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van Beilen, J.W.A.; Brul, S.

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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

**Johan W. A. van Beilen and Stanley Brul**

Department of Molecular Microbial Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, Netherlands

**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 prokaryotic organisms, the pH of the cell's interior can be measured using 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 and the activity of the probe itself in a cell as well as the detection method desired. The probe used to measure pH should maintain accuracy over the pH range assessed. In addition, both the presence and the activity of the probe itself in a cell as well as the detection method should have minimal effect on cell physiology. Currently used techniques include the distribution of radiolabeled membrane-permeant weak acids, 31P nuclear magnetic resonance.

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 (lpHluorin) of the ratiometric, pH-sensitive fluorescent protein pHluorin by extending it at the 5' end with the first 24 bp of corG&. 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&g&G) or during sporulation (P&spoIIA, P&spoIIID, and P&spoEI). Our results show strong, compartment-specific expression of lpHluorin 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 pH of 6.0 to 6.3. Upon full germination the pH rose steeply (T=0–1.5h). The presence of sorbic acid in the germination medium inhibited such effects were absent when acetic was added at identical concentrations.

Keywords: bacterial spore formers, spores, spore germination, intracellular pH, GFP, pHluorin, weak organic acids, uncouplers
(NMR), fluorescent dyes (e.g., carboxyfluorescein, carboxyfluorescein diacetate, and succinimidyl ester; Ugarbli et al., 1978; Booth, 1985; Balthius et al., 1993; Magill et al., 1994; Boozer 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 (Stosiekowski et al., 2009). Weak acid dyes or reporters may alter the pH, and are therefore difficult to use accurately, and may require many treatment and incubation steps before measurement. 13P NMR and radiolabeled 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 (Ori et al., 2011; Ubha et al., 2012), and more recently also in B. subtilis (Ver Beck, 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 (YFP). The pH-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-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.

### 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 (M3G; 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 pPG148-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.

#### SPORULATION OF B. subtilis STRAINS

Spores of B. subtilis were prepared by glucose depletion of defined liquid medium (M3S, which is M3G 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) tot = 1.

#### CLOSING 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, with an ATG start codon, were fused to pHluorin by a polymerase chain reaction (PCR) with Pfu polymerase using primers IpHlu_F_2010_FW and IpHlu_F_2010_RV. This sequence was subsequently extended with a standard Shine–Dalgarno (SD) region (AAAGGAGGAAGCAGGT; Joseph et al., 2001) using primers IpHlu pdGA_FW. This SD-improved pHluorin (IpHluorin) was

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

| Strains | Genotype | Reference or construction |
|---------|----------|--------------------------|
| E. coli MC1061 | Cloning host; F− araD139 araE19 galU galK hsdR2 mcrA mcrB1 rpsL | Casadaban and Cohen (1980); C.W. Price |
| Bacillus subtilis | | |
| PB2 | trpC; 168 wild-type | This work |
| PB2 pDG148 | trpC; pDG148 | This work |
| PB2 Pspxl-pHluorin | trpC; amyES'; ppc Pspxl-pHluorin amyES' | This work |
| PB2 Pspxl-pHluorin | trpC; amyES'; ppc Pspxl-pHluorin amyES' | This work |
| PB2 pDG-pHluorin | trpC; pDG-pHluorin | This work |
| PB2 pDG-IPHluorin | trpC; pDG-IPHluorin | This work |
| PB2 Pspxl-IPHluorin | trpC; amyES'; ppc Pspxl-IPHluorin amyES' | This work |
| PB2 Pspxl-IPHluorin | trpC; amyES'; ppc Pspxl-IPHluorin amyES' | This work |
| PB2 Pspxl-IPHluorin | | This work |
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 P\textsubscript{xyi} promoter. To monitor the pH\textsubscript{i} 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 (P\textsubscript{xyi}), specific for pre-septum, sporulating cells, P\textsubscript{spoIId}, a forespore-specific gene, and P\textsubscript{spoIia}, a mother cell-specific promoter of B. subtilis were selected for their expression levels (Steil et al., 2005; Venema 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 promoter sites (Steil 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-1-thiogalactopyranoside (IPTG).

For calibration of the pH\textsubscript{i}, expression of ratiometric pHluorin and IpHluorin was induced for 2.5 h by the addition of 1 mM IPTG. At OD\textsubscript{600} = 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 K\textsubscript{2}HPO\textsubscript{4}. 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. OD\textsubscript{600} 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 OD\textsubscript{600} 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 SPORULATION, GERMINATION, AND OUTGROWTH**

To monitor pH\textsubscript{i} 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 OD\textsubscript{600} = 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. OD\textsubscript{600} 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 Beek, 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. pH\textsubscript{i} was measured in the presence of the acid and glucose for 1 h after resuspension of the spores in the assay medium.

| Plasmid     | Genotype                           | Reference or construction |
|-------------|------------------------------------|---------------------------|
| pDG148      | bla, ble, kan, lac, Pspac           | Strager et al. (1988)     |
| pDG-pHluorin| pDG148, pHluorin                    | This work                 |
| pDG-IpHluorin| pDG148, IpHluorin                   | This work                 |
| pSG1729     | bla amyE3, aec Pgyl-pGfpmut1 amyE3′ | Lewis and Marston (1999)  |
| pSG-pHluorin| bla amyE3, aec Pgyl-pHluorin amyE3  | This work                 |
| pSG-IpHluorin| bla amyE3, aec Pgyl-IpHluorin amyE3′| This work                 |
| pSGPxyi-IpHluorin| bla amyE3, aec Pxyi-IpHluorin amyE3′| This work                 |
| pSGPxyi-pHluorin| bla amyE3, aec Pxyi-pHluorin amyE3  | This work                 |
| pSGPxyi-AvrII-IpHluorin| bla amyE3, aec Pxyi-AvrII-IpHluorin amyE3′| This work                 |
| pSGPxyi-AvrII-pHluorin| bla amyE3, aec Pxyi-AvrII-pHluorin amyE3| This work                 |

Table 2 | Plasmids used in this study.
concentrations. To trigger germination, 5 μl 20× concentrated AGFK (10 mM l-asparagine, 10 mM d-glucose, 1 mM d-fructose, 1 mM KC3, 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 UPLAFN 100 x/1.3 oil objective (Japan) with a 41017 ×/1.3 oil objective (Japan) with a 41017

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**RESULTS**

**IMPROVED EXPRESSION OF pHluorin**

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 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 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 transf...
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 B. subtilis 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 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.

### 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 decrease in pHi 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, PspoIIID, and PspoIIE starts after the drop in OD600. The surviving cells may prepare for diauxic growth or sporulation (Veenings 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 PspoIIE are lower than of PptsG, but are still reliable and strong enough to allow pHi 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.

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

| Promoter | Regulator | Corresponding growth phase |
|----------|-----------|-----------------------------|
| PptsG    | σ^A       | Growth on glucose (Botella et al., 2010) |
| PspoIIA  | SpoOA, σ^IIA | Early sporulation (Wang et al., 2006) |
| PspoIIID | σ^E       | Early sporulation, mother cell-specific (Wang et al., 2006) |
| PspoIIE  | σ^F       | Late sporulation, spore-specific (Wang et al., 2006) |

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Figure 1 | Improved expression of pHluorin in B. subtilis.
(A) Schematic overview of the improved pHluorin construct: SD – Shine–Dalgarno sequence, comGA – the first 24 bp of the Shine–Dalgarno sequence, comGA – the first 24 bp of the Shine–Dalgarno sequence. comGA – the first 24 bp of the Shine–Dalgarno sequence, comGA – the first 24 bp of the Shine–Dalgarno sequence.

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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) PpsG-IpHluorin-expressing cells; (E,F) sporulating cells expressing PspoID-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 (Veenig 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 fore-spore (our unpublished observations; Veenig et al., 2006). Expression of genes in the mother cell regulated by \( P_{spoIID} \) follows that of those regulated by \( P_{spoIIA} \) as expected, but because expression levels of \( P_{spoIID} \)-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 \( P_{ptsG} \)-IpHluorin strain. The mother cell (\( P_{spoIID} \)-IpHluorin) had a pH of 7.8 after 17 h of incubation. IpHluorin expressed from the spore-specific promoter \( P_{spspE} \) 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 fore-spore (\( P_{spspE} \)-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 fore-spore is 7.4, as reported by \( P_{spspE} \)-IpHluorin. After 40 h, the pH value of 6.8 reported by \( P_{spspE} \)-IpHluorin is approaching the reported value for Bacillus spores (\( pH_i \approx 6.0 \pm 0.3 \); 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 fore-spore-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 \( P_{spspE} \) in Figure 5 and beyond).
As described above, IpHluorin expressed from PsspE accumulates in mature spores. Germination and outgrowth were monitored used 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 OD$_{600}$ of the spore crop drops, because the refractile spores turn phase-darker, due to water uptake. Simultaneously, the spore’s pH$_i$ rises. Depending on the medium pH, the pH$_i$ 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 pefC 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 CO$_2$ (Russell and Dietz-Gonzalez, 1999; Oriol, 2010). After approximately 60 min, there is a sudden drop in pH and OD$_{600}$ as described above (Figure 5).

Germination with medium pH = 7.4 shows a more rapid decrease in OD$_{600}$ and an equally faster rise in pH$_i$. Also, when outgrowth commences, pH$_i$ 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 (Correzzino et al., 2004; Van Melis et al., 2011). When spores start to germinate, they release protons and the pH$_i$ 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 Brul, 2010; Ullah et al., 2012). While both WOAs have a similar pK$_a$ 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 pH$_i$ increase. In controls the pH$_i$ 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 pH$_i$ which gradually decreased from pH$_i = 7.4$ to 7.2 at t = 11 h (Figure 6A). Twenty-five millimolar of K-acetate allowed a rapid increase in pH$_i$ during germination.

The pH$_i$ 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 (30 mM) is shown to delay the maximum drop in OD$_{600}$ indicative for spore germination. The data in Figure 4B show a drop from OD$_{600}$ 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 pH$_i$ 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 dependant reduction in OD$_{600}$ drop-rate as well as a reduced pH$_i$ increase-rate. The OD drop-rate decreased from 80 $\times$ 10$^{-3}$ to 40 $\times$ 10$^{-3}$ OD$_{600}$/min when 0.5 mM undissociated sorbic acid was present (Figure 8B). Such effects were not seen with acetic acid at identical concentrations, which

The pH$_i$ 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 (30 mM) is shown to delay the maximum drop in OD$_{600}$ indicative for spore germination. The data in Figure 4B show a drop from OD$_{600}$ 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 pH$_i$ 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 dependant reduction in OD$_{600}$ drop-rate as well as a reduced pH$_i$ increase-rate. The OD drop-rate decreased from 80 $\times$ 10$^{-3}$ to 40 $\times$ 10$^{-3}$ OD$_{600}$/min when 0.5 mM undissociated sorbic acid was present (Figure 8B). Such effects were not seen with acetic acid at identical concentrations, which
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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 pHi from approximately 200 min. Data are from a representative example. (A) Germination and outgrowth of B. subtilis spores at an external pH = 6.4 with 25 mM KAc. (B) Germination and outgrowth of B. subtilis spores at an external pH = 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 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 pHi measurements of the B. subtilis spore. During spore germination and outgrowth, the signal from IpHluorin, expressed from PsspE, overlaps slightly in time with PspGIpHluorin expression, thus allowing continuous pHi 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 pHi approaches pH = 8. The pHi 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 PspGIpHluorin) 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

FIGURE 8 | OD600 and internal pH during germination of B. subtilis PB2 PsspE-IpHluorin spores in medium without glucose (pHo = 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.
IpHluorin in individual (growing, sporulating, and germinating) cells.

We have observed clear differences in pH between Pmg-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 and pH. This heterogeneity cannot be clearly monitored in that of exponentially growing cells. This is particularly the case for WOAs sorbic acid and acetic acid.

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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intracellular pH measurement in growing and sporulating batch without the use of more specific promoters or single cell observations. Also during sporage germination such heterogeneity is seen (Smoll et al., 2010), so our results show the average of a germinating population.

During spore germination, the pH increases due to release of protons (Swedlow et al., 1991). This process follows the drop in pH that of exponentially growing cells. This is particularly the case for 3-PGA store (Magill et al., 1994). pH can reactivate PGM, thus allowing the utilization of the spore’s protons (Swerdlow et al., 1981). This process follows the drop in pH during sporulation, while the PsspE-IpHluorin strain may give more insight in compartments during sporulation, while the PsspE-IpHluorin strain may also help understanding spore germination characteristics in the presence of potential growth 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 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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