Molecular physiology of weak organic acid stress in Bacillus subtilis
van Beilen, J.W.A.

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CHAPTER 3

SORBIC ACID AND ACETIC ACID HAVE DISTINCT EFFECTS ON THE ELECTROPHYSIOLOGY AND METABOLISM OF *Bacillus subtilis*  

J.W.A. van Beilen, M.J. Teixeira de Mattos, K.J. Hellingwerf, S. Brul
ABSTRACT
Sorbic and acetic acid are amongst the most commonly used weak organic acid preservatives to improve the microbiological stability of foods. Both have a similar pKₐ value but sorbic acid is a far more potent preservative. Weak organic acids are most effective at low pH. Under these circumstances, they are assumed to diffuse over the membrane as neutral undissociated acids. We show here that the level of initial intracellular acidification depends on the concentration of undissociated acid and less on the nature of the acid. Recovery of the internal pH depends on the presence of an energy source, presumably ATP, but acidification of the cytosol causes a decrease in catabolism. Furthermore, sorbic acid is a more potent uncoupler of the membrane potential than acetic acid. This may slow down the rate of ATP synthesis, necessary for recovery, and may (partially) explain its effectiveness. We also show that sorbic acid may slow down spore germination at an early stage, already interfering with the development of a pH gradient.
INTRODUCTION
Various small weak organic acids (WOA) have been used as food preservatives for a very long period of time. These weak acids slow down growth of various spoilage bacteria, yeasts and moulds without overt undesired effects on taste or being toxic to the consumer. The undissociated states of the WOA preservatives are more effective in slowing growth than the dissociated form, although the latter may have some level of toxicity. As such, WOAs are most effective when applied at low pH values, below their pKₐ value (66, 108). Under these conditions, the neutral acid is assumed to diffuse across the plasma membrane and dissociate in the cytosol which generally has a higher pH. In this way, the proton gradient over the membrane is depleted and the anion may accumulate to potentially toxic levels inside the cell. This is known as the classical “weak-acid preservative” theory (161). Commonly used WOA preservatives include sorbic and acetic acid, which have a similar pKₐ value of 4.76 but a dissimilar octanol: water partition coefficient (log Kₒₒ) of 1.33 and -0.17 respectively (162). This means that at a particular pH and the same total concentration, concentrations of both undissociated acids are the same, but sorbic acid has a higher affinity for a hydrophobic (membrane) environment. Sorbic acid is clearly the more potent preservative of the two, but the exact reason why is still not fully clear (122, 163).

It is important to distinguish the different modes of action that WOAs may have on cell physiology. The classical “weak-acid preservative” theory (161) only assumes entry of the undissociated acid, dissociation in the cytosol and cytosolic acidification. While this is sometimes described as uncoupling, we will use this latter phrase for compounds that shuttle protons across the membrane and are thus protonophoric uncouplers. Others have also pointed to potentially toxicity of accumulated anions (108, 112, 164). If we assume that only the undissociated acid diffuses across the membrane, it follows from the Henderson–Hasselbalch equation that \( \Delta pH = \log \frac{[A^-]_{\text{in}}}{[A^-]_{\text{out}}} \), and thus the internal concentration of the anion may become very high when a high \( \Delta pH \) remains present. Also, specific binding of sorbate to cysteine (117) has been shown and is proposed as an explanation for the higher toxicity of sorbic acid.

Several resistance mechanisms against WOAs have been reported for yeasts like Saccharomyces cerevisiae, Zygosaccharomyces bailii and Z. rouxii. These organisms induce the expression of H⁻-ATPases to regulate their cytosolic pH. S. cerevisiae uses a dedicated ATP binding cassette (ABC) transporter (Pdr12) to prevent accumulation of the anion (122). Also S. cerevisiae plasma membrane components are likely to play an important role in the modulation of the influx of lipophilic weak organic acids (124, 125). Furthermore, Z. rouxii and Aspergillus niger have been shown to degrade sorbic acid to 1,3-pentadiene (126, 127).
The responses and potential resistance mechanisms of bacteria against weak acids have not been so well-described as for yeasts (130, 165). The level of growth reduction has been modelled some 30 years ago (108) and the effect of sorbic acid on the membrane potential in *Escherichia coli* membrane vesicles has been described (109). More recently Ter Beek et al. (66) performed a microarray study on *B. subtilis* exposed to sorbic acid. Their results showed a broad transcriptomic response resembling a pattern typical for cells responding to nutrient limitation. The authors observed an upregulation of genes encoding potential efflux pumps as well as genes involved in remodelling of the plasma membrane. The latter was also functionally assessed and recently corroborated by direct membrane phospholipid analysis in the sorbic acid hypersensitive *rodZ B. subtilis* mutant strain (Chapter 4 of this thesis).

*Bacillus subtilis* has been the Gram positive model organism for decades, because of its GRAS-status, genetic accessibility and fully sequenced genome. It also forms heat resistant spores and is as such a recognized spoilage organism (8). Spores of several related bacterial species are of great concern to the food industry because they are highly resistant to most preservation techniques and once germinated can cause food spoilage through growth of vegetative cells that may produce toxins (165).

To comprehensively elucidate the physiological effects of WOAs on *B. subtilis*, we measured the effects of sorbic and acetic acid on the chemical (ΔpH) and electrical (ΔΨ) components of the proton motive force (PMF). With a depleted PMF, we speculated that the cell might alter its energy needs in terms of glucose consumption and availability of its terminal electron acceptor (i.e. O₂), which were hence determined. We assessed the rate and extent of change in pH caused by these two weak acids using *B. subtilis* cells that were either directly exposed to both WOAs or had been pre-exposed to sorbic acid and re-exposed to WOAs. The latter experiment was done because we inferred from the previously collected micro-array data that cells elicit an adaptive response to these compounds (66). Finally, because sorbic acid has been suggested to interfere with spore germination of *B. subtilis* and *B. cereus* (106, 166), we also monitored pH, during germination and outgrowth of *B. subtilis* spores under weak organic acid stress.

**METHODS**

**GENERAL GROWTH CONDITIONS**

For general purposes *B. subtilis* PB2 strains were grown in Lysogeny Broth (LB). For weak acid stress experiments and fluorescence measurements, *B. subtilis* strains were grown in defined liquid medium (M3G; 84) set at pH 5.5, 6.4 (buffered with 80 mM MES), as well as 7.0 and 7.4 (buffered with 80 mM MOPS). The medium contained 5 mM glucose, 10 mM glutamate, and 10 mM NH₄Cl as carbon and nitrogen sources. All cultures were grown at 37°C, under continuous agitation at 200 rpm. When required, the following antibiotics
were added: 10 μg/ml kanamycin for strains carrying pDG148-derived plasmids, and 50 μg/ml spectinomycin for strains carrying pSG1729 derived inserts.

**Sporulation of B. subtilis strains**

Sporules of *Bacillus subtilis* were generated by depletion of defined liquid medium (M3S, which is M3G without Na-glutamate), at pH = 7.0. Cultures were incubated for 4 days at 37°C under continuous agitation (200 rpm). Spores were harvested and purified by extensive washing with MilliQ water at 4°C. The spore crops were inspected by phase-contrast microscopy and were free (>99%) of vegetative cells, germinating spores, and debris. Spores were stored for up to 1 week in MilliQ water at 4°C at OD₆₀₀ = 1.

**Cloning of promoter fusions with IpHluorin**

Strains used are listed in table 1. All strains expressing IpHluorin were constructed as described in chapter 2.

**Table 1.** Strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>trp2C; 168 wild-type</td>
<td>Gift from C.W. Price</td>
</tr>
<tr>
<td>PB2</td>
<td>trp2C, amyE3'spc IpHluorin amyE5'</td>
<td>Chapter 2 of this thesis</td>
</tr>
<tr>
<td>PB2 PsspE-IpHluorin</td>
<td>trp2C, amyE3'spc IpHluorin amyE5'</td>
<td>Chapter 2 of this thesis</td>
</tr>
</tbody>
</table>

**Calibration of IpHluorin**

The internal pH was measured in strains expressing IpHluorin as described in chapter 2.

**Internal pH measurements**

The internal pH was measured as described in chapter 2. All strains were grown in M3G at pH = 6.4. To compare cells for adaptation responses, cultures were split and 3 mM potassium-sorbate (K-S) was added to half of the cultures. Cells were left to grow for at least 2 h or 2 doublings in OD₆₀₀. When OD₆₀₀ = 0.4 was reached, cells were harvested by centrifugation (10 minutes, 7500 x g at room temperature), resuspended in medium with or without glucose and with 10 μg/ml chloramphenicol to inhibit growth during the experiment. For rapid-exposure WOA stress experiments, K-S and potassium acetate (K-Ac) were used at 250 mM, dissolved in M3G medium without glucose. 2-10 μl of these solutions were injected into the cell suspensions at 310 μl/s using the injector of the FluoStar Optima (BMG Labtech, Germany). As a control experiment, the same concentrations of either KCl or NaCl were injected into the microtiter wells. For 50% and 80% growth inhibition experiments, 3 and 11 mM K-sorbate or 25 and 80 mM K-acetate were used, respectively.
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BATCH MEASUREMENTS OF pH\textit{I} DURING GERMINATION AND OUTGROWTH

All \textit{B. subtilis} strains with IpHLuorin fused to endogenous promoters were grown as described, in M3S, pH 7.0, to an OD\textsubscript{600} = 0.1 in an incubator at 37°C under continuous agitation (200 rpm). Cell suspensions were diluted two-fold by adding 50 µl of culture to 50 µl of medium in black microtiter plates. For spore germination, spores were heat activated (30 min, 70°C, then cooled on ice) and subsequently mixed 1:1 with 2x concentrated M3G with or without glucose, containing weak organic acid in predetermined concentrations. To trigger germination, 5 µl 20x concentrated AGFK (10 mM L-asparagine, 10 mM D-glucose, 1 mM D-fructose, 1 mM KCl) (87) was added. Microtiter plates were placed in a FluoStar Optima (BMG Labtech, Germany) at 37°C and shaken between measurements (200 rpm). Growth was monitored for 2-12 h, with pH and OD\textsubscript{600} measurements taken every 10 min.

CELL COUNTS AND PROTEIN MEASUREMENTS

In order to compare results per cell from the different experiments, cell counts were performed with a CASY counter (Roche, Germany) equipped with a 60 µm tube. 4-10x10\textsuperscript{6} cells per ml were counted. Protein concentrations were determined using a BCA kit (Thermo Scientific) according to the manufacturer’s instructions. Finally, the OD\textsubscript{600} of the cultures was measured with a FluoStar Optima.

MEMBRANE POTENTIAL MEASUREMENTS

\textit{B. subtilis} PB2 and \textit{B. subtilis} P\textsubscript{psg}–IpHLuorin were grown as described in M3G, pH = 6.4, to OD\textsubscript{600} = 0.4. Cells were harvested by centrifugation and resuspended in 1/10 volume M3G without glucose. To inhibit growth and protein synthesis between experiments, 10 µg/ml chloramphenicol was added. Cells were stored at 37°C. The membrane potential (ΔΨ) was measured using a Tetra Phenyl Phosphonium ion (TPP\textsuperscript{+}) electrode (World Precision Instruments, Inc., USA) filled with 1 mM TPP\textsuperscript{+}. All measurements were performed in a warmed (37°C), 2-ml measuring cell, containing a TPP\textsuperscript{+} electrode, reference electrode and an oxygen sensor (see below). The cell suspension was stirred magnetically and aerated by a continuous flow of compressed air, so that at least 120 µM oxygen was present when Δψ was measured. One ml of cell suspension was added to 1 ml medium with glucose; subsequently, weak organic acids and a TPP\textsuperscript{+} calibration series (1, 1, 2, 4 and 8 µl of 1 mM TPP\textsuperscript{+}) were added. The addition of weak organic acid did affect the offset, but not the slope of the calibration response. Stress conditions tested were: 3 and 11 mM K-S and 25 and 80 mM K-Ac. These conditions were compared to non-exposed cells. The membrane potential was calculated as described (167–170) using de-energized cells (incubated for 10 min at 70°C) as reference for non-specific binding of the probe.

The electric potential was calculated using the following equation (167, 170):

\[
\Delta \psi = Z \cdot \log \left[ \frac{C_0(C_e - 1)^{-1} + x(1 - \frac{1}{2}f_{cm}K_{cm})}{x(1 + \frac{1}{2}f_{cm}K_{cm})} \right]
\]
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Where

\[ Z = \frac{RT}{nF} \]

\( \Delta \psi \) = transmembrane electrical potential (mV, \( \psi_{\text{in}} - \psi_{\text{out}} \))

\( Z \) = Conversion factor

\( F \) = Faraday’s constant (96.6 J mV-1 mol⁻¹)

\( R \) = universal gas constant (8.31 J K⁻¹ mol⁻¹)

\( n \) = charge of the translocated ion

\( T \) = absolute temperature (K)

\( C_0 \) = Probe concentration in the medium without addition of cells

\( C_e \) = Extracellular probe concentration

\( f_{cm} \) = Ratio of fractional cytoplasmic membrane and intracellular volume

\( K_{cm} \) = Cytoplasmic membrane partition coefficient

\( x \) = Fractional internal volume

The factor \( f_{cm}K_{cm} \) was determined by a probe binding assay (167) to be 14. In this (simplifying) approach one assumes that the amount of TPP⁺ bound to extra-cytoplasmic components of the cell (like parts of the cell wall) is equal to the amount of probe that binds to intracellular components like nucleic acids. At the end of the experiment, 1.5-ml samples were collected for protein quantification as described above. Three biological replicates were measured for each condition.

**Oxygen consumption measurements**

Oxygen consumption rates were measured simultaneously with the \( \Delta \psi \), in the same measuring cell using a Neofox fiber optic oxygen sensor (Ocean Optics Inc., USA). Oxygen concentrations were measured every second from the maximally aerated to the fully oxygen depleted state. The slope of the straight part of the plot (at least 15 seconds) was used to derive the oxygen consumption rate. At the end of the experiment, 1.5-ml samples were collected for protein quantification as described above. Rates were normalized to protein content.

**Glucose consumption and metabolite measurements**

*B. subtilis* PB2 and *B. subtilis* P_{pHluorin} were grown as described in M3G, pH = 6.4, to \( \text{OD}_{600} = 0.8 \). The cultures were split and exposed to different stress conditions (3 and 11 mM K-sorbate or 25 and 80 mM K- Ac), and 10 mM glucose was added to each culture. A high \( \text{OD}_{600} \) was required to observe a significant decrease in glucose concentrations within the timeframe of the experiment.

Samples, taken every 30 minutes, were snap frozen in liquid nitrogen. The protein concentration of each sample was measured as described above. Samples were further processed for HPLC analysis; a 1 ml sample was mixed with 100 µl 35% perchloric acid and subsequently 55 µl 7 M KOH was added. Filtered supernatants were analysed for glucose consumption and fermentation products. Glucose, succinate, lactate, acetate, 2,3-
butanediol and ethanol contents were determined by HPLC (LKB) with a REZEX organic acid analysis column (Phenomenex) at 45°C with 7.2 mM H₂SO₄ as the eluent, using an RI 1530 refractive index detector (Jasco) and AZUR chromatography software for data integration. All measurements were performed with two biological replicates. From the obtained metabolite fluxes, a carbon balance was calculated with the assumption that similar molar amounts of CO₂ were produced per amount of O₂ consumed plus similar molar amounts of CO₂ were produced per mole of acetate produced and per mole of 2,3-butanediol, 2 moles of CO₂ were produced. Oxygen consumption was corrected for directly related acetate and 2,3-butanediol production.

\[ C_b(\%) = 100\% \cdot \left( 2q_{\text{Acetate}} + \frac{5}{2}q_{\text{2,3-Butanediol}} + q_{\text{O2}} \right) / 6q_{\text{Glucose}} \]

RESULTS

INTERNAL PH DURING GROWTH UNDER WOA STRESS REMAINS LOW

Weak organic acids have been shown to lower the internal pH of microorganisms (110, 132). Also, several WOA-resistance mechanisms have been proposed. It is clear that the collapse of the PMF is of great concern for a bacterial cell (108, 110, 158), so we measured pH, during growth with a number of WOA stresses. To reduce growth rate by approximately 50%, 3 mM K-sorbate or 25 mM K-acetate was used. To reduce growth rate by 85%, 10 and 80 mM were used, respectively.

Growth and pH, were monitored for 6 hours (Fig. 1A and 1B respectively). The internal pH of non-stressed cultures dropped from pHᵢ = 7.5 to 7.2 during this time. The internal pH of sorbic acid-stressed cultures continued to drop from t = 0 until the end of the experiment. With 80 mM K-Ac, this is not seen, and pH remained stable around pH = 7.3. These results show that there is considerable acidification of the cell during growth and that there is no recovery of the pH during extended exposure to WOAs, not even when growth resumes.

WEAK ORGANIC ACIDS CAUSE RAPID DROP IN INTERNAL PH

Long term (6 h) exposure to WOAs as described above, showed that no long-term recovery of the pH of the cells and the largest drop in pH, upon weak acid exposure occurs within the first minutes after addition. To investigate the influx rate of weak organic acid preservatives and their dependence on the metabolic activity of the cell, we investigated the short term effects of WOA injections into the medium. Also, because an adaptive response has been described (66) and changes in B. subtilis’ membrane composition have been observed (Chapter 4 of this thesis), we tried to identify mechanisms of resistance by comparing non-stressed cells with cells pre-exposed for 2 hours to 3 mM of K-sorbate. Cells were grown to mid-exponential phase with and without K-sorbate. To starve the cells for glucose and inhibit protein synthesis, they were resuspended in medium lacking glucose for at least 1 h and supplemented with 10 μg/ml chloramphenicol.

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**Figure 1.** Growth curve (A) and internal pH (B) of *B. subtilis* PB2 pDG-lpHluorin monitored for 6 h under various stress conditions. Medium pH = 6.4. In M3G, the growth rate is highest, but a continuous decrease in pHi is seen. Sorbic acid stress causes the growth rate to be reduced as well as a continuous decrease in internal pH. Acetic acid stress has a similar effect on growth rate, but pH remains constant.

Before acid addition, the pHi of starved wild-type *B. subtilis* was 7.30 ± 0.05. Injection of either KCl or NaCl at identical concentrations as the weak acids had no effect on pHi within one minute after injection (not shown). Upon glucose injection, the pHi rapidly increased to 7.5 within 1 minute (not shown). When WOAs were injected, the pH dropped to its lowest point within 1-4 s. In starved cells, this pH remained stable, but in the presence of glucose, pHi recovered quickly to a new equilibrium (Fig. 2A-D).
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A

B

C

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**Figure 2.** Acidification and recovery upon weak organic acid addition to *B. subtilis* PB2 P$_{inco}$pHluorin. Sorbic acid (A and B) or acetic acid (C and D) is injected at $t = 0$ s. Medium pH = 6.4. The internal pH drops to its lowest point within seconds (A and C) and recovers fast when glucose is available (B and D). The pH recovers to higher values with acetic acid stress. Data from typical examples is shown.

The acidification of starved cells was fitted with a first order kinetic equation

$$pHi(t) = pHi(0) - \Delta pHi(e^{-kt} - 1)$$

where $(B_i)$ is the amplitude factor ($\Delta pHi$/mM), indicating the intracellular buffering capacity, $[HA]$ is the concentration of undissociated weak acid (in mM), $(k)$ is the rate constant (s$^{-1}$) and $pHi(0)$ is the offset (pH, at $t=0$, before injection). Using this equation, values for $(B_i)$ and $(k)$ were determined for sorbic and acetic acid. The value for $\Delta pHi$ or the amplitude of acidification has a linear relation with the concentration of undissociated acid in the medium (Fig. 3). These data (with starved cells) show that sorbic acid causes a similar change in $pHi$ as acetic acid does. The rate of acidification is high and similar for both acids, $1.29 \pm 0.09$ s$^{-1}$ for sorbic acid and $1.27 \pm 0.33$ s$^{-1}$ for acetic acid. However, with a measuring frequency of one per second, this rate is most likely set by our detection system and the actual rate is likely higher. Cells pre-exposed to sorbic acid had similar values as non-stressed cells for $B_i$ and $k$ for either WOA, showing no sign of adaptation at this level (Table 2). With glucose added to the cultures, recovery of the pH started immediately after the injection of weak acid. This curve too was fitted, but with an additional factor describing recovery.

$$pHi(t) = pHi(0) - B_i[HA](e^{-kt} - 1) + B_r[HA](e^{-kr} - 1)$$
Where $B_r$ is the amplitude of recovery and $k_r$ is the rate constant of the pH recovery.

### Table 2. Rate constants ($k (s^{-1})$) and amplitudes ($B$ (pH/mM)) of acid influx ($i$) and recovery ($r$) for sorbic and acetic acid. PS = pre-stressed

<table>
<thead>
<tr>
<th>K-sorbate</th>
<th>Starved</th>
<th>PS + Starved</th>
<th>Glucose</th>
<th>PS + Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_i$</td>
<td>1.29±0.09</td>
<td>1.39±0.96</td>
<td>1.87±0.34</td>
<td>1.70±0.57</td>
</tr>
<tr>
<td>$B_i$</td>
<td>0.87±0.07</td>
<td>0.82±0.26</td>
<td>0.78±0.33</td>
<td>0.41±0.29</td>
</tr>
<tr>
<td>$k_r$</td>
<td>0.08±0.04</td>
<td>0.10±0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$B_r$</td>
<td>0.30±0.06</td>
<td>0.26±0.03</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>K-Acetate</th>
<th>Starved</th>
<th>PS + Starved</th>
<th>Glucose</th>
<th>PS + Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_i$</td>
<td>1.27±0.33</td>
<td>1.48±0.02</td>
<td>2.20±0.19</td>
<td>2.21±0.53</td>
</tr>
<tr>
<td>$B_i$</td>
<td>0.66±0.11</td>
<td>0.59±0.10</td>
<td>0.59±0.16</td>
<td>0.56±0.06</td>
</tr>
<tr>
<td>$k_r$</td>
<td>0.05±0.01</td>
<td>0.06±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$B_r$</td>
<td>0.39±0.06</td>
<td>0.36±0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recovery kinetics do not seem to change between unexposed and pre-exposed cultures. There is however a clear difference between sorbic and acetic acid. The pH of acetic acid stressed cultures recovers to a higher pH than sorbic acid stressed cultures (Fig. 4 and table 2). This is most evident in pre-stressed cultures. The amplitude of recovery also appears to have a linear relation with the acid concentration. The amplitude of initial acidification of pre-stressed cells with glucose is also lower than with non-stressed cells. This suggests that there might be some changes in intracellular buffer capacity. However, we do not see such a difference in starved cells. We do observe, however, that the pH of pre-stressed cells before acid exposure is slightly lower than that of non-stressed cultures. This may either indicate a lower metabolic rate or represent a stress-coping strategy. The acquired constants allow predictions for both acidification and subsequent recovery (Fig. 5).

**SORBIC ACID AFFECTS BOTH Δ$\text{pH}$ AND ΔΨ**

Proton translocation by the electron transport chain through the cell membrane results in both a gradient in the chemical potential of protons (i.e. $\Delta \text{pH}$), as well as an electrical potential ($\Delta \Psi$). This electrochemical proton gradient exerts an inward-directed proton motive force or PMF (26, 27). The PMF can drive protons back into the cell via the $F_1F_0$-ATPase for ATP synthesis, and is also required for various membrane transport processes and for flagellar rotation.
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Figure 3. Amplitude of acidification by addition of sorbic or acetic acid. Data from experiments at an extracellular pH=5.5 and 6.4 are combined. A linear trend can be observed between ΔpH and −log[HA] ([HA] in M).

Figure 4. Acidification and recovery upon weak organic acid addition to B. subtilis PB2 P_\text{pnu}-\text{ip}Hluorin. Acetic or sorbic acid is injected at t = 10s. Medium pH = 6.4. The internal pH drops to its lowest point within seconds and recovers fast when glucose is available. With glucose available, the pH at t = 0 is also higher. The pH recovers to higher values with acetic acid stress. Data from a typical example is shown.

The PMF is defined as (e.g. (18)):

\[ \Delta p = \mu_H^+ \cdot F^{-1} = \Delta \psi - Z \cdot \Delta pH \]

Where

\[ Z = 2.303 \cdot \frac{RT}{nF} \]
Δp= Proton motive force, PMF, (mV)
ΔµH⁺ = transmembrane electrochemical proton potential (J/mol)
F = Faraday’s constant (96.6 J mV⁻¹ mol⁻¹)
Δψ = transmembrane electrical potential (mV, ψₐₐₐ - ψₐₐₐₐ)
Z = Conversion factor
ΔpH = transmembrane pH gradient (pHₐₐₐ - pHₐₐₐₐ)
R = universal gas constant (8.31 J K⁻¹ mol⁻¹)
T = absolute temperature (K)

In our experiments, which were performed at 37°C, Z = 61.4 mV. Very little research has been published on the effects that WOA preservatives have on the membrane potential (19, 109, 112, 158, 171) of microorganisms. Our measurements with TPP⁺ show that under non-stressed conditions with extracellular pH = 6.4, Δψ = 103 ± 9 mV and the pHᵢ = 7.78 ± 0.05. This thus gives a Z*ΔpH value of 84 ± 3 mV and a total PMF of 188 ± 9 mV (Fig. 6). This value for Δψ is similar to earlier reported values (158, 169) and the pHᵢ value is slightly higher (158). Sorbic acid has a severe effect on Δψ, reducing it with 64% even at 3 mM K-S. Acetic acid, however, does not deplete Δψ as strongly. With 25 mM K-Ac, the Δψ is 17% lower and with 80 mM only a 45% reduction is seen. It appears that sorbic acid acts more as an uncoupler than acetic acid does.

Together, Δψ - Z*ΔpH comprise the proton motive force. In total, WOA stress causes a dissipation from 188 ± 9 mV in non-stressed cells to 83 ± 5 mV with 11 mM K-S and 108 ± 7 mV with 80 mM K-Ac. This too shows that sorbic acid has a stronger effect on the electrochemical gradient for protons than acetic acid does at concentrations that lead to similar growth inhibition (Table 3).

Table 3. The effect of weak organic acids on ΔpH, Δψ and proton motive force.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Z*ΔpH (mV)</th>
<th>Δψ (mV)</th>
<th>PMF (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3G, pH=6.4</td>
<td>84.4 ± 3.2</td>
<td>103.9 ± 9.1</td>
<td>188.3 ± 9.6</td>
</tr>
<tr>
<td>3 mM K-S</td>
<td>66.5 ± 0.3</td>
<td>37.8 ± 2.7</td>
<td>104.3 ± 2.7</td>
</tr>
<tr>
<td>11 mM K-S</td>
<td>58.9 ± 0.6</td>
<td>24.8 ± 5.4</td>
<td>83.7 ± 5.4</td>
</tr>
<tr>
<td>25 mM K-Ac</td>
<td>61.1 ± 0.4</td>
<td>87.2 ± 4.4</td>
<td>148.3 ± 4.4</td>
</tr>
<tr>
<td>80 mM K-Ac</td>
<td>50.6 ± 0.5</td>
<td>58.2 ± 6.5</td>
<td>108.8 ± 6.5</td>
</tr>
</tbody>
</table>

**Oxygen consumption is reduced upon addition of high WOA concentrations**

Because of the depleted PMF, the cell may experience energy depletion stress, since the F₄Fₒ ATPase depends on the proton gradient for its activity. It is possible that the cell tries to compensate for the decrease in PMF by increasing the flux through the electron transfer chain and increase proton pumping activity. This also requires a terminal electron acceptor (e.g. O₂). We therefore measured the oxygen levels during our PMF experiments and made sure that at least 120 μM O₂ was measured when Δψ was measured. Non-
Sorbic acid and acetic acid have distinct effects on the electrophysiology and metabolism of Bacillus subtilis.

Figure 5. Actual data (open figures) and modelled acidification (closed figures) and recovery upon weak organic acid injection in B. subtilis PB2 P$_{enr}$-I$p$Hluorin. Acetic or sorbic acid is injected at t = 1 s. Medium pH = 6.4.

Figure 6. Differential effects of sorbic and acetic acid on the proton motive force. Sorbic acid stress affects both ΔpH as well as Δψ. Acetic acid affects ΔpH, but has little effect on Δψ. Data are from cultures exposed to weak acids for approximately 5 min. Results are based on 3 biological replicates. Error bars indicate standard deviation.
Figure 7. Weak organic acid stress reduces respiration. Respiration was monitored for 1 min or the time it took to reduce O2 levels to 0 µmol. Results are based on 3 biological replicates. Error bars indicate standard deviation.

Stressed cells consumed oxygen at a rate of 7.4 mmol s⁻¹ (mg protein)⁻¹. Sorbic acid stress reduced the rate of oxygen consumption to 3.9 and 1.5 mmol s⁻¹ (mg protein)⁻¹ with 3 and 11 mM of K-sorbate respectively. 25 mM of K-acetate had an insignificant effect on the oxygen consumption rate, but 80 mM reduced it to 1.1 mmol s⁻¹ (mg protein)⁻¹ (Fig. 7). These effects took place immediately after addition of the acid.

These observations show that the cell consumes less O₂ when faced with weak organic acid stress. This suggests that there are fewer electrons to feed the electron transport chain and that metabolism may be affected by acidification of the cytosol.

**Glucose metabolism is affected by weak organic acid stress**

Because weak organic acids partially dissipate the PMF, the amount of ATP that the cells can generate through the F₁F₀-ATPase is considerably smaller (as shown above). So, to produce a sufficient amount of ATP to restore pH homeostasis and proliferate, the cell might alter its glucose metabolism to generate ATP through substrate level phosphorylation. Also because increased energy needs have been observed in WOA stressed yeast (172, 173) and a starvation-like response was reported for sorbic acid-stressed *B. subtilis* (66), we decided to measure the glucose consumption rate as well as the production of fermentation products.
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Figure 8. Increased weak organic acid stress lowers glucose consumption rate. The rate of glucose consumption compared to the rate of acetate and 2,3-butanediol production. Results are based on two biological replicates. Error bars indicate standard deviation.

Table 4. The effects of weak organic acid stress on glucose metabolism and respiration. All fluxes are in mmol h⁻¹ (mg protein)⁻¹. The carbon balance was calculated with the assumptions stated in the materials and methods.

<table>
<thead>
<tr>
<th></th>
<th>qGlucose</th>
<th>qAcetate</th>
<th>qButanediol</th>
<th>qO₂</th>
<th>C-balance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3G, pH=6.4</td>
<td>7.80</td>
<td>5.27</td>
<td>0.73</td>
<td>27.87</td>
<td>90.7</td>
</tr>
<tr>
<td>3 mM K-S</td>
<td>3.36</td>
<td>2.43</td>
<td>0.00</td>
<td>14.13</td>
<td>88.9</td>
</tr>
<tr>
<td>11 mM K-S</td>
<td>2.58</td>
<td>1.97</td>
<td>0.00</td>
<td>5.52</td>
<td>61.2</td>
</tr>
<tr>
<td>25 mM K-Ac</td>
<td>2.08</td>
<td>0.54</td>
<td>0.95</td>
<td>22.66</td>
<td>231.9</td>
</tr>
<tr>
<td>80 mM K-Ac</td>
<td>1.45</td>
<td>0.09</td>
<td>0.70</td>
<td>3.92</td>
<td>91.5</td>
</tr>
</tbody>
</table>

Glucose consumption was highest for non-stressed cells, reaching 7.8 mmol s⁻¹ (mg protein)⁻¹. Higher concentrations of weak acid lowered glucose consumption rates, with 80 mM K-Ac resulting in 1.45 mmol s⁻¹ (mg protein)⁻¹ (Fig. 8 and Table 4).

Acetate was produced under all conditions tested and already present in low amounts at the start of the experiment. Non-stressed cultures produced acetate at the highest rate, together with the K-sorbate exposed cells. Addition of K-sorbate lowered the synthesis rate of acetate, possibly due to growth reduction or a decrease in glycolytic activity. Acetic acid-stressed cells had the lowest rate of acetic acid production. These cultures redirected fermentation routes by switching to 2,3-butanediol fermentation. Some 2,3-
butanediol was also found in the final sample of non-stressed cells, but not with sorbic acid-stressed cells.

**Carbon Flux**

When confronted with a lack of electron acceptor, *B. subtilis* employs a mixed acid fermentation, and has been shown to produce lactate, acetate, acetoin, ethanol, 2,3-butanediol and succinate as fermentation products when grown on glucose (174), but also produces a lot of acetate as a result of overflow metabolism (22, 175). In our experiments, only acetate and 2,3-butanediol were found.

Although batch-cultures are not ideally suited for accurate carbon flux determinations, our data does seem to be in line with reported values for wild-type *B. subtilis* (22). With the assumptions made above regarding O₂ utilization, our data (Table 4) for WOA stressed cells shows reasonably close C-balances for all stresses, apart from 25 mM K-Ac, which appears to use about 3.5x as much O₂ as estimated based on carbon fluxes based solely on glycolysis and fermentation. This increase in respiration may indicate that acetic acid is metabolized through the citric acid cycle.

The calculated qATP based on qAcetate and q2,3-Butanediol (qATP = 2*qAcetate + 2*q2,3-Butanediol) through glycolysis and fermentation displayed a similar WOA concentration dependent behaviour as the qGlucose. That is, increasing concentrations of weak acid cause a reduction in fluxes. This behaviour seems analogous to the effect on pHᵢ, which depends more on the concentration than the nature of the acid (as shown above). Indeed, there appears to be a linear relation between pHᵢ and qGlucose, as well as between pHᵢ and the calculated amounts of ATP generated via production of qAcetate and q2,3-Butanediol (Fig. 9A and B). When looking at correlations between Δψ and qGlucose, such a correlation does not exist for both WOA. Only for sorbic acid might a linear correlation be observed, but more experiments will be needed to confirm this relationship. The relation between qO₂ and decrease in pHᵢ seems rather constant, while a decreasing qO₂ seems to correlate with a decreasing Δψ. However, more experiments will be needed for an accurate description.

**Internal pH during Spore Germination with Weak Acid Stress**

Dormant spores are highly resistant to antimicrobial treatment, but also metabolically inert (165). When germination is triggered, the spore becomes more sensitive. Also, it has been observed that germination of *Bacillus* spores can be inhibited by various preservatives (106, 166). When spores start to germinate, they release protons and the internal pH rises (90). Also, during this stage water is taken up and metabolism should be We compared the effects of sorbic and acetic acid on germination and outgrowth by using concentrations of both acids that had a similar effect on growth rate (103). Low concentrations of both acids reduced the exponential growth rate by approximately 50%. Spores germinating in medium (pH = 6.4) with 3 mM K-sorbate had a decreased rate of pHᵢ.
Sorbic acid and acetic acid have distinct effects on the electrophysiology and metabolism of *Bacillus subtilis*.

**Figure 9A.** Glucose flux versus internal pH due to weak organic acid stress (no stress, 3 and 11 mM K-S, 25 and 80 mM K-Ac). A decrease in pH due to WOA stress is accompanied by a decrease in glucose flux.

**Figure 9B.** Calculated ATP flux based on fermentation products (acetate and 2,3-butanediol) versus internal pH due to weak organic acid stress (no stress, 3 and 11 mM K-S, 25 and 80 mM K-Ac). A decrease in pH due to WOA stress is accompanied by a decrease in calculated ATP flux restarted. These processes might be a target moment for weak organic acids to halt outgrowth of the germinating spore.
Figure 10. Internal pH during spore germination and outgrowth in M3G. IpHluorin accumulated in the spores (from expression controlled by PsspE) allows pH measurements from t = 0 to approximately 300 min. Expression of IpHluorin from PptsG allows calculation of the pH from approximately 200 min. Germination was triggered by AGFK. Data are from a representative example.

A. Germination and outgrowth of *B. subtilis* spores at an external pH = 6.4 with 3 mM K-sorbate

B. Germination and outgrowth of *B. subtilis* spores at an external pH = 6.4 with 10 mM K-Sorbate
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*Bacillus subtilis*

![Graphs showing internal pH during spore germination and outgrowth in M3G.](image)

**Figure 11.** Internal pH during spore germination and outgrowth in M3G. IpHluorin accumulated in the spores (from expression controlled by PsspE) allows pH measurements from t=0 to approximately 300 min. Expression of IpHluorin from PptsG allows calculation of the pHi from approximately 200 min. Germination was triggered by AGFK. Data are from a representative example. A. Germination and outgrowth of *B. subtilis* spores at an external pH = 6.4 with 25 mM K-Acetate. B. Germination and outgrowth of *B. subtilis* spores at an external pH = 6.4 with 80 mM K-Acetate.

 increase, and also, the OD<sub>600</sub> dropped slower. At the onset of the exponential phase, the pH, which gradually decreased from pH = 7.4 to 7.2 at t = 11 h (Fig. 10A). 25 mM K-Ac allowed a more rapid increase in pH, during germination. The pH, during exponential growth remained stable at 7.2 during the experiment (Fig. 11A). High concentrations of
weak organic acids were selected to reduce growth by 85%. \( \text{P}_{\text{pro}} \)-driven expression of IpHluorin is delayed under these conditions, while spore specific IpHluorin can be observed for longer periods of time because the signal is not diluted out. Again, K-S (10 mM) is shown to slow the drop in OD\(_{600}\) and the rise of the pH. This effect was not seen with 80 mM K-Ac, although the reduction in growth rate is similar (Fig. 10B and 11B).

High growth rate could be associated with high pH, (Fig. 12A and 12B). This also holds for mildly stressed cultures and could also be recognized in more severely stressed cultures.
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Figure 13. OD600 and internal pH during germination of B. subtilis PB2 PsspE-lpHluorin spores in medium without glucose (pH=6.4). Data are from a representative example.

A. Germination with AGFK.
B. Germination with AGFK in 0.5 mM sorbic acid.
C. Germination with AGFK in 0.5 mM acetic acid.
In the latter case, pH, and growth rates were generally lower and less divergence in growth rate within one experiment was observed (Fig. 12B). To further confirm the observation that sorbic acid inhibited the development of a positive inside pH gradient, spores of B. subtilis PB2 Pspa^−Ipfluorin were incubated with identical concentrations of either sorbic or acetic acid in medium without glucose other than present as germinant. When germination was triggered by addition of AGFK, spores incubated with sorbic acid showed a clear concentration dependant reduction in OD_{600} drop-rate as well as a reduced pH, increase-rate (Fig. 13A and 13B). The OD drop rate decreased from 80x10^{-3} OD_{600}/min to 40x10^{-3} OD_{600}/min when 0.5 mM undissociated sorbic acid was present. Such effects were not seen with acetic acid at identical concentrations, which behaved virtually identical to non-stressed germinating spores (Fig. 13A and 13C). These observations are in agreement with earlier reports stating that sorbic acid can specifically inhibit germination of B. cereus and B. subtilis, likely by interacting with germinant receptors (106, 166).

DISCUSSION AND CONCLUSIONS

Both sorbic and acetic acid lower pHi and reduce the growth rate of B. subtilis. When growing with WOA stress, the internal pH is lower than that of cells not exposed to WOAs during the early exponential phase and the internal pH does not seem to recover, even after 6 h of growth without additional glucose. To reduce the growth rate to a similar level, more acetic acid is required than sorbic acid at an identical external pH value. Given that the pK₅ for both weak acids is almost the same, this makes sorbic acid a more effective preservative.

To elucidate why there is such a difference in effectiveness, we set out to measure several relevant physiological parameters that might be influenced by the addition of weak organic acid preservatives. Even though in the first minute after WOA exposure, some recovery takes place, no full recovery is seen that would have pointed to resistance to WOA stress within the timeframe of our experiments. This, together with the observation that there is no difference between non-stressed and pre-stressed cells regarding acidification rates by sorbic acid and acetic acid, suggests that changes in gene expression upon sorbic acid stress should be interpreted as a ‘coping with stress’ - or adaptation to lower growth rate - mechanism rather than a resistance mechanism (defining a resistance mechanism as a response to eliminate or prevent interaction of a toxin with the cell and a coping mechanism as a response that operates to restore homeostasis to a new optimum without directly dealing with the toxin). This contrasts with the reports of uncoupler-resistant mutants of B. subtilis (176, 177), which show that resistance to the classic uncoupler CCCP is due to alterations in membrane composition by mutations of the fatty acid desaturase, that may decrease the effectiveness of uncouplers. Noticeably, Ter Beek et al. (66) observed no change in desaturase (Des) (51) transcripts in cells exposed to sorbic acid. The only indication of a putative adaptation mechanism involves the lower pHi.
observed in pre-stressed cells with glucose. With a lower pHi, less acid anion may accumulate in the cytosol, which may limit the uncoupling effect of sorbic acid.

Optimal proton extrusion depends clearly on the presence of an energy source (i.e. glucose) in the medium, albeit that recovery of the pHi does not reach the original values. It appears that a new ΔpH equilibrium is established within a few minutes, i.e. before most transcriptomic changes take effect. The level of cytosolic acidification after weak acid injection into the medium is very similar for both acids. This suggests that in starved cells, an equilibrium for acid dissociation is established. The ability to restore pHi differs between sorbic acid and acetic acid-treated cells. Since the only factor measured in this regard is pH or [H⁺], the anion accumulation level is unknown. It can be estimated if it is assumed that $\Delta pH = \log \frac{[A^-]_{in}}{[A^-]_{out}}$. The cell therefore, has to establish a new equilibrium with a ΔpH low enough to reduce anion concentrations, but high enough to allow metabolism.

Another possibility is that this inability to recover pHi during sorbic acid stress reflects the ability of sorbate to act as a classic uncoupler, shuffling protons over the membrane whereas the less lipophilic acetic acid may do this to a lesser extent. This latter possibility is corroborated by the greater effect sorbic acid has on the membrane potential, while acetic acid only carries bulk volume protons across the membrane until a steady state is reached, allowing Δψ to remain relatively unaffected. Even though some authors have pointed to the similar effects of the uncoupler 2,4-dinitrophenol and sorbic acid on growth and metabolism (115–117), no quantitative measurements of the effect that sorbic acid has on Δψ have been reported thus far to our knowledge. Such anion efflux from the cytosol would be driven by the remaining Δψ. This effect was shown clearly with the permeant ion picrate, which only acts as an uncoupler in everted cell membranes (178). It has also been observed that lipophilic compounds such as ethanol can stimulate leakage of protons over the membrane of S. cerevisiae (179, 180). However, recent results from our group show this not to be the case for sorbic or acetic acid (122).

Other commonly used preservatives such as lactic, benzoic and propionic acid are also expected to diffuse and dissociate in agreement with the Henderson – Hasselbalch equation. Future experiments should establish to what extent other preservatives such as lactic acid, benzoic acid and propionic acid have similar uncoupling properties, and what the quantitative effect of Δψ depletion on growth rate is. It is likely that their log $K_{OW}$ values (Table 1, Chapter 1 of this thesis) will be related to this, but also their ability to cross the aqueous phase / membrane barrier will play a role, as seems to be the case with sorbic acid (181, 182).

On the metabolic level, a similar picture emerges as with pHi, glucose metabolism slows down to the same extent as pHi, in the presence of WOA, depending on the concentration
of undissociated acid as has also been described for E. coli and Saccharomyces cerevisiae (17, 110, 111). This results in a linear relation between pHi and qGlucose as well as between pH, and qATP based on acetate and 2,3-butanediol synthesis via glycolysis and fermentation. Glycolytic flux can be controlled, amongst other factors by pH (157), affecting phosphofructokinase (Pfk) activity (16), or specifically in the case of Bacillus species, phosphoglycerate mutase activity (183), which is also very sensitive to pH changes. The observation that acetic acid continues to be produced when cells are exposed to sorbic acid (2 mmol h⁻¹ (mg protein)⁻¹ with 11 mM K-sorbate), may be an extra stress factor for cells growing under these conditions, and may be part of the explanation for the observed continued decrease in pH during sorbic acid stress.

Glucose is generally assumed to be the preferred carbon and energy source for B. subtilis which is taken up via the phosphoenolpyruvate-sugar phosphotransferase system (PTS). It is subsequently converted into fructose 1,6-bisphosphate (FBP) by Pfk. FBP is required for phosphorylation of CcpA, one of the main repressors in carbon catabolite repression (CCR) in B. subtilis. This way, FBP availability through CCR which causes repression of the use of less preferred substrates (13) at sufficient concentrations of the preferred substrate. Also, CCR inhibits expression of citric acid cycle-enzymes when glucose concentrations are high. When a decrease of the internal pH reduces the activity of Pfk, the concentration of FBP is reduced, CcpA (a global regulator (inhibitor) of many carbon metabolism routes(14)) activity is reduced and CCR is released. This may explain how in earlier studies (66) a starvation-like response could be observed in B. subtilis upon sorbic acid stress.

With high concentrations of acetic acid, it is likely that fermentation routes towards acetate are diverted to 2,3-butanediol. Synthesis of 2,3-butanediol depends on bdhA, encoding acetoin reductase (174), a gene that has SigB-controlled expression. Acetic acid stress can trigger stressosome activity and indeed shows upregulation of acetoin reductase (Unpublished results by A. Ter Beek, (103)). Weak organic acid stress also causes an upregulation of citric acid cycle enzymes (66, 103). This route may also consume added acetic acid, thereby providing an extra carbon source and eliminating this weak organic acid. The high oxygen consumption rate that we observed in the presence of 25 mM and 80 mM K-Ac, may be explained by the utilization of acetate. While growth rates are reduced by 80% with both 11 mM K-sorbate and 80 mM K-acetate, the glucose consumption rate with 80 mM K-acetate is almost half of that with 11 mM K-sorbate. Future experiments should be conducted in a turbidostat setup and include CO₂ measurements to allow a direct measurement of the carbon balance under these conditions.

Oxidative phosphorylation is an important source of ATP for aerobic, non-stressed B. subtilis cells. The O₂ consumption rates follow a similar trend as qGlucose, qAcetate and q2,3-Butanediol, apart from the 25 mM K-Ac stress. With the WOA stress applied in our
experiments WOA stress may leave too little PMF to allow $F_1F_0$-ATPase to produce ATP. Also the succinate dehydrogenase of *B. subtilis* requires a PMF to function (184). We did not observe release of succinate in the medium in our experiments, but it may have accumulated in the cell’s cytosol.

Once the primary acid influx has equilibrated, the differential effects of the specific acids start to play a role. Some, such as sorbic acid can likely also act as an uncoupler and the rate with which it does so determines the rate of the secondary acidification and depletion of the PMF. Sorbic acid has a similar effect as acetic acid on pH$_i$, but is more effective in depleting $\Delta\psi$. The depletion of the membrane potential may have a plethora of effects, ranging from reduced transporter activity (185) to destabilization of the bacterial cytoskeleton (28), although we have not seen evidence for this latter effect in these experiments. This may partially explain the observation that sorbic acid is a more potent preservative than acetic acid.

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