STUDYING THE EFFECT OF HETEROGENEOUS CHEMICAL ENVIRONMENTS ON BACTERIA USING MICROFLUIDIC DEVICES

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Our research laboratory employs the technique of microfluidics to study bacterial populations from various aspects. In our experiments microfluidic devices are utilized as bacterial culture vessels or habitats that are carefully designed and engineered to ensure precisely controlled physical and chemical microenvironments for bacterial colonies. Therefore, we can study how particular environmental factors affect the life of bacterial populations.

The focus of the current research was to investigate the effect of gradients of antibiotics and signal molecules involved in cell-cell communication (e.g. quorum sensing) on growth and motility of *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*) bacteria.

Substantial progress was made regarding the research aims as described below. However, we had some challenges, and certain experiments proceeded somewhat slower than planned. Therefore, we are still working on some parts and writing manuscripts that are going to publish results generated during this proposal. These results and challenges are explained in greater detail below.

1. *Eschericia coli* in gradients of signalling molecules

Intra- and interspecies interactions are the main driving force behind the formation of microbial communities. Therefore, we aimed to study how various signalling molecules affect the behavior of *E. coli* cells. To study bacterial motility in chemical concentration gradients, we have fabricated and experimentally characterized a microfluidic system that creates temporally stable chemical concentration gradient in a flow-free environment. We have used this device earlier to study interacting populations of *E.coli*, but the detailed characterization of gradient formation, and the questions below were investigated during this project.

The device was fabricated of poly(dimethylsiloxane) (PDMS). It consists of two large reservoirs and a shallow observation channel between them. A porous membrane separates the reservoirs and the central channel: chemicals get through the membrane but bacteria don't. Diffusion of molecules from one reservoir to the other through the membrane and the central channel leads to a concentration gradient across the width of the central channel, and this gradient remains stable for hours. Bacteria are inoculated in the central channel, and their spatial distribution is monitored by using fluorescence time-lapse microscopy. To quantify the spatial distribution (chemotactic response) of bacteria in the device we introduced the *Asymmetry index* (*A*) that is calculated based on the fluorescence intensity measured at left and right side of the observation channel.

We studied the chemoeffector potential of quorum sensing signalling molecules of *P. aeruginosa* on the swimming behaviour of *E. coli* bacteria. Our findings are published in the journal "*Biomicrofluidics*" together with other results coming from earlier experiments [1].

1.1. Chemoeffector potential of homoserine lactones (HSL-s) on *E. coli*

The two main quorum sensing (QS) systems of *P. aeruginosa*, the Las and Rhl systems, employ 3-oxo-C12-HSL and C4-HSL as signal molecules. *E. coli* cells were investigated in our microfluidic device in the presence of gradients of these molecules. The maximum concentration of the HSLs in each experiment was 100 μM. A clear positive, but transient chemotactic response was found both in the C4-HSL and the 3-oxo-C12-HSL gradients. It is confirmed by the initial positive values of the calculated asymmetry index, as well. When non-chemotactic mutant cells were used, bacteria stayed equally distributed over the channel, no accumulation occurred during the experiment.

1.2. Chemotaxis response of *E. coli* to gradients of secondary metabolites produced by *P. aeruginosa*

The secretion of pyocyanin and pyoverdine, secondary metabolites of *P. aeruginosa*, is regulated by the quorum sensing system. The main functions of these molecules are well-known, pyocyanin has a toxic effect

to other species and pyoverdine have important role in the iron homeostasis. *E. coli* cells were exposed to a linear chemical gradient of these molecules. Surprisingly, pyocyanin proved to be a weak attractant while pyoverdine are repellent. As control, we tested non-chemotactic mutant bacteria that showed no chemotactic response to these chemicals, i.e. remained evenly distributed in the observation channel.

1.3. Interaction of physically separated, but chemically coupled *P. aeruginosa* and *E. coli* populations

Loading *E. coli* in the observation channel and *P. aeruginosa* in one of the reservoirs resulted in a complex, dynamic behaviour of *E. coli* population monitored in the channel.

2. *Escherichia coli* in antibiotic gradients

Antibiotics are very important drugs in human healthcare, and are a subject of intensive research. However, it has not been studied before how bacteria behave in a landcape of heterogeneous antibiotic distribution, although it is expected that in their natural environments (and in the human body) bacteria are exposed to such conditions.

We started a systematic study on the swimming behaviour of *E. coli* in the presence of different antibiotics. We selected three antibiotics with different mode of actions:

- ampicillin (inhibitor of cell wall synthesis),
- gentamicin (inhibitor of protein synthesis)
- and ciprofloxacin (inhibitor of cell division).

2.1. The effect of antibiotics on the motility of *E. coli*

Motility is the main process that shapes the spatial distribution of cells on shorter timescales (compared to the generation time). Therefore, we wanted to explore how antibiotics affect the swimming motility of *E. coli* cells.

We studied the concentration dependence of the effect of these antibiotics in homogenous environments by tracking the movement of cells. For this purpose we put the cells into nutrient-free media (so called chemotaxis buffer) supplemented with antibiotics at different concentrations, and recorded videos of the swimming cells. The used antibiotic concentrations were chosen based on the minimal inhibitory concentration (MIC) of ampicillin (32 μg/ml), gentamicin (16 μg/ml) and ciprofloxacin (60 ng/ml) that was previously measured for the used bacterial strains. The typical trajectory and the swimming speed were determined from these experiments (Figure 1.).

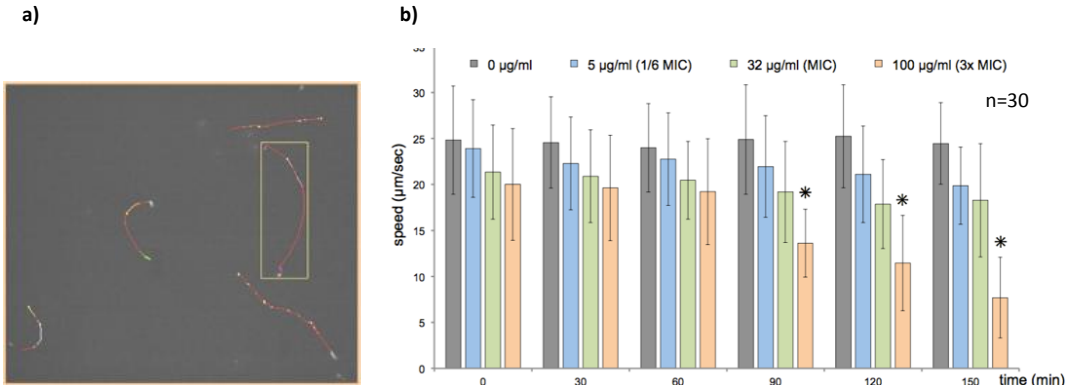


Figure 1. *a) Typical cell trajectories analyzed by ImageJ software. b) The changes of calculated swimming speed of E. coli in media supplemented with different concentrations of ampicilln.*

Figure 1. shows a typical image and data measured on wild type *E. coli* strain in the presence of ampicillin. Here I present only the effect of ampicillin, but our experiments proved that all the selected antibiotics have similar concentration-dependent effect on *E. coli*. Within the timescale of the experiments they possess significant effect on the swimming speed of bacteria only at high concentrations. Bacteria slow down if the antibiotic dose is higher than the MIC value. However, this change in the swimming speed appears only after an hour, and little changes could be detected at concentrations below and around the MIC. These experiments were performed both with wild type chemotactic strains and non-chemotactic mutants. Our non-chemotactic strain (with a mutation ΔCheY) showed no difference to the wild type one in this sense.

2.2. Chemotactic response of *E. coli* in antibiotic gradients

Bacteria were exposed to antibiotic gradients, and the spatial distribution of cells was determined. For these experiments the same chemotaxis buffer and the same antibiotic concentrations were used as in the above detailed tracking experiments. We selected concentrations below, around and above the MIC value. Fluorescent time-lapse microscopy was used to follow the motion of bacteria in the microfluidic gradient generator device. To quantitatively describe the spatial distribution of bacteria with simple quantities, we used two parameters. The *Asymmetry index* (*A*) gives us the contrast of the fluorescence intensity (i.e. cell number) in the left and right side of the channel, while the *Side-central index* (*SC*) gives the contrast at the centre and the edges of the channel The two parameters together describe precisely the different patterns observed in the channel. In case of equal distribution both values are closed to zero. Figure 2. shows the calculation of these parameters.

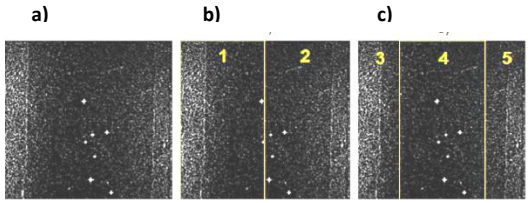
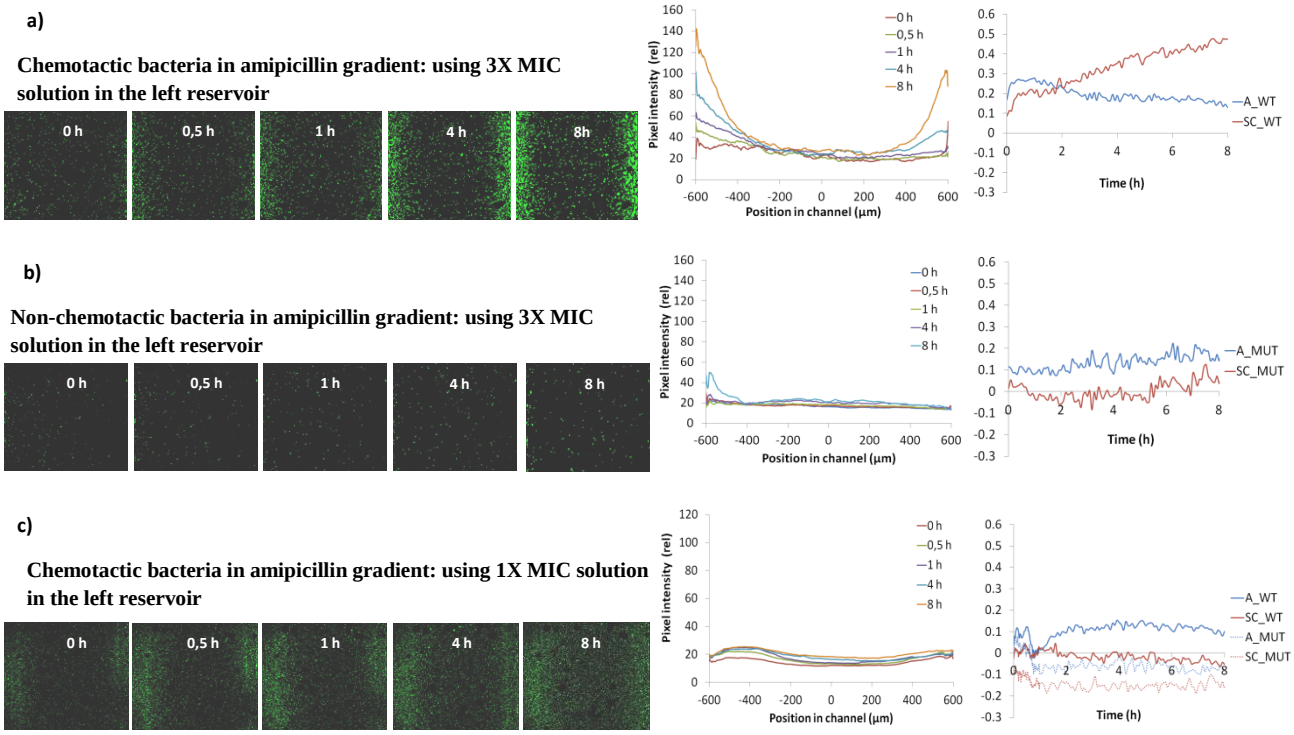


Figure 2. *Microscope images of bacteria in the observation channel demonstrating the division of snapshots for the calculation of quantitative parameters describing the cell distribution: a) whole channel b) division in 1:1 ratio for calculating A c) division in 1:2:1 ratio to calculate SC d) equations of A and SC.*

The introduction of the *SC* index was necessary because *A* alone was not enough to characterize some distributions observed in certain cases. Our experiments showed that *E. coli* populations distribute unevenly in ampicillin gradients. A special pattern formed in time within the device: accumulation of bacteria appeared on the edges of the observation channel, where the antibiotic concentration is minimal (right edge) and maximal (left edge). *A* did not change in time, but *SC* was increasing (Figure 3a). The observed response seems to be specific for high antibiotic concentrations (>MIC), and for chemotactic strains (Figure 3). This indicates that chemotaxis may be important in this behaviour.



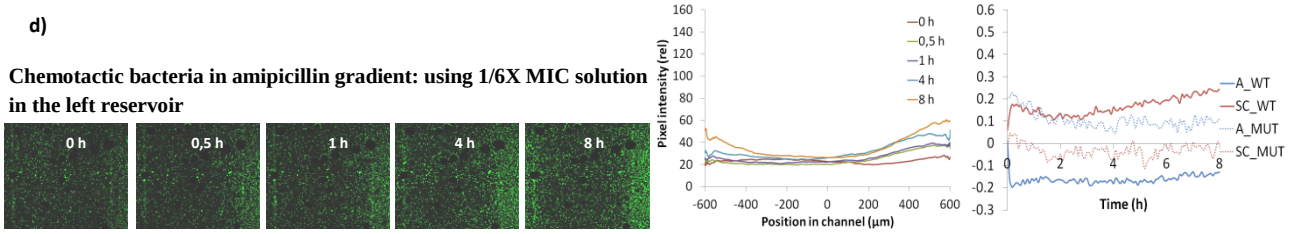


Figure 3. *Characteristic E. coli responses to ampicillin gradients of different steepness. a) Snapshots, corresponding fluorescence intensity profiles and calculated A and SC values of chemotactic bacteria in gradient formed by using 3x MIC ampicillin solution. b) Snapshots, corresponding fluorescence intensity profiles and calculated A and SC values of non-chemotactic mutant bacteria in gradient formed by using 3x MIC ampicillin solution. c) Snapshots, corresponding fluorescence intensity profiles of chemotactic bacteria in gradient formed by using 1x MIC ampicillin solution, A and SC values are presented both for wild type chemotactic bacteria (WT, solid lines) and the mutant strain as well (MUT, dashed lines). d) Snapshots, corresponding fluorescence intensity profiles of chemotactic bacteria in gradient formed by using 1/6x MIC ampicillin solution, A and SC values are presented both for wild type chemotactic bacteria (WT, solid lines) and the mutant strain as well (MUT, dashed lines).*

We carried out the same set of experiments with ciprofloxacin. I show here a characteristic response of *E. coli* when using high concentration of ciprofloxacin solution in the left reservoir (Figure 4a.). We saw a similar response from the chemotactic bacteria as in case of ampicillin gradients, but it is less intense. Within an hour we can see bacteria accumulated at the edges of the observation channel, but later this distribution is suppressed by stronger accumulation of bacteria at the high antibiotic concentration side of the channel (*A* is increasing during the whole experiment, while *SC* remains stable at a low positive value). Controls with non-chemotactic mutants were carried out.

In case of gentamicin we didn't observe any response either from the chemotactic wild type strain, or the non-chemotactic mutant *E. coli* populations to the gradients (Figure 4b).

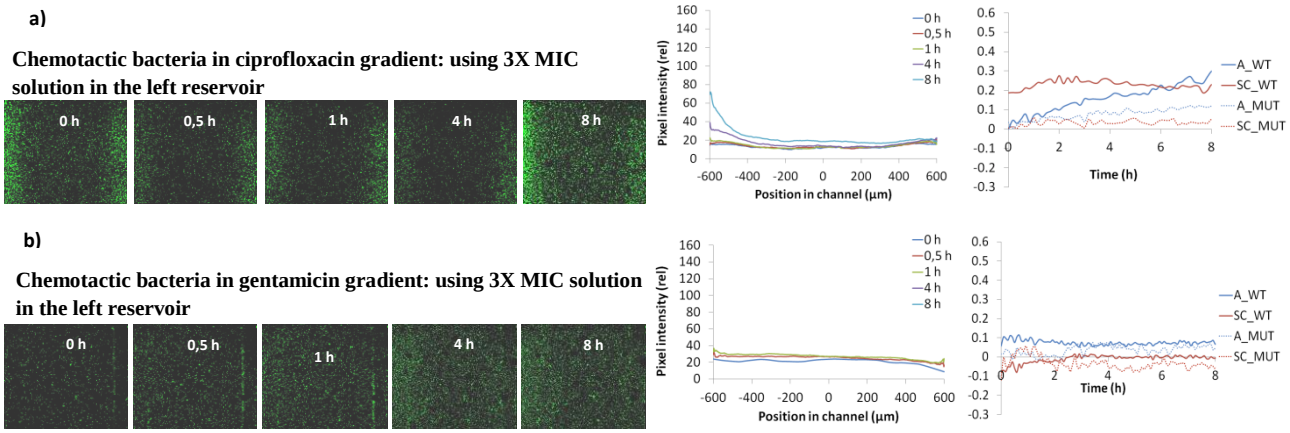


Figure 4. *Characteristic E. coli responses to ciprofloxacin and gentamicin gradients using 3x MIX solutions of the antibiotics in the left reservoir. a) Snapshots, corresponding fluorescence intensity profiles of chemotactic bacteria in gradient formed by using 3x MIC ciprofloxacin solution, A and SC values are presented both for wild type chemotactic bacteria (WT, solid lines) and the mutant strain as well (MUT, dashed lines). b) Snapshots, corresponding fluorescence intensity profiles of chemotactic bacteria in gradient formed by using 3x MIC gentamicin solution, A and SC values are presented both for wild type chemotactic bacteria (WT, solid lines) and the mutant strain as well (MUT, dashed lines).*

We have performed a large set of experiments using linear distribution of antibiotics with different slopes across the central channel of the microfluidic device together with tracking experiments. Based on our results we can conclude that certain antibiotics do have significant effect on the swimming behaviour of bacteria. In case of amipicillin and ciprofloxacin gradients, we detected a complex response, where we revealed the role of chemotaxis. However, we still need more experiments to understand the details about the observed phenomena. The accumulation of bacteria at the high antibiotic concentration side of the channel is still not explored from all aspects. Besides a chemotactic response (positive and negative), other mechanisms may contribute to this characteristic distribution, such as the concentration dependent decrease of swimming speed (as shown on Figure 1.) or surface adhesion of cells. We are conducting further experiments to explore the possible role of these processes. Furthermore, last year we acquired specific receptor mutant strains to

identify which chemotaxis receptor is responsible for the observed phenomena. However, yet, we didn't manage to modify them for GFP-expression (without obtaining antibiotic resistance as well). We are working on this problem, and we also started to further develop our device in order to be able to monitor bacteria without expressing fluorescent proteins (See Section 4.1). We plan to publish our results with the relevant receptors identified.

2.3. The effect of gradients on the evolution of antibiotic resistance

Since one expect antibiotics to be heterogeneously distributed in the human body after a treatment or in natural bacterial habitats, we were wondering if antibiotic gradients have any effect on the evolution of antibiotic resistance. Therefore, we collected bacteria from the microfluidic device after the experiments. We found no change in the measured MIC values upon the above presented antibiotic treatments. However, we noticed that changing the chemotaxis buffer to nutrient-reach media causes dramatic changes in the behaviour of the population. If bacteria were left in the gradient for 1-3 days a resistant sub-population emerged that quickly colonized the channel. The MIC of the strain increased, as well. We carried out experiments to define the specific circumstances that contribute to the observed response of the population and explore the biological mechanisms behind.

We performed the same set of experiments (using the same linear antibiotic distributions) as in Section 2.2., but in nutrient rich LB media. The first antibiotic we tested was ampicillin. In figure 5. I present the result of a typical experiment using 3x MIC ampicillin concentration in the left reservoir of the device. The emergence and extreme fast spread of resistant populations within the channel can be observed both on the microscope images and on the fluorescence intensity profiles.

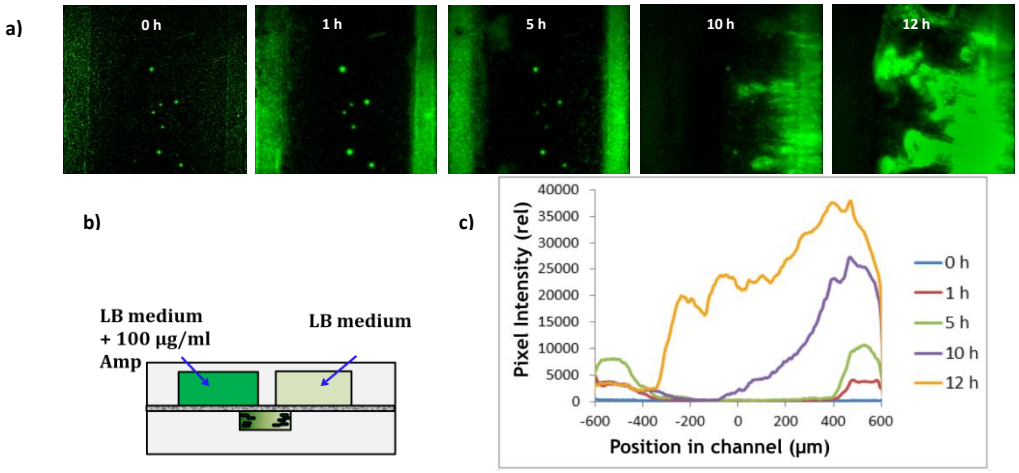


Figure 5. *Chemotactic wild type E. coli population in ampicillin (3x concentration at the left side) gradient in nutrient rich LB medium. a) microscope images taken at different time points during the experiment. b) Schematic cross sectional view of the experiment layout. c) Intensity profiles taken at different time points showing the extreme growth of the population.*

This behaviour of *E. coli* in ampicillin gradients seems to be quiet robust. We performed the same experiment but with less number of cells, where we could detect the exact locations from which bacteria spread all over the channel (Figure 6.).

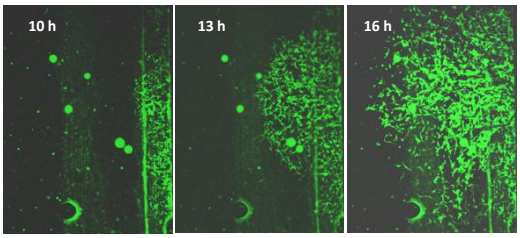


Figure 6. *Zooming in the microfluidic channel. Microscope images show the emergence and spread of resistant bacteria from the low-antibiotic concentration part of the channel.*

These resistant sub-populations almost always appear on the low antibiotic concentration side of the observation channel. To understand what exactly happens (e.g. what kind of morphological changes can be

identified, or detection of persistence cells) at these specific locations we would need high resolution microscopy, and the present form of our microfluidic device does not support the use of high magnification objectives. However, new hydrogel-PDMS hybrid device that we are developing will allow such experiments as well.

We tested whether the observed chemotactic response to ampicillin gradients (described in section 2.2) contribute to the appearance of resistant sub-populations in the microchannel. For this purpose we repeated the experiments with loading non-chemotactic mutant strains in ampicillin gradient. So far, we found that the observed spatial response is similar in case of non-chemotactic mutant cells. However, the propagating fronts seem to appear much later in time and spread slower (Figure 7.).

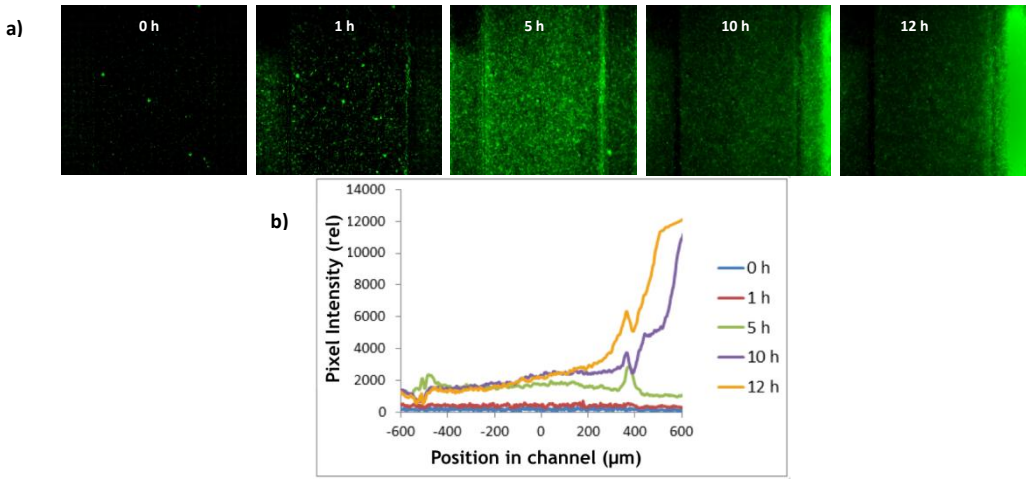


Figure 7. *Non-chemotactic mutant E. coli population in ampicillin (3x MIC concentration at the left side) gradient in nutrient rich LB medium. a) microscope images taken at different time points during the experiment. b) Corresponding intensity profiles across the channel.*

Similar responses of *E. coli* populations were found in case of ciprofloxacin gradients (Figure 8).

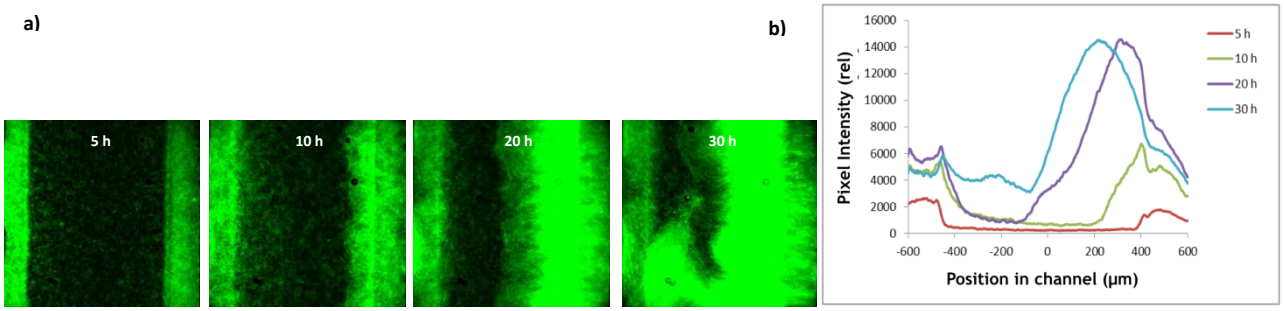


Figure 8. *Chemotactic wild type E. coli population in ciprofloxacin (3x concentration at the left side) gradient in nutrient rich LB medium. a) microscope images and b) corresponding fluorescence profiles taken at different time points during the experiment.*

We carried out experiments with different gradient steepness and different incubation times. We collected the cells from the microfluidic device after 1-2-3 days of exposure to the antibiotic gradients. Then, we measured the MIC changes upon the treatment. We found 4-100 fold increase in the MIC. We already performed numerous experiments, and are continuing to do so in order to collect statistically relevant datasets: the MIC changes seem to be quite diverse between the experiments. This is likely due to the stochastic nature of the mechanisms behind the increase of tolerance (e.g. appearance of mutations).

We can conclude that the timescale of the emergence of the resistant population is an order of magnitude faster compared to experiments carried out using traditional cell culturing methods using shaken tubes, flasks or agar plates. We found a proportional relation between the incubation time the population spent in the device and the measured increase in MIC. Currently we are preparing for the genome sequencing of the strains collected from the experiments, that is needed before publication.

There are still a lot of open questions about the observed phenomena, which we plan to explore. Since habitats of bacteria (e.g. the human body) are often compartmentalized and chemically heterogeneous, such

gradients of antimicrobials also form in natural conditions and can contribute to the evolution of antibiotic resistance. Because of its medical relevance, it is upmost important to better understand the underlying processes behind this complex response and fast evolution. I'm going to work on this project in the next years within the confines of another postdoctoral grant.

2.4. The effect and mechanism of antimicrobial peptides on bacteria

Antimicrobial peptides are promising candidates to use in the pharmaceutics as substitutes of antibiotics. Therefore, understanding their detailed mode of action is significant for further applications. We were planning to subject bacteria to gradients of antimicrobial peptides, as well. However, first we wanted to know more about their mechanisms. For this purpose, we performed atomic force microscopy studies and analyzed membrane potential changes upon treatments of small cationic antimicrobial peptides (NCR247 and NCR335). These studies were carried out in co-operation with the Laboratory of Plant Genomics, Biological Research Centre (Attila Kereszt and Éva Kondorosi). We identified morphological changes (increasing surface roughness) in the bacterial cell wall of *E. coli* and *Sinorhizobium meliloti*. We also showed that NCR peptides cause damages both in the outer and inner membranes but at different extent and it results in the loss of membrane potential that could be the primary reason of their antimicrobial activity. These results are published separately in the journals "*General Physiology and Biophysics*" [2] and "*Annals of Clinical Microbiology and Antimicrobials*" [3].

3. Dynamics of quorum sensing in *Pseudomonas aeruginosa*

Quorum sensing is a fundamental type of bacterial communication. We know a lot about the basic molecular mechanisms involved in this complex communication system (e.g. genes, receptors, hierarchy). However, we still lack information about its dynamics (both on population and single cell level) and its effect on the motile behaviour of bacteria. We studied these phenomena during the research project. *P. aeruginosa* has a hierarchic quorum sensing system, specialized for specific homoserine lactone molecules. Using mutants (that cannot produce these specific molecules), containing fluorescence reporter plasmid (pKRC12), we can follow how QS "turns on" at cellular level upon adding exogenous HSL. For this purpose we used a ΔlasI mutant strain (containing the reporter plasmid), that is able to sense, but is not able to produce 3-oxo-C12-HSL molecules.

3.1. The effect of QS signal molecules on the growth of bacteria

We tested if the presence of HSL molecules has an effect on the growth rate of *P. aeruginosa*. We measured the optical density of the growing population in plastic tubes (in shaker incubator, 30 °C, 200rpm) at 600 nm in nutrient rich LB media and M8 media. We found that in case of the presence of HSL the cells consume almost all of their energy to operate their QS processes. Fitting exponentials to the initial parts of the measured growth curves we got time constants 4 times higher in case the culture media contained 1 μM oxo-C12-HSL. We found a relation between the applied HSL concentration and the increase in the time constant: a higher amount of signal molecules (ranging from 0.01 μM to 1 μM) in the culture media caused proportionally slower growth rate in both LB and M8 media (data not shown).

3.2. Dynamics of the onset of QS state

We studied the effect of flow rate (of culture media), HSL concentration and nutrient supply on bacterial growth and the dynamics of the onset of quorum sensing. For this purpose, we have designed and created a microfluidic device, in which we can observe surface-adhered bacteria under controlled physical and chemical conditions (Figure 9a). The chip was made of PDMS that was bound to a microscope glass slide by oxygen plasma treatment. The device consisted of 6 parallel channels coated with poly-L-lysine (PLL) to promote the attachment of bacteria to the surface. We loaded bacteria in the microchannel at low concentration (below OD<0.1). Different flow rates of culture media were provided by syringe pumps. We performed time-lapse microscopy experiments in each channel to follow how QS "turns on" in time. We took phase contrast and fluorescence images in parallel (Figure 9b). With this setup the same population of bacteria, and even single cells can be studied for a long period of time. We measured the area occupied by cells, the total fluorescence intensity and the average fluorescence intensity per cell by using ImageJ [8].

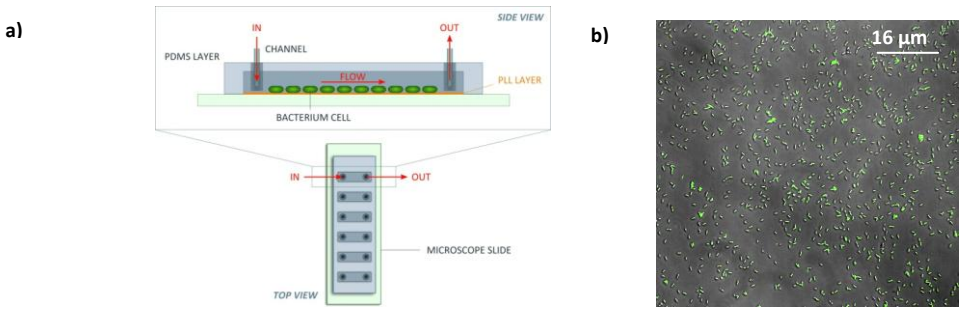


Figure 9. *a) Schematic drawing of microfluidic device with six parallel channels for studying QS dynamics of P. aeruginosa. b) Phase contrast and fluorescence image together of P. aeruginosa in QS active state upon exogenous HSL treatment.*

We tested whether flow rate (shear stress) has any effect on the growth of bacteria within our microfluidic setup. Therefore, we circulated culture media without signal molecules. We found that bacterial colonies grew somewhat slower in the microchannels than in plastic tubes, however considerable differences were detected only in case of high flow rates (>500 µl/h). In Figure 10. the behaviour of *P. aeruginosa* ΔlasI population in the microchannel is shown under different flow rates of M8 media.

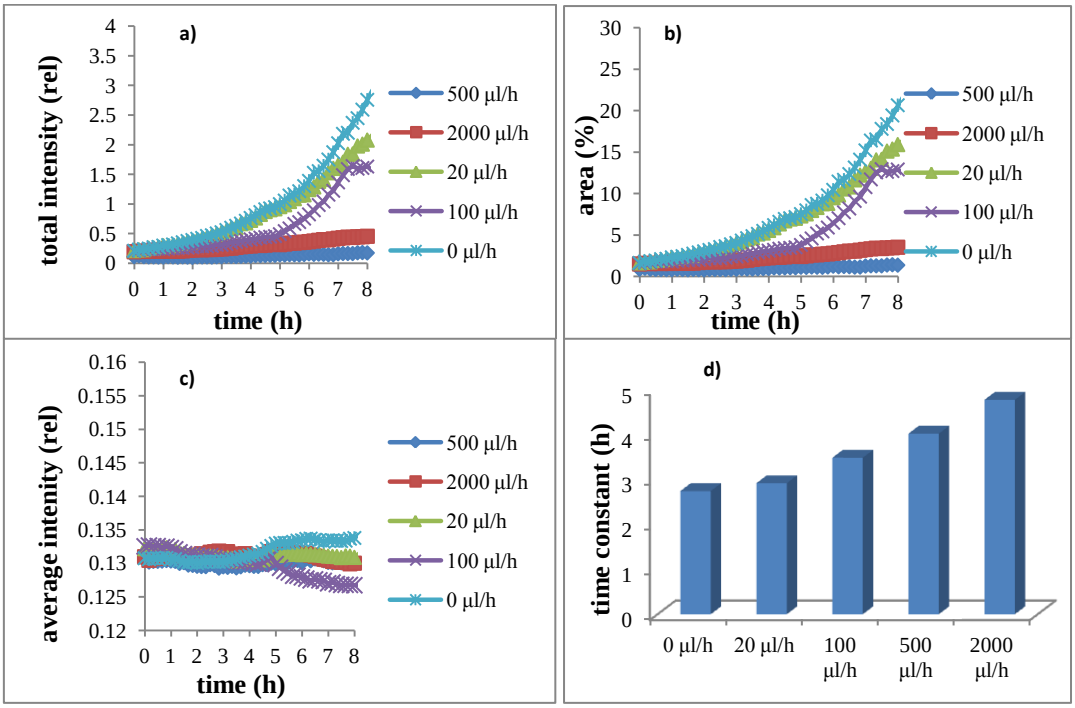


Figure 10. *The behaviour of P. aeruginosa ΔlasI in microchannels in M8 media. a) The measured fluorescence intensity in time. b) The changes of the area occupied by bacteria in time (growth curves). c) Average fluorescence intensity. d) Time constants calculated from fitting exponentials on the initial part of the growth curves.*

We studied the effect of signal molecules on the dynamics of the onset of QS state in our system by circulating M8 media supplemented with 3-oxo-C12-HSL molecules. We used two different concentrations of this molecule: 1 µM and 0.1 µM. We found similar responses in both cases: the fluorescence intensity of the cells reached a maximum value within few hours (figure 11a). The flow of the culture media resulted in slower response of the population that is shown in the measured average intensity values. Figure 11. shows a characteristic experiment when 1 µM HSL is added to the system. We can see that without flow cells turned on their QS state faster, and the average intensity reached higher values than in case of media flow. The slope values calculated from fitting the initial part of the intensity curves also confirms this. We haven't seen any relevant differences between the dynamics under various shear stresses (flow rates). Applying 0.1µM HSL induced responses similar to those in case of 1 µM HSL.

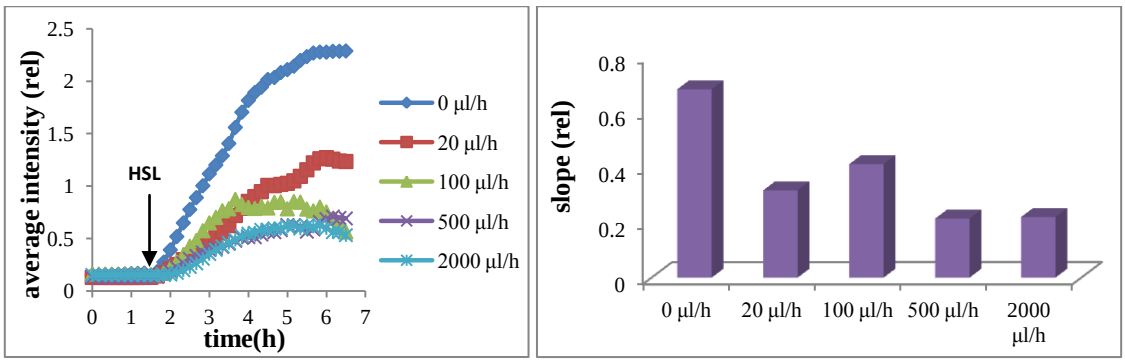


Figure 11. *The onset of quorum state upon the addition of 1 µM exogenous HSL. a) The changes of the average fluorescence intensities of cells in time in case of different flow rates of M8 media supplemented with HSL. b) Slopes obtained by linear fittings of the initial part of the curves.*

We are also working on evaluating our experiments and results not only on population level, but single cell level as well. We are continuously working on the development of our image analysis in order to get data of individual cells. A cell-to-cell variability in the fluorescence intensities (indicating the QS state) was detected (examples are presented in Figure 12.), but we need to work on the automation of our system to get statistically relevant results (hundreds of cells per experiment).

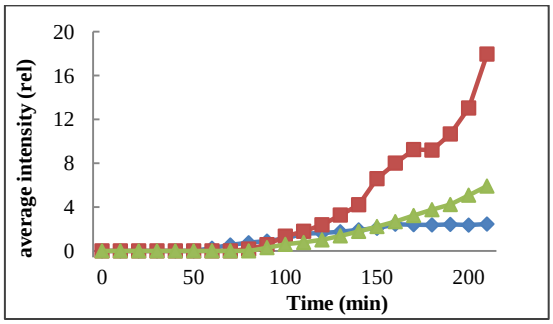


Figure 12. *a) The onset dynamics of three individual P. aeruginosa cells upon the addition of 1µM 3-oxo-C12-HSL.*

Besides studying the effect of flow rate on the dynamics of QS, we also carried out experiments where we removed the signal molecules from the system. We found that *P. aeruginosa* remained in an active QS state for many hours, in most cases even 12 hours of washing away the HSL from the system. It is interesting because maintaining an active QS state is resource consuming for the cells. We expected QS turning off faster (considering the short lifetime of the GSP used). However, this sustained QS state may increase the robustness of the system and make it stable against fluctuations of the QS signal.

These experiments mimic certain aspects of natural environments, such as liquid flow, that can be present in soil, the natural environments of *P. aeruginosa*. Our results suggest that flow rate (shear stress) may have significant effect on bacterial growth and the dynamics of the onset of QS state as well. We also revealed that behind the average population response there is a variety of responses from individual cells.

3.3. Exposing cells to gradients of signal molecules

In the original research plan we proposed to study the chemotactic response of *P. aeruginosa* to HSL gradients and other QS regulated secondary metabolites. Unfortunately these experiments proved to be difficult to perform due to the robust adherence of bacteria to different surfaces. We also had problems with the detection of bacteria within different devices. We acquired a signal negative strain that continuously expresses RFP for the purpose of these chemotaxis studies, but its fluorescence was too weak to detect with our current experimental system.

I have tried several microfluidic setups and surface modification methods to carry out chemotaxis experiments with *P. aeruginosa*. Three devices had been tested: a flow-based microfluidic gradient generator device (well-known in the literature), our flow-free setup in its present form, and another device that is commercially available from IBIDI. These devices all turned out to be insuitable for these studies.

- In the flow-based device we had difficulties when using optimal flow rate for gradient formation, bacteria still tended to attach to the surface of the chip and already in the loading plastic tube as well.

This method is also not as sensitive as using flow-free devices. The detection of bacteria without expressing fluorescence protein was also challenging.

- In our device we have difficulties with brightfield or phase contrast imaging of bacteria because of the aluminium-oxide membrane used.
- In the IBIDI system the chambers are not separated in a well defined way physically. This caused problems when detached cells colonize the reservoirs outside the observation area.

Due to the above difficulties we decided to develop a new microfluidic system that could be used for these experiments (section 4.1).

4. Microfluidic device development

4.1. PDMS-hydrogel hybrid gradient generator device to study bacteria without fluorescent protein expression

We started to work on a new hybrid gradient generator microfluidic device made of PDMS and incorporating hydrogel (PEGDA) walls to separate the reservoirs and the observation channel. With this setup we have all the capabilities of the previous chips for gradient generation along with extended microscopic observation possibilities. One of the main advantages of this type of device is the possibility to study bacteria without expressing fluorescent proteins, and we can even take higher magnification images looking through a thin glass slide (instead of the thick glass-PDSM layer of the previous sandwich-like device). The gradient is expected to be constant by continuous slow liquid flow through the reservoirs. By eliminating the need for an aluminium oxide membrane and using only PDMS, glass and hydrogel we expect the surface modifications to be more effective in preventing adhesion of cells to the device.

We have designed the device for this setup and performed computer simulations to study the gradient formation (Figure 13). We also prepared photomasks for the photolithographic fabrication. Fabricating the master mold for casting the PDMS for the microfluidic channels and reservoirs was successful, however we experienced that the PEGDA hydrogel walls were not stable when fabricated by standard photolithography. Therefore we worked out a method to expose the PEGDA hydrogel in a microscope using a 40x objective and a DAPI filter set and fabricate the wall structures by moving the sample in the microscope. Creating a good contact and seal between the PDMS and the hydrogel structures was not trivial, and required a careful tuning of the fabrication parameters and prior surface treatments.

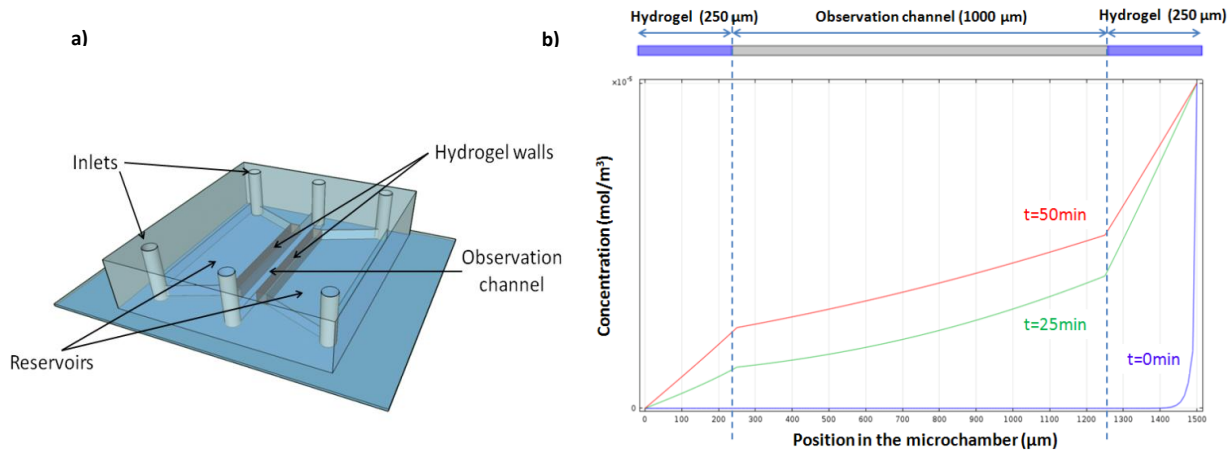


***Figure 13**. a) Schematic drawing of the hydrogel-PDMS hybrid device. b) Computer simulation of gradient formation in the device.*

A gradient formed within the device by using fluorescent rhodamine 6G dye is shown in Figure14. The amount of pohotoiniciator and the exposure time influences the physical properties of the hydrogel structure and therefore, the gradient formation. We are currently performing a detailed characterization of the device in terms of gradient formation and stability before publication and routine use.

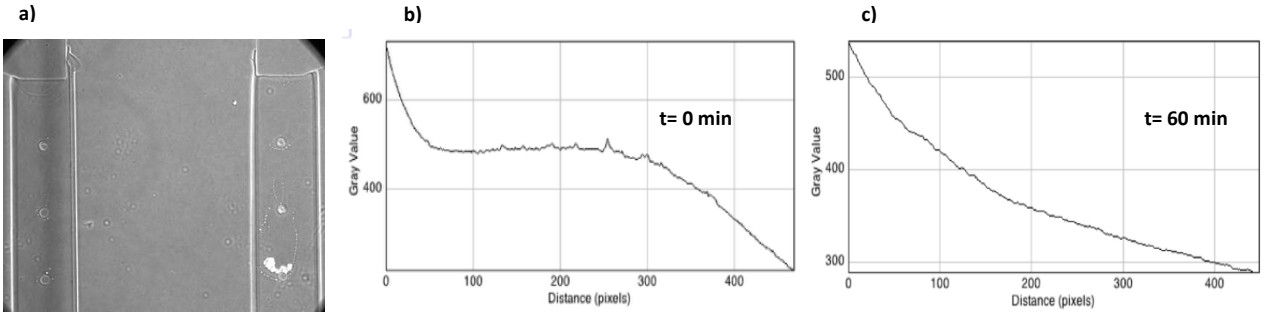


Figure 14*. a) Phase-contrast image of the fabricated hydrogel walls within the device, b) Fluorescence intensity profile just after filling the left side with rhodamine 6G solution, c) gradient formed of rhodamine 6G across the channel after an hour.*

4.2. Electrochemical flow-cell to monitor redox processes

During the second year of this grant, I was involved in a project (in cooperation with the Department of Medical Physics and Biophysics, University of Szeged (László Nagy), where we designed a new arrangement for sensing herbicides by specific reactions with bacterial photosynthetic reaction centers bound to working electrode of electrochemical cells. In our lab, we created a microfluidic electrochemical flow-cell with classical electrochemical arrangement. This microfluidic device enables us to reduce the dimensions of the sensor and also the sample quantity and to simplify the electrode arrangement. By adjusting the flow rate, we could follow in real time the degradation and recovery of the photochemical activity of the protein after successive intermittent inhibitor treatments in real time. These results are published in the journal of "*Photosynthesis Research*" [4]. We are planning to further develop this sensor, and use it for example to sense bacterial secondary metabolites under quorum sensing control (e.g. pyocyanin) based on their redox activity.

5. Interaction of bacteria and cancer cells with brain perycites and endothelial cells

We extended our research, and started a co-operation with the "*Physiology and Pathology of the Blood-Brain-Barrier Research Group*" (led by István Krizbai) of the Institute of Biophysics, Biological Research Centre. The technology we use for creating specific microenvironments can be modified to culture mammalian cells, as well. Therefore, it provides a unique possibility to generate devices in which we mimic certain characteristics of the human body, such as blood-stream.

In the focus of our interest was to study how bacteria and cancer cells interact with and disrupt the blood-brain-barrier (BBB) layer of cerebral endothelial cells. Understanding these cell-cell interactions and the adhesion and migration of cancer cells and bacteria under dynamic conditions have significant medical relevance: 1) brain metastases are common and devastating complications of breast cancer and melanoma 2) bacterial meningitis infection is caused by circulating bacteria that cross the BBB. In both cases we lack a detailed understanding of the pathogenesis of the diseases.

5.1. Culturing endothelial cells in microfluidic devices

We created biocompatible PDMS-based microchips as the model of the brain capillary network, in which the adhesion, growth and migration of D3 human microvascular cerebral endothelial cells can be followed under flow conditions. The formation of endothelial monolayer and the behaviour of melanoma and breast cancer cells have been studied in this device and results were compared to those coming from experiments carried out under static conditions. Our first results are summarized in a publication appeared in the journal "*Cell Adhesion & Migration*" [5].

5.2. Interaction of *E.coli* with brain pericytes and endothelial cells

Before applying microfluidics, we started to study bacteria-BBB interactions under static conditions in petri dishes and well plates. We co-cultured *E. coli* and perycites/endothel cells and analyzed what kind of mechanisms had been activated in the mammalian cells upon bacteria treatment. We found bacteria-induced inflammasome activation in human brain vascular pericytes. Confocal microscopy identified bacteria phagocyted by perycites. We also isolated outer membrane vesicles produced by *E. coli*, and found similar

responses, probably caused by the lipopolysaccharide content of vesicles. Our results have been published this year in "*Brain Behaviour and Immunity*" [6]. The detailed characterization of *E. coli* –endothelial cell interaction in well plates is still under examination. Then, we are planning to use a microfluidic model to study these interactions in dynamic environments as well.

SUMMARISING THE MAIN CHALLANGES OF THE RESEARCH PLAN AND THE SUBSEQUENT DIFFERENCES IN THE REALIZED WORK PLAN

During the last three years we focused our interest on the main questions described in the research plan, however due to some unexpected factors there are some slight differences in a few points compared to the original plan.

- We planned to analyze the results of antibiotic chemotaxis experiments carried out on *E. coli* from the evolution of antibiotic resistance point of view. However, we found that the experiments carried out in chemotaxis buffer had proved to be unsuitable for evolution experiments. Instead, changing to nutrient rich media, we observed interesting phenomena and new unexpected questions arose. We started to explore the conditions required for the fast evolution of resistance, but new set of experiments were needed and these evolution experiments run on longer timescale as well. Therefore, these experiments took considerable more time and effort than planned before. And to analyze our results we also need genome sequencing of the collected strains.
- The chemotaxis experiments with the quorum sensing signal molecules on *P. aeruginosa* and *E. coli* were carried out only on *E. coli*, because of the experimental difficulties when using *P. aerugonosa* cells. In order to solve these problems we are developing a new microfluidic platform.
- We had challenges with the detection of bacteria without fluorescence protein expression. The new microfluidic gradient generator device in development is suitable for non-fluorescent cells too, and can be used with the chemorecteptor mutants.

IMPACT AND RELEVANCE OF OUR RESULTS

Heterogeneity is a typical characteristic of natural habitats where microbes live. Microbial communities continuously adapt to survive and thrive in changing environmental conditions that is often manifested in the dynamic nature of populations. Different organisms live together and often compete for nutrients and space in a dynamic, heterogeneous environment, where the distribution of various compounds (e.g. nutrients, signal molecules, toxins) in space (and time) is uneven. The dynamic behaviour of populations is often led by the inhomogeneous distribution of chemicals. Our results on bacterial orientation, chemotaxis, communication and evolution may help in the better understanding of basic interactions of microbial communities in spatially heterogeneous environments. We have submitted a minireview in "*Frontiers in Microbiology*" including some of our results in this topic. [7].

Talks and conferences:

I participated and presented my results at several conferences. I had an invited talk at the Humboldt-University of Berlin (Department of Chemistry, group of Prof. Dr. Janina Kneipp). I gave 2 talks at international conferences, and other 3 at national conferences. I had more than 10 poster presentations.

Involvement of undergraduate and graduate students

During the first year of the project a PhD student was involved in the bacterial chemotaxis experiments, and those results also became part of her doctoral thesis:

- **Orsolya Sipos** (Dec. 2015, supervisor: Péter Galajda): Studying physical and biochemical interactions in bacterial communities using microfabricated devices

Two other PhD. students were also involved in this research. Orsolya Hodula started to work under my guidance on the antibiotic resistance experiments, but she left the PhD program of the University of Szeged, after the second year, and went on a maternal leave However, Ágnes Ábrahám joined our lab in 2016, and she is also involved in this research.

Four bachelor students were working in the lab and wrote their diploma thesis under my joint supervision:

- **József Balog** (Dec. 2014): The effect of antibiotics on the swimming motility of bacteria, University of Szeged, Molecular Bionic BSc.
- **Renáta Elek** (Dec. 2014): Designing hydrogel-based microchip for chemical gradient generation, University of Szeged, Molecular Bionic BSc.
- **Barbara Dukic** (Dec. 2015): The effect of antibiotic concentration gradients on *Escherichia coli* bacteria, University of Szeged, Molecular Bionics BSc.
- **Vanda Zsiros** (Dec. 2015): Studying the dynamics of quorum sensing in *Pseudomonas aeruginosa* bacteria, University of Szeged, Molecular Bionics BSc.

A student from Szent István University, Budapest spent his summer practice in our lab working on finding the optimal conditions to generate hydrogel structures in PDMS devices.

- **Balázs Kis** (2016): Developing hybrid microfluidic devices, Szent István University, Bioengineer BSc.

Honours and awards related to the project:

- Our students participated in 2017 at the *National Scientific Students' Associations Conference* (in the section of Biophysics), where *Vanda Zsiros won second prize*, while *Barbara Dukic got special prize* for their essays and presentations related to the above topics.
- In 2016 I have been awarded the *Kovács Tibor Prize* from the *Romhányi György Foundation* and gave a talk at the annual Membrane Transport Conference (Sümeg, Hungary) about my results on the effect of heterogeneous antibiotic distribution on bacteria.
- In 2017 I have been awarded the *Ernst Jenő Prize* from the *Hungarian Biophysical Society* for my work on bacterial chemotaxis, and I gave a talk at the XXVI. congress of the society.
- Based on my results on the fast evolution of antibiotic resistance in chemical concentration gradients, I got a new postdoctoral grant from the National Research, Development and Innovation Office starting from Oct. 2017. to explore in details the phenomena.

RELATED PUBLICATIONS

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

2. K. Nagy, K. R. Mikuláss, A. G. Végh, A. Kereszt, É. Kondorosi, G. Váró, Z. Szegletes (2015): Interaction of cysteine-rich cationic antmicrobial peptides with intact bacteria and model membranes, *General Physiology and Biophysics*, **34**. 135-144.

3. K. R. Mikuláss, K. Nagy, B. Bogos, Z. Szegletes, E. Kovács, A. Farkas, G. Váró, É. Kondorosi, A. Kereszt (2016): Antimicrobial nodule-specific cysteine-rich peptides disturb the integrity of bacterial outer and inner membranes and cause loss of membrane potential, *Annals of Clinical Microbiology and Antimicrobials*, **15**. p.43.

4. T. Szabó, R. Csekő, K. Hajdú, K. Nagy, O. Sipos, P. Galajda, G. Garab, L. Nagy (2017): Sensing photosynthetic herbicides in electrochemical flow cell, *Photosynthesys Research*, **132**. 127-134.

5. J. Molnár, C. Fazakas, J. Haskó, O. Sipos, K. Nagy, Á. Nyúl-Tóth, A. E. Farkas, A. G. Végh, G. Váró, P. Galajda, I. A. Krizbai, I. Wilhelm (2016): Inhibition of Rac or PI3K decreases the transmigration rate of melanoma and breast cancer cells through the brain endothelium, *Cell Adhesion & Migration*, **10**. 269-281.

6. Á. Nyúl-Tóth, M.Kozma, P. Nagyőszi, K. Nagy, C. Fazakas, J. Haskó, K. Molnár, A. E. Farkas, A. G. Végh, G. Váró, P. Galajda, I. Wilhelm, I. A. Krizbai (2017): Expression of pattern recognition receptors and activation of the non-canonical inflammasome pathway in brain pericytes, *Brain Behaviour and Immunity*, **64**. 220-231.

7. K. Nagy, Á. Ábrahám, P. Galajda (2017): Application of microfluidics to microbial ecology: the importance of being spatial, *Frontier in Microbiology*, submitted

Conference abstract in referred journal:

8. K. Nagy, V. Zsiros, E. Csákvári, O. Hodula, P. Galajda (2017): The dynamics of onset of AHL-mediated quorum sensing in *Pseudomonas aeruginosa*, *European Biophysical Journal*, **46**. Suppl. 1. S378, P-1001.