that delineated eight culture areas of 7 by 4 mm. The polymerized latex was not strongly autofluorescent: illumination in the 515- to 730-nm range required exposures of >5 seconds to saturate the charge-coupled device camera. Anopore strips were placed on an appropriate nutrient agar base (5), inoculated on the upper surface at a density of 100 to 2,000 CFU/mm^2, and then incubated. Microcolonies were stained by transfer of these strips right-side up to a microscope slide covered with a 1-mm-thick film of 1% (wt/vol) solidified low-melting-point agarose (Sigma, The Netherlands) containing 10 μM Syto-9 dye plus 40 μM propidium iodide (PI) or 5 μM hexidium iodide (HI) for bacteria and 20 μM Fun-1 for yeasts (7) (all dyes were from Invitrogen, The Netherlands). Staining was for 20 min at room temperature for bacteria and 30°C for yeasts. These procedures allowed staining of the organisms on the Anopore surface through the pores without disruption of the microcolonies. Strips were then imaged directly (without coverslip, immersion oil, or fixative) using an Olympus BX-41 fluorescence microscope equipped with U-MWIBA filters (excitation spectrum of 460 to 490 nm, dichroic mirror splitting at 505 nm, and an emission spectrum of 515 to 550 nm, used for Syto9 and Fun-1 dyes), U-M41007 (530 to 560 nm excitation, 565 nm splitting, and 575 to 645 nm emission, used for PI and HI dyes), and U-M41008 (590 to 650 nm emission, 660 nm splitting, and 665 to 735 nm excitation) (Olympus, Japan). Scanning and examination of microcolonies were performed with 4× and 10× UMPlanF1 objective lenses, and observation of individual cells used a 50× UMPlanF1 objective (Olympus, Japan). Growth of a wide range of microorganisms was possible (Table 1), and all organisms tested grew. Growth was detected after one to two rounds of division by the formation of microcolonies (Fig. 1A and B). Formation of visible colonies on Anopore generally occurred in a similar time frame as directly on the same nutrient agar; for example, colonies of *Escherichia coli* 2613 were visible with an overnight incubation on Anopore on a variety of media (Table 1). The culturability of mid-log- and stationary-phase cultures of *Escherichia coli* 2613 and *Staphylococcus aureus* 1101711 was similar on Anopore placed on Mueller-Hinton or blood agar as on these media directly (assessed by viable count). Cell morphology and development were as expected; for example, *Candida albicans* formed germ tubes within 1.5 to 2.5 h after inoculation of the yeast form on Anopore on blood agar (Fig. 1D). Syto-9/PI staining (7) of the distribution of cells within compartments suggested that the latex barrier was neither toxic nor bacteriostatic for *E. coli* 2613, *Enterobacter aerogenes* 1499, or *S. aureus* 1101711.

The doubling time of *E. coli* 2613 on Anopore was assessed by Syto-9 staining at different time points, followed by a manual count of the number of cells per microcolony (Fig. 2). After a lag phase of 25 min, the increase in cell number was exponential, with a doubling time of 27 min for growth on Anopore

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TABLE 1. Bacterial and yeast strains cultured on Anopore

Species	Strain[a]	Growth conditions[b]
Bacteria		
Actinomyces meyeri	2498	BA and SC 37°C An
Aerococcus viridans	344	BA 37°C CO_2
Aeromonas hydrophila	2324	MH 37°C CO_2
Aeromonas veronii	1289	MH 30°C
Bacillus cereus	2380	MH 37°C CO_2
Bacillus licheniformis	377	BA 37°C
Bacillus mycoides	24-1	BA 37°C
Bacillus subtilis	168	LA and 2TY 37°C
Bacteroides fragilis	ATCC 25285	BA 37°C An
Brevundimonas versicularis	2412	MH 37°C CO_2
Campylobacter jejuni	ATCC 11392	CA and MH 43°C Micro
Citrobacter divers (koseri)	711	MH and BA 37°C CO_2
Citrobacter freundi	2452	MH and BA 37°C CO_2
Clostridium perfringens	M2	MH and BA 37°C AN
Corynebacterium aquaticum	231	MH 37°C CO_2
Enterobacter aerogenes	X1468	MH and BA 37°C $\pm CO_2$
Enterobacter cloacae	U2364	MH and BA 37°C $\pm$ CO_2
Enterococcus faecalis	2113	BA 37°C $\pm$ CO_2
Escherichia coli	XL2 Blue	MH and LA 37°C
Escherichia coli	2613	MH and BA 37°C CO_2
Haemophilus influenzae	ATCC 49247	CH 37°C CO_2
Hafnia alvei	981	MH and BA 37°C CO_2
Klebsiella pneumoniae	2600	BA 37°C CO_2
Legionella pneumophila	643	MH-IH 37°C CO_2
Listeria monocytogenes	1444	MH 37°C CO_2
Neisseria gonorrhoeae	7	TM 37°C
Nocardia asteroides	168-1	BA 37°C
Pantoae agglomerans	X28	BA and MH 37°C CO_2
Proteus mirabilis	2612	BA and MH 37°C CO_2
Proteus vulgaris	1790	BA and MH 37°C CO_2
Providencia rettgeri	2699	BA 37°C
Pseudomonas aeruginosa	ATCC 27853	MH 37°C CO_2
Pseudomonas stutzeri	52-2	MH 37°C CO_2
Ralstonia pickettii	112	BA and MH 37°C
Salmonella enteritidis	2627-2	BA 37°C CO_2
Serratia marcescens	2553	BA and MH 37°C CO_2
Shigella flexneri	M1	MH and BA 37°C CO_2
Shigella sonnei	2627-1	MH and BA 37°C $\pm$ CO_2
Staphylococcus aureus MRSA	110117	MS, BA and MH 37°C $\pm$ CO_2
Staphylococcus epidermidis	159	MH 37°C $\pm$ CO_2
Stenotrophomonas maltophilia	4089	BA 37°C CO_2
Streptococcus agalactiae	725	BA 37°C CO_2
Streptococcus pneumoniae	ATCC 49619	BA 37°C CO_2
Streptococcus pyogenes	*74*	BA 37°C CO_2
Vibrio parahaemolyticus	265	TCBS 37°C
Yersinia enterolitica	2636	BA 30°C CO_2
Yeasts		
Candida albicans	32	BA and SB 37°C $\pm$ CO_2
Candida krusei	ATCC 6258	SB and BG 37°C
Candida tropicalis	876	SB and BG 37°C

[a] Strain numbers refer to the strain collection of the Jeroen Bosch Hospital.

[b] Medium abbreviations: MH, Mueller-Hinton agar; MH-IH, MH plus IsovitaleX and hemoglobin; TM, Thayer-Martin agar; TCBS, thiosulfate citrate bile sucrose agar; BA, sheep's blood agar; LA, Luria agar; 2T, 2TY agar; SC, Schaedler agar; CH, chocolate agar; CY, charcoal yeast agar; SB, Sabouraud agar; MS, mannitol salt agar; BG, biggy agar. Atmosphere abbreviations: An, anaerobic; CO_2, aerobic culture in a CO_2 incubator; $\pm$ CO_2 growth both with and without CO_2; Micro, microaerobic culture. If atmosphere is not noted, incubation was aerobic without additional CO_2.

(Mueller-Hinton agar at 37°C). This compared to a doubling time of 23 min for the same strain grown in shake flasks in Mueller-Hinton broth at 37°C, with growth monitored by the change in optical density at 600 nm. The doubling time was 24 min at 37°C on Anopore on sheep's blood agar, a medium too turbid for the conventional monitoring of growth in broth culture.

To test a practical application of these chips, a dilution series (a 1/20 and 1/400 dilution of visibly turbid samples, undiluted, and a 1/20 dilution of other samples) of each of 15 potentially infected urine samples was inoculated on both Anopore chips (7- by 4-mm culture areas, 5 μl per area; two to four replicates) and directly on blood agar and incubated at 37°C for 4 and 18 h, respectively. The standard lab method was visual identification of differences in colony morphology to indicate a mixed culture. A combination of selective culture, Gram stain

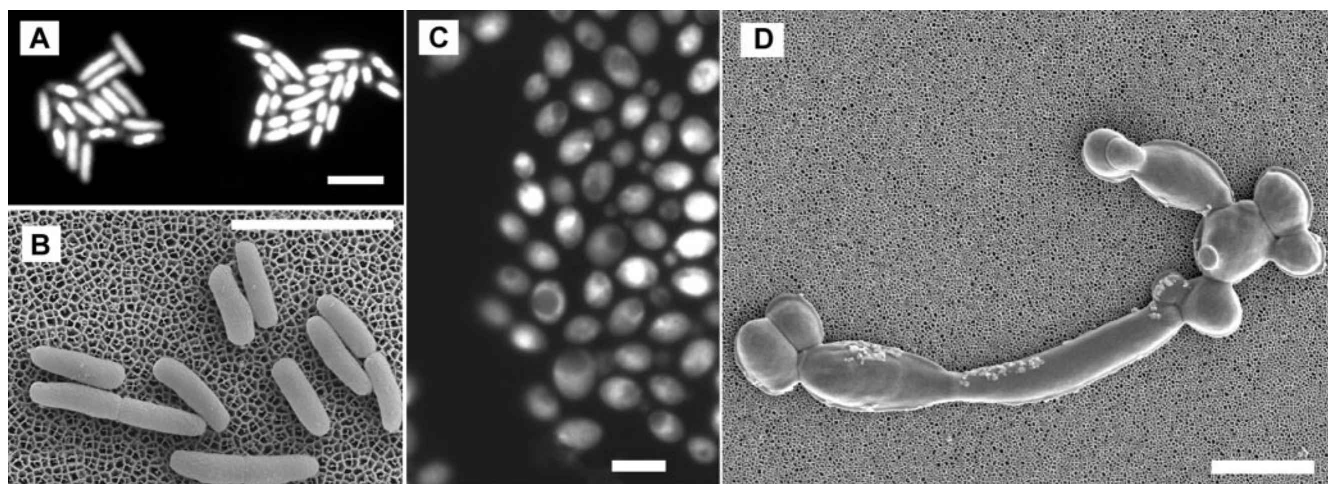

FIG. 1. Examples of microorganisms grown and imaged on Anopore placed on agar plates. (A) *Escherichia coli* 2613 stained by Syto-9 and imaged by fluorescence microscopy. (B) *Shigella sonnei* 2627-1 imaged by scanning electron microscopy, showing the Anopore structure. (C) *Candida tropicalis* stained with Fun-1 dye. (D) Scanning electron microscopy of *Candida albicans* showing germ tube outgrowth on Anopore. Bars, 5 μm.

and Vitek2 identification (BioMerieux, The Netherlands) was then used to assess if the samples on Anopore and agar were correctly assigned. Both Anopore and agar scored the same 10/15 samples as infected at a high level, with $>10^5$ CFU per ml on agar or $>10^5$ microcolonies per ml on Anopore. The agar plates scored only 2/10 samples as mixed species on the basis of colony appearance. Imaging both cell and microcolony morphology on Anopore, combined with HI/Syto-9 staining, suggested that 6/10 samples (including the same 2 identified on agar) were mixed cultures (i.e., a minority population of at least 0.5%). One mixed sample was missed by both methods and picked up in the subsequent analysis. These data suggest that a rapid microcolony approach could rapidly determine whether a urine sample required further investigation and so target therapy, possibly combined with antibiotic sensitivity testing and/or a Gram stain.

To create a format capable of more highly multiplexed analysis, the number of compartments on an 8- by 36-mm strip of Anopore was increased to 336 (an 8-by-42 array). Printing of this fine grid was done using an automated syringe (I&J Fisnar) with a 100-μm-diameter needle moving at 18 mm/second.

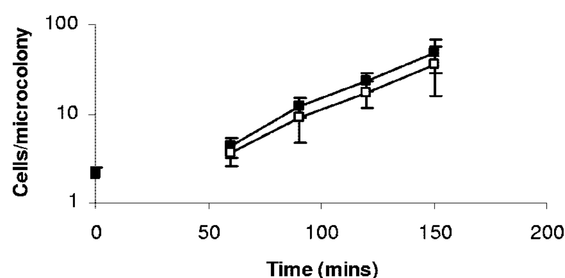

FIG. 2. Growth curve of *E. coli* 2613 after inoculation on Anopore and incubation on Mueller-Hinton (□) or sheep's blood agar (■); each time point sample was then stained with Syto-9 and imaged. A total of 160 microcolonies were analyzed per data point; error bars indicate standard deviations from the mean values.

Gridlines were printed at a pitch of 0.7 mm with the latex dispensed under 1 bar of pressure. The rigidity of Anopore and flatness permitted precise and reproducible printing, with the needle operating 5 to 10 μm above the surface. The rate of latex polymerization was slowed by the addition of 10% (vol/vol) ethylene glycol (Sigma, The Netherlands) to facilitate dispensing. After delivery and partial polymerization of the latex, the chips were heated to 60°C (10 min) or washed in 96% ethanol to complete curing. The rigidity and limited expansion or contraction of Anopore (4, 6) allowed wetting and heating treatments to be made without distorting the grid. The result was a highly uniform barrier on the upper surface of the Anopore. The latex grid penetrated the pores of the Anopore where printed, anchoring it in place. The compartments were 0.3 by 0.4 mm, and the barrier was 0.3 mm wide and high (Fig. 3A). Attempts to reproduce this grid pattern on nylon and polycarbonate membranes failed. This was partly due to difficulties in maintaining these flexible membranes sufficiently flat during printing and processing (especially with wetting and temperature changes) and also due to poor anchoring of the grid. Inoculation of bacteria (<20 CFU) into a compartment with a needle and subsequent growth was demonstrated (Fig. 3B). Even after 48 h of growth there was no spread from the target compartment into adjacent compartments, indicating effective segregation. Spread-plating each of three chips with *E. coli* 2613 (10 μl, 10^4 CFU/ml) using a small sterile glass spreader resulted in growth in all compartments. Spread-plating each of 10 336-well chips with an average of 30.2 CFU of *E. coli* resulted in growth in 14.1 ± 3.4 compartments per chip (47% culturability). In order to test segregation and detection of different species, 10 chips (3,360 compartments) were spread-plated with a mixture of *E. coli* 2613 (1.1 CFU/compartment) and *S. aureus* 110117 (0.09 CFU/compartment). After incubation for 4 h on Muller-Hinton agar and staining with HI and Syto-9 (7), growth occurred in 38% of compartments (n = 1,277). Compartments containing bacteria that preferentially stained with HI (indicating they were likely to be gram

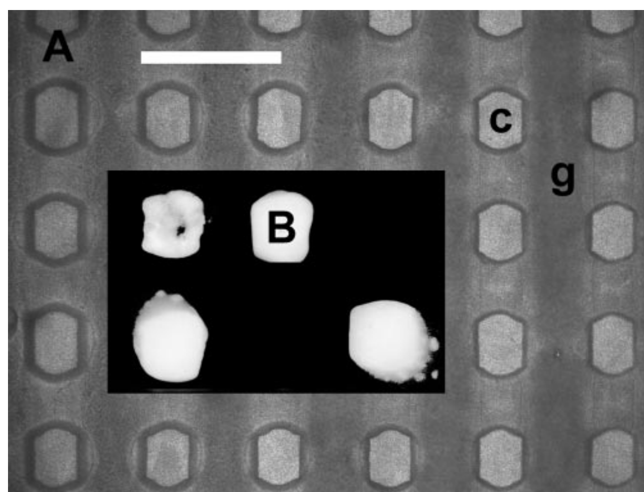

FIG. 3. Detail of 336-compartment Anopore chips. (A) Transmission light microscopy image of uninoculated chip. c, Anopore compartment; g, grid barrier. Bar, 0.8 mm. (B) Fluorescence microscopy image of *E. coli* 2613 inoculated and cultured in four compartments and subsequently stained with Syto-9.

positive) were detected in 3.0% of compartments ($n = 99$). Confirmation that *S. aureus* was being detected was done by imaging cell morphology; 56 compartments contained a mixed population, 42 contained *S. aureus* alone, and 1 was a false positive (no cocci).

Anopore chips have been fabricated that allow a higher density of culturing than most microtiter plates, without problems of desiccation or poor aeration, and which are accessible to robotics and imaging. Observation of microcolonies gives a speed advantage and when combined with the fluorescent dyes, which can be used in small quantities, an information advantage is gained over conventional culture. This format has applications in many areas of microbiology, including high-throughput screening within the antimicrobials and food industries, environmental monitoring, and rapid diagnostics. The inertness, flatness, and low thermal expansion coefficient of Anopore (4, 6, 9) are key. These properties may facilitate microengineering techniques for the creation of more chips capable of even more highly multiplexed assays (1). The thermal and chemical stability of Anopore (4) offers other interesting possibilities too, such as in extremophile culture and in the low-temperature storage of "ready-to-use" strain libraries on chips. Finally, the application of Anopore in flowthrough microarrays (11) suggests the potential for the integration of microbial culture with molecular assays.

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