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pH of the Cytoplasm and Periplasm of *Escherichia coli*: Rapid Measurement by Green Fluorescent Protein Fluorimetry[▽]

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Cytoplasmic pH and periplasmic pH of *Escherichia coli* cells in suspension were observed with 4-s time resolution using fluorimetry of TorA-green fluorescent protein mutant 3* (TorA-GFPmut3*) and TetR-yellow fluorescent protein. Fluorescence intensity was correlated with pH using cell suspensions containing 20 mM benzoate, which equalizes the cytoplasmic pH with the external pH. When the external pH was lowered from pH 7.5 to 5.5, the cytoplasmic pH fell within 10 to 20 s to pH 5.6 to 6.5. Rapid recovery occurred until about 30 s after HCl addition and was followed by slower recovery over the next 5 min. As a control, KCl addition had no effect on fluorescence. In the presence of 5 to 10 mM acetate or benzoate, recovery from external acidification was diminished. Addition of benzoate at pH 7.0 resulted in cytoplasmic acidification with only slow recovery. Periplasmic pH was observed using TorA-GFPmut3* exported to the periplasm through the Tat system. The periplasmic location of the fusion protein was confirmed by the observation that osmotic shock greatly decreased the periplasmic fluorescence signal by loss of the protein but had no effect on the fluorescence of the cytoplasmic protein. Based on GFPmut3* fluorescence, the pH of the periplasm equaled the external pH under all conditions tested, including rapid acid shift. Benzoate addition had no effect on periplasmic pH. The cytoplasmic pH of *E. coli* was measured with 4-s time resolution using a method that can be applied to any strain construct, and the periplasmic pH was measured directly for the first time.

In order to colonize the human gastrointestinal tract, the enteric bacterium *Escherichia coli* must be able to grow between pH 4.5 and pH 9 (7). Over this wide pH range, *E. coli* preserves enzyme activity, as well as protein and nucleic acid stability, by maintaining the cytoplasmic pH in the range from pH 7.2 to 7.8 (26, 27, 32). *E. coli* responds rapidly to intracellular pH change; after acidification of the external environment, the intracellular pH of *E. coli* begins to recover within 1 min, and full recovery occurs within 5 min (28). The efficiency with which *E. coli* maintains pH homeostasis has been attributed to a combination of constitutive and regulated mechanisms, but the essential requirements remain poorly understood (7, 9, 14, 18, 28). Some components of pH homeostasis act in the presence of chloramphenicol, whereas others require ongoing protein synthesis (10).

Previously, cytoplasmic pH has been measured using ^{31}P nuclear magnetic resonance (NMR) of titratable phosphate and methylphosphonate (28) and through transmembrane equilibration of radiolabeled permeant acids (32). Both methods have limitations. Radiolabeled permeant acids have low sensitivity, and they measure only the transmembrane pH difference; they do not measure cytoplasmic pH independent of external pH. ^{31}P NMR requires highly concentrated cell suspensions, typically suspensions with optical densities at 600 nm (OD_{600}) of 20 to 200.

The advent of highly pH-sensitive fluorescent proteins offers new possibilities for measuring cellular pH (11, 13, 16, 22, 24). Fluorescent proteins provide highly sensitive detection, do not require indicator loading, and lack phototoxicity (5, 11, 20, 23). Derivatives of the green fluorescent protein (GFP) and yellow fluorescent protein (YFP) are pH indicators ideally suited for kinetic studies (11, 13, 15, 22, 24). After acidification of the protein's environment, the fluorescence signals of both GFP and YFP decrease in less than 1 ms, allowing rapid detection of pH change (11, 15). This rapid change in fluorescence intensity is based on the occurrence of a simple protonation reaction (11, 15, 24). Thus, GFP and YFP are sensitive pH indicators and can rapidly detect changes in bacterial cytoplasmic pH.

In previous reports, the pH-dependent changes in fluorescence have been observed using fluorescence microscopy (22). Microscopy, however, necessitates cumbersome quantitation procedures that introduce error and limit the time scale of the observable signal. An alternative approach is fluorescence spectroscopy or fluorimetry, a sensitive technique that allows observation of a live cell culture in liquid medium. Fluorimetry has not yet been widely used to measure changes in the intracellular pH of cell suspensions (1), in part because of the need for highly sensitive instrumentation which has only recently become available.

In order to measure the pH of *E. coli*, we devised a procedure based on fluorimetry of plasmid-expressed GFP mutant 3* (GFPmut3*) and YFP gene fusions. The GFPmut3* fusion strain was expressed with a TorA signal peptide that can either retain the fusion protein in the cytoplasm or direct its transport to the periplasm (3, 17, 30). To our knowledge, there has been no previous report of measurement of periplasmic pH.

MATERIALS AND METHODS

Strains and growth conditions. All strains were derivatives of *E. coli* K-12. Strain W3110 was transformed with pSL38-YFP (12) to form strain JLS0617. Strains MC4100AR Δ*tatABCDE*/pBAD TorA-GFPmut3* and MC4100/pBAD TorA-GFPmut3* were obtained from Mullineaux et al. (17).

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Strain JLS0617 was cultured overnight in buffered M63 salts medium [20 mM homopiperazine-*N*,*N*′-bis-2-(ethanesulfonic acid) (HOMOPIPES), pH 7.5] containing 1.5% casein hydrolysate, 0.8% glycerol, and 100 μg/ml. ampicillin. The overnight culture was diluted 25-fold in the same medium and cultured to late log phase (OD_{600}, 0.8 to 0.9) at 37°C with aeration.

The MC4100 strains containing pAra TorA-GFPmut3* were grown overnight in potassium-modified Luria broth with NaCl replaced by 100 mM KCl (designated LBK medium) as described by Maurer et al. (14). The LBK medium was buffered with 20 mM HOMOPIPES, pH 7.5. To maintain the plasmid, media included 50 μg/ml ampicillin. Each overnight culture was diluted 1,000-fold into the same medium with 200 μM L-arabinose. Bacteria were cultured to late log phase (OD_{600}, 0.8 to 0.9) at 37°C with aeration.

For cytoplasmic pH measurement, the cultures were resuspended to an OD_{600} of 0.4 in M63 medium containing 1.5% casein hydrolysate, 0.8% glycerol, and HOMOPIPES (5 to 50 mM depending on the experiment). The pH was adjusted with KOH to pH 5.5 to 8.0, depending on the experiment. For periplasmic pH measurement, cultures of the MC4100/pAra TorA-GFPmut3* strain (with a functional *tat* system) were washed and resuspended to an OD_{600} of 0.5 in 20 ml of buffered LBK medium (20 mM HOMOPIPES, pH 7.5) containing ampicillin (50 μg/ml) and no arabinose. The cultures were incubated at 37°C for a further 3 h to allow complete transport of TorA-GFPmut3* to the periplasm (3). Then cultures were resuspended in M63 medium (supplemented as described above) at an OD_{600} of 0.4. All resuspended cultures were stored on ice until fluorimetry.

Fluorescence spectroscopy. Excitation spectra were recorded using a Fluoromax-3 spectrofluorimeter (Horiba Jobin Yvon). This instrument has a high signal-to-noise ratio (Water Raman signal-to-noise ratio, 3,000:1) and an emission detector employing photon counting in order to detect low light levels. A cell suspension (3 ml) was placed into a Starna Spectrosil quartz cuvette with a path length of 10 mm. Aliquots of solution were added through an injection port using a 1-ml syringe. The temperature of the chamber was adjusted to 30°C, and aeration was provided by stirring. GFPmut3* excitation was measured from 480 to 510 nm (slit width, 2 nm), using an emission wavelength of 545 nm (slit width, 20 nm) (6). YFP excitation was measured from 450 to 520 nm (slit width, 2 nm), using an emission wavelength of 550 nm (slit width, 20 nm) (15). Spectra were recorded for three biological replicates at each pH. For time course experiments, continuous excitation spectra were obtained every 4 s for 1 min before aliquot addition (time zero) and then for 5 min after addition.

Data were fitted to a standard curve correlating internal pH with fluorescence intensity. The standard curve was determined for each fluorophore (GFPmut3* and YFP) by obtaining fluorescence measurements of samples resuspended at pH 5.5, 6.0, 7.0, 7.5, and 8.0, with inclusion of 20 mM sodium benzoate, a permeant acid that equilibrates cytoplasmic pH with external pH. For GFP fluorescence, an equation was fitted to the curve of GFPmut3* signal intensity (GFPmut3* signal intensity = $-2.2 \times 10^{10} + 5.8 \times 10^{9} \times$ pH) and was used to convert time course signal intensities (sum of 480 nm to 510 nm) to pH units. For YFP, the equation was as follows: YFP signal intensity = $-3.91 \times 10^{8} + 9.4 \times 10^{7} \times$ pH. For each fluorophore, the equation was used to fit a standard curve to the data of a given experiment, interpolating between intensities at pH 5.5 and intensities at pH 7.5.

The pH curve was modified for experiments in which a permeant acid was added to cultures in medium with an external pH of 7.0. In this case, linear interpolation was conducted between pH 7.6 and pH 7.0. The fluorescence corresponding to pH 7.0 was determined by adding an additional 20 mM benzoate to each test sample at the end of the time course.

Osmotic shock. To permeabilize the outer membrane and remove the periplasmic contents, osmotic shock was performed. The cultures were spun down and resuspended in buffered sucrose-EDTA (20% sucrose, 33 mM morpholinepropanesulfonic acid [MOPS] [pH 7.3], 100 μM EDTA) and then were held at room temperature for 10 min. The cultures were resuspended in ice-cold 500 μM $MgCl_2$. Cells were washed and resuspended to an OD_{600} of 0.4 in buffered (5 mM HOMOPIPES) supplemented M63 medium adjusted to pH 5.5 or to pH 7.5.

RESULTS

pH dependence of GFPmut3* and YFP in the living cell. The fluorescence excitation spectra of cytoplasmic TorA-GFPmut3* (17) and TetR-YFP (12) were observed as a function of cytoplasmic pH (Fig. 1A and B). Previously, GFP has been used as a probe for determination of bacterial pH by fluorescence microscopy but not by fluorimetry of cell suspensions

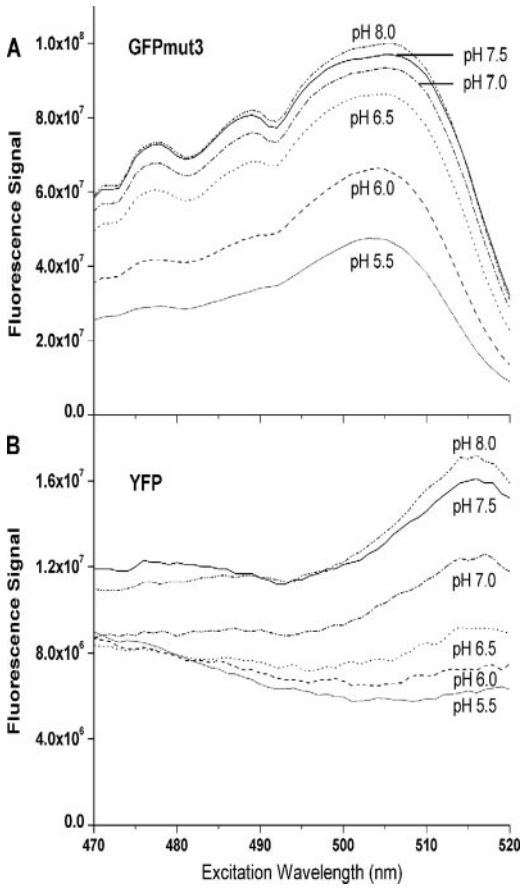

FIG. 1. Excitation spectra of cytoplasmic GFPmut3* and YFP as a function of pH. *E. coli* cells were suspended at different pH values in the presence of 20 mM benzoate. The strains used were (A) MC4100AR Δ*tatABCDE* TorA-GFPmut3* and (B) JLS0617.

(22). Cells of strains MC4100AR Δ*tatABCDE* TorA-GFPmut3* and JLS0617 (W3110/pSL38_YFP) were suspended in buffered M63 medium with supplements, adjusted to pH values in the range from pH 5.5 to 8.0. The permeant acid benzoate (20 mM) was included in order to equalize the cytoplasmic and extracellular pHs.

The excitation spectra for cell suspensions containing TorA-GFPmut3* and YFP peaked at excitation wavelengths of approximately 511 nm and 514 nm, respectively (Fig. 1A and B). These values correspond to the peak excitation wavelengths reported for GFPmut3* and YFP in vitro (6, 15). Overall, for both strains, the fluorescence signal increased with increasing pH over the range from pH 5.5 to 8.0. In the pH range relevant to cytoplasmic pH perturbation, typically pH 7.0 to 8.0, the YFP signal showed greater variation than the GFPmut3* signal. The GFPmut3* signal, however, showed fivefold-greater intensity than the YFP signal.

For both fluorophores, the fluorescence signal was found to be critically dependent on cell density. When the cell density was too low (OD_{600}, <0.3), the fluorescence signal intensity was undetectable. At higher cell densities, however (OD_{600}, >0.4), the scattering of the excitation or emission beam from the concentrated sample reduced the apparent signal intensity.

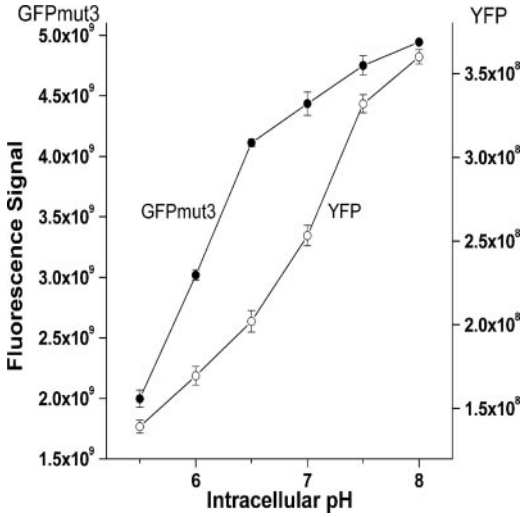

FIG. 2. Standard curves of cytoplasmic pH as a function of fluorescence signal. The fluorescence signal was measured and summed as described in Materials and Methods. The strains used were (A) MC4100 Δ*tatABCDE* TorA-GFPmut3* and (B) JLS0617. The error bars represent standard errors of the means ($n = 3$).

For all our fluorescence observations, the cells were resuspended at an OD_{600} of 0.4.

To use the GFPmut3* and YFP signals as a measure of cytoplasmic pH, standard curves were generated for pH dependence. For each excitation spectrum, the fluorescence intensities were summed over a peak wavelength range. The peak fluorescence signal was observed for cultures suspended in medium over a range of pH values in the presence of the permeant acid benzoate (20 mM) in order to peg the cytoplasmic pH to the pH of the medium (Fig. 2). The intensity of the fluorescence signal increased with increasing pH over the range expected for each fluorophore, including the range of interest for *E. coli* growth, pH 5.5 to 8.0. The YFP signal varied more than the GFPmut3* signal over the cytoplasmic range of pH 7.0 to 8.0. The GFPmut3* signal, however, showed nearly 10-fold-greater intensity than the YFP signal; thus, overall the change in intensity over the cytoplasmic range from pH 7.0 to 8.0 was fivefold greater for GFPmut3* than for YFP.

The regression equations generated from these standard curves were used to convert signal intensities (defined as the sum of intensities at 480 nm to 510 nm) to pH units in order to estimate the cytoplasmic pH values during time course experiments. The equations were fitted to the data by interpolating between fluorescence intensities observed in cells with the cytoplasmic pH depressed by 20 mM benzoate so as to equal the extracellular pH, at external values of pH 5.5 and pH 7.5.

Cytoplasmic pH shift. A time course analysis was conducted in order to observe cytoplasmic pH and recovery following rapid acidification of the external medium (Fig. 3). Strains MC4100AR Δ*tatABCDE* TorA-GFPmut3* and JLS0617 were suspended in mildly buffered M63 medium (5 mM HOMOPIPES) adjusted to pH 7.5. At time zero, HCl (8.5 mM) was added in order to lower the external pH to about pH 5.5. After the completion of the time course, the external pHs of the cultures were measured and found to be between pH 5.5 and 5.7.

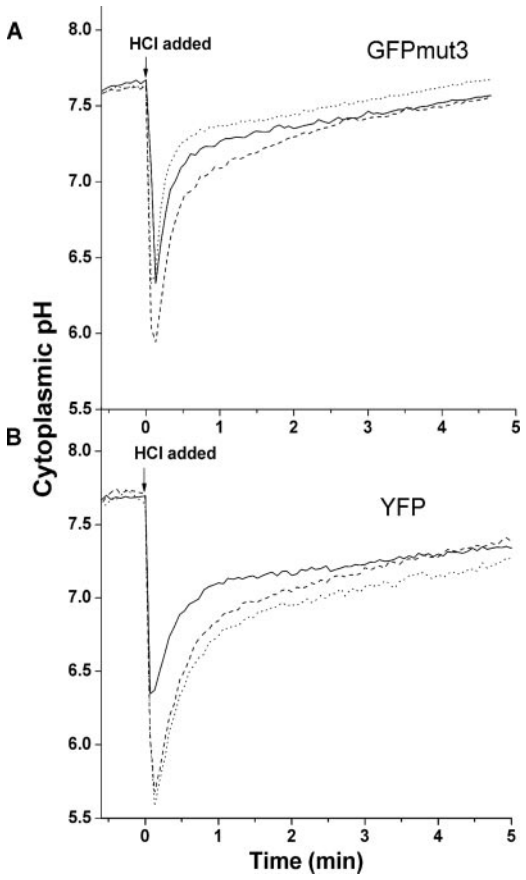

FIG. 3. Effect of external acid shift on cytoplasmic pH. Cultures were suspended at pH 7.5 in supplemented M63 minimal media (5 mM HOMOPIPES). Fluorescence was converted to pH units using the standard curves (Fig. 2) as described in Materials and Methods. At time zero, 8.5 mM HCl was added to shift the external pH from pH 7.5 to 5.5. The strains used were (A) MC4100AR Δ*tatABCDE* TorA-GFPmut3* and (B) JLS0617. For each strain, three independent cultures were tested.

Before HCl addition, the intracellular pH of all cultures was pH 7.6, as determined based on a standard curve obtained using cells from the same culture. After HCl addition, the cytoplasmic pH of each sample fell within 10 to 20 s to pH 5.6 to 6.5. Recovery began within 4 s after the lowest point (Fig. 3A and B). The extent of internal pH change varied similarly between the strains and between the biological replicates. In both strains (one strain containing GFPmut3* and one strain containing YFP), rapid recovery occurred until about 30 to 60 s after HCl addition and was followed by slower recovery over the next several minutes.

Cytoplasmic pH shift in the presence of permeant weak acids. The effect of permeant acid addition on cytoplasmic pH was tested. A permeant weak acid dissociates upon entering the cell, partly equilibrating cytoplasmic and external pHs and partly impairing pH homeostasis; the precise balance is poorly understood and is a complex function of cell buffering capacity and ion transport (25, 26, 28).

The effects of acetate on cell pH are of particular interest because *E. coli* produces acetate rapidly during log-phase aerobic growth on most substrates (29), as well as during fermen-

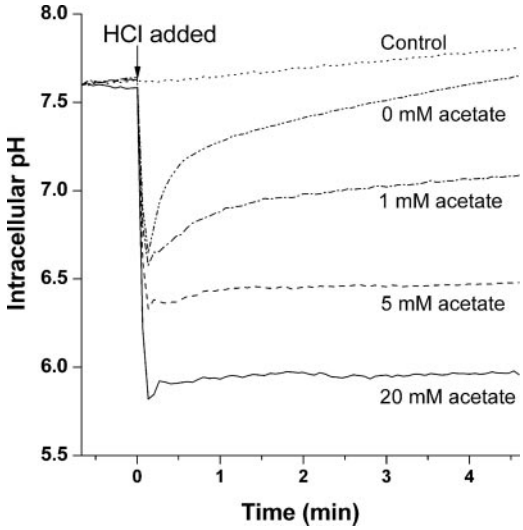

FIG. 4. Acetate amplifies acid stress on cytoplasmic pH. The strain observed was MC4100AR Δ*tatABCDE* TorA-GFPmut3*; fluorescence was converted to pH units as described in Materials and Methods. Signals were averaged for three independent cultures for each condition. Cells were suspended in supplemented M63 medium (pH 7.5; 5 mM HOMOPIPES) containing 0, 1, 5, and 20 mM sodium acetate. At time zero, the pH of the external environment was shifted from 7.5 to 5.5 using 8.5 mM HCl. For the control, KCl (8.5 mM; pH 7.5) was added instead of HCl.

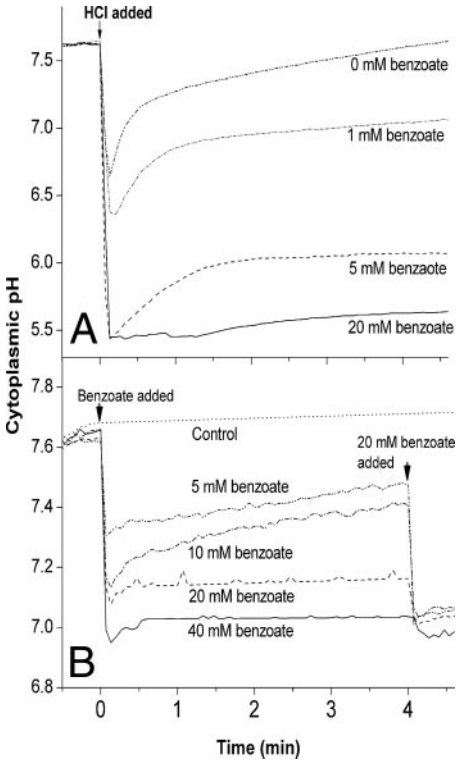

FIG. 5. Benzoate amplifies acid stress on cytoplasmic pH. The experiment was conducted as described in the legend to Fig. 4, with an acid shift in the presence or absence of benzoate. (A) Cells were suspended in supplemented M63 medium (pH 7.5; 5 mM HOMOPIPES) containing 0, 1, 5, and 20 mM sodium benzoate. At time zero, the pH of the external environment was shifted from 7.5 to 5.5 using 8.5 mM HCl. (B) Cells were suspended in M63 medium (pH 7.0; 50 mM HOMOPIPES). At time zero, the pH of the internal environment was shifted by adding 5, 10, 20, and 40 mM benzoate. For the control, 50 mM HOMOPIPES (pH 7.0) buffer was added instead of HCl. Benzoate (20 mM) was added at 4.1 min for pH calibration, as described in Materials and Methods. For each condition, results from three independent cultures were averaged.

tation (25). Cultures of strain MC4100AR Δ*tatABCDE* TorA-GFPmut3* were suspended in LBK medium weakly buffered at pH 7.5 in the presence of 0, 1, 5, and 20 mM acetate (Fig. 4). At time zero, addition of HCl (8.5 mM) caused a rapid fall in the cytoplasmic pH, followed by a relatively slow partial recovery of the cytoplasmic pH in the presence of acetate.

A similar experiment was conducted to observe the time course of cytoplasmic acidification in the presence of 0, 1, 5, and 20 mM benzoate (Fig. 5A). Following HCl addition, the cytoplasmic pH of all cultures fell within 10 to 15 s to the low point. The initial rates of recovery were similar over the range from 0 to 5 mM benzoate. The subsequent slow recovery, however, was strongly dependent upon the benzoate concentration, which ranged from 0 mM to 20 mM. The cytoplasmic pH of the cultures suspended in the absence of benzoate dropped to approximately pH 6.5 and then recovered fully, whereas the internal pH of the cultures containing 20 mM benzoate dropped to pH 5.5 (the pH of the external medium) and showed minimal recovery.

In another experiment, benzoate (5, 10, and 20 mM) or pH 7.0 buffer was added to cultures buffered at pH 7.0 without changing the external pH (Fig. 5B). To calibrate the pH, a final addition of 20 mM benzoate was made, which set the cytoplasmic pH at or near the pH of the external medium (pH 7); then the final fluorescence intensity was used to calibrate pH 7.0. The initial addition of benzoate transiently acidified the cytoplasm (Fig. 5B). No rapid short-term recovery was observed (Fig. 3 and 4A). The rate of long-term recovery was dependent on the benzoate concentration. In the presence of 5 mM benzoate, the cytoplasmic pH fell to pH 7.3, close to the pH of the external medium (pH 7.0), and made nearly a full recovery. The presence of 10 and 20 mM benzoate, however, lowered the cytoplasmic pH (Fig. 5B). In the presence of these higher concentrations of benzoate the cytoplasmic pH exhibited minimal recovery. Changes in the signal unrelated to benzoate were controlled for by the addition of pH 7.0 buffer at time zero (Fig. 5B), which caused no significant change in pH.

Controls for chloride and buffer change. Control experiments were designed to eliminate the possibility of effects on the fluorescence signal unrelated to pH change. The factors tested include chloride ion concentration, which is known to quench the fluorescence signal of some GFP derivatives (31); buffer addition at near-cytoplasmic pH (pH 7.5); and buffer addition at low pH (pH 5.5). Strain MC4100AR Δ*tatABCDE* TorA-GFPmut3* was suspended in strongly buffered M63 minimal medium adjusted to either pH 5.5 or pH 7.5. At time zero, either KCl (8.5 mM, pH 7.5) or HOMOPIPES (50 mM, pH 7.5) was added to cells suspended at a pH near the cytoplasmic pH. Neither addition caused a significant change in the fluorescence signal. HOMOPIPES (50 mM, pH 5.5) addition was also tested in medium buffered at pH 5.5, again with no effect on the fluorescence signal.

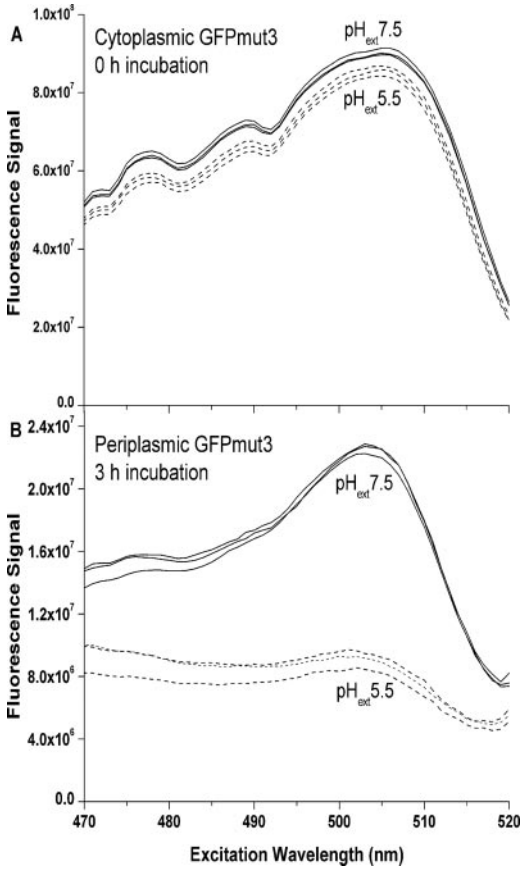

FIG. 6. Cytoplasmic and periplasmic fluorescence observed at external pH 7.5 and at pH 5.5. Strain MC4100AR TorA-GFPmut3* was cultured as described in Materials and Methods. Cells were harvested following (A) no further incubation and (B) 3 h of incubation in the absence of arabinose. Cultures were suspended in supplemented M63 medium (50 mM HOMOPIPES) at pH 7.5 and at pH 5.5. For each condition, three independent cultures were tested. pH_{ext}, external pH.

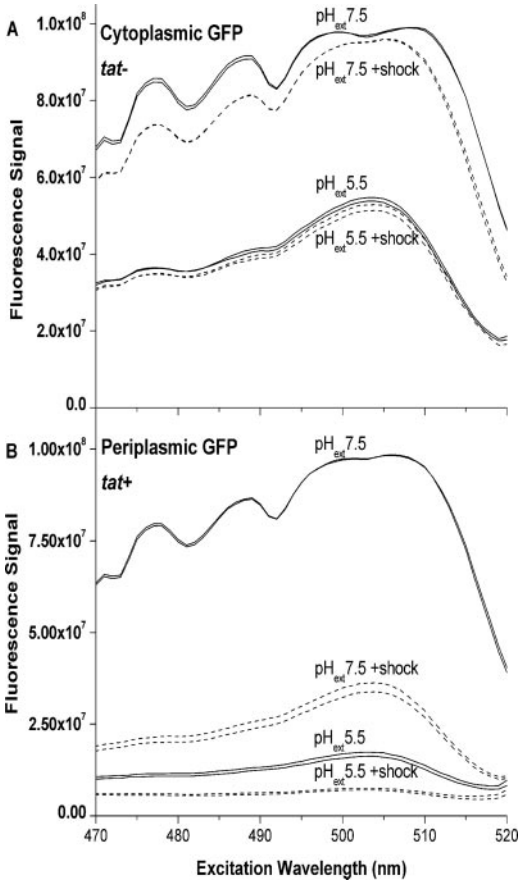

FIG. 7. Effect of osmotic shock on cytoplasmic and periplasmic fluorescence. The strains observed were (A) MC4100AR Δ*tatABCDE* TorA-GFPmut3* and (B) MC4100AR TorA-GFPmut3* (*tat*$^+$) incubated for 3 h in the absence of arabinose. Osmotic shock was performed as described in Materials and Methods. Both shocked (+shock) and nonshocked cultures were suspended in supplemented M63 medium (50 mM HOMOPIPES) buffered at pH 7.5 or pH 5.5. For each condition, two independent cultures were tested. pH_{ext}, external pH.

pH dependence of periplasmic GFPmut3*. GFP fusion proteins are incapable of folding within the periplasm; thus, GFP functioning in the periplasm requires a system capable of transporting the fully folded protein into the periplasmic space (8, 30). In bacteria, fully folded proteins are transported into the periplasm using the *tat* pathway (4, 30). By fusing GFP to a protein normally exported by *tat*, fully folded active GFP can be transported into the periplasm (3, 17, 30). For our observations of periplasmic pH, we used an MC4100 construct with a functional *tat* system to transport TorA-GFPmut3* to the periplasm (17).

Strain MC4100AR TorA-GFPmut3* was cultured to late log phase in the presence of L-arabinose. One half of the cell culture was spun down, washed, and suspended in the absence of L-arabinose. The culture without L-arabinose was incubated for a further 3 h to allow complete transport into the periplasm (3). Each culture was suspended in supplemented M63 medium at pH 5.5 or at pH 7.5. The excitation spectra were obtained for cultures harvested without incubation (Fig. 6A) or after 3 h of incubation (Fig. 6B). Without the extra incubation, the cytoplasmic (nontransported) TorA-GFPmut3* maintained nearly the same fluorescence level at an external pH of pH 5.5 or pH 7.5 (Fig. 6A). This observation is consistent with little or no TorA-GFPmut3* transport into the periplasm. After 3 h of incubation without L-arabinose, however, the excitation intensity showed a substantial dependence on the external pH (Fig. 6B). This finding is consistent with the transport of all or most of the TorA-GFPmut3* into the periplasm, whose pH is presumed to remain at or near the pH of the external medium (19).

Osmotic shock of *tat* and *tat*$^+$ strains. In order to demonstrate that GFPmut3* was successfully exported into the periplasm, cells incubated without arabinose were subjected to osmotic shock (Fig. 7). Osmotic shock permeabilizes the outer membrane and causes the periplasmic contents to leak into the extracellular space, while the inner membrane and cytoplasm remain intact (2). Osmotic shock was performed on a *tat*$^+$ strain (MC4100AR TorA-GFPmut3*) and on a *tat* deletion strain (MC4100AR Δ*tatABCDE* TorA-GFPmut3*) in which the TorA-GFPmut3* remains in the cytoplasm. One half of each culture was subjected to osmotic shock, and one half was left unshocked. All cultures were suspended to the same

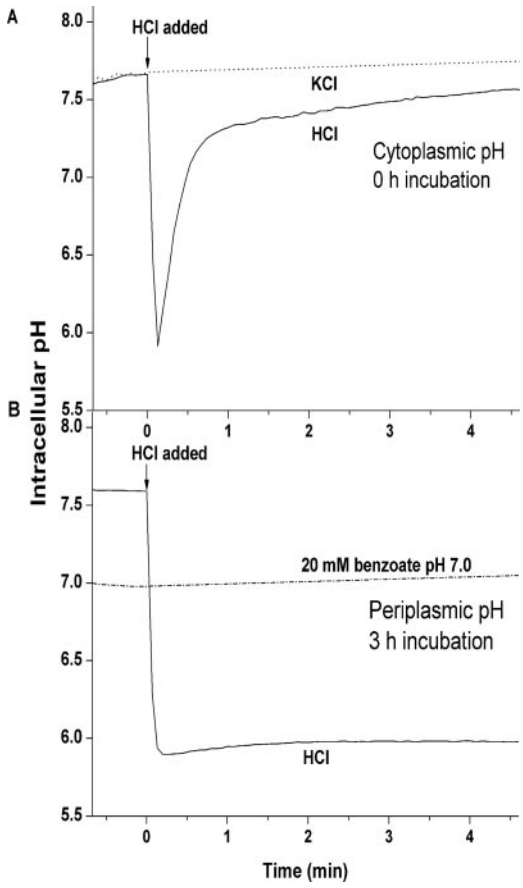

FIG. 8. Effect of external acid shift on cytoplasmic pH and periplasmic pH. Strain MC4100AR TorA-GFPmut3* was cultured as described in Materials and Methods. Cultures were tested (A) without further incubation and (B) after 3 h of incubation in the absence of arabinose. Cultures were suspended in pH 7.5 M63 medium (5 mM HOMOPIPES). At time zero, the external pH was shifted from pH 7.5 to 5.5 by adding 8.5 mM HCl. For the control, at time zero, (A) 8.5 mM KCl (pH 7.5) was added or (B) 20 mM sodium benzoate (pH 7.0) was added to cultures suspended in pH 7.0 M63 medium (50 mM HOMOPIPES). The fluorescence signals from three independent cultures were averaged for each condition.

optical density in buffered M63 minimal medium at pH 5.5 or at pH 7.5.

At both pH 5.5 and 7.5, the shocked *tat* cultures (no periplasmic transport) showed little or no loss of fluorescence signal compared to the nonshocked cultures (Fig. 7A). This observation is consistent with retention of most or all TorA-GFPmut3* within the cytoplasm. The shocked *tat*$^+$ cultures, however, showed a significant loss of signal intensity compared to the nonshocked cultures, indicating that the majority of the TorA-GFPmut3* was successfully exported into the periplasm (Fig. 7B). In both the *tat* and *tat*$^+$ cultures, there was a significant difference between the signal intensities of the pH 5.5 and pH 7.5 cultures, thus demonstrating that the pH dependence of GFPmut3* is not affected by osmotic shock (Fig. 7).

Periplasmic pH shift. A time course analysis was conducted in order to compare the perturbation and recovery of cytoplasmic and periplasmic pHs following rapid external acidification (Fig. 8). Cultures of MC4100AR TorA-GFPmut3* incubated for 3 h without L-arabinose (periplasmic GFPmut3*) were compared to nonincubated cultures (cytoplasmic GFPmut3*). In each case cells were resuspended at pH 7.5, and HCl was added in order to lower the external pH to pH 5.5.

Before HCl addition, the cytoplasmic and periplasmic pHs of all cultures were about pH 7.5 to 7.6. After HCl addition, the pHs observed both for cytoplasm (Fig. 8A) and periplasm (Fig. 8B) fell to just below pH 6.0. The cytoplasmic pH began to recover within 4 s (Fig. 8A). The results were similar to those shown in Fig. 3, obtained using the *tat* strain. This observation is consistent with the report that the TorA-GFPmut3* remains in the cytoplasm as long as the arabinose inducer is present (17). The cultures with periplasmic GFPmut3*, however, showed minimal recovery of the pH (Fig. 8B). In addition, we tested the response of the periplasmic pH to a permeant acid without shifting the external pH. Benzoate (20 mM) was added to 3-h-incubated cultures (periplasmic TorA-GFPmut3*) suspended at pH 7.0 (Fig. 8B). Benzoate addition caused no significant change in the observed pH of the periplasm (pH 7.0). This observation confirms that essentially all the functional reporter protein was in fact transported to the periplasm.

DISCUSSION

We found that fluorescence spectroscopy of pH-sensitive GFP family proteins in cell suspensions can detect pH changes in the cytoplasm and periplasm on a 4-s time scale. Previous studies used fluorescence microscopy (11, 13, 16, 22, 24) and did not examine rapid pH homeostasis. Rapid pH perturbation is of particular interest with respect to colonization of the enteric tract, as enteric bacteria must survive rapid pH change at entry and during passage through the stomach. Thus, because of its rapid resolution our method may be a useful tool for investigation of the regulatory networks employed by enteric bacteria during the early response to pH stress.

Fluorescence probes for cytoplasmic and periplasmic pHs. The reliability of our method was strengthened by the use of two different gene fusion constructs, TorA-GFPmut3* and TetR-YFP. The two probes showed significant differences in pH titration (Fig. 2), and the GFPmut3* fluorophore showed fivefold-greater signal intensity than YFP; these effects were consistent with previously observations of GFPmut3* and YFP (6). Nevertheless, the two probes generated essentially the same profiles of cytoplasmic pH response to an acid shift (Fig. 3).

The Tat-transported TorA-GFPmut3* fusion enabled pH measurement in the periplasm, a cell compartment where pH has not been measured previously (Fig. 8). The direct measurement of periplasmic pH is of interest because the periplasm is the gram-negative cell's frontline defense against pH change, containing major pH stress proteins such as the HdeA and HdeB acid-dependent periplasmic chaperones (19, 21, 29). These chaperones are needed to respond to protein misfolding during acid stress.

Previously, GFP fusion proteins have not been observed in the periplasm because the protein fails to fold properly after transport and is thus inactive (8). However, the Tat twin-arginine translocase pathway, unlike the Sec pathway, allows the transport of fully folded globular proteins into the periplasm

(4, 30). Thus, the GFPmut3* exported to the periplasm in folded form via this pathway is as active as the GFPmut3* located within the cytoplasm. Periplasmic transport requires removal of arabinose; in the presence of arabinose, the fusion protein remains in the cytoplasm. Thus, our approach allows measurement of cytoplasmic pH and periplasmic pH in the same Tat^+ strain.

Regulation of cytoplasmic pH after rapid external acid shift. Previous kinetic studies of intracellular pH regulation used ^{31}P NMR of titratable phosphates, a method that requires highly concentrated cell suspensions (27, 28). In these experiments, cytoplasmic pH does not begin to recover until approximately 2 min after HCl addition. The initial dip before recovery lasts significantly longer than the 10 to 20 s that was reported in the present study. A possible reason for this difference may be the relatively stressed condition of the cells in the NMR experiment, which were harvested in late log phase and resuspended at high density.

The conditions of the experiments in the present study provided sufficient resolution to distinguish two phases of cytoplasmic pH recovery: the initial recovery, within 1 min after the dip, followed by a slower recovery over several minutes. We showed that the fast recovery is absent when pH is perturbed by a permeant acid such as benzoate, while partial slow recovery remains. Future experiments will enable us to test the two phases of pH recovery in various mutant backgrounds under different physiological conditions.

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