Visualization of Cell Morphology and Cytoplasmic pH of *Escherichia coli* Biofilms Using Fluorescence Microscopy

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Abstract

The Gram-negative bacterium *Escherichia coli* inhabits the gastrointestinal tracts of many species, including humans, and plays an important role in the digestion of certain foods. *E. coli* have been found to exist in nature as both free-floating, planktonic cells as well as biofilms. A biofilm is a collection of individual bacterial cells that adhere to a surface and produce an extracellular matrix. In this study, the pH survival range of *E. coli* biofilms was tested through observation of cytoplasmic pH using fluorescence microscopy involving a ratiometric green fluorescent protein (GFP) derivative. Because *E. coli* is a weak acidophile, it was expected that *E. coli* biofilms would have the most growth around a neutral pH (pH 7). Biofilms grown at pH 4.5 produced long, filamented cells with distinct compartments of cytoplasm, whereas biofilms grown at higher pHs (pH 9) produced short, spherical bodies which did not fluoresce.

Introduction

• **A biofilm is a collection or deposit of organized bacteria which adhere to a particular surface and establish a community**.
Cells within a biofilm are encased in extracellular matrix (ECM), composed of various sugars, proteins, and nucleic acid fragments. This extracellular matrix creates a microenvironment around the bacteria and can act as a barrier or protection from harmful environments or molecules, such as antibiotics. It also controls the flow into the matrix of elements such as oxygen to the cells in the biofilm. A major factor in determining a cell’s microenvironment is cell location within the three-dimensional structure of the biofilm. As biofilms are common in many environments and biological systems, including humans, it is important to know how this life mode affects such bacteria.

•**We investigated the pH range at which a biofilm will grow and observed cytoplasmic pH within individual cells in the biofilm fluorescence microscopy techniques.**
The ability to regulate cytoplasmic pH is a good measure for cell health and viability because the ability to maintain a functioning internal environment despite harsh external conditions is essential for cell life. The ability of cells in a biofilm to regulate their internal cytoplasmic pH under the stress of a highly acidic or basic environment may give insight into unknown mechanisms of pH regulation or cell signaling for cells that live in biofilms.

•**pH regulation can be measured through fluorescence microscopy; in this experiment, GFP was expressed by a gene cloned into *E. coli* using a plasmid.**
The GFP emits light at two wavelengths, and the ratio between the two is calculated and represented as a color in Metafluor. Using ratiometric fluorimetry, the calculated ratio can be correlated to a pH value using a standard curve. The derivative of green fluorescent protein (GFP) used in this project is called pHluorin (4).

Methods

1. Bacterial Growth
Luria broth supplemented with KCl (LBK) was adjusted to the desired pH using 5 M KOH or HCl, and various buffers (pH 4.5, 100 mM HOMOPIPES; pH 5, 5.5, and 6, 100 mM MES; pH 7, 100 mM MOPS; pH 8, 100 mM TAPS; pH 9 and 9.5, 100 mM AMPSO; pH 10, 100 mM CAPS). Overnight cultures of *E. coli* strain JLS1105 were grown in 2 ml of LBK with 50 µg/ml ampicillin. A final concentration of 0.2% l-arabinose was used to induce the plasmid containing the ratiometric GFP derivative pHluorin. Overnights were grown in rotating metal cap tubes for 15-16 hours at 37 °C. After rotating, 10 µl of overnight culture was spotted onto a 40 mm round glass coverslip coated with 0.01% poly-l-lysine, to promote adherence and biofilm growth. The coverslip was inserted into a flow cell chamber (Bioptechs FCS3) with a total volume of 250 µl, and perfused for 15-16 hours with buffered LBK media at the same pH as the overnight growth (4).

2. Fluorescence Microscopy
After overnight biofilm growth was completed, biofilm morphology was observed on an Olympus BX61WIF-5 microscope using a 100x objective lens (oil immersion). The flow cell was perfused with 50 mM buffered minimal media (M63A) with the same pH as the growth media. Metafluor for Olympus was used to record images and the fluorescence intensity ratio at two excitation wavelength ranges (400-425 nm and 460-480 nm). Individual cells were traced and the ratio within each cell was then converted to a cytoplasmic pH value for each time point in the experiment using a standard curve in Microsoft Excel. Flow cell chamber pH was checked using a mix of pH indicator dyes that were added to the LBK media and checking for appropriate color in the flow cell chamber (4). A pH shift from 7.5 to 5.5 was performed for the biofilm grown at neutral pH. The purpose of the pH shift is to test the ability of cells grown at one pH to recover and maintain cytoplasmic pH after undergoing a quick change in external pH.

Results

pH 4.5

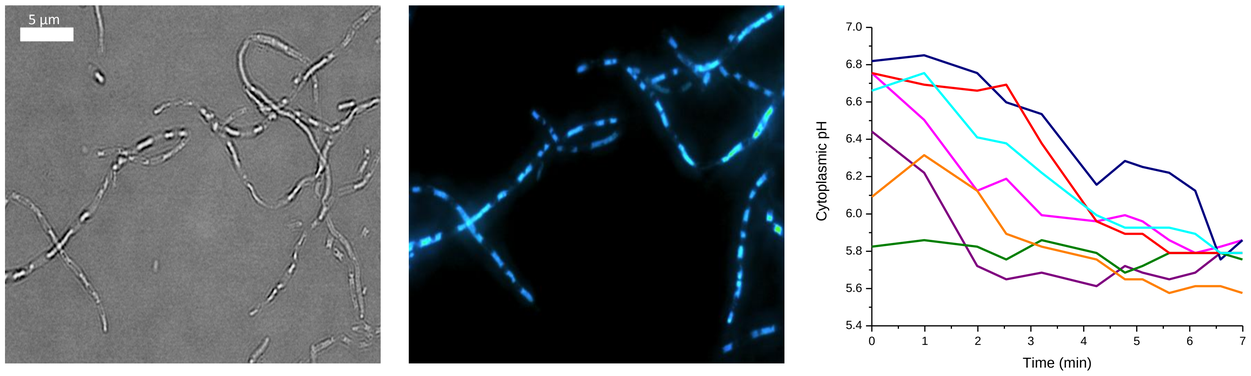

Figure 1. Brightfield and fluorescent images of an *E. coli* biofilm grown in LBK at pH 4.5. Cells show filamenting behavior, with distinct regions of cytoplasm. Color shown is the ratio between the two excitation peaks, not the actual fluorescence. Not all observed cells fluoresced. The graph shows representative cells’ cytoplasmic pH over the observation time. Four samples were observed.

pH 7.5

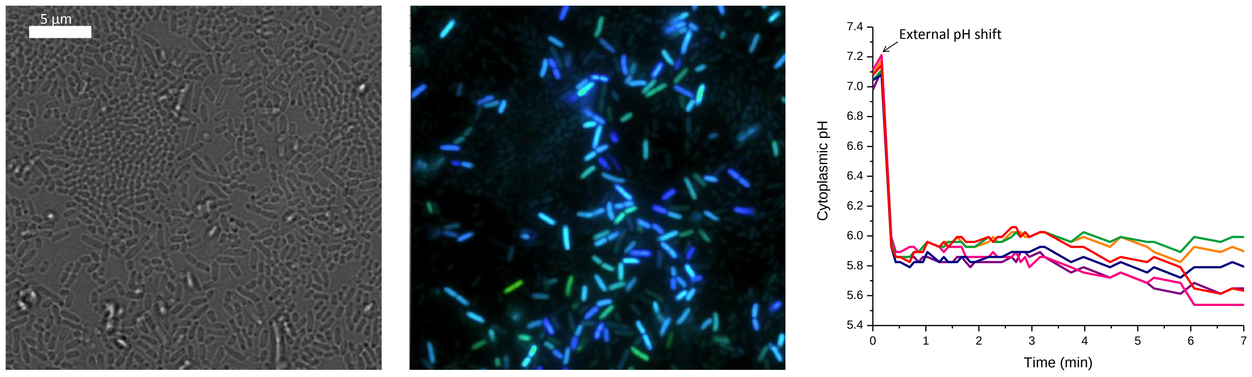

Figure 2. Brightfield and fluorescent images of an *E. coli* biofilm shift from pH 7.5 to pH 5.5, performed on Feb 15, 2012. Individual cell responses to rapid change in pH is variable. Some cells are able to maintain approximately neutral cytoplasmic pH (fluorescing green), while the majority of cells respond with a drop in internal pH, as shown by the cells fluorescing blue. On far right, the rapid drop around 0.5 minutes represents when the flow media was switched from pH 7.5 to 5.5.

pH 9

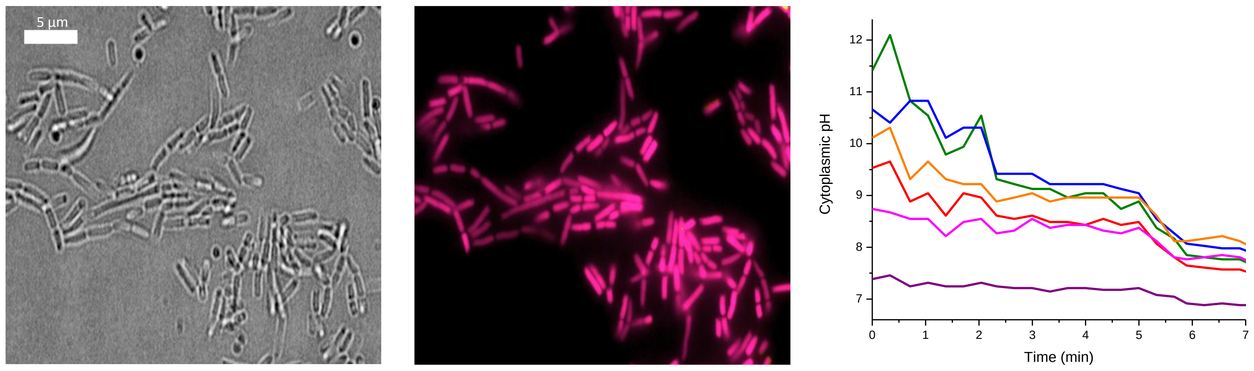

Figure 3. *E. coli* biofilms grown at pH 9 show a ratio between excitation peaks that is coded as a pink-magenta color. Color Small, dark, spherical minicells are visible on the bright field image (far left), which do not fluoresce (middle image). Some filamenting cells are also observable, but do not show the regions lacking cytoplasm which was observed at pH 4.5 (see **Figure 1**). Not all observed cells fluoresced. The graph shows representative cells’ cytoplasmic pH over the observation time. Six samples were observed.

pH	**4.5**	**5**	**5.5**	**6**	**7.5**	**8**	**9**
Average Cytoplasmic pH	5.82	5.81	6.42	6.66	7.14	7.24	7.7

Table 1. The average cytoplasmic pH for individual *E. coli* cells in biofilms was calculated by averaging the pH for all timepoints for 50 cells from one film for each pH., with the exception of pH 7.5, which only used timepoints prior to the media change. pH values were rounded to two decimal places. No data was able to be calculated for pH 9.5 and pH 10 due to the lack of biofilm growth and florescence observed. Average cytoplasmic pH was always closer to neutral than the external pH, suggesting regulation of cytoplasmic pH, even under extreme acid or base stress.

Discussion

•**Continuous biofilm growth is not observed at either pH 4.5 or pH 9.**
While some cell growth did occur at either end of the pH scale, biofilms were substantially more sparse than the continuous mats of bacterial growth observed around neutral pHs (6, 7, and 8). Cell morphology at either end of the pH scale was more varied than cell morphology observed around pH 7. Due to the inherent variability of biofilm growth, no significant pattern of growth or difference in morphology was identified between the two extremes (pH 4.5 and pH 9). Typically, 4 biofilms were grown at each pH.

•**Presence of filamentous *E. coli* and minicells at extreme pHs.**
At the extremes of the pH scale, both filamenting cell growth and smaller, spherical cell bodies were observed at pH 4.5, and pH 9.0 and above. Such cell types have been observed elsewhere, and are suspected to be miniature *E. coli* cells which do not contain DNA (1, 2). Minicells did not fluoresce at any pH. A possible explanation is that the minicells have lost the plasmid containing the GFP and therefore can no longer produce the protein (2).

•**Fluorescence levels decreased at extreme pHs**.
Lower intensities of emitted light were observed at the extremes of the pH scale, particularly in the upper reaches (pH 9 and above). Not all cells had fluorescence intensities high enough to be detected by Metafluor with an exposure time of 250 ms. Some films fluoresced with increased exposure times, but this leads to lower quality images from too much stimulation (4). One possible explanation for the relatively low levels of fluorescence observed at higher pHs is that the modified GFP, pHluorin, degrades or does not functional optimally outside of a certain pH range, and so the excitation intensity is significantly less.

•**Future experiments**
One possible future experiment is to test whether or not the minicells observed at pH extremes contain any DNA. By using a fluorescent dye which binds to DNA, such as DAPI, it would be possible to see if the minicells contain no DNA, or contain DNA but do not fluoresce. If the latter is the case, then biofilm growth at extreme pHs may be selectively losing the plasmid. Another direction of exploration involves looking at genes that are involved in separation and septum formation in *E. coli,* such as *ftsK* (7), in order to determine the environmental effect of extreme pH on cell division, particularly in the formation of filamentous cells. Another important question is to test if *E. coli* biofilms grown at a non-neutral pH are capable of surviving a pH shift, suggesting some plasticity or adaptability. It is possible that the same mechanisms which provide resistance to harsh external pHs also give cells resistance to change in the external pH.

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