Live-imaging of Bacillus subtilis spore germination and outgrowth

Pandey, R.

Citation for published version (APA):
Chapter 6

Intracellular pH response to weak acid stress in individual *Bacillus subtilis* vegetative cells: Use of IpHluorin for live-imaging

Manuscript in preparation: Rachna Pandey, Norbert O.E. Vischer, Jan P.P.M. Smelt, Winnok H. De Vos, Alex Ter Beek, Stanley Brul, Erik M.M. Manders; Supplementary materials are available upon request at the department of Molecular Biology & Microbial Food Safety, contact s.brul@uva.nl
6.1 Abstract

To monitor the intracellular pH ($pH_i$) of *Bacillus subtilis* cells during its growth phase, an improved version of the genetically encoded ratiometric pHluorin (IpHluorin) was used. The new version of IpHluorin showed an approximate 40% increase in fluorescence intensity per cell. The gene encoding IpHluorin which was expressed from the native *B. subtilis* promoter and specifically active during vegetative growth on glucose ($P_{pts,G}$). Ratio imaging was set up and allowed us to resolve the population data at single cell level. A calibration curve comparing the fluorescence ratio with pH was obtained at an external pH range of 5.0 to 8.5 with uncouplers that breakdown the transmembrane pH difference. *B. subtilis* cells were stressed with 3 mM sorbic acid (KS) and 25 mM acetic acid (KAc) in a chemically defined medium (MOPS). In the presence of sorbic acid a decrease in $pH_i$ and increase in generation time of growing cells were observed. Similar effects were observed when cells were stressed with acetic acid but at a higher concentration (25 mM). This shows that sorbic acid (KS) lowers the $pH_i$ more effectively than acetic acid (KAc). Cells with a lower $pH_i$ all showed a lower growth rate.
6.2 Introduction

The food industry uses different preservation techniques to ensure that manufactured foods remain safe and unspoiled for long periods of time. Weak acids such as sorbic acid, acetic, lactic and benzoic acid are commonly used as food preservative in the food industry. These molecules inhibit the outgrowth of both bacterial and fungal cells (Krebs et al., 1983). Sorbic acid and its salts inhibit the growth of various bacteria, including sporeformers, at various stages of their life cycle (germination, outgrowth and cell division)(Sofos et al., 1986). The weak acids inhibit the growth of microorganisms in a number of ways such as membrane disruption, inhibition of essential metabolic reactions (Bracey et al., 1998; Krebs et al., 1983), stress on intracellular pH homeostasis and the accumulation of toxic anions (Bracey et al., 1998; Eklund 1985). Studies by Holyoak et al. (1996) and Bracey et al. (1998) showed that in yeast the inhibitory action of weak acid preservatives could be an energetically expensive stress response. The attempts to restore homeostasis resulting in a drop of available energy pools for growth and other essential metabolic functions. In nature, microorganisms have evolved different resistance mechanism to combat the weak organic acid effect. For example *Saccharomyces cerevisiae* have an efflux system, presumably membrane localized, that removes accumulated anions from inside the cell (Henriques et al., 1997; Piper et al.,1998).

Microbes have evolved to grow within a particular range of $pH_i$. The $pH_i$ affects many biological activities such as enzyme activity, reaction rates, protein stability and structure of different molecules such as nucleic acids. Many intracellular enzymes show optimal activity and stability in a narrow pH range near neutrality. Shioi et al., (1980) has shown that during optimal growth conditions the *Bacillus subtilis* cell maintains its cytoplasmic pH within the range of 7.4-7.8. Van Beilen and Brul recently (2013) corroborated this data. Thus the intracellular pH of the bacteria is very important to ensure optimal growth. This pH effect is explored by the food industry for food preservation.

Sorbic and acetic acids are common preservatives that are known to permeate over the plasma membrane in their undissociated form. Inside they encounter near neutral pH values and dissociate during pHi down. The $pH_i$ of the cells can be measured by various methods such as $^{31}$P NMR, fluorescent dyes (carboxyfluorescein, carboxyfluorescein diacetate, and succinimidyl ester) and the distribution of radiolabeled membrane-permiable weak-acids (Ugurbil et al., 1978; Bulthuis et al., 1993; Magill et al., 1994; Breeuwer et al., 1996; Leuschner et al., 2000). The advantage of these methods is that they do not require genetic modification. In the case of fluorescent dyes, single cell measurements are possible (Slonczewski et al., 2009). The disadvantage of using weak organic acid dyes is that they may
They themselves alter the $pH_i$. The disadvantage of the $^{31}P$ NMR and radiolabeled compounds are that they require extensive cell handling and high cell density, which also disturbs the cell’s physiology. Fluorescent proteins (green fluorescent protein (GFP) derivatives) are another important way for measurement of the internal pH of the bacterial cell. GFP extracted from jellyfish (Aequorea victoria) is widely used as a noninvasive fluorescent marker for gene expression, protein localization, and intracellular protein targeting (Gerdes et al., 1996; Cubitt et al., 1995). A modified version of GFP sensitive to pH is called ratiometric pHluorin (Miesenböck et al., 1998). This ratiometric pHluorin uses the cell’s protein biosynthesis apparatus to produce the fluorescent probe and therefore does not require artificial staining procedures, which could affect cell physiology. The importance of this modified ratiometric GFP is that it allows direct, fast, and localized pH measurements. It has been successfully used our laboratory in $S$. cerevisiae (Orij et al., 2011; Ullah et al., 2012) and more recently in $B$. subtilis (Martinez II et al., 2012; Van Beilen and Brul, 2013; Ter Beek et al., 2014). IpHluorin has been used to probe the cytosolic and organellar pH (mitochondria and Golgi apparatuses) of $S$. cerevisiae. In several studies, pHluorin have been used to describe the cell’s response to various growth conditions, glucose pulses, respiratory chain inhibitors and other treatments (Martinez-Munoz et al., 2008; Orij et al., 2009).

In this paper, an improved version of ratiometric pHluorin (IpHluorin) was used to study the effect of sorbic and acetic acid on the $pH_i$ of individual $B$. subtilis cells using live-imaging. We also present an image analysis tool, called “Multichannel-SporeTracker” (semi-automated). It calculates the internal pH (based on the ratio of the intensities of the two wavelengths at 390 and 470 nm that has emission at 510 nm) and generation time of exponentially growing $B$. subtilis $P_{pts}$-IpHluorin vegetative cells.

### 6.3 Materials and Methods

#### 6.3.1 Growth conditions

To monitor the internal pH ($pH_i$) of the exponentially growing $B$. subtilis cell for a long period of time, the $B$. subtilis $P_{pts}$-IpHluorin (trp2C; amyE3’ spcR $P_{pts}$-IpHluorin amyE5’) construct was used (Van Beilen and Brul, 2013). This construct consists of the pHluorin gene (Miesenböck et al., 1998), which was inserted after the first 24 bp of comGA adjacent to the promoter $P_{pts}$G. This encodes the glucose-specific enzyme called phosphotransferase system II, which allows the expression of IpHluorin in vegetative cells growing on glucose containing medium. The $B$. subtilis 168 laboratory wild-type strain $PB_2$ and $B$. subtilis $P_{pts}$-IpHluorin were grown exponentially in Luria Broth (LB) at 37°C, under continuous agitation.
6.3. Materials and Methods

at 200 rpm. The exponentially growing cells were re-inoculated in minimal defined NKDM medium buffered with 80 mM MOPS (3-(N-morpholino) propanesulfonic acid) as described previously (Kort et al., 2005) to pH 7.4. Hereafter referred to as MOPS medium. The MOPS medium contained 50 μg/ml spectinomycin, and cells were grown until exponential phase at 37°C, under continuous agitation at 200 rpm. The optical density at 600 nm (OD$_{600}$) was measured in time to check whether the cells were in the exponential phase. Cells in the early exponential growth phase (OD$_{600}=0.2$) were used for time-lapse microscopy experiments (see below). In stress experiments, 3 mM sorbic acid (KS) and 25 mM acetic acid (KAc) at pH 6.4 were used to test for their effect on the growth and pH$_i$ of exponentially growing bacteria.

### 6.3.2 Slide preparation and its settings for fluorescent time-lapse imaging

A closed air-containing chamber (Pandey et al., 2013) was used for time-lapse fluorescence microscopy. The chamber was prepared by attaching a Gene Frame® to a standard microscope slide and cover slip. In this chamber a thin (160 μm), semisolid matrix pad of 1% agarose-medium was made. The pad was loaded with exponentially growing vegetative cells (1μl). Time-lapse series were made by making use of a temperature-controlled boxed incubation system for live imaging set at 37°C. The specimens were observed with a 100X/1.3 plane apochromatic objective (Axiovert-200 Zeiss, Jena, Germany), a GFP filter set (Chroma) for excitation at 390 nm and 470 nm and a 510 LP-filter for fluorescence emission. Images were taken by a CoolSnap HQ CCD camera (Roper Scientific), using Metamorph software 6.1 (Molecular Devices). For control experiments, the time-lapse series of phase-contrast and fluorescence images were recorded at a sample frequency of 1 frame per 10 min for 5 hours and for stress experiments the cells were imaged for 10 hours (also 1 frame per 10 min). Two biological replicates and 15-30 technical replicates (recorded fields of view on one slide) were recorded in parallel per experiment. In every field of view (technical replicate) 2-8 vegetative cells were identified and followed in time. This resulted in the analysis of approximately 30-60 vegetative cells from the start of each imaging experiment per biological replicates.

### 6.3.3 Phototoxicity measurements

Phototoxicity is a detrimental phenomenon in life-cell imaging, which occurs upon repeated exposure of fluorescently labeled cells to intense light. In order to check the effect of phototoxicity on vegetative cells, exponentially growing B. subtilis PB$_2$ and B. subtilis P$_{pt}$G-IpHluorin cells (grown in MOPS medium) were repet-
Chapter 6: **Intracellular pH response to weak acid stress in individual Bacillus subtilis vegetative cells: Use of IpHluorin for live-imaging**

...exposed to excitation light of two different wavelengths (390 nm and 470 nm) with an exposure time of 100 ms and 30 ms, respectively for a period of 5 h with time a interval of 5 min and 10 min. The generation times of the cells were calculated with multichannel-SporeTracker over a period of 5 h. The total number of cells assessed for *B. subtilis PB2* cells grown in absence of fluorescent light was 107 and for *B. subtilis P_pts*G-IpHluorin cells in the absence and presence of fluorescent light was 164, 77, and 92 respectively. The effect of phototoxity on the cells was regarded as negligible when there was no measurable cell death.

### 6.3.4 Calibration of $pH_i$

*B. subtilis P_pts*G-IpHluorin cells were grown to exponential phase in MOPS medium to pH 7.4 containing 50 µg/ml spectinomycin. At $OD_{600nm}=0.4$ the cells were centrifuged (4000 rpm; 10 min) and re-suspended in phosphate-citrate buffers (0.1 M citrate and 0.2 M $K_2HPO_4$) with pH values ranging from 5.5 to 8.5. The cells were then permeabilized with vallinomycin (1 µl) and nigericin (1 µl) (Breeuwer et al., 1996). This treatment makes pores in the cell membrane and therefore, allows equilibrating on the intracellular pH with the externally set pH. Subsequently, cells in phosphate-citrate buffer of different pH (5.5 to 8.5) were transferred to agarose pads of the corresponding pH value and in closed air-containing chambers. For each pH, fluorescence images were recorded and around 200 cells were analyzed with Multichannel-SporeTracker to construct a calibration curve. This curve represents the relationship between the ratio of the 510 nm emission intensities of IpHluorin upon excitation at respectively 390 and 470 nm ($E_{390}/E_{470}$) and the $pH_i$. The curve was fitted with a Henderson-Hasselbalch equation, which describes the relation between the ratio of the intensity of wavelengths ($E_{390}/E_{470}$) and $pH_i$. It took the form as: $E_{390}/E_{470}=(10^{pH-pKa})/(10^{pH-pKa}+1)(b+a)$.

The performance of the model is shown in Figure 6.3 and parameter and its estimated value are $pKa=7.18$, $b=1.61$ and $a=0.66$. The curve could also be fitted with a sigmoid equation of the same of $pH = B*log(((A-D)/(ratio-D))-1)+C$, with: $A = 0.67$, $B = 0.42$, $C = 7.2$, $D = 2.25$.

### 6.3.5 $pH_i$ measurements in a microcolony and in single cells within a microcolony

For $pH_i$ measurements two data analysis tools were used: one for analysis at the microcolony level and another for analysis at the single cell level. “Multichannel-SporeTracker” [http://simon.bio.uva.nl/objectj/examples/sporetracker/SporeTracker.htm], was developed for $pH_i$ measurements at the microcolony level. This program runs in combination with ObjectJ, [http://simon.bio.uva.nl/objectj/], which is a plugin for ImageJ [http://imagej.nih.gov/ij/]. It calculates the gener-
ation time by calculating the growing area \((\log_2)\) of cells with time and the \(pH_i\) of vegetative cells as a function of time. \(pH_i\) measurements are based on the ratio of the fluorescence emission at 510 nm after excitation at 390 nm and 490 nm respectively \((E_{390}/E_{470})\). To calculation the \(pH_i\) and generation time of the cell, the cell’s IpHluorin intensity was probed for a fluorescent channel. The fluorescence images were aligned with the corresponding phase contrast images in time. Before measuring the fluorescence intensity of the cells, the background of the fluorescent images was made to nearly zero by subtracting the  mode value per frame throughout the movie. The mode value is the pixel intensity number, which is repeated for maximum number of time while calculating the background of the fluorescent images. The fluorescence intensities of IpHluorin expressing cells were measured by making a region of interest around the cells. The \(E_{390}/E_{470}\) of IpHluorin expressing cells was calculated. By correlating the ratio with the calibration curve (mentioned below) the \(pH_i\) of the cell was determined. After obtaining the data from the Multichannel SporeTracker, differences in variance were tested with F-tests. Depending on the results of the F-tests the appropriate t-tests were performed to test differences in the average.

For \(pH_i\) measurements at the single cell level the Fiji plugin ColiMetrics.ijm was used (http://fiji.sc and www.limid.ugent.be/downloads). In brief, bacteria were first segmented on the DIC channel by maxima finding and conditional region growing on the inverted images, using a noise tolerance of 4%, object size range in between 50 and 500 pixels and circularity in between 0.15 and 0.80. After refining the segmented objects, the regions of interest delineating the bacteria were used to measure the signal intensities in the two fluorescence channels (390 and 470). 390/470 intensity ratio’s were calculated and represented in the form of intensity-normalized ratio images, i.e. HSV images in which the Hue represents the ratio of both fluorescence channels and the Value the product of both channels as described before (Back et al., 2012 ). To convert ratios to pH, a calibration curve was established in which bacteria were grown in media of fixed pH (see below).

### 6.4 Results

In order to ensure the unbiased growth of aerobic bacteria, a closed air-containing chamber (Pandey et al., 2013) was used. In this chamber cells were sandwiched between the glass coverslip and a thin (160 \(\mu\)m) agarose-medium pad to ensure their immobilization in the presence of sufficient culture medium and enough oxygen for undisturbed growth. The automated program “Multichannel-SporeTracker” allows accurate measurements of the intensity of IpHluorin in the cell, calculates
the ratio \( \frac{E_{390}}{E_{470}} \) of IpHluorin and deduces the \( pHi \) and the generation time of the vegetative cells growing into a microcolony in any desired time frame (Figure 6.1).

### 6.4.1 Phototoxicity measurements

The phototoxicity is an important problem in fluorescence live-cell imaging. It often occurs upon repeated exposure of fluorescently labeled cells to light, which is induced to excite the fluorophores. In their excited state, fluorescent molecules are inclined to react with molecular oxygen to produce free radicals that can damage cellular components compromising cell vitality. In order to measure phototoxicity in the bacterial cells, \( B. subtilis \) wild-type strain \( PB_2 \) and IpHluorin expressing strain \( P_\text{pts}G\text{-IpHluorin} \) were grown in an air-containing chamber on MOPS medium to pH 6.4 for 5 h and 10 h, respectively. During growth the cells were exposed to light of two wavelengths (390 nm and 470 nm, for 100 and 30 ms, respectively) with 5 min or 10 min intervals between fluorescence measurements. The generation time of exponentially growing cells was calculated by using the Multichannel-SporeTracker. Figure 6.2 shows the effect of 390 nm and 470 nm excitation light on \( B. subtilis \) \( P_\text{pts}G\text{-IpHluorin} \) cells. The cells grown in the presence of light of the two wavelengths, 390 nm at 100 ms and 470 nm at 30 ms) with either 5 min or 10 min interval, have similar generation times 115.71±3.27 min and 114.44±21.07 min respectively. The generation times of \( B. subtilis \) cells grown in the absence of excitation light were (92.36±13.63 min). Noteworthy, cell-death has not been observed within the time frame of the experiment using either 5 or 10 min exposure intervals (data not shown). The generation time of wild-type \( B. subtilis \) \( PB_2 \) cells grown in absence of excitation light (92.93±12.73 min) is similar to the generation time of the IpHluorin expression cells grown in the absence of excitation light (92.36±13.63 min). Therefore we conclude that IpHlorin expresion is not harmful to the cells. Although an increase in generation time was seen when IpHluorin expressing cells were exposed to excitation light, the effect was not lethal and we conclude that our settings are acceptable for long term (10 h) \( pHi \) live-imaging experiments.

### 6.4.2 Calibration of \( pHi \) measurements in \( B. subtilis \) cells at single cell resolution

Population level studies on cytoplasmic pH measurement account for average values (Kitko et al., 2009), therefore overseeing the individual cell heterogeneity in a population. Moreover the single-cell response is important because every bacterial cell behaves differently in growth and division, resulting in a physiologically diverse population. The ability to maintain pH homeostasis is an important pa-
6.4. Results

Figure 6.1: Multichannel-SporeTracker output for $pH_i$ measurements in growing *B. subtilis* cells. Shown here are collective plots of 4 individual starting cells measured every 5 min for 5 hours. Bottom to top: $\log_2$(area of cells); pixel intensity (for spore germination, the column is blank as only vegetative cell were analysed); fluorescence intensities measured at 510 nm when excited at 390 and 470 nm, respectively; ratio of the excitation wavelength (390 and 470 nm) of fluorescence intensities and $pH_i$. 
Chapter 6: Intracellular pH response to weak acid stress in individual Bacillus subtilis vegetative cells: Use of IpHluorin for live-imaging

Figure 6.2: The effect of fluorescent light (excitation at 390 nm and 470 nm and emission at 510 nm) on B. subtilis PptsG-IpHluorin. Movies of B. subtilis PB2 cells grown in absence of fluorescent light and B. subtilis PptsG-IpHluorin cells in absence and presence of fluorescent excitation light (390 and 470 nm) with time interval of either 5 min or 10 min were made during 5 h. The data was analysed for generation time by Multichannel-SporeTracker. The total number of cells assessed for B. subtilis PB2 cells grown in absence of fluorescent light was 107 and for B. subtilis PptsG-IpHluorin cells in absence and presence of fluorescent light was 164 (5 min, in absence of light), 77 (10 min, in presence of light), and 92 (5 min, in presence of light).

rameter in this regards (Stewart et al., 2005). Therefore to measure the internal pH within B. subtilis PptsG-IpHluorin cells a calibration curve was developed using fluorescent microscopy (Figure 6.3). Fluorescence intensity ratios of ~200 cells per externally set pH were calculated in permeabilized B. subtilis PptsG-IpHluorin cells. The data was fitted to a Henderson-Hasselbalch equation as given in materials and methods.

6.4.3 Effect of sorbic and acetic acids on pHi and growth of growing B. subtilis vegetative cells

Weak acids are frequently used as preservative in the food industry. Sorbic and acetic acid have effects on bacterial cells. Sorbic acid is lipophilic whereas acetic
Figure 6.3: Calibration curve of B. subtilis P\textsubscript{pts} G-IpHluorin, which describes the relation between the ratio of the intensity after excitation at 390 and 470 nm respectively ($E_{390}/E_{470}$) and pH\textsubscript{i}. The B. subtilis P\textsubscript{pts} G-IpHluorin cells were permeabilized using nigericin and valinomycin and immobilized on an agarose slide with set pH values ranging from 5.5 to 8.5. The cell fluorescence emission intensities were measured and the ratio ($E_{390}/E_{470}$) were plotted against pH\textsubscript{i}. At least 200 cells were measured per data point. Error bars indicate the standard deviation. The squares represent measured data points and the black line gives the fit according to the Henderson-Hasselbalch equation.

acidity is hydrophilic in nature even though both have a similar pKa (4.76). Here the effect of 3 mM sorbic acid and 25 mM acetic acid on vegetative cells was studied at single cell level. Growth rate of B. subtilis cells is on average reduced by 30% under these conditions (Ter Beek, A., 2009). B. subtilis P\textsubscript{pts} G-IpHluorin cells were grown on defined minimal medium containing 3 mM sorbic acid and 25 mM acetic acid (stress) as well as without the acid stress (control) for either 10 h or 5 h respectively and the fluorescence was recorded at using 10 min intervals between fluorescence measurements, at 100 and 30 ms exposure times respectively. Figure 6.4 shows the effect of sorbic and acetic acid on the pH\textsubscript{i} and generation time of B. subtilis P\textsubscript{pts} G-IpHluorin vegetative cells. In sorbic acid stressed cells, the internal pH decreased from 7.1 to 6.7 (Figure 6.4A, Table 6.1) and the generation time
increased significantly (Figure 6.4B, Table 6.1). In 25 mM acetic acid stressed cells, a similar decrease in internal pH and increase in generation time was observed (Figure 6.4C and 6.4D, Table 6.1). The average $pH_i$ of cells grown on defined minimal medium with and without 3 mM sorbic, 25 mM acidic acid stress and unstressed cells cultured under the microscope were $6.78 \pm 0.14$, $6.76 \pm 0.11$ and $7.10 \pm 0.17$, respectively. Interestingly there is no significant difference in $pH_i$ and generation time of the cell grown in sorbic acid (3 mM) as compared to cells stressed with acetic acid (25 mM) (Table 6.1). Figure 6.5). This shows that these concentrations of both sorbic and acetic acids reduce the $pH_i$ and the growth rate to a similar level. Thus this result corroborates that the sorbic acid is a more effective preservative. The $pH_i$ can be a good indication of the health status of the bacteria. Orij et al., (2011) and van Belein and Brul, (2013) showed that in population level the growth rate and $pH_i$ can be correlated. Van Belein and Brul, (2013) in microiter plate experiment showed that high $pH_i$ ($\sim 8$) correlates with high growth rates whereas cells with low $pH_i$ display low growth rates.

We observed an increase in fluorescence of IpHluorin in exponentially growing cells likely due to constant production of the fluorescent protein (data not shown). In order to facilitate the image analysis process for the calculation of $pH_i$ of single cells within a microcolony a program (macro) was written. This program defines an image analysis process in Fiji, a plugin for ImageJ (see Materials and Methods). The macro calculates the $pH_i$ of the cells and is visually represented by a color-code. Figure 6.6 shows a ratiometric HSV representation of two images of growth and division of $B. subtilis$ $P_{pts}G$-IpHluorin vegetative cells in the presence (stress) and absence (control) of sorbic and acetic acid (see Movie S1, S2, and S3). Panel A shows the control and panel B and C show the sorbic and acetic acid stress condition respectively. In the control condition the cells appeared blue, which indicates (color bar) that these cells have a higher $pH_i$. In contrast in sorbic and acetic acid stress conditions, the cells appeared in pink color which indicates that they have lower $pH_i$ than the control (Figure 6.6). There is some heterogeneity in the intensity of the IpHluorin amongst the observed cells despite of the fact that IpHluorin is present in genome of the $B. subtilis$ cells. Since there is no indication for a different glucose availability to the cells, difference in $P_{pts}G$-IpHluorin expression are the likely caused by stochastic effect.

6.4.4 Discussion

Here we deployed a derivative of green fluorescent protein (GFP), IpHluorin, to probe at single cell level the intracellular pH of $Bacillus subtilis$ cells. The GFP (green fluorescent protein) from the jellyfish Aequorea victoria is highly fluorescent and stable under many assay conditions (Cubitt et al., 1995). Studies on its
Table 6.1: Mean values and standard deviation of internal pH and generation time of individual B. subtilis \(P_{\text{pts}}\)G-IpHluorin vegetative cells in the presence and absence of sorbic acid and acetic acid\(^a\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(pH_i)</th>
<th>Generation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.10 ± 0.17((n = 151))</td>
<td>83.57 ± 10.74((n = 145))</td>
</tr>
<tr>
<td>Sorbic acid (3 mM)</td>
<td>6.78 ± 0.14((n = 205))#*</td>
<td>304.63 ± 109.70((n = 109))#*</td>
</tr>
<tr>
<td>Acetic acid (25 mM)</td>
<td>6.76 ± 0.11((n = 131))#*</td>
<td>286.13 ± 80.78((n = 122))#*</td>
</tr>
</tbody>
</table>

\(a\) B. subtilis \(P_{\text{pts}}\)G-IpHluorin vegetative cells were stressed in MOPS medium with or without sorbic acid (3 mM) and acetic acid (25 mM). \(pH_i\) and generation time of individual vegetative cell were calculated as described in the Materials and methods.

\(b\) Mean time of \(pH_i\) and generation time is given including the standard deviation. The amount of cells analysed from \(pH_i\) and generation time are gathered from two (control, sorbic acid, acetic acid) microscopy experiments, which is given in brackets. The star indicates that the variance of the distributions between the stress and control experiment are significantly different (\(P<0.05\)). The hash indicates that the mean of the distributions between the stress and control experiment are significantly different (t-test, \(P<0.05\)).
Chapter 6: Intracellular pH response to weak acid stress in individual Bacillus subtilis vegetative cells: Use of IpHluorin for live-imaging

Figure 6.4: Analysis of B. subtilis P_{pts}G-IpHluorin vegetative cells growing into microcolonies with Multichannel-SporeTracker shows that pH$_i$ and generation time of sorbic acid and acetic acid-treated are affected. Movies of sorbic acid (3 mM) and acetic acid (25 mM) and un-treated cells were analyzed with Multichannel-SporeTracker for 5 hrs for control and 10 hrs for sorbic acid and acetic acid stressed cells. Frequency distributions of sorbic acid and acetic acid-stressed (Black) and un-stressed cells (control, white) were calculated. Depicted are the frequency distributions of (A and C) the pH$_i$ after sorbic acid and acetic acid stress respectively, (B and D) the generation time of B. subtilis P$_{pts}$G-IpHluorin cells under sorbic acid and acetic acid stress in a microcolony. pH$_i$ and generation time for both the stress were compared with un-stressed cell (control). The total number of spores assessed for pH$_i$ in sorbic acid, acetic acid and control were 205, 131 and 151, whereas for generation time, total number of cells in sorbic acid, acetic acid and control were 109, 122 and 145 respectively.

expression in heterologous systems made it a unique reporter gene (Chalfie et al., 1994.). The advantage of GFP is that its expression has a low toxicity to cells and is known not to interfere with normal cellular function. Moreover, GFP is easily detectable and quantifiable by fluorescence microscopy and FACS analysis (Cheng et al., 1996.). Noticeably, GFP requires molecular oxygen to form the protein’s fluorophores (Heim et al., 1994). Thus this is a disadvantage of GFP in biological
systems where oxygen is limiting such as in the study of Clostridium spp. Here we used the expression of IpHluorin, Bacillus optimized pH sensitive GFP derivative that allows ratiometrical probing of the internal pH of Bacillus cells (Van Beilen and Brul, 2013). Ratiometric IpHluorin is a GFP variant that displays a bimodal excitation spectrum with peaks at 390 and 470 nm and an emission maximum at 510 nm. Upon acidification, IpHluorin emission upon excitation at 390nm decreases with a corresponding increase in the emission upon excitation at 470 nm. Phototoxicity often occurs upon repeated exposure of fluorescently labeled cells to light from high-intensity arc-discharge lamps. In their excited state, fluorophores of GFPs tend to react with molecular oxygen to produce free radicals called reactive oxygen species (ROS). The ROS react with oxidizable components in systems where oxygen is limiting such as in the study of Clostridium spp. Here we used the expression of IpHluorin, Bacillus optimized pH sensitive GFP derivative that allows ratiometrical probing of the internal pH of Bacillus cells (Van Beilen and Brul, 2013). Ratiometric IpHluorin is a GFP variant that displays a bimodal excitation spectrum with peaks at 390 and 470 nm and an emission maximum at 510 nm. Upon acidification, IpHluorin emission upon excitation at 390nm decreases with a corresponding increase in the emission upon excitation at 470 nm. Phototoxicity often occurs upon repeated exposure of fluorescently labeled cells to light from high-intensity arc-discharge lamps. In their excited state, fluorophores of GFPs tend to react with molecular oxygen to produce free radicals called reactive oxygen species (ROS). The ROS react with oxidizable components in

Figure 6.5: Growth rate vs. pH$_i$ of B. subtilis $P_{pt3}$ G-IpHluorin cells growing into microcolonies in un-stressed and cell stressed with sorbic acid and acetic acid. Movies of cells stressed with sorbic acid (3 mM) and acetic acid (25 mM) and un-treated cells were analyzed for pH$_i$ and generation time with Multichannel-SporeTracker. The pH$_i$ of B. subtilis $P_{pt3}$ G-IpHluorin cells was plotted against the growth rate. Cells with a lower pH$_i$, set with 3mM sorbic acid or 25 mM acetic acid, all showed a lower growth rate. The squares represent measured data points and the black line gives the fit according to the Henderson-Hasselbalch equation.
Chapter 6: Intracellular pH response to weak acid stress in individual *Bacillus subtilis* vegetative cells: Use of IpHluorin for live-imaging

Figure 6.6: Time-resolved (I) phase contrast and (II) fluorescent images showing growth and division of *B. subtilis* *P*<sub>ptpG</sub>-IpHluorin vegetative cells in absence (control) and presence of sorbic acid (3 mM) and acetic acid (25 mM). Exponentially growing cells were spotted on minimal defined medium to pH 6.4 with and without sorbic and acetic acids. Control shown in panel A and sorbic and acetic acid stressed cells are shown in panel B and C, respectively. The cells are followed in time using fluorescent microscopy and analyzed by a macro written in Fiji (see Materials and Methods). The color code pH<sub>i</sub> scale is shown at the bottom of the image.
6.4. Results

the cells, such as proteins, nucleic acids and lipids thereby damaging subcellular structures of cells (Wright et al., 2002; Jakubowski et al., 1997; Foyer et al., 1994) often compromising cell vitality and viability (Vrouenraets et al., 2003; Dixit et al., 2003; Martin et al., 2005; Heim et al., 1999). Such ROS-mediated phototoxicity is mainly dependent on photochemical properties of the fluorophores (Sugden et al., 2004) and the dose of excitation light (Foyer et al., 1994). To minimize the phototoxicity, the dose of the excitation-light can be minimized. This decrease of excitation-light causes a reduced fluorescence signal, which obviously negatively influences the image quality of the fluorescent microscope by decreasing the signal to noise ratio (S/N) (Sheppard et al., 1995). Thus the quest is always to find the proper balance between light dose and cell viability. The closed air-containing chamber described in chapter 3 (Pandey et al., 2013) was used for fluorescent microscopy. In this chamber cells were sandwiched between a glass coverslip and a thin (160 µm) agarose-medium pad to ensure their immobilization and the supply of sufficient culture medium and enough oxygen for unperturbed growth. Pandey et al. (2013) showed that in this chamber the generation time of the bacteria was in good agreement with the generation time of cells grown in a well aerated shake flask. This shows that the developed chamber is well suited for the study of growth dynamics of aerobic bacteria. The automated programs multichannel-sporetracker allowed for an effective data analysis. The program measures the $pH_i$, a crucial cellular parameter involved in growth physiology, by ratiometric fluorescence measurement of IpHluorin emission at 510 nm after excitation at 390 and 470 nm respectively. This as well as the generation time of the exponentially growing vegetative cells can be determined in any desired time frame. The data is relevant for the food industry as it gives information about the effect of different preservatives on the intracellular pH at single cell level thereby allowing it to be linked to a stochastic analysis of bacterial growth in cellular populations. Such analysis is useful for the food industry in their risk assessment procedures for microbiological food stability.

Weak acids are naturally occurring preservatives that are commercially used in the food industry. They extend shelf life of food products by inhibiting microbial growth. The widely accepted theory of weak acid preservative action suggests inhibition of growth through lowering of the internal pH ($pH_i$). According to the theory undissociated acid molecules pass readily through the plasma membrane by diffusion. In the cytoplasm (pH 7.0) the acid molecules dissociate into charged anions and protons. These cannot pass across the lipid membrane and hence accumulate in the cytoplasm, lowering there the internal pH ($pH_i$) of the cell. The acidification of the cytoplasm in turn inhibits metabolism. A recent study by van Beilen et al. (2014, in press) shows that sorbic acid has an ability to act as a classical uncoupler, transporting protons over the membrane whereas acetic
acid, which is less lipophilic, does so to a much lesser extent. This is corroborated by the fact that sorbic acid has a greater effect on the membrane potential, while acetic acid only carries bulk volume protons across the membrane until a steady state is reached. Direct measurement of the internal pH may be used as a proxy for cellular metabolism and thereby provide rapid insight in survival strategies at the single cell level. In this study we analysed the effect of sorbic and acetic acid on vegetative cells grown in defined minimal medium. At low concentration of sorbic acid, the $pH_i$ decreases with increase in generation time. Similar results were obtained from the analysis of acetic acid treated cells, albeit at higher acid concentrations. Clearly distribution of generation times widened.

In conclusion, the single-cell analysis techniques can enhance the mechanistic basis of food preservation affecting the bacterial growth. The closed air-containing chamber and image analysis tool can be used to study the effect of different stresses, on internal pH and growth rate of vegetative cells. Future experiments involve the study of higher concentration of sorbic and acetic acid as well as other weak-acids such as lactic and benzoic acid for effects on the internal pH and explore the quantitative effect on growth rate of *B. subtilis*. The analyses can be extended to the ratiometric assessment of the dynamics of the internal pH of spores during germination and outgrowth and resulting vegetative cells growth. It will allow us to point out the phase where the weak acids have maximum effect and also could provide key information about the timing of weak organic acid action when it enters in individual germinating/outgrowing spores. This information can be coupled to risk management of unwanted growth of bacteria in food and hence can help in combating the spoilage of food products in the food industry. Eventually for instance a micro-fluidics variant of the system could be used to perform live-imaging. This would allow researchers to change the growth media during experiments and to monitor the subsequent dynamics of spore germination and outgrowth as well as vegetative growth. Thus such experiments should provide ways to deconvolute the population data with respect to effects of different consecutive or combined stresses on the germination and (out)growth efficiency of *B. subtilis* spores.

### 6.4.5 Acknowledgments

We thank Johan van Beilen from university of Amsterdam, for providing *B. subtilis* $P_{pts}$G-IpHluorin construct.
6.4.6 References


Henriques, M., Quintas C. and Loureiro-Dias M.C. (1997). Extrusion of benzoic
Chapter 6: **Intracellular pH response to weak acid stress in individual Bacillus subtilis vegetative cells: Use of IpHluorin for live-imaging**

acid in Saccharomyces cerevisiae by an energy-dependent mechanism. Microbiology.143 (6), 1877-83. doi: 10.1099/00221287-143-6-1877


secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 394, 192-195.10.1038/28190.
doi:10.1038/28190
Orij, R., Brul, S and Smits, G.J. (2011). Intracellular pH is a tightly con-
trolled signal in yeast. Biochimica et Biophysica Acta 1810(10), 933-944. doi:
10.1016/j.bbagen.2011.03.011
Orij, R., Postmus, J., Ter Beek, A., Brul, S. and Smits, G.J. (2009). In vivo
measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP deriva-
tive in Saccharomyces cerevisiae reveals a relation between intracellular pH and
growth. Microbiology (UK) 155(1) 268-278. doi:10.1099/mic.0.022038-0.
Pandey, R., Ter Beek, A., Vischer, N. O. E., Smelt, J., Brul, S. and Manders,
E. M. M. (2013). Live cell imaging of germination and outgrowth of individ-
ual Bacillus subtilis spores; the effect of heat stress quantitatively analyzed with
SporeTracker. PLoS ONE 8:e58972. doi: 10.1371/journal.pone.0058972
Piper, P., Mahe, Y., Thompson, S., Pandjaitan, R., Holyoak, C., Egner, R.,
required for the development of weak organic acid resistance in yeast. The EMBO
Journal. 17(15),4257-4265. doi: 10.1093/emboj/17.15.4257
focal microscopes. In Handbook of Biological Confocal Microscopy, edn. 2 (ed.
Shioi, J. I., Matsuura, S. and Imae, Y. (1980). Quantitative measurements of
measurement and homeostasis in bacteria and archaea. Advance in microbial
Physiology 317(55),1-79. doi: 10.1016/S00065-2911(09)05501-5
of sorbic acid on bacterial cells and spores. International Journal of Food Micro-
biology. 3(1),1-17. doi: 10.1016/0168-1605(86)90036-X
an organism that reproduces by morphologically symmetric division. PLoS One
3:295-300 doi: 10.1371/journal.pbio.0030045
Sugden, J.K (2004). Photochemistry of dyes and fluorochromes used in biol-
ogy and medicine: some physicochemical background and current applications.
Biotech. Histochem. 79(2), 71-90. doi:10.1080/10520290412331292400
Ter Beek, A. (2009). Weak organic acid stress in Bacillus subtilis. University of
Amsterdam
Ter Beek, A., Janneke, G.E., Wijman, Zakrzewska, A., Orij, R., Smits, G.J. and
organic acid stress in Bacillus subtilis. Food Microbiology. 1-12.doi:10.1016/j.fm
.2014.02.013
Chapter 6: Intracellular pH response to weak acid stress in individual Bacillus subtilis vegetative cells: Use of IpHluorin for live-imaging


