Use of fluorescence ratio imaging for intracellular pH determination of individual bacterial cells in mixed cultures

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The development of a rapid method for measuring intracellular pH (pHi) in single bacterial cells is described. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Listeria innocua* were used as test organisms. The method is based upon fluorescence microscopy and ratio imaging of cells stained with carboxyfluorescein succinimidyl ester. After staining, the bacteria were immobilized on a membrane filter and transferred to a closed perfusion chamber, allowing control of the cell environment during analysis. The set-up was optimized with regard to the use of neutral-density filters and background subtraction, for determining the excitation ratio 490 nm/435 nm (R_{490/435}) independent of the excitation light intensity, and to reduce photobleaching. This allowed for time-lapse studies with multiple exposures. To study the pHi of *Lb. delbrueckii* subsp. *bulgaricus* and *L. innocua* in response to different extracellular pH (pHex) values, an in vivo calibration curve was constructed in the pH range 5.0-8.5. Distinct differences between the two cultures were observed. *L. innocua* maintained a near-neutral pHi almost independently of pHex (5.0-8.0), whereas the pHi of *Lb. delbrueckii* subsp. *bulgaricus* decreased with decreasing pHex. In pure and mixed cultures at pHex 5.0, the pHi values of *Lb. delbrueckii* subsp. *bulgaricus* and *L. innocua* were 6.1±0.2 and 7.5±0.2, respectively. This difference in pHi may explain how *Lb. delbrueckii* subsp. *bulgaricus* obtains a competitive advantage over *L. innocua* at low pHex.

Keywords: intracellular pH, ratio imaging, single cells, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Listeria innocua*

INTRODUCTION

Most bacteria maintain an intracellular pH (pHi) close to neutral within fairly narrow limits (Padan et al., 1981), because this enables metabolic reactions to occur even under unfavourable extracellular pH (pHex) conditions. Bacteria can be divided into three groups with regard to pH homeostasis: neutrophiles, acidophiles and alkalophiles. These groups differ in requirement for pHex, but all groups maintain a pHi between 6.5 and 9.5 (White, 1995). Acidophiles maintain a large gradient between pHi and pHex (ΔpH), but are restricted to growth in very acidic environments due to an inverted membrane potential (White, 1995). In contrast, a large group of acid-tolerant fermentative bacteria grows at pH values ranging from neutral to pH 3.5 (Kashket, 1987; McDonald et al., 1990; Russell, 1991a). This group consists of certain ruminal bacteria (Russell, 1991b) and various species of lactic acid bacteria (Kashket, 1987). A common feature is the ability to decrease their pHi with pHex during growth (Russell & Hino, 1985; Nannen & Hutkins, 1991a; Cook & Russell, 1994), and therefore this group of bacteria does not comply with the conventional classification of pH homeostasis. The pronounced organic acid production of these bacteria creates an environment unfavourable for most other organisms (Russell, 1992), which is the basis of many methods of food preservation by fermentation.

Food fermentations are often carried out by a concerted or sequential microbiota as the substrate changes. This
is the case in a two-culture system like yoghurt (Auclair & Accolas, 1983) or more complex systems like cheese, where several micro-organisms interact (Hansen & Jakobsen, 1997). In fermented vegetables, growth of Lactobacillus plantarum follows growth of Leuconostoc mesenteroides. The microbial succession can be explained by differing levels of acid tolerance and regulation of pH, for the two organisms (McDonald et al., 1990).

Similar differences could be important for microbial interactions between lactic acid bacteria and food-borne pathogens such as Listeria monocytogenes, a Gram-positive organism physiologically and phylogenetically related to lactic acid bacteria (Holzapfel et al., 1990). The pathogenic Escherichia coli O157:H7 has been shown to decrease its pH, and thereby resist high levels of acetic acid (Diez-Gonzales et al., 1995). The microbial succession can be explained by the high acid tolerance of this serotype compared to laboratory strains like E. coli K-12.

In the present work we used fluorescence microscopy and ratio imaging to measure the pH, of Lactobacillus delbrueckii subsp. bulgaricus and Listeria innocua. Lb. delbrueckii subsp. bulgaricus is known to decrease pH, in response to pHox (Kashket, 1987), while L. innocua maintains a near-neutral pH, at acidic pHox (Breeuwer et al., 1996). For the first time we report a rapid method for time-lapse studies of pH, in immobilized single cells of pure and mixed cultures of bacteria.

**METHODS**

**Chemicals.** All chemicals were analytical grade from Merck, unless otherwise stated.

**Bacterial strains and growth conditions.** Lactobacillus delbrueckii subsp. bulgaricus (NCFP 2772, kindly provided by Dr Gert Grobben, Groningen University, The Netherlands) was grown overnight at 42 °C in MRS broth (Difco). Listeria innocua [AJ 1-3, provided by the Alfred Jorgensen Laboratory, Copenhagen, Denmark] was grown overnight at 30 °C in BH1 broth (Difco).

**Buffer solutions and staining solutions.** Potassium phosphate buffers, adjusted to a given pH by mixing 50 mM solutions of KH₂PO₄ or KH₂HPO₄, were used in the pH range 6.0–8.5. Below pH 6.0, buffers consisting of a mixture of citric acid (25 mM) and KH₂HPO₄ (50 mM) were used. When cells were energized, a 1 M glucose stock solution was added to the respective buffer to a final concentration of 10 mM. A 5 μM solution of S(6)-carboxyfluorescein (Sigma) was prepared from a concentrated stock solution (10 mM in DMSO) by dilution in potassium phosphate buffer pH 8.0.

**Fluorescent staining of cells.** The following method was modified from Breeuwer et al. (1996). Cells were harvested by centrifugation (10000 g, 2 min) and resuspended in 50 mM potassium phosphate buffer, pH 7.0, to an OD₆50 of 0.6. They were then incubated in the presence of 10 μM S(6)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes) at 37 °C for 15 min (Lb. delbrueckii subsp. bulgaricus) or 30 min (L. innocua and mixed-culture experiments). Afterwards, cells were washed by centrifugation (10000 g, 2 min) and resuspended in equivalent volumes of the buffer indicated in each experiment. Cells not analysed immediately were stored on ice in the dark for a maximum of 1 h.

**Equilibration of pH.** Calibration points for Lb. delbrueckii subsp. bulgaricus and L. innocua were determined in buffers ranging from pH 5.0 to 8.5. After staining and resuspension of the cultures, the pH, and pHox, were equilibrated by addition of valinomycin (Sigma) and nigericin (Molecular Probes) to a final concentration of 1 μM each, followed by a 10 min incubation at 37 °C.

**Immobilization of cells for microscopical analysis.** Stained cells were diluted 100-fold in the appropriate buffer, and 200 μl aliquots were drawn through a 45 μm membrane filter (Schleicher & Schuell, ME 25/31). The portion of the membrane containing cells was excised (diameter 0.6 cm) and mounted in a perfusion chamber system with a nominal volume of 250 μl, previously described by Guldfeldt & Arneborg (1998). After addition of 250 μl of the appropriate buffer, a small piece of large-pored foam rubber (~0.5 × 0.5 × 0.5 cm) was inserted between the pressure cap and the backside of the filter to prevent movement of the membrane filter inside the chamber.

**Fluorescence microscopy.** The set-up was the same as described by Guldfeldt & Arneborg (1998), and consisted of a monochromator (Monochromator B, TILL Photonics) with a 75 W xenon lamp to provide the two excitation wavelengths (490 nm and 435 nm). The inverted epifluorescence microscope (Zeiss Axiovert 135 TV) was equipped with a Zeiss Fluor 10× objective (numerical aperture 1.3), a dichroic mirror (510 nm) and an emission bandpass filter (515–565 nm). Fluorescence emission was recorded on a cooled CCD camera (EEV 512 × 1024, 12 bit frame transfer camera, Princeton Instruments).

Focusing was done with epifluorescent excitation (450 nm), in order to reduce photobleaching, as ordinary observation by transmitted light microscopy was not possible because of the experimental set-up.

**Data analysis.** Images were stored on a Pentium PC using Metafluor 3.0 (Universal Imaging). Data analysis was carried out on the saved experiment, and to analyse single cells, regions were drawn along the perimeter of the cell. The region data were directly logged into a spreadsheet. Cells were randomly selected on the 435 nm image (in order to avoid selection biased by a high intensity on the 490 nm image, which is pH dependent). Twenty cells were analysed in each experiment, unless stated otherwise. The R₄⁹⁰₄₃⁵ calculation was performed by dividing the intensity of individual pixels on the 490 nm image by the intensity of the corresponding pixels on the 435 nm image.

A calibration curve was constructed by plotting R₄⁹⁰₄₃⁵ versus pH of equilibrated cells in the range pH 5.0–8.5, using linear interpolation between points. In order to convert the standard deviations from R₄⁹⁰₄₃⁵ to pH, the minimal and maximal R₄⁹⁰₄₃⁵ were calculated (mean ± standard deviation) and converted to minimal and maximal pH, Due to linear interpolation between the calibration points, the minimal or maximal pH, values were, in a few cases, calculated from a slightly different equation (max. error ≤0.05 pH units). In all cases, the largest value was chosen to represent a given pH.

The ratio images were generated using the Intensity Modulated Display (IMD) mode of Metafluor. The settings for IMD were 16 ratios with 16 intensities. The range of colours in the image spans the R₄⁹⁰₄₃⁵ values of the apparent minimum and maximum pH, in the respective image. The pH scale bar was generated in Metafluor and representative pH values were
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added next to the colour of the corresponding $R_{490/435}$. A thorough description of the IMD mode used to generate these images is given by Tsien & Harootunian (1990). In short, this display mode takes the intensity of individual pixels into account when creating the ratio image, thereby creating a uniform black background.

RESULTS

Definition of experimental parameters for fluorescence ratio imaging

A solution of 5 μM carboxyfluorescein in phosphate buffer pH 8.0 was used to examine the response of the system. Fig. 1 shows the fluorescence intensities at 490 nm (pH-dependent) and 435 nm (pH-independent) and the corresponding ratio ($R_{490/435}$), at various intensities of excitation light controlled by a range of neutral-density filters. The pH and concentration of carboxyfluorescein were selected to obtain a strong signal and to avoid quenching (Guldfeldt & Arneborg, 1998). It can be seen that the emission intensities at both wavelengths as well as $R_{490/435}$ change proportionally to the altered light intensity. As the emission returns to the original level when the light intensity is increased again, photobleaching is negligible in this experiment.

The observation that $R_{490/435}$ changes with excitation light intensity was also made with immobilized cells of Lb. delbrueckii subsp. bulgaricus, stained with carboxyfluorescein succinimidyl ester. However, subtraction of the background intensity (representative region with no cells) from both images, i.e. at 490 nm and 435 nm, prior to the calculation rendered $R_{490/435}$ practically independent of the intensity of excitation light (Fig. 2).

To minimize photobleaching of the stained cells, a neutral-density filter with 2.5 % throughput was chosen to reduce the excitation light intensity. This also reduced the emission and it was therefore necessary to increase the acquisition time to 3 s for both wavelengths to obtain a sufficient signal.

After determination of the optimal experimental parameters, it was possible to perform long-term experiments with multiple exposures and a constant $R_{490/435}$. Fig. 3 shows the result from following 10 randomly selected cells of Lb. delbrueckii subsp. bulgaricus over a period of 12 min. Even though the intensities decrease, $R_{490/435}$ is not significantly affected. The small variations in $R_{490/435}$ appear to be caused by focus drift, as refocusing (indicated by arrows) restores the ratio.
decreased slowly but steadily throughout the experiment, whereas \( R_{490/435} \) remained almost constant. The slight variations in \( R_{490/435} \) are primarily due to focus drift, as refocusing restored \( R_{490/435} \) to its original value, indicated by the arrows in Fig. 3. The emission intensities did not increase when the sample was refocused, which might be due to a slight bleaching during focusing.

**Calibration curve for measuring pH in single cells**

The calibration curve of valinomycin- and nigericin-treated cells of *Lb. delbrueckii* subsp. *bulgaricus* suspended in buffers of different pH is shown in Fig. 4. The relationship between \( R_{490/435} \) and \( pH_i \) is non-linear and calculation of \( pH_i \) values from the calibration curve was performed by linear interpolation. The sensitivity of the probe is greatest at pH 5.5–8.0. The slope levels off in the low-pH range, and no determinations below pH 4.5 were attempted. The calibration points of *Lb. delbrueckii* subsp. *bulgaricus* (Fig. 4), which was used for both species.

The accuracy of this method is described by the observed standard deviations, corresponding to errors smaller than 0.2 pH unit in the \( pH_i \) range from 5.5 to 8.5. This also includes the relatively large standard deviation of *L. innocua* at pH 8, which results in an error of 0.15 pH units. The calibration points were reproducible in repeated determinations (results not shown).

**Effect of \( pH_{ex} \) on \( pH_i \) of *Lb. delbrueckii* subsp. *bulgaricus* and *L. innocua***

The difference in pH regulation between *Lb. delbrueckii* subsp. *bulgaricus* and *L. innocua* in the \( pH_{ex} \) range 5–8 is illustrated in Fig. 5. The cultures were examined in the presence of glucose (10 mM), and the values therefore represent the \( pH_i \) regulation of metabolically active cells. Each point indicates the mean and standard deviation of \( pH_i \) of 20 individual cells calculated from the respective \( R_{490/435} \) values. The \( \Delta pH \) in *Lb. delbrueckii* subsp. *bulgaricus* is suspended at pH 8, where the \( pH_i \) is actually lower than the \( pH_{ex} \), and \( \Delta pH \) increases steadily with decreasing \( pH_{ex} \) until a \( pH_{ex} \) of 6. The decrease in \( pH_i \) from \( pH_{ex} \) 6 to \( pH_{ex} \) 5 is more pronounced, and the \( \Delta pH \) is approximately 1 pH unit. In *L. innocua*, the decrease in \( pH_i \) is linear but minor, and the \( \Delta pH \) increases at low \( pH_{ex} \). The \( pH_i \) in single cells can also be presented by ratio images with a colour-coded pH scale, as can be seen in Fig. 6(a, b). The non-linear pH scale is numerically equivalent to Fig. 4, and the \( pH_i \) is described by the colour hues.

The mean \( pH_i \) values of a population of *Lb. delbrueckii* subsp. *bulgaricus* and *L. innocua*, at a \( pH_{ex} \) of 7.0, are 7.7 and 8.0, respectively. The images in Fig. 6(a, b) are from a different experiment than the results presented in Fig. 5, but the results are identical, confirming the reproducibility of the method applied.

**Determining pH in a mixed culture**

Mixing *Lb. delbrueckii* subsp. *bulgaricus* and *L. innocua* prior to staining allowed us to determine \( pH_i \) in single cells within a mixed culture. The two species still regulated \( pH_i \) differently at both high and low \( pH_{ex} \) (Fig. 6c and d, respectively). *L. innocua* maintains a considerably higher \( pH_i \) than *Lb. delbrueckii* subsp. *bulgaricus* regardless of \( pH_{ex} \). Furthermore, the \( pH_i \) of the species in a mixture at \( pH_{ex} \) 7 (Fig. 6c) corresponds well to the experiments performed on the pure cultures at the same \( pH_{ex} \) (Fig. 6a, b).
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**Fig. 6.** Ratio images of *Lb. delbrueckii* subsp. *bulgaricus* and *L. innocua* in the presence of 10 mM glucose. (a, b) Pure cultures of *Lb. delbrueckii* subsp. *bulgaricus* (a) and *L. innocua* (b) at pH₇, 7. (c, d) A mixed culture of *Lb. delbrueckii* subsp. *bulgaricus* (large rods in pairs and chains) and *L. innocua* (small rods) at pH₇, 7 (c), and after further incubation with 10 mM glucose for 15 min at pH₅, 5 (d). Bar, 10 μm. A colour-coded pH scale is shown in each panel.

**DISCUSSION**

In contrast to previous methods based upon ion distribution of radiolabelled cells (Rottenberg, 1979), ³¹P-NMR (Foucaud et al., 1995) or spectrofluorometry (Molenaar et al., 1991; Breeuwer et al., 1996; Aono et al., 1997), our experimental approach allows a rapid pH measurement of single bacterial cells in time-lapse studies.

Problems inherent to fluorescence microscopy of low-light-level objects have been overcome, and the fluorescent probe gives a good estimation of pH between 5.5 and 8.5. The useful range of fluorescent pH indicators is mostly determined by their pKₐ, and probes with lower pKₐ are commercially available (see Molecular Probes at http://www.probes.com). However, these probes generally suffer from lack of sensitivity in the near-neutral pH range, and therefore it is important to establish the pH range of a study prior to selection of probes. The presence of a succinimidyl ester group reduces the efflux significantly (Breeuwer et al., 1996), and continuous background subtraction eliminates errors from potential extracellular carboxyfluorescein.

In a study by Cimprich et al. (1995) on yeast cells, it was stated that the emission from stained cells was sufficient to neglect background subtraction. The smaller size of the bacteria in our study, and the necessity of reducing...
the excitation intensity in order to minimize photobleaching, reduces the overall fluorescence intensity of the cell. In addition, the CCD camera produces a small overall background that is not linked to the fluorescent probe. As the fluorescence intensity of cells decreases, the relative contribution from this overall background increases, rendering background subtraction necessary, as performed in the present work.

The design of the closed perfusion chamber has the potential for dynamic measurements of pH in a changing environment. Due to the presence of liquid in the chamber, the micro-organisms are not likely to suffer from nutrient exhaustion as in experiments with cells localized between slide and coverslip.

It is accepted that Lb. delbreuckii subsp. bulgaricus does not maintain a pH close to neutrality when pHex decreases (Kashket, 1987), but the pH is clearly regulated as a function of pHex. From pHex 8 to 6, there is an increase in ΔpH in Lb. delbreuckii subsp. bulgaricus to approximately 1 unit. This gradient is kept constant when pHex is reduced to 5, which is in agreement with earlier reports stating that acid-tolerant bacteria, such as lactobacilli, maintain a constant ΔpH of approximately 1 at low pHex (Kashket, 1987; Russell, 1992; Hutkins & Nannen, 1993). It has been suggested that pH decreases only upon energy limitation (Poolman et al., 1987), but our results on Lb. delbreuckii subsp. bulgaricus as well as earlier reports on Streptococcus bovis and Lactococcus lactis (Cook & Russell, 1994) demonstrate that pH decreases as a function of pHex in the presence of glucose. As bacteria maintaining a constant ΔpH rather than a constant pH are not pH-homeostatic in the classical sense (Padan et al., 1981), these bacteria might be recognized as a distinct class.

H⁺-ATPase has been shown to be the key enzyme in regulating pH in Enterococcus faecalis (Kobayashi et al., 1986), and it is also assumed to regulate pH in lactic acid bacteria (Nannen & Hutkins, 1991b). In Lb. delbreuckii subsp. bulgaricus, the onset of the constant ΔpH at pHex 6 coincides with the optimal pHex for growth of 5.8 (Beal et al., 1989), indicating a tighter control of pH at acidic pHex. This is further supported by the significantly lower pH optimum for H⁺-ATPase (5.6) of an acid-tolerant bacterium, Lactobacillus casei (Bender & Marquis, 1987), compared to the less acid-tolerant Streptococcus thermophilus and Lactococcus lactis with a pH optimum of 7.5 (Nannen & Hutkins, 1991b). The presence of short fatty acid anions such as lactate and acetate is also involved in the regulation of pH in acid-tolerant bacteria (Russell, 1991a; Cook & Russell, 1994); however, this factor has not been investigated in the present work.

The much smaller size of L. innocua is a challenge when measuring pH in single cells, and the lower fluorescence intensity is probably the reason for the somewhat larger standard deviations observed for this bacterium, even when pH and pHex are equilibrated (Fig. 4). L. innocua has previously been investigated with regard to pH by spectrofluorometry (Breeuwer et al., 1996), and the pH results on single cells of L. innocua obtained in this study are in agreement with the results obtained on a population of the same bacteria.

The accuracy of the method (to within 0.2 pH units) is determined by the standard deviations in the calibration curve (Fig. 4). A change in focus alters R490/435 slightly (Fig. 3), and some of the equilibrated cells used for calibration may have been slightly out of focus, thus causing the deviations in R490/435. However, visual examination of the images does not reveal the examined cells to be obviously out of focus (results not shown).

To our knowledge, there are no other published reports describing the pH variation within a bacterial population. The populations investigated in the present study revealed pH standard deviations of 0.1–0.3 units (Fig. 5); this is only slightly higher than accounted for by the calibration accuracy (Fig. 4). Results published on the yeast Saccharomyces cerevisiae have reported standard deviations in pH of 0.15–0.25 (Cimprich et al., 1995; Guldfeldt & Arneborg, 1998), but the influence of method artefacts (e.g. focus drift) on deviations in pH was not assessed.

As shown in the present study, we are now able to measure pH in individual bacterial cells in mixed cultures. In most natural habitats, bacteria are exposed to more stressful conditions than investigated here. For example, bacterial populations present in food (desired micro-organisms as well as natural contaminants) are generally heterogeneous, and differences in physiological state will probably be reflected in variations of pH and ΔpH. Work is in progress on studying pH of individual cells in natural environments. Additionally, we expect to be able to monitor pH in micro-organisms on solid substrates such as cheese or meat surfaces.

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