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Intracellular pH response to weak acid stress in individual vegetative *Bacillus subtilis* cells

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Running title: Intracellular pH response to weak acid in single cells

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Abstract

The intracellular pH (pH$_i$) critically affects bacterial cell physiology. Hence, a variety of food preservation strategies aim at perturbing pH$_i$ homeostasis. Unfortunately, accurate pH$_i$ quantification with existing methods is suboptimal since measurements average across populations of cells, not taking into account inter-individual heterogeneity. Yet, physiological heterogeneity in isogenic populations is well known to be responsible for differences in growth and division kinetics of cells in response to external stressors. To assess in this context the behavior of intracellular acidity, we have developed a robust method to quantify pH$_i$ at single-cell levels in *Bacillus subtilis*. Bacilli spoil food, cause disease and are well known for their ability to form highly stress-resistant spores. Using an improved version of the genetically encoded ratiometric pHluorin (IpHluorin), we have quantified pH$_i$ in individual *B. subtilis* cells, cultured at an external pH of 6.4, in the absence or presence of weak acid stresses. In the presence of 3 mM potassium sorbate, a decrease in pH$_i$ and an increase in the generation time of growing cells were observed. Similar, effects were observed when cells were stressed with 25 mM potassium acetate. Time-resolved analysis of individual bacteria in growing colonies shows that after a transient pH decrease, long-term pH evolution is highly cell-dependent. The heterogeneity at single cell level shows the existence of subpopulations that might be more resistant and contribute to population survival. Our approach contributes to an understanding of pH$_i$ regulation in individual bacteria and may help scrutinizing effects of existing and novel food preservation strategies.
1. Introduction

Microbes have evolved to maintain a narrow range of optimal intracellular pH (pH\textsubscript{i}) values. For instance, during optimal growth conditions \textit{Bacillus subtilis} maintains its cytoplasmic pH at neutral or slightly higher values, the exact range depending somewhat on the measurement tool used (compare ref. 1 with our data). The pH\textsubscript{i} affects many biological processes such as enzyme activity, reaction rates, protein stability, and the structure of different molecules such as nucleic acids. Thus, pH\textsubscript{i} of bacteria is very important to ensure optimal growth, and conversely, perturbing the physiological pH\textsubscript{i} is a strategy that is often exploited by the food industry for preservation purposes. Weak acids such as sorbic, acetic, lactic and benzoic acids are naturally occurring preservatives that are commercially used in the food industry. These molecules are long known to inhibit the outgrowth of both bacterial and fungal cells (2), thereby allowing for the extension of the shelf-life of food products. Sorbic acid and its salts inhibit the growth of various bacteria including sporeformers, at various stages of their life cycle including spore germination, outgrowth, and vegetative cell division (3). The widely accepted theory of weak acid preservative action suggests inhibition of growth through lowering the pH\textsubscript{i}. According to the theory, undissociated acid molecules pass depending on their lipophilicity more or less readily through the plasma membrane by diffusion. In the cytoplasm (pH> 7.5) the acid molecules dissociate into charged anions and protons. These cannot pass across the lipid membrane and hence accumulate in the cytoplasm, lowering the pH\textsubscript{i} of the cell. The acidification of the cytoplasm, in turn, inhibits metabolism. A recent study by van Beilen et al. (4) 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. Studies by Holyoak et al. and Bracey et al. (5, 6) showed that in yeast the inhibitory action of weak acid preservatives evokes an energetically expensive stress response. This response in *S. cerevisiae* is based on a membrane localized efflux system that removes both the accumulated anions as well as the excess protons, from inside the cell (7, 8). The attempts to restore homeostasis, however, require significant amounts of ATP hence resulting in a drop of available energy pools for growth and other essential metabolic functions. In summary, weak acids inhibit the growth of microbes in a number of ways including through membrane perturbation, inhibition of essential metabolic reactions (6, 3), and stress on pH, homeostasis as well as the accumulation of toxic anions (6, 9).

Direct measurement of the pH, may be used as a proxy for cellular metabolism and thereby provide rapid insight in survival strategies at the single-cell level. The pH, of the cells can be measured by various methods such as $^{31}$P NMR, fluorescent dyes (most noticeably 5 (and 6-) carboxyfluorescein diacetatesuccinimidyl ester) and the distribution of radio-labeled membrane-permeable weak-acids (10-14). The advantage of these methods is that they do not require genetic modification. In the case of fluorescent dyes, single cell measurements are possible (15). The disadvantage of using weak organic acid dyes is that they may themselves alter the pH, The disadvantage of the $^{31}$P NMR and radio-labeled compounds are that they require extensive cell handling and high cell density, which also disturbs the cell’s physiology. Fluorescent proteins make an attractive, non-invasive alternative for measuring the pH, of the bacterial cell though
obviously here genetic modification is a prerequisite. pHluorin, a ratiometric, pH-sensitive GFP variant (16), allows direct, fast, and localized pH$_i$ measurements. It has been successfully used in *S. cerevisiae* (17, 18) and more recently in *B. subtilis* (19-21), to probe cellular responses to various growth conditions, glucose pulses, respiratory chain inhibitors, and a few other treatments. A specific advantage of a fluorescence-microscopy based method is that it can provide information with (sub)cellular resolution (22,23). This allows for the capturing of inter-individual phenotypic heterogeneity that arises from factors such as differential growth kinetics and stochastic effects at the level of gene expression and protein activity. Taking advantage of this added value, we analyzed the effect of sorbic and acetic acid on the perturbation of the pH$_i$ of *Bacillus subtilis* vegetative cells using an improved IpHluorin reporter.
2. Materials and Methods

2.1 Growth conditions

To monitor the pH\(_i\) of exponentially growing \textit{B. subtilis} cells for a long period of time, the \textit{B. subtilis} PptsG-IpHluorin (\textit{trp}2\textit{C}; \textit{amy}E3' \textit{spcR} PptsG-\textit{IpHluorin amy}E5') construct was used (20). This construct consists of the IpHluorin gene (16), which was inserted after the first 24 bp of \textit{comGA} adjacent to the promoter PptsG. This promoter drives the expression of the gene encoding the glucose-specific phosphotransferase system II. Thus we were able to obtain expression of IpHluorin in vegetative cells growing on a glucose-containing medium. The \textit{B. subtilis} 168 laboratory wild-type strain PB2 and \textit{B. subtilis} PptsG-IpHluorin were grown exponentially in Luria Broth (LB) at 37°C, under continuous agitation at 200 rpm. The exponentially growing cells were re-inoculated in a minimal defined medium with 80 mM MOPS (3-(N-morpholino) propanesulfonic acid) (26), here buffered to pH 7.4; hereafter referred to as MOPS medium. The MOPS medium contained spectinomycin (50 μg/ml) when appropriate, and cells were grown until exponential phase at 37°C, under continuous agitation at 200 rpm. The optical density at 600\text{nm} (OD\(_{600\text{nm}}\)) was measured in time to check whether the cells were in the exponential phase. Cells in the early exponential growth phase (OD\(_{600\text{nm}}\) = ~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.

2.2 Phototoxicity measurements
Phototoxicity is a detrimental phenomenon in live-cell imaging, which occurs upon repeated exposure of fluorescently labeled cells to intense light. In order to test for possible phototoxicity, exponentially growing *B. subtilis* PB2 and *B. subtilis* PptsG-IpHluorin cells (grown in MOPS medium under live-imaging conditions, see below) were repetitively 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 an interval of 5 min and 10 min. The exposure time was chosen in such a way that the bleaching in each channel has the same rate. The generation time of the cells was calculated with a home-written script for ImageJ (http://imagej.nih.gov/ij/) (25), multichannel-SporeTracker. The total number of cells assessed for *B. subtilis* PB2 cells grown in the absence of fluorescent light was 107 and for *B. subtilis* PptsG-IpHluorin cells cultured in the absence and presence of fluorescent light between 77 and 164. The effect of phototoxicity on the cells was regarded as negligible when the generation times of vegetative cells did not differ significantly (t-test, p> 0.05).

2.3 Fluorescence time-lapse microscopy (live-imaging)

In order to ensure the unbiased growth of aerobic bacteria, a closed air-containing chamber that has been described previously (25) was used for time-lapse fluorescence microscopy. In this chamber, cells were sandwiched between the glass coverslip and a thin (160 μm) 1% agarose-medium pad, molded in a Gene Frame®, to ensure their immobilization in the presence of sufficient culture medium and enough oxygen for undisturbed growth. The pad was loaded with 1 μl of exponentially growing vegetative
cells (OD$_{600\text{nm}}$ = ~2). 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 oil objective mounted on a Zeiss wide field fluorescence microscope (Axiovert-200 Zeiss, Jena, Germany) controlled by Metamorph 6.1 software. For ratiometric imaging, light from a Xenon arc lamp was filtered by a monochromator (Optoscan, Cairn Research Ltd, UK) and tuned to 390 nm or 470 nm, each with a bandwidth of 30 nm. The microscope was equipped with a standard GFP filter cube (Chroma) with 510 nm LP emission filter. Images were acquired with a CoolSnap HQ CCD camera (Roper Scientific). For phase contrast imaging, a red filter (610 nm LP; Schott AG, Germany) was placed in the light path to protect the cells from phototoxicity. For control experiments, the time-lapse series of phase-contrast and fluorescence images were recorded at a sampling frequency of 1 frame per 10 min (see Results section for the final choice of 10 min intervals) 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 replicate.

2.4 pH$_{i}$ measurements in a microcolony and in single cells within a microcolony

For pH$_{i}$ measurements, two image analyses tools for ImageJ were used. “Multichannel-SporeTracker”
was developed for pHᵢ measurements at the microcolony level. This program runs in combination with ObjectJ, a plugin for ImageJ. ObjectJ supports graphical vector objects that non-destructively mark images on a transparent layer. It provides back-and-forth navigation between results and images. The results table supports statistics, sorting, color-coding, qualifying, and macro access.

Multichannel-SporeTracker allows accurate measurements of the intensity of IpHfluorin in the cell, calculates the ratio \( \frac{E_{390}}{E_{470}} \) of IpHfluorin and deduces the pHᵢ and the generation time of the vegetative cells growing into a microcolony at any desired time frame (Figure 1). The generation time is calculated from the area \( \log_2 \) of the growing cell population. The pHᵢ measurements are based on the ratio of the fluorescence emission at 510 nm after excitation at 390 nm and 470 nm respectively \( \left( \frac{E_{390}}{E_{470}} \right) \). To calculate the pHᵢ, fluorescence images were first aligned with the corresponding phase contrast images. Before measuring the fluorescence intensity of the cells, temporal intensity fluctuations were buffered by subtracting the mode (most frequent) value per frame throughout the time-lapse image stack. Then, the fluorescence intensities of IpHfluorin expressing cells were measured in both fluorescence channels within cellular regions of interest and the \( \frac{E_{390}}{E_{470}} \) ratio was calculated. By correlating the ratio with a calibration curve (mentioned below), the pHᵢ of the cell was determined.

For pHᵢ measurements at the single cell level, a custom-made script for IJ/FIJI ColiMetrics.ijm was used. The algorithm segments individual bacteria and tracks them through time. It also converts the two-channel fluorescence image (excitation at 390 nm and 470 nm) into a color-coded image
representing the pH\textsubscript{i} for every individual cell in a micro colony (i.e. pH maps). pH\textsubscript{i} maps are HSV (Hue, Saturation, Value) images, in which the hue represents the ratio of both fluorescence channels, converted into a pH\textsubscript{i} value according to a sigmoidal fit of the calibration curve, and the value the average intensity of both channels (expansion of a macro described before. See reference 27). For single cell analysis, individual bacteria were tracked through time up to the point of cell division. Ratio values E\textsubscript{390}/E\textsubscript{470} were measured per cell and plotted as a function of time.

2.5 Calibration of pH\textsubscript{i}

*B. subtilis* PptsG-IpHluorin cells were grown to exponential phase in MOPS medium to pH 7.4 containing spectinomycin (50 μg/ml). At an optical density of 0.4 (OD\textsubscript{600nm}), the cells were centrifuged (1073 g; 10 min) and resuspended in phosphate-citrate buffers (0.1 M citrate and 0.2 M K\textsubscript{2}HPO\textsubscript{4}) with pH values ranging from 5.5 to 8.5 as described previously by us (20). The cells were then permeabilized with the potassium ionophore valinomycin (1 μM) and the protonophore nigericin (1 μM) (13). This treatment allows for the equilibration of the pH\textsubscript{i} with the externally set pH. Subsequently, cells in the phosphate-citrate buffer of different pH (5.5 to 8.5) were transferred to agarose pads with the ionophores at 1μM and of the corresponding buffered pH values in closed air-containing chambers (20). For each pH, fluorescence images of ~200 cells were analyzed with Multichannel-SporeTracker to construct a calibration curve (Figure 2). This curve represents the relationship between the ratio of the 510 nm emission intensities of IpHluorin upon excitation at respectively 390 nm and 470 nm (E\textsubscript{390}/E\textsubscript{470}) and the pH\textsubscript{i}.
The data was fitted to a slightly modified Henderson-Hasselbalch equation. It describes the relation between the ratio of the intensity of wavelengths \( R = \frac{E_{390}}{E_{470}} \) and pHi. Here, \( R = \frac{(10^{\text{pHi}-\text{pK}_a})}{(10^{\text{pHi}-\text{pK}_a}+1))} \times b + a \). \( \text{pK}_a \) is the negative 10 logarithm of the dissociation constant, \( b \) and \( a \) are parameters without physiological meaning but they are used to enable a quantitative fit of the \( R \) values. The fitting procedure was conducted as follows: In total 876 observations were available; 23 observations almost equally distributed across the different pH values were either negative of far beyond the set (3 standard deviations) to which they belonged. After elimination of these outliers the average of each local clusters was taken. In total 62 average values were obtained. To test the robustness of the model 20 average values were selected randomly and left out. The 42 remaining values were fitted according to our modified Henderson-Hasselbalch equation. The estimates of the parameters including confidence intervals are shown in Table 1 and Table 2. For nonlinear regression models the correlation coefficients between the parameters can be considered as acceptably low (28). The average residual sum of squares (RSS) was calculated for each pH value that was studied. No significant correlation was found between averages RSS and pH. The average sum of squares was 0.009688.

3. Results

3.1 Long-term ratiometric imaging of IpHluorin expressing cells is not phototoxic

A typical fluorochrome can only withstand a limited number of excitation cycles. Excessive illumination eventually leads to irreversible loss of fluorescence (photobleaching) and the production of free radicals that can damage cellular components
compromising cell viability (phototoxicity). The combined effect, i.e. photodamage, restricts long-term fluorescence live-cell imaging. Photodamage can be mitigated by the parsimonious use of illumination light but cannot be eliminated completely (29). To assess whether our imaging conditions allowed for monitoring bacterial cells without excessive photodamage, we compared the generation time with and without fluorescence illumination. In addition, we assessed the effect on cell growth of IpHluorin expression.

The generation time of wild-type *B. subtilis* PB2 cells grown in the absence of excitation light (93±13 min) was similar to the generation time of the IpHluorin-expressing cells grown in the absence of excitation light (92±17 min) (Figure 3). Therefore, we concluded that IpHluorin expression is not harmful to the cells under the tested conditions. Next, cells were exposed to sequential pulses of light of two different wavelengths (390 nm and 470 nm, for 100 and 30 ms, respectively) with 5 min or 10 min intervals between fluorescence measurements. Figure 3 also shows the effect of repeated exposure of cells to 390 nm and 470 nm excitation on the generation time of the *B. subtilis* PptsG-IpHluorin expressing cells. Both conditions, i.e. 5 min and 10 min intervals, resulted in a slight increase in generation time (116±3 min resp. 114±21 min) without causing cell death (no cell lysis was observed whilst analyzing either of the incubations microscopically). We conclude that our settings are suitable for up to 5 hours of pHl live-imaging and went for the longer 10 min interval in further experiments.

### 3.2 Sorbic and acetic acid impact on pHl and growth of *B. subtilis* cells

Sorbic and acetic acid have detrimental effects on bacterial cells. Both have a similar pKa of 4.76, but sorbic acid is lipophilic whereas acetic acid is hydrophilic in nature. Here the effect of exposure at pH 6.4 to 3 mM potassium sorbate and 25 mM potassium acetate
was studied in vegetative *B. subtilis* cells at single cell level. Figure 4 shows the effect of sorbic and acetic acids on the pH\textsubscript{i} and generation time of *B. subtilis* PptsG-IpHluorin vegetative cells growing in microcolonies. In sorbic acid-stressed cells, the average internal pH of the microcolonies decreased from 7.2 to 6.8 (Figure 4A, Table 3) and the generation time increased significantly (Figure 4A, Table 3). In 25 mM potassium acetate-stressed cells, a similar trend in internal pH and increase in generation time was observed (Figure 4A, Table 3). Figure 4B gives the correlation graph between the average internal pH of the microcolonies and generation time for control cells as well as cells stressed with the two acids. PptsG-IpHluorin expressing cells are typically lowered 50% in their growth-rate by the addition of 3mM potassium sorbate or 25 mM potassium acetate to liquid cultures whilst monitoring at population level pH\textsubscript{i} (4). Here, growth-rate inhibition by these concentration of both weak organic acids is more and the observed standard deviation for the population is high. This might reflect some light sensitization by the weak organic acid stresses. However, we have also shown previously that in the MOPS buffered defined medium used here, there is a \textasciitilde30% increase in generation time of wild-type *B. subtilis* cells compared to liquid culture conditions (25). Hence, though we cannot exclude that under our live-imaging conditions weak acid stress response may sensitize the PptsG-IpHluorin expressing cells to light, it will not be the major response seen.

In conclusion, acid stressed cells and control populations displayed both for pH\textsubscript{i} and growth-rate significant differences (p< 0,001). Figure 4C shows selected time-points from movies (see Videos S1, S2, S3 and Table S1) of *B. subtilis* PptsG-IpHluorin vegetative cells in the presence and absence of 3 mM potassium sorbate and 25 mM
potassium acetate, color coded by their pHᵢ. Noticeably, as was observed previously by
van Beilen et al. (figure 1 b in ref. 4), pHᵢ of control cells started in some at values above
8, indicating a somewhat stalled metabolic activity at the onset of imaging. In this regard
we noted a clear batch variation between the two biological repeats shown. Growth-rate
and average colony pHᵢ calculations with Multichannel-SporeTracker were always
performed from the time-point where clearly detectable surface increase, i.e. growth, had
resumed. pHᵢ was then generally ~7.5, values seen previously by van Beilen et al. in
liquid populations (4, 20).

3.3 Lineage tracing of individual cells in microcolonies reveals pHᵢ heterogeneity.

As noted in Methods, single *B. subtilis* cells grow and divide to form micro colonies. We
observed that within a developing microcolony the E₃₉₀/E₄₇₀ fluorescence ratio of
individual *B. subtilis* cells differs. This shows that there is heterogeneity in pH between
individual *B. subtilis* cells in a microcolony at a given time-point of culture. Figure 5
shows for control conditions a typical example of tracking individual cells, growing from
a single cell up to a microcolony. It became clear that under those conditions, after a
transient drop in pHᵢ presumably due to the increased levels of acetate made by the
growing bacteria themselves (see e.g. ref. 4), individual bacteria are likely able to mount
to varying extent a response that allows them to finally again raise their internal pH albeit
to varying degrees. Such variation in pHᵢ is also observed for the intentionally, at time
zero, weak organic acid stressed cells. This is in particular true for those exposed to
sorbic acid (Figure 4A, B). Under 3mM potassium sorbate stress conditions strikingly,
microcolonies emerged with pHᵢ values well within the range of those from control cells
as well as well below. While $\text{pH}_i$ is clearly a major determinant for growth-rate it is
definitely not the only one, certainly not under sorbic acid stress.

### 4. Discussion

Here, we deployed a derivative of green fluorescent protein (GFP), IpHluorin, to probe at a single cell level the $\text{pH}_i$ of *B. subtilis* cells. The use of this genetically encoded reporter holds various advantages such as the inherent labeling, strong signal-to-noise ratio, and concentration-independence. A potential disadvantage is its requirement for molecular oxygen, precluding its use in anaerobic species such as *Clostridium* spp.

Using stably expressing IpHluorin *B. subtilis*, we have established a robust microscopy-based assay for simultaneously measuring $\text{pH}_i$ and generation time. We have first tuned the imaging conditions so as to minimize phototoxicity. Subsequently we have established a calibration curve showing strong correlation of the fluorescence ratio with the externally adjusted pH ranging from 5.5 to 8.5. Once optimized, we have benchmarked our assay using two well-known weak acid preservatives, namely sorbic and acetic acid. Also, to analyze the microscopy images we developed a semi-automated image analysis tool based on the previously published Spore-Tracker (25), called “Multichannel-SporeTracker”. This tool calculates the internal pH and the generation time of exponentially growing *B. subtilis* PptsG-IpHluorin vegetative cells. It allows us to monitor individual cells and subpopulations and deconvolute the population level information at single cell level.

The analysis of the effect of sorbic acid and acetic acid on vegetative cells showed that at low concentration of sorbic acid, the generation time increases with decreasing $\text{pH}_i$. 
Similar results were obtained from the analysis of acetic acid treated cells, albeit at higher acid concentrations and a wider distribution of generation times is seen. Thus, at the selected concentrations both acids reduce the pH$_i$ and the growth-rate to a similar extent. This result corroborates the notion that sorbic acid is the more effective preservative of the two. Van Beilen et al. showed that sorbic acid is unable to recover pH$_i$ during acid stress. These observations reflect the notion that sorbate acts as a classical uncoupler, which shuttles protons over the membrane whereas, acetate is believed to do this to a much lesser extent (4). The data of van Beilen et al. show that sorbic acid has an effect on the membrane potential while acetic acid carries only bulk volume protons across the membrane until a steady state is reached leaving Δψ relatively unaffected. In line with this, Orij et al. and van Beilen et al. (4, 17) have shown at the population level that the growth-rate and pH$_i$ can be correlated (4). We now also demonstrate a similar correlation at the single cell level, demonstrating that pH$_i$ can be assessed as a good indicator of individual ‘bacterial health’. From this knowledge of pH$_i$ one could infer at the individual cell level the activity of metabolic pathways key to cellular energy conversion. Such data may be used by food microbiologists to feed contemporary models that aim at quantitatively predicting microbial food stability. The study suggests that heterogeneity at the individual cell level is prominent with important implications for weak organic acid based food preservation strategies. We can now, through lineage tracing, also start to verify mechanistically whether under long term weak organic acid stress conditions, subpopulations of *Bacillus* cells arise that might be more able to restore their pH$_i$ hence explaining their better survival in foods and outgrowth potential to new (micro-)colonies
that can spoil foods. It may also be applied to the analysis of other potential food spoilage organisms, such as e.g. *Zygosaccharomyces bailii* (30).

In conclusion, our microscopy-based single-cell analysis technique effectively allows for gauging pH$_i$ and relating it to generation time. In doing so, the method can further mechanistic insight in the principles of existing and novel food preservation strategies. The analysis can be extended to the ratiometric assessment of the dynamics of the pH$_i$ of spores during germination and outgrowth. It will allow one to point out the phase where weak acids have a maximum effect and also could provide key information about the timing of weak organic acid action on individual germinating and outgrowing spores. This information can be coupled to risk management of the unwanted growth of bacteria in food and hence help food industry to combat food spoilage. An attractive platform for implementing such an assay would be a microfluidics-based lab-on-chip (31). Such a platform allows for rapid measurement of pH$_i$ values under dynamically changing conditions (change of media types or supplements) coupled to monitoring of the dynamics of spore germination and outgrowth. This type of experiments should provide ways to deconvolute the population data with respect to effects of different sequences of stresses on the germination and (out-)growth efficiency of *B. subtilis* spores.

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27. **Motulsky, H. and Christopoulos, A.** eds. 2004. Fitting models to biological data


Figure Legends

Figure 1: Multichannel-SporeTracker output for pH\textsubscript{i} measurements in growing \emph{B. subtilis} cells. Shown here are collective plots of 4 individual cells measured every 5 min for 5 hours. Bottom to top: Log\textsubscript{2} (surface area occupied by cells); fluorescence intensities measured at 510 nm when excited at 390 nm (Fluor A) and 470 nm (Fluor B), respectively; the ratio of the excitation wavelength (390 nm and 470 nm) of fluorescence intensities (2\textsuperscript{nd} panel from the top) and pH\textsubscript{i} (top panel). Note that here 4 cells are shown
from the batch that starts at pH$_i$ > 8; in cells from other batches pH$_i$ was lower (~7.5) at the onset of imaging (see text and figure 5 lineage tracking for comparison).

**Figure 2:** Calibration curve of *B. subtilis* PptsG-IpHluorin, which describes the relation between the ratio of the emission intensity at 510 nm after excitation at 390 nm and 470 nm respectively (E$_{390}$/E$_{470}$) and pH$_i$. The *B. subtilis* PptsG-IpHluorin cells were permeabilized using 1 μM nigericin and 1 μM valinomycin and immobilized on agarose pads containing both compounds and of the set pH values ranging from 5.5 to 8.5. The cell fluorescence emission intensities were measured and the ratio (E$_{390}$/E$_{470}$) was plotted against pH$_i$. At least 200 cells were measured per data point. Error bars indicate the standard deviation. The figure gives a comparison between the observations that were fitted (observed 1 Δ), the observations that were not used for fitting (♦) and the actual fit according to the Henderson-Hasselbalch equation based on observed 1 (---).

**Figure 3:** 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 the absence of fluorescent light and *B. subtilis* PptsG-IpHluorin cells in the absence and presence of fluorescent excitation light (390 nm and 470 nm) with a time interval of either 5 min or 10 min were made during 5h. Generation time analyzed by Multichannel-SporeTracker. The total number of cells assessed for *B. subtilis* PB2 cells grown in absence of fluorescent light was 107, for *B. subtilis* PptsG-IpHluorin cells in absence of fluorescent light 164 and for regularly illuminated PptsG-IpHluorin cells 77 (for the
specimens inspected every 10 minutes), and 92 (for those illuminated every 5 minutes).

No cell lysis was observed whilst analyzing either of the incubations.

Figure 4: Analysis of *B. subtilis* PptsG-IpHluorin vegetative cells growing into microcolonies with Multichannel-SporeTracker shows that pH$_i$ and generation time of sorbic acid and acetic acid-treated cells are affected. (A) pH$_i$ frequency distributions of microcolonies of sorbic and acetic acid-stressed (black) as well as control (gray) *B. subtilis* PptsG-IpHluorin cells were calculated per 0.05 pH unit bin from data obtained in two biological repeats. Depicted are the frequency distributions as well as the generation time of *B. subtilis* PptsG-IpHluorin cell microcolonies exposed to respectively 3 mM potassium sorbate or 25 mM potassium acetate at pH 6.4. (B) Growth-rate vs. pH$_i$ of *B. subtilis* PptsG-IpHluorin cells growing in microcolonies for unstressed cells and cells stressed with sorbic acid and acetic acid. Between the acid stressed cells and the control populations both the mean and the variance of pH$_i$ and growth-rate are significantly different (t-test p< 0.01). (C) Still images at set time-points of (I) phase contrast and (II) fluorescence data showing growth and division of *B. subtilis* PptsG-IpHluorin vegetative control cells (Top row) and cells grown in the presence of 3 mM potassium sorbate (Middle row) and 25 mM potassium acetate (Bottom row) at an external pH of 6.4. The acids were included in the agarose slides from the onset of the experiment. Imaging was done as described in Materials and Methods with a 10 min illumination interval.

Figure 5: Time-resolved ratiometric image showing growth and division of single *B. subtilis* PptsG-IpHluorin vegetative cell in the absence of stress at an external pH of 6.4.
(a) Montage of the ratio image in which the color represents the pH; (b) The pH profiles of individual color-coded cells superimposed on the standard deviation of the mean signal of the entire microcolony in grey; (c) Lineage tracking and ratio changes in grey scale.

Tables

Table 1 Parameter estimates of the slightly modified Henderson-Hasselbalch model (see Materials and Methods section 2.6) describing the relation between pH, and the ratio of the IpHluorin fluorescence emission intensity upon excitation at wavelengths 390/470 nm (R = E\textsubscript{390}/E\textsubscript{470}).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKa</td>
<td>7.112</td>
<td>0.055</td>
<td>7.001 - 7.223</td>
</tr>
<tr>
<td>b</td>
<td>1.625</td>
<td>0.048</td>
<td>1.528 - 1.722</td>
</tr>
<tr>
<td>a</td>
<td>0.703</td>
<td>0.040</td>
<td>0.621 - 0.785</td>
</tr>
</tbody>
</table>

Table 2 Correlations of parameter estimates reported in table 1.

<table>
<thead>
<tr>
<th></th>
<th>pKa</th>
<th>b</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKa</td>
<td>1.000</td>
<td>-0.047</td>
<td>0.625</td>
</tr>
<tr>
<td>b</td>
<td>-0.047</td>
<td>1.000</td>
<td>-0.681</td>
</tr>
<tr>
<td>a</td>
<td>0.625</td>
<td>-0.681</td>
<td>1.000</td>
</tr>
</tbody>
</table>

The average RSS = 0.00968

Table 3: Mean values and standard deviation of internal pH and generation time of individual B. subtilis PptsG-IpHluorin vegetative cells in the presence and absence of sorbic acid and acetic acid\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Mean(min)±SD\textsuperscript{b}</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Potassium sorbate</td>
</tr>
</tbody>
</table>

\textsuperscript{a} sorbic acid and acetic acid
<table>
<thead>
<tr>
<th>pH&lt;sub&gt;i&lt;/sub&gt;</th>
<th>7.20±0.24 (n=151)</th>
<th>6.78±0.14 (n=205)&lt;sup&gt;**&lt;/sup&gt;</th>
<th>6.76±0.11 (n=131)&lt;sup&gt;**&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation time (min)</td>
<td>114±21 (n=164)</td>
<td>304.63±109.70 (n=109)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>286.13±80.78 (n=122)&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a B. subtilis PptsG-IpHluorin vegetative cells were grown in MOPS medium stressed with or without 3 mM potassium sorbate and 25 mM potassium acetate.

b Standard deviation.

* Indicates the variance of the distributions between the stress and control experiment are significantly different (p<0.01).

# Indicates the mean of the distributions between the stress and control experiment are significantly different (t-test, p<0.01).

The amount of cells analyzed for pH<sub>i</sub> and generation time determination are gathered from two (control, sorbic acid, acetic acid) microscopy experiments and given in brackets.

**Supplementary Data**

**Video S1.** Growth of B. subtilis PptsG-IpHluorin vegetative cells in defined minimal (MOPS-buffered) medium (pH 6.4). The video shows three movies of respectively the phase contrast image as well as the fluorescent emission images upon excitation at 390 nm and 470 nm.

**Video S2.** Growth of B. subtilis PptsG-IpHluorin vegetative cells in defined minimal (MOPS-buffered) medium (pH 6.4) containing 3 mM potassium sorbate. The video
shows three movies of respectively the phase contrast image as well as the fluorescent emission images upon excitation at 390 nm and 470 nm.

Video S3. Growth of *B. subtilis* PptsG-IpHluorin vegetative cells in defined minimal (MOPS-buffered) medium (pH 6.4) containing 25 mM potassium acetate. The video shows three movies of respectively the phase contrast image as well as the fluorescent emission images upon excitation at 390 nm and 470 nm.

Table S1. Results obtained from Multichannel-SporeTracker of growth of *B. subtilis* PptsG-IpHluorin vegetative cells at single cell level. Exponentially growing *B. subtilis* PptsG-IpHluorin vegetative cells were inoculated in defined minimal (MOPS-buffered) medium (pH 6.4) supplemented with (A) nothing (control), (B) 3 mM potassium sorbate and (C) 25 mM potassium acetate. Note that as was observed previously by van Beilen et al. (figure 1 b in ref. 4) pH$_i$ may start at values above 8 likely indicating a stalled metabolic activity of the (control) cells at the start of imaging. Growth-rate and average colony pH$_i$ calculations with Multichannel-SporeTracker were always performed from the time-point where discernible surface increase, i.e. growth, had resumed. pH$_i$ values at the start of our observations differed between both batches of *B. subtilis* cells shown.
IpHfluorin pH calibration curve

- \( \frac{E_{390\text{nm}}}{E_{470\text{nm}}} \) vs pH

- \( \triangle \): observed 1
- \( \bullet \): observed (left out in fitting)
- Dashed line: fitted based on observed 1