Spatial distribution of cells over the process of biofilm formation in a *Methylophilus* strain

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Keywords*:* cell organization, biofilm development, phase-contrast microscopy, pellicle biofilms

Abstract

Understanding spatial organization of individual cells in biofilms is challenging and only recent studies started to clarify this aspect for only a few model organisms. Therefore, when considering the wide diversity of biofilm-forming species in the environment, this information is even less understood. Here, we characterize the process of biofilm formation for a methanol-oxidizing bacterium isolated from Trunk River. Sequencing of the 16S rDNA and phylogenetic analyses identified the bacterium as *Methylophilus* sp strain LM11. Individual cells in mature biofilms are usually evenly spaced 1.5-3.5 μm from each other and seem to be mainly vertically oriented in the biofilm matrix. This pattern of organization is consistent in biofilms formed in glass-chambers slides and non-shaking tubes, indicating reproducibility under different methods. Future analysis of cells spatial organization for other species should reveal if this is a phenomenon unique to *Methylophilus* sp. or if this is a general pattern that can be found in other biofilm-forming species.

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Introduction

Biofilms are aggregates of cells embedded in a matrix of extracellular polymeric substances (EPS). This form of life is spread in nature and is the way microorganisms usually colonize the environment, being determinant for several unique physiological characteristics (physiological aspects for cells within a biofilm varies significantly when compared to free-living stages). Furthermore, biofilms are important in a variety of human-related issues, including infections (resistance to antibiotics), water contaminations (biofilms are present on pipes, affecting water quality) and many others (Flemming et al. 2016). EPS matrix is self-produced by the species forming the biofilm and is composed mainly by polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender, 2010).

Despite advances on our understanding of chemical (i.e. matrix composition), physiological (i.e. expression levels of cells) and ecological (i.e. inter and intra species interactions) aspects of biofilms, clarifying how individual cells are organized within biofilms has been a technical challenge (Neu and Lawrence, 2015). Recently, it was shown stages of biofilm development in *Vibrio cholerae* considering 3D positions for individual cells (Drescher et al. 2016). To our knowledge, this was the first time a systematic characterization under individual cell level was performed. Besides identifying phases of biofilm development, the authors also provided information about cell shape and size over the development. Studying *E. coli* biofilms and electron microscopy, it was shown that cells can be spatially segregated and display distinctive morphology under complex EPS structures (Hung et al. 2013).

As mentioned, our understanding of cell spatial organization in biofilm is limited and only a few model systems have been studied so far, with limited information available for other species present in nature. During the first three weeks of the Microbial Diversity course 2016, a biofilm-forming bacterial species was isolated and initial microscopic analyses indicated an intriguing spatial organization of the cells. During this mini-project, we further characterized this organization as well as the process of biofilm formation for this strain isolated from Trunk River (Woods Hole – MA).

Material and methods

Enrichment, isolation and phylogenetic analysis

For enrichment of methanol oxidizers, we used the "*Hyphomicrobium* medium" prepared for the course: Fresh water base, 10 mM MOPS (pH 7.2), 10mM Na_2SO_4, 1mM KPhos (pH 7.2), KNO_3 (5g/L), Trace elements solution and methanol [2.5mL/L] as carbon source. Sample used for enrichment came from the "sediment under the lemonade", from Trunk River. One gram of sediment material was added to a flask containing 50mL of medium and incubated at 30 ºC (not shaking). After a 4 days, a transfer was made for a new flask and the process was repeated three times. After three transfers, 10 µL of the enrichment was used for a streak in agar plates (same medium) and isolated colonies were picked. Three new streaks from these isolated colonies were prepared for obtaining pure cultures.

After isolation of a pure culture, 16S rDNA was amplified by single-colony PCRs and sequenced using generic bacterial primers. Nucleotide-Blast searches indicated the strain was a member of the genus *Methylophilus* (top hit: *Methylophilus methylotrophus* NR_041257, 99% when searching for only type material). For confirmation, a phylogenetic analysis was performed. In summary, 16S sequences of top hits from the NCBI-GenBank searches were selected; sequences were aligned using MAFFT v.7 (Katoh and Standley, 2013) and a phylogenetic analysis was performed using Bayesian inference (software MrBayes (Ronquist, 2013)). The nucleotide substitution model used was HKY+I+G; two independent runs were performed with standard conditions in the software (described in the software manual). Each run consisted of Markov Chain Monte Carlo (MCMC) sampling for 1 million generations. The tree was edited in FigTree v.1.4.0.

Biofilm development visualization

For visualization of biofilm development, we used 4-well chambers slides with cover slips on the bottom filled with liquid medium and an inoculum of 10 µL of an overnight culture grown in shaker and diluted to OD_{600} 0.1 before inoculation. To fill the chamber, we varied the amount of liquid from 200-400 µL in different replications. The chambers were incubated at room temperature and at 30 ºC, with the second resulting in faster biofilm growth. Every day, samples were visualized on an inverted microscope to check the biofilm development. We also performed time-lapse experiments, where a new z-stack (ranging from the bottom of the chamber until the

top of liquid medium-air interface) was taken every 20 min, and it was possible to observe the development of biofilms both in the bottom and top (pellicle).

Finally, we also imaged pellicles developed in non-shaking tubes. After three days of growth and visible pellicle development, tubes were moved to 55 ºC incubator for 1h for fixation. This process made the pellicle more rigid and enabled its manipulation (i.e. to cut small pieces and use in slides preparation). Slides were then analyzed using phase-contrast microscopy and z-stacks were obtained for 3D reconstructions and image analysis.

Quantification of cells spatial positions

We observed that cells seemed to be regularly spaced in mature biofilms formed by *Methylophilus* sp. LM11 and we decided to apply a quantification method to determine how far from each other cells were positioned in the biofilms. For this, a MATLAB script developed by Georgia Squyres (Harvard) was used. The software first takes all frames of a full z-stack and use a threshold tool for separation of cells from the background transforming the images in a binary (black and white) image. The threshold can be manually selected for better results. After that, with cells particles already separated for each frame of the z-stack, the position in the center of each cell (centroid) was determined and used for plotting of 2D and 3D (in these plots it is possible to visualize cell density and positions). Also, for each frame it was calculated the distance to the nearest-neighbor cell and the distribution of the values was plotted in histograms. The software counts the number of cells present in each frame and simulates a random arrangement for the same number of cells in a simulation analysis, then calculating the nearest-neighbor distances for each of these randomly positioned cells and plotting them in a histogram for comparison with the observed “real” data. This pipeline was used for analysis of biofilms formed both in chamber and tubes.

Scanning Electron Microscopy (SEM)

Aiming to visualize the cells and biofilms in more detail, SEM analyses were performed. A three-day old biofilm formed in non-shaking tubes was disrupted in vortex and fixed with 4% formaldehyde for 2 hrs. After fixation, the sample was filtered in a 0.2 nm membrane, washed with PBS buffer and taken to the microscopy facility for SEM preparation. Samples were dehydrated sequentially through a series of ethanol concentrations (30, 50, 70, 90 and 100%, 15 min on each)

and dried until the critical point using liquid CO_2. After this step, stubs were mounted, sputtered with platinum and examined using Environmental scanning electron microscope available in the lab. A similar process was done for intact biofilm pellicles (not disrupted) for visualization of biofilm structure.

Results and discussion

Enrichment, isolation and identification of a methanol-oxidizing bacterium

During our enrichments for methanol-oxidizing bacteria performed in the first three weeks, different morphotypes appeared in the liquid cultures (Figs. 1A and B). Although all the main morphotypes showed were still present after three transfers to new flasks, the more abundant was certainly the bacterium forming a pellicle biofilm in the interface between the liquid and water (Figs. 1C and D). Due to characteristic of forming thick biofilm and the fact that it showed a fast growth rate (i.e. enrichment flasks became turbid overnight, even with the oligotrophic environment), we decided to focus on isolation of this bacterium. Thus, after streaking 10uL of the enrichment's liquid medium on plates containing the same medium with agar, colonies arose and were re-streaked for isolation of pure cultures. We sequenced the partial 16S rDNA and our strain grouped in the *Methylophilus* genus, close related to *Methylophilus methylotrophus*, *M. glucosoxydans*, *M. rhizosphaerae* and *M. quaylei* (Fig. 2). All these species together with other species in the genus *Methylotenera*, *Methylobacillus* and *Methylovorus* are methylotrophs, specialized in the use of methanol as carbon source (Jenkins et al. 1987; Doronina et al. 2005). We tried to grow the strain LM11 in LB agar, 5YE and STAT media and no growth was observed. Interestingly, *Methylophilus* was previously detected by sequencing in an enrichment in the course (Lee, 2006), but in that occasion a pure culture was not obtained.

The process of biofilm formation

As mentioned before, a particular and interesting characteristic of *Methylophilus* sp. LM11 is the quick formation of a thick pellicle biofilm. Initial observations under the microscope during the isolations showed that many times the cells in these pellicles did not seem to be in direct contact with each other. Because of this observation, we decided to investigate the process of biofilm formation in this strain and characterize the spatial distribution on the cells in intact biofilms. In

order to accomplish this aim, we used 4-well chamber slides with a cover slip in the bottom (https://www.thermofisher.com/order/catalog/product/155360) and visualized biofilm formation and growth (see material and methods for details). We observed biofilm formation both in the bottom of the chamber and in the top of the liquid medium (i.e. interface between medium and air, similar to the pellicle observed in tubes - Fig. 1D). Right after inoculation, the system is very dynamic, with the majority of the cells moving around the chamber. With time, cells start to attach to the surface in the bottom and cell division can be observed. Cells extend during division, sometimes releasing a new cell to the liquid medium, but in many cases it can be observed an extension of cells (filaments) that starts to divide and form agglomerates, originating micro-colonies in the bottom of glass (Fig. 3A). By performing time-lapse experiments, we could visualize the growth of these micro-colonies until different colonies merged into a thick biofilm layer containing cells and EPS (Fig. 3B). Interesting, the pellicle formation in the chambers also occurs quickly. Around nine hours after inoculation, it was already possible to see cells floating in the liquid-air interface and starting to form aggregates (Fig. 3C). With around 12 hours, there almost no space anymore and thin layer of cells is formed. Later, after around 24 hours, a thick layer of cells and EPS can be visualized, with no free space in the surface and this layer gets thicker with time (Fig. 3D).

We also wanted to visualize the structure of a "mature biofilm" in a chamber, specifically considering special organization of cells within the biofilm EPS matrix. As mentioned before, initial microscopy during enrichments indicated that cells could be spaced and relatively separated from each other. Surprisingly, when analyzing a mature biofilm (i.e. around 4-6 days), an interesting spatial organization was detected. Both in the bottom and in the top biofilms, cells were not only separated from each other in the matrix, but also most of them displayed a vertical orientation (although there were still cells horizontally oriented in the biofilms). This organization was reproducible in at least two other trials, but time for formation varies with incubation conditions: ideal temperature for growth and biofilm formation seems to be ~ 30 ºC. There is also a liquid portion between the top and bottom biofilms, when cells at this stage are floating and present lesser density and higher space between each other. Figure 4A-C shows frames from a z-stack from the top and bottom biofilms, as well as the liquid portion between both of them.

Quantification of cell spatial distribution in chambers

Interested in quantifying cell organization of this intriguing biofilm, we applied a method for measuring distances between cells. A script in MATLAB was written by Georgia Squyres and applied here (for details in the script pipeline, see methods). In summary, we identified the center of all cells (centroids) present in each of the frames in a z-stack of the entire biofilm and plotted them in 2D and 3D dimensions to visualize cells distributions. This is a clear way to visualize how cells are concentrated in the bottom and top of the chamber, when compared to the middle (where cells are spaced and floating in liquid and not immersed in a biofilm matrix, Fig. 5A-B). We also calculated the distance of each centroid to the centroid of the closest neighbor cell in the same frame. As expected, cells from the bottom and top biofilms were more close to each other than cells floating the liquid in the middle (Fig. 4). Distances were around 1.5-3.5 μm between cells in both the bottom and top biofilms, and 3-8 μm for the middle of the chamber. We also simulated a random organization for the same number of cells detected in each frame and plotted the distribution of the closest neighbor for these randomly positioned cells. As displayed in Fig. 4, we could detect a slight shift to the right (i.e. more distant) in the distribution of the observed data compared to the distribution of simulated positioning for bottom and top biofilms, but not for the middle. This could indicate that in biofilms cells are more distant from each other than they would be in a biofilm with random organization of cells, indicating an "organized" pattern in the biofilm. However, this still need to be statistically tested in future analysis to support such differences in the distributions. In future analyses we intend to combine all frames in each of the three sections of the biofilm (bottom, middle, top) and visualize their distribution of neighbor distances.

Spatial distribution of cells in pellicles formed in tubes

Despite being able to quantify cells distributions in biofilms formed in glass chambers, we also wanted to analyze cells positions in the thick pellicle formed in non-shaking tubes (Fig. 1D), which was the first feature that called our attention when isolating this bacterium (note: in tubes, there is no biofilm in the bottom, probably because *Methylophilus* is a strict aerobe and O_2 concentration in the bottom of non-shaking tubes is too low). However, despite thick, the pellicles are usually fragile and it is easy to disrupt their structure when trying to remove them for the tubes. During the course, the temperature of the incubator was accidently changed from 30 ºC to ~ 55 ºC. This happened for approximately 2h and it despite apparently killing most of the cells, it turned

out to be a great fixation method for pellicles. After incubation of previously formed pellicles at 55 ºC, the structure became much more rigid (jelly consistency) and it was relatively easy to remove it from the tubes, cut and analyze it under the microscope in the exact position that it was formed (Fig. 6A).

We performed phase-contrast imaging in pellicles and characterized cell spatial distribution as done by glass-chamber biofilms. The distances between cells were very close to what we observed for the bottom and top biofilms in the chambers (Fig. 6B), and the general structure was also very similar (i.e. cells mainly in vertical position, immersed in a matrix and regularly spaced from each other). The similarity of these results compared to what we found for glass-chamber biofilms suggests that heating at 55 ºC did not cause major modifications in cell organization and might be a good method for fixing these pellicles for microscopic observations.

Scanning electron microscopy (SEM) analysis

We performed SEM aiming to visualize pellicle biofilm structure and cell shapes in high resolution. It is very challenging to keep pellicle structure over the process of sample preparation for SEM, but fixation at 55 ºC worked relatively well. It is possible to see cells in the thick EPS matrix, and they do not seem to be at very high density (Fig. 7A-B). This is even more evident when analyzing images from biofilm stuck in 0.2 nm filters. For this sample, pellicle biofilm formed in tubes were disrupted by vortexing and subsequently filtered in 0.2 nm pore membranes. These membranes were prepared for SEM and the result can be seen in Fig. 7C-D. Interestingly, the SEM confirmed that a considerable amount of EPS matrix is present with low density of cells (Fig. 7C). This corroborates what we observed with phase-contrast microscopy for late stages biofilms, where cells are relatively distant from each other.

Conclusion

Methylophilus sp. LM11 forms an intriguing biofilm with cells regularly spaced and mainly oriented in vertical position. This was consistent in replicates, and happens both in the bottom and top of biofilms formed in chambers. A similar pattern could be observed in thick pellicles formed non-shaking tubes. Based on all the observations, we propose a simple model for biofilm

colonization (Fig. 8). The reason why cells are spaced this way remains unknown and should be investigated in future studies.

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Figures

Fig. 1. Diversity of morphotypes in methanol-oxidizers enrichment (**A**-**B**) and pellicle biofilm formation (**C**: enrichment; **D**: *Methylophilus* sp. LM11 pure culture).

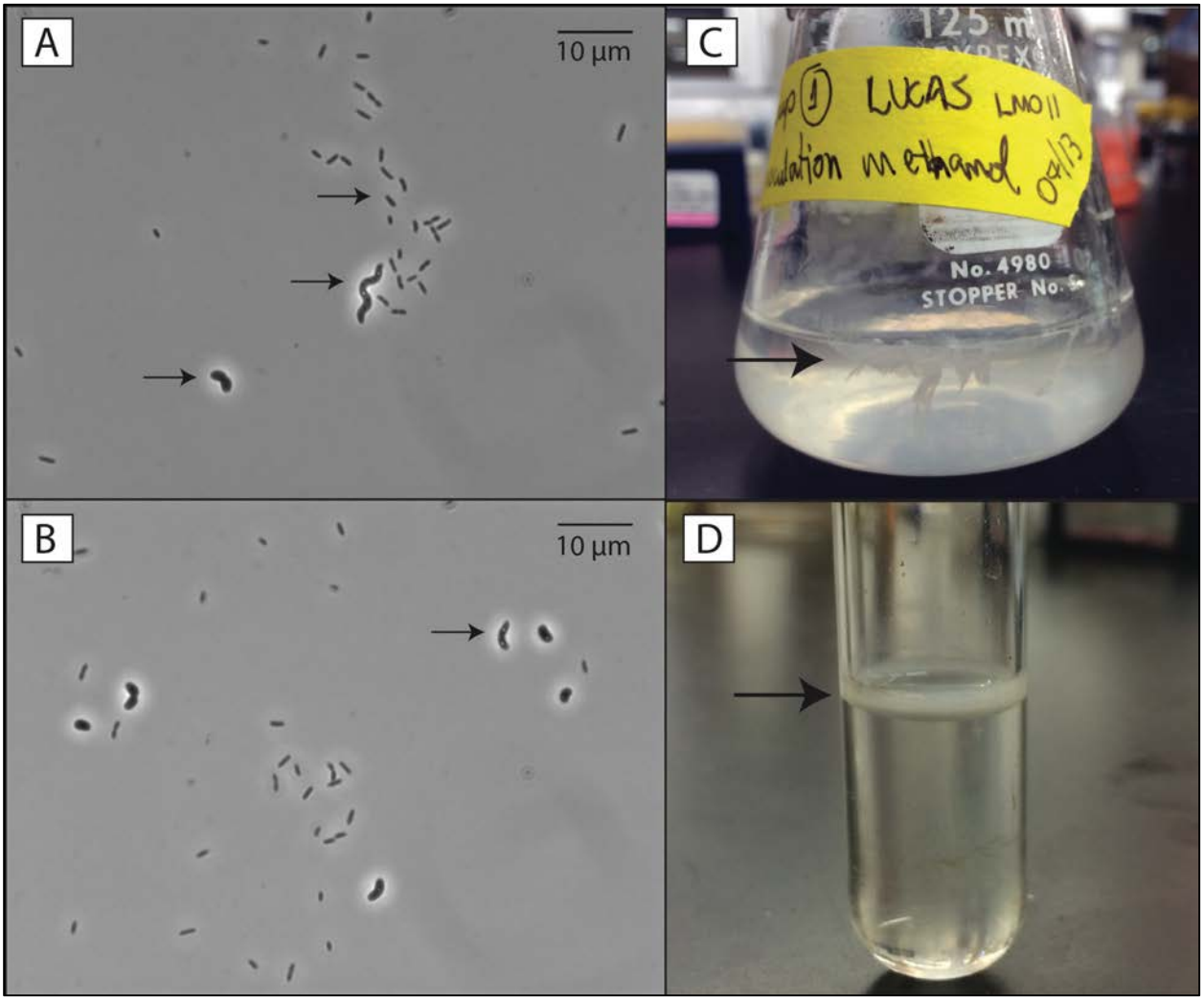

Fig. 2. Phylogenetic position of *Methylophilus* sp. LM11 within *Methylophilus* and other close-related genera. Tree was reconstructed using Bayesian inference and our strain is highlighted in bold-red.

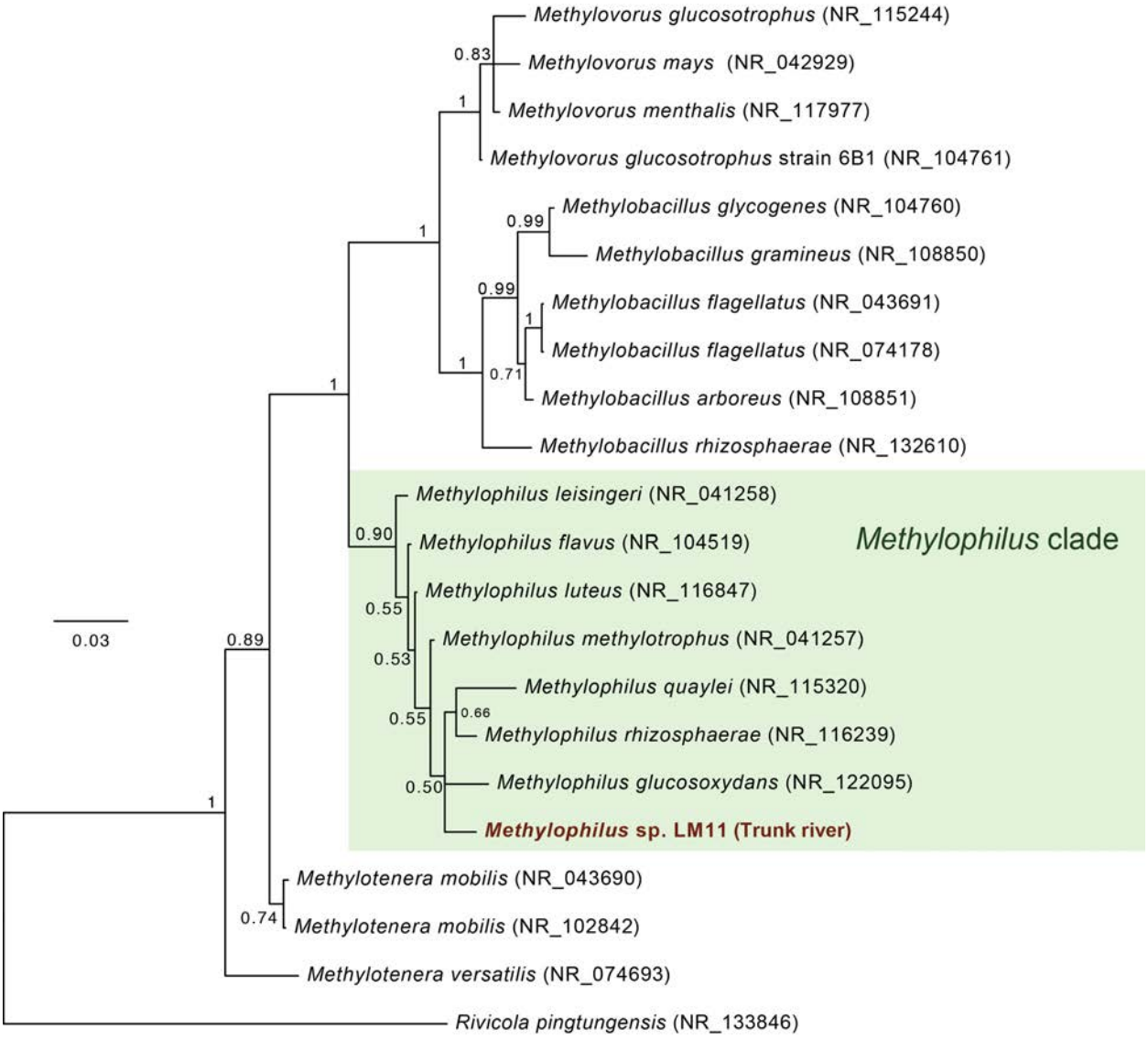

Fig. 3. Biofilm development on the bottom (A-B) and top (i.e. liquid-air interface, C-D) of chambers. Note micro-colonies merge after 30hrs on the bottom and formation of a pellicle biofilm on the top.

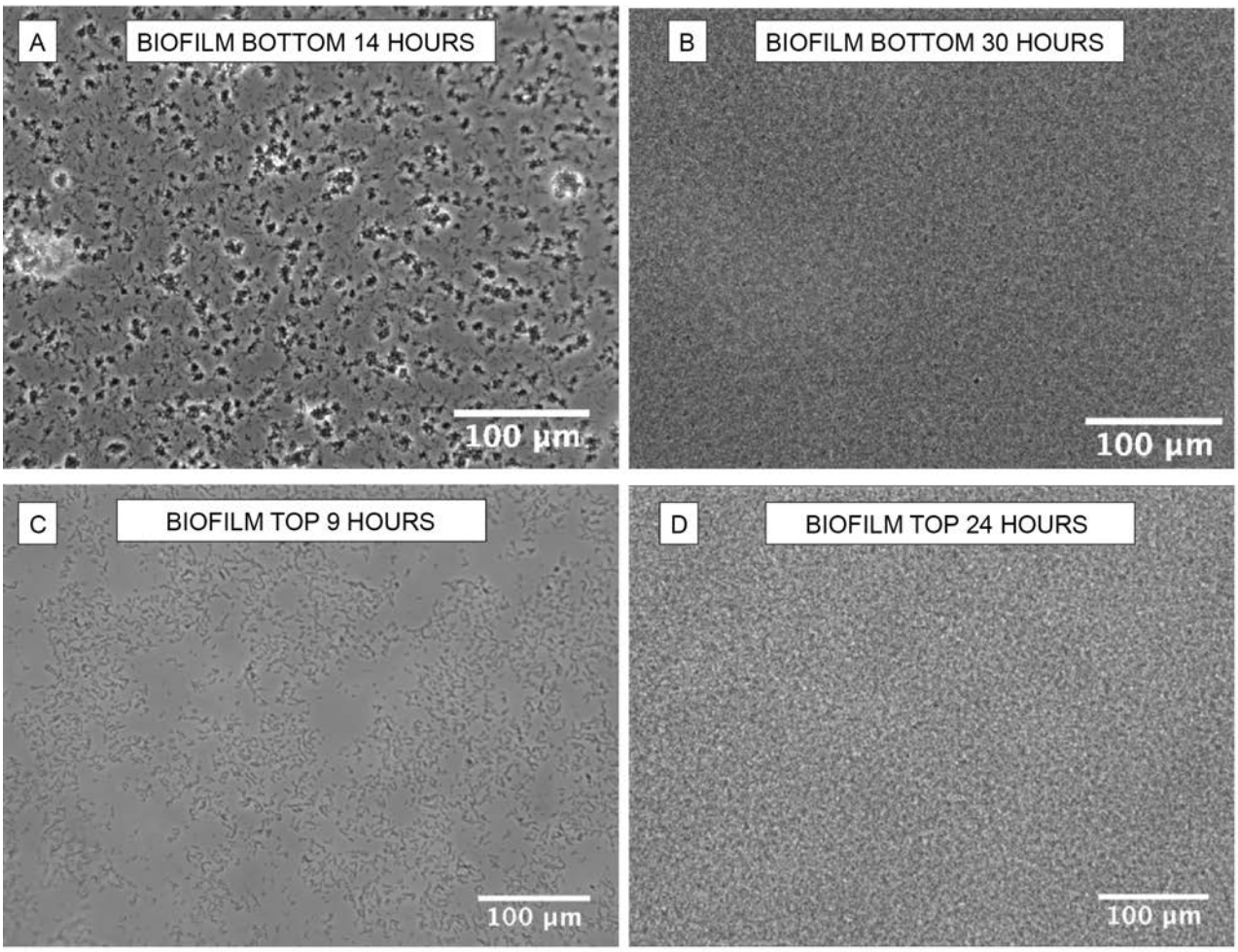

Fig. 4. Different frames of the z-stack of a mature biofilm formed in chambers. Each frame is a representative of the general structure found in each of these sections of the biofilm. On the left is shown a phase-contrast image and on the right its respective distribution of values for distances of nearest-neighbor cells. Frequency = number of occurrences for a determined distance.

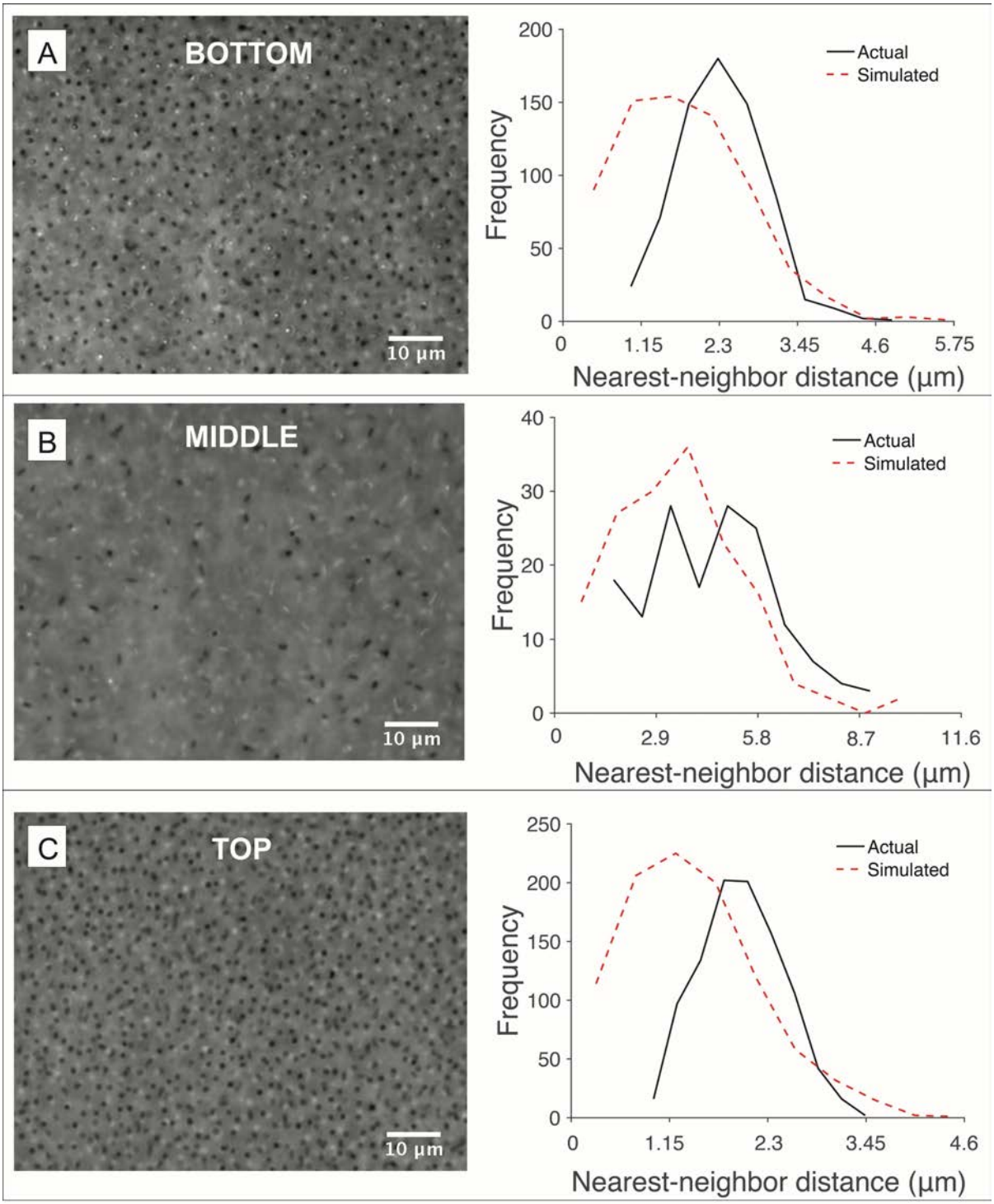

Fig. 5. 3D plotting of the centroids of cells forming chamber biofilms. Note the higher concentration on the bottom and top of the biofilm compared to the middle (area where cells were floating and not embedded in an EPS matrix).

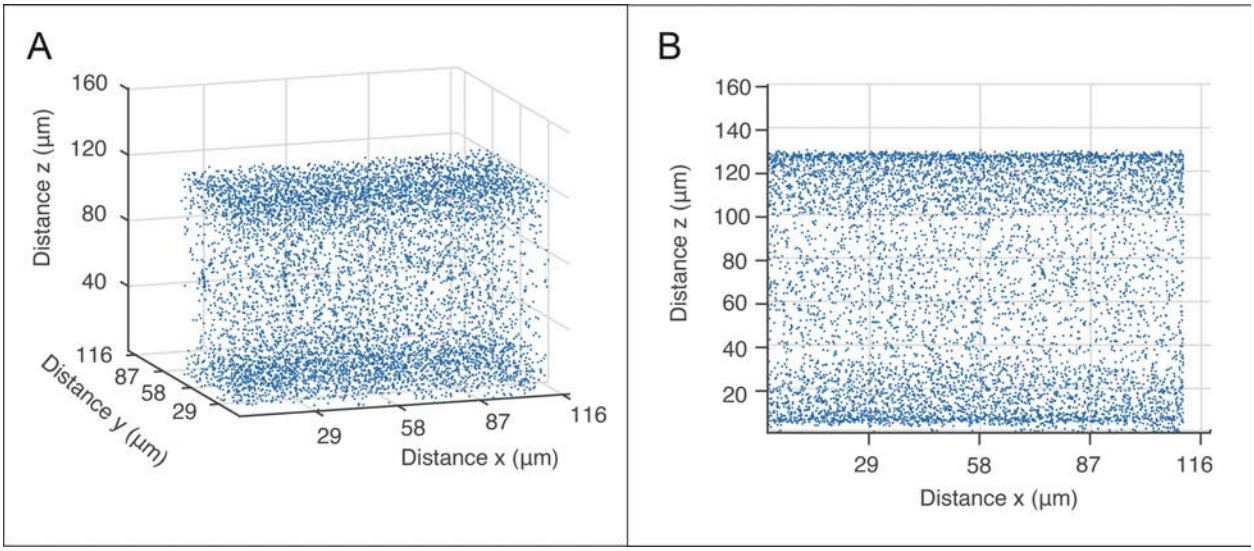

Fig. 6. Analysis on pellicle biofilms formed in non-shaking tubes. **A**: Jelly structured of pellicle after fixed in 55 ºC floating in PBS; a piece of the biofilm was cut in the center and analyzed in a microscope slide. **B**: Nearest-neighbor distances histogram as calculated for chambers biofilms in Fig. 4 (note that values are very similar to distances found for bottom and top biofilms in chambers). **C** and **D**: 3D positioning plots without and with zoom, respectively (note that concentration of cells in the tube pellicle is similar to the ones found on the top and bottom of chamber biofilms).

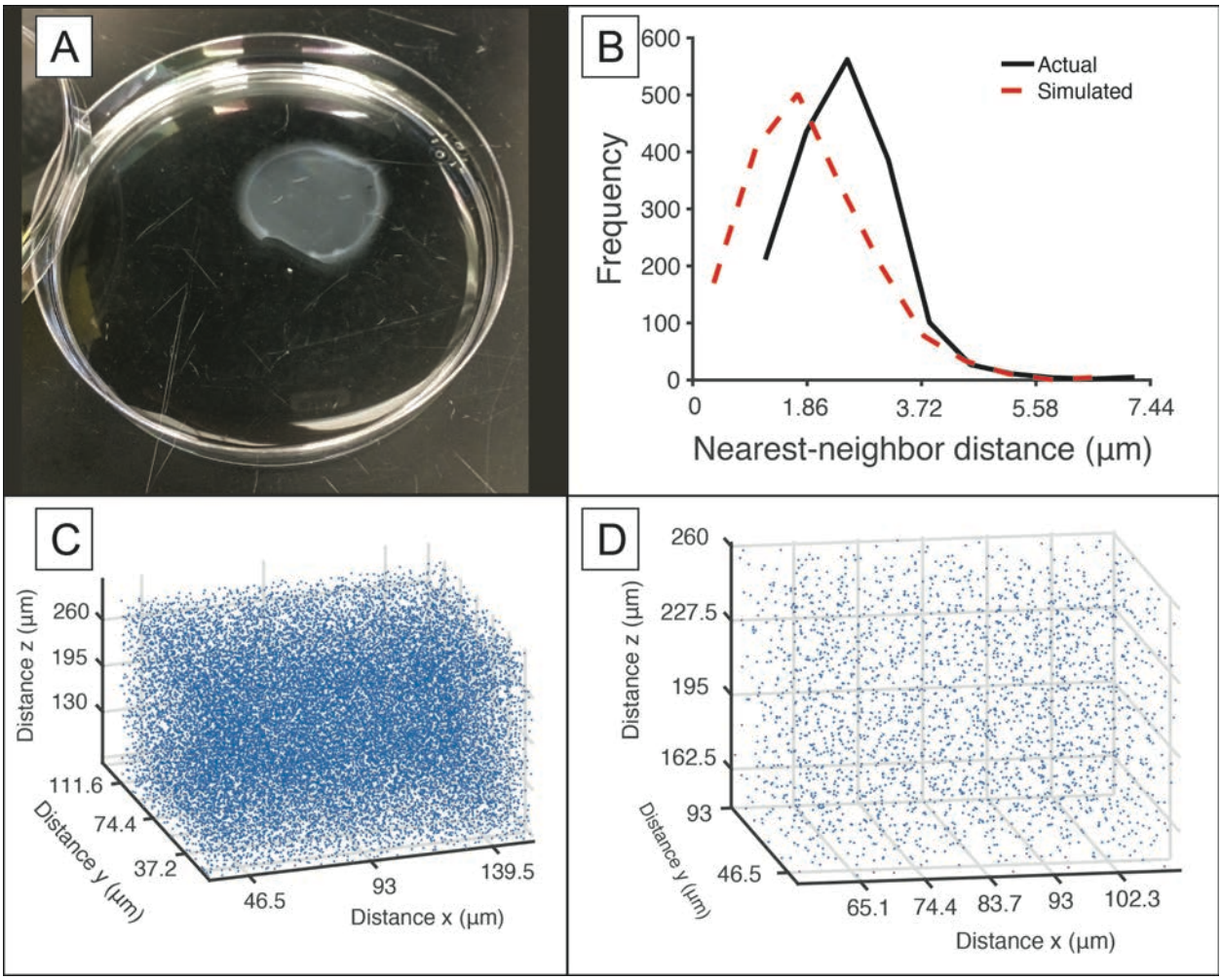

Fig. 7. SEM images for non-disrupted (**A-B**) and disrupted (**C-D**) biofilms. Red arrows in A point to some of the many cell-like structures found in biofilm layers. Note the high amount of EPS compared to cells in **C**, supporting our phase-microscopy images. **D** shows a rare region of the filter where cells in apparent free-living stages could be found.

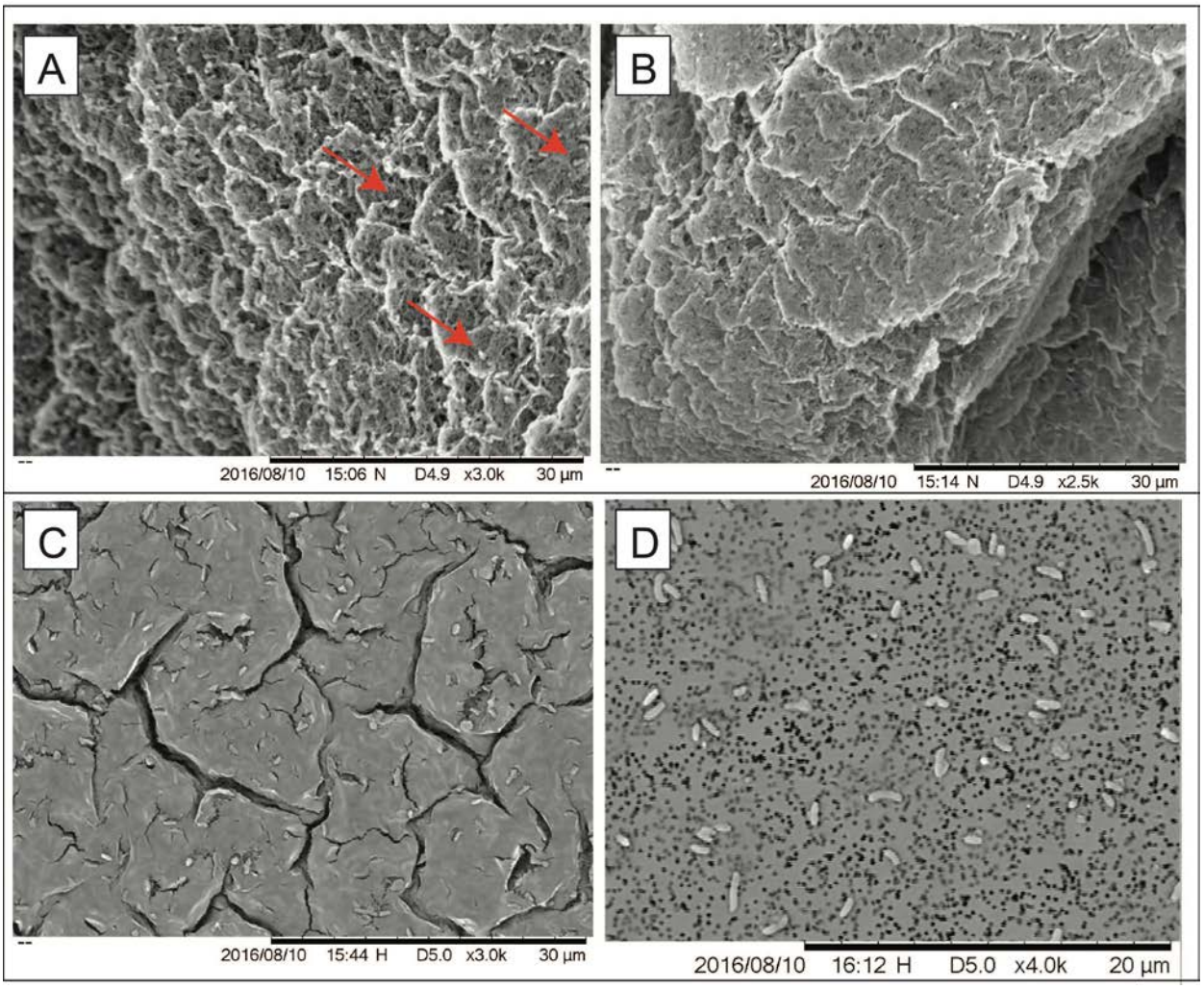

Fig. 8. General model for biofilm formation in chambers here proposed.

Phase 1: cells are dispersed and swimming (represented by the presence of a flagellum); few cells start to attach to the bottom and many cells try to stay on the liquid-air interface where oxygen is more abundant.
Phase 2: cells that were attached start cell division process originating filaments and cell aggregates; some of the new cells from the bottom can de-attach and assume swimming state; thin layer of cells starts to form on the liquid-air interface.
Phase 3: cell aggregates on the bottom form micro-colonies containing EPS (represented in yellow); layer of cells in the top covers completely the interface forming an initial pellicle; only a few swimmers are found on the middle section.
Phase 4: Micro-colonies merge on the bottom and form a thick biofilm, with many cells oriented in vertical position; only floating cells in the middle (no swimmers); pellicle gets thicker on the top and cells are also mainly vertically oriented.