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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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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 (Krubwich et al., 1998, 2011; Sionczewski et al., 2009). In the model eukaryote Saccharomyces cerevisiae, pH was found to be a signal controlling growth (Oijj et al., 2011). Gene expression as a response to glucose starvation was found to be mediated by changes in the pHi, through the protonation state-dependent binding of a transcription factor to membrane-associated phosphatidic acid (Young et al., 2010). In multicellular eukaryotes pH is thought to be important during growth and differentiation (Crumit et al., 2010). In prokaryotic organisms, the relationships between pH and growth and development have not been studied extensively (Padan and Schuldiner, 1987).

Because of its various well-described differentiation modes, B. subtilis is generally considered to be the bacterial model organism for cellular differentiation. The best described mode of differentiation of this Gram-positive prokaryote is sporulation, with the pathways controlling sporulation understood in great molecular detail (Eichenberger et al., 2004; Steil et al., 2005; Wang et al., 2006). Germination is less well understood, but Keijser et al. (2007) have shown that this too, is a carefully orchestrated process. We reasoned that in analogy to eukaryotes, pH could be a global regulator, as well as an indicator of the metabolic and energetic state of the cell. To gain further insight in the putative pH dynamics of these differentiation processes, we studied the pHi of the mother cell and forespore independently. During sporulation the development of the pHi in the mother cell and the nascent forespore may also give insight in the level of independence of the two cells.

In B. subtilis, the pH of the developing pre-spore is generally assumed to drop to pH = 6.0-6.4 during sporulation (Magill et al., 1994). The drop in pH causes a decrease in activity of phosphoglycerate mutase (PGM), which catalyses the conversion of 3-phosphoglycerate (3-PGA) to 2-phosphoglycerate. The reduced activity of PGM causes the accumulation of 3-PGA in the pre-spore (Singh et al., 1977). 3-PGA is metabolically dormant and contain no measurable ATP or glucose that could act as energy source during sporulation (Singh et al., 1977; Magill et al., 1980). It is assumed that the accumulated 3-PGA serves as an initial carbon and energy source for the cell.

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.
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>araD139 (ara-leu)7696 (lac)X74 galU galK hsdR2 mcrA mcrB1 rspL</td>
<td>Casadaban and Cohen (1980)</td>
</tr>
<tr>
<td>B. subtilis PB1</td>
<td>trpC2; 168 wild-type</td>
<td>C.W. Price</td>
</tr>
<tr>
<td>B. subtilis PB2</td>
<td>pDG148</td>
<td>This work</td>
</tr>
<tr>
<td>B. subtilis PB2 PyrI pHluorin</td>
<td>trpC2; amyE5′ recPspIIA–IpHluorin amyE5′</td>
<td>This work</td>
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<td>This work</td>
</tr>
<tr>
<td>B. subtilis PB2 pDG pHluorin</td>
<td>trpC2; pDG–IpHluorin</td>
<td>This work</td>
</tr>
<tr>
<td>B. subtilis PB2 PspII–IpHluorin</td>
<td>trpC2; amyE5′ recPspIId–IpHluorin amyE5′</td>
<td>This work</td>
</tr>
<tr>
<td>B. subtilis PB2 PspIL–IpHluorin</td>
<td>trpC2; amyE5′ recPspIIId–IpHluorin amyE5′</td>
<td>This work</td>
</tr>
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</table>

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 (MDG; Keijser et al., 2007) buffered at pH 5.5 or 6.4 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 pPJ148-derived plasmids; 10 μg/ml for *B. subtilis* strains, 50 μg/ml for *E. coli* strains, spectinomycin for strains carrying pG1729-derived plasmids or genomic inserts (50 μg/ml). The strains used in this study are listed in Table 1.

SPORELATION OF *B. subtilis* STRAINS

Spores of *B. subtilis* were prepared by glucose depletion of defined liquid medium (MS, which is M63 without soybean 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)600 = 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 fused to IpHluorin with an AUG start codon, were fused to IpHluorin with the signal strength of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The pH-dependent ratiometric fluorescent properties of IpHluorin were not affected by this fusion. To monitor the pH, of both the mother cell and the pre-spore, recombinant *B. subtilis* strains were grown in Lysogeny broth (LB).

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-spore, mother cell and mature spore, as well as during spore germination. Effects of the addition of sorbic and acetic acid on the pH, of germinating spores are described.

Table 1 | Strains used in this study.

<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 pBG-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 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 (*PxyI*, specific for vegetative cells growing on glucose, *Pspac*, specific for pre-septum, sporulating cells, *PpapA*, a fore-sphere-specific gene, and *PpapD*, a mother cell-specific promoter) of *B. subtilis* were selected for their expression levels (Steil et al., 2004; Veenming et al., 2006a). Approximately 500 bp upstream of the start codons 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 *amylE* locus as described (Lewis and Marston, 1999). All plasmids and oligonucleotides used in this study are listed in Tables 2 and 3.

### 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 ble kan lac Pspac</td>
<td>Stragier 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 amylE′, esp PxyI-pG3mut1′ amylE′</td>
<td>Lewis and Marston (1999)</td>
</tr>
<tr>
<td>pSG-pHluorin</td>
<td>bla amylE′, esp PxyI-pHluorin amylE′</td>
<td>This work</td>
</tr>
<tr>
<td>pSG-IpHluorin</td>
<td>bla amylE′, esp PxyI-IpHluorin amylE′</td>
<td>This work</td>
</tr>
<tr>
<td>pSGpxyl-pHluorin</td>
<td>bla amylE′, esp PxyI-pHluorin amylE′</td>
<td>This work</td>
</tr>
<tr>
<td>pSGpxyl-ipHluorin</td>
<td>bla amylE′, esp PxyI-IpHluorin amylE′</td>
<td>This work</td>
</tr>
<tr>
<td>pSGpxylsp-pHluorin</td>
<td>bla amylE′, esp PxyI-SPspE-pHluorin amylE′</td>
<td>This work</td>
</tr>
<tr>
<td>pSGpxylsp-ipHluorin</td>
<td>bla amylE′, esp PxyI-SPspE-IpHluorin amylE′</td>
<td>This work</td>
</tr>
</tbody>
</table>

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 (Brenner 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 Brink, 2009). For spore germination, washed spores were heat activated (30 min, 70°C, then cooled on ice) and subsequently mixed 1:1 with 2× concentrated M3 with or without glucose, containing weak organic acid (WOA) in predetermined
concentrations. To trigger germination, 5 μL 20% concentrated AGFK (10 mM L-asparagine, 10 mM D-glucose, 1 mM D-fructose, 1 mM KC3, Wall 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 IpHfluorin 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 UPLANL 100×/1.3 oil objective (Japan) with a 410 nm excitation, 510 nm fluorescence emission at 390 and 470 nm. The three strains compared had identical growth rates 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 GFP and YFP in B. subtilis (Veening et al., 2004). We used this approach to construct improved pHfluorin (IpHfluorin, Figure 1A).

To analyze fluorescence intensity of Bacillus cells harboring pDG148, pDG-pHfluorin, or pDG-IpHfluorin cells were transfected 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 IpHfluorin had the highest signal intensity at 1 mM IPTG, approximately 40% higher than pHfluorin under the same conditions. The most important characteristic of pHfluorin is its shift in excitation wavelength depending on the pH of its surroundings. To establish if the addition of eight amino acids at the N-terminus would alter these characteristics, calibration curves for pHfluorin and IpHfluorin were made (Figure 1C) and depended on the concentration of pHfluorin. Cells expressing IpHfluorin had the highest signal intensity at pH 7.0, but at pH 6.0 the signal was lower by a factor of 20.

**RESULTS**

**IMPROVED EXPRESSION OF pHfluorin**

Many microorganisms have an internal (cytosolic and/or mitochondrial) pH between 7 and 8 (Ury et al., 2009; Słonczewski 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 pHfluorin, 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 GFP and YFP in B. subtilis (Veening et al., 2004). We used this approach to construct improved pHfluorin (IpHfluorin, Figure 1A).

**INTERNAL pH VARIATES 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
genes to IpHluorin. This allowed us to measure pHi of we fused promoters of strongly expressed, growth phase-specific (Shioi et al., 1980; Slonczewski et al., 2009). Expression levels from PspoIIA, PspoIIID, and PsspE starts after the drop in OD600. The surviving cells may prepare for diauxic growth or sporulation (Veenin 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, PspoIIID, and PsspE 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.

**Table 4 | Promoters used for IpHfluorin expression.**

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Regulator</th>
<th>Corresponding growth phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PptsG</td>
<td>σ^A</td>
<td>Growth on glucose (Botella et al., 2010)</td>
</tr>
<tr>
<td>PspoIIA</td>
<td>SpoTA, α^H</td>
<td>Early sporulation (Wang et al., 2006)</td>
</tr>
<tr>
<td>PspoIIID</td>
<td>σ^B</td>
<td>Early sporulation, mother cell-specific (Wang et al., 2006)</td>
</tr>
<tr>
<td>PsspE</td>
<td>σ^C</td>
<td>Late sporulation, spore-specific (Wang et al., 2006)</td>
</tr>
</tbody>
</table>

**SPORULATION-SPECIFIC EXPRESSION OF IpHfluorin**

Sporulation of B. subtilis is a well-documented, 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 IpHfluorin. 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, PspoIIID, and PsspE starts after the drop in OD600. The surviving cells may prepare for diauxic growth or sporulation (Veenin 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, PspoIIID, and PsspE 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) PspgG-IpHluorin-expressing cells; (E,F) sporulating cells expressing PspoIID-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 (Veenin 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; Veenin 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 IpHluorin 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 spore-specific promoter PspE 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 (PspE-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 PspE-IpHluorin. After 40 h, the pH value of 6.8 reported by PspE-IpHluorin is approaching the reported value for Bacillus spores (pH = 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 pH of B. subtilis spores also indicated values around 6.0 ± 0.3 (see, e.g., pHluorin data of time point 0 obtained with IpHluorin driven by PspE in Figure 5 and beyond).
INTERNAL pH DURING SPORE GERMINATION AND OUTGROWTH

As described above, IpHluorin expressed from PsspE accumulates in mature spores. Germination and outgrowth were monitored using B. subtilis PB2 PptsG-IpHluorin and B. subtilis PB2 Pptse2-IpHluorin. When germination is triggered by addition of a mixture of asparagine, glucose, fructose and potassium (AGFK), the pHpse of the spore crop drops, because the refractile spores turn phase-dark, due to water uptake. Simultaneously, the spore’s pHr rises. Depending on the medium pH, the pHr 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 ptsG 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 pHr. Also, when outgrowth commences, pHr of these cells is higher, but follows a similar trend as with medium at pH = 6.4.

INTERNAL pH DURING SPORE GERMINATION 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 (Correzzetto et al., 2004; Van Melis et al., 2011). When spores start to germinate, they release protons and the pHr 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 Beck and Brul, 2010; Ullah et al., 2012). While both WOAs have a similar pKₐ 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 Beck, 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 pHr increase. In controls the pHr 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 pHr, which gradually decreased from pH = 7.4 to 7.2 at t = 11 h (Figure 6A). Twenty-five millimolars of K-acetate allowed a rapid increase in pHr during germination.

The pHr 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 6B 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 pHr 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 PptsG-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 pHr increase-rate. The OD drop-rate decreased from 80 × 10⁻³ to 40 × 10⁻³ 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
Intracellular pH measurement in Bacillus

FIGURE 7 Internal pH during spore germination and outgrowth. IpHluorin accumulated in the spores (from expression controlled by PsspE) allows pH measurements from \( t = 0 \) to approximately 300 min. Expression of IpHluorin from PsspE allows calculation of the pH from approximately 200 min. Data are from a representative example. (A) Germination and outgrowth of B. subtilis spores at an external pH \( \geq 6.4 \) with 25 mM KAc. (B) Germination and outgrowth of B. subtilis spores at an external pH \( \geq 6.4 \) with 80 mM KAc.

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 pH 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 genetically integrated constructs with endogenous promoters for the expression of IpHluorin gene during extended periods of growth (not shown). The use of genetically 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 pH 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 PsspE-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 pH during exponential growth (as observed with PsspE-IpHluorin) further corroborates the accuracy of our method.

Other methods to measure pH generally involve compounds that are hydrophobic and have WOA groups and may act as uncouplers, thereby depleting the \( \Delta pH \) and influencing \( \Delta \Psi \) 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 Preg-IpHluorin and sporulation-specific IpHluorin. It has been shown that within a growing population of B. subtilis cells, differentiation occurs (Smelt et al., 2008) and this may affect metabolic state and this heterogeneity may be maintained throughout batch without the use of more specific promoters or single cell observations. Also during sporulation such heterogeneity is seen (Smelt et al., 2000), so our results show the average of a heterogenous population.

During spore germination, the pH increases due to release of protons (Swedlow et al., 1991). This process follows the drop in OD600, and results from H2O uptake and release of EPA. Our results show that a pH is established rapidly. Such an increased pH can reactivate PGM, thus allowing the utilization of the spore’s 3-PGA store (Magill et al., 1994).

Taken together, our results show accurate, long-term pH monitoring in growing and sporulating B. subtilis cultures as well as during spore germination. The pH of sporulating cells is as high as that of exponentially growing cells. This is particularly the case for the mother cell. The pre-pH6 drop to pH 6.0, however, the Preg-IpHluorin strain can be used for many experiments where the pH 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 Preg-IpHluorin strain may also help understanding spore germination characteristics in the presence of potential outgrowth inhibitors such as the WOAs sorbic acid and acetic acid.

Clearly, because not all cells are in exactly the same state, these data represent the average value of the pH in the population studied. To analyze the heterogeneity single-spore pH measurements are needed. Currently we are extending our single cell live imaging tool “SporeTracker” (Pandey 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. Received: 05 April 2013; accepted: 30 May 2013; published online: 18 June 2013. Citation: van Beilen JWA and Brul S (2013) Compartment-specific pH monitoring in Bacillus subtilis using fluorescent sensor proteins: a tool to analyze the antibacterial effect of weak organic acids. Front. Microbiol. 4:157. doi: 10.3389/fmicb.2013.00157

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