UNIVERSITY OF SZEGED
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# Studying physical and biochemical interactions in bacterial communities using microfabricated devices

Summary of doctoral thesis

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**Publications related to the thesis:**

I. K. Nagy, **O. Sipos**, E. Gombai, A. Kerényi, S. Valkai, P. Ormos, and P. Galajda.
Interaction of bacterial populations in coupled microchambers.
*Chem. Biochem. Eng. Q.* **28**, 225 (2014)

II. **O. Sipos**, K. Nagy, and P. Galajda.
Patterns of collective motion of swimming and non-swimming bacteria in microfluidic devices.
*Chem. Biochem. Eng. Q.* **28**, 233 (2014)

III. **O. Sipos**, K. Nagy, R. Di Leonardo, and P. Galajda.
Hydrodynamic trapping of swimming bacteria by convex walls.
*Phys. Rev. Lett.* **114**, 258104 (2015)

IV. K. Nagy*, **O. Sipos***, S. Valkai, É. Gombai, O. Hodula, Á. Kerényi, P. Ormos, and P. Galajda.
Microfluidic study of the chemotactic response of *Escherichia coli* to amino acids, signaling molecules and secondary metabolites.
*Biomicrofluidics* **9**, 044105 (2015)

** Both authors contributed equally to this work.*

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**Other publications:**

V. Gy. M. Szabo, **O. Haja**[**], K. Szatmary, A. Pal, and L. L. Kiss.
Limits on Transit Timing Variations in HAT-P-6 and WASP-1.
*Information Bulletin on Variable Stars* **5919**, 1 (2010)

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# 1. Introduction

Microbes can form complex functional communities through vivid physical and biochemical interactions with their microenvironment and other cells, which can lead to complex social behavior (biofilm formation, bacterial infections, etc.). To understand these structured, functional microbial communities, we should study the physical and biochemical cell-cell and cell-habitat interactions on the single cell and population level.

*E. coli*, our model organism, is a rod-shaped peritrichously flagellated bacterium. The swimming motility of *E. coli* includes periods of straight swimming ("run") and instantaneous stops followed by random changes in swimming direction ("tumble"). This so-called "run and tumble" motion is eventually a three dimensional random walk, that enables bacteria to effectively explore their environment. The random bacterial swimming motility can be affected by numerous environmental stimuli. We shortly review some of those important physical, chemical and biological interactions with the local environment that can profoundly influence bacterial swimming behavior.

It has been observed that swimming bacteria are attracted to solid surfaces and this phenomenon results in a notable cellular accumulation near solid boundaries. It was proposed that the observed wall entrapment during swimming may have a purely hydrodynamic origin, however, more recently the role of hydrodynamic interactions has been questioned. Despite the extensive theoretical and numerous experimental works in the hydrodynamics of bacterial swimming, a straightforward, unambiguous and direct identification of the main mechanism responsible for wall entrapment is still lacking. All the existing models capture different aspects of the problem, and they are all capable of justifying the accumulation effects observed at flat walls. Understanding cell-surface interactions have a substantial biological importance too. Biofilms are multicellular (and often multispecies) communities that develop on surfaces. Surface colonization by biofilm-forming bacteria is initiated by contact and adhesion to the surface, in which hydrodynamic trapping can play a crucial role. The subsequent biofilm growth

can cause highly resistant bacterial infections on medical implants and catheters, or impaired industrial equipment.

Direct physical interactions between individual cells can also shape the swimming behavior of bacterial populations, if the distance between bacteria is greatly reduced. In dense bacterial suspensions whirlpools, vortices and jets are formed by groups of adjacent cells that aligned their swimming speed and direction. Without any geometrical constriction these emerging coherent formations are transient, thus, bacteria display fast, constantly fluctuating swimming patterns. The synchronization of active motion of bacteria leads to the enhancement of diffusion. This can provide several benefits for the whole population (e.g. better nutrient availability or faster biochemical signal propagation). Beside the extensive fundamental experimental and theoretical work on the physics of collective swimming behavior in high-density bacterial cultures, engineering applications have also started to appear recently.

In their natural habitat, bacteria can sense and respond to a vast range of environmental signals, such as the intensity of light, nutrients, pH, temperature, osmolarity and oxygen levels. Using an elaborate chemosensory system, bacteria often respond to chemical environmental clues by changing their swimming motility patterns via chemotaxis. The past decades revealed a new group of important chemicals for bacteria: small secreted communication signals. The bacterial communication phenomenon is called quorum sensing because the concentration of the secreted signal molecules, which is proportional to the local cell density, must reach a minimal threshold level to elicit an appropriate response. These signals can trigger a wide range of important biological mechanisms, such as biofilm formation, virulence, sporulation, antibiotic resistance, colony formation, exoenzyme production, cell differentiation, and antibiotic production.

Gram-negative bacterial strains use a characteristic set of acyl-homoserine lactons (AHLs) as signal molecules. Therefore, these chemicals are mainly treated in the literature as intraspecies communication signals. Nevertheless, it has been proved that other species are able to sense and respond to them as well. For example, SdiA was identified in *E. coli* as a receptor protein, which can be only used for AHL signal interception, as *E. coli* does not produce any AHL molecules. While these signaling molecules are natural clues for bacteria, it is an

interesting question if they could be chemoeffectors (even for other species) at the same time. The relation between quorum sensing and chemotaxis seems particularly important if we consider that both phenomena play a crucial role in bacterial infections.

Recently microtechnology revolutionized the traditional techniques used in biological research. Microfluidics is a multidisciplinary scientific field, which deals with the operation and manipulation of liquids (pumping, mixing) in the spatial dimension of 1-1000 μm. Microfluidics became a primary technology in applications of miniaturized systems currently used in biological, chemical and medical research. It provides an outstanding opportunity to design and build precisely controlled microenvironments for microbiological studies. Using microfabricated microhabitats we can obtain deeper understanding of the physical and biochemical interactions between bacteria and their local environment.

In this work we used microfluidic devices, as precisely controlled microhabitats, to study the effects of physical and biochemical interactions between bacteria and their local environment on bacterial swimming motility.

## 2. Aims of this study

The aim of our experiments is to understand the effects of physical and biochemical interactions between swimming bacteria and their local environment on bacterial swimming motility. These phenomena may play important roles in the development of complex microbial communities, biofilm formation and bacterial infections. We address the following questions about the physical aspects of bacterial swimming behavior:

- What is the main mechanism behind reversible wall entrapment?
- Does the surface geometry affect cell adhesion?
- How do the geometric constrictions (solid boundaries, walls, chambers) affect the swimming behavior of bacteria in high-density cultures?
- Can we shape and stabilize emerging swimming patterns by physical constrictions?

For studying bacterial communication and chemotaxis, we aim to develop a novel microfluidic device, in which we are able to quickly establish a stable linear chemical gradient in a flow-free environment. This device can be used to study the chemotaxis response of *E. coli* towards several chemical compounds involved in bacterial communication. In this thesis we focus on the following interesting biological problems:

- Can we find evidence of direct interactions between chemotaxis and quorum sensing?
- Can we use our novel microfluidic device to show chemotactic effects of canonical quorum sensing signals or molecules under quorum sensing regulation?
- Can we study the complexity of chemical communication between adjacent bacterial populations in our microfluidic device?

# 3. Materials and methods

## 3.1 Microfabrication

In our experiments we used polydimethylsiloxane-based (PDMS) microfluidic devices, which were manufactured using standard soft lithography techniques. The main steps of the microfabrication process were (i) designing the microstructures, (ii) fabrication of the photomasks, (iii) production of the master molds, (iv) creating the elastomer based microdevices by replica molding. Blueprints for the photolithography masks were printed onto emulsion film or chromium-based photomasks. For creating the master molds we applied 40-75 μm photoresist layers (SU-8 2015, SU-8 2050 (MicroChem Corp.)) onto 100 mm diameter silicon wafers or microscope slides by spin coating. The layers were baked on a level hot plate, and they were exposed to UV light through the photomasks. After post exposure bake, the unexposed and non-polymerized parts of the SU-8 layers were removed by immersing the wafers into SU-8 developer solution. Positive replicas of the master molds were made by PDMS (Sylgard 184, Dow Corning Corp.) casting. To prevent the attachment of the molds to the PDMS, they were treated with silane (Gelest Inc.) under vacuum for 4 hours. Uncured PDMS was poured onto the

master molds and the devices were baked at 40-90°C in an oven for overnight. The polymerized microchips were peeled off the master molds, and inlet holes were created. Depending on the actual microchip design, the PDMS devices were bound to coverslips, using oxygen plasma treatment, or to ~ 60 μm thick aluminium-oxide membranes (Anodisc 47, Whatman). The attachment of the microchips to the membranes was achieved by a stamping method, using a thin (6-10 μm) layer of PDMS.

3.2 Cell culturing

Our experiments were performed using the *E. coli* HCB33 and HCB437 strains carrying the pMPMA2-GFPmut2 plasmid, the *E. coli* W3110 strain (bearing a lac promoter inserted together with a GFP (green fluorescent protein) encoding gene), and the *P. aeruginosa* PUPa3 strain.

Bacteria were grown overnight in 3 ml of LB (lysogeny broth) media (Sigma-Aldrich) at 30°C in a shaker incubator, shaken at 200 RPM. The overnight bacterial cultures were diluted 1000 times on the morning of the experiment, and bacteria were grown at the same conditions until they reached optical density 0.5-0.9 at 600 nm ($OD_{600}$).

3.3 Microscopy

During all the experiments a Nikon Eclipse Ti-E epiflourescence microscope (Nikon Inc.) equipped with a 10× Plan Fluor, and a 40× S Plan Flour objective, a fluorescence filter set for GFP (Chroma Inc.), a Prior Proscan III microscope stage, and a Prior Lumen 200Pro metal arc lamp (Prior Scientific Inc.) was used for imaging. The microscope setup was controlled by the Nikon NIS Elements AR microscopy software (Nikon Inc.). During time-lapse video recording, a Rolera em-c2 digital EM-CCD camera (QImaging Corp.) or an Andor NEO sCMOS (Andor Technology Ltd.) camera was used.

3.4 Gradient characterization and model calculations

Pyranine (Sigma-Aldrich), a water soluble fluorescent dye, was used to characterize the chemical gradient in our no-flow gradient generator device. Pyranine was solved in phosphate buffered saline at 0.1 μM and 1 μM concentrations. Pyranine solution was loaded into one reservoir, the

other one was filled with pure buffer, and the observation channel was filled with a 1:1 mixture of the buffer and the dye solution. Fluorescence time-lapse imaging was used to characterize the gradient formation process in our microfluidic device. During the calibration experiments, we considered the measured fluorescence intensity as a direct representation of the pyranine concentration.

A two dimensional simulation was done using the "Transport of Diluted Species" model of Comsol Multiphysics 4.3a software. We simulated the gradient establishment via diffusion of the pyranine molecules, and studied the stability of the gradient over time.

### 3.5 Data analysis

Fiji, an open source software package, and Matlab (MathWorks Inc.) were used for image processing and data analysis.

## 4. Results

1. We studied the hydrodynamic interactions between swimming *E. coli* cells and nearby solid surfaces in PDMS-based microfluidic devices. We found that more than 90% of the bacteria swam along planar walls after collision, while the fraction of bacteria that followed the surface of micropillars was 60-90%, when the radius of curvature was larger than 50 μm. We found that bacteria tend to spend more time swimming around pillars with larger radii, and swimming velocity near solid walls was reduced. Although, we did not find any significant variation in the average swimming speed near different pillars. We observed that cells usually did not align their body axis along the surface, but swam with a finite average angle to the surface plane. The typical average angle of orientation for an individual bacterium was around 5°. Our findings strongly indicate the presence of a hydrodynamic trapping effect that acts on the bacterial cells swimming near a solid surface. We studied the role of surface geometry in cellular adhesion, and we found that bacteria tend to adhere to surfaces with less curvature, as the hydrodynamic trapping effect keeps them near flat surfaces for the longest period of time. Our results indicate

that hydrodynamic entrapment may play a role in biofilm formation via cell adhesion and surface colonization.

Our collaborator, Roberto Di Leonardo from Sapienza University of Rome, created a theoretical model of the physical interactions between a swimming bacterial cell and a nearby surface. Our experimental results are in an excellent agreement with the model predictions.

2. We examined the physical characteristics of swimming motility patterns in high-density cultures of *E. coli* in a PDMS-based microfluidic chip, which contained several small round chambers. In droplets of dense bacterial suspensions, we observed whirlpools, vortices and jets, formed by groups of adjacent cells that aligned their swimming speed and direction. We found that the characteristic size of these transient structures was about 10-30 μm, and they persisted only for a couple of seconds on average. In our experiments we were able to stabilize, shape, and characterize the emerging bacterial vortices inside microfabricated chambers.

3. We designed and fabricated a new flow-free chemical gradient generator microfluidic platform, in which we were able to quickly generate (3-5 minutes) and precisely control linear chemical gradients. Moreover, we were able to maintain nearly the same gradient for at least 24 hours. We demonstrated the usability of our device in chemotaxis experiments with well-known chemoeffectors for *E. coli* (aspartate and nickel). Our results showed that the amino acid L-lysine acts as a weak attractant, while L-arginine does not induce a chemotactic response in *E. coli*.

4. The two main quorum sensing systems of *P. aeruginosa*, the Las and Rhl systems, employ N-(3-oxododecanoyl)-homoserine lactone (oxo-C12-HSL) and N-(butryl)-homoserine lactone (C4-HSL) signal molecules, respectively. We found that *E. coli* exhibits a positive, but transient chemotactic response to these signal molecules. Furthermore, we showed that pyocyanin, a secondary metabolite and virulence factor of *P. aeruginosa* is a weak attractant, while pyoverdines, extracellular siderophores of iron metabolism, are repellents for *E. coli*.

5. We showed that our microfluidic device is a suitable and versatile experimental platform to investigate the complex behavior of multispecies microbial communities. We performed co-culturing experiments in our new microfluidic device, in which the bacterial populations in the different chambers were physically separated, but chemically coupled. In this configuration linear gradients of signal molecules and other secreted biochemical factors may form through the central channel, which enables us to study the chemotactic response of bacteria inside this channel. We showed that metabolic products or signaling molecules (i. e. ethanol, acetate, indole), secreted by an *E. coli* population growing in nutrient rich environment, act as chemorepellents and increase the cell adhesion of a neighboring *E. coli* population. We also demonstrated that a *P. aeruginosa* population produces chemical compounds that vastly increase cellular adhesion in an adjacent *E. coli* population. On the other hand unidentified chemorepellents were secreted by the *P. aeruginosa* population, which induced negative chemotactic response of a small planktonic subpopulation of *E. coli*. Our results suggest that communication signal molecules and secondary metabolites connected to quorum sensing could be among the potentially important chemicals that may act throughout the induction of a chemotactic response between adjacent bacterial populations.

## 5. Conclusions

In this work we examined several seemingly distinct biological processes, such as cell adhesion, chemotaxis and bacterial communication. As all of them significantly contribute to biofilm formation and bacterial infections, our findings help us to build a more complex picture and gain a better understanding of these medically important phenomena. Our results demonstrated the significance of both physical and biochemical interactions in biofilm formation and bacterial infections and provided further evidence of the interconnections of chemotaxis and bacterial communication.

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