

Bacterial Printing Press that Regenerates Its Ink: Contact-Printing Bacteria Using Hydrogel Stamps

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This paper describes the use of micropatterned agarose stamps prepared by molding against PDMS masters to print patterns of bacteria on agar plates. Topographically patterned agarose stamps were inked with suspensions of bacteria; these stamps generated patterns of bacteria with features as small as 200 μm over areas as large as 50 cm^2 . Stamps with many small features ($>200\ \mu\text{m}$) were used to study patterns of bacteria growing on media containing gradients of small molecules; stamps with larger features ($>750\ \mu\text{m}$) were used to print different strains of bacteria simultaneously. The stamp transfers only a small percentage of cells that are on its surface to the agar at a time; it is thus possible to replica-pattern hundreds of times with a single inking. The use of soft stamps provides other useful functions. Stamps are easily customized to provide a range of patterns. When culture media is included in the agarose stamp, cells divide and thrive on the surface. The resulting "living stamp" regenerates its "ink" and can be used to pattern surfaces repetitively for a month. This method is rapid, reproducible, convenient, and can be used to control the pattern, spacing, and orientation between colonies of different bacteria.

Introduction

This paper describes the use of soft lithography¹ using agarose stamps to pattern bacteria on agar surfaces. The method prints patterns of bacteria with features as small as 200 μm over areas as large as 50 cm^2 (that is, the size of a 10 cm Petri dish). Patterned agarose stamps can be used to pattern several different strains of bacteria simultaneously; the same stamp can be used repeatedly. This technique is parallel, rapid, and reproducible. We believe that this method will be useful to a broad range of scientists (chemists, biologists, and microbiologists) interested in generating patterns of bacteria on cell-culture media or other surfaces for studying organism–organism, organism–small molecule, and organism–surface interactions.

Patterns of bacteria have been used in microbial genetics^{2,3} and are of growing interest in biofilm formation and the broader area of microbial ecology.^{4,5} Several methods have been used to pattern bacteria in parallel. One of the most widely used techniques—replica plating—was developed by Lederberg et al. using surfaces coated with pile fabric to transfer colonies from one plate to another.^{6,7} Each stamping event transfers only a portion

of cells from the fabric to the new surface; it is thus possible to generate many replicas from a master plate. Colonies of bacteria have also been patterned using arrays of pins that transfer cells from liquid suspensions in 96 well plates to culture plates.^{8,9} These pins are a fixed distance apart, typically with a pitch identical to that of a 96 well plate ($\sim 9\ \text{mm}$). Each pin transfers a small but variable amount of liquid, making it difficult to control the geometry of features due to wetting. As a consequence, this method is limited to patterns that have low resolution. It would be useful to have a method that allows cells to be patterned over relatively large areas with control over the size, shape, and pattern of colonies.

We have explored the possibility of patterning bacteria by microcontact-printing (μCP). μCP is a parallel method of patterning molecules or suspensions of colloidal particles on surfaces. It can be used to deposit $>10\ 000$ spots/ cm^2 over areas as large as 50 cm^2 .¹⁰ Microfabricated poly-(dimethylsiloxane) (PDMS) stamps have been those most commonly used in μCP . These stamps have been used, for example, to pattern organic thiols on the surfaces of gold and palladium films. The resulting patterns define regions selective for the adhesion of mammalian cells.¹¹ Instead of PDMS stamps, we describe the use of topographically patterned hydrogel stamps fabricated out of agarose for μCP .¹²

In this paper, we explore two applications using agarose stamps to pattern bacteria on soft substrates (nutrient agar): (i) printing a single strain of bacteria with stamps

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having a large (>100) number of posts/features—this technique has allowed us to study the growth of patterns of bacteria printed on media containing chemical gradients at the surface and (ii) printing different strains of bacteria simultaneously with stamps containing features $>750\ \mu\text{m}$ to study interactions between different bacteria. We also demonstrate that stamps prepared using cell-culture media provide a surface upon which cells grow—a “living stamp”; the “ink” is thus, in a sense, self-regenerating. The stamp can be likened to a bacterial printing press that regenerates its own ink. After printing bacterial colonies with an agarose stamp containing cell-culture media, stamps were incubated in a warm, sterile environment to allow cells remaining on the stamp to divide. We were able to use a single stamp to print bacterial colonies over the course of a month before the stamp became contaminated from periodic handling. Finally, agarose stamps are “soft”, and patterns on stamps can be easily reconfigured after their initial preparation and use. Following the transfer of an initial pattern of colonies to a substrate, we used a scalpel blade to remove raised features from a stamp in order to create a new, reconfigured pattern.

Patterned agarose stamps (3% w/v) were prepared by casting hot solutions of agarose onto PDMS molds. We “inked” stamps by applying a suspension of cells to the surface of the stamp; the stamp absorbed water, leaving bacteria deposited on the surface of the stamp. These stamps transferred patterns of bacterial cells onto agar plates containing cell-culture media. Cells printed onto agar plates divided and grew into patterns of bacteria.

This procedure generated patterns of bacteria (triangles, lines, grids, text, etc.) with features as small as $200\ \mu\text{m}$ (with a pitch of $300\ \mu\text{m}$) or as large as $8\ \text{mm}$ (with a pitch of $11.2\ \text{mm}$). We used this method to pattern colonies of bacteria from a variety of genera onto agar growth media (1.5% agar) and swarming bacteria onto softer substrates (0.5% agar). Agarose stamps can be used to print bacterial colonies repetitively; a single “inking” with cells makes it possible to print a pattern at least 250 times onto agar cell-culture media.

Agarose stamps have several characteristics that make them useful for patterning bacteria: (1) Stamps are easily prepared and can be made with posts as small as $2\ \mu\text{m}$.¹² (2) They are biocompatible and may be prepared out of solid culture media. (3) They absorb excess liquid, but not bacteria, during inking; this property makes it practical to ink stamps with suspensions of cells. (4) They are soft and easily customized using a scalpel blade to reconfigure patterns. (5) They transfer only a fraction of the bacteria on their surfaces on each contact with agar; this characteristic makes it possible to prepare replicas of a particular pattern without intermediate reinking. (6) They transfer cells from stamps to the surface of culture plates without distortion of the pattern.

We have demonstrated that this method is capable of producing patterns of bacteria not possible by any other techniques and have studied how patterns of bacteria change in response to gradients of small molecules.

Results

Fabricating Molds and Stamps. The molds we used for casting stamps were (i) commercially available (for example, a 1536 well plate), (ii) prepared by microfabrication using standard photolithographic techniques, or (iii) fabricated using a new procedure that generates features in epoxy ($\geq 100\ \mu\text{m}$, with an aspect ratio of >1)

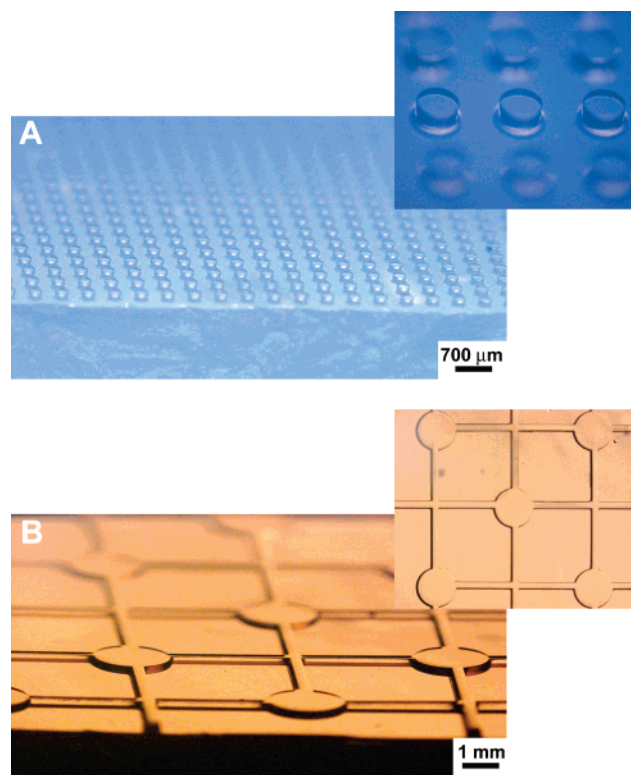


Figure 1. Agarose stamps used to contact-print patterns of bacterial colonies. (A) An image of an agarose stamp prepared using a PDMS mold cast from a photolithographically patterned silicon wafer. Agarose (3% w/v) was melted in nutrient media and cast onto the PDMS mold. Gelation was allowed to proceed at $4\ ^\circ\text{C}$; the mold was then peeled away from the stamp carefully. The stamp consisted of posts that were $190\ \mu\text{m}$ wide, $140\ \mu\text{m}$ tall, with a pitch of $400\ \mu\text{m}$. (B) An image of an agarose stamp consisting of circular features ($2\ \text{mm}$ wide, $500\ \mu\text{m}$ tall) connected by lines ($500\ \mu\text{m}$ wide, $500\ \mu\text{m}$ tall).

on glass or silicon substrates without the need of a cleanroom.¹³

We fabricated agarose stamps with features in *bas*-relief by casting a hot solution of high-gel-strength agarose (3%) in nutrient broth against PDMS molds. Sterile stamps were prepared by autoclaving media and PDMS molds. After the agarose had gelled, we carefully removed the PDMS mold to release the stamps and used a scalpel to trim the stamps (Figure 1).

Inking Stamps with Cells and Printing. We used three methods to ink agarose stamps: (i) by placing the stamp directly into contact with a lawn of bacteria grown on an agar plate, (ii) by inking posts individually with a suspension of cells using a micropipet, or (iii) or by spreading a suspension of bacteria uniformly over the surface of the entire stamp. In the last two methods, we used liquid cell cultures that were grown to log phase. The stamp rapidly absorbed excess liquid, leaving cells deposited on the features of the stamp. After allowing the stamp to absorb the excess liquid, we printed colonies by bringing the stamp into contact with an agar surface for 5 s (Figure 2). The capability of agarose to absorb excess liquid made it possible to produce well-defined patterns, presumably because it limited the spreading of liquid during stamping.

Patterns of *Vibrio fischeri* Colonies. To explore patterns of bacteria on agar surfaces, we used *Vibrio fischeri*—a bacterium that produces photoluminescence

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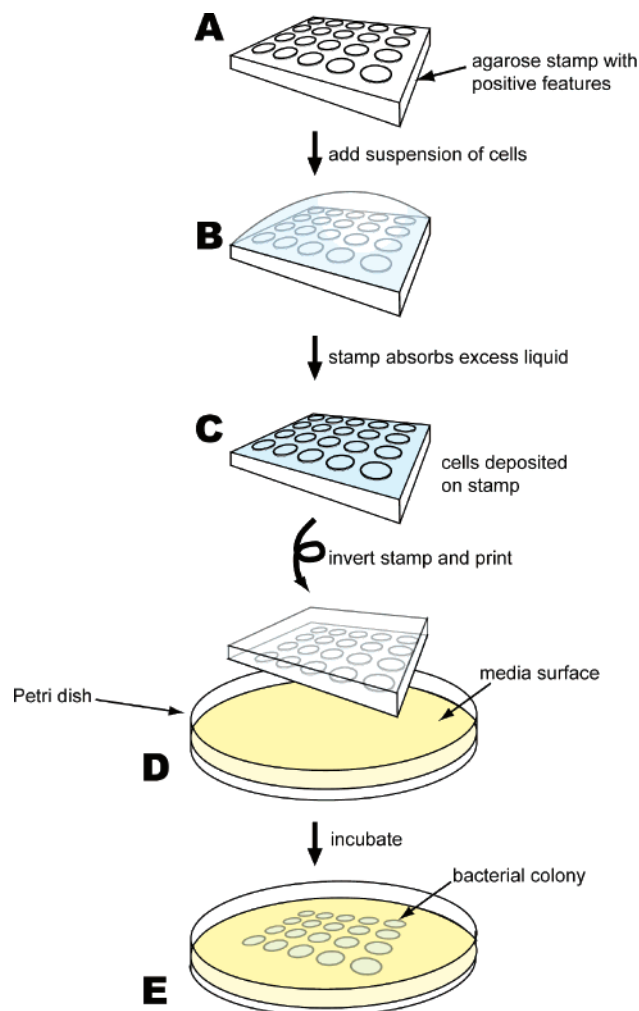


Figure 2. A schematic depicting bacterial microcontact-printing. (A) A cartoon of an agarose stamp with embossed features. (B) A suspension of cells is added to a 3% agarose stamp. (C) The stamp absorbs the excess liquid, leaving cells deposited on the surface of the stamp. (D) The stamp is inverted and brought into contact with cell-culture media (containing 1.5% agar). (E) The plate is incubated and patterns form.

($\lambda_{\max} \approx 540$ nm) based on cell density or quorum sensing—as a model organism.^{14,15} We used *V. fischeri* primarily because bacterial luminescence is easy to image. We were also interested in the possibility of using patterns of *V. fischeri* as relatively narrow bandwidth light sources. Figure 3 illustrates the growth of a colony of *V. fischeri* over time at 25 °C, by contact-printing cells with an agarose stamp, containing embossed lines with a zigzag geometry, (500 μm diameter lines, 500 μm tall, 5 mm pitch) onto a Good Vibrio Media (GVM)-agar substrate. The patterns began to luminescence after 12 h and faded after 80 h; this was presumably due to quorum sensing.

Figure 4 illustrates different patterns of colonies of *V. fischeri* possible with this technique. The shapes of the bacterial colonies were well defined and expanded with time. Colonies at the perimeter of patterns typically spread further outward than colonies within a pattern (Figure 4F) and eventually made contact. We found that the distortion of patterns of *V. fischeri* was not due to cell swarming on agar plates. Outward spreading could be

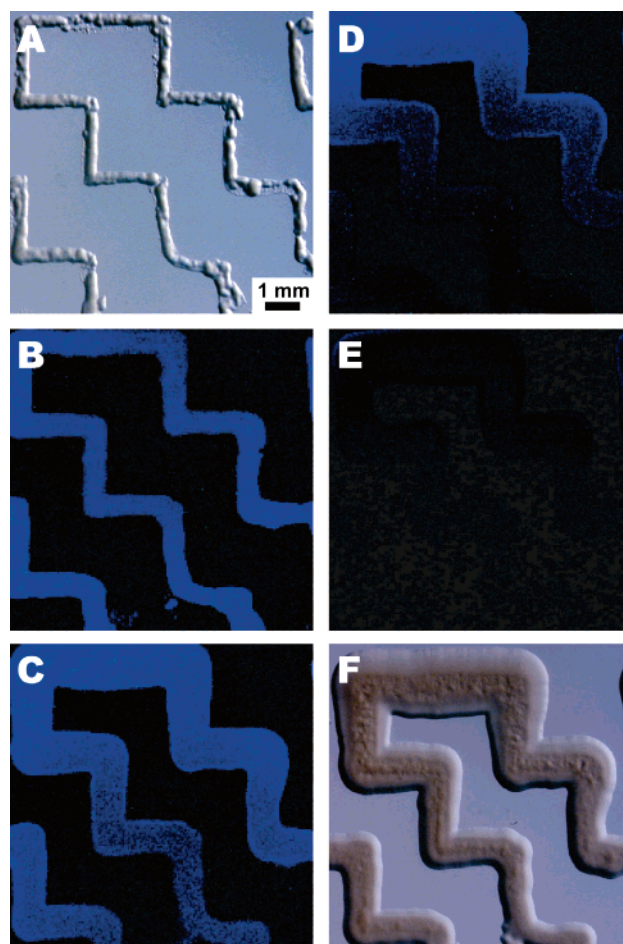


Figure 3. Images illustrating the growth of a pattern of *V. fischeri* over time. An agarose stamp (3% w/v in GVM: 10 g/L tryptone, 5 g/L casamino acids, 25 g/L NaCl, 4 g/L MgCl_2 , 1 g/L KCl) containing embossed lines (500 μm wide, 500 μm tall) with a zigzag geometry was used to pattern *V. fischeri* on GVM-agar plates. We inked the stamp by applying a suspension of cells of *V. fischeri* to the top face of the stamp; after the stamp had adsorbed the excess liquid, we used it to contact-print patterns of cells. The stamp was brought into contact with the surface of the plates for 5 s and carefully removed; the resulting plates incubated at 25 °C. We imaged the top, left corner of a larger, repeating pattern. Images were recorded, unless otherwise noted, by measuring photoluminescence collected in a dark room over an 8 s exposure. Images A–F depict the growth of the patterns of bacteria over time: (A) bright-field image after 10 h of growth (no photoluminescence was detected); (B) 20 h; (C) 40 h; (D) 60 h; (E) 80 h; (F) A bright-field image after 80 h. After 60 h, no change in colony size or shape occurred.

controlled by the geometry of the pattern; when bacteria were printed on a plate *within* a second pattern that had grown for 24 h, 1 mm diameter lines of *V. fischeri* that formed a continuous perimeter, the inner pattern did not spread, even after 6 days of growth (Figure 4G). The preferential outward spreading of colonies at the perimeter of patterns was either due to cells moving to higher nutrient concentrations or to avoid the accumulation of metabolic waste in the media.

Reconfigurable Agarose Stamps. Agarose stamps are easily modified with a sharp tool, such as a scalpel blade. Agarose stamps containing a rectangular (13 \times 9) array of posts (1 mm diameter, 1 mm deep, 2.5 mm pitch) were prepared and inked with a liquid suspension of *V. fischeri*, and the pattern was transferred to a GVM-agar substrate (Figure 5A) via contact-printing. Without re-inking, a sterile scalpel blade was used to reconfigure the

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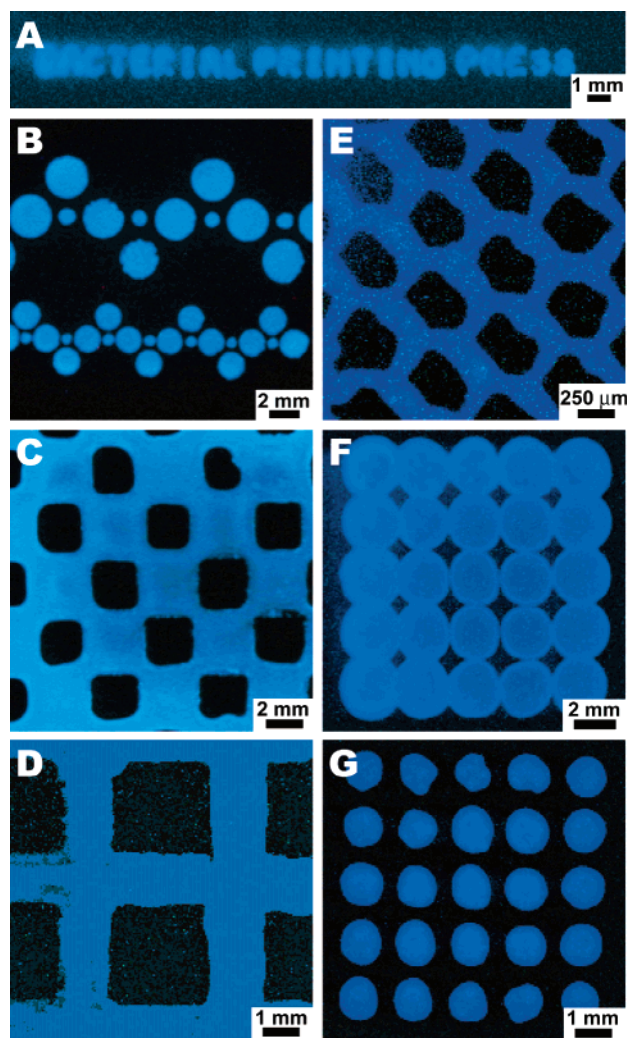


Figure 4. Images of different patterns of *V. fischeri* prepared by contact-printing onto GVM-agar using the procedure described in Figure 3. Agarose stamps (3% w/v in GVM) with different embossed patterns were used to pattern to GVM-agar plates; the contact time between the stamp and the surface of the plates was 5 s. After the plates were incubated for 60 h at 25 °C, no additional colony growth was visible and the plates were imaged. (A) "Bacterial printing press". (B) Alternating small and large circles. (C) A checkerboard pattern. (D) A grid of straight lines. (E) A honeycomb pattern. (F) An array of 5 × 5 circular colonies. (G) An array of 5 × 5 circular colonies stamped inside a second pattern consisting of a continuous border of lines; the lines were positioned ~4–5 mm from the outermost colonies in the image shown. All images record colony luminescence averaged over an 8 s exposure on a CCD. Images were collected in a dark room.

stamp into a checkerboard pattern by removing posts. The resulting pattern was transferred to GVM-agar media via contact-printing (Figure 5B). The stamp was configured once again to produce lines that were transferred to GVM-agar media (Figure 5C).

Replica Stamping. An agarose stamp containing GVM inked with bacteria can be used to transfer a pattern multiple times without reinking. To explore the limit of repetitive contact-printing of bacteria, we inked an agarose stamp containing a rectangular array of 1 mm diameter posts (2.5 mm pitch) with a suspension of *V. fischeri*, and used the stamp to pattern successive GVM-agar plates (245 mm × 245 mm Petri dishes). With only a single inking, we were able to transfer the pattern over 250 times without a significant loss in the quality of the pattern (Figure 5D). The time between successive printings was ~5 s; the time

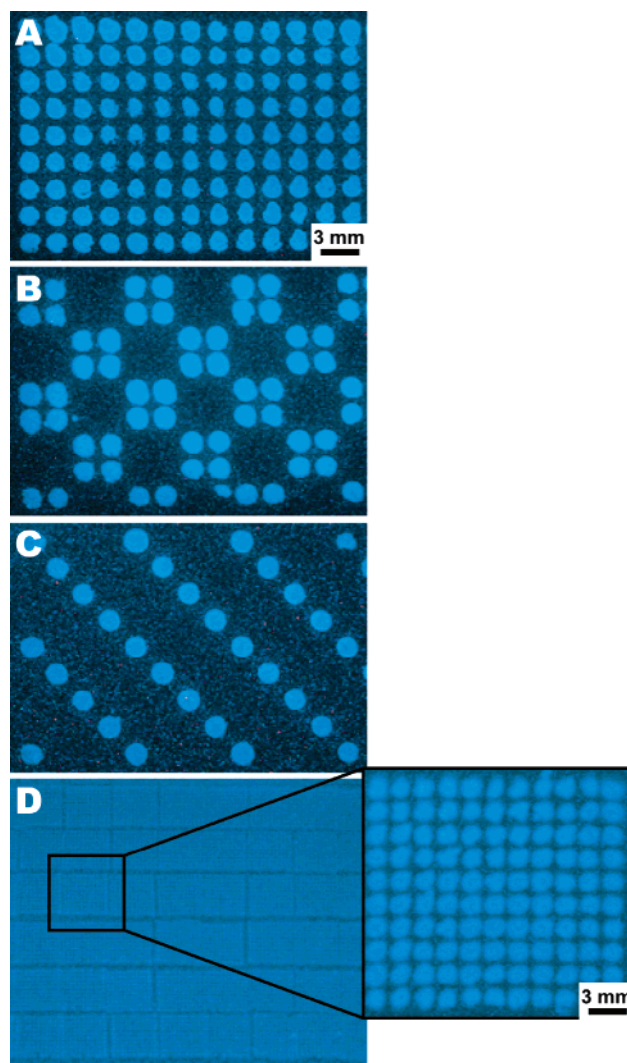


Figure 5. Reconfigurable agarose stamps and replica stamping. (A) An agarose stamp (3% w/v in GVM media) containing a rectangular (13 × 9) array of circular posts (1 mm diameter, 2.5 mm pitch) was inked with a suspension of cells of *V. fischeri*; after the stamp had adsorbed excess liquid, it was used to transfer the pattern of bacteria to a GVM agar plate (1.5% agar) by contact-printing. The contact time between the stamp and the surface of the plate was 5 s. (B) The stamp was reconfigured into a checkerboard pattern by removing posts with a sterile scalpel, and without reinking, was transferred to a GVM agar plate by contact-printing. (C) The stamp was reconfigured into a pattern of lines, and without reinking, transferred to a GVM plate by contact-printing. (D) A rectangular pattern of circular posts used to repetitively pattern GVM-agar plates (245 mm × 245 mm, 1.5% agar). The stamp was inked with a suspension of cells of *V. fischeri* and used to transfer the pattern to the surface of GVM-agar plates 250 times with a single inking. The contact time between the stamp and the surface of the plates was 5 s, and the time that had elapsed between stamping sequential patterns was ~5 s (the time elapsed between the first and the 250th print was ~20 min). The large image shows a sequential series of patterns (prints 142–190) made by contact-printing with the same stamp; the image inset shows a pattern at higher magnification. After print 250, we incubated the stamp in a warm, sterile environment for one week to allow the "ink" to be regenerated and then printed more patterns. This sequence could be repeated for over a month. All images record colony luminescence averaged over an 8 s exposure on a CCD. Images were collected in a dark room. Plates were incubated at 25 °C.

that had elapsed between the first and the 250th print was ~20 min. We currently do not know the limit of repetitive patterning.

After a stamp was used to pattern bacteria on plates, we incubated the stamp in a warm, sterile environment for a week and then used it to print more patterns of bacteria. This sequence could be repeated for several weeks before the stamp became contaminated from handling.

Patterning other Bacterial Genera. To determine the scope of this method and as the basis for experiments that explore the interactions of multiple microorganisms, we used agarose stamps to pattern different genera of bacteria, including swarming and nonswarming strains of *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*. All bacteria, with the exception of a swarming strain of *E. coli*, were printed on Lauria–Bertani (LB) agar plates (1.5% agar); swarming *E. coli* strain AW405 was printed on swarmer plates (0.5% agar).^{16,17} Over time, the bacteria grew into the pattern defined by the features of the stamp (Figure 6A); the fidelity of the pattern depended on the strain of bacteria. Both *B. cereus* and swarming *E. coli* strain AW405 grew beyond the pattern defined by the 5 × 5 array of circular features (1 mm diameter, 2.5 mm pitch) embossed on the stamp; other bacteria grew into the expected pattern. Colonies of *B. cereus* grew rapidly, fused, and formed a lawn of cells from which the pattern was barely recognizable (Figure 6B). We were surprised to find that colonies on the inside of the pattern of swarming *E. coli* strain AW405 grew only slightly larger than the posts used to pattern them, while colonies at the perimeter of the pattern grew to the approximate size of the posts, and then swarmed outward forming a dense mat of cells (Figure 6C).

Patterning Multiple Bacteria on a Single Stamp. We explored whether this method might be useful for studying the interaction of many different bacterial colonies—the basis for microbial ecology—by patterning multiple types of bacteria simultaneously using a single stamp. A micropipet was used to deliver a small droplet (~1 mL) of a liquid culture of bacteria to each of the 16 circular posts (1 mm diameter, 2.5 mm pitch) of a stamp. We used the following six strains of bacteria: *B. subtilis*, an *E. coli* clone that produces an *N*-acyl amino acid antibiotic against *B. subtilis*,¹⁸ and four *E. coli* clones that produce colored colonies (brown, dark blue, light blue, and purple).¹⁹ After the stamp had absorbed the excess liquid, the cells were transferred to LB–agar plates and incubated at 25 °C. Patterns of bacteria were imaged periodically over the course of 6 days to investigate the interactions between different types of bacteria (Figure 6D–G). Colonies within the pattern fused to adjacent colonies positioned along horizontal and vertical axes but did not contact adjacent, diagonal colonies. Although we positioned a strain of *E. coli* that produces an antibiotic against *B. subtilis* adjacent to colonies of the latter, we were unable to detect the inhibition of growth of *B. subtilis*. This is likely due to a lag phase before the concentration of the antibiotic in the medium is high enough to inhibit the growth of *B. subtilis*.

Patterning Bacteria on Gradients of Ampicillin. We patterned colonies of bacteria onto cell-culture media containing a gradient of ampicillin (amp)—a bacteriocidal agent—to explore how the phenotype might change across

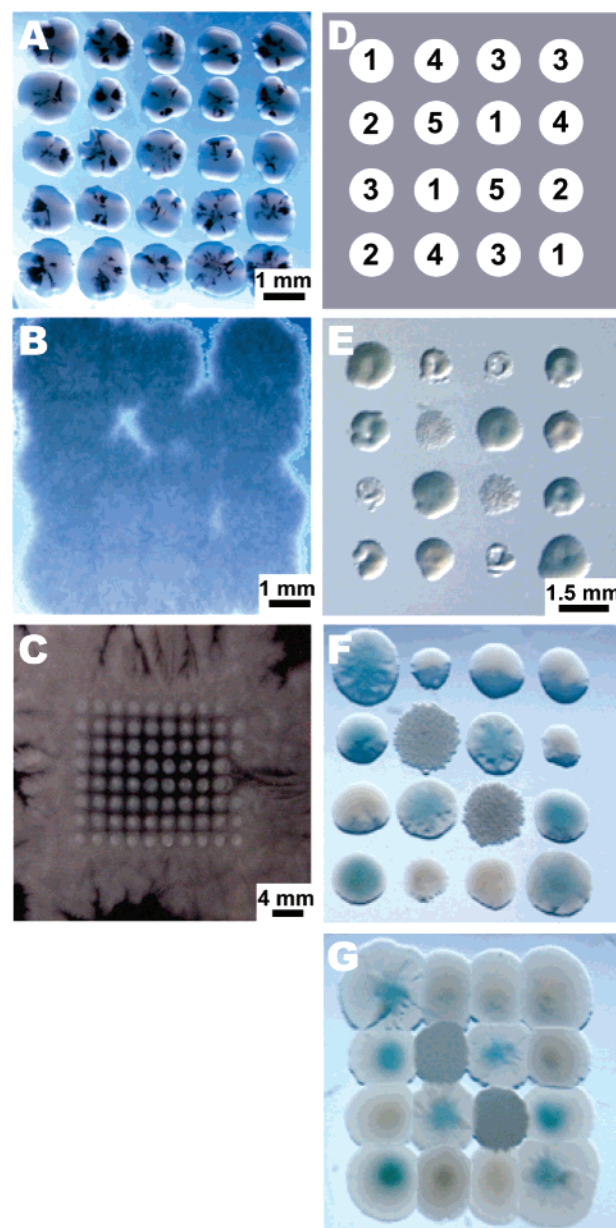


Figure 6. Growth of patterns of different organisms. Agarose stamps (3% w/v) containing culture media with a square (5 × 5) array of circular posts (1 mm diameter, 2.5 mm pitch) were used to pattern different organisms. Stamps were inked with suspensions of bacteria; after adsorbing the excess liquid, the stamps were used to transfer patterns of bacteria. The contact time between the stamp and the surface of the plates was 5 s. (A) A blue strain of *E. coli* patterned on LB–agar; in the absence of ampicillin, the strain rapidly loses its plasmid, and only some cells produce indigo. The progeny of these cells also produce indigo, and the feature of bacteria consequently contains streaks of indigo. (B) *B. cereus* patterned on LB–agar. (C) Swarming *E. coli* AW405 patterned on swarming media (0.5% agar). (D) Individual posts of an agarose stamp containing a square array (4 × 4) of circular posts (1 mm diameter, 2.5 mm pitch) were inked with five different bacteria using a micropipet to deliver 1 μ L of a suspension of cells to each post. After the excess liquid had been adsorbed by the stamp, a second aliquot (1 μ L) of cells was added to the posts. The stamp was used to pattern LB–agar. (E) A schematic showing the location of different bacteria on the agar plate: (1) an *E. coli* clone that produces *N*-acyl amino acid antibiotics against *B. subtilis*, (2) an *E. coli* clone that produces indigo (blue), (3) an *E. coli* clone that produces violacein (purple), (4) an *E. coli* clone that produces melanin (brown), and (5) *B. subtilis*. (F) An image of the colonies after 16 h at 25 °C. (G) An image of the colonies after 96 h at 25 °C.

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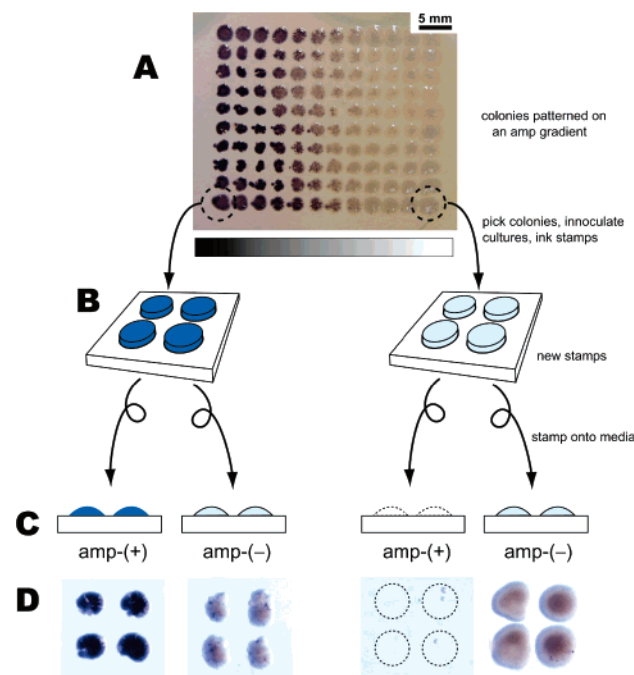


Figure 7. The growth of patterns of bacteria on media containing a gradient of ampicillin. Nutrient plates (LB-agar) were prepared containing a transient gradient of amp at the surface. An agarose stamp (3% w/v) containing LB and with array of 12×10 posts embossed on its surface (1 mm diameter posts) was inked with a solution of a strain of *E. coli* that produces indigo. The stamp adsorbed the excess liquid and was used to pattern bacteria on the plates; the plates were incubated at 25 °C for 4 days and imaged. (A) An image of the resulting pattern of colonies. The gradient key under the image depicts high concentrations of amp (dark) and lower concentrations of amp (light). Cells from both ends of the pattern (white and blue) were picked and used to inoculate a liquid culture (LB broth). (B) The resulting cultures were used to ink two separate stamps consisting of an array of 2×2 posts (1 mm diameter) and patterned onto LB plates by contact-printing; the contact time between the stamp and the surface of the plates was 5 s. The plates were incubated at 25 °C. (C) A cartoon depicting the resulting colonies on plates. Symbols indicate the presence of ampicillin (+) in the media, and the absence of ampicillin (-). (D) Images of the resulting colonies after 6 days at 25 °C. In the presence of ampicillin, colonies grown from cultures prepared from the blue colonies remained blue while those from white colonies did not grow. In the absence of ampicillin, colonies grown from cultures prepared from the blue colonies turned white while those from white colonies remained white.

a pattern of bacteria exposed to a selective pressure. To visualize changes in the phenotype, we followed the loss of an amp-resistant plasmid by an *E. coli* clone containing a plasmid encoding both the genes for amp resistance and indigo biosynthesis.¹⁹ In the presence of amp, colonies of this bacteria appeared dark blue due to the expression of indigo by cells. In the absence of amp, many cells lost the plasmid and appeared white; the resulting colonies were marbled as the number of cells retaining the plasmid decreased.

Slant plates of amp-LB-agar were prepared by pouring a layer of amp-LB-agar in the bottom of Petri dishes. After the agar had gelled, the dishes were tilted at a 10° angle and a solution of LB-agar (55 °C) was poured on top; the plates remained tilted as the top layer solidified, creating a substrate consisting of an upper layer of LB-agar with a slanted profile positioned above a flat layer of amp-LB-agar. The resulting plates contained a transient gradient of amp at the surface that was produced by the diffusion-limited transport of amp from the bottom

layer to the surface of a top layer that varied in thickness. As soon as the top layer had solidified, we patterned colonies of the blue *E. coli* clone onto the plates using a stamp consisting of a 12×10 array of circular posts (1 mm diameter, 2.5 mm pitch) (Figure 7). After incubating the plates for several days at 25 °C, we observed that the pattern of bacterial colonies formed a gradient of color—blue at one end and white at the other. Colonies in the middle of the pattern grew lighter in color; as the concentration of amp decreased, the ratio of blue to white cells in each colony decreased.

We picked blue cells from the portion of the plate containing a high concentration of amp and white cells from the portion containing a low concentration of amp and used them to inoculate two separate liquid cultures. The bacterial cultures were used to ink stamps consisting of a 2×2 array of posts (1 mm in diameter, 2.5 mm pitch), and the stamps were used to pattern both LB and LB-amp plates. On LB, the culture derived from the blue colony turned white as the plasmid was lost, while on LB-amp it turned dark blue (Figure 7D). The culture derived from the white colony grew into white colonies when patterned on LB but did not grow on LB-amp, presumably because the cells had lost the plasmid *before* being patterned again. These experiments suggest that patterns of bacteria on gradients of small molecules may provide a method for rapidly identifying and isolating strains of bacteria that display interesting phenotypes, including antibiotic resistance. This method should also be useful for assaying the stability of plasmids over a range of antibiotic concentrations.

Discussion

Patterns of bacteria can be transferred to cell-culture media by contact-printing cells using agarose stamps with embossed features. This method should be broadly useful in screening, and in studying microbial ecology, interactions between bacteria and small molecules and surfaces, as well as the movement of organisms on patterns of bacteria.

The use of hydrogel stamps with embossed features represents a conceptually new approach to patterning bacteria. There are currently no other parallel techniques that make it practical to pattern bacteria on soft surfaces and that can simultaneously control the size, shape, and pattern of colonies. There are several advantages of this technique: (1) it uses inexpensive, readily available material; (2) it can be used to pattern bacteria over relatively large areas ($\geq 50 \text{ cm}^2$); (3) it can generate patterns composed of colonies of different bacteria; (4) with a single inking, hundreds of patterns can be generated from one stamp; (5) cells thrive on the surface of stamps containing media, making it possible to prepare stamps that “regenerate” their own ink; and (6) the patterns on stamps are easily customized with a scalpel or other mechanical devices for removing posts.

Experimental Section

Materials. High-gel-strength Gibco BRL agarose (the gel strength of a 1.5% solution, reported by the manufacturer, is $> 3200 \text{ g/cm}^2$) and PDMS (Dow Corning Sylgard 184) were used as received.

Organisms. *E. coli* swarming strain AW405 was used in experiments with swarming bacteria. Nonswarming *E. coli* clones with four easily discernible phenotypes were used in this study: (i) antibacterial activity (production of *N*-acyl amino acids),¹⁸ (ii) purple (production of violacein),¹⁹ (iii) blue (production of indigo), and (iv) brown (production of melanin). Each of the clones was originally identified in a cosmid-based genomic library con-

structed from DNA extracted directly from soil. In these studies, DNA extracted from an environmental sample (environmental DNA, eDNA) was cloned in a model bacterial host and eDNA libraries, containing large inserts of DNA, were screened for the heterologous expression of natural product phenotypes. Details of this procedure have been described elsewhere.^{18–20} In brief, bacteria present in the environmental samples were lysed in situ by heating in the presence of a detergent, and high-molecular-weight DNA was collected by precipitation with alcohol from a centrifuge-clarified crude lysate. High-molecular-weight DNA purified by gel electrophoresis was blunt-ended, ligated into a cosmid vector, packaged into lambda phage, and used to transfect *E. coli*. The eDNA libraries were screened using a variety of assays to identify clones that produced interesting phenotypes.

With the exception of strain AW405, all other strains of *E. coli* contained plasmids that conferred them resistant to amp. Other organisms used in this work, include *B. subtilis* (ATCC 6633), *V. fischeri* (strain MJ1),²¹ *Vibrio harveyi* (strain B392),²² *B. cereus* (ATCC 10876), *P. aeruginosa* (PA01), and *S. aureus* (ATCC 6538P).

Bacterial Cultures. We prepared liquid cultures of *E. coli*, *B. subtilis*, *B. cereus*, *P. aeruginosa*, and *S. aureus* by picking single colonies from plates, and used them to inoculate LB broth. For *E. coli* clones, the broth contained amp (50 mg/L). *V. fischeri* was grown in GVM (10 g/L tryptone, 5 g/L casamino acids, 25 g/L NaCl, 4 g/L MgCl₂, 1 g/L KCl). *B. subtilis* and *V. fischeri* were grown at 25 °C; the other cultures were grown at 32 °C. *E. coli* strain AW405 was grown on swarmer plates (0.5% agar, 0.3 g/L beef extract, 10 g/L peptone, 5 g/L NaCl); liquid cultures were grown in LB broth. Bacterial cultures were grown to log phase before using them to ink agarose stamps. Cells of *V. fischeri* (MJ1) did not swarm on growth media containing concentrations of agar ranging from 0.5 to 1.5% (w/v).

Fabrication of Stamps. Stamps were prepared from molds fabricated in PDMS. Molds containing posts 1 mm in diameter were prepared by a recently published method.¹² Other PDMS molds were prepared by casting PDMS prepolymer onto a master fabricated out of photoresist or epoxy on a silicon wafer or glass slide using photolithography. After the PDMS was cured thermally, the mold was peeled away from the master, treated with an oxygen plasma, and immersed in water to render the PDMS hydrophilic. The PDMS molds were placed face-up in the bottom of a well (6 cm × 6 cm × 2.3 cm), cut out of a large block of PDMS (8.5 cm × 8.5 cm × 3 cm), and sterilized. We found that PDMS molds could be autoclaved >50 times without any detrimental effects to the mold. A hot, sterile solution of 3% (w/v) high-gel-strength agarose (Gibco BRL) in PBS buffer or cell-culture media was poured into the block of PDMS, covering the PDMS master. The agarose solution wet the surface of the PDMS and displaced air trapped in the features of the mold. If the mold was not oxidized before each use, the hot solution of agarose did not wet the surface completely, making it necessary to remove air bubbles trapped in the wells by placing the mold in a vacuum or by aspiration with a micropipet. The agarose stamps were transferred to a refrigerator (4 °C) where they solidified within 30 min. After solidification, the agarose stamp and PDMS mold were removed together from the PDMS block and the mold peeled away from the agarose stamp. A scalpel was used to cut the stamp to the desired size.

Procedure for Inking Stamps. We inked stamps by placing freshly cast agarose stamps upside down (posts up) in a Petri dish and added suspensions of cells from liquid cultures grown to log phase to the top face of the stamp; we added a volume of liquid that was sufficient to immerse the features of the stamp. After allowing ~5–10 min for the stamp to absorb the liquid completely, another aliquot of a liquid culture was added to the face of the stamp. After an additional 10 min, all of the liquid

had been absorbed. To ink stamps with different bacteria, we added <1 μ L of cell suspensions to individual posts of an agarose stamp with a micropipet. After ~2–5 min, another aliquot of cells was added. Individual posts of the stamp could be inked manually, provided the diameter of the posts was $\geq 500 \mu$ m. Stamps could also be inked directly from a lawn of bacteria. To ink from a lawn, we placed stamps, feature-side-down, into contact with a lawn of bacteria for 3 min. The stamp was removed and then used to directly pattern substrates.

Stamping. Freshly cast and inked agarose stamps were blotted to remove excess liquid from the features by holding the stamp with tweezers and bringing it into contact with a sterile, polycarbonate surface approximately five times. Patterns of cells were transferred from the stamp to cell-culture media plates by bringing the stamp gently into contact with the plate surface for ~5 s. For large stamps (>4 cm), we found it most convenient to place the stamps feature-side-up in a large Petri dish. We used these stamps to pattern substrates by inverting cell-culture media plates, bringing them gently into contact with the stamp for 5 s then lifting them off carefully. Stamps made of hydrophilic PDMS were also capable of patterning bacterial cells. The surface of PDMS stamps was rendered hydrophilic by plasma oxidation, and stamps were inked by pressing the stamp into contact with a lawn of bacteria.

Imaging. Patterns of bacterial colonies <10 mm were imaged using bright field microscopy using a Nikon DXM1200 digital camera connected to a Leica MZ12 microscope. Patterns of *V. fischeri* colonies <10 mm were imaged using bright field microscopy and by measuring photoluminescence averaged over a collection period of 60 s on a CCD in a dark room. Patterns of bacterial colonies >10 mm were imaged using a Sony digital camera. Images of photoluminescence emitted from patterns of *V. fischeri* colonies were collected using an exposure time of 8 s.

Patterns of Bacteria on Gradients of Ampicillin. We prepared cell-culture plates containing a gradient of amp by casting a layer of LB-agar of varying thickness above a layer of LB-agar containing amp of fixed thickness. Specifically, 15 mL of LB-agar containing amp (0.11 mg/mL) was poured into the bottom of 3.25 in. Petri dishes and allowed to solidify; the resulting layer was 5 mm thick. We propped plates at an angle of ~10° and added 20 mL of LB-agar (cooled to 55 °C). The plates remained tilted as the top layer solidified. The resulting substrate consisted of an upper layer of LB-agar with a slanted profile—11 mm at the tallest point, as measured above the bottom layer—situated above a flat layer of amp-LB-agar. A transient gradient of amp at the surface was produced by the diffusion-limited transport of amp from the bottom layer to the surface of the top layer. After the top layer of agar had solidified, we immediately used the plates; patterns of cells (*E. coli* K12-derived strain containing a plasmid conferring ampicillin resistance and indigo biosynthesis) were stamped onto the surface and incubated at 25 °C. The growth of colonies and phenotypic differentiation were observed after 4 days.

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Supporting Information Available: A table of different strains of bacteria patterned using this technique and their sources are described. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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