Weak organic acid stress in Bacillus subtilis

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Chapter 5

In vivo Measurement of Intracellular pH using a pH-Sensitive GFP Derivative in Bacillus subtilis Reveals Acidification of the Cytosol upon Weak Organic Acid Stress and Hyperosmotic Shock

Manuscript in preparation:
Alex Ter Beek, Janneke G. E. Wijman, Rick Orij, Gertien J. Smits, and Stanley Brul
5.1. Abstract

We constructed a plasmid (pDG148-pHluorin) to measure in vivo the intracellular pH (pHᵢ) of Bacillus subtilis. With this inducible ‘pH meter’ we tested the effect of weak organic acids and hyperosmotic shock on the pHᵢ of B. subtilis. The commonly used food preservatives potassium -sorbate (KS), -acetate (KAc), and -benzoate (KB), all three lowered the pHᵢ of B. subtilis. The uncoupler carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) and hyperosmotic shock induced by NaCl conferred a decline in pHᵢ as well. The main drop in pHᵢ occurred for all stresses within the first six minutes and no recovery to the control value of pH 7.3 ± 0.1 was observed during 2 h of stress. The growth inhibition percentage caused by all stresses related to the drop in pHᵢ. However, no linear correlation was found, likely due to the buffering capacity of the cell. Comparison of the stresses (when taking into account the growth inhibition caused by each stress) revealed that CCCP gave the highest, and hyperosmotic shock the lowest pHᵢ drop. The preservatives KS, KAc and KB showed intermediate values. A clear threshold value of the growth inhibition caused by NaCl was needed before inducing a drop in pHᵢ. These results indicate that the growth-inhibitory effect of NaCl is not initially and solely acidification of the pHᵢ, yet this is more the case for the tested weak organic acids, and primarily for CCCP.
5.2. Introduction

The intracellular pH (pHi) affects many cellular processes such as DNA transcription, protein synthesis and folding, enzyme activities, redox state and transport over the membrane (Beck & Jahns, 1996, Cotter & Hill, 2003, Foster, 2004, Veine et al., 1998). Reliable and fast pHi measurements in living cells are therefore crucial to the understanding of stress response and adaptation. Current techniques used to measure the pHi are $^{31}$P NMR (Ogino et al., 1983), probing with pH-sensitive fluorescent dyes (Breeuwer et al., 1995, Fang et al., 2004), and deploying radioactively labelled membrane-permeable weak acids or bases (Siegumfeldt et al., 2000, ten Brink & Konings, 1982). However, the disadvantages of these techniques are the limited time resolution and extensive manipulation of cells, which itself can affect pHi (Karagiannis & Young, 2001). A promising method for pHi measurements is based on in situ expression of the pH-sensitive GFP ratiometric pHluorin, which adjusts its excitation spectrum according to the pH of the surrounding environment (Miesenbock et al., 1998). Using this protein researchers were able to determine the pHi of *Escherichia coli*, *Lactococcus lactis*, and *Mycobacterium tuberculosis* (Olsen et al., 2002, Schuster et al., 2005, Vandal et al., 2008). Recently, we introduced ratiometric pHluorin into *Saccharomyces cerevisiae* and measured the cytosolic and mitochondrial pH (Orij et al., 2009).

One class of compounds that is thought to lower the pHi are weak organic acids (Beales, 2004, Davidson, 2001). These acids are most effective at external pH ($pH_{ex}$) conditions close to or below their pKₐ value. The undissociated form of the molecule is thought to diffuse freely over the membrane. Inside the cell the acid dissociates due to the higher existing pHi and releases protons into the cytosol. Consequently, the proton gradient dissipates and, depending on the buffering capacity of the cell, acidification of the cytosol may take place. It has been reported that this affects oxidative phosphorylation, the transport of nutrients and many other metabolic functions (Bauer et al., 2003, Brown & Booth, 1991, Ronning & Frank, 1987, Salmond et al., 1984, Sheu et al., 1975).

The food industry commonly utilizes weak organic acids, and their anionic salts, like potassium -sorbate (KS), -acetate (KAc), and -benzoate (KB), as they inhibit the growth of spoilage bacteria, yeasts, and moulds (Beales, 2004, Davidson, 2001, Piper et al., 2001). *Bacillus subtilis* is one of the organisms that causes food spoilage and its growth is inhibited by weak organic acids (Eklund, 1983, Ter Beek et al., 2008). Thus, we set out to construct a plasmid to measure the pHi of *B. subtilis* upon weak organic acid stress. In this study we expressed ratiometric pHluorin in *B. subtilis* and demonstrated that this allows direct and time-resolved monitoring of pHi in *B. subtilis* cells. We show that weak organic acids, as well as the uncoupler CCCP, and NaCl cause a drop in pHi. Our results further illustrate that CCCP more efficiently decreases the pHi than the tested food preservatives and that acidification of the cytosol is not the initial effect of hyperosmotic stress caused by NaCl.
Chapter 5

5.3. Materials and Methods

5.3.1. Strains, plasmids, and culture media

Bacterial strains and plasmids used in this study are listed in Table 5.1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C. When appropriate, ampicillin (100 µg/ml) or kanamycin (50 µg/ml) was used as antibiotic. *B. subtilis* strains were grown in LB or a defined minimal medium (Ter Beek et al., 2008) and supplemented with 10 or 5 µg/ml kanamycin when required. The defined minimal medium contained 25 mM glucose, 10 mM NH₄Cl and 10 mM glutamate (final concentrations) as carbon and nitrogen sources. Where indicated, the LB and defined minimal medium were buffered with 80 mM 3-(N-morpholino)propanesulfonic acid (MOPS), the pH was set to 6.4 with KOH, and isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>General cloning host</td>
<td>Stratagene</td>
</tr>
<tr>
<td>TOP10</td>
<td>General cloning host</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>MC1061</td>
<td>F' araD139Δ(ara-leu)7696 Δ(lac)X74 galU galK hsdR2 mcrA mcrB1 rspL</td>
<td>Casadaban and Cohen (1980)</td>
</tr>
<tr>
<td><strong>B. subtilis strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A700</td>
<td>trpC2; 168 wild-type</td>
<td>BGSC⁵⁵</td>
</tr>
<tr>
<td>ATB031</td>
<td>trpC2 pDG148; KmR</td>
<td>pDG148 → 1A700; this study</td>
</tr>
<tr>
<td>ATB032</td>
<td>trpC2 pDG148-pHluorin; KmR</td>
<td>pDG148-pHluorin → 1A700; this study</td>
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<td><strong>Plasmids</strong></td>
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<tr>
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<td>PCR cloning vector; KmR</td>
<td>Invitrogen</td>
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<tr>
<td>pDG148</td>
<td>Pspac; ApR, KmR</td>
<td>Stragier et al. (1988)</td>
</tr>
<tr>
<td>pDG148-pHluorin</td>
<td>Ratiometric pHluorin gene cloned into</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>pDG148</td>
<td></td>
</tr>
</tbody>
</table>

⁵⁵ KmR, kanamycin resistance; ApR, ampicillin resistance.

⁵⁵ B. subtilis Genetic Stock Center (http://www.bgsc.org/).

⁵⁵ Arrow indicates transformation of donor plasmid into recipient.

5.3.2. Construction of pDG148-pHluorin and transformation to B. subtilis

Standard molecular genetics techniques were used as described by Sambrook et al. (1989). Restriction enzymes, *Pfu* DNA polymerase and T4 DNA ligase were obtained from Fermentas. Plasmids were isolated from *E. coli* using a QIAprep Spin Miniprep Kit (Qiagen) and DNA fragments were isolated from agarose gels with a QIAEX II Gel Extraction Kit (Qiagen).

The ratiometric pHluorin gene (GenBank accession no. AF058694) was PCR amplified using primers pHBSF (5'-AAG CTT AAG GAG GAA GCA GGT AGT AGT AAA GGA GAA GAA C-3') and pHBSR (5'-C GTC GAC TTA TTT GTA TAG TTC ATC CAT GCC ATG TG-3') (Isogen Life Science) on pYES2-P<sub>ACT1</sub>-pHluorin (Orij et al., 2009) introducing HindIII and SalI restriction sites as well as a ribosome binding site. The pYES2-P<sub>ACT1</sub>-pHluorin plasmid
contains the gene coding for a functional ratiometric pHluorin without the L220F mutation described in Miesenböck et al. (1998), as well as a start and stop codon (Orij et al., 2009). The resulting 745-bp PCR product was cloned into the pCR-Blunt II-TOPO vector and transformed to competent *E. coli* TOP10 cells using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). DNA sequence analysis (BaseClear) confirmed the correct DNA sequence (data not shown). Next, the TOPO vector was digested with *Hind*III and *Sall* and the fragment containing ratiometric pHluorin gene was inserted into the *Hind*III and *Sall*-digested pDG148. The resulting plasmid pDG148-pHluorin was transformed to *E. coli* XL1-Blue and subsequently to MC1061 cells. Finally, we isolated the plasmid from *E. coli* MC1061 and transformed it to competent 1A700 *B. subtilis* cells as described previously (Kunst & Rapoport, 1995).

5.3.3. Validation of ratiometric pHluorin expression using fluorescence microscopy

1A700 *B. subtilis* cells harbouring the pDG148-pHluorin (ATB032) or the empty pDG148 plasmid (ATB031) were grown exponentially in shake-flasks in LB medium of pH 6.4, containing 10 µg/ml kanamycin at 37°C. Upon reaching an optical density measured at 600 nm (OD<sub>600</sub>) of 0.2, the production of ratiometric pHluorin was induced by the addition of 1 mM IPTG. The cells were maintained in the exponential phase by diluting the cultures in fresh medium containing 1 mM IPTG when necessary, such that an OD<sub>600</sub> of 1 was not exceeded. After 2.5 h of exponential growth the cells were harvested for microscopic analysis. Cells were immobilized on agarose slides as described by Van Helvoort et al. (1998) and photographed with a PowerShot A640 digital camera (Canon) attached to an Axiovert 40 CFL fluorescence microscope (Zeiss). In all experiments, the cells were photographed first in the phase-contrast mode and then using filter set 38 (excitation: 450 – 490 nm; emission: 500 – 550 nm) (Zeiss).

5.3.4. Calibration of pH

*B. subtilis* strains ATB031 and ATB032 (1A700 containing empty pDG148 or pDG148-pHluorin, respectively) were grown exponentially in shake-flasks in defined minimal medium of pH 6.4 containing 5 µg/ml kanamycin. At an OD<sub>600</sub> of 0.2 the production of ratiometric pHluorin was induced for 2.5 h by the addition of 1 mM IPTG. The cells were maintained in the exponential phase by diluting the cultures in fresh medium containing 1 mM IPTG when necessary, such that an OD<sub>600</sub> of 1 was not exceeded. At an OD<sub>600</sub> of 0.4 the cells were centrifuged and resuspended in buffers with pH values ranging from 5.6 to 8.2 prepared from 0.1 M citric acid and 0.2 M KH<sub>2</sub>PO<sub>4</sub>. The pH<sub>nex</sub> and pH<sub>i</sub> were equilibrated by the addition of 1 µM valinomycin and 1 µM nigericin (Breeuwer et al., 1995). The fluorescence was measured in a SpectraMax Gemini XS microtiter plate spectrofluorometer (Molecular Devices Corp.) and the ratio of emission intensity at 512 nm resulting from emission at 390 and 470 nm was calculated as described previously (Orij et al., 2009). The strain containing the empty pDG148
plasmid was used for background fluorescence. The calibration curve was determined by fitting the data of three independent biological replicates, each consisting of three technical replicates, with a polynomial curve of the fifth order.

5.3.5. Stress experiments and pH measurements

Cells containing empty pDG148 and pDG148-pHluorin were grown exponentially and ratiometric pHluorin expression was induced with 1 mM IPTG for at least 2.5 h as described above for the calibration of pH. At an OD<sub>600</sub> of 0.35 cells were transferred into two microtiter plate readers, measuring the fluorescence and optical density (SpectraMax Plus, Molecular Devices Corp.). At an OD<sub>600</sub> of 0.16 in the microtiter plate readers (which corresponds to an OD<sub>600</sub> of 0.4 in a 1-cm-path-length spectrophotometer), the cells were stressed with the following chemicals: KS (0.5, 1, 3, or 10 mM), KAc (5, 25, or 80 mM), KB (2 or 6 mM), CCCP (0.25, 0.5, 0.85, or 2 μM) and NaCl (0.1, 0.25, 0.5, 0.6, 0.75, or 1 M). Cells were cultivated in the microtiter plate readers under rigorous shaking at 37°C for 120 min. The percentages of growth inhibition (GI %) were calculated in the exponential phase from the increase in the optical density between 58 and 120 min of the control (no addition of stress) and the stressed culture. All conditions were tested in the plate readers four times, and biologically independent experiments were performed at least in duplicate.

5.4. Results

5.4.1. Construction of pDG148-pHluorin and expression in B. subtilis

Since the pH<sub>i</sub> is an important indicator for the fitness of a microorganism, we wanted to measure in vivo the pH<sub>i</sub> of B. subtilis cells under varying environmental conditions. Miesenböck et al. (1998) constructed a pH-sensitive version of GFP (ratiometric pHluorin), which allows for in situ expression and in vivo pH measurements. Therefore, we set out to clone the gene coding for ratiometric pHluorin into the pDG148 expression vector for B. subtilis (Stragier et al., 1988). This commonly used vector, utilized to overexpress promotorless genes in B. subtilis, contains an IPTG inducible spac promoter, the replicon from pUB110 for multiplication in B. subtilis, and a ble and a kan gene for selection by phleomycin and/or kanamycin. It also possesses the replicon of pBR322 and a bla gene for the replication and ampicillin selection in E. coli. After PCR amplification the ratiometric pHluorin gene including a start and stop codon, as well as a ribosome binding site was cloned into pDG148. The resulting plasmid pDG148-pHluorin was transformed to competent 1A700 wild-type B. subtilis cells.

We checked the expression of ratiometric pHluorin in the transformed B. subtilis strain using fluorescence microscopy. An exponentially growing culture of WT strain 1A700 harbouring pDG148-pHluorin (ATB032) was induced with 1 mM IPTG. After 2.5 h of induction and exponential growth the cells were harvested for inspection by fluorescent microscopy.
Fig. 5.1. Validation of ratiometric pHluorin expression using fluorescent microscopy. Exponentially growing 1A700 cells harbouring pDG148-pHluorin (A) and pDG148 (B) were monitored by phase-contrast microscopy (top panel) and fluorescence microscopy (bottom panel). Production of ratiometric pHluorin was visualized with fluorescence microscopy, as described in the Materials and Methods.

using a GFP filter. As a negative control we used the WT strain containing the pDG148 plasmid without the pHluorin insert (ATB031). A clear GFP signal was observed in the pDG148-pHluorin containing strain and not in the strain harbouring the empty vector (Fig. 5.1). Additionally, no GFP fluorescence could be observed for the strain containing pDG148-pHluorin that was not induced with IPTG (our unpublished data).

5.4.2. Calibration of pH$_i$

To measure the actual pH$_i$ using ratiometric pHluorin, the measured fluorescence signal needs to be calibrated with known pH values. Therefore, strains ATB031 and ATB032 were grown exponentially and induced with 1 mM IPTG to start production of ratiometric pHluorin. At an OD$_{600}$ of 0.4, cells were resuspended in buffers of known pH in the range of 5.6 – 8.2, permeabilized with a combination of valinomycin and nigericin, and their fluorescence was measured. A combination of the ionophores valinomycin (K$^+$ uniporter) and nigericin (electroneutral K$^+$/H$^+$ antiporter) is commonly used to fully de-energize (dissipate both the
5.4.3. Weak organic acid stress and hyperosmotic shock lower the pH\textsubscript{i}

Weak organic acids are thought to lower the pH\textsubscript{i} (Beales, 2004, Davidson, 2001). We recently showed using pHluorin that sorbic acid lowers both the cytosolic and mitochondrial pH in \textit{S. cerevisiae} (Orij \textit{et al.}, 2009). Olsen \textit{et al.} (2002) also observed a pH\textsubscript{i} drop upon organic acid stress in \textit{L. lactis} using pHluorin. Now that we were able to determine the pH\textsubscript{i} of \textit{B. subtilis} cells in vivo we set out to measure, in a time resolved manner, the pH\textsubscript{i} of \textit{B. subtilis} cells stressed with different levels of weak organic acid. In unstressed cells we measured a pH\textsubscript{i} of 7.3 ± 0.1 at a pH\textsubscript{ex} of 6.4. We chose to test the anionic salt of the following weak organic acids commonly used as food preservatives: sorbic-, acetic- and benzoic- acid.

Although the side chains of these carboxylic acids differ significantly, sorbic- and acetic- acid have a similar pK\textsubscript{a} of 4.76, and benzoic acid, containing an aromatic side chain, has a pK\textsubscript{a} value of 4.20. All three preservatives clearly lowered the pH\textsubscript{i} in a concentration dependent manner (Fig. 5.3ABC). Adding higher concentrations of the potassium-salt of the weak organic acid led to a lower pH\textsubscript{i}. The maximal pH\textsubscript{i} drop seemed to occur already within the first ten minutes of stress and did not recover to control levels (further discussed below). The stresses applied did not lead to a loss in OD\textsubscript{600} and the cells continued growing albeit at a reduced rate. Depending on the concentration of weak organic acid used (mM range) the growth rate was reduced between 30 and 60% (further discussed below). During incubation, control cells (no addition of stress) showed a very small drop in pH\textsubscript{i} (Fig. 5.3).
We also tested the uncoupler CCCP, which is actually in itself a weak organic acid. This protonophore (pK_a = 5.95) is a known powerful uncoupling agent that very efficiently destroys the proton gradient. As expected, very low concentrations of CCCP (μM range) clearly lowered the pH_i (Fig. 5.3D). Stress caused by 0.85 and 2 μM CCCP resulted in a lowering of the growth rate by 25 and 49%, respectively. Finally, we tested the effect of hyperosmotic stress by the addition of NaCl, probably the oldest known food preservative. Salt inhibited the growth rate by 48 and 79% when 0.75 or 1 M NaCl was applied, respectively. Although NaCl is chemically unrelated to weak organic acids it also caused a significant lowering of the pH_i.
when applied in relative high concentrations (molar range) (Fig 5.3E). It was recently reported by using 5,6-carboxyfluorescein succinimidyl ester (cFSE) that hyperosmotic stress caused by either NaCl or sorbitol causes a drop the pH\textsubscript{i} of *Listeria monocytogenes* (Fang *et al*., 2004). As also seen for the pH\textsubscript{i} in weak organic acid stressed cells, the maximal pH\textsubscript{i} drop seemed to occur already within the first ten minutes of stress and the pH\textsubscript{i} did not recover during the timeframe of the experiments (further discussed below).

### 5.4.4. Comparison of ΔpH\textsubscript{i} and GI % values reveal differences between various weak organic acids, CCCP and NaCl

Since the pH\textsubscript{i} of the control cells showed a slight decrease during incubation (Fig. 5.3) we calculated the difference between the pH\textsubscript{i} for each stress and the pH\textsubscript{i} of the control cells. Small variations in pH\textsubscript{i} before the addition of stress were eliminated by subtracting the ΔpH\textsubscript{i} data from an average value of the ΔpH\textsubscript{i} prior to stress (t = 0). It can be clearly seen that the main drop in pH\textsubscript{i} (large ΔpH\textsubscript{i} value) is already reached after the first 6 min of stress for all stresses applied, which indicates that the effect of the tested compounds is relatively fast (Fig. 5.4). Only the ΔpH\textsubscript{i} addition of 1 M NaCl seemed to increase slowly over time. Since the ΔpH\textsubscript{i} at 2 min is smaller for all stresses than after 6 min of stress, the time resolution of the

![Figure 5.4](image-url)

**Fig. 5.4.** The drop in pH\textsubscript{i} caused by weak organic acids, CCCP or NaCl occurs mainly within the first 6 min. Cells were stressed with the indicated concentrations of chemical. The ΔpH\textsubscript{i} (pH\textsubscript{i,control} − pH\textsubscript{i,stress}) profiles (subtracted from ΔpH\textsubscript{i} at t = 0) for are shown. Note that at the addition of stress (t = 0) the values are zero by definition. Values represent the average of four technical replicates and error bars indicate the standard error.
conducted experiments seemed sufficient. Remarkably, longer incubations (2 h) did not reveal a recovery from the drop in pH values obtained in the first 30 min after stress (our unpublished results). Instead, small increases in ΔpH were observed for KS, KB and salt stress, but not for KAc or CCCP independent of the concentration used (our unpublished data).

**Fig. 5.5.** Comparison of ΔpH and GI % values reveals differences between the various weak organic acids, CCCP and NaCl. (A) The average ΔpH was calculated between 2 and 30 min after stress with the indicated concentrations of KS (blue), KAc (red), KB (green), CCCP (purple), and NaCl (orange). (B) Percentages of growth inhibition caused by the indicated stresses. (C) The average ΔpH (from A) normalized by the GI % (from B). Values represent the average of minimally two biological independent experiments, each based on four technical replicates. The error bars indicate the standard deviation.
From Fig. 5.3 and 5.4 it is clear that all the different stresses applied cause a drop in pH\textsubscript{i}. However, different concentration ranges (from µM for CCCP to molar for NaCl) are needed to achieve lowering of the pH\textsubscript{i}. To be able to compare the different stresses applied we normalized the data to the GI % that each stress caused. We chose this parameter to normalize the data, because it represents a common denominator for the effectiveness of the stress factor. Molecule numbers for instance are not suitable for normalization, since different forms of the molecule (e.g. undissociated or dissociated) may have different modes of action and therefore, effectiveness. For instance, it has been shown before that weak organic acids are most effective in their undissociated form, be it that the (dissociated) anion also significantly contributes to growth inhibition (Davidson, 2001, Eklund, 1983, Ter Beek et al., 2008). The average ΔpH\textsubscript{i} for the first 30 min of stress was calculated and divided by the GI % that each stress caused (Fig. 5.5 and Table 5.2). The data showed for 0.85 µM of CCCP an average drop in pH\textsubscript{i} of 0.14 ± 0.01 in the first 30 min, while 0.75 M of NaCl lowered the pH\textsubscript{i} by only 0.08 ± 0.03 (Fig. 5.5A). The growth rates were reduced by the stresses by 25 ± 7% and 48 ± 6%, respectively (Fig. 5.5B). Therefore, 0.85 µM CCCP clearly resulted in a larger ΔpH\textsubscript{i} / GI % value than 0.75 M NaCl did (0.53 ± 0.10 and 0.16 ± 0.04, respectively) (Fig. 5.5C). It can be clearly seen that CCCP gave the largest pH\textsubscript{i} drop and salt the lowest when normalized by the GI %. The three weak organic acids (KS, KAc and KB) showed intermediate values.

Since a difference is observed between the pH\textsubscript{i} drop caused by CCCP, NaCl and the three weak organic acids, we wondered whether threshold levels of GI % are actually needed before a drop in pH\textsubscript{i} can be observed and, if so, do these threshold levels differ between the various stresses? Therefore, we tested concentrations of stress causing a relative small

<p>| Table 5.2. Comparison of GI % and ΔpH\textsubscript{i} caused by weak organic acids, CCCP and NaCl. |
|-----------------|-----------------|---------------|---------------|---------------|---------------|</p>
<table>
<thead>
<tr>
<th>Stress</th>
<th>pK\textsubscript{a}</th>
<th>Concentration (mM)</th>
<th>GI %\textsuperscript{a}</th>
<th>ΔpH\textsubscript{i}\textsuperscript{b}</th>
<th>ΔpH\textsubscript{i} / GI %</th>
<th>GI % threshold\textsuperscript{c}</th>
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<tr>
<td>KS</td>
<td>4.76</td>
<td>3</td>
<td>0.067</td>
<td>34 ± 6\textsuperscript{d}</td>
<td>0.07 ± 0.02</td>
<td>0.22 ± 0.07 \textsuperscript{e}</td>
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<td></td>
<td></td>
<td>10</td>
<td>0.224</td>
<td>59 ± 3</td>
<td>0.19 ± 0.02</td>
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<tr>
<td>KAc</td>
<td>4.76</td>
<td>25</td>
<td>0.560</td>
<td>31 ± 2</td>
<td>0.086 ± 0.006</td>
<td>0.28 ± 0.04</td>
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<tr>
<td></td>
<td></td>
<td>80</td>
<td>1.792</td>
<td>47 ± 3</td>
<td>0.176 ± 0.002</td>
<td>0.37 ± 0.03</td>
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<tr>
<td>KB</td>
<td>4.20</td>
<td>2</td>
<td>0.013</td>
<td>40 ± 2</td>
<td>0.093 ± 0.009</td>
<td>0.27 ± 0.06</td>
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<td></td>
<td></td>
<td>6</td>
<td>0.0376</td>
<td>61 ± 7</td>
<td>0.22 ± 0.01</td>
<td>0.36 ± 0.04</td>
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<tr>
<td>CCCP</td>
<td>5.95</td>
<td>0.00085</td>
<td>0.000223</td>
<td>25 ± 7</td>
<td>0.138 ± 0.006</td>
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<td></td>
<td>0.002</td>
<td>0.000524</td>
<td>49 ± 5</td>
<td>0.30 ± 0.03</td>
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<tr>
<td>NaCl</td>
<td>-</td>
<td>750</td>
<td>-</td>
<td>48 ± 6</td>
<td>0.08 ± 0.03</td>
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<td></td>
<td></td>
<td>1000</td>
<td>-</td>
<td>79 ± 11</td>
<td>0.17 ± 0.04</td>
<td>0.22 ± 0.02</td>
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</table>

\textsuperscript{a} The percentages of growth inhibition were calculated from the increase in the optical density between 58 and 120 min of the control (no addition of stress) and the stressed culture (obtained from Fig. 5.5).

\textsuperscript{b} The ΔpH\textsubscript{i} (pH\textsubscript{i,control} – pH\textsubscript{i,stress}) value is the calculated average between 2 and 30 min after stress (obtained from Fig. 5.5).

\textsuperscript{c} GI % threshold value corresponds to the value of GI % needed to measure a ΔpH\textsubscript{i} value that is significantly different from zero (obtained from Fig. 5.6).

\textsuperscript{d} Values including their standard deviation are shown.

\textsuperscript{e} ND, Not determined.
inhibition of the growth rate and measured the pH\textsubscript{i} (Fig. 5.6 and Table 5.2). Clearly, it can be seen that for salt a threshold level of the GI % was needed to cause lowering of the pH\textsubscript{i}. Even 21 \pm 3\% of growth inhibition caused by 0.5 M NaCl did not result in a measurable drop in pH\textsubscript{i}. The errors in the negative ΔpH\textsubscript{i} values at small GI % for salt are too large to make a statement about a possible alkalinization and remains to be further investigated. Interestingly, an alkalinization of the pH\textsubscript{i} of \textit{E. coli} was reported within the first 10 min after 0.5 M NaCl stress (Dinnbier et al., 1988). Unfortunately, higher concentrations of salt were not tested in this study. A smaller threshold level could be derived from the data for KS (Fig. 5.6 and Table 5.2). For CCCP and KAc likely an even lower threshold level or no threshold level at all is needed. However, to elucidate this, lower GI % caused by CCCP and KAc need to be tested.

Concentrations of KB causing small inhibitions of the growth rate were not tested. These results further imply that the initial inhibitory effects caused by hyperosmotic shock and sorbic acid are not primarily caused by acidification of the cytosol.

\textbf{Fig. 5.6.} Threshold levels of GI % are required before a drop in pH\textsubscript{i} caused by osmotic shock and sorbic acid is observed. The ΔpH\textsubscript{i} was plotted as a function of calculated GI % caused by 0.5, 1, or 3 mM KS (blue diamonds), 5, 25, or 80 mM KAc (red squares), 0.25, 0.5, or 0.85 μM CCCP (green triangles), and 0.1, 0.25, 0.5, 0.6, or 0.75 M NaCl (purple crosses). Values represent the average of minimally two biological independent experiments, each based on minimally two technical replicates. The error bars indicate the standard deviation.
5.5. Discussion

We successfully constructed a plasmid for the overexpression of ratiometric pHluorin to measure the pH\textsubscript{i} in \textit{B. subtilis}. Due to limitations of the fluorescent protein the pH values can be measured reliably between 5.8 and 8.2 (Fig. 5.2). A pH\textsubscript{i} of 7.3 ± 0.1 was measured in exponentially growing cells when cultured in a defined minimal medium of pH 6.4. This value is slightly lower than the pH\textsubscript{i} range (7.5 – 8.0) reported for neutrophiles using other methods (Booth, 1985). Using cFSE Breeuwer \textit{et al.} (1995) even reported for \textit{B. subtilis} a pH\textsubscript{i} of around 7.6 at a pH\textsubscript{ex} of 6 and a pH\textsubscript{i} of around 8 at a pH\textsubscript{ex} of 7 when grown in peptone broth. The differences between reported values may be due to the different growth conditions (e.g. rich vs. defined minimal medium) and/or the different techniques used (e.g. shake flask vs. microtiter plate). Olsen \textit{et al.} (2002) reported for \textit{L. lactis} grown at a pH\textsubscript{ex} of 6.5, a pH\textsubscript{i} of around 7.4 using pHluorin and a pH\textsubscript{i} of around 7.7 using cFSE. They refer to the fact that both methods rely on standard curves and may therefore introduce variation.

We used the \textit{B. subtilis} strain harbouring the constructed plasmid to measure the pH\textsubscript{i} in weak organic acid stress and hyperosmotic shock induced by NaCl. Also the protonophore CCCP was tested. Clearly, the tested food preservatives KS, KAc, KB, as well as CCCP and NaCl lowered the pH\textsubscript{i} in a concentration dependent manner (Fig. 5.3). Addition of the tested compounds to the medium, as well as the growth of the control cells in itself did not affect the pH\textsubscript{ex} of 6.4 during the time course of the experiment (our unpublished data). During incubation of the control cells (no addition of stress) a minimal drop of the pH\textsubscript{i} was observed (Fig. 5.3). This may be due to different growth conditions of the pre-cultures, which were grown either in a microtiter plate reader or in shake-flasks. Since the drop was minimal (around 0.1 pH unit) and ΔpH\textsubscript{i} (pH\textsubscript{i,control} − pH\textsubscript{i,stress}) values were calculated, we believe the set-up of the experiments was adequate.

Salt is not a weak acid and consequently in itself no potential carrier of protons over the membrane. Therefore, the lowering of pH\textsubscript{i} upon hyperosmotic shock was rather surprising. However, hyperosmotic shock studies in \textit{L. monocytogenes} with NaCl and in \textit{S. cerevisiae} and \textit{Zygosaccharomyces mellis} with sorbitol and sucrose also revealed an acidification of the pH\textsubscript{i} (Fang \textit{et al.}, 2004, Vindelov & Arneborg, 2002). To the contrary, one study performed in \textit{E. coli} reported an alkalization upon 0.5 M NaCl stress (Dinnbier \textit{et al.}, 1988). Our data showed there is no drop in pH\textsubscript{i} observed at NaCl concentrations of 0.5 M and lower (Fig. 5.6). Although no firm statement can be made at this point, the data also tend to a possible alkalization caused by the addition of low concentrations of NaCl (Fig. 5.6).

NaCl initially causes cell shrinkage and induces changes in lipid composition, which likely alters the membrane permeability (Beales, 2004, Lopez \textit{et al.}, 2006, Wood, 1999). Recently, we also observed changes in the lipid composition of \textit{B. subtilis} when stressed with sorbic acid (our unpublished results). Expression of the fatty acid biosynthesis genes were clearly induced upon sorbic acid stress (Ter Beek \textit{et al.}, 2008). Interestingly, while transcriptome...
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data of NaCl and sorbic acid stressed cells clearly differ from another (Steil et al., 2003, Ter Beek et al., 2008), a proteomics study on salt stressed cells showed increased concentrations of the fatty acid biosynthesis enzymes FabF and FabHB (Hopper et al., 2006). Hyperosmotic shock is also suggested to increase the membrane permeability for protons (Vindelov & Arneborg, 2002). Since the cells were grown at a pH<sub>ex</sub> of 6.4, which is lower than the measured pH<sub>i</sub> of 7.3, protons may leak more easily into the cell and cause the observed acidification upon NaCl stress. In the studies showing an acidification of the pH<sub>i</sub> upon hyperosmotic shock (Fang et al., 2004, Vindelov & Arneborg, 2002), including ours, the pH<sub>i</sub> never dropped below the pH<sub>ex</sub>, which supports the assumption that acidification is caused by leakage instead of transport of the protons into the cell by a carrier. The addition of 200 mM KAc, 30 mM KB, or 4 uM CCCP, causing around 90% growth inhibition, did lower the pH<sub>i</sub> below the pH<sub>ex</sub> of 6.4 (our unpublished data).

Interestingly, the main pH<sub>i</sub> drop induced by all tested chemicals is already established within 6 min after addition of the chemicals (Fig. 5.4). Transport of protons over the membrane by molecules is expected to be relatively fast (depending on diffusion rates and solubility of the carrier in the membrane). Remarkably, no clear recovery of the pH<sub>i</sub> was observed even after 120 min for all concentrations of the stresses tested (Fig. 5.3, 5.4 and our unpublished results). Fang et al. (2004) also reported no pH<sub>i</sub> recovery back to control values during 120 min of salt stress.

By normalizing the obtained data against the growth inhibition caused by the applied stress, we could make a distinction between the effects of CCCP, the food preservatives and hyperosmotic shock on the pH<sub>i</sub> (Fig. 5.5). The percentages of growth inhibition were all calculated from the increase in the optical density between 56 and 120 min, since reliable growth rates could not be calculated from earlier time-points due to resolution of the OD<sub>600</sub> measurements. However, the outcome did not change significantly in later time-frames (our unpublished data). As expected, CCCP induced the highest drop in pH<sub>i</sub> (Fig. 5.5). CCCP is a weak acid that is able to destroy the proton gradient very efficiently. The negative charge of the anion is delocalized over the entire molecule and therefore it may be assumed that, besides the neutral molecule, also the anion is, able to diffuse into the membrane more easily than e.g. the acetate anion. Lower ΔpH<sub>i</sub> / GI % values were observed for the food preservatives KS, KAc, and KB and the lowest for NaCl (Fig. 5.5). Together with the finding that a clear threshold in GI % caused by salt is needed to induce a lowering of the pH<sub>i</sub> (Fig. 5.6), this indicates the growth-inhibitory effect of NaCl is not initially and solely acidification of the pH<sub>i</sub>. This is more the case for the tested weak organic acids, and mostly for CCCP (Fig. 5.6). While no significant distinction can be observed between the food preservatives on the basis of the calculated ΔpH<sub>i</sub> / GI % values, the different GI % threshold levels required for lowering the pH<sub>i</sub> reveal a difference in the effects of KS and KAc (Fig. 5.3C, 5.6 and Table 5.2).
Although a relationship was found (higher GI % induced larger ΔpHi), no linear correlation between GI % and the drop in pHi was observed (Fig. 5.5 and 5.6). Likely, the buffering capacity of the cell (also illustrated by the observed threshold levels derived from Fig. 5.6) is different for each type of stress and may change upon increased concentrations of stress. The latter may be the consequence of (high) energy requiring processes being induced only upon exceeding a certain threshold level of stress. For instance, in *B. subtilis* only severe sorbic acid stress leads to the induction of the SigB mediated general stress response (Ter Beek *et al.*, 2008). Interestingly, a recent study by Marles-Wright *et al.* (2008) showed that a threshold level of ~0.3 M NaCl is needed before activating the general stress response. However, no threshold level of azide was needed to activate SigB. Azide is both a respiratory inhibitor and an uncoupler, and is reported to lower the pHi (Lohmeier-Vogel *et al.*, 1989, Thevelein *et al.*, 1987). These observations also support our findings that a threshold level of salt was needed before reducing the pHi and this was not the case when the uncoupler CCCP was added.

In this study we measured the average fluorescence signal of whole cell populations grown in microtiter plates. As can be seen from Fig. 5.1, the expression of ratiometric pHluorin is heterogeneous. Cells expressing more pHluorin will therefore contribute more to the average fluorescence signal measured than low pHluorin expressing cells. The origin of this heterogeneity in pHluorin expression is not known. At this point it is unclear whether cells differentially expressing pHluorin also exhibit different stress resistant properties. Single-cell analysis is required to further investigate this phenomenon.

The pHi is an important parameter for the fitness of a cell. Using pHluorin, the changes in pHi in *B. subtilis* can now be measured accurately (<0.1 pH unit), fast (minutes, even within seconds possible), at low cell densities (OD600 = 0.4) and *in vivo*. Therefore, the strain constructed can be used as a tool to screen for new antimicrobials.

### 5.6. Acknowledgements

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