Molecular physiology of weak organic acid stress in Bacillus subtilis
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CHAPTER 6

SINGLE CELL INTRACELLULAR pH MEASUREMENTS OF Bacillus Subtilis

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ABSTRACT

Within a growing bacterial culture, considerable heterogeneity may exist. This makes predictions of microbial stability of food products exceedingly difficult. Spore-forming bacteria pose additional problems as they are more resistant to various food preservation methods than vegetative cells. To gain insight in the physiology of the more robust sub-population, the internal pH (pHi) is an important parameter for vegetative cells because of its role in the cellular metabolic state. Here we provide our initial results of monitoring this physiological parameter on a single cell level. We have used to that end the ratiometric GFP “pHluorin” to measure the cytoplasmic pH of individual B. subtilis cells during 6 hours of live imaging. The pH, varied between 7.5 and 8.2 in growing cells, and was readily resolved into distinct sub-populations. pH correlated with growth rate with relatively fast-growing cells and micro-colonies tending to have a relatively high pH. This method may allow us to specifically look at cells capable of growth under stress by appropriate modulation of the internal pH value.
INTRODUCTION

*Bacillus subtilis* has been a Gram positive model organism for decades. These days mostly because of its genetic accessibility, but also as a model for cellular differentiation, sporulation and spore germination. Related organisms such as *B. cereus* and the anaerobic spore former *Clostridium botulinum* pose serious threats to public health, being the causative agents of potentially deadly forms of food poisoning. The spores of these microorganism especially pose problems, because the metabolically inactive survival capsules have an extreme resistance towards environmental stresses. It is therefore vital to understand how bacterial spore formers respond to various antimicrobial treatments, which cells survive and how they deal (cope) with the damage and stress.

Common industrial heat treatments are known to cause an increase in the lag phase of spore outgrowth and variance therein (67, 92). Weak organic acid preservatives also cause in increased variance in growth rate (Chapter 5 of this thesis). These two factors together complicate predictive modeling of bacterial growth and reinforce the question what makes the surviving sub population so robust. Studies on sporulation and heterogeneity (21, 95) have shown that within micro-colonies, several phenotypes of a single species may develop. In addition it was shown that medium composition plays a critical role in the number of germinant receptors on bacterial spores (222). These examples show some of the origins of heterogeneity within bacterial populations that may exist in food product.

Monitoring physiological parameters such as growth rate and internal pH (pHi), may give clues to understand why certain sub-population may be most robust to anti-microbial treatments. With such knowledge available, it may be possible to inhibit the development of such a sub-population, or specifically target surviving sub-populations. The pHi is an important parameter for vegetative cells, both to understand the cellular metabolic state as well as being a messenger for the cell itself, potentially steering developmental fate, similar to the situation in yeast (140, 223). The fact that weak organic acids alter (uncouple) the ΔpH interferes with the homeostasis of cellular metabolism and hence with cellular function.

It has been reported that the pHi of *Bacillus* spores lies around 6.3 (90). Data from population studies reported in chapter 2 of this thesis corroborate these observations. Magill *et al.* have shown that this lowering of the pHi, interrupts metabolism in the prespore, which allows accumulation of 3-phospho-glycerate to provide a carbon and energy source during the initial phase of spore germination (89). Around the same time as metabolism is reinitiated, a membrane potential should be reestablished, consisting of an electric potential and a pH gradient.

Using our recently published methods, we combined the compartment specific expression of (∆pH)luorin (133, 134) with single cell observations of *Bacillus subtilis* (92). Here, we
show that our method allows single cell and micro-colony measurements of the internal pH during growth for up to 6 h. In the near future, this may allow us to measure the pH of individual *B. subtilis* cells during various phases of growth and in specific compartments. Also the pH, during spore germination may be measured and can be used to pinpoint the moment that a ΔpH is established as well as the moment that the cell membrane becomes permeable to weak organic acids.

**METHODS**

**GENERAL GROWTH CONDITIONS**

For general purpose growth *B. subtilis* PB2 and *B. subtilis* Pgars-IpHfluorin (Chapter 2 of this thesis; table 1) were grown in Lysogeny Broth (LB). For experiments and fluorescence measurements, *B. subtilis* strains were grown in defined liquid medium (M3G;84) set at pH 7.0 and 7.4 (buffered with 80 mM MOPS). The medium contained 5 mM glucose, 10 mM glutamate, and 10 mM NH₄Cl as carbon and nitrogen sources. All cultures were grown at 37°C, under continuous agitation at 200 rpm. When required, 50 µg/ml spectinomycin was added.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB2</td>
<td>trpC; 168 wild-type</td>
<td>Gift from C.W. Price</td>
</tr>
<tr>
<td>PB2 Pgars-IpHfluorin</td>
<td>trpC, amyE3' spc' Pgars-IpHfluorin amyE5'</td>
<td>Chapter 2 of this thesis</td>
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**SLIDE PREPARATION AND TIME-LAPSE MICROSCOPY**

Essentially the same setup was used as described by Pandey *et al.*, 2013 (92). In brief: a closed air-containing chamber, was prepared by attaching a 65 µl volume Gene Frame™ (1.5 x 1.6 cm, Thermo Scientific) to a standard microscope slide. A thin (~160 µm), semisolid matrix pad (1 x 1 cm) with different concentrations of growth medium was made by adding 1% agarose (Sigma - Aldrich) to the medium in a 1:1 ratio and spreading the final solution onto a siliconized glass cover-slip (24 x 32 mm, Thermo Scientific). Exponentially growing vegetative cells or spores (1 µl) were loaded onto the pad and then the pad was transferred upside down onto another glass cover slip (18 x 18 mm, Thermo Scientific). This glass cover slip was placed on the Gene Frame™ and pressure was applied on the cover slip along the Gene Frame™ for complete sealing. The resulting chamber was used for time-lapse microscopy.

Time-lapse series were made by making use of a temperature-controlled boxed incubation system for live imaging set at 37°C and observing the specimens with a 100X/1.3 plane Apochromatic objective (Axiovert-200 Zeiss, Jena, Germany). Images were taken by a CoolSnap HQ CCD camera (Roper Scientific), using Metamorph software 6.1 (Molecular...
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Devices. Phase-contrast and fluorescence time-lapse series were recorded at a sample frequency of 1 frame per 5 min for 5.5 h. Maximally 9 different fields of view were recorded in parallel per experiment.

CALIBRATION OF IPHLUORIN

*B. subtilis* P<sub>ptsG</sub>-IpHluorin was grown to exponential phase in M3G at pH 7.0 containing 50 μg/ml spectinomycin. At OD<sub>600</sub> = 0.4 the cells were centrifuged and resuspended in buffers with pH values ranging from 5.0 to 8.5 prepared from 0.1 M citric acid and 0.2 M K<sub>2</sub>HPO<sub>4</sub>. The intracellular and extracellular pH were equilibrated by the addition of 1 μM valinomycin and 1 μM nigericin (146). Cells were transferred to microscope slides as described above, with agarose pads prepared using the calibration buffers. Fluorescence and phase contrast images were taken directly after proper positioning of the slide. The ratio of emission intensity at 510 nm resulting from excitation at 390 and 470 nm was calculated as described previously (137). Background fluorescence was subtracted at individual wavelengths before calculating the ratio. At least 100 cells were observed per pH value on the calibration curve.

DATA ANALYSIS

Phase-contrast time-lapse series were aligned using custom-build software, based on “SporeTracker”<http://simon.bio.uva.nl/objectj/examples/sporetracker/SporeTracker.htm>. This macro runs in combination with ObjectJ <http://simon.bio.uva.nl/objectj> which is a plugin for ImageJ <http://rsb.info.nih.gov/ij>.

The Align Fluorescence Channels tool for ObjectJ was used to align the fluorescent images with corresponding the phase contrast image. This tool aligns both phase contrast images in time as well as fluorescent images with the corresponding phase contrast image. Phase contrast channel is converted to a mask, so objects have intensity = 1 and background has intensity 0. This mask is multiplied with the underlying fluorescence channel, and the total fluorescence of the product is measured. By shifting the mask in x and y direction, this value will change. Those offsets that yield the maximum result will be used for alignment. Using FFT (Fast Fourier Transformation) correlation, this process can be done in a single step.

RESULTS

CALIBRATION AND VALIDATION OF SINGLE CELL INTRACELLULAR PH MEASUREMENTS IN *B. subtilis*

To ensure optimal growth conditions, that allow comparable growth and pH measuring conditions as were described in chapter 2 of this thesis, the criteria set by Pandey *et al.* (92) should be met. Apart from these, the fluorescence measurements should be conducted in such a way as not provide too much additional stress (phototoxicity).

Expression of IpHluorin from native promoters in *B. subtilis* was high in batch experiments (chapter 2 of this thesis). Expression from P<sub>ptsG</sub> is intrinsically heterogeneous, but 113
nevertheless proved never the less to be strong enough in most cells to allow accurate single cell pH measurements (Fig. 1 and 3). The calibration curve for IpHluorin in B. subtilis in our microscopy setup has a similar profile as in batch, although specific settings regarding exposure time, light intensity and gain affect fluorescent intensity values obtained for each wavelength. The current calibration curve, based on approximately 100 cells per pH value does allow pH measurements with approximately 0.5 pH units accuracy.

**Figure 1.** Representation of ratiometric fluorescence microscopy. Per time point, three images are taken. The phase – contrast image serves both for the growth – rate determination as well as a mask for the fluorescence images. Images are artificially coloured and are based on actual data.

**Distribution of internal pH between microcolonies**

As a result of the pH – fluorescence ratio profile (Fig. 2), variation at the extreme ends of the calibration curve tend to result in similar a pH. This may result in an overestimation of cells with pH values around the extreme values of the calibration curve.
Figure 2. Ratiometric fluorescence microscopy calibration curve of \textit{B. subtilis} \text{P}_{\text{rec}}\text{lpHluorin}. \textit{B. subtilis} cells were immobilized on agarose slides prepared with set pH buffer and permeabilized using nigericin and valinomycin. This resulted in rapid equilibration of the external and internal pH. The calibration curve and pH measurements were performed as described in the materials and methods section. At least 100 cells were measured per data point. Error bars indicate the standard deviation.

The internal pH of \textit{B. subtilis} is not constant and was shown to fluctuate in batch cultures between different growth phases. The growth rate and pH\text{\textsubscript{I}} can be correlated, with high pH\text{\textsubscript{I}} (~8) populations correlating with high growth rates, and cultures with low pH\text{\textsubscript{I}} displaying low growth rates in batch cultures (Chapter 2 of this thesis). The distribution of pH\text{\textsubscript{I}} (Fig. 4) shows that pH\text{\textsubscript{I}} of individual growing cells lies between 7.4 and 8.1. Based on Fig. 3, it appears that there are two groups, one with cells displaying a high pH\text{\textsubscript{I}} and one with cells with low individual pH\text{\textsubscript{I}} values. Hence, in this preliminary work, we show that also on a single cell level, a high pH\text{\textsubscript{I}} correlates with a high growth rate. The pH\text{\textsubscript{I}} of the fast – growing population drops during the first hour of growth. It is very possible that this decrease corresponds with a lag phase due to a change in growth conditions from shake flask to microscope slide. It is quite remarkable that the same sub population that starts with a high pH\text{\textsubscript{I}}, also return to a high pH again, while cells with a low pH\text{\textsubscript{I}} keep this low pH.
**Figure 3.** Examples of the internal pH of 9 micro-colonies of *B. subtilis* PptsG-IpHluorin, observed using ratiometric fluorescence microscopy. The internal pH was calculated based on the calibration curve (figure 2).

**Figure 4.** Histogram of the distribution in internal pH of *B. subtilis* PptsG-IpHluorin during the first 60 minutes of growth.

**DISCUSSION**

Understanding heterogeneity within a population of cells can best be tackled using a single cell (microscopy) approach. Following single cells in time is required to understand the behaviour of cells that survive antimicrobial treatments. As weak organic acid preservatives are assumed to act primarily by decreasing the internal pH value, single
Figure 5. The internal pH (during the first 60 min) of 28 micro colonies of B. subtilis PptsG-IpHluorin, plotted against the growth rate of the first 60 minutes of growth. The straight line represents a linear fit with coefficient of determination. This results in a significant correlation between pH and growth rate (P < 0.01).

cell pH measurements will be highly beneficial to understand how individual cells are affected by these compounds. The internal pH may give clues on metabolic activity ([111] and Chapter 3 of this thesis) and thereby provide insight in survival strategies. We show here in a first set of experiments that pH can be monitored for extended periods in single cells and growing micro colonies and that the internal pH correlates with growth rate. The pH of the micro colonies decreased over time. Future experiments should establish if this is caused by a decrease in all cells or only in a sub population within the micro colony. The setup used has been shown to allow growth rates highly similar to batch culturing [92] and pH values that closely resemble those obtained from batch cultures (Chapter 2 of this thesis). The calibration curve results in some bias at the extreme values and might be expanded with higher pH values and more cells to make the obtained curve more accurate.

Two related major issues exist regarding single cell pH measurements as performed with our current setup. Photobleaching results in a decreasing signal intensity after prolonged exposure. This is the result of damage to the fluorescent protein. Prolonged exposure to high light intensity also results in phototoxicity, decreasing growth rate and causing cell lysis. This is likely caused by the formation of reactive oxygen species (ROS) as well as damaged fluorescent proteins. These two parameters were, albeit only partially, addressed in a recent paper concerning the internal pH of individual B. subtilis and Escherichia coli cells by the Slonczewski group [224].
Several future experiments involving the response to weak organic acid stress may be envisaged. Weak organic acids have been shown to decrease the internal pH of *B. subtilis*. While this is initially caused by the weak organic acid, the (level of) subsequent recovery of pH may be a resistance mechanism (112). In batch cultures, the fastest growing cells emerge and dominate the average observed value for pH. It is possible that on a single cell level, a different distribution exists in internal pH values, with some cells raising their pH to a higher level, resulting in an increased toxicity and/or uncoupling rate by the accumulating acid anion, thereby slowing growth of this sub-population. It is possible that within a biofilm, cells are shielded from the surrounding acid, as cells with a high pH will accumulate the dissociated acid. This then may alter the local acid concentration.

The spores of *B. subtilis* have a low pH (~6.2) (Chapters 2 and 3 of this thesis). This likely causes a decrease in 3-phosphoglycerate mutase activity, and thus 3-PGA accumulates in the developing spore. It is assumed that this serves as an energy reservoir, required for early metabolism during germination (89). It will be interesting to see how pH develops during germination and outgrowth, especially as long as no glucose is metabolized. Germination may also be a target moment to inhibit outgrowth of *B. subtilis*. This method may therefore also allow us to pinpoint the timing of WOA entry in the individual germinating/outgrowing spore and couple it to the risk of unwanted growth hence the spoilage of food products.