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 (pH,) 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 (R490/435) independent of the excitation light intensity, and to reduce photobleaching. This allowed for time-lapse studies with multiple exposures.

To study the pH, 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 pHex range 5-0-8-5. Distinct differences between the two cultures were observed. L. innocua maintained a near-neutral pH, almost independently of pHex (5-0-8-0), whereas the pH, of Lb. delbrueckii subsp. bulgaricus decreased with decreasing pHex. In pure and mixed cultures at pHex 5-0, the pH, values of Lb. delbrueckii subsp. bulgaricus and L. innocua were 6-1±0-2 and 7-5±0-2, respectively. This difference in pH, 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 (∆pHi), 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 pH, 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 & Wood, 1995). The pathogenic Escherichia coli O157:H7 has been shown to decrease its pH and thereby resist high levels of acetic acid (Díez-Gonzales & Russell, 1997), offering an explanation of 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 pH_{ex} (Kashket, 1987), while L. innocua maintains a near-neutral pH at acidic pH_{ex} (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 (NCIB 2772, kindly provided by Dr Gert Grobben, Groningen University, The Netherlands) was grown overnight at 42 °C in MRS broth (Difco). Listeria innocua [AJL 1-3, provided by the Alfred Jorgensen Laboratory, Copenhagen, Denmark] was grown overnight at 30 °C in BHI broth (Difco).

**Buffers and staining solutions.** Potassium phosphate buffers, adjusted to a given pH by mixing 50 mM solutions of KH_{2}PO_{4} or K_{2}HPO_{4}, 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 KH_{2}PO_{4} (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 5(6)-carboxyfluorescein (Sigma) was prepared from a concentrated stock solution (10 mM in DMSO) by dilution in potassium phosphate buffer pH 6.0.

**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 OD_{560} of 0.6. They were then incubated in the presence of 10 μM 5(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 L. 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 pH_{ex} 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 100x 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 Metaflour 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 R_{490:435} 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 R_{490:435} 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 R_{490:435} to pH, the minimal and maximal R_{490:435} 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 Metaflour. The settings for IMD were 16 ratios with 16 intensities. The range of colours in the image spans the R_{490:435} values of the apparent minimum and maximum pH, in the respective image. The pH scale bar was generated in Metaflour 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 with more than 20 subsequent exposures. It can be seen that emission intensities
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 $pH_i$ in single cells

The calibration curve of valinomycin- and nigericin-treated cells of *Lb. delbrueckii* subsp. *bulgaricus* suspended in buffers of different $pH_i$ 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-$pH_i$ range, and no determinations below $pH_i$ 5 are possible. The decrease in $pH_i$ is linear but minor, and the $ApH$ in $pH_i$ from $pH_{ex}$ 6 to $pH_{ex}$ 5 is more pronounced, and the $ApH$ 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 *Lb. 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 $pH_i$ in a mixed culture

Mixing *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. 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). *Lb. 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).

**Fig. 4.** Calibration curve of *Lb. delbrueckii* subsp. *bulgaricus* (■) and *L. innocua* (○): $R_{490/435}$ versus $pH_i$. The $pH_i$ was equilibrated to $pH_{ex}$ by incubation with 1 μM valinomycin and 1 μM nigericin. Each point represents the mean of 20 individual cells, with error bars indicating the standard deviation.

**Fig. 5.** $pH_i$ of pure cultures of *Lb. delbrueckii* subsp. *bulgaricus* (■) and *L. innocua* (○) in the presence of 10 mM glucose at different $pH_{ex}$. Each point represents the mean of 20 individual cells, with error bars indicating the standard deviation. The dashed line represents $pH_i = pH_{ex}$.
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 pH<sub>i</sub> 7. (c, d) A mixed culture of *Lb. delbrueckii* subsp. *bulgaricus* (large rods in pairs and chains) and *L. innocua* (small rods) at pH<sub>i</sub> 7 (c), and after further incubation with 10 mM glucose for 15 min at pH<sub>i</sub> 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; Breuwer et al., 1996; Aono et al., 1997), our experimental approach allows a rapid pH<sub>i</sub> 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<sub>i</sub> between 5.5 and 8.5. The useful range of fluorescent pH indicators is mostly determined by their pK<sub>a</sub>, and probes with lower pK<sub>a</sub> 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 (Breuwer 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\textsubscript{i} 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 \textit{Lb. delbrueckii} subsp. \textit{bulgaricus} does not maintain a pH\textsubscript{i} close to neutrality when pH\textsubscript{ex} decreases (Kashket, 1987), but the pH\textsubscript{i} is clearly regulated as a function of pH\textsubscript{ex}. From pH\textsubscript{ex} 8 to 6, there is an increase in \Delta pH in \textit{Lb. delbrueckii} subsp. \textit{bulgaricus} to approximately 1 unit. This gradient is kept constant when pH\textsubscript{ex} is reduced to 5, which is in agreement with earlier reports stating that acid-tolerant bacteria, such as lactobacilli, maintain a constant \Delta pH of approximately 1 at low pH\textsubscript{ex} (Kashket, 1987; Russell, 1992; Hutkins & Nannen, 1993). It has been suggested that pH\textsubscript{i} decreases only upon energy limitation (Poolman et al., 1987), but our results on \textit{Lb. delbrueckii} subsp. \textit{bulgaricus} as well as earlier reports on \textit{Streptococcus bovis} and \textit{Lactococcus lactis} (Cook & Russell, 1994) demonstrate that pH\textsubscript{i} decreases as a function of pH\textsubscript{ex} in the presence of glucose. As bacteria maintaining a constant \Delta pH rather than a constant pH, are not pH-homeostatic in the classical sense (Fadan et al., 1981), these bacteria might be recognized as a distinct class.

H\textsuperscript{+}-ATPase has been shown to be the key enzyme in regulating pH\textsubscript{i} in \textit{Enterococcus faecalis} (Kobayashi et al., 1986), and it is also assumed to regulate pH\textsubscript{i} in lactic acid bacteria (Nannen & Hutkins, 1991b). In \textit{Lb. delbrueckii} subsp. \textit{bulgaricus}, the onset of the constant \Delta pH at pH\textsubscript{ex} 6 coincides with the optimal pH\textsubscript{ex} for growth of 5.8 (Beal et al., 1989), indicating a tighter control of pH\textsubscript{i} at acidic pH\textsubscript{ex}. This is further supported by the significantly lower pH optimum for H\textsuperscript{+}-ATPase (5.0) of an acid-tolerant bacterium, \textit{Lactobacillus casei} (Bender & Marquis, 1987), compared to the less acid-tolerant \textit{Streptococcus thermophilus} and \textit{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\textsubscript{i} 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 \textit{L. innocua} is a challenge when measuring pH\textsubscript{i} 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\textsubscript{i} and pH\textsubscript{ex} are equilibrated (Fig. 4). \textit{L. innocua} has previously been investigated with regard to pH\textsubscript{i} by spectrofluorometry (Breeuwer et al., 1996), and the pH\textsubscript{i} results on single cells of \textit{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 R\textsubscript{490/435} slightly (Fig. 3), and some of the equilibrated cells used for calibration may have been slightly out of focus, thus causing the deviations in R\textsubscript{490/435}. 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\textsubscript{i} variation within a bacterial population. The populations investigated in the present study revealed pH\textsubscript{i} 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 \textit{Saccharomyces cerevisiae} have reported standard deviations in pH\textsubscript{i} 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\textsubscript{i} was not assessed.

As shown in the present study, we are now able to measure pH\textsubscript{i} 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\textsubscript{i} and \Delta pH. Work is in progress on studying pH\textsubscript{i} of individual cells in natural environments. Additionally, we expect to be able to monitor pH\textsubscript{i} in micro-organisms on solid substrates such as cheese or meat surfaces.

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