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_i) 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 pH_i of *Lb. delbrueckii* subsp. *bulgaricus* and *L. innocua* in response to different extracellular pH (pH_{ex}) values, an *in vivo* calibration curve was constructed in the pH_i range 5.0–8.5. Distinct differences between the two cultures were observed. *L. innocua* maintained a near-neutral pH_i almost independently of pH_{ex} (5.0–8.0), whereas the pH_i of *Lb. delbrueckii* subsp. *bulgaricus* decreased with decreasing pH_{ex} . In pure and mixed cultures at pH_{ex} 5.0, the pH_i values of *Lb. delbrueckii* subsp. *bulgaricus* and *L. innocua* were 6.1 ± 0.2 and 7.5 ± 0.2 , respectively. This difference in pH_i may explain how *Lb. delbrueckii* subsp. *bulgaricus* obtains a competitive advantage over *L. innocua* at low pH_{ex} .

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

INTRODUCTION

Most bacteria maintain an intracellular pH (pH_i) close to neutral within fairly narrow limits (Padan *et al.*, 1981), because this enables metabolic reactions to occur even under unfavourable extracellular pH (pH_{ex}) conditions. Bacteria can be divided into three groups with regard to pH homeostasis: neutrophiles, acidophiles and alkalophiles. These groups differ in requirement for pH_{ex} , but all groups maintain a pH_i between 6.5 and 9.5 (White, 1995). Acidophiles maintain a large gradient between pH_i and pH_{ex} (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 pH_i with pH_{ex} 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

Abbreviations: CCD, charge-coupled device; pH_i , intracellular pH; pH_{ex} , extracellular pH; ΔpH , $pH_i - pH_{ex}$; $R_{490/435}$, excitation ratio 490 nm/435 nm.

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_i 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_i and thereby resist high levels of acetic acid (Diez-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_i of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Listeria innocua*. *Lb. delbrueckii* subsp. *bulgaricus* is known to decrease pH_i in response to pH_{ex} (Kashket, 1987), while *L. innocua* maintains a near-neutral pH_i at acidic pH_{ex} (Breeuwer *et al.*, 1996). For the first time we report a rapid method for time-lapse studies of pH_i 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* (NCFB 2772, kindly provided by Dr Gert Grobbee, Groningen University, The Netherlands) was grown overnight at 42 °C in MRS broth (Difco). *Listeria innocua* (AJL 1-3, provided by the Alfred Jørgensen 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_2PO_4 or K_2HPO_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 K_2HPO_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 μ M solution of 5(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 OD_{600} of 0.6. They were then incubated in the presence of 10 μ M 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_i . 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_i and pH_{ex} were equilibrated by addition of valinomycin (Sigma) and nigericin (Molecular Probes) to a final concentration of 1 μ M 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 μ l aliquots were drawn through a 45 μ m 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 μ l, previously described by Guldfieldt & Arneborg (1998). After addition of 250 μ l 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 Guldfieldt & 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 100× 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 $R_{490/435}$ 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_i , the minimal and maximal $R_{490/435}$ were calculated (mean ± standard deviation) and converted to minimal and maximal pH_i . Due to linear interpolation between the calibration points, the minimal or maximal pH_i 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 $R_{490/435}$ values of the apparent minimum and maximum pH_i in the respective image. The pH scale bar was generated in Metafluor and representative pH values were

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 μ M 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

Fig. 1. Influence of excitation light intensity on emission of a solution of carboxyfluorescein (5 μ M). Emission intensities at excitation wavelengths of 490 nm ($\square$) and 435 nm ($\triangle$), and $R_{490/435}$ ($\bullet$), were recorded at 15 s intervals, while different neutral-density filters (2.5%, 6% or 30% throughput) were inserted into the excitation light path.

Fig. 2. Influence of background subtraction on $R_{490/435}$ of *Lb. delbrueckii* subsp. *bulgaricus* stained with carboxyfluorescein succinimidyl ester, at different levels of excitation light intensity (100% and 2.5% throughput), before ($\bullet$) and after ($\circ$) background subtraction. Each point represents the mean of 20 individual cells, with error bars indicating the standard deviation.

Fig. 3. Emission intensities at 490 nm ($\square$) and 435 nm ($\triangle$), and $R_{490/435}$ ($\bullet$), for 10 randomly selected cells of *Lb. delbrueckii* subsp. *bulgaricus*, excited more than 20 times 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.

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

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 μ M valinomycin and 1 μ M nigericin. Each point represents the mean of 20 individual cells, with error bars indicating the standard deviation.

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 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 5.0 were attempted. The calibration points of *L. innocua* are almost superimposed on the calibration curve 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

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 $pH_i = pH_{ex}$.

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 ΔpH in *Lb. delbrueckii* subsp. *bulgaricus* is suspended at pH 8, where the pH_i is actually lower than the pH_{ex} , and ΔpH 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 ΔpH is approximately 1 pH unit. In *L. innocua*, the decrease in pH_i is linear but minor, and the ΔpH 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_i 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).

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_{ex} 7. (c, d) A mixed culture of *Lb. delbrueckii* subsp. *bulgaricus* (large rods in pairs and chains) and *L. innocua* (small rods) at pH_{ex} 7 (c), and after further incubation with 10 mM glucose for 15 min at pH_{ex} 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), ^{31}P -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_i 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_i between 5.5 and 8.5. The useful range of fluorescent pH indicators is mostly determined by their pK_a , and probes

with lower pK_a 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 photo-bleaching, 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_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 *Lb. delbrueckii* subsp. *bulgaricus* does not maintain a pH_i close to neutrality when pH_{ex} decreases (Kashket, 1987), but the pH_i is clearly regulated as a function of pH_{ex} . From pH_{ex} 8 to 6, there is an increase in ΔpH in *Lb. delbrueckii* subsp. *bulgaricus* to approximately 1 unit. This gradient is kept constant when pH_{ex} 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 pH_{ex} (Kashket, 1987; Russell, 1992; Hutkins & Nannen, 1993). It has been suggested that pH_i decreases only upon energy limitation (Poolman *et al.*, 1987), but our results on *Lb. delbrueckii* subsp. *bulgaricus* as well as earlier reports on *Streptococcus bovis* and *Lactococcus lactis* (Cook & Russell, 1994) demonstrate that pH_i decreases as a function of pH_{ex} in the presence of glucose. As bacteria maintaining a constant ΔpH rather than a constant pH_i 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_i in *Enterococcus faecalis* (Kobayashi *et al.*, 1986), and it is also assumed to regulate pH_i in lactic acid bacteria (Nannen & Hutkins, 1991b). In *Lb. delbrueckii* subsp. *bulgaricus*, the onset of the constant ΔpH at pH_{ex} 6 coincides with the optimal pH_{ex} for growth of 5.8 (Beal *et al.*, 1989), indicating a tighter control of pH_i at acidic pH_{ex} . This is further supported by the significantly lower pH optimum for H^+ -ATPase (5.0) 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_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 *L. innocua* is a challenge when measuring pH_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_i and pH_{ex} are equilibrated (Fig. 4). *L. innocua* has previously been investigated with regard to pH_i by spectrofluorometry (Breeuwer *et al.*, 1996), and the pH_i

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 $R_{490/435}$ slightly (Fig. 3), and some of the equilibrated cells used for calibration may have been slightly out of focus, thus causing the deviations in $R_{490/435}$. 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_i variation within a bacterial population. The populations investigated in the present study revealed pH_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 *Saccharomyces cerevisiae* have reported standard deviations in pH_i of 0.15–0.25 (Cimprich *et al.*, 1995; Guldeldt & Arneborg, 1998), but the influence of method artefacts (e.g. focus drift) on deviations in pH_i was not assessed.

As shown in the present study, we are now able to measure pH_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_i and ΔpH . Work is in progress on studying pH_i of individual cells in natural environments. Additionally, we expect to be able to monitor pH_i in micro-organisms on solid substrates such as cheese or meat surfaces.

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