Compartment-specific pH monitoring in Bacillus subtilis using fluorescent sensor proteins: a tool to analyze the antibacterial effect of weak organic acids.
van Beilen, J.W.A.; Brul, S.

Published in:
Frontiers in Microbiology

DOI:
10.3389/fmicb.2013.00157

Citation for published version (APA):
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 (IpHluorin) of the ratiometric, pH-sensitive fluorescent protein pHluorin by extending it at the C' end with the first 24 bp of corA6. 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 (PspφIIA, PspφIIID, and PspφIIε) or during sporulation (PspφIIA, PspφIIID, and PspφIIε). Our results show strong, compartment-specific expression of IpHluorin 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 development of the pHi in the mother cell and the nascent 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 pHi 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. B. subtilis spores are metabolically dormant and contain no measurable ATP or glucose that could act as energy source during germination (Singh et al., 1977; Magill et al., 1994). 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., 1990), to minimize the effect of the cell’s surrounding (unless desired). The probe used to measure pHi 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; Ugurbil et al., 1978; Booth, 1985; Bulthuis et al., 1993; Magill et al., 1994; Breeseover et al., 1996; Leuchner and Lifford, 2000). These methods have the advantage that no genetic modification is required and in the case of fluorescent dyes, single cell measurements are possible (Skoczowska 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. 1P 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 (Orti et al., 2011; Ullah et al., 2012), and more recently also in B. subtilis (van 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 (Veenings et al., 2004). We therefore fused the first eight amino acids of the codon usage of pHluorin was not optimized for use in B. subtilis and more recently also in B. subtilis (van 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 (Veenings et al., 2004). We therefore fused the first eight amino acids of comGA to pHluorin (Veenings 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 pHluorin were not affected by this fusion.

Expression of pHluorin 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 were 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 (MMG; 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 pJS148-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 MMG without sodium glutamate), at pH = 7.0. Cultures were incubated for 4 days at 37°C under continuous agitation (200 rpm). S pores 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 optimal density (OD600 = 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, with an ATG start codon, were fused to pHluorin by a polymerase chain reaction (PCR) with Fnu polymerase 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 (IpHlu) was

---

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>F' araD139 araE73998 lacY1 galU3 galK1 recA1 mcrA1 mcrB1 rpsL</td>
<td>Catanasis and Cohen (1980)</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB2</td>
<td>topC2, 188 wild-type</td>
<td>C.W. Price</td>
</tr>
<tr>
<td>PB2 pDG148</td>
<td>topC2, pDG148</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PhylHun</td>
<td>topC2, amyE5' ncp Phyl-pHluorin amyE5'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PhylHun</td>
<td>topC2, amyE5' ncp Phyl-pHluorin amyE5'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 pDG-pHun</td>
<td>topC2, pDG-pHluorin</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 pDG-pHun</td>
<td>topC2, pDG-pHluorin</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PptsG-pHun</td>
<td>topC2, amyE5' ncp PptsG-pHluorin amyE5'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PaplA-pHun</td>
<td>topC2, amyE5' ncp PaplA-pHluorin amyE5'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PaplD-pHun</td>
<td>topC2, amyE5' ncp PaplD-pHluorin amyE5'</td>
<td>This work</td>
</tr>
<tr>
<td>PB2 PaplD-pHun</td>
<td>topC2, amyE5' ncp PaplD-pHluorin amyE5'</td>
<td>This work</td>
</tr>
</tbody>
</table>

---

"fmicb-04-00157" — 2013/6/15 — 11:19 — page 2 — #2
inserted between the HindIII and SalI sites of pDG148. This con-
struct, pDG-Iphluorin, was transformed into *B. subtilis* PB2 and
compared with PB2 carrying pDG-pHluorin to analyze expres-
sion levels and pH-dependent characteristics of pHluorin and
Iphluorin. Also, a xylose-inducible, genome-integrated expres-
sion 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
Pמה promoter.

To monitor the pH of *B. subtilis* for extended periods of time
in different phases of its life cycle, without the need for exter-
nally supplied expression inducers, the promoter region of several
growth phase-specific genes (*Pmae*; specific for vegetative cells
growing on glucose, *Ppae*, specific for pre-septum, sporulating cells, *Pspo*, a
fore-spore-specific gene, and *PspoD*, a mother cell-specific pro-
motor) of *B. subtilis* were selected for their expression levels (Steil
et al., 2005; Veening 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 plas-
mids 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-D-1-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 extra-
cellular 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 Flu-
osoArt Optima. OD600 was measured before start of the experiment.
The ratio of emission intensity at 510 nm resulting from excita-
tion at 390 and 470 nm (with photomultiplier gain set to 2,000)
was calculated as described previously (Orji et al., 2011). Fluores-
cence and OD600 were monitored for 30 min, with measurements
taken every 5 min. Calibration curves for pHluorin and Iphluo-
rin were identical, with only minor fluctuations in fluorescence
in time observed with pHluorin at pH = 8.5. From this, we con-
cluded that the intracellular and extracellular pH had equilibrated
rapidly. *B. subtilis* PB2 carrying pDG148 was measured for back-
ground fluorescence. Background fluorescence was subtracted at
individual wavelengths before calculating the ratio. The calibra-
tion 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, 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 Flu-
osoArt Optima BMG (Labbach, 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 Beek, 2009). For spore germination, washed
spores were heat activated (50 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

| Table 2 | Plasmids used in this study |
|-----------------|------------------|------------------|
| **Plasmid**     | **Genotype**     | **Reference or construction** |
| pDG148          | blg blb lacI lacZ pijac | Stoger et al. (1988) |
| pDG-pHluorin    | pDG148                                      | This work          |
| pDG-Iphluorin   | pDG148                                      | This work          |
| pSG1729         | blg amyE5′   apr gfpmut1′ amyE5′           | Lewis and Marston (1999) |
| pSG-PspoiD-Iphluorin | amyE5′      | This work          |
| pSG-PspoiA-Iphluorin | amyE5′      | This work          |
| pSG-PspoiA-Iphluorin | amyE5′      | This work          |
| pSG-PspoiA-Iphluorin | amyE5′      | This work          |
concentrations. To trigger germination, 5 μl 20% concentrated AGFK (10 mM l-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 OD₅₆₀ 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 UPLAN1 100×/1.3 oil objective (Japan) with a 41017×1.3 oil objective (Japan) with a 41017

**RESULTS**

**IMPROVED EXPRESSION OF pHluorin**

Many microorganisms have an internal (cytosolic and/or mitochondrial) pH between 7 and 8 (Uji 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 (Misenbök 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 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 most important characteristic of pHluorin 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 pHluorin and IpHluorin were made (Figure 1C), and depended on the concentration of pHluorin. Cells expressing IpHluorin had the highest signal intensity at 1 mM IPTG, approximately 40% higher than pHluorin under the same conditions. The most important characteristic of pHluorin 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 pHluorin and IpHluorin were made (Figure 1C), and depended on the concentration of pHluorin. Cells expressing IpHluorin had the highest signal intensity at 1 mM IPTG, approximately 40% higher than pHluorin under the same conditions. The most important characteristic of pHluorin 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 pHluorin and IpHluorin were made (Figure 1C), and depended on the concentration of pHluorin. Cells expressing IpHluorin had the highest signal intensity at 1 mM IPTG, approximately 40% higher than pHluorin under the same conditions. The most important characteristic of pHluorin 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 pHluorin and IpHluorin were made (Figure 1C), and depended on the concentration of pHluorin. Cells expressing IpHluorin had the highest signal intensity at 1 mM IPTG, approximately 40% higher than pHluorin under the same conditions. The most important characteristic of pHluorin 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 pHluorin and IpHluorin were made (Figure 1C), and depended on the concentration of pHluorin. Cells expressing IpHluorin had the highest signal intensity at 1 mM IPTG, approximately 40% higher than pHluorin under the same conditions. The most important characteristic of pHluorin 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 pHluorin and IpHluorin were made (Figure 1C), and depended on the concentration of pHluorin. Cells expressing IpHluorin had the highest signal intensity at 1 mM IPTG, approximately 40% higher than pHluorin under the same conditions.

**THE INTERNAL pH VARIATES WITH GROWTH PHASE**

The cytosolic pH is a crucial parameter for bacteria because it modulates the activity of many enzymes (Vojinovic and Von Stockar, 2009) and in many species plays a crucial role in generating the
gene to IpHluorin. This allowed us to measure pHi of cells fused promoters of strongly expressed, growth phase-specific promoters, a cut-off of 1,000 arbitrary units in the 390 to 510 nm fluorescence channel was used for pHi calculations. Reported values ranging from pH = 7.8 to 8.1 (Setlow and Setlow, 1986; 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 step decrease in pH, 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 PpreA-driven IpHluorin may also get trapped in sporulating cells so that the observed pH from PpreA-driven IpHluorin is the average of sporulating and non-sporulating cells as well as 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 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 pH of both cells separately. Expression from PspoIIA, PpreA, and PptsG starts after the drop in OD600. The surviving cells may prepare for diacitic growth or sporulation (Vermeij et al., 2008). This characteristic allowed us to measure differences in pH, in both mother cell and pre-spore in the subpopulation that initiates sporulation. Expression levels from PspoIIA, PpreA, and PptsG are lower than of PpreA, 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 pH calculations.

![Figure 1](https://example.com/figure1.png)  
**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 comGA, with the start codon converted to ATG. (B) Expression levels of pHluorin and improved pHluorin after induction with IPTG. Fluorescence (AU, arbitrary units) at 510 nm from excitation at 390 nm was measured after 2.5 h of induction and normalized to OD600. Diamonds, control (pDG148); squares, improved pHluorin after induction with IPTG; triangles, IpHluorin (pDG-IpHluorin). Error bars indicate standard deviations (n = 3).

![Figure 2](https://example.com/figure2.png)  
**Figure 2A–H** | Expression levels of pHluorin and IpHluorin.  
(A) Expression from PspoIIA, PspoIIID, and PsspE starts after the drop in OD600. The surviving cells are, for instance, smaller than exponentially growing cells. Hence, after sporulation commences, the pH values observed with PspoIIA-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 PpreA-driven IpHluorin may also get trapped in sporulating cells so that the observed pH from PpreA-driven IpHluorin is the average of sporulating and non-sporulating cells as well as 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>
<thead>
<tr>
<th>Promoter</th>
<th>Regulator</th>
<th>Corresponding growth phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcomGA</td>
<td>σA</td>
<td>Growth on glucose (Botella et al., 2010)</td>
</tr>
<tr>
<td>PspoIIAD</td>
<td>Spo0A, σE</td>
<td>Early sporulation (Wang et al., 2006)</td>
</tr>
<tr>
<td>PpreA</td>
<td>σE</td>
<td>Early sporulation, mother cell-specific (Wang et al., 2006)</td>
</tr>
<tr>
<td>PptsG</td>
<td>σG</td>
<td>Late sporulation, spore-specific (Wang et al., 2006)</td>
</tr>
</tbody>
</table>

---

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

---

**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) PptsG-IpHluorin-expressing cells; (E,F) sporulating cells expressing PspoIIID-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., 2006). 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 PspoIIIG follows that of those regulated by PspoIIA, as expected, but because expression levels of PspoIIIG-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 (PspoIIIG-IpHluorin) had a pH of 7.8 after 17 h of incubation. IpHluorin expressed from the spore-specific promoter PspoIIIG revealed pH values of 7.4. The mother cell-specific expression of IpHluorin from the spoIIIG 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 (PspoIIIG-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 PspoIIIG-IpHluorin. After 40 h, the pH value of 6.8 reported by PspoIIIG-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 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 PspoIIIG 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 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-dark, 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 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, 1998; Orij, 2010). After approximately 600 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 fast 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 (Cortezzo 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 Beck 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 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 pH_i increase. In controls the pH_i increase started between the start of germination and t = 90 min was 1.4 units whereas 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 millimolars 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%. PsspE-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 6B 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 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 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 7B). 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 pH$_i$ 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 pH$_i$ measurements during sporulation. The IpHluorin that accumulates in the spore under control of PsspE allows pH$_i$ 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$_i$ 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$_i$ approaches pH = 8. The pH$_i$ 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$_i$ during exponential growth (as observed with PptsG-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 Δ$p$H 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...
IpHfluorin in individual (growing, sporulating, and germinating) cells.

We have observed clear differences in pH between Fp-ac- IpHfluorin and sporulation-specific IpHfluorin. 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(s). This heterogeneity is monitored in batch without the use of more specific promoters or single cell observations. Also during spore germination such heterogeneity is seen (Smelt et al., 2011), 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 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 stored energy. This process follows the drop in pH that of exponentially growing cells. This is particularly the case for the mother cell. The pre-spore pH drops to pH 6.0, however. The Fp-ac-IpHfluorin 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 IpHfluorin-expressing strains may give more insight in compartmentalization during sporulation, while the Fp-ac-IpHfluorin strain may also help understanding spore germation 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.

ACKNOWLEDGMENTS

Tanneke den Blaauwen and Jolanda Verheul are thanked for usage of and assistance with the microscopy facilities of the department of Molecular Cytology at the University of Amsterdam. Frans Klin and Gertien Smits are acknowledged for critically reading initial versions of the manuscript. Alex Ter Beek and Jannike Wijman are acknowledged for initial experiments with pHluorin and many stimulating discussions on Bacillus subtilis acid stress physiology.

REFERENCES


Cruciat, C.-M., Ohkawara, B., Ace- brom, S. P., Karabacak, E., Reinhard, C., Ingheling, D., et al. (2010). Requirement of proton receptor and vacuolar H-ATPase-mediated compartment bundling, and is essential for differentiating cell type during sporulation, while the PsspE-IpHluorin strain can be used for many experiments.


Fukuda, M., Inagaki, T., Takami, S., and Watanabe, H. (2006). A novel method for studying the relationship between ATPase-mediated compartment bundling and is essential for differentiating cell type during sporulation, while the PsspE-IpHluorin strain can be used for many experiments.


Koppelman, C.-M., Aarsman, M. E. G., Huis, D. M., and van der Spek, H., et al. (2004). R174 plasmid bundling, and is essential for differentiating cell type during sporulation, while the PsspE-IpHluorin strain can be used for many experiments.


"fmicb-04-00157" — 2013/6/15 — 11:19 — page 10 — #10


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: 04 April 2013; accepted: 30 May 2013; published online: 18 June 2013.

© 2013 van Beilen and Brul. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums, provided the original authors and source are credited and any changes are properly attributed, is permitted.