The Application of pH-sensitive Fluorescent Dyes in Lactic Acid Bacteria Reveals Distinct Extrusion Systems for Unmodified and Conjugated Dyes

Erwin Glaasker, Wil N. Konings, and Bert Poolman

Published in: Molecular Membrane Biology 13, 173-181 (1996)
ABSTRACT.

Extracellular pH in bacteria can be measured efficiently between internal pH values of 6.5 and 8.5 with the fluorescent pH indicator 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF). A new fluorescent pH probe with a lower pK_a(app) than BCECF was synthesized from fluorescein-isothiocyanate and glutamate. The new probe, N-(fluorescein thio-ureanyl)-glutamate (FTUG), was much less sensitive to changes in concentrations of KCl than BCECF. Similar to BCECF, an efflux of FTUG independent of the proton motive force but dependent on ATP was observed both in Lactobacillus plantarum and Lactococcus lactis. Corrections for probe efflux allowed accurate measurements of the pH_{in}. Similar intracellular pH values were determined with FTUG and BCECF in the range where both probes can be applied and the pH values correlated well with those estimated from the distribution of radio-labelled benzoic acid. Since FITC can easily be coupled to substrates containing an amino group, it is possible to develop other FITC-derivatives as well. The mechanisms of probe excretion and the nature of the excreted product(s) were studied in further detail for BCECF and FTUG. BCECF was excreted from wild-type L. lactis in an unmodified form as was determined by chromatographic and mass spectrometry analysis. In case of FTUG, the excreted product was a conjugated derivative. Genuine FTUG was not excreted although it was present in cellular extracts from L. lactis. Exit of BCECF was completely inhibited in a BCECF efflux mutant (Bef^-) of L. lactis, whereas FTUG-conjugate efflux in this mutant was similar to the wild-type. Addition of indomethacin, a known inhibitor of BCECF efflux in human epithelial cells resulted in complete inhibition of BCECF efflux in wild type L. lactis, whereas FTUG-conjugate exit was only slightly affected. The results of the mutant and inhibitor studies suggest that FTUG-conjugate and BCECF efflux in L. lactis are mediated by different ATP-driven extrusion systems for organic anions.

INTRODUCTION

The cytoplasmic pH of bacterial and eukaryotic cells determines to a large extent their physiological activity (5, 22, 26). pH sensitive fluorescent dyes have been applied for measuring intracellular pH (pH_{in}) in bacteria and eukaryotic cells (12). Conventional methods to measure pH_{in} are based upon the distribution of radioactively labeled weak organic acids and bases over the cytoplasmic membrane.
Use and efflux of pH sensitive fluorescent dyes

(15, 30). Hence, equilibration of the radioactive probes across the cytoplasmic membrane and subsequent separation of the cells from the medium by centrifugation or filtration is necessary. Another method for measuring pH\textsubscript{in} uses nuclear magnetic resonance spectroscopy and is based on the pH dependence of the chemical shift of inorganic phosphate or other compounds present in the intra- and extracellular compartments (8, 20). Disadvantages of this method relate to the high cell densities that are required, the relatively long time intervals that are needed for each measurement, and the disturbance of the measurement by paramagnetic ions (e.g. Mn\textsuperscript{2+}) that are abundant in the cytoplasm of most bacteria. The use of fluorescent probes offers the advantage that pH\textsubscript{in} can be measured continuously with a high time resolution and sensitivity.

Eukaryotic cells can be loaded efficiently with fluorescent indicators by adding membrane permeable acetoxymethyl ester-forms of the probe to the cells (6, 34). Intracellular hydrolysis of the probe will occur due to the action of esterases. Bacterial cells do not hydrolyze esterified fluorescent pH indicators rapidly or, alternatively, may excrete the esterified fluorescent indicator prior to trapping of the free acid in the cytoplasm (4, 13). Another method to load bacterial cells with fluorescent indicators comprises a lowering of the external pH of a dense cell suspension for a short period of time in the presence of the probe (18). In this way the negative charges of the probes are neutralized, allowing the acid form of the probe to enter the cytoplasm. Once inside, the probe is rapidly deprotonated and captured due to a higher pH\textsubscript{in}. Other methods that have been described are covalent coupling of carboxyfluorescein succinimidyester to amino groups in proteins (7), and electroporation of the cells in the presence of the membrane impermeable fluorescent indicator pyranine (25) or dextran conjugated probes, but these methods can be detrimental for the organisms.

Most pH\textsubscript{in} measurements with fluorophores have been performed with carboxyfluorescein or its derivatives. Especially 2',7'-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein (BCECF) is frequently used as a fluorescent pH indicator. In bacteria that are loaded with BCECF, an apparent efflux of the probe is observed due to the presence of excretion system(s) with specificity for BCECF (19). When the rate kinetics of probe efflux are relatively simple, e.g., pseudo-first order, a correction of the fluorescence signal for the probe efflux can be made (18).

In general the pH sensitivity of fluorophores is limited to the range pK\textsubscript{a(app)}±1. Since BCECF has a pK\textsubscript{a(app)} of 7.5, this indicator is not suitable for determination of the cytoplasmic pH below values of 6.5 which is often encountered in fermentative bacteria. Several new fluorescent indicators with a lower pK\textsubscript{a} have
been developed and applied in eukaryotic cells, e.g. 5-(and-6)-carboxy-2',7'-dimethyl-3'-hydroxy-6'-N-ethylaminospiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one (DM-NERF), 5-(and-6)-carboxy-2-chloro-3'-hydroxy-1,2,3,4-tetrahydropropyridino[5,6]-spiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one (Cl-NERF), 5-(and-6)-carboxyfluorescein diacetate (CFDA), lissamine-rhodamine B sulfonyl chloride, and carboxy-seminaphthorhodafluor-1 (C-SNARF-1) (2, 17, 37). Unfortunately, these fluorescent indicators were not suitable for application in bacteria due to a high rate of passive leakage from the cells (unpublished results). To expand the application of fluorescent pH indicators to more acidic pH values, a fluorescein-isothiocyanate (FITC) derivative, that is well retained by bacterial cells, has been synthesized. FITC has been coupled to glutamate yielding a new pH sensitive fluorescent dye N-(fluorescein thio-ureanyl)-glutamate (FTUG), which has a pKₐ(app) of 6.9. The new pH sensitive dye was used to determine the pHᵢₙ of *Lactococcus lactis* and *Lactobacillus plantarum* over a wide pH range, and the results were compared with values obtained by the distribution of ¹⁴C-benzoic acid and BCECF fluorescence.

The kinetics of probe efflux was studied for FTUG and BCECF in *Lactobacillus plantarum*, *Lactococcus lactis*, and a mutant of *Lactococcus lactis*, deficient in BCECF efflux (Bef⁻). The results indicate that probe efflux in bacteria has features in common with BCECF efflux in human epithelial cells as well as conjugated drug transport in mammalian cells such as hepatocytes (14, 23, 29). These putative anionic drug excretion systems are different from the previously described extrusion systems for cationic drugs in *L. lactis* (3, 3a, 4, 36).

**MATERIALS AND METHODS**

**Media and strains.** *Lactobacillus plantarum* VBLLAC11-10 (typed by the American Type Culture Collection) was kindly provided by Unilever Research Laboratories. *Lactococcus lactis* subspecies *lactis* ML3 and the BCECF efflux mutants (Bef⁻) have been described previously (19). All organisms were grown at 30°C on a modified MRS medium (10), containing per liter 10 g of tryptone, 10 g of LabLemco powder (Oxoid), 5 g of yeast extract, 2 g of K₂HPO₄, 0.2 g of MgSO₄.7H₂O, 0.035 g of MnSO₄.H₂O, and 1 ml of Tween 80, adjusted to pH 6.6 with HCl. The medium was supplemented with separately sterilized 0.5% (w/v) glucose.

**Synthesis of N-(fluorescein thio-ureanyl)-glutamate (FTUG).** Fluorescein isothiocyanate (final concentration, 11 mM) was freshly prepared in 90 ml of 50 mM bis-Tris-Propane, pH 10.0. The reaction was initiated by adding 10 ml of 4 M K-glutamate, pH 10.0. The reagents were mixed thoroughly and kept at 4°C in the dark for 2-18h to complete the reaction. The mixture was directly used.

**Loading of bacteria with BCECF or FTUG.** Cells were harvested in the logarithmic phase of
growth at an $A_{660}$ of 0.6, washed twice with 50 mM potassium phosphate, pH 7.0, and resuspended to a final protein concentration of 10-50 mg/ml. The fluorescent indicator (BCECF or FTUG) was added to these cells to a final concentration of 1 mM. The cells were loaded by lowering the external pH to approximately 1 for 5 min by adding a small aliquot of 0.5 M HCl (18). Routinely, the amount of acid used was 2.5 ml of 0.5 M HCl per 20 ml of concentrated cell suspension (50 mg/ml). Subsequently, the cells were washed four times with 50 mM potassium phosphate, pH 7.0.

**Internal pH measurements.** Fluorescence measurements on dye-loaded cells were performed in 30 mM citric acid, 30 mM $K_2HPO_4$, 30 mM 2-(N-cyclohexylamino)-ethanesulfonic acid (CHES), adjusted to pH 5.0 - 7.0 with KOH (CKC buffer). The final protein concentration during the measurement was 10-25 mg/ml. Excitation and emission monochromator wavelengths for BCECF were 502 and 518 nm, with slit widths of 5 and 15 nm, respectively. For FTUG excitation and emission wavelengths were 495 and 518 nm, with slit widths of 2.5 and 20 nm, respectively. The fluorescence signal was averaged over time intervals of 1 s. The cytoplasmic pH was calculated from the fluorescence data with the Henderson-Hasselbach equation: $pH = pK_a (\text{app}) + \log (F - F_{\text{min}}/F_{\text{max}} - F)$ in which $F$, $F_{\text{min}}$, and $F_{\text{max}}$ correspond to the actual fluorescence signal that is measured, the minimal fluorescence that is measured at low pH (<5.0) in uncoupled cells (in the presence of 1 mM valinomycin, 1 mM nigericin, plus 70 mM N-carbonyl cyanide m-chlorophyl hydrzone (CCCP)), and the maximal fluorescence that is measured at high pH (>10.0) in uncoupled cells. The signal was corrected for probe efflux according to Molenaar et al. (18): $F_{\text{cor}} = (e^{kt}/q_0) \cdot (F - (1-q_0 \cdot e^{kt}) \cdot F_0)$ in which $F_{\text{cor}}$ is the fluorescence signal that was corrected for probe efflux, $F$ is the total fluorescence signal that is measured, $F_0$ is the fluorescence when all fluorescent indicator is located extracellularly (determined by the addition of 0.2% Triton X-100), $k$ is the first order rate constant for efflux (determined by plotting $\ln |F - F_0|$ against time), and $q_0$ is the fraction of the indicator that was located internally at time zero (which is close to 1). The fluorescent techniques to determine pH in cells were compared with the distribution of $^{14}$C-benzoic acid (specific activity 50 mCi/mmole) over the cytoplasmic membrane using silicone oil centrifugation to separate the cells from the surrounding medium (32).

**Efflux assays.** Cells, loaded with BCECF or FTUG, were diluted into 2 ml CKC buffer of the desired pH to a final protein concentration of 0.5-2 mg/ml at 30 °C. After 1 min of incubation, the assays were initiated by adding glucose (final concentration, 10 mM) with or without 1 mM indomethacin or 1 mM each of valinomycin plus nigericin. Aliquots of 200 ml were withdrawn and the cells were spun down within 20 s in a microfuge at 14,000 x g. The supernatant (180 ml) was removed carefully and the fluorescence corresponding to extracellular probe was measured after a 16.7-fold dilution with bis-Trispropane, pH 10.0. The pellet was resuspended in 160 ml 0.2 % Triton X-100, and the suspension was kept at room temperature for 30-60 min to release internal fluorescent dyes. Subsequently, the cell debris was spun down for 5 min in a microfuge and the fluorescence was determined as before. Alternative methods to permeabilize the cells (e.g., sonication, higher concentrations of detergent) confirmed that 0.2% Triton X-100 was sufficient to permeabilize the cells.

**HPLC analysis.** BCECF and FTUG solutions (50 ml) were applied to an Econosphere C18 reversed phase column using fluorescence detection (excitation at 500 nm, emission at 520 nm). The probes were eluted with a gradient of 0.4 % glacial acetic acid, pH 4.9, and acetonitril. The elution was initiated (t=0) with 0% acetonitril, subsequently the concentration of acetonitril was increased to 15% at t=20 min and 30% at t=30 min; elution with 30% acetonitril was continued.
for 10 min, whereafter the column was equilibrated with buffer (0% acetonitril) for 15 min. Cellular extracts and excreted dyes were obtained from dye-loaded cells (see above) in 50 mM potassium phosphate, pH 6.5, at a final protein concentration of about 50 mg/ml, essentially as described in the section “efflux assays”. Non-energized cells (control) and cells that were actively excreting the dyes for 20 min by energization with 0.2 % glucose at 30°C were collected by centrifugation at given times. Supernatants were analyzed directly, and the pellets were extracted by adding 300 ml of methanol, followed by 5 min incubation at room temperature and removal of cellular debris by centrifugation. Mass spectrometry analysis of BCECF and FTUG solutions was performed by direct flow-injection.

**Flow cytometry.** Cells were loaded with BCECF and FTUG as described above, and immediately after loading, 1 mg/ml of the membrane impermeable DNA stain propidiumiodide was added to the cell suspension. To increase the sensitivity of the flow cytometric detection, the cells were counter-stained with the membrane permeable DNA stain Hoechst (1 mg/ml). Flow cytometric analysis was performed approximately 3 h after loading.

**Miscellaneous.** Protein was determined by the method of Lowry *et al.* (16) with bovine serum albumine as a standard. Distributions of 14C-benzoic acid were calculated using water-accessible cytoplasmic volumes of 2.5 and 2.9 ml/mg of protein for *Lactobacillus plantarum* and *Lactococcus lactis*, respectively (28, 33).

**Chemicals and equipment.** Fluorescent dyes were obtained from Molecular probes, Eugene, OR, USA. BCECF solutions were prepared as described by Molenaar *et al.* (18) and stored at -20°C in the dark. Radiochemicals were purchased from Amersham (Buckinghamshire, England). All other chemicals were reagent grade and obtained from commercial sources. The fluorimeter for pH determinations was a Perkin-Elmer LS50 with computer controlled data aquisition and storage. The flow cytometer was a Fluorescence Activated Cell Sorter II (FACS II).

**RESULTS**

**Loading of bacterial cells with fluorescent dyes.** The procedure to load bacterial cells with fluorescent pH indicators consists of treating a dense cell suspension with acid in the presence of the probe (18). This procedure was succesfully applied in a number of lactic acid bacteria, including *Lactococcus lactis*, *Listeria innocua*, *Leuconostoc oenos*, *Lactobacillus helveticus*, *Lactobacillus sanfransisco* and *Lactobacillus plantarum*. The efficiency and reliability of the procedure was tested in detail for *L. plantarum* and *L. lactis*. Putative lysis of the cells was measured by using the membrane impremerant DNA stain propidiumiodide (PI), which becomes fluorescent upon intercalating with DNA following entry into the cells. For *L. plantarum* and *L. lactis* less than 3 % cell lysis was observed in several independent experiments. Flow cytometry was used to monitor the loading of individual cells, and to show, by means of propidiumiodide fluorescence, whether lysis had occurred. In case of *L. plantarum* and *L. lactis* approximately 1.5% of the cell population did not contain any fluorescent indicator, 95.5% was loaded with BCECF, and 3% showed propidiumiodide fluorescence (data not shown). When these cells were kept
on ice in the absence of glucose, the leakage of BCECF or FTUG was insignificant for periods up to 8h. The results show that the acid shock procedure can be used for efficient loading of bacterial cells with weakly acidic fluorescent dyes without much effect on the cell integrity.

**Development of FTUG.** To expand the application of fluorescent pH probes to more acidic pH values, a new pH sensitive fluorescein-isothiocyanate derivative was synthesized. The new probe was generated by coupling of fluorescein-isothiocyanate (FITC) to glutamate; a thio-urea bond is formed between the thiocyanate group of FITC and the amino group of glutamate (Fig. 1). A 40-fold excess of the amino-group containing substrate (glutamate) was added to remove the highly reactive FITC completely. The remaining products in the reaction mixture are glutamate and its derivate with FITC, that was termed N-(fluorescein thio-ureanyl)-glutamate (FTUG). In comparison to FITC, FTUG has two extra negatively charged carboxylic groups, which should minimize passive diffusion of FTUG across the cytoplasmic membrane. The synthesis reaction was followed by HPLC analysis using fluorescence detection. A good separation of FTUG (retention time 19 min), and FITC (retention time 37 min) was obtained (Fig. 2A). The reaction was complete (>99% of the FITC had reacted to a product with a retention time of 19 min) after 2h of incubation at 4°C in the dark. The chemical nature of FTUG was confirmed by mass spectrometry, demonstrating the conversion to a product with a molecular mass of 536 g/mol.

**Characteristics of FTUG and BCECF.** The fluorescence of FTUG was measured as a function of pH and compared with BCECF in dye-loaded cells of *L. plantarum* (Fig. 3) and *L. lactis* (not shown) to determine the pH range in which FTUG can be used to monitor the changes in cytoplasmic pH. The ionophores valinomycin and nigericin plus the protonophore CCCP were added to these cells to maintain an equal internal and external pH. The titration curves show a lower pKₐ(app) for FTUG (6.9) than for BCECF (7.5) both in *L. plantarum* (Fig. 3) and *L. lactis* (data not shown). Some variation in pKₐ(app) values was observed, especially between different batches of BCECF (7.3 to 7.7) and to a lesser extent also for FTUG (6.9 to 7.0). The variation within one batch of probe was limited to approximately 0.05 pH unit. Since a pH sensitive fluorescent dye might also respond to cations other than protons, the influence of sodium and potassium ions on the fluorescence quantum yield of BCECF and FTUG was tested next. To investigate the effect of ionic strength on the pKₐ(app) of BCECF and FTUG, titration curves were made in the presence and absence of 1 M KCl, a physiological concentration in the cytoplasm of lactic acid bacteria (11, 27). The pKₐ(app) of FTUG was lowered from
6.9 to 6.7, whereas that of BCECF was lowered from 7.5 to 6.7.

**Intracellular pH measurements.** Upon addition of glucose to dye-loaded cells, the fluorescence increases which corresponds with an increase in pH\(_\text{in}\). After this initial increase, the fluorescence decreases slowly in time as a result of probe efflux (see below) and falls back to the initial values upon dissipation of the pH gradient by the ionophore nigericin. To test whether dye-loading affects the pH\(_\text{in}\) of *L. lactis* ML3, the pH\(_\text{in}\) of unloaded cells was compared with that of BCECF- and FTUG-loaded cells by measuring the distribution of \(^{14}\text{C}\)-labelled benzoic acid across the membrane. Loading of *L. lactis* ML3 with FTUG or BCECF had no significant effect on the pH\(_\text{in}\) values (Fig. 4; closed bars). Moreover, a good correlation (especially at lower pH values) was observed of the absolute pH values obtained from the distribution of \(^{14}\text{C}\)-benzoic acid and from the fluorescence of BCECF and FTUG over the pH\(_\text{ex}\) range 5.0-7.0 (Table I). The results demonstrate that FTUG can be used for the measurement of pH\(_\text{in}\) values that are approximately 0.5 pH unit lower than the pH values that can be measured with BCECF and that FTUG is less sensitive to changes in potassium concentration than BCECF.

**Efflux of FTUG and BCECF.** The kinetics and energetics of the efflux of FTUG and BCECF in *L. plantarum* were studied in the presence and absence of glucose. In contrast to energy-depleted cells, glycolyzing cells excreted both probes on a 10 min time scale, indicating that excretion is a metabolic energy requiring process. Manipulation of the proton motive force by the addition of valinomycin and/or nigericin did not have any effect on the rate of BCECF and FTUG efflux in *L. plantarum* (Fig. 5). This suggests that excretion of FTUG and BCECF is most likely ATP-driven in *L. plantarum*, as was suggested previously for BCECF efflux in *L. lactis* (19). To investigate whether BCECF and FTUG excretion are mediated via the same system, the efflux of both dyes was further characterized in *L. lactis* ML3 since an isogenic BCECF efflux mutant (Bef\(^{-}\)mutant) was available for this strain (19). Although the Bef\(^{-}\) mutant grows equally well as the parent ML3 strain (19), it was observed that the mutant maintains the pH\(_\text{in}\) at a value approximately 0.4 pH units higher than the wild-type (Fig. 4). Since the defect of this mutant in BCECF efflux was also observed under conditions that pH\(_\text{in}=\text{pH}_{\text{out}}\), it is unlikely that the reduced efflux rate is a direct consequence of the higher pH\(_\text{in}\). The efflux of FTUG was not affected in the Bef\(^{-}\) mutant (Fig. 6), whereas BCECF efflux was slowed down more than five-fold. This suggests that FTUG is excreted by a separate efflux system, or, alternatively, that the apparent affinity constant for BCECF is increased in the Bef\(^{-}\) mutant. Excretion of BCECF occurred at a higher rate than FTUG in *L. lactis*, whereas the opposite was observed in *L. plantarum* (Fig. 5 and 6).
Chemical nature of intracellular FTUG and BCECF. To establish whether or not the fluorescent probes are modified, the chromatographic behaviour of excreted products was compared with standard solutions of BCECF and FTUG. These experiments showed that the excreted product from BCECF loaded *L. lactis* (dotted line) eluted at similar retention times as authentic BCECF (straight line). BCECF from cytoplasmic extracts of *L. lactis* (dashed line) was also found to be identical to the original probe (Fig. 2C). About 70% of the internal FTUG, however, eluted at different retention times than the original probe, mostly at 17.5 (13%; peak I), 23.5 (20%; peak III), and 34.0 min (28%; peak IV). The product eluting at 34 min (peak IV) is a contamination that is also present in FITC and FTUG standard solutions. One fluorescent FTUG-derivative, that was present in a low concentration in cellular extracts (±3% of the total area; peak II), was preferentially excreted by *L. lactis* (Fig. 2B).

**TABLE I. The intracellular pH of *L. lactis* and *L. plantarum* determined by the distribution of 14C-benzoic acid and the fluorescence of BCECF and FTUG.**

<table>
<thead>
<tr>
<th></th>
<th>External pH</th>
<th>Internal pH</th>
<th>14C-benzoic acid distribution</th>
<th>FTUG fluorescence</th>
<th>BCECF fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L. lactis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>7.02</td>
<td>7.00</td>
<td>7.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>7.11</td>
<td>7.10</td>
<td>7.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>7.36</td>
<td>7.53</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>7.68</td>
<td>7.81</td>
<td>7.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>L. plantarum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
<td>6.40</td>
<td>n. d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>-</td>
<td>7.05</td>
<td>7.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>-</td>
<td>7.33</td>
<td>7.35</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The internal pH measurements were performed in CKC buffer of the desired pH at a final protein concentration of 0.78 mg/ml (distribution of benzoic acid) and 8 µg/ml (fluorescence measurements).
Mass spectrometry analysis confirmed that excreted BCECF corresponds to a compound with a molecular weight of 520 g/mol, which is identical to the calculated $M_w$ of BCECF. The commercially available BCECF, however, consists of two isomers that were separated by HPLC (Fig. 2C). The peaks could not be assigned to either $2',7'$-bis-(2-carboxyethyl)-5-carboxyfluorescein or $2',7'$-bis-(2-carboxyethyl)-6-carboxyfluorescein, because it is not possible to obtain optically pure BCECF. The ratio between the two isomers with retention times of 17.2 and 11.5 min, respectively, decreased from 0.85 for free BCECF to 0.28 in cytoplasmic extracts of *L. lactis*, and remained the same after extrusion from the cytoplasm into the surrounding medium (Fig. 2C). This indicates that the cells are more efficiently loaded with one of the isomers of BCECF, but that the efflux system does not discriminate between the two isomers.

**Inhibitor studies.** Indomethacin, which is known to abolish BCECF efflux in human epithelial cells (1, 9), was used to characterize the efflux of BCECF and FTUG-derivative further. Addition of 1 mM indomethacin completely inhibited BCECF excretion in *L. lactis* ML3, whereas only a 50% reduction of “FTUG” efflux was observed under these conditions (Fig. 7). Indomethacin decreased the $pH_{in}$ of glycolizing cells by 0.25 pH unit (not shown), but this effect is unlikely to be the cause for the inhibition of probe efflux as BCECF excretion is slightly lowered and “FTUG” excretion is not affected by changes in $pH_{in}$ (Fig. 5 and 6).

**DISCUSSION**

In this paper, the synthesis and characterization of N-(fluorescein thio-ureanyl)-glutamate (FTUG), a new pH sensitive fluorescent indicator with a $pK_{a(app)}$ of 6.8, is described. The probe is readily synthesized from fluoresceine isothiocyanate plus glutamate, and its retention by *L. plantarum* and *L. lactis* is comparable to that of BCECF. After correction for probe efflux, the fluorescence signal can be transformed to cytoplasmic pH values via a calibration curve as was previously described for BCECF (18). In *L. lactis*, the $pH_{in}$ values estimated from the distribution of $^{14}$C-benzoic acid and the fluorescence of both BCECF and FTUG were similar. Such a good correlation between $^{14}$C-benzoic acid distribution and BCECF fluorescence data was not concluded from previous experiments with *L. lactis*; the pH values obtained from BCECF fluorescence were 0.3 pH unit higher than those obtained from the $^{14}$C-benzoic acid distribution (18). It seems likely that
these differences are due to variations in the physiological state of the cells, as these comparisons were made between cells that were not cultivated in an identical manner. In our experiments the internal pH values derived from the fluorescence and benzoic acid data were obtained with the same batches of cells. The fluorescence changes of BCECF and FTUG were tested for potential artifacts, such as sensitivity to cations and variations in different batches of probes that could affect the pH\textsubscript{in} determinations. A lowering of the pK\textsubscript{a}(app) from 7.5 to 6.7 in the presence of 1 M KCl was observed for BCECF. The effects of K\textsuperscript{+} on the pK\textsubscript{a}(app) of FTUG were much less: the pK\textsubscript{a}(app) decreased from 6.9 to 6.7 when KCl was added at 1 M. This makes FTUG a more reliable probe for the estimation of cytoplasmic pH values, as changes in potassium concentration are frequently associated with changes in pH\textsubscript{in} (5). Moreover, the pKa(app) of BCECF varied considerably between different batches of probe (from 7.3 to 7.7), whereas the pKa(app) of various batches of FTUG was quite constant (6.9 - 7.0). In parallel with the difference in pKa(app), the ratio between the two isomers of BCECF also varied between different batches of BCECF [the primary eluting isomer (fig. 2C) constituted between 40 and 55% of the total BCECF]. Both observations were confirmed by Molecular Probes Inc. The manufacturer, however, was not able to provide information about the pKa(app) of the two different isomers. It seems likely that the variation in pKa(app) between separate batches of BCECF is caused by a different pKa(app) of the two isomers.

Although both FTUG and BCECF are excreted by \textit{L. lactis}, \textit{L. plantarum}, and other bacteria (unpublished results), this efflux does not seriously hamper the use of the probes as indicators of pH\textsubscript{in}. In \textit{L. lactis} the efflux rate of BCECF was somewhat lowered upon the addition of valinomycin plus nigericin. This is most likely due to a decrease in the activity of the extrusion system at the lower pH\textsubscript{in} values. The efflux of FTUG was not affected by valinomycin plus nigericin, which is consistent with a separate efflux system for this probe (see below). In \textit{L. plantarum}, efflux of BCECF and FTUG is initially slow which corresponds with a slow start of glycolysis, and consequently a lag in the production of ATP. Since efflux of BCECF and FTUG is independent of a proton motive force in both \textit{L. lactis} and \textit{L. plantarum} but dependent on metabolic energy, we speculate that the extrusion of both compounds is driven by ATP directly.

To investigate whether or not BCECF and FTUG are modified prior to excretion from the cells, HPLC chromatograms of free BCECF (or FTUG) in solution and excreted products were compared. Excreted BCECF as well as BCECF in cytoplasmic extracts of \textit{L. lactis} eluted at similar times as genuine BCECF in
solution. However, the retention time of 70% of the FTUG in cytoplasmic extracts from dye-loaded *L. lactis* did not correlate with that of a standard solution, and one of these products was preferentially excreted. As already suggested by the different sensitivity towards a lowering of pH in (Fig. 6), this could indicate that the mechanisms of BCECF and FTUG efflux are different. Further support for this notion came from studies in the BCECF efflux mutant of *L. lactis* ML3 (Bef⁻) (19), in which, in contrast to excretion of BCECF, the efflux of the FTUG-conjugate was identical to the parent ML3 strain. In mammalian cells excretion of various organic anions occurs via the Multispecific Organic Anion Transporter (MOAT), also termed multidrug resistance-associated protein (MRP) (21, 23, 24, 35). The MOAT protein resembles P-glycoprotein in its primary sequence as well as function; both proteins are ATP dependent export systems which are regulated by phosphokinase c and pump a wide variety of organic compounds out of the cell against high concentration gradients (23, 29, 31). However, P-glycoprotein excretes cationic and neutral substrates, whereas MOAT is specific for anions (cysteiny1 leukotrienes, glutathione S-conjugates, and other amphiphilic anions). Typical substrates/inhibitors of P-glycoprotein [e. g. daunorubicin, cyclosporin A (analogs) and verapamil] do not affect the export of organic anions via cMOAT (14). The observation that FTUG is excreted in a modified form is reminiscent of the transport of (mostly) glutathione conjugates by MOAT in mammalian cells. Unfortunately, it was not possible to determine the molecular masses of the FTUG-derivates by mass spectrometry or to isolate these compounds for structure analysis. *In vitro* assays to conjugate FTUG to components of the cellular extract or glutathione failed thusfar, most likely due to the absence of essential cofactor(s). The identification of FTUG-derivates will be the topic of future research.

In human epithelial cell lines the pharmacological profile of the inhibition of BCECF efflux was found to be unique, i.e., not resembling the inhibitors for P-glycoprotein or MOAT mediated efflux (1, 9). A potent and specific inhibitor of BCECF efflux is indomethacin. When indomethacin was added to *L. lactis* cells excreting BCECF or FTUG, the efflux of FTUG was somewhat lower, but BCECF efflux was almost completely inhibited. Although the affinity constants for BCECF and “FTUG” excretion and indomethacin inhibition have not been determined, also this result is consistent with the presence of two distinct extrusion systems for organic anions in *L. lactis*. The efflux of BCECF may occur via a similar system as present in human epithelial cells, whereas FTUG-conjugate efflux might be related to the MOAT (or MRP) efflux system that is observed in various mammalian cells.

In conclusion the characterization of the bacterial retention of a new fluorescent pH
Use and efflux of pH sensitive fluorescent dyes

indicator has led to the identification of an efflux activity that may represent part of a hitherto not foreseen drug detoxification mechanism in bacteria. The isolation of a FTUG efflux mutant and determination of the nature of the excreted product should form important steps forwards in the further unraveling of this excretion mechanism.

ACKNOWLEDGEMENTS

This research was funded by Unilever Research Laboratories, Vlaardingen, The Netherlands. The authors would like to thank Drs J. J. P. M. Smelt and P. F. ter Steeg for stimulating discussions, A. Bos and J. Kei for assistance with the flow cytometry measurements, TNO Rijswijk for providing the flow cytometer facilities, M. Jansen for assistance with the HPLC, and Dr. A. P. Bruins and C. M. Jeronimus-Stratingh for performing the mass spectrometry analysis.

REFERENCES

Chapter 7

Use and efflux of pH sensitive fluorescent dyes


