Journal of Industrial Microbiology & Biotechnology (1998) 21, 65–74

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Acid-base response of bacterial suspensions

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The response of suspensions of non-proliferating bacteria to external pH ranging from 3.6 to 9.9 was examined. The acid-base response is affected by the number of viable cells in the suspensions and culture conditions (aerobiosis or anaerobiosis) but not by culture time, composition of the culture medium or the age of suspensions. The results with carbonylcyanide *m*-chlorophenylhydrazone-and *N*, *N*′- dicyclohexylcarbodiimide-treated cells indicated that passive proton conductance and proton translocation by ATP could be involved in this response. pH profiles of suspensions of 18 chemoheterotrophic Gram-negative and Gram-positive bacteria support the hypothesis that the proton gradient generated by an alkali or an acid shift could be dissipated by bacteria with respiratory metabolism.

Keywords: acid-base response; bacterial suspensions; chemoheterotrophic bacteria

Introduction

The mechanisms by which bacteria respond to external pH have not been well established. The requirement for a high internal pH for growth has been used for many years in the preservation of food from microbial spoilage. Acidic conditions are used to preserve cell suspensions from contamination by undesirable microorganisms [2]. The ability of bacteria to adapt to low pH has been stressed as a potential problem in the design of food preservation regimes [3,11,12].

Non-growing bacteria are important in biotechnology in general, including processes based on the fermentation of food products [18]. Non-proliferating cell suspensions are widely used for studying the synthesis and regulation of secondary metabolites. They are also used for studying the influence of environmental conditions upon uptake and accumulation of heavy metals by microorganisms. It has been reported that the pH of non-proliferating cell suspensions did not remain constant during experiments [14,16,29,31,32]. For example, Williams *et al* [32] and Solé *et al* [29] reported that the pH of suspensions of non-proliferating cells of *Serratia marcescens* rapidly reached pH values between 7.3 and 8.3, irrespective of the initial pH, but we have not found any direct studies on pH changes in cell suspensions.

The aim of this work was to study the response of bacterial suspensions to external pH ranging from 3.6 to 9.9, and to determine the factors that influence such a response. We compared the pH changes in non-proliferating cell suspensions of 18 chemoheterotrophic Gram-negative and Gram-positive bacteria which differ in metabolism, optimal pH for growth and buffering capacity. There are seven obligately aerobic bacteria that have strictly respiratory metabolism: *Aquaspirillum serpens* ATCC 11335, which is microaerophilic, *Pseudomonas aeruginosa* PAO 1, *Halobacterium halobium* CCM 2090 which is extremely halophilic, two acetic acid bacteria, *Acetobacter aceti* subsp *aceti* ATCC 15973 and *Gluconobacter oxydans* subsp *suboxydans* ATCC 621, *Alcaligenes faecalis* ATCC 19018 and *Bacillus alcalophilus* ATCC 27647, which is alkalophilic. Three bacteria have fermentative metabolism, *Zymomonas mobilis* subsp *mobilis* ATCC 29191, *Enterococcus faecalis* ATCC 19433 and a lactic acid bacterium *Lactobacillus acidophilus* ATCC 11506, which is acidophilic. The other bacteria studied are all facultatively anaerobic: *Escherichia coli* ATCC 10536, *Salmonella typhimurium* Mutton (ATCC 13311), *Serratia marcescens* ATCC 274, *Serratia marcescens* GP (a non-pigmented mutant of *S. marcescens* ATCC 274 [19], *Proteus mirabilis* NCTC 5887, *Aeromonas hydrophila* subsp *hydrophila* NCIB 9233, *Staphylococcus aureus* ATCC 9144 and *Bacillus subtilis* ATCC 6633.

Materials and methods

Bacterial strains and growth conditions

The organisms and growth conditions are listed in Table 1. Unless noted otherwise, all the bacterial strains were grown in aerobiosis. Microorganisms were maintained as agar slope cultures stored at 4°C with a weekly transfer to fresh medium.

The influence of the composition of the medium on the pH of suspensions was analysed in *S. marcescens* cells grown in four different liquid media: (i) PYG medium [21]; (ii) trypticase soy broth (TSB; BBL, Cockeyswille, MD, USA); (iii) nutrient broth (NB; Oxoid, Basingstoke, UK); and (iv) a mineral salts medium (GLN), containing (per liter distilled water): K_2HPO_4, 8 g; NH_4Cl, 1.28 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $Fe(NO_3)_3 \cdot 9H_2O$, 0.02 g; NaCl, 0.5 g; glycerol, 8 ml. The pH was adjusted to 7.2 using 0.1 M NaOH before the medium was autoclaved. The influence of cultural conditions (aerobiosis and anaerobiosis) on pH was determined in cells grown on PYG and GLN only.

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Received 2 June 1998; accepted 6 August 1998

Table 1 Bacterial species used and their growth conditions

Organism	Temp (°C)	Medium [Ref]
Aquaspirillum serpens ATCC 11335	27	MPSS [8]
Pseudomonas aeruginosa PAO 1	37	TSB
Halobacterium halobium CCM 2090	37	M_1 [10]
Acetobacter aceti ATCC 15073	27	MYP [23]
Gluconobacter oxydans ATCC 621	27	MYP [23]
Alcaligenes faecalis ATCC 19018	37	NB
Escherichia coli ATCC 10536	37	TSB
Salmonella typhimurium Mutton	37	TSB
Serratia marcescens ATCC 274	37	PYG [21]
Serratia marcescens GP	37	PYG [21]
Proteus mirabilis NCTC 5887	37	TSB
Aeromonas hydrophila NCIB 9233	27	TSB
Zymomonas mobilis ATCC 29191	27	SM [30]
Staphylococcus aureus ATCC 9144	37	TSB
Enterococcus faecalis ATCC 19433	37	TSB
Bacillus subtilis ATCC 6633	27	NB
Bacillus alcalophilus ATCC 27647	30	HA [5]
Lactobacillus acidophilus ATCC 11506	37	MRS [15]

TSB: Trypticase Soy Broth (BBL, Becton Dickinson and Co, Cockeysville, MD, USA); NB: Nutrient Broth (Oxoid, Unipath Ltd, Basingstoke, UK).

Bacteria were grown under anaerobic conditions in the GasPak anaerobic system (BBL, Becton Dickinson and Co).

Enumeration

Culturable cells (CFU) were counted in triplicate by standard plate count procedures on the media and at the temperature used for growth (Table 1). Bacterial suspensions were diluted in 0.85% NaCl (0.15 M) solution and CFU were counted after 48 h of incubation. *H. halobium* suspensions were diluted in 4 M NaCl and plates were incubated for 5 days.

Measurement of cell protein

Protein content was determined according to Lowry *et al* [13].

Chemicals and enzymes

Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and *N*, *N*′-dicyclohexyl carbodiimide (DCCD) were from Merck (Darmstadt, Germany). CCCP was used at a final concentration of 0.5 mM and DCCD at 5 mM. These agents were added to cell suspensions as small volumes of concentrated stocks in acetone; the final acetone concentration did not exceed 0.2%. Carbonic anhydrase was from Sigma Chemical Co (St Louis, MO, USA) and was prepared at 20 mg ml^{-1} in 0.15 M NaCl.

Preparation of non-proliferating cell suspensions (NPCS)

Whatever the culture conditions, cells were harvested at the end of the exponential phase of growth (20 h in aerobiosis, unless otherwise stated, and 4 days in anaerobiosis) and washed three times in 0.15 M NaCl. NPCS of *H. halobium* were prepared in 4 M NaCl and samples were kept in the dark for the time required for collection of data.

To test the effect of viability of cell suspensions on pH, *S. marcescens* suspensions were heated for 15 min in boiling water, cooled, and used for the experiments.

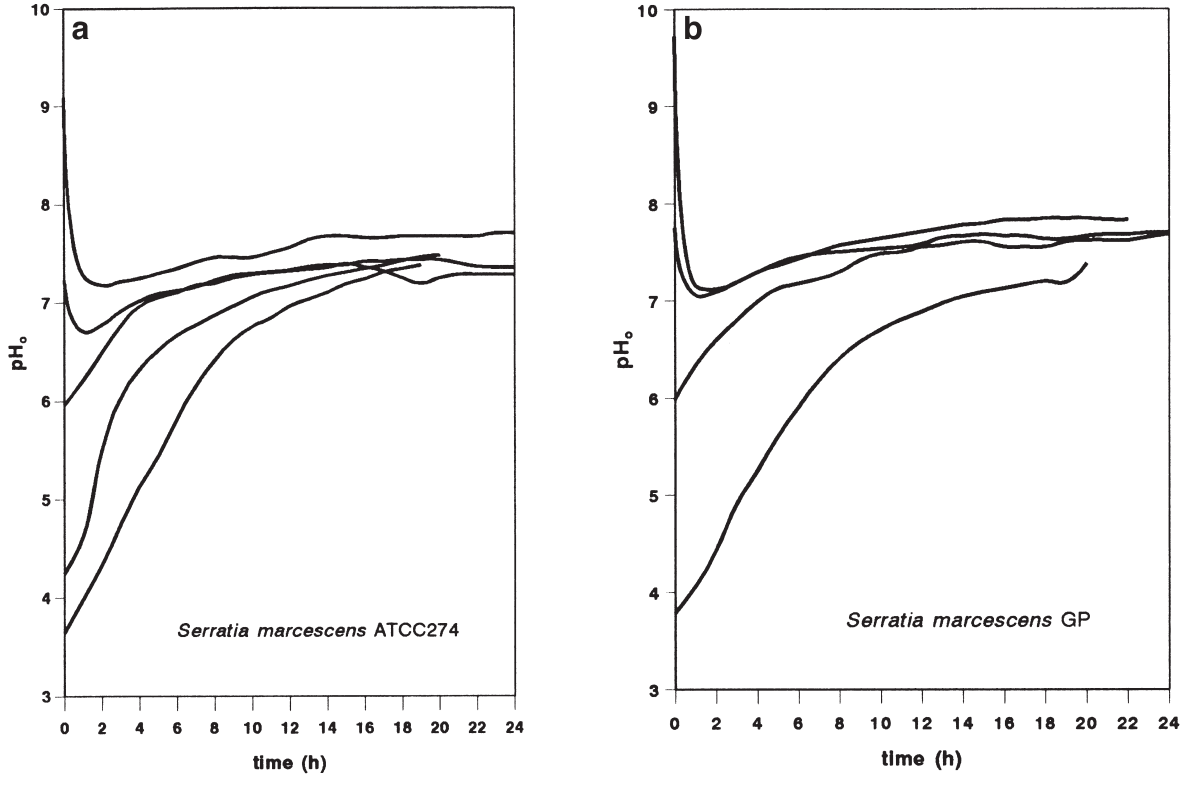

Figure 1 pH profiles of NPCS of (a) *S. marcescens* ATCC 274 and (b) *S. marcescens* GP.

To study the effect of carbonic anhydrase on the pH of cell suspensions, 2 ml of freshly prepared carbonic anhydrase solution was added inmediately before the assay.

The effect of CCCP and DCCD on the external pH of cell suspensions was determined in cell suspensions permeabilized with EDTA (applied as sodium salt). Cells washed three times in 0.15 M NaCl were centrifuged, resuspended in 20 ml of 0.15 M NaCl, and placed in a reciprocal shaker maintained at 37°C, and EDTA (3 mM final concentration, pH 6.2) was added. Five min later, the cells were diluted 10-fold with the same medium that was used to prepare them and centrifuged at room temperature. The pellet was resuspended in 0.15 M NaCl. CCCP or DCCD was added to bacterial suspensions inmediately after the desired initial pH was attained. Controls were prepared following the same procedure but omitting the CCCP and DCCD. There was no difference in pH profiles between the suspensions permeabilized with EDTA and those without EDTA treatment.

Measurement of extracellular pH profiles

External pH was measured with an Orion pH meter (SA 720), equipped with a combined glass electrode (ROSS 81–03 sc). An Omniscribe recorder, series D5000, was employed.

Experiments were conducted on 60-ml samples of cell suspensions in 100-ml glass bottles, which were magnetically stirred at room temperature. The pH of such suspensions was between 6.4–6.8. Changes in external pH were recorded for 20–24 h.

When assays were performed at an initial pH below or above 6.8, an initial pH adjustment was done before recording. In these cases, additions of aliquots of 1 M NaOH or 1 M HCl sterile solutions were made until the desired pH was attained. All experiments were performed two or three times, except for *S. marcescens* strains which were performed five to seven times, and the reported data are means of replicate samples.

Potassium, phosphate and OD at 260 nm and 280 nm of the supernatant phases

At desired intervals, 10 ml of bacterial suspensions were removed and centrifuged at 6000 × *g* for 10 min (Kontron Centrikon T-124 with an A-8.24 rotor), samples of supernatant were filtered twice through Sartorius 0.22-μm pore size filters. The residual solution was kept in sterile tubes at 4°C until use. The amount of potassium ions was determined by air-acetylene flame atomic absorption spectrophotometry (AAS), with a Varian model AA875 spectrophotometer, under the conditions recommended by the instrument manufacturer. The phosphate content of the supernatant phase was determined following the Phosphat Aquamerck 8046 Kit (Merck). OD at 260 nm and 280 nm were measured with a Kontron Uvikon 810 spectrophotometer.

Results and discussion

Figure 1 shows the evolution of the external pH of *S. marcescens* suspensions. The final pH of these suspensions was between 7.3 and 7.8. The pH of suspensions with an initial pH above 7.0 decreased during the first 2 h and then

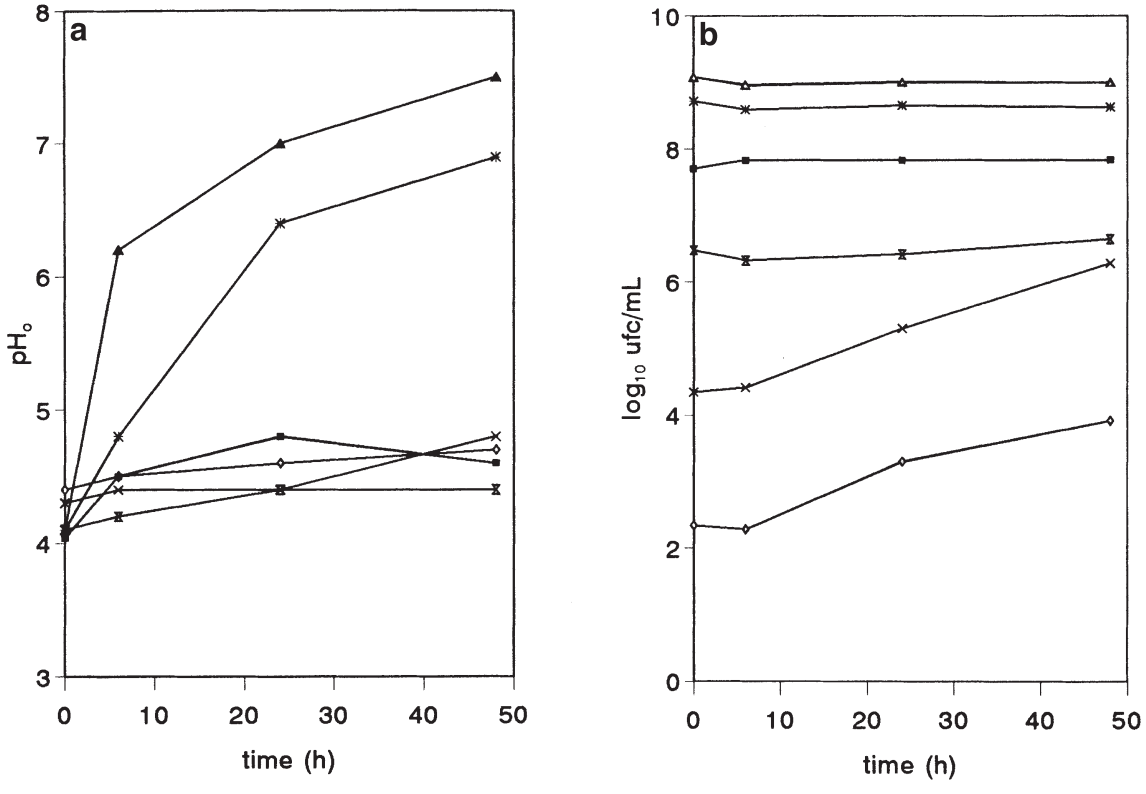

Figure 2 (a) Influence of the number of viable cells of *S. marcescens* on the external pH of NPCS after an acid shift. (b) Comparative survival of the NPCS of *S. marcescens* ATCC 274. –▲– 1.2 E9; –✳– 5.2 E8; –■– 5.0 E7; –⊠– 3.0 E6; –✕– 2.2 E4; –◇– 2.2 E2.

Table 2 Amount of potassium ions and phosphate and OD at 260 nm and 280 nm of supernatant phases of *S. marcescens* ATCC 274 suspensions at initial pH values of 3.9 and 7.1

Time (h)	pH	K^+ (ppm)	PO_4^{3-} (ppm)	OD	
				260 nm	280 nm
0	3.9 ± 0.1	6.4 ± 0.3	5.7 ± 0.7	0.092 ± 0.025	0.041 ± 0.055
2	5.1 ± 0.1	4.6 ± 0.2	7.8 ± 0.1	0.224 ± 0.015	0.150 ± 0.067
6	6.2 ± 0.4	4.6 ± 0.2	9.2 ± 1.5	0.424 ± 0.251	0.351 ± 0.269
24	7.2 ± 0.2	6.4 ± 0.3	17.9 ± 1.3	1.150 ± 0.277	0.813 ± 0.203
0	7.1 ± 0.2	4.1 ± 0.2	1.8 ± 0.4	0.196 ± 0.053	0.198 ± 0.075
2	6.5 ± 0.0	5.5 ± 0.3	5.1 ± 0.7	0.353 ± 0.127	0.346 ± 0.100
6	6.8 ± 0.1	4.6 ± 0.2	7.6 ± 0.6	0.536 ± 0.124	0.480 ± 0.146
24	7.4 ± 0.2	5.5 ± 0.3	16.9 ± 5.3	1.249 ± 0.571	0.940 ± 0.470

increased slowly. The pH of acidic suspensions increased rapidly during the first 6 h and then increased slowly. *S. marcescens* ATCC 274 and *S. marcescens* GP, a nonpigmented mutant of *S. marcescens* ATCC 274 [19], showed similar pH curves indicating that prodigiosin was not involved in external pH changes of cell suspensions.

We attempted to identify the factors that may affect the pH of bacterial suspensions. We first studied the effect of cellular viability on pH changes. When *S. marcescens* suspensions were subjected to a heat-shock, no cells could be cultured, and the pH of these suspensions did not change after 24 h of incubation (data not shown). Similar observations were reported by Marqués *et al* [16] with *Pseudomonas* sp suspensions.

Figure 2a shows that the extent of external pH change was influenced by the number of viable cells in the suspension. *S. marcescens* suspensions with 5.2×10^8 CFU ml^{-1} and 1.2×10^9 CFU ml^{-1}, at an initial pH of 4.1, reached a pH of 6.4 and 7.0 respectively. The pH of NPCS with 5×10^7 CFU ml^{-1} or less did not change more than one pH unit after 48 h of incubation. We also found a significant increase in the number of viable cells when suspensions were prepared with 2.2×10^4 CFU ml^{-1} or less (Figure 2b). No contamination was observed in any case.

In order to rule out the influence of cell lysis on pH changes we measured the amount of potassium ions and phosphate in the supernatant phase of bacterial suspensions at initial pH values of 3.9 and 7.1. We also measured OD of the supernatants at 260 nm and 280 nm. As shown in Table 2, pH is not directly mediated by potassium or phosphate released by cell lysis. OD at 260 nm and 280 nm increased with time in both suspensions, irrespective of the initial pH.

Since physiological state can affect pH, the effect of the age of the culture on the external pH of NPCS was studied. Experiments were carried out by preparing suspensions of *S. marcescens* ATCC 274 with 6- and 20-h-old cells in 0.15 M NaCl solution (2.2×10^9 CFU ml^{-1}). The pH of these suspensions was adjusted to 6.0. After 48 h, the pH of all suspensions was 7.8. This indicates that the age of cells did not influence the pH of cell suspensions.

Catalysis by the carbonate/CO_2 equilibration with carbonic anhydrase at a final concentration of 0.67 mg ml^{-1} did not influence the pH of cell suspensions (Figure 3). During the first 4 h, both suspensions, with and without carbonic anhydrase, presented the same pH evolution. After 20 h of incubation the pH of cell suspensions treated with carbonic anhydrase was not significantly different (0.24 pH units higher) than pH of non-treated suspensions.

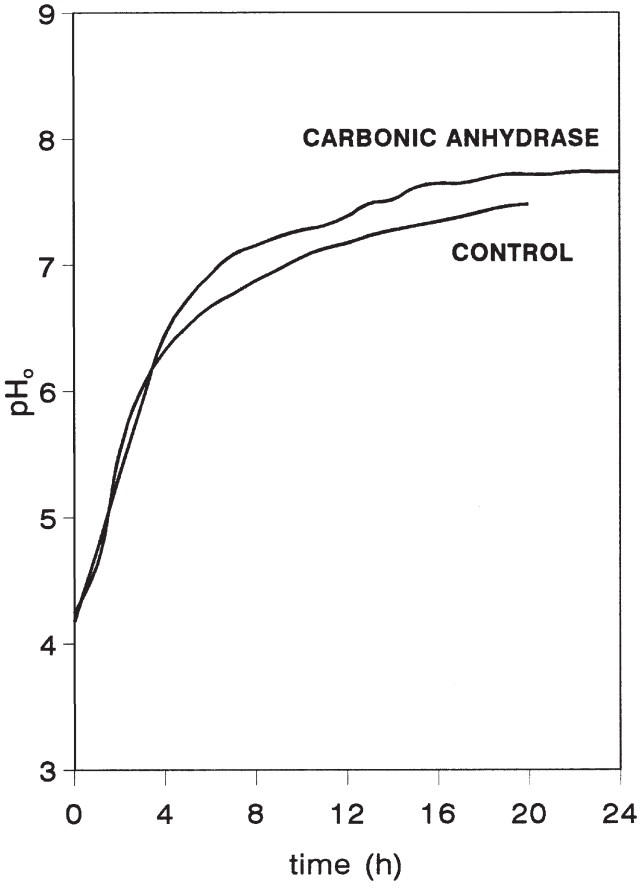

Figure 3 Effect of carbonic anhydrase on the external pH response of NPCS of *S. marcescens* ATCC 274.

On the basis of our results, we conducted all experiments with NPCS prepared from 20-h-old cultures, with 2×10^9 CFU ml^{-1}, and no carbonic anhydrase was added.

It has been shown that several polypeptides were induced in *E. coli* during starvation and that starvation protein synthesis began within minutes of the start of starvation and lasted for 2–4 h [17]. NPCS of *S. marcescens* ATCC 274

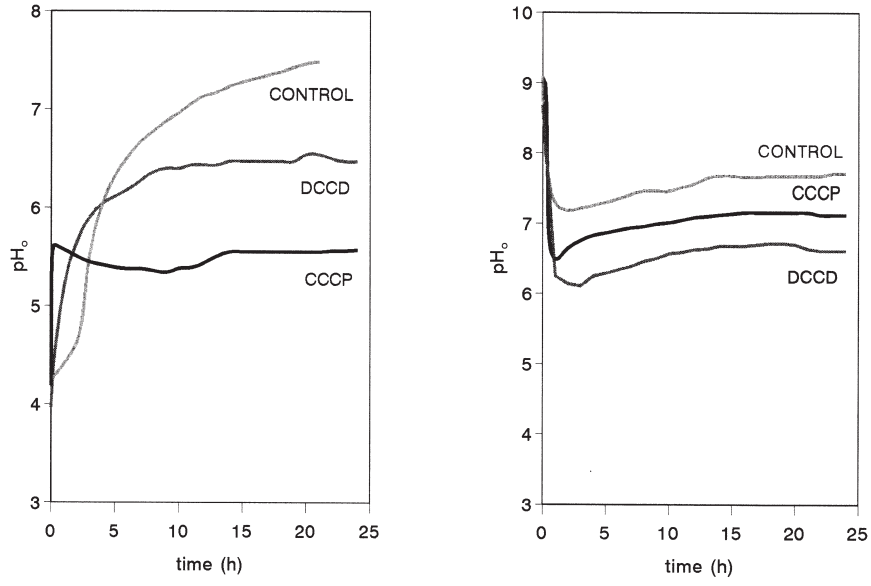

Figure 4 Influence of CCCP and DCCD on the acid-base response of suspensions of *S. marcescens* ATCC 274.

in 0.15 M NaCl solution were prepared and stored 0, 13 and 20 h at 38°C. Then pH was adjusted to 5.5. After 24 h at room temperature, the pH of these suspensions was 7.4, 7.6 and 7.5, respectively. These results suggested that starvation protein synthesis was not involved in external pH changes of bacterial suspensions.

The experiments shown in Figure 4 compare the behaviour of control and CCCP- or DCCD-treated bacteria cells at about pH 4 and 9. The presence of CCCP, a protonophore, had a marked effect on pH modification. The pH of acidic suspensions increased rapidly and reached an equilibrium at pH 5.6 after 12 min due to CCCP. The pH of control suspensions was 7.5 after 24 h of incubation. At an initial pH 9.1, pH of CCCP-treated cells decreased to 6.5 in 1 h and then increased slowly to 7.1. Final pH of control suspensions was 7.7 with a minimum pH of 7.2 after 2 h of incubation.

Suspensions treated with DCCD exhibited similar behaviour to CCCP-treated cells. The pH of suspensions exposed to DCCD increased from 4 to 6 in 4 h and then reached 6.5 after 14 h of incubation. The pH of basic suspensions decreased from 8.7 to 6.1 in 3 h and then increased slowly to 6.6.

These results suggested that passive proton conductance and proton translocation by ATP were involved in the modification of external pH of NPCS.

The treatment with CCCP or DCCD did not affect the viability of bacteria; the CFU ml^{-1} of cell suspensions at the end of each experiment was the initial CFU ml^{-1} ± 10%.

The composition of the culture medium did not influence

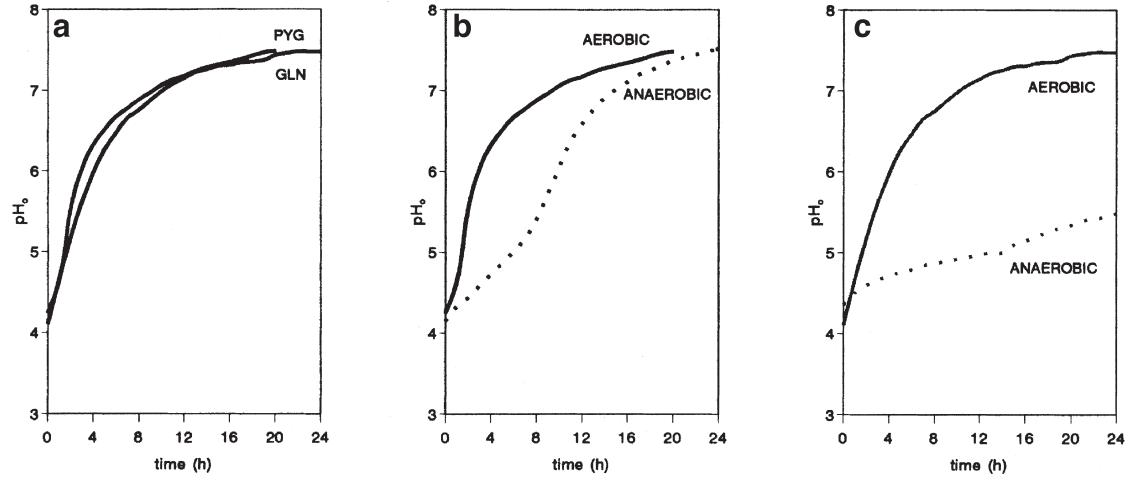

Figure 5 Effect of the composition of the medium and the culture conditions on the kinetics of the pH change in *S. marcescens* ATCC 274 suspensions. (a) Cells were grown in a complex (PYG) or a minimal (GLN) bacteriological medium aerobically. (b) NPCS prepared with cells grown in complex medium (PYG). (c) NPCS from cultures in minimal medium (GLN).

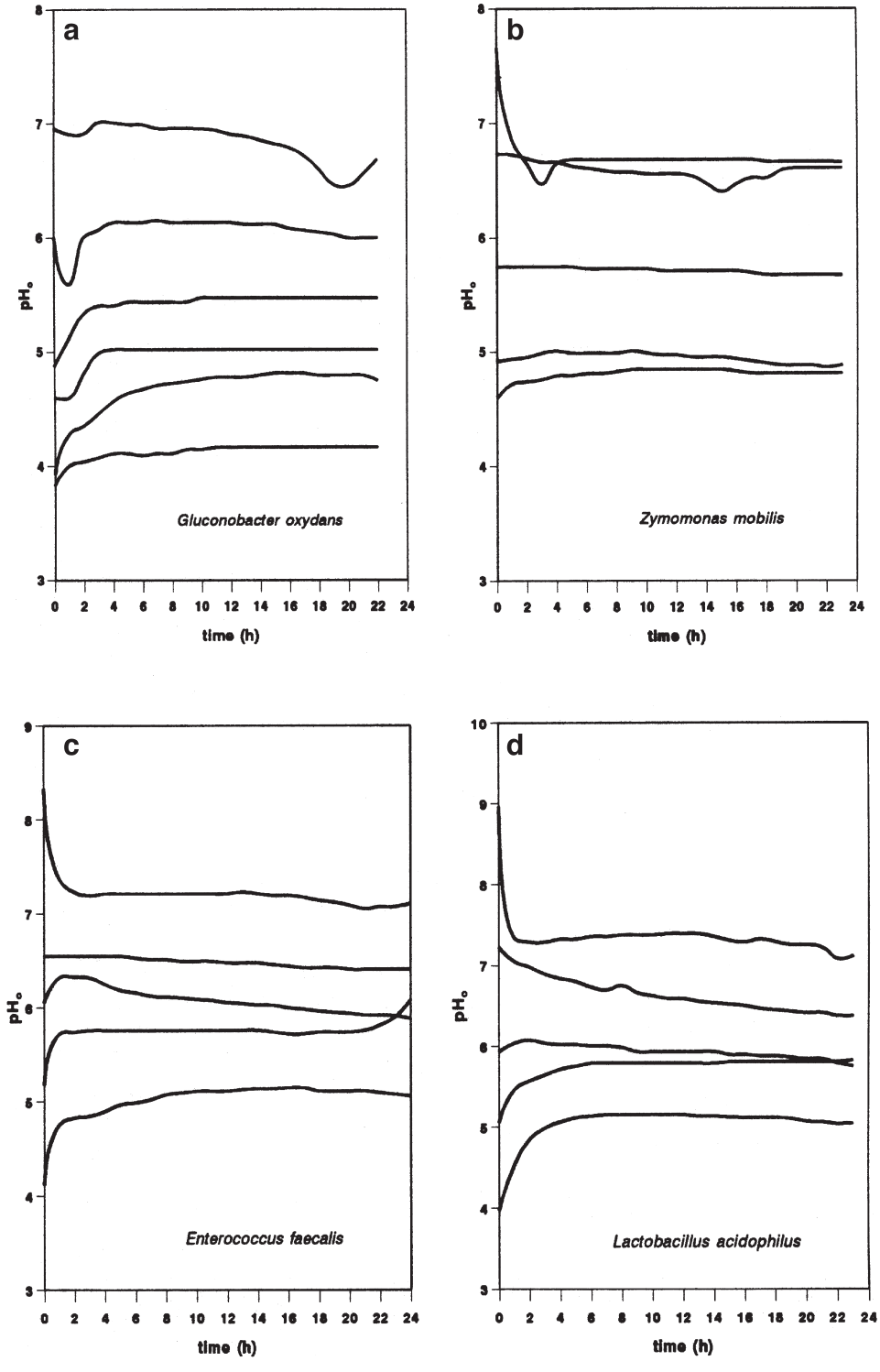

Figure 6 pH profiles of suspensions of (a) *Gluconobacter oxydans* subsp *suboxydans* ATCC 621, (b) *Zymomonas mobilis* subsp *mobilis* ATCC 29191, (c) *Enterococcus faecalis* ATCC 19433 and (d) *Lactobacillus acidophilus* ATCC 11506.

the response of NPCS. Suspensions of *S. marcescens* grown aerobically in four different media showed the same behaviour. Figure 5a shows pH evolution of NPCS from cells grown in complex (PYG) and minimal (GLN) media, both media containing glycerol as carbon source. Similar results were obtained with cells grown in nutrient broth (NB; Oxoid) and trypticase soy broth (TSB; BBL), complex bacteriological media which contain no sugar or glucose, respectively (data not shown). However culture conditions, aerobic or anaerobic, did affect the pH evolution of bacterial suspensions. The pH of NPCS of *S. marcescens* grown in complex media under anaerobic conditions

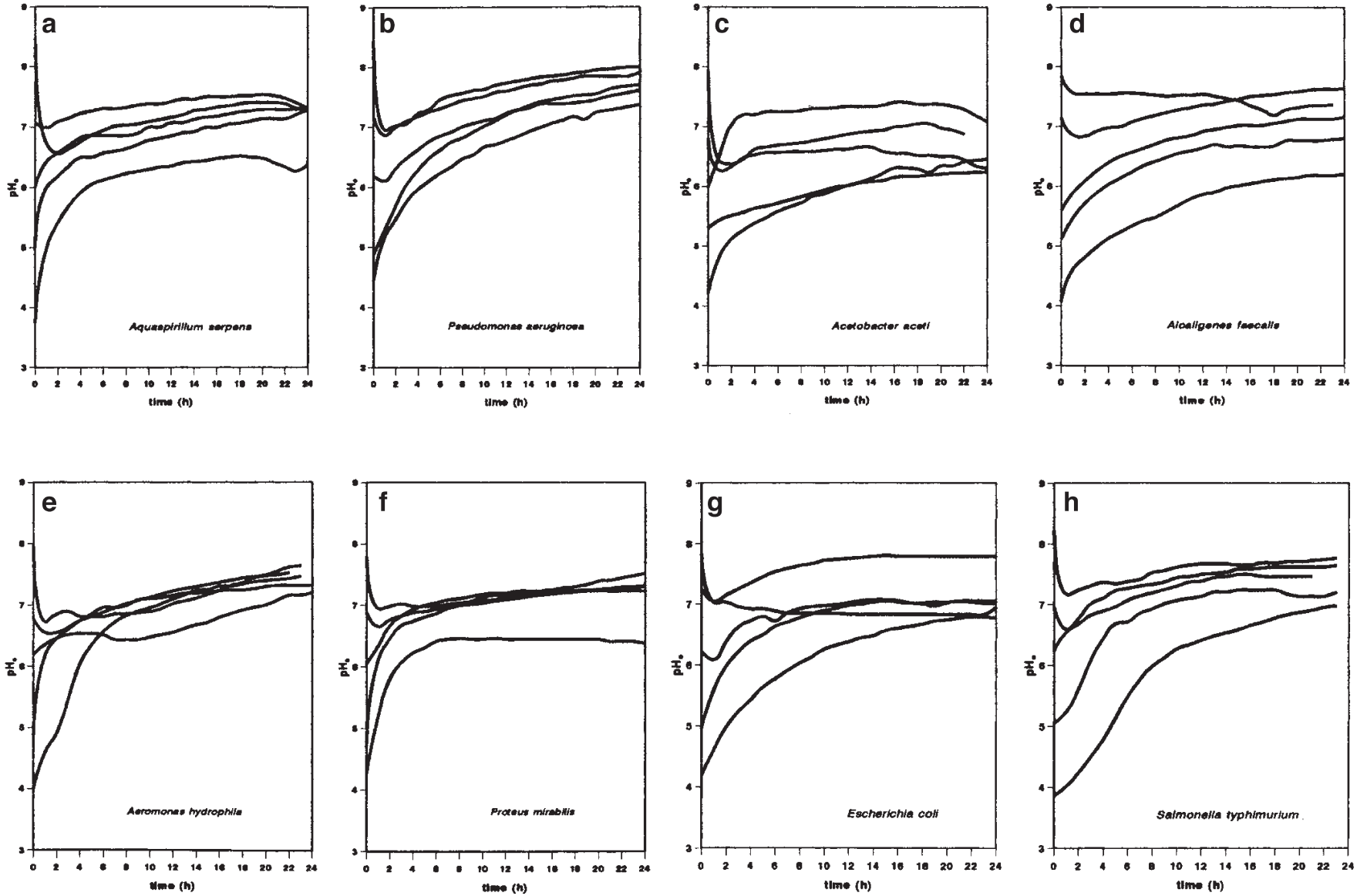

Figure 7 pH changes of suspensions of (a) *Aquaspirillum serpens* ATCC 11335, (b) *Pseudomonas aeruginosa* PAO 1, (c) *Acetobacter aceti* subsp *aceti* ATCC 15973, (d) *Alcaligenes faecalis* ATCC 19018, (e) *Aeromonas hydrophila* subsp *hydrophila* NCIB 9233, (f) *Proteus mirabilis* NCTC 5887, (g) *Escherichia coli* ATCC 10536 and (h) *Salmonella typhimurium* Mutton.

increased more slowly than the pH of NPCS from aerobic cultures (Figure 5b). The final pH of both suspensions was 7.5.

The effect of culture conditions was significantly higher when cells were grown in minimal medium (Figure 5c). The pH of suspensions prepared with cells grown under anaerobic conditions increased about one pH unit.

These results indicate that the metabolic activity of cells can modify the external pH of non-proliferating cells extruding and uptaking protons. To confirm this, we studied the pH changes of NPCS of 18 chemoheterotrophic bacteria prepared with cells grown under aerobic conditions. Included are Gram-negative and Gram-positive bacteria, seven of which are obligately aerobic possessing a strictly respiratory type of metabolism, and three of which possess a fermentative type of metabolism and the rest being facultatively anaerobic. Two types of pH response were observed. One type consisted of either no change, or a pH modification by about one unit. The three obligately fermentative bacteria studied and *G. oxydans* showed this type of response (Figure 6).

Z. mobilis, *E. faecalis* and *L. acidophilus* are obligately fermentative bacteria. It has been reported that fermentative organisms exhibit a greater range of intracellular pH (pH_i) over which growth will occur than do organisms which have a respiratory mode of metabolism. Initially, the growing fermentative cells have a pH gradient of 0.8–1.0, alkaline inside, which causes the acids to accumulate inside cells and reduces the intracellular pH_i. In spite of the fact that the pH_i falls, cells continue to grow [1,4,7,28]. Thus

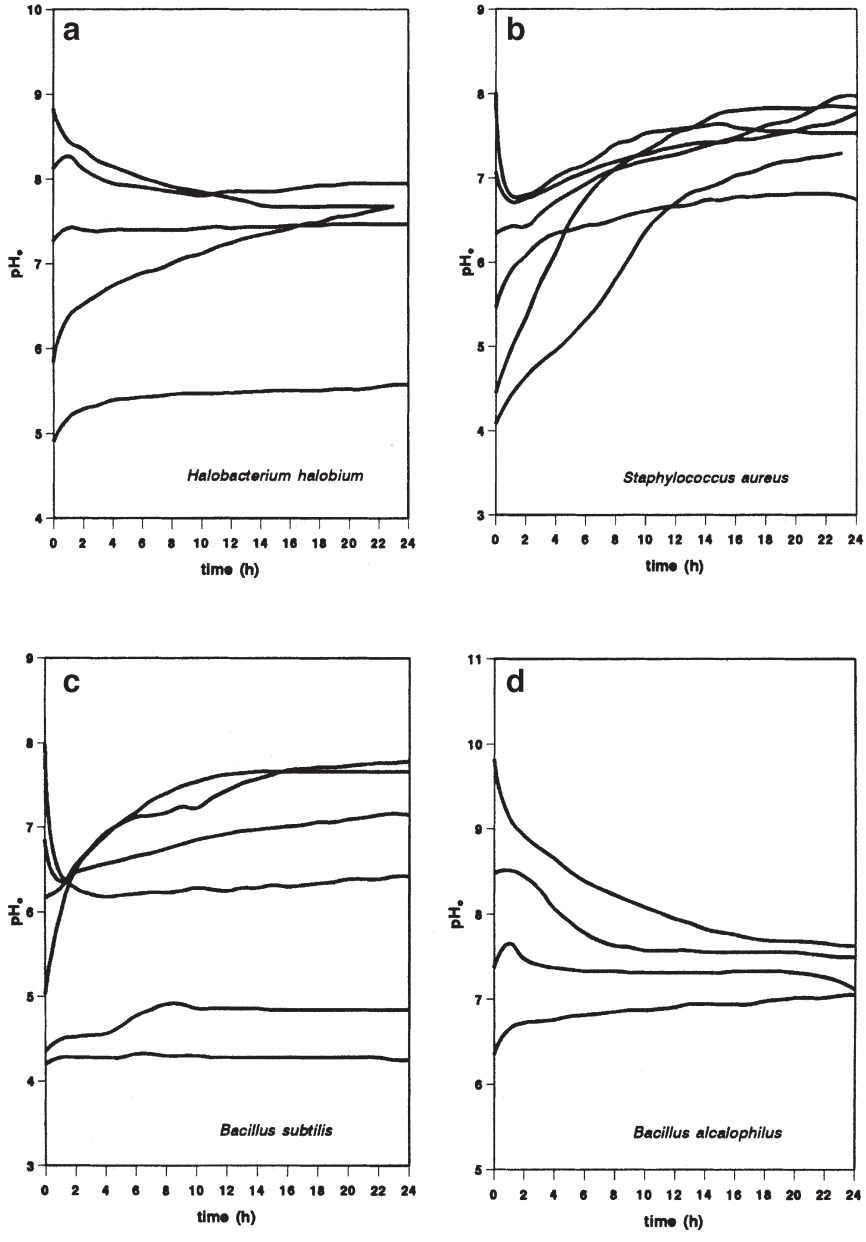

Figure 8 pH profiles of suspensions of (a) *Halobacterium halobium* CCM 2090. (b) *Staphylococcus aureus* ATCC 9144, (c) *Bacillus subtilis* ATCC 6633 and (d) *Bacillus alcalophilus* ATCC 27647.

these organisms can adapt to the environmental consequences of their metabolism. Lactobacilli can lower their internal pH to 4.4–4.8 and survive well at an external pH of 3.5 [6,17]. Our results showed that the obligately fermentative bacteria studied and *G. oxydans*, which oxidises sugars and alcohols to acids and accumulates them, can survive acidic conditions at or near 100% viability for at least 24 h. The pH of alkaline suspensions of these bacteria decreased to a value near neutrality, suggesting that they do not tolerate basic conditions.

The second kind of pH response consisted of a rapid or moderate initial pH change followed by a slow change as shown for *S. marcescens* (Figure 1). Figure 7 shows the pH changes of suspensions of *A. serpens*, *P. aeruginosa*, *A. aceti*, *A. faecalis*, *A. hydrophila* and the *Enterobacteriaceae* studied other than *S. marcescens*. Data presented here show that the external pH of suspensions of non-proliferating bacteria changed in 24 h, irrespective of the initial pH of the medium. Our results agree well with those reported for *P. aeruginosa* and *S. marcescens* [16,25,29].

Figure 8 presents pH changes of cell suspensions of *H. halobium*, *S. aureus*, *B. subtilis* and *B. alcalophilus*. The final pH of *H. halobium* suspensions at an initial pH from 5.8–8.8 was 7.5–7.9. *H. halobium* suspensions at initial pH 4.9 reached a stable value of 5.6, after 2 h of incubation. *S. aureus* suspensions exhibited similar pH profiles as the rest of facultatively anaerobic bacteria studied. The final pH of these suspensions was between 6.7 and 8.0.

Suspensions of *B. subtilis* at an initial pH below 5.0 modified external pH less than 0.5 pH units. There was an inverse linear relation between initial pH and final pH of suspensions of these bacteria at an initial pH over 5.0 and below 8.0. The higher the initial pH, the lower the final pH. The final pH of these suspensions was between 6.4 and 7.8. The external pH of suspensions of *B. alcalophilus* at an initial pH of 6.3–9.9 was between 7.0 and 7.9.

Bacteria possess a very high buffering capacity [9,19–27]. Our results cannot be explained only in terms of the buffering power of bacteria. *L. acidophilus*, an obligately fermentative bacterium which does not modify external pH, showed higher buffering capacity than some facultatively anaerobic bacteria which modify the pH of their suspensions more than one pH unit [21,22,24]. The pigmented strain *S. marcescens* ATCC 274 had a higher buffering capacity than its non-pigmented mutant *S. marcescens* GP, but both strains showed similar pH profiles when suspended at different pHs [21].

Dense cell suspensions present biochemical properties which can be used in industrial and food microbiology. Acidification is used extensively to preserve cell suspensions from contamination by other microorganisms, but bacteria have developed mechanisms to sense and respond to external pH. It has also been reported that acid adaptation cross-protected cells against distinct environmental stresses [12]. Sjogren and Gibson [28] suggested that certain enteric bacteria are able to survive in an aqueous environment by using a proton gradient generated by lowering the pH of the environment. Although additional studies are necessary to confirm such a response, we show here that the proton gradient generated by an alkali or acid shift to the cell suspensions could be dissipated by bacteria with a respiratory type of metabolism. Obligately fermentative bacteria and *G. oxydans*, well adapted to acidic conditions, slightly modified the pH of their suspensions.

Acknowledgements

We are specially grateful to Colección Española de Cultivos Tipo (CECT) for providing samples of bacteria. We thank Serveis Cientifico-Tècnics de la Universitat de Barcelona for technical assistance. We are grateful to Robin Rycroft for expert criticism during the preparation of this manuscript.

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