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Noninvasive Measurement of Bacterial Intracellular pH on a Single-Cell Level with Green Fluorescent Protein and Fluorescence Ratio Imaging Microscopy

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We show that a pH-sensitive derivative of the green fluorescent protein, designated ratiometric GFP, can be used to measure intracellular pH (pH_i) in both gram-positive and gram-negative bacterial cells. In cells expressing ratiometric GFP, the excitation ratio (fluorescence intensity at 410 and 430 nm) is correlated to the pH_i, allowing fast and noninvasive determination of pH_i that is ideally suited for direct analysis of individual bacterial cells present in complex environments.

Bacteria are often subjected to various forms of environmental stress, and in recent years, there has been increasing focus on the different mechanisms they employ to protect themselves against these environmental changes. One of the most extensively studied stress responses is the ability of bacteria to survive low pH by adjusting their intracellular pH (pH_i) in response to changes in extracellular pH (pH_{ex}) (1, 4). Several methods have been developed for measuring bacterial pH_i (2, 14, 17, 19). However, as these techniques require radioactive labeling and time-consuming staining procedures, noninvasive methods for continuous measurement of pH_i in bacteria are in demand. Since the discovery of green fluorescent protein (GFP), a wide range of mutant variants has been created. In eukaryotic cells, several GFP variants with altered excitation and emission spectra have been examined for their use as pH_i probes (8, 11, 12, 13, 16). One of these GFP variants, ratiometric GFP, was obtained by introducing specific amino acid substitutions to the chromophore, causing the resulting protein to alter its excitation spectrum according to the pH of the surrounding environment (13).

In order to express the ratiometric GFP protein in both gram-positive and gram-negative bacterial cells, we inserted the corresponding gene downstream of the P32 promoter in the chloramphenicol-resistant expression vector pMG36c, which replicates in both cell types (20). The ratiometric *gfp* gene (GenBank accession no. AF058694) was amplified by PCR by using the oligonucleotides C-GFP (5′-TAT CCC AAG CTT TTA TTT GTA TAG TTC ATC CAT GCC ATG TG-3′) and N-GFP (5′-TGC TCT AGA GTA ATA AGG AGG AAA AAA TAT GAG TAA AGG AGA AGA ACT TTT CAC TGG AGT TGT CCC-3′) (DNA Technology, Århus, Denmark) that additionally introduced an initiating ATG codon as well as a ribosomal binding site (5). The resulting 750-bp DNA fragment was inserted into the *Xba*I- and *Eco*RI-digested pMG36c, and the correct DNA sequence of the ratiometric *gfp* gene present in the resulting plasmid (pGFPratiometric) was confirmed by DNA sequence analysis (data not shown).

When we introduced pGFPratiometric in the gram-positive bacterium *Lactococcus lactis* subsp. *lactis* CNRZ 157 as previously described (9) and grew cells at 30°C in M17 broth containing 0.5% (wt/vol) glucose and 5 μg of chloramphenicol per ml, we found that excitation at 410 nm gave a strong pH-dependent fluorescent signal. Furthermore, a pH-independent isosbestic point was seen at 430 nm, which is in accordance with previous results obtained in mammalian cells (13 and data not shown). It was therefore concluded that the excitation ratio, fluorescence intensity at 410 and 430 nm ($R_{410/430}$), is suitable as a measure of pH_i.

To correlate $R_{410/430}$ with pH_i, *L. lactis* cells carrying pGFPratiometric were permeabilized with nisin (3, 7), which disrupts the proton gradient across the cytoplasmic membrane and allows the internal pH to become identical to the external pH. Correspondingly, *Escherichia coli* TOP 10 cells (Invitrogen) were grown at 37°C in Luria-Bertani broth, transformed with pGFPratiometric (15), and pH-equilibrated by use of carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) (6). Subsequently, overnight cultures expressing ratiometric GFP were harvested by centrifugation (10,000 × *g* for 5 min), washed twice in potassium phosphate buffer (pH 7.5) containing glucose (10 mM), and finally resuspended in potassium phosphate buffer (10 mM glucose) with appropriate pH for 30 min. One hundred microliters of the bacterial suspension (approximately 10^8 cells) was applied to a coverslip coated with 0.01% (wt/vol) poly-L-lysine (Sigma) and allowed to settle. Prior to being used, the coverslips were cleaned by overnight submersion in chrome sulfuric acid (Sigma) and then rinsed in distilled water and stored in 70% (vol/vol) ethanol. Unattached bacteria were removed by rinsing with buffer. The fluorescence microscopy setup has previously been described (19), except that the emission was recorded on a Coolsnap fx charge-coupled device camera (Roper Scientific, Trenton, Pa.). The dichroic mirrors were 380 and 510 nm for ratiometric GFP and cFDAse [fluo-

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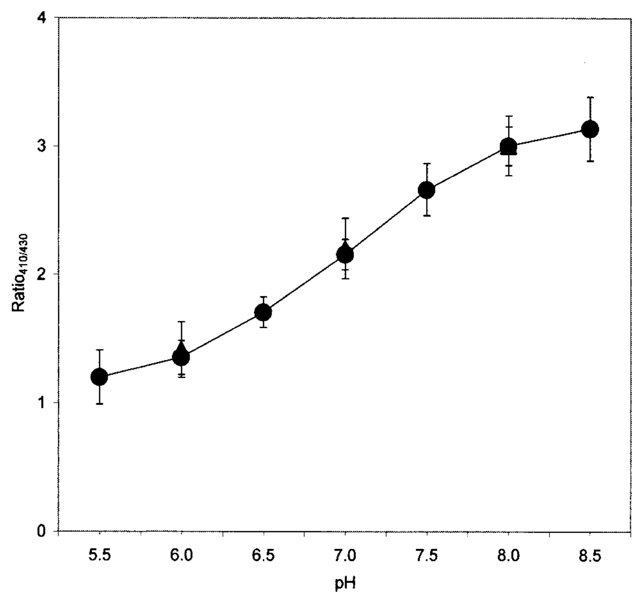

FIG. 1. Correlation between pH_i and $R_{410/430}$ of ratiometric GFP in pH-equilibrated cells. *L. lactis* and *E. coli* single cells were equilibrated with 10 kIU of nisin ml^{-1} (closed circles) and 10 μM CCCP (closed triangles), respectively. Each point represents the mean value for 20 individual cells, with error bars indicating the standard deviations.

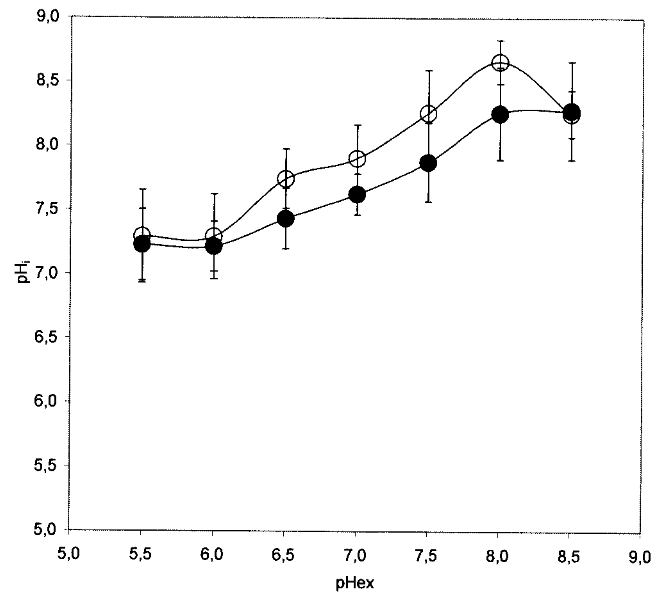

FIG. 2. Determination of *L. lactis* pH_i as a function of pH_{ex} by use of ratiometric GFP and cFDAse. The pH_i of cells transformed with ratiometric GFP (closed circles) was obtained by measuring the $R_{410/430}$ and converting the obtained value to the correlating pH_i. For validation, pH_i was also obtained by staining the cells with cFDAse (open circles) and measuring the $R_{490/435}$. Each point represents the mean value for 20 individual cells, with error bars indicating the standard deviations.

rochrome 5(6)-carboxyfluorescein diacetate succinimidyl ester], respectively, and the emission band-pass filters were 500 to 530 nm and 515 to 565 nm for ratiometric GFP and cFDAse, respectively. Images were stored on a personal computer by using Metafluor 4.5 (Universal Imaging, Downingtown, Pa.), and data from regions of interest (i.e., single cells) were logged into a spreadsheet (Excel). In each experiment, at least 20 single cells were analyzed. Each experiment was carried out in duplicate.

On the basis of the results obtained with nisin- or CCCP-treated cells, a calibration curve was constructed by plotting $R_{410/430}$ versus the pH of equilibrated cells in the pH range of 5.5 to 8.5 (Fig. 1). When *L. lactis* cells were permeabilized with nisin, we found that the relationship between $R_{410/430}$ and pH_i was similar to that observed when *E. coli* cells treated with CCCP were investigated.

Subsequently, we used the calibration curve (Fig. 1) to determine pH_i in single bacterial cells that had been resuspended in 50 mM potassium phosphate buffers with various pHs (Fig. 2). Examination of the pH_i of *L. lactis* cells revealed that the pH gradient ($\Delta pH = pH_i - pH_{ex}$) is approximately 1.8 at a pH_{ex} of 5.5. Increasing the pH_{ex} caused a decrease in the ΔpH, which finally became negative at a pH_{ex} of 8.5. In order to validate these results, the pH measurements were also carried out by using the conventional pH-sensitive cFDAse. *L. lactis* cells stained with cFDAse were analyzed as previously described (19), except that the pH equilibration was performed with nisin as described above.

The results presented in Fig. 2 show a good correlation ($R = 0.987$) between the pH responses obtained with the two methods. The observed deviations possibly result from the fact that both methods rely on a calculation of pH_i based on standard curves, which introduces a variation of 0.1 to 0.2 pH units (18). In addition, heterogeneities in the populations will influence the standard deviation. Therefore, we conclude that ratiometric GFP can be used as a pH_i probe for bacterial cells. When pH_i was measured in *E. coli* cells exposed to various pH values, we found that a large ΔpH of approximately 1.5 was maintained at a low pH_{ex}, gradually decreasing with increasing pH_{ex} until finally becoming 0 at a pH_{ex} of 8 (Fig. 3). For both bacteria, we obtained similar standard deviations (Fig. 2 and 3), suggesting that ratiometric GFP is equally applicable for measurements with gram-negative and gram-positive bacteria.

To ensure that the presence of pGFPratiometric did not affect the growth of bacterial cells, we followed the growth of *L. lactis* and *E. coli* cells in the presence or absence of the plasmid and found no effect with *L. lactis*, while the presence of the plasmid in *E. coli* cells slightly reduced the growth rate (data not shown). We also investigated the stability of pGFPratiometric as previously described (10) and found that 74% of *L. lactis* and 54% of *E. coli* cells retained the plasmid after 60 generations, demonstrating that studies can be conducted in the absence of antibiotic selection of the plasmid. Furthermore, we investigated whether the type of acid used for acidification affects the ratiometric GFP excitation spectrum when expressed in *L. lactis* cells and found the same excitation spectrum with acetic, citric, or formic acid as we found with hydrochloric acid (data not shown). This result indicates that the method is also applicable when the pH is adjusted with organic acids.

In conclusion, we have applied a pH-sensitive derivative of the GFP, ratiometric GFP (13), for measurement of pH_i in single bacterial cells in the pH range of 5.5 to 8.5. While this

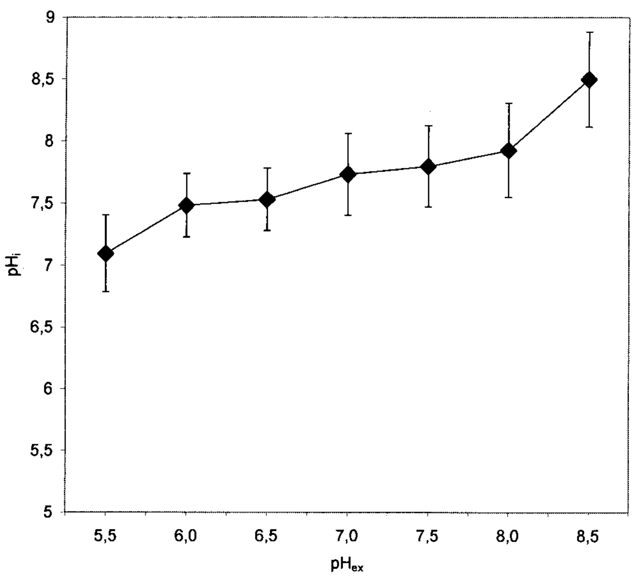

FIG. 3. Determination of *E. coli* pH_i as a function of pH_{ex}. Cells expressing ratiometric GFP were resuspended in phosphate buffers at pHs ranging from 5.5 to 8.5. The $R_{410/430}$ was determined and was transformed to the corresponding pH_i. Each point represents the mean value for 20 individual cells, with error bars indicating the standard deviations.

technique requires that the cells of interest express the ratiometric GFP protein, it provides a fast, noninvasive, and continuous determination of pH_i which is ideally suited for kinetic studies of both gram-negative and gram-positive bacteria. As the ratio between the fluorescence intensities at two different excitation wavelengths is used, the determination is independent of the individual concentration of ratiometric GFP in each cell. Furthermore, we show that the excitation spectrum of ratiometric GFP when cells were acidified with various organic acids was identical to that when cells were acidified with hydrochloric acid, suggesting that the ratiometric GFP is well suited for studies of the pH_i in bacteria residing in natural environments such as food products.

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