

Interaction of Bacterial Populations in Coupled Microchambers

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Micro- and nanotechnology offer tools that enable new approaches in microbiology and biotechnology. With microfluidic technologies we are able to manipulate liquids in a precise, well-controlled manner in microscopic dimensions. A microfluidic setup for studying interacting bacterial populations is presented in this work. Bacterial cultures were grown in a device within distinct microchambers and channels that are separated by porous membranes. This membrane acts as a physical boundary for the populations in each chamber, nevertheless it enables chemical coupling between them. The free diffusional transport of nutrients and secreted products throughout the microfluidic chip results in a chemical anisotropy within the bacterial microhabitats. Such heterogeneities affect the development and spatial distribution of the cell populations. We observed attractive and repulsive interactions between the bacterial populations and showed that chemotaxis and likely intercellular signaling play a fundamental role in these phenomena. Our results show that at population level biochemical interactions have to be considered when using microreactors or microchambers to culture bacteria.

Key words:

microfluidics, bacterial communication, microbial interaction, chemotaxis, cell motility

Introduction

When culturing bacteria in artificial microhabitats, such as in microfluidic devices, microchambers and microreactors, the ecological and microbiological aspects must be considered. In such devices and experiments cells often live in structured environments that from an ecological viewpoint could be considered “patchy” (i.e. with strong spatial heterogeneities and variations in suitability for the cells).¹ In such artificial habitats, the formation of a metapopulation, a set of interacting subpopulations, is observed along with a complex population dynamics.² The ecological aspects are also manifested in the fact that cells compete for resources,³ which sometimes results in unexpected spatial distribution and growth of cells.⁴

In such biological scenarios, cell-cell communication is important.⁵ The complexity of the evolved communication mechanisms among prokaryotes can be distinguished by the various chemical signals used by the different bacterial species. Beside the known quorum sensing signaling molecules (homoserine lactones and oligopeptides^{6,7}), bacteria use toxins (antibiotics, bacteriocins⁸), antimicrobial peptides,⁹ amino acids,¹⁰ exopolysaccharides,¹¹ or metabolic waste products (indole¹¹) as signaling molecules. These chemical signals have

distinguishable targets and functions (intra-, or interspecies communication, inter-kingdom signaling), and they have a key role in the communication of bacterial populations in natural habitats.

Motile bacteria have the great advantage of being able to explore the heterogeneous environment. By a mechanism called chemotaxis bacteria are able to sense concentration changes of certain chemicals, and swim towards increasing or decreasing concentrations of chemoattractants or chemorepellent molecules, respectively.^{13,14} It has been shown that signaling and chemotaxis may be coupled, and signaling molecules may act as chemoeffectors.⁵

Although traditional microbiology techniques enable us to study the interactions of bacterial communities on a large scale (such as co-culturing bacteria on agar plates or in shaken flasks), these traditional tools do not allow us to follow the dynamics and the fundamental mechanisms on single cell level. In the last few decades, the development of microengineering and nanotechnology has revealed new directions in traditional microbiology. Microfluidics has provided excellent tools for studying bacteria in controlled environments.^{15–18} Here we present experiments performed with microfluidic devices to study the interaction of physically separated but chemically coupled bacterial populations. These populations, growing in microchambers and channels separated by porous membranes, exhibit dynamic spatial rearrangements as a result of secre-

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tion, diffusion and sensing of metabolic products and/or signaling molecules. Our results show that chemotaxis, metabolism and signaling together determine the distribution of bacteria in structured microdevices such as microchambers and microreactors.

Materials and methods

Strains and cultures

The strains used were kindly provided by Karen Fahrner and Howard C. Berg. We used the *Escherichia coli* (*E. coli*) HCB33 (equal to RP437 which is widely used in chemotaxis studies) strain which is considered wild type for chemotaxis.¹⁹ This strain was transformed to carry the plasmid pMPMA2-GFPmut2,²⁰ resulting in a fluorescent chemotactic strain. Control experiments were carried out using HCB437 *E. coli* strain that is a non-chemotactic mutant¹⁹ derived from the HCB33 strain. This strain was also transformed using the abovementioned plasmid yielding a fluorescent variant. All media used contained 50 $\mu\text{g mL}^{-1}$ ampicillin (A9518, Sigma-Aldrich Kft., Hungary) and 50 $\mu\text{g mL}^{-1}$ streptomycin (S6501, Sigma-Aldrich Kft., Hungary). Prior to the experiments, bacteria were grown overnight in 3 mL lysogeny broth (LB) medium (L3022, Sigma-Aldrich Kft., Hungary) supplemented with antibiotics at 30 °C in plastic tubes shaken at 200 rpm. Overnight cultures were diluted back in the morning, and cul-

tured further to reach an OD_{600} (optical density measured at 600 nm) between 0.5–0.8. Then, 1 mL of the cultures was centrifuged (3000 rpm, 10 minutes), the supernatant disposed, and the sedimented cells resuspended in LB medium containing 40 mg mL^{-1} bovine serum albumin (A7906, Sigma-Aldrich Kft, Hungary) to yield a 1 mL volume. As a reference medium, motility medium (10 mM potassium phosphate buffer, 0.1 mM EDTA, 40 mg mL^{-1} bovine serum albumin (A 7906, Sigma-Aldrich Kft., Hungary) was used in all experiments.

Fabrication of the microfluidic chips

Microfluidic chips were fabricated from polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning Corp.) using standard photolithography techniques.²¹ Imprints of the microdevices were created by using SU8–2015 and SU8–2050 photoresists (MicroChem Corp.) on silicon wafers and glass slides. Fig. 1A,B shows the schematic representation of the device. Practically, a porous membrane with 100 nm pores (Anodisc, Whatman Ltd.) was sandwiched between two blocks of PDMS, patterned with channels and reservoirs, and having inlet holes punched into them. The alignment of the components was done under a stereoscope, and the accuracy was measured to be better than 10 μm . In this sandwich structure, there are two large reservoirs (8 mm \times 8 mm \times 1 mm size, 64 μL volume

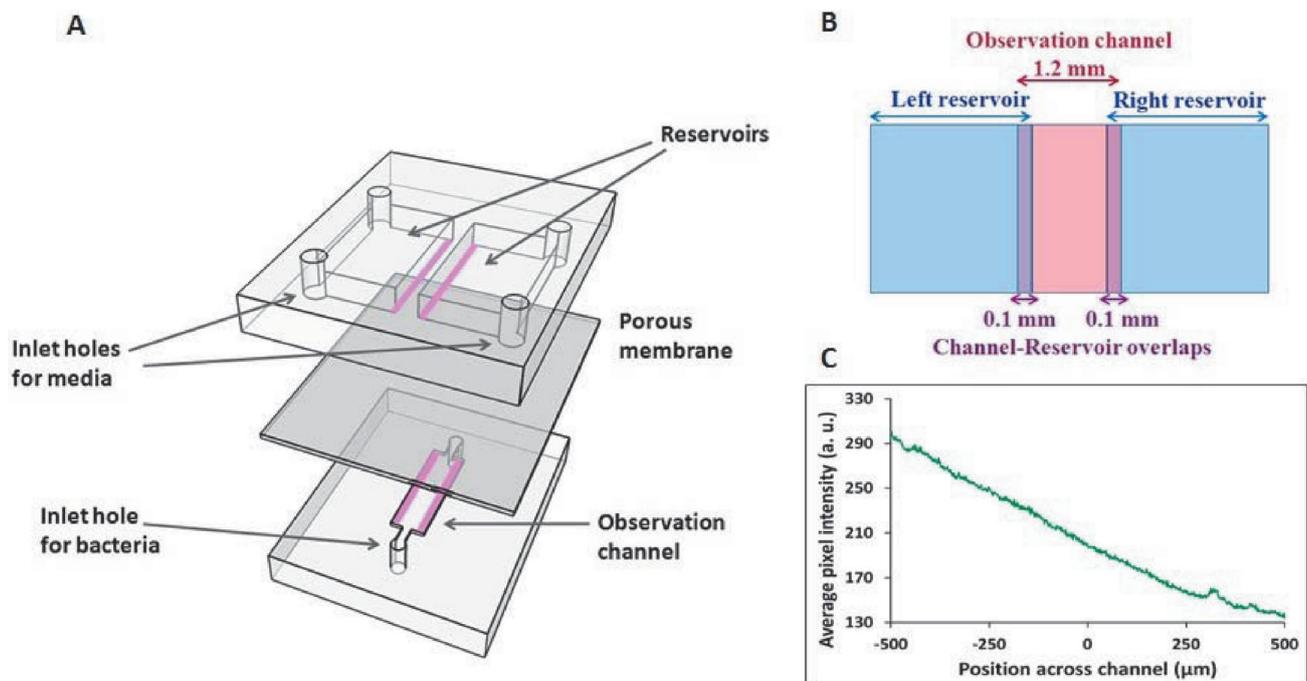


Fig. 1 – (A) Schematic drawing of the microfluidic device (not to scale). The overlapping regions of the channel and the reservoirs are highlighted. (B) Schematic drawing showing the overlap of the central channel and the reservoirs from a top view. (C) We demonstrated the formation of chemical gradient by filling one of the reservoirs with a fluorescein dye solution. The average fluorescence intensity measured across the width of the observation channel is plotted.

each) on one side of the membrane, and a narrow observation channel (10 mm × 1.2 mm × 0.04 mm size 0.48 μL volume) on the other side with 100 μm wide overlapping regions between them.

Filling the microfluidic chip

First, the shallow observation channel was filled with bacterial culture. Then one of the reservoirs was filled with motility medium, and the other one with LB medium containing bacteria as well. The initial concentration of bacteria in the channel was half the concentration of bacteria in the reservoir. The appropriate concentration was achieved by diluting the culture in a 1:1 ratio with motility medium before loading the channel. All the inlet holes of the device were closed off using uncured PDMS.

Microscopy

Fluorescence microscopy was used for imaging the bacteria in the microfabricated channels. The experiments were done at 30 °C using a Nikon Eclipse Ti-E inverted microscope (Nikon Inc.) equipped with a home built incubator set to 30 °C and a 10× Nikon Plan Fluor objective, a GFP fluorescence filter set (49002 filter set, Chroma Inc.), a Prior Proscan II motorized stage (Prior Scientific Ltd.) and a LUMEN 200Pro metal arc lamp (Prior Scientific Ltd.) as a fluorescence excitation source. Time-lapse imaging of the bacteria was done using an Andor NEO sCMOS camera (Andor Technology plc.). The image acquisition and the microscope control were done using the NIS Elements Ar software (Nikon Inc.). The microfluidic device was imaged every 10 minutes during the experiments. We used a solution of 1 mM fluorescein in NaHCO₃ buffer in one reservoir of the device and plain buffer in the reservoir to demonstrate the gradient formation in the microfluidic device.

Image processing and analysis

Matlab 2013a (MathWorks Inc.) and ImageJ,²² an open source software package was used for data processing and image analysis. A background correction was performed on each frame using the “rolling ball” algorithm.²³ Manufacturing artifacts of the membranes showed up as small bright spots on the fluorescence images. These spots were masked out and excluded from the image analysis.

We introduced the asymmetry index A to quantify the (horizontal) spatial distribution of the bacteria across the width of the observation channel. The axis of the observation channel was vertical on our images. To calculate A , first the center of the 1.2 mm wide observation channel was determined on all fluorescence images. The average pixel values were

calculated from the grayscale fluorescence images for the left and right half of the channel (with respect to the center): all pixel values from the left side of the channel were averaged resulting I_- , while the average from the pixels of the right side of the channel is denoted by I_+ . Then the asymmetry index was calculated as given by the following equation:

$$A = \frac{I_- - I_+}{I_- + I_+}. \quad (1)$$

Results and discussion

We used a microfluidic device shown in Fig. 1A,B to culture bacteria in microchambers and study the interaction between distinct populations. Bacteria may grow in this device in a flow-free environment making heterogeneities in the spatial distribution of chemicals possible. The compartments in the device are physically separated by a porous membrane. Bacteria cannot cross the membrane but molecules can diffuse through. Due to the overlapping areas of the reservoirs and the channel, chemicals may diffuse between the reservoirs and the channel. As a result, there is a chemical coupling between the compartments. These compartments therefore may be utilized to culture bacteria, and observe the interaction of the bacterial populations developing in the chambers and the channel of the device. In this case the interaction is purely (bio)chemical as no physical contact between the cells of different populations is possible.

Such a flow-free microfluidic arrangement allows the development of chemical heterogeneities in the device. Molecules may diffuse from one reservoir to the other only by passing across the channel. Therefore when there is a concentration difference of some chemical compound between the reservoirs, there will be a net transport by diffusion from the high concentration reservoir to the low concentration reservoir. As a result, a concentration gradient develops between the reservoirs across the width of the channel. We have demonstrated such chemical gradients in the device using fluorescent dyes (Fig. 1C).

Furthermore, an important characteristic of the PDMS material used to build the chip is its gas permeability.²⁴ This means that cells cultured in the device are continuously provided with oxygen from the atmosphere surrounding the device.²⁵ On the whole, we could say the microfluidic device is a suitable and versatile experimental platform to investigate microbial ecosystems where i) the interaction of microbial populations, and/or ii) the effect of a chemically heterogeneous environment is under study. Our particular experiments indeed aim the above.

At the start of the experiments we filled the central channel with a 1:1 mixture of motility medium (based on potassium phosphate buffer) and a culture of fluorescent wild type chemotactic *E. coli* bacteria. One of the reservoirs (on the right in Fig. 1A,B) was filled with motility medium. This medium lacks carbon sources (although we put bovine serum albumin in the medium to prevent sticking of the cells, previous studies suggest that it is not metabolized²⁶), so bacteria are not able to grow in it, but contains essential salts for the cells to maintain swimming motility for several hours. The second reservoir (on the left in Fig. 1A,B) was filled up with LB medium containing non-fluorescent wild type chemotactic *E. coli* bacteria (the measured optical density of the solution was $OD_{600} = 0.5$). As opposed to motility medium, LB medium is a rich nutrient source that is able to support fast growth and reproduction of bacteria while the nutrients last. By observing the fluorescent bacteria in the central channel we saw multiple dynamic rearrangements of the spatial distribution of bacteria during the ~ 4 h time course of the experiments (Fig. 2). In parallel, we observed a monotonic increase in the overall fluorescence intensity in the channel suggesting an increase in biomass.

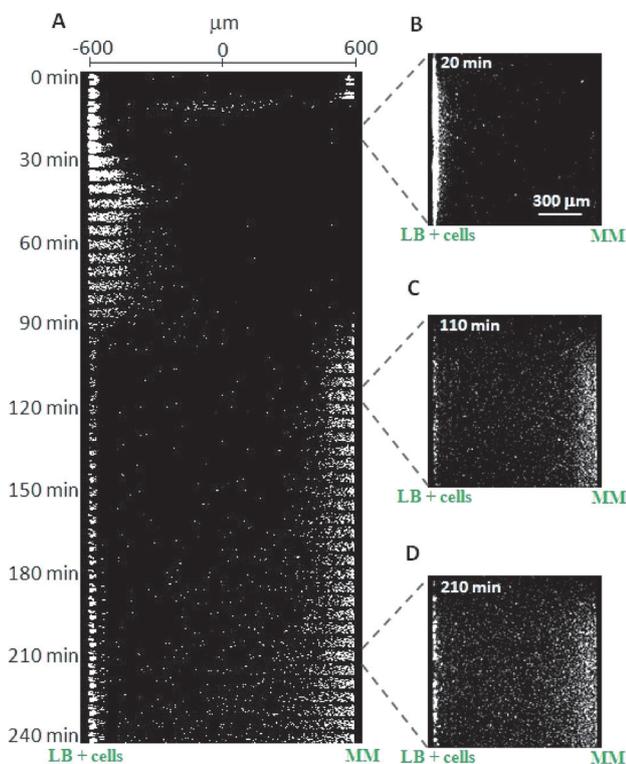


Fig. 2 – (A) Kymograph of a typical 4-hour experiment. Fluorescence microscopy images of a time lapse recording of the observation channel were compressed vertically. Subsequent images were pasted one beneath the other. (B, C, D) Background corrected fluorescence images of the observation channel at representative time points (20 min, 110 min, 210 min).

Initially, after filling the device there were about the same number of cells on the left and right half of the channel. Due to the filling process, cells were not spread homogeneously in the channel, however, their distribution was symmetric (see at $t = 0$ min in Fig. 2A). Soon after filling, cells started to accumulate on the left side of the channel (where the channel overlaps with the LB-containing reservoir). This accumulation was a result of the active movement of the cells. Bacteria that were initially on the right edge of the channel moved across the middle and to left side, as may be seen on the kymograph in Fig. 2A. This accumulation process led to a sharp asymmetric distribution of bacteria as may be seen in Fig. 2B. At 20 minutes, nearly all cells were on the left side. This distribution was quite stable for some time; however, a monotonic increase in cell number (and overall fluorescence intensity) was observed due to the growth of cells. The spatial distribution of cells at this phase is also shown in Fig. 3, where the average pixel intensity on the images (corresponding to the indicated experimental times) are plotted as a function of the distance from the midline of the channel. This cell distribution is caused by the formation of a concentration gradient of nutrients across the channel and the resulting chemotactic response of the cells. We observed identical events in experiments where the left channel was filled with pure LB medium without bacteria mixed in (data not shown). As nutrients diffused from the left reservoir towards the other, the chamber containing motility medium, a nutrient gradient formed across the width of the channel ($t = 0$ –15 min in Fig. 2A). Swimming cells followed this gradient by positive chemotaxis, and moved towards the side of the channel with higher nutrient concentration. LB is a rich medium with many components that might induce this response. Aspartate and serine for example are known to be strong chemoattractants for *E. coli*²⁷ so these amino

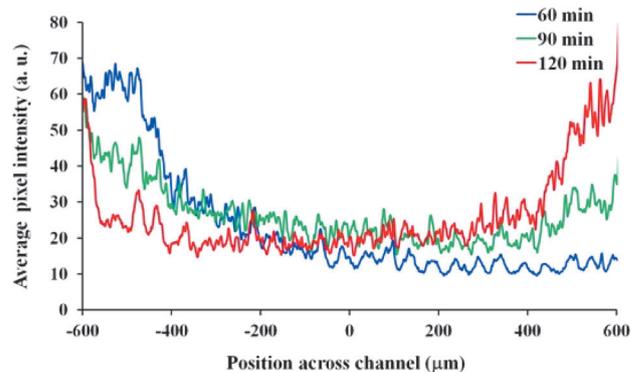


Fig. 3 – Average intensity profiles measured across the observation channel at representative time points (60 min, 90 min, 120 min) of the experiment

acids may be candidates to play a role in this initial phase of the experiment.

After about 90 minutes into the experiments, we saw a rapid, synchronized relocation of cells from the left side of the channel to the right ($t = 90$ min in Fig. 2A,C and Fig. 3). This event is quite striking as the majority of cells crossed the channel; however, there were non-motile bacteria stuck to the surface of the membrane that stayed on the left edge of the channel. These stuck cells may be seen on the kymograph in Fig. 2A ($t > 90$ min) and also on the cross-sectional intensity profile corresponding to 120 minutes in Fig. 3.

Although we did not monitor directly the change of the bacterial population inside the left, LB-medium containing reservoir, we could assume that similar to the bacteria in the observation channel, the population in the left reservoir also grew during the initial phase of the experiment (this was confirmed at the end when we saw that the medium in this reservoir turned opaque due to the increased cell density). Since the volume of the reservoir was about 130 times larger than the channel volume, the population grown there had many times more cells than what we observed in the channel thus they had an impact on the medium composition both in the reservoir and the channel. The growth of the reservoir population had two consequences: i) the depletion of nutrients in the reservoir, and ii) accumulation of metabolic products and signaling molecules in the medium. This led to a change in the chemical composition and an alteration of the spatial distribution of chemicals inside the central observation channel too. Such changes induced a chemotactic response resulting in the migration of the bacteria to the right side. What specific chemicals and what kind of gradients triggered this response that led to the late accumulation of cells on the right side? On one hand, it is possible that we saw a positive chemotaxis toward attractant compounds that had an increasing concentration toward the right. On the other hand, perhaps there was a negative chemotaxis from chemical agents that were more concentrated on the left side (and in the left reservoir). Further experiments discussed below suggest the latter.

We have repeated the experiment with the following modification. The reservoirs were filled up as before, however we injected a 1:1:2 mixture of non-fluorescent chemotactic wild type cell culture, fluorescent non-chemotactic mutant cell culture, and fresh LB medium. During these experiments, we imaged the fluorescent non-chemotactic mutant bacteria in the channel. This mutant population did not gather on either side of the channel. They did not assemble on the left at the early phase, and did not show an en masse migration to the right side at the later phase of the experiment. The difference in

the behavior of the mutant and the wild type chemotactic bacteria is shown clearly by the different dynamics of the asymmetry index of the cellular distribution across the channel (Fig. 4). The dimensionless asymmetry index A is a normalized difference between the average pixel intensities of the left and right side of the channel images (Eq. 1). This measure gives information on the distribution of cells. In general, the value of A may vary between -1 (all cells on right) and 1 (all cells on left). We get $0 < A \leq 1$ or $-1 \leq A < 0$ for the asymmetry index when there are more cells (and hence more intense fluorescence) on the left or right half of the channel, respectively. An even distribution of cells yield $A = 0$. Fig. 4 shows that for the wild type cells there was an early fast increase in the asymmetry index corresponding to the initial accumulation of cells on the left near the nutrient-rich reservoir. The cell distribution more or less stabilized then, but after about an hour, a sharp decrease of the asymmetry index and a change of its sign correspond to the collective migration of bacteria to the right side of the channel. These two sudden changes in A were a result of the quick change in the cellular distribution due to active swimming (and chemotaxis) of the bacteria. A location dependent growth could not explain such sudden changes that take place in about 20 minutes. This timescale is considerably faster than the typical generation time of *E. coli* at 30°C (~ 50 min). However, in the last phase of the experiment we saw a slow gradual increase in A for the wild type cells. This slow change could be a result of differential growth within the channel: the reproductive rate of cells was location-dependent ($t > 120$ min), i.e. it was not the same for bacteria on the left or right part of the channel. This was due to the uneven distribution of available nutrients across the channel. The slow increase in A suggests that the cell growth was faster on the left, so the nutrients were more available there. This indicates that, at this late phase, there still was a nutrient con-

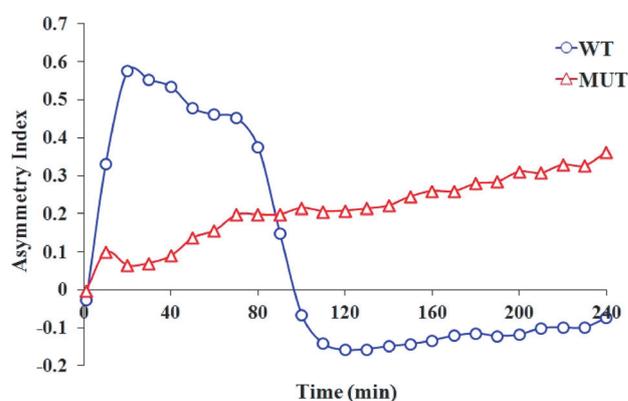


Fig. 4 – Temporal variation of the asymmetry index calculated for the wild type (blue line) and non-chemotactic mutant (red line) bacterial populations during a 4-hour experiment

centration gradient similar to the early section of the experiment, and there was no complete resource depletion.

The lack of sudden extensive increase or decrease in the asymmetry index corresponding to the mutant populations is due to the fact that there were no dynamic, motility-related rearrangements of the bacterial distribution within the channel. The slow monotonic increase in the *A* index is explained by the differential growth of the population (similarly to the slowly increasing *A* at later times for the wild type cells, as described before). The mutant cells reproduced faster on the left side of the channel than on the right.

This differential growth phenomenon of the mutant cells (and the wild type cells later in the experiments) indicates an important characteristic of the nutrient conditions. One may imagine the following. There was a constant transport of media components between the two reservoirs by diffusion. At the early phase of the experiments, this means the nutrients diffused into the motility medium filled reservoir and accumulated there. Meanwhile the population growing in the left LB-rich reservoir may have depleted the nutrients there. A fast and complete depletion could possibly lead to a reversed situation with more resources being in the right reservoir rather than in the left one. This would have led to a nutrient gradient formation in the channel that is the opposite in direction compared to the gradient in the initial phase of the experiment. Such an effect could have led to bacteria gathering on the right side of the channel after some time by positive chemotaxis for nutrients. However, the differential growth observed does not support this scenario. Instead, it shows that the direction of the nutrient gradient stayed the same for the whole experiment: cells grew more on the left because there were more resources available there earlier on and later also. Therefore, we can exclude the possibility of nutrient-based positive chemotaxis causing bacteria migrating to the right side of the channel during the experiment.

The observed migration phenomenon was due to the bacterial population growth in the reservoir containing LB medium. During this growth process, the nutrients were consumed and metabolic products were released. The latter may also act as repellents. There are several secreted products of *E. coli* – such as ethanol, acetate, and indole – that accumulate in batch cultures and have repellent chemoeffector activity. For example, a similar repulsion phenomenon of *E. coli* populations has been observed on agar plates using the chemical in plug method.²⁸ The exact chemical signal inducing the negative chemotactic response is not known. We speculate that indole may be a good candidate for

this. It is a product of tryptophan metabolism, and is produced by *E. coli* and many other Gram-positive and Gram-negative bacteria. Therefore, its presence is quite common in natural habitats. However, recent studies have shown that several biological functions may be assigned to this compound. It has been suggested that indole is an intercellular and/or inter-species signal molecule.^{29,30} It has been reported that *E. coli* start to produce indole in the early exponential phase and in a rich medium (like LB), and it may accumulate in concentrations of about 0.6 mM.³¹ At this concentration, indole is a repellent for *E. coli*.^{28,32} Furthermore at this concentration indole does not seem to affect growth, much higher concentrations (> 2mM) are necessary for a negative effect.^{33,34} All the above results from other works seem to support the possibility of indole accumulating in our microfluidic chip and forming a concentration gradient left to right across the observation channel. This could have induced a negative chemotactic response, resulting in the migration of bacteria to the right side of the channel in the later phase of the experiment. This however does not exclude the possibility that other chemical compounds (such as the above mentioned acetate) play a role in our experiments.

Conclusions

Microfluidic chips were used to culture bacteria in microchambers. We studied the interaction of physically separated but chemically coupled bacterial populations. Chemical heterogeneities formed in the device that induced changes in the spatial distribution of cells by a chemotactic response, and by differential growth. The observed population of *E. coli* cells moved to nutrient rich regions in the chip. In a later phase of the experiment the grown adjacent cell population had a repellent effect and caused the observed cells to migrate and accumulate on the opposite side of their habitat. We confirmed that this effect was not caused by a reversal of nutrient gradients. Furthermore, we showed that although differential growth is present in the device, the fast and dynamic cell migrations are the result of chemotaxis.

We suggest that indole secreted by bacteria may play an important role in the abovementioned effect, and could act as a chemical agent through which bacterial cells and populations interact. Our results show that chemotaxis, metabolism, and signaling together determine the distribution and growth of bacteria, and these mechanisms have to be considered when using or culturing bacteria in structured microdevices, such as microchambers and microreactors.

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