Compartment-specific pH monitoring in Bacillus subtilis using fluorescent sensor proteins: a tool to analyze the antibacterial effect of weak organic acids.

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Compartment-specific pH monitoring in *Bacillus subtilis* using fluorescent sensor proteins: a tool to analyze the antibacterial effect of weak organic acids

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The internal pH (pHi) of a living cell is one of its most important physiological parameters. To monitor the pH inside *Bacillus subtilis* during various stages of its life cycle, we constructed an improved version (IpHiLuorin) of the ratiometric, pH-sensitive fluorescent protein pHiLuorin by extending it at the 5′ end with the first 24 bp of comGA. The new version, which showed an approximate 40% increase in fluorescence intensity, was expressed from developmental phase-specific, native promoters of *B. subtilis* that are specifically active during vegetative growth on glucose (P_{S+G}) or during sporulation (P_{PS+G}, P_{SP}, and P_{I}). Our results show strong, compartment-specific expression of IpHiLuorin that allowed accurate pH measurements of live cultures during exponential growth, early and late sporulation, spore germination, and during subsequent spore outgrowth. Dormant spores were characterized by an pHi of 6.0 ± 0.3. Upon full germination the pH rose dependent on the medium to 7.0–7.4. The presence of sorbic acid in the germination medium inhibited a rise in the intracellular pH of germinating spores and inhibited germination. Such effects were absent when acetate was added at identical concentrations.

**Keywords**: bacterial spore formers, spores, spore germination, intracellular pH, GFP, pHiLuorin, weak organic acids, uncouplers

**INTRODUCTION**

The internal pH (pHi) of living cells plays a fundamental role in many chemical reactions. Many intracellular enzymes show optimal activity and stability in a narrow pH range near neutrality. Furthermore, in many organisms proton gradients are required for the greater part of ATP synthesis while uptake systems often depend on the proton gradient over the cell membrane (Krulwich et al., 1998, 2011; Slonczewski et al., 2009). In many chemical reactions. Many intracellular enzymes show optimal activity and stability in a narrow pH range near neutrality. Furthermore, in many organisms proton gradients are required for the greater part of ATP synthesis while uptake systems often depend on the proton gradient over the cell membrane. The cell's pHi can be measured with various methods. Ideally, intracellular pH measurements should be conducted in systems containing Good's buffers (Good et al., 1966; Ferguson et al., 1980), to minimize the effect of the cell's surrounding (unless desired). The probe used to measure pH should maintain accuracy over the pH range assessed. In addition, both the presence of the probe itself in a cell as well as the detection method applied should have minimal effect on cell physiology. Currently used techniques include the distribution of radiolabeled membrane-permeant weak acids, 31P nuclear magnetic resonance.
(NMR), fluorescent dyes (e.g., carboxyfluorescein, carboxyfluorescein diacetate, and succinimidyl ester; Ugarbli et al., 1978; Booth, 1985; Baltzias et al., 1993; Magill et al., 1994; Breese et al., 1996; Leuschner and Lillford, 2000). These methods have the advantage that no genetic modification is required and in the case of fluorescent dyes, single cell measurements are possible (Stenczewska et al., 2009). Weak acid dyes or reporters may alter the pHi and are therefore difficult to use accurately, and may require many treatment and incubation steps before measurement. 31P NMR and radiosolated compounds require extensive cell handling and high cell density, which also disturb cell physiology. Another useful method is the use of fluorescent proteins (green fluorescent protein (GFP) derivatives). This does require the organism to be genetically accessible but allows direct, fast, and localized pH measurements. In our lab, we have successfully used ratiometric pHluorin (Miesenböck et al., 1998) for a number of years in S. cerevisiae (Orji et al., 2011; Ullah et al., 2012), and more recently also in B. subtilis (Yer Beek, 2009). However, the codon usage of pHluorin was not optimized for use in B. subtilis. Our initial experiments suggested that our results might benefit from an increase in fluorescence intensity. This might be achieved by improving translation initiation (Veening et al., 2004). We therefore fused the first eight amino acids of comGA to pHluorin (Veening et al., 2004), as this was shown to improve the signal strength of cyan fluorescent protein (CFP) and yellow fluorescent protein (YPF). The pHi-dependent ratiometric fluorescent properties of IpHluorin were not affected by this fusion. Expression of IpHluorin resulted in strong, compartmentalized, and cell-type-specific signals. This allowed us to monitor the pH during growth and sporulation, in both pre-spor, mother cell and mature spore, as well as during spore germination. Effects of the addition of sorbic and acetic acid on the pHi of germinating spores are described.

### Materials and Methods

#### Strains and Growth Conditions

For general purpose growth, Escherichia coli MC1061 and B. subtilis PB2 strains were grown in Lysogeny broth (LB). For fluorescence measurements, B. subtilis strains were grown in defined liquid medium (MG; Keijser et al., 2007) buffered at pH = 7.0 or 7.4 with 80 mM 2-(N-morpholino)ethanesulfonic acid (MES), or at pH = 7.0 or 7.4 with 80 mM 3-(N-morpholino)propanesulfonic acid (MOPS). All cultures were grown at 37°C, under continuous agitation at 200 rpm. When required, the following antibiotics were added: kanamycin for strains carrying pDG148-derived plasmids; 10 μg/ml for B. subtilis strains, 50 μg/ml for E. coli strains, spectinomycin for strains carrying pSG1729-derived plasmids or genomic inserts (50 μg/ml). The strains used in this study are listed in Table 1.

#### Sporulation of B. subtilis Strains

Spores of B. subtilis were prepared by glucose depletion of defined liquid medium (MS, which is MG without sodium 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 optical density (OD)~1.

#### Cloning of Promoter Fusions with pHluorin

Our initial experiments suggested that the accuracy of pH measurements might benefit from increased expression of pHluorin. To improve translation efficiency, the first 24 bp of comGA were extended with a standard Shine–Dalgarno (SD) region (AAGGAGGAAGCAGGT; Joseph et al., 2001) using primers IpHlu_2010_FW and IpHlu_2010_RV. This sequence was subsequently extended with a standard Shine–Dalgarno (SD) region (AAGGAGGAAGCAGGT; Joseph et al., 2001) using primers IpHlu_pDGA_FW. This SD-improved pHluorin (IpHluorin) was used in this study.

### 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>E. coli MC1061</td>
<td>Cloning host, F' araD139 (ara-leu)7696 lacZ Δ (lac)X74 galU galK hsdR2 mcrA mcrB1 rpsL recA 43 strA2 mbl lacZ57 galU54BAD3 maltose 55274 5877/7</td>
<td>Casadaban and Cohen (1980)</td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td>C. W. Price</td>
</tr>
<tr>
<td>PB2</td>
<td>trpC2C, 168 wild-type</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 δG148</td>
<td>trpC2C, δG148</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 Pxyhluorin</td>
<td>trpC2C, amyE3' pyc Pxyhluorin amyE3'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 Pxyhluorin</td>
<td>trpC2C, amyE3' pyc Pxyhluorin amyE3'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 δGhluorin</td>
<td>trpC2C, δGhluorin</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 δGhluorin</td>
<td>trpC2C, δGhluorin</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PptsGhluorin</td>
<td>trpC2C, amyE3' pyc PptsGhluorin amyE3'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PptsGhluorin</td>
<td>trpC2C, amyE3' pyc PptsGhluorin amyE3'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PpstIPhluorin</td>
<td>trpC2C, amyE3' pyc PpstIPhluorin amyE3'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PsstIPhluorin</td>
<td>trpC2C, amyE3' pyc PpstIPhluorin amyE3'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PsstIPhluorin</td>
<td>trpC2C, amyE3' pyc PpstIPhluorin amyE3'</td>
<td>This work</td>
</tr>
</tbody>
</table>

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inserted between the HindIII and SalI sites of pDG148. This construct, pDG-IpHluorin, was transformed into B. subtilis PB2 and compared with PB2 carrying pDG-pHluorin to analyze expression levels and pH-dependent characteristics of pHluorin and IpHluorin. Also, a xylose-inducible, genome-integrated expression system was constructed. To this end, IpHluorin was inserted in pSG1729, between the AvrII and HindIII sites, thereby replacing the GFP and placing IpHluorin under control of the xylose-inducible Pxy promoter.

To monitor the pH of B. subtilis for extended periods of time in different phases of its life cycle, without the need for externally supplied expression inducers, the promoter region of several growth phase-specific genes (PpreC), specific for pre-septum, sporeulating cells, Pspc, a fore-sphere-specific gene, and PpreOIII, a mother cell-specific promoter) of B. subtilis were selected for their expression levels (Steil et al., 2005; Veenings et al., 2006a). Approximately 500 bp upstream of the start codon were selected for cloning. By standardizing the SD region, we aimed to increase and standardize the expression levels of poorer promoter sites (Ozbudak et al., 2002; Botella et al., 2010). The promoter and SD-IpHluorin sequences were fused by a PCR and inserted in pSG1729, between the AvrII and HindIII sites, thereby replacing the GFP and placing IpHluorin under control of a B. subtilis promoter. All enzymes used were obtained from Fermentas (Thermo Fisher Scientific).

Bacillus subtilis PB2 was used as target for our transformations. B. subtilis cells were made transformation-competent as described before (Kunst and Rapoport, 1995). The newly constructed plasmids were integrated in the amyE locus as described (Lewis and Marston, 1999). All plasmids and oligonucleotides used in this study are listed in Tables 2 and 3.

CALIBRATION OF IpHluorin

Bacillus subtilis PB2 containing either pDG148, pDG-pHluorin or pDG-IpHluorin were grown to exponential phase in M3G at pH 7.0 containing 10 μg/ml kanamycin. Bacterial growth and expression levels of ratiometric pHluorin and IpHluorin were monitored in a Fluostar Optima (BMG Labtech, Germany) for 3 h after addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

For calibration of the pH, expression of ratiometric pHluorin and IpHluorin was induced for 2.5 h by the addition of 1 mM IPTG. At OD600 = 0.4 the cells were centrifuged and resuspended in buffers with pH values ranging from 5.0 to 8.5 prepared from 0.1 M citric acid and 0.2 M K2HPO4. The intracellular and extracellular pH were equilibrated by the addition of 1 μM valinomycin and 1 μM nigericin (Bremer et al., 1996). Cells were transferred to black-walled microtiter plates and incubated at 37°C in a Fluostar Optima. OD600 was measured before start of the experiment. The ratio of emission intensity at 510 nm resulting from excitation at 390 and 470 nm (with photomultiplier gain set to 2,000) was calculated as described previously (Orij et al., 2011). Fluorescence and OD600 were monitored for 30 min, with measurements taken every 5 min. Calibration curves for pHluorin and IpHluorin were identical, with only minor fluctuations in fluorescence in time observed with pHluorin at pH = 8.5. From this, we concluded that the intracellular and extracellular pH had equilibrated rapidly. B. subtilis PB2 carrying pDG148 was measured for background fluorescence. Background fluorescence was subtracted at individual wavelengths before calculating the ratio. 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 third order.

BATCH MEASUREMENTS OF pH, DURING SPOORULATION, GERMINATION, AND OUTGROWTH

To monitor pH, during growth and sporulation, all B. subtilis strains, wild type (WT) (PB2) and those with IpHluorin fused to endogenous promoters were grown as described, in M3S without antibiotics, pH 7.0, to an OD600 = 0.1 in an incubator at 37°C under continuous agitation (200 rpm). Cell suspensions were diluted twofold by adding 50 μl of culture to 50 μl of medium in black microtiter plates which were then monitored in a Fluostar Optima BMG (Labtech, Germany) at 37°C. OD600 and pH measurements were taken every 10 min for 48 h. The plates were shaken (200 rpm) in between measurements thus ensuring optimal growth (Ter Beck, 2009). For spore germination, washed spores were heat activated (30 min, 70°C, then cooled on ice) and subsequently mixed 1:1 with concentrated M3 with or without glucose, containing weak organic acid (WOA) in predetermined

### Table 2 | Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Reference or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDG148</td>
<td>bla blw lacI Pspac</td>
<td>Stenger et al. (1988)</td>
</tr>
<tr>
<td>pDG-pHluorin</td>
<td>pDG148, pHluorin</td>
<td>This work</td>
</tr>
<tr>
<td>pDG-IpHluorin</td>
<td>pDG148, IpHluorin</td>
<td>This work</td>
</tr>
<tr>
<td>pSG1729</td>
<td>bla amyE′ apr Psychilammut1′ amyE′</td>
<td>Lewis and Marston (1999)</td>
</tr>
<tr>
<td>pSG-amyE′</td>
<td>apr Psychilammut1′ amyE′</td>
<td>This work</td>
</tr>
<tr>
<td>pSG-plasmid</td>
<td>apr Psychilammut1′ amyE′</td>
<td>This work</td>
</tr>
<tr>
<td>pSGp5 promoter–IpHluorin</td>
<td>apr Psychilammut1′ amyE′</td>
<td>This work</td>
</tr>
<tr>
<td>pSGp5 promoter–IpHluorin</td>
<td>apr Psychilammut1′ amyE′</td>
<td>This work</td>
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<tr>
<td>pSGp5 promoter–IpHluorin</td>
<td>apr Psychilammut1′ amyE′</td>
<td>This work</td>
</tr>
<tr>
<td>pSGp5 promoter–IpHluorin</td>
<td>apr Psychilammut1′ amyE′</td>
<td>This work</td>
</tr>
</tbody>
</table>
concentrations. To trigger germination, 5 μl 20% concentrated ASFK (10 mM D-asparagine, 10 mM D-glucose, 1 mM D-fructose, 1 mM KCl; Wax and Freese, 1968) 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 OD600 measurements taken every 10 min.

MICROSCOPY
To verify if expression of IpHluorin was correctly localized, B. subtilis cells were cultured as described above for batch measurements at pH = 7.0. All strains were grown as described to exponential phase or for 16–24 h to observe sporulating cells. Cells were immobilized on 1% agarose (Koppelman et al., 2004), and photographed with a CoolSnap fx (Photometrics) charge-coupled device (CCD) camera mounted on an Olympus BX-60 fluorescence microscope through an UPlanFLN 100×/1.3 oil objective (Japan) with a 410 nm excitation bandpass filter (Chroma Technology Corp., Bellows Falls, VT, USA).

RESULTS
IMPROVED EXPRESSION OF pHluorin
Many microorganisms have an internal (cytosolic and/or mitochondrial) pH between 7 and 8 (Uti et al., 2009; Slonczewski et al., 2009) during optimal growth and maintaining pH homeostasis is of vital importance for most, including B. subtilis where pH differences have been inferred for its various developmental phases. We now used the pH-sensitive GFP pHluorin, developed for yeast (Miesenböck et al., 1998), to directly measure on-line the pH dynamics in B. subtilis. Codon usage of this GFP was not optimized for B. subtilis and our initial experiments suggested that expression might be improved. It was shown previously that addition of the first eight amino acids of comGA improved translation initiation efficiency of CFP and YFP in B. subtilis (Veening et al., 2004). We used this approach to construct improved pHluorin (IpHluorin, Figure 1A).

To analyze fluorescence intensity of Bacillus cells harboring pDG148, pDG-pHluorin, or pDG-IpHluorin cells were transferred to microtiter plates containing 0–1 mM IPTG to induce expression. Cell growth was monitored for 3 h, together with fluorescence emission at 510 nm upon excitation at 390 and 470 nm. The three strains compared had identical growth rates (not shown). Fluorescence intensity after 2.5 h is shown in Figure 1B, and depended on the concentration of IPTG. Cells expressing IpHluorin had the highest signal intensity at 1 mM IPTG, approximately 40% higher than pHluorin under the same conditions.

The internal pH varies with growth phase
The cytosolic pH is a crucial parameter for bacteria because it modulates the activity of many enzymes (Vojnovic and Von Stockar, 2009) and in many species plays a crucial role in generating the
proton-motive force (Shioi et al., 1980; Slonczewski et al., 2009). To monitor pHi during various stages of growth in Bacillus, we fused promoters of strongly expressed, growth phase-specific genes to IpHluorin. This allowed us to measure pHi of vegetative cells without addition of inducers such as IPTG or xylose (Figures 2A–H). The selected promoters and their specific expression phase are shown in Table 4.

To monitor the pHi during growth in minimal medium with glucose as the only carbon source, we used the promoter of ptsG, which encodes the glucose-specific enzyme II of the carbohydrate-phosphotransferase system to drive IpHluorin expression. PptsG is a strong promoter during vegetative growth on glucose (Botella et al., 2010). Expression of IpHluorin from the PptsG promoter follows the growth curve closely (Figure 3A). When the cells die or move into stationary phase (after 7.5 h), the signal intensity remains high and stable. The sporulation-specific promoters (Figure 4B) are activated after the drop in OD600, signifying the onset of sporulation.

The pHi of B. subtilis reaches its highest value of around 8 during exponential growth. This value is in agreement with earlier reported values ranging from pH = 7.8 to 8.1 (Setlow and Setlow, 1980; Magill et al., 1994). At the drop in OD600, cells either die or differentiate and initiate sporulation or remain in stationary phase. This was accompanied by an apparent steep decrease in pHi, to 7.0 in vegetative cells expressing IpHluorin from PptsG. Likely, this at least partially is indicative for cell lysis as a strong fluorescent signal could also be detected in the medium after spinning down the cells. Additionally, it is possible that morphological changes of the cell affect their optical properties. Sporulating cells are, for instance, smaller than exponentially growing cells. Hence, after sporulation commences, the pH values observed with PptsG-driven IpHluorin can no longer be considered an accurate estimate of the intracellular pH in vegetative cells. Apart from aberrant values due to cell lysis, the PptsG-driven IpHluorin may also get trapped in sporulating cells so that the observed pH from PptsG-driven IpHluorin is the average of sporulating and non-sporulating cells as well as the medium. Subsequently, the OD600 rose again slowly and the apparent pH increased to 7.4 (Figure 3). We do not know from which cells this signal originates as it may represent the average of various differentiation types, all expressing IpHluorin. To deconvolute these signals, single cell measurements are needed.

### Table 4 | Promoters used for IpHluorin expression.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Regulator</th>
<th>Corresponding growth phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PptsG</td>
<td>σ⁺</td>
<td>Growth on glucose (Botella et al., 2010)</td>
</tr>
<tr>
<td>PspoIIA</td>
<td>Spo0A, σH</td>
<td>Early sporulation (Wang et al., 2006)</td>
</tr>
<tr>
<td>PspoIID</td>
<td>σ⁺</td>
<td>Early sporulation, mother cell-specific (Wang et al., 2006)</td>
</tr>
<tr>
<td>PspoIFE</td>
<td>σ⁺</td>
<td>Late sporulation, spore-specific (Wang et al., 2006)</td>
</tr>
</tbody>
</table>

SPORULATION-SPECIFIC EXPRESSION OF IpHluorin

Sporulation of B. subtilis is a well-described, carefully orchestrated process where a number of different sigma factors are activated during subsequent stages (Wang et al., 2006). It has been reported that the pHi of Bacillus spores is lower than that of vegetative cells (Magill et al., 1994). We set out to measure the pH in spores and at what stage in sporulation the drop in pH starts and when the pH would rise again during germination. For this purpose, we constructed strains with early and late (pre)spore-specific expression of IpHluorin. We selected promoters that would be active in the pre-spore and mother cell at different times during sporulation to monitor pHi of both cells separately. Expression from PspoIIA, PspoIIIE, and PspoIIIF starts after the drop in OD600. The surviving cells may prepare for diauxic growth or sporulation (Veening et al., 2008). This characteristic allowed us to measure differences in pHi in both mother cell and pre-spore in the subpopulation that initiates sporulation. Expression levels from PspoIIA, PspoIIIE, and PspoIIIF are lower than of PptsG, but are still reliable and strong enough to allow pH monitoring (Figures 3A,B). For the sporulation-specific promoters, a cut-off of 1,000 arbitrary units in the 390 to 510 nm fluorescence channel was used for pHi calculations.
van Beilen and Brul

Intracellular pH measurement in Bacillus

FIGURE 2 | Expression of IpHluorin in B. subtilis PB2.

Left panels: phase-contrast images; right panels: corresponding fluorescent signals.

(A,B) Non-transformed cells; (C,D) PtpG-ipHluorin-expressing cells; (E,F) sporulating cells expressing PspolIIID-ipHluorin; (G,H) sporulating cells expressing PB2 PsspE-ipHluorin.
SpoIIA is activated by high levels of activated Spo0A and its presence was shown to be a reliable indicator for cells that initiate sporulation (Veenings et al., 2005). Indeed, a fluorescent signal of a GFP reporter under control of the SpoIIA promoter can be found in both mother cell and forespore (our unpublished observations; Veenings et al., 2006b). Expression of genes in the mother cell regulated by PspoIIID follows that of those regulated by PspoIIA, as expected, but because expression levels of PspoIIID-controlled lpHluorin are higher, reliable pH measurements can be obtained earlier with the latter. Initially, the sporulating subpopulation had a pH that closely resembled the pH of exponentially growing cells measured with the PptsG-IpHluorin strain. The mother cell (PspoIIID-IpHluorin) had a pH of 7.8 after 17 h of incubation. IpHluorin expressed from the forespore-specific promoter PspoIIA revealed pH values of 7.4. The mother cell-specific expression of IpHluorin from the spoIIID promoter decreased after 25 h of culture. At that time point and from then onward, an apparent decrease measured with the mother cell-specific promoter driving IpHluorin expression was observed. This data, however, may at least partially be influenced by mother cell lysis and release of IpHluorin into the medium. The inferred pH at 40 h of culture closely resembled medium pH, corroborating this notion.

The decrease in pH in the forespore (PspoIIA-IpHluorin) drops below the medium pH and its fluorescent signal can clearly be observed inside maturing spores (Figure 2H). Noticeably, spores have a very low water activity and optical properties dissimilar from vegetative cells, which may obscure the pH as defined as the number of free protons (Sunde et al., 2009). Our data indicates that at 17.5 h of culture, the pH of the forespore is 7.4, as reported by PspoIIA-IpHluorin. After 40 h, the pH value of 6.8 reported by PspoIIA-IpHluorin is approaching the reported value for Bacillus spores (pHi = 6.0 ± 0.3; Barton et al., 1980; Setlow and Setlow, 1980; Magill et al., 1994, 1996). Likely, because at this time point the population is still a mix of some forespore-containing cells as well as many free spores, the observed pH is slightly higher than the reported values for isolated Bacillus spores. Corroborating this, when we washed and isolated the spores our pHluorin-based measurement of the pHi of B. subtilis spores also indicated values around 6.0 ± 0.3 (see, e.g., pHluorin data of time point 0 obtained with lpHluorin driven by PspoIIA in Figure 5 and beyond).
As described above, IpHluorin expressed from PB2 PsspE accumulates in mature spores. Germination and outgrowth were monitored using B. subtilis PB2 PsspE-IpHluorin and B. subtilis PB2 PptsG-IpHluorin. When germination is triggered by addition of a mixture of asparagine, glucose, fructose and potassium (AGFK), the OD₆₀₀ of the spore crop drops, because the refractile spores turn phase-dark, due to water uptake. Simultaneously, the spore’s pHᵢ rises. Depending on the medium pH, the pHᵢ rises to 7.0–7.4 (Figure 5 and our unpublished observations for germination at pH 7.4, respectively). In the case of germination at pH 6.4, this indicates the establishment of a pH gradient. Not all spores germinate at the same time, and significant heterogeneity can be observed in the timing of germination and outgrowth (Smelt et al., 2008). Since this is a mixed population, consisting of phase-bright and germinating spores, the actual pH change in individual germinating spores may differ.

During the lag phase between germination and outgrowth, the pteG promoter is activated. Parallel expression of IpHluorin from this promoter shows that the pH measured this way lies between 7.5 and 7.8. This range of pH values is maintained during exponential growth. A generally observed slow decrease in pH may be due to acidification of the medium by acetic acid or CO₂ (Russell and Dietz-Gonzalez, 1998; Ori, 2010). After approximately 600 min, there is a sudden drop in pH and OD₆₀₀ as described above (Figure 5).

Germination with medium pH=7.4 shows a more rapid decrease in OD₆₀₀ and an equally faster rise in pHᵢ. Also, when outgrowth commences, pHᵢ of these cells is higher, but follows a similar trend as with medium at pH=6.4.

**Internal pH during spore germination and outgrowth with weak acid stress**

Dormant spores are highly resistant to antimicrobial treatment, but also metabolically inert (Brul and Coote, 1999). 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 (Cortezzo et al., 2004; Van Melis et al., 2011). When spores start to germinate, they release protons and the pHᵢ rises. Also, during this stage water is taken up and metabolism should be restarted. These processes might be a target moment for WOAs to halt outgrowth of the germinating spore.

Acetic and sorbic acid are amongst the most commonly used food preservatives (Stratford et al., 2009; Ter Beek and Bruil, 2010; Ullah et al., 2012). While both WOAs have a similar pKᵢ value, sorbic acid is clearly the more potent antimicrobial compound. 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 (Ter Beek, 2009). 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 increase. In controls the pHᵢ increase between the start of germination and t = 90 min was 1.4 units whilst with 3 mM K-sorbate this was 0.7 units. At the onset of the exponential phase, the pHᵢ gradually decreased from pHᵢ = 7.4 to 7.2 at t = 11 h (Figure 6A). Twenty-five millimolars of K-acetate allowed a rapid increase in pHᵢ during germination.

The pHᵢ during exponential growth remained stable at 7.2 during the experiment (Figure 7A).

High concentrations of WOAs were selected to reduce growth by 85%. PptsG-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. K-sorbate (10 mM) is shown to delay the maximum drop in OD₆₀₀ indicative for spore germination. The data in Figure 4B show a drop from OD₆₀₀ 0.13 to 0.08 in 216 min rather than from 0.13 to 0.07 in 84 min as was seen in the control shown in Figure 5. The rise of the pHᵢ was here similarly delayed as was the case with 3 mM K-sorbate. Such effects were not seen with 80 mM K-acetate, although the reduction in growth rate is similar (Figure 7B).

To further confirm the observation that sorbic acid inhibited the development of a positive inside pH gradient, spores of B. subtilis PB2 PsspE-IpHluorin 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 dependent reduction in OD₆₀₀ drop-rate as well as a reduced pHᵢ increase-rate. The OD drop-rate decreased from 80 × 10⁻⁶ to 40 × 10⁻⁶ OD₆₀₀/min when 0.5 mM undissociated sorbic acid was present (Figure 8B). Such effects were not seen with acetic acid at identical concentrations, which
behaved virtually identical to non-stressed germinating spores (Figures 8A,C). 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 (Cortezzo et al., 2004; Van Melis et al., 2011).

**DISCUSSION**

We show here that IpHluorin is an accurate, versatile probe to investigate the pHi of *B. subtilis*. We were able to improve expression of pHluorin by fusion of the first 24 bp of comGA with the pHluorin-encoding gene. Genomic integration of IpHluorin resulted in more homogeneous expression levels compared to a multi-copy plasmid. It also resulted in a more stable construct, not requiring antibiotics for maintenance of the IpHluorin gene during extended periods of growth (not shown). The use of genomically integrated constructs with endogenous promoters for the expression of IpHluorin resulted in a strong enough signal for accurate pH measurements during exponential growth on glucose as well as compartment-specific pHi measurements during sporulation. The IpHluorin that accumulates in the spore under control of PsspE allows pH measurements of the *B. subtilis* spore. During spore germination and outgrowth, the signal from IpHluorin, expressed from PsspE, overlaps slightly in time with PptsG-IpHluorin expression, thus allowing continuous pH monitoring during germination and outgrowth in batch. The pH values we have observed here closely resemble those found with other methods. During exponential growth, the pH approaches pH = 8. The pH of *B. subtilis* spores was also found to lie at approximately pH = 6. Despite the fact that expression levels of IpHluorin are much lower in spores, the pH value observed again closely corresponds to earlier reported values. The notion that during outgrowth a pH is observed that closely resembles the pHi during exponential growth (as observed with PptsG-IpHluorin) further corroborates the accuracy of our method.

Other methods to measure pHi generally involve compounds that are hydrophobic and have WOA groups and may act as uncouplers, thereby depleting the ΔpH and influencing ΔΨ over the membrane. They are also more labor-intensive when high temporal resolution is required and except for fluorescent dyes do not allow cell type-specific pH measurements. However, these methods require long-term incubation with the dye plus extensive washing, taking up to 20 min to prepare the sample. Future studies will have to determine the phototoxicity and bleach rate of...
IpHluorin in individual (growing, sporulating, and germinating) cells.

We have observed clear differences in pH, between Pmuc-IpHluorin and sporulation-specific IpHluorin. It has been shown that within a growing population of B. subtilis cells, differentiation occurs (Veening et al., 2006a,b) and this may affect metabolic state, which indicates that pHi is not constant. This heterogeneity cannot be clearly monitored in batch without the use of more specific promoters or single cell observations. Also during spore germination such heterogeneity is seen (Smith et al., 2010), so our results show the average of a germinating population.

During spore germination, the pHi increases due to release of protons (Swerdlow et al., 1981). This process follows the drop in pH to 6.0, however. The Pmuc-IpHluorin strain can be used for many experiments where the pHi needs to be measured in cells growing on glucose, without the need for additional inducers like IPTG. Also, antibiotics are not strictly necessary. The sporulation-specific IpHluorin-expressing strains may give more insight in compartmentalization during sporulation, while the Pmuc-IpHluorin strain may also help understanding spore germination characteristics in the presence of potential outgrowth inhibitors such as the WA6 sorbic acid and acetic acid.

Clearly, because not all cells are in exactly the same state, these data represent the average value of the pHi in the population studies. To analyze the heterogeneity single-spore pH measurements are needed. Currently we are extending our single cell live imaging tool “SporeTracker” (Paisley et al., 2013) to that end.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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