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# A Novel Method for Continuous Determination of the Intracellular pH in Bacteria with the Internally Conjugated Fluorescent Probe 5 (and 6-)-Carboxyfluorescein Succinimidyl Ester

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**A novel method based on the intracellular conjugation of the fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE) was developed to determine the intracellular pH of bacteria. cFSE can be taken up by bacteria in the form of its diacetate ester, 5 (and 6-)-carboxyfluorescein diacetate succinimidyl ester, which is subsequently hydrolyzed by esterases to cFSE in the cytoplasm. When *Lactococcus lactis* cells were permeabilized with ethanol, a significant proportion of cFSE was retained in the cells, which indicated that cFSE was bound intracellularly. Unbound probe could be conveniently extruded by a short incubation of the cells in the presence of a fermentable sugar, most likely by exploiting an active transport system. Such a transport system for cFSE was identified in *L. lactis*, *Listeria innocua*, and *Bacillus subtilis*. The intracellular pH in bacteria can be determined from the ratio of the fluorescence signal at the pH-sensitive wavelength (490 nm) and the fluorescence signal at the pH-insensitive wavelength (440 nm). This cFSE ratio method significantly reduced problems due to the efflux of fluorescent probe from the cells during the measurement. Moreover, the method described was successfully used to determine the intracellular pH in bacteria under stress conditions, such as elevated temperatures and the presence of detergents.**

The $pH_{in}$ is critical for the control of many cellular processes, such as DNA transcription, protein synthesis, and enzyme activities. To study regulation of the $pH_{in}$, a reliable method to measure the $pH_{in}$ is of utmost importance. Currently, the most frequently used method to measure $pH_{in}$ in bacteria is determination of the distribution of radiolabeled weak acids or bases in combination with silicon oil centrifugation (2, 3, 6, 8, 13, 25). The disadvantages of this technique are the limited time resolution and the potential negative effects of weak acids on cell metabolism. Another common method is the $^{31}P$ nuclear magnetic resonance technique (24), but its application is limited by the very high cell densities required, the limited time response, and the expensive equipment necessary.

Application of fluorescence techniques for $pH_{in}$ measurements have the advantage of high time resolution and simplicity of use (16, 26). However, until now, a significant drawback had been leakage of the probe to the external environment (3, 16, 19), which could impair measurement by creating a high level of background. A strategy to prevent this problem is to employ more polar fluorescent compounds, such as BCECF and 8-hydroxy-1,3,6-pyrene-trisulfonic acid (pyranine) (11, 21), which, because of their polarity, are less likely to leak from the cell. Such an approach is, however, hampered by the inability of bacterial cells to take up such negatively charged molecules. This problem can be avoided by incorporating fluorescent probes as (nonfluorescent) acetoxymethyl or diacetyl esters (11, 26). These esters are membrane permeable and cleaved in the cytoplasm by esterases, which results in accumulation of the fluorescent form. Other methods which have been used to incorporate negatively charged fluorescent probes include electroporation (21) or a short acid shock (16).

Given that different bacteria (such as acidophiles, neutrophiles, and alkalophiles) can exhibit a wide range of $pH_{in}$ values (typically from 5.6 to 9) (2, 3), it is impossible to employ a single pH probe which can cover the entire pH range. For general purposes, the $pK_a$ of the probe should ideally be around pH 7. Currently, the most commonly used fluorescent probe for $pH_{in}$ measurements is BCECF, which has a $pK_a$ of 6.97 (23). This derivative of fluorescein has four to five negative charges at physiological pH, which enhances intracellular retention compared with fluorescein or carboxyfluorescein (11). It has been shown recently, however, that BCECF, like other fluorescent probes, may also be actively extruded from cells by transport systems (1, 5, 17). To minimize resulting background problems, chemical elimination of extracellular probe (19) and mathematical correction of the fluorescent signal for efflux (16) have been exploited. Nevertheless, there is still an enormous demand for a simple, direct, and high-time-resolution measurement of the $pH_{in}$ of bacteria without the need for extensive corrections.

In this study, the fluorescent probe cFSE was evaluated for determination of the $pH_{in}$ of bacteria. cFSE has previously been used to determine cell division in lymphocytes (15, 28) and bacteria (27), but it can potentially be applied as a pH probe, because its fluorescence is pH dependent. cFSE can be easily taken up by bacteria during incubation with its diacetate ester, cFDASE. Once it is incorporated, it is thought that its succinimidyl group forms conjugates with aliphatic amines (10, 28). Fluorescence can be detected after intracellular esterase activity. This approach should avoid problems due to leakage or active efflux of the probe and allow accurate calibration of

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the fluorescence signal. The method has been successfully applied to determine the $pH_{in}$ of *Lactococcus lactis*, *Bacillus subtilis*, *Listeria innocua*, and *Escherichia coli*. It was also demonstrated that the $pH_{in}$ of bacteria under severe stress conditions, such as elevated temperatures and the presence of detergents, could be measured by the cFSE method.

## MATERIALS AND METHODS

**Abbreviations.** BCECF, 2′,7′-bis-(2-carboxyethyl)-5 (and 6-)-carboxyfluorescein; cF, 5 (and 6-)-carboxyfluorescein; cFDASE, 5 (and 6-)-carboxyfluorescein diacetate succinimidyl ester; cFSE, 5 (and 6-)-carboxyfluorescein succinimidyl ester; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Zwittergent 3-12, *N*-dodecyl-*N*,*N*-dimethyl-3-ammoniopropane sulfonate; $pH_{in}$, intracellular pH; $pH_{out}$, extracellular pH; BSA, bovine serum albumin.

**Organisms and growth conditions.** *L. lactis* subsp. *lactis* (ATCC 19435 [the type strain]) and *E. coli* B104 (Chemunex S.A.) were grown overnight at 30°C in M17 broth (with 0.5% [wt/vol] lactose) and brain heart infusion broth (OXOID Ltd.), respectively. *L. innocua* 56 (provided by the Alfred Jørgensen Laboratory, Copenhagen, Denmark) and *B. subtilis* (ATCC 6051 [the type strain]) were grown overnight in brain heart infusion broth at 30°C and peptone broth in a shaking water bath at 37°C, respectively. The overnight cultures were diluted five times in fresh medium and incubated for an additional 3 h before they were harvested (at an optical density at 620 nm of approximately 0.6).

**Loading of cells with fluorescent probe.** Harvested cells were washed and resuspended in 50 mM potassium HEPES buffer, pH 8.0. Subsequently, the cells were incubated for 10 min at 30°C in the presence of 1.0 μM cFDASE, washed, and resuspended in 50 mM potassium phosphate buffer, pH 7.0 (unless indicated otherwise). To eliminate nonconjugated cFSE, glucose (final concentration, 10 mM) was added and the cells were incubated for an additional 30 min at 30°C (*L. lactis* was energized with lactose instead of glucose). The cells were then washed twice, resuspended in 50 mM phosphate buffer (pH 7.0), and placed on ice until required. With *E. coli*, the cFSE incorporation was accomplished by supplementing the HEPES buffer with 5 mM EDTA and using phosphate buffer supplemented with 10 mM $MgCl_2$.

**Efflux of cFSE.** cFSE efflux assays were performed in phosphate buffer, pH 7.0, in the absence and presence of 10 mM glucose (lactose in the case of *L. lactis*). At time zero, the tubes were placed in a water bath at 30°C, and at various times, samples (200 μl) were withdrawn and put in Eppendorf tubes, which were placed on ice. The cells were spun down immediately for 3 min in an Eppendorf centrifuge, and 150 μl of the supernatant was carefully pipetted off and diluted with 750 μl of potassium phosphate buffer, pH 7.0, in a 1-ml cuvette. Fluorescence was measured at excitation and emission wavelengths of 490 and 520 nm, respectively (at 5-nm slit widths), in a spectrofluorometer (Perkin-Elmer LS 50B, with a 50-Hz pulsed xenon lamp as the light source). Total fluorescence (100%) was estimated by using 150 μl of uncentrifuged sample (supernatant and cells) after incubation.

**Measurement of $pH_{in}$.** Cells containing fluorescent probe were diluted to a concentration of approximately $10^7$ cells per ml in a 3-ml glass cuvette and placed in the stirred and thermostated cuvette holder of the spectrofluorometer. Fluorescence intensities were measured at excitation wavelengths of 490 and 440 nm by rapidly alternating the monochromator between both wavelengths. The emission wavelength was 525 nm, and the excitation and emission slit widths were 5 and 10 nm, respectively. The 490-to-440-nm ratios were corrected for background signal due to buffer. The incubation temperature was 30°C (unless indicated otherwise). At the end of each assay, the extracellular fluorescence signal (background) was determined by filtration of the cell suspension through a 0.22-μm-pore-size membrane filter and measurement of the filtrate.

**Calibration of $pH_{in}$.** Calibration curves for *L. lactis*, *B. subtilis*, and *L. innocua* were determined in buffers with pH values ranging from 4 to 10. Buffers were prepared from glycine (50 mM), citric acid (50 mM), $Na_2HPO_4 \cdot 2H_2O$ (50 mM), and KCl (50 mM); the pH was adjusted with either NaOH or HCl. The $pH_{in}$ and $pH_{out}$ were equilibrated by addition of valinomycin (1 μM) and nigericin (1 μM), and the ratios were determined as described previously.

## RESULTS

**Effect of cFSE incorporation on cell viability.** The influence of cFSE incorporation on the viability of *L. lactis* was determined by a plate count (M17 agar). No reduction in the number of CFU was observed after the protocol (cFDASE treatment followed by incubation with a carbon source and washing) was carried out compared with the number of CFU found by plate counts of cell samples prior to cFDASE treatment or after application of the protocol without cFDASE treatment (data not shown).

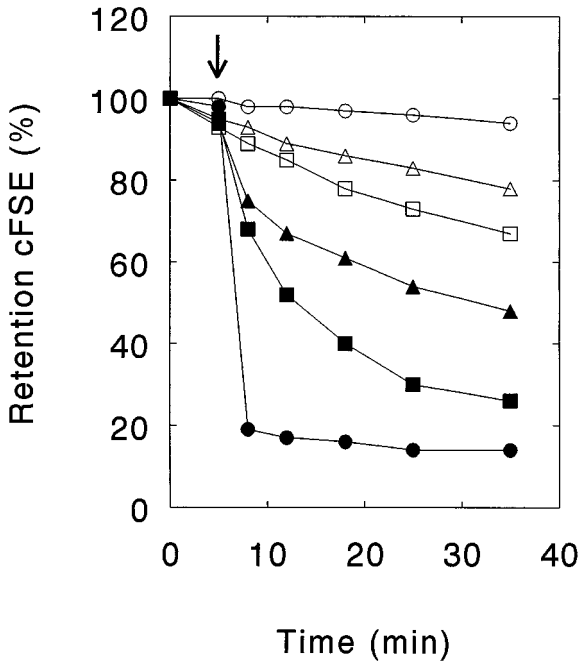


FIG. 1. Retention of cFSE in *L. lactis* (circles), *B. subtilis* (squares), and *L. innocua* (triangles). The cells were loaded with cFSE by incubation at 30°C with 1 μM cFDASE. The efflux of cFSE was measured at 30°C in 50 mM potassium phosphate buffer (pH 7.0) in the absence (solid symbols) and presence (open symbols) of 10 mM glucose (*B. subtilis* and *L. innocua*) or lactose (*L. lactis*), which was added at the time indicated by the arrow.

**Efflux of cFSE.** *L. lactis* did not extrude cFSE in the absence of a carbon source, but upon addition of lactose (10 mM), efflux was strongly stimulated (Fig. 1). Similarly, with *B. subtilis* and *L. innocua*, efflux in the absence of a carbon source was slow and was significantly stimulated upon addition of glucose (10 mM), but it was slower compared with that with *L. lactis*. The percentages of probe retained in *B. subtilis*, *L. innocua*, and *L. lactis* after 1-h incubations at 30°C in the presence of a carbon source were approximately 20, 50, and 15%, respectively.

**Conjugation of cFSE and elimination of free probe.** The succinimidyl group of cFSE is thought to form stable conjugates with (intracellular) aliphatic amines. In the *L. lactis* suspension containing cFSE, the relative fluorescence signal at 440 nm was 46.8, of which 3.5 was extracellular (Table 1). Upon the addition of lactose (10 mM) or ethanol (26%, vol/vol) to the cuvette, most cFSE was detected in the filtrate (extracellular) after filtration of the cell suspension. Nevertheless, a significant percentage of cFSE (approximately 20%) was not lost from the cells and apparently conjugated. When the cells were energized with lactose (10 mM) for 30 min and washed, only a small quantity of cFSE was lost from the cells after the postwashing addition of lactose or ethanol. In contrast, other analogs of fluorescein, cF and BCECF, were lost almost completely (>98%) from previously treated cells after the 30-min preincubation with lactose.

**Measurement of $pH_{in}$.** The $pH_{in}$ was determined from the ratio of the pH-sensitive wavelength (490 nm) and the pH-insensitive wavelength (440 nm). The measurement of the 490-to-440 ratio was performed during a 25-min assay (Fig. 2). Immediately after the cFSE-stained *L. lactis* cells were washed, the fluorescence intensities at 490 and 440 nm decreased. This phenomenon is most likely due to photofading. At the end of

TABLE 1. Retention of cFSE, cF, and BCECF in *L. lactis*

| Probe | Relative fluorescence at 440 nm | | | | | |
|---|---|---|---|---|---|---|
| | Cells loaded with fluorescent probe[a] | | | Cells loaded with fluorescent probe and incubated 30 min at 30°C in the presence of lactose (10 mM) | | |
| | Total | Intracellular | Extracellular | Total | Intracellular | Extracellular |
| cFSE | 46.8 | 43.3 | 3.5 | 8.6 | 8.4 | 0.2 |
| cFSE + lactose | 46.8 | 9.9 | 36.9 | 8.6 | 7.1 | 1.5 |
| cFSE + ethanol (33%)[b] | 46.8 | 10.3 | 36.5 | 8.6 | 7.1 | 1.5 |
| cF | 165.3 | 139.5 | 25.8 | 2.0 | 2.0 | 0.0 |
| BCECF | 53.3 | 40.7 | 12.6 | 0.3 | 0.3 | 0.0 |

[a] Before measurement, the cells are washed twice and resuspended in potassium phosphate buffer, pH 7.
[b] Values are corrected for the effects of ethanol on the fluorescence signal.

each assay, the $pH_{in}$ and $pH_{out}$ were equilibrated and the sample was filtered. When *L. lactis* cells were not energized with lactose, the unbound probe in the filtrate, which was leaked and/or exported from the cells, represented more than 90% of the total signal (Fig. 2A). However, after incubation with lactose and subsequent washing, the extracellular fluorescence signal (background), which was determined at 440 nm after the filtration, was reduced to only 11% of the total signal (Fig. 2B).

The $pH_{in}$ of *E. coli* was assessed after EDTA treatment to facilitate staining. A small pH gradient ($pH_{in}$ 7.3 to $pH_{out}$ 7.0 at 30°C) could be observed after the addition of glucose (10 mM) (data not shown). This suggests that the vitality of this organism is decreased, since previous studies have indicated that *E. coli* has a $pH_{in}$ of approximately 7.8 at an external pH of 7.0 (3).

**Calibration of $pH_{in}$.** In bacteria, the $pK_a$ values derived from the 490-to-440-nm ratios of intracellular cFSE were increased in pH by about 0.6 compared with the effective $pK_a$ of 6.5 for free cFSE (Fig. 3). The ratios after the equilibration of $pH_{in}$ and $pH_{out}$ could not be fitted adequately with the Henderson-Hasselbalch equation (9, 12) but could be fitted with a four-parameter sigmoid function, $y = a + b/\{1 + \exp[-(x - c)/d]\}$.

The excitation spectrum of intracellular cFSE at pH 7 after equilibration with the $pH_{out}$ exhibited a red shift of about 5 nm (Fig. 4). Potassium glutamate (0.5 M), which is present in cells at high levels, decreased the fluorescence intensity, but a red shift was not observed. The effect of some proteins on the spectrum of cFSE was also investigated. When cFSE was incubated in the presence of BSA (0.3%, vol/vol), a small red shift of the spectrum was observed and the fluorescence intensity decreased. This red shift was apparently not influenced by the pH of the incubation buffer (data not shown). β-Casein (0.03%, vol/vol) had no effect at all on the fluorescence spectrum of cFSE (data not shown).

**Effect of $pH_{out}$, elevated temperatures, and detergents on the $pH_{in}$ of bacteria.** The $pH_{in}$ values of energized *L. lactis*, *B. subtilis*, and *L. innocua* were measured by the cFSE ratio method during incubation in buffers with pH values ranging from 5 to 9 (Fig. 5). The observed $pH_{in}$ values for *B. subtilis* and *L. innocua* were remarkably similar, while that of *L. lactis* was lower.

Furthermore, $pH_{in}$ measurements with *L. innocua* were performed at various temperatures, and these experiments showed that at 30°C, a clear pH gradient (higher $pH_{in}$) of approximately 1 was generated after the addition of potassium and glucose (Fig. 6). At 45°C in this organism, the $pH_{in}$ initially decreased, but addition of potassium and more especially glucose caused a subsequent increase in the $pH_{in}$ to about 7.7. However, the cells were not able to maintain this gradient. At 53°C, the $pH_{in}$ was reduced and a reversed pH gradient (lower $pH_{in}$) resulted. Addition of potassium increased the $pH_{in}$ somewhat, but the $pH_{in}$ still remained lower than the $pH_{out}$. Subsequent addition of glucose had no effect.

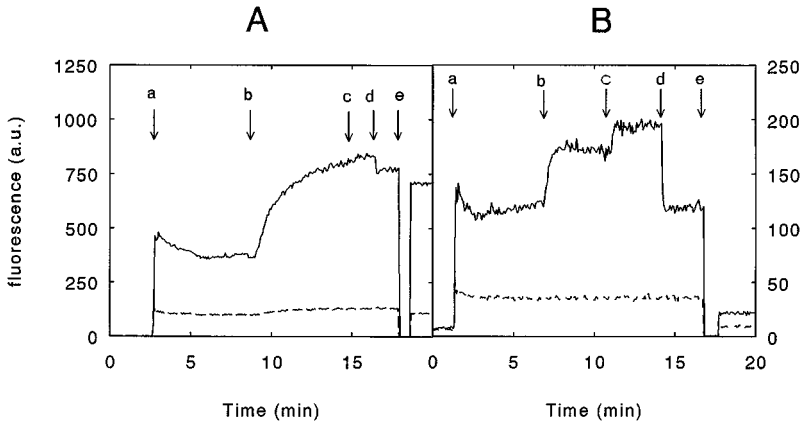


FIG. 2. Fluorescence intensity of *L. lactis* cells measured at excitations of 490 nm (——) and 440 nm (------). The cells were preincubated in the absence (A) or presence (B) of lactose (10 mM). The emission wavelength was 525 nm. The cells were loaded with cFSE as described in Materials and Methods. Measurements in the cuvette were performed at 30°C in 3 ml of 50 mM potassium phosphate buffer, pH 7.0. The following additions were made at the times indicated by the arrows: a, cell suspension (100 μl); b, lactose (10 mM); c, valinomycin (1 μM); and d, nigericin (1 μM). At arrow e, the cuvette was removed for a short time from the spectrofluorometer and the filtrate was measured after filtration of the cell suspension through a disposable disc filter (0.22 μm pore size). a.u., arbitrary units.

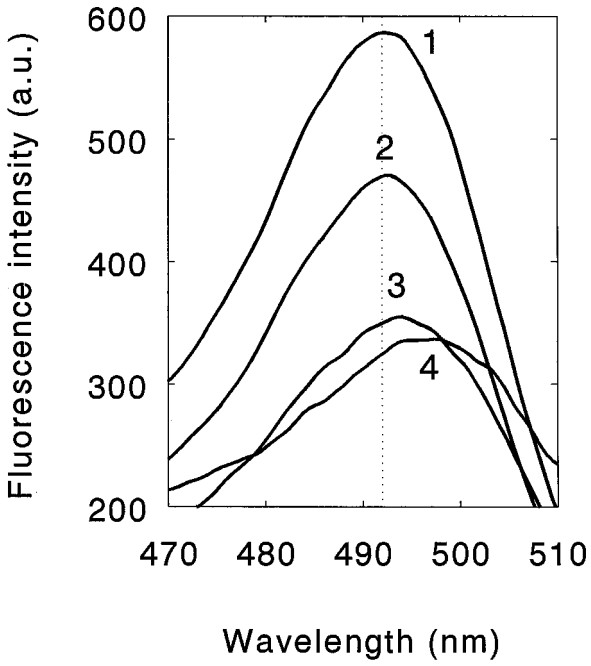


FIG. 3. Excitation spectra of cFSE in buffer (1), in the presence of 0.5 M potassium glutamate (2) or 0.3% (vol/vol) BSA (3), and when incorporated in *L. lactis* cells (4). The cFSE spectra were corrected for the autofluorescence of BSA. The measurements were performed in 50 mM potassium phosphate buffer, pH 7.0, at room temperature. The emission wavelength was 525 nm. The fluorescence intensities of curves 1, 2, and 3 cannot be directly compared with the fluorescence intensity of curve 4. a.u., arbitrary units.

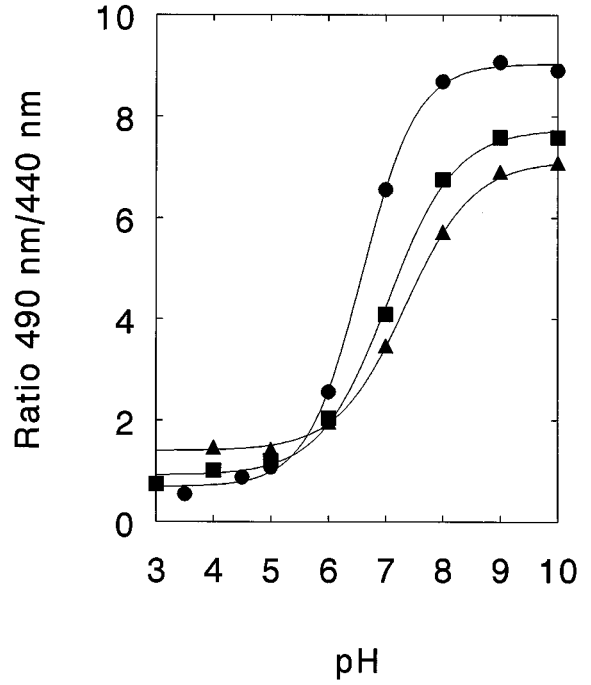


FIG. 4. The relationship between the pH and the ratio (490 nm to 440 nm) of cFSE in buffer (●), in *B. subtilis* cells (■), and in *L. lactis* cells (▲). $pH_{in}$ and $pH_{out}$ were equilibrated by incubation with valinomycin (1 μM) and nigericin (1 μM).

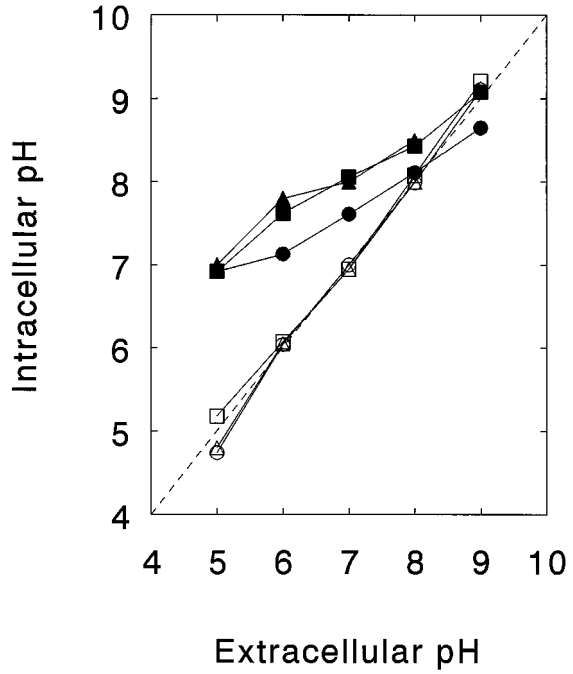


FIG. 5. Effect of $pH_{out}$ on the $pH_{in}$ of energized *L. lactis* (circles), *B. subtilis* (squares), and *L. innocua* (triangles) cells in the absence (solid symbols) or presence (open symbols) of valinomycin (1 μM) and nigericin (1 μM). Assays were performed at 30°C. The buffers were prepared from glycine (50 mM), citric acid (50 mM), $Na_2HPO_4 \cdot 2H_2O$ (50 mM), and KCl (50 mM); pH was adjusted with either NaOH or HCl.

In the presence of various concentrations of detergent, the pH gradient (at an external pH of 7.0) in *B. subtilis* was completely dissipated by Zwittergent 3-12 and Triton X-100 at 0.025 and 0.006%, respectively (Fig. 7). These results indicate that permeabilization of the cytoplasmic membrane by detergents can be determined accurately by the cFSE method.

## DISCUSSION

This report describes a novel technique for measuring the $pH_{in}$ of bacteria. This technique is based on the intracellular conjugation of cFSE in the cytoplasm of cells and then the elimination of free probe by a short incubation in the presence of a fermentable sugar. The principle advantage of such a system is that leakage of fluorescent probe is minimal, being less than 25% for cFSE after 20 min at 30°C, compared with over 90% for other analogs of fluorescein, such as cF and BCECF. The method takes advantage of the supposed conjugation of the succinimidyl group of cFSE with the aliphatic amines of intracellular proteins (10). Significantly, incorporation and conjugation of cFSE in the cytoplasm of the cells does not affect the viability of *L. lactis*. The system is further enhanced by the ability of bacteria to eliminate unconjugated probe. Mammalian cells as well as fungi and bacteria are all known to actively extrude fluorescein and related analogs cF and BCECF (1, 5, 14, 17). In this study, three gram-positive bacteria were found to efficiently extrude cFSE after the addition of an appropriate carbon source. Efflux is most likely catalyzed by an ATP-driven extrusion system, because efflux proceeds in the presence of ionophores valinomycin (1 μM) and nigericin (1 μM) (4). This system might be similar to that previously described for BCECF in *L. lactis* (17). In such gram-negative bacteria as *E. coli*, the use of the cFSE method is

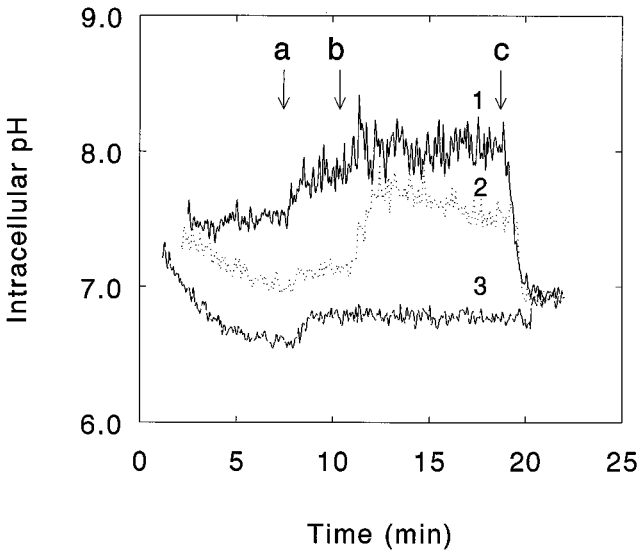


FIG. 6. Effect of temperature on the $pH_{in}$ of *L. innocua*. The assays were performed at 30 (1), 45 (2), and 53°C (3) in 50 mM sodium phosphate buffer, pH 7.0. The following additions were made at the times indicated by the arrows: a, potassium chloride (100 mM); b, glucose (10 mM); and c, Zwittergent 3-12 (0.1%).

complicated by the inability of the prefluorochrome cFDASE (molecular weight, 557) to pass the outer membrane in the gram-negative cell wall. For this species, a short incubation with EDTA overcame this problem. However, the EDTA treatment may well interfere with the active efflux of unbound cFSE and the generation of a significant pH gradient. Hence, application of this protocol for gram-negative genera needs further attention.

Generally, the background levels of extracellular probe were about 10 to 20%, which, while very low compared with those of other fluorescent probes, such as cF and BCECF, are still significant. The occurrence of this background could not be avoided, but the resulting error can be minimized by performing the calibration under identical conditions with comparable background levels.

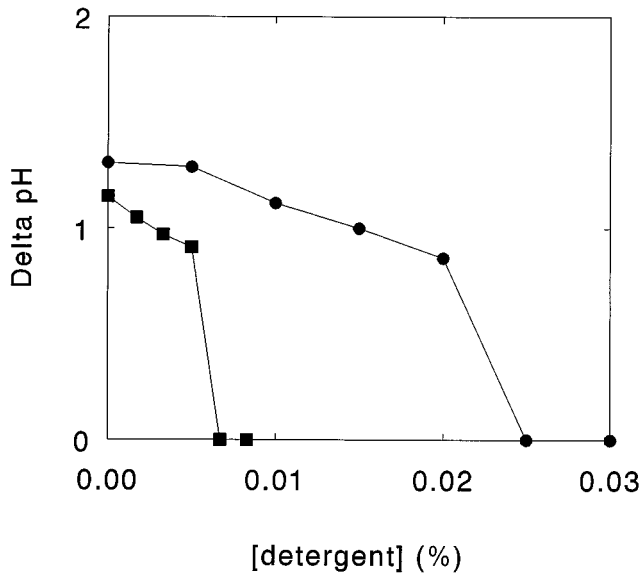


FIG. 7. ΔpH of *B. subtilis* in the presence of increasing concentrations of Triton X-100 (●) and Zwittergent 3-12 (■). Assays were performed at 30°C in 50 mM potassium phosphate buffer, pH 7, with glucose (10 mM).

The observed intracellular pK shift following the uptake of cFSE has been reported previously for other probes (7, 9, 16), but no clear reason for this phenomenon was provided. It is unlikely that this pK shift resulted from high probe concentrations or a Donnan potential (7, 16). Recently, it was suggested that interaction of the probe with lipophilic cell compartments, such as the cytoplasmic membrane (20), was responsible for a pK shift with the fluorescent pH indicator carboxy-seminaphthorhodafluor. The quantum yield of cFSE might be affected significantly by the conjugation of the molecule. This was illustrated in vitro by the addition of potassium glutamate and BSA, both of which reduced the fluorescence intensity and/or excitation maximum. Changes in the spectral properties of cFSE inside the cells might explain why the ratio can not be fitted adequately with the Henderson-Hasselbalch equation. The ratio could, however, be fitted with a four-parameter sigmoid function. The $pH_{in}$ values observed for *L. lactis* by the cFSE ratio method were comparable to the values reported by Molenaar et al. (17) and Poolman et al. (22). For other bacteria (e.g., *B. subtilis* and *L. innocua*), the measured $pH_{in}$ values were also comparable to those reported in the literature (3, 4, 6).

The advantage of using a conjugated probe for the measurement of the $pH_{in}$ is most clearly illustrated by the ability to measure $pH_{in}$ under conditions that may permeabilize the cell envelope. Convential methods are unable to determine the $pH_{in}$ under such conditions. Indeed, the cFSE approach was able to accurately show the effect of elevated temperatures on the pH gradient. This offers exciting new possibilities for the study of the impact of heat shock on pH homeostasis in bacteria. In fact, preliminary results indicated that *L. lactis* cells exposed to a heat shock could maintain a higher pH gradient than control cells (4). Moreover, the application of flow cytometry with fluorescent cFSE-treated cells allows the determination of the $pH_{in}$ of individual cells within a population. This ability could prove useful for the study of population effects after the exposure of bacteria to various stress conditions. The approach can also be exploited in the investigation of low $pH_{in}$ in such bacteria as acidophiles, in which case cFSE may be replaced with 5 (and 6-)-carboxy-2′,7′-dichlorofluorescein succinimidyl ester, which has a $pK_a$ of 3.9 in buffer and 4.9 when incorporated in bacterial cells (4, 18).

In conclusion, the cFSE ratio method allows sensitive and continuous measurement of the $pH_{in}$ in bacteria. Leakage of fluorescent probe, which is one of the major problems with measurements with such molecules as cF and BCECF, is avoided by intracellular conjugation of cFSE and subsequent elimination of free probe. It thus offers the ability to investigate the important physiological responses to $pH_{in}$ variations in bacteria, even with bacteria experiencing such severe stress conditions as elevated temperatures and exposure to detergents.

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## REFERENCES

1. **Allen, C. N., E. S. Harpur, T. J. B. Gray, N. L. Simmons, and B. H. Hirst.** 1990. Efflux of bis-carboxyethylcarboxyfluorescein (BCECF) by a novel ATP-dependent transport mechanism in epithelial cells. Biochem. Biophys. Res. Commun. **172:**262–267.

2. **Bakker, E. P.** 1990. The role of alkali-cation transport in energy coupling of neutrophilic and acidophilic bacteria: an assessment of methods and concepts. FEMS Microbiol. Rev. **75:**319–334.
3. **Booth, I. R.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49:**359–378.
4. **Breeuwer, P.** Unpublished data.
5. **Breeuwer, P., J. L. Drocourt, F. M. Rombouts, and T. Abee.** 1994. Energy-dependent, carrier-mediated extrusion of carboxyfluorescein from *Saccharomyces cerevisiae* allows rapid assessment of cell viability by flow cytometry. Appl. Environ. Microbiol. **60:**1467–1472.
6. **Bruno, M. E. C., A. Kaiser, and T. J. Montville.** 1992. Depletion of proton motive force by nisin in *Listeria monocytogenes* cells. Appl. Environ. Microbiol. **58:**2255–2259.
7. **Chaillet, J. R., and W. Boron.** 1985. Intracellular calibration of a pH-sensitive dye in isolated perfused salamander proximal tubules. J. Gen. Physiol. **86:**765–794.
8. **Cook, G. M., and J. M. Russell.** 1994. The effect of extracellular pH and lactic acid on pH homeostasis in *Lactococcus lactis* and *Streptococcus bovis*. Curr. Microbiol. **28:**165–168.
9. **Graber, M. L., D. C. DiLillo, B. L. Friedman, and E. Pastoriza-Munoz.** 1986. Characteristics of fluoroprobes for measuring intracellular pH. Anal. Biochem. **156:**202–212.
10. **Haugland, R. P.** 1992. Succinimidyl esters and carboxylic acids, p. 24–33. *In* K. D. Larison (ed.), Handbook of fluorescent probes and research chemicals. Molecular Probes Inc., Eugene, Oreg.
11. **Haugland, R. P., and A. Minta.** 1990. Design and application of indicator dyes, p. 1–20. *In* J. K. Foskett and S. Grinstein (ed.), Noninvasive techniques in cell biology. Wiley-Liss, New York.
12. **James-Kracke, M. R.** 1992. Quick and accurate method to convert BCECF fluorescence to $pH_{in}$: calibration in three different types of cell preparations. J. Cell. Physiol. **151:**596–603.
13. **Kashket, E. R.** 1985. The proton motive force in bacteria: a critical assessment of methods. Annu. Rev. Microbiol. **39:**219–242.
14. **Kondo, M., and M. Araie.** 1994. Movement of carboxyfluorescein across the isolated rabbit iris-ciliary body. Curr. Eye Res. **13:**251–255.
15. **Lyons, A. B., and C. R. Parish.** 1994. Determination of lymphocyte division by flow cytometry. J. Immunol. Methods **171:**131–137.
16. **Molenaar, D., T. Abee, and W. Konings.** 1991. Continuous measurement of the cytoplasmic pH in *Lactococcus lactis* with a fluorescent pH indicator. Biochim. Biophys. Acta **1115:**75–83.
17. **Molenaar, D., H. Bolhuis, T. Abee, B. Poolman, and W. N. Konings.** 1992. The efflux of a fluorescent probe is catalyzed by an ATP-driven extrusion system in *Lactococcus lactis*. J. Bacteriol. **174:**3118–3124.
18. **Nedergaard, M., S. Desai, and W. Pulsinelli.** 1990. Dicarboxy-dichlorofluorescein: a new fluorescent probe for measuring acidic intracellular pH. Anal. Biochem. **187:**109–114.
19. **Noël, J., A. Tejedor, P. Vinay, and R. Laprade.** 1989. Fluorescence measurement of intracellular pH on proximal tubule suspensions. Renal Physiol. Biochem. **12:**371–387.
20. **Opitz, N., E. Merten, and H. Acker.** 1994. Evidence for redistribution-associated intracellular pK shifts of the pH sensitive fluoroprobe carboxy-SNARF-1. Pflügers Arch. **427:**332–342.
21. **Peña, A., J. Ramírez, G. Rosas, and M. Calahorra.** 1995. Proton pumping and the internal pH of yeast cells, measured with pyranine introduced by electroporation. J. Bacteriol. **177:**1017–1022.
22. **Poolman, B., A. J. M. Driessen, and W. N. Konings.** 1987. Regulation of solute transport in streptococci by external and internal pH values. Microbiol. Rev. **51:**498–508.
23. **Rink, T. J., R. Y. Tsien, and T. Pozzan.** 1982. Cytoplasmic pH and free $Mg^{2+}$ in lymphocytes. J. Cell Biol. **95:**189–196.
24. **Salhany, J. M., T. Yamane, R. G. Shulman, and S. Ogawa.** 1975. High resolution $^{31}P$ nuclear magnetic resonance studies of intact yeast cells. Proc. Natl. Acad. Sci. USA **72:**4966–4970.
25. **Ten Brink, B., and W. N. Konings.** 1982. The electrochemical proton gradient and lactate concentration gradient in *Streptococcus cremoris* grown in batch culture. J. Bacteriol. **152:**682–686.
26. **Thomas, J. A., R. N. Buchsbaum, A. Zimniak, and E. Racker.** 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry **18:**2210–2218.
27. **Ueckert, J., P. Breeuwer, T. Abee, P. Stephens, G. Nebe von Caron, and P. F. ter Steeg.** Flow cytometry applications in physiological study and detection of foodborne microorganisms. Int. J. Food Microbiol., in press.
28. **Weston, S. A., and C. R. Parish.** 1990. New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. J. Immunol. Methods **133:**87–97.