Measurement of cytoplasmic pH of the alkaliphile *Bacillus lentus* C-125 with a fluorescent pH probe

Rikizo Aono, Masahiro Ito and Koki Horikoshi

Author for correspondence: Rikizo Aono. Fax: +81 45 924 5819. e-mail: raono@bio.ritech.ac.jp

Department of
Bioengineering, Faculty
of Bioscience and
Biotechnology, Tokyo
Institute of Technology,
Nagatsuta 4259,
Yokohama 226, Japan

A method was established to measure the cytoplasmic pH of the facultative alkaliphilic strain, *Bacillus lentus* C-125. The bacterium was loaded with a pH-sensitive fluorescent probe, 2',7'-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein (BCECF), and cytoplasmic pH was determined from the intensity of fluorescence of the intracellular BCECF. The activity of the organism to maintain neutral cytoplasmic pH was assessed by measuring the cytoplasmic pH of the cells exposed to various pH conditions. The cytoplasmic pH maintenance activity of C-125 increased with increasing culture pH, indicating that the activity was regulated in response to the culture pH.

Keywords: alkaliphile, *Bacillus lentus*, cytoplasmic pH, pH homeostasis

INTRODUCTION

The facultative alkaliphile, *Bacillus lentus* C-125, grows between pH 6.8 and 10.8 (Aono & Horikoshi, 1983; Aono, 1995; Aono *et al.*, 1995). It has been reported that a $\Delta\psi$ -dependent Na^+/H^+ antiporter in the cell membrane helps to maintain the cytoplasmic pH around neutrality. This activity is increased with increasing culture pH (Kitada *et al.*, 1994). In addition, structural components of the cell surface of the organism are changed and respiratory activity increases in response to the culture pH (Aono & Horikoshi, 1983; Aono, 1985; Aono *et al.*, 1995, 1996).

Microbial cytoplasmic pH has been measured from the distribution of weak acids or weak bases after equilibration of the electrolytes between intracellular and extracellular compartments (Zilverstein *et al.*, 1982; Padan & Schuldiner, 1986) or absorption or fluorescence intensity of a pH-sensitive dye trapped intracellularly (Dencher *et al.*, 1986; Tsujimoto *et al.*, 1988). However, distribution of weak electrolytes might be affected by a high density of negative charges fixed on the cell surface of alkaliphiles (Aono & Horikoshi, 1983). Moreover, the cytoplasmic ionic strength of the alkaliphile is unknown and dissociation constants of weak electrolytes depend on ionic strength. If the dissociation constants differ between the cytoplasm and external milieu, accurate cytoplasmic pH cannot be calculated theoretically. We therefore tried to measure cytoplasmic

pH of *B. lentus* C-125 using the pH-sensitive fluorescent probe, 2',7'-bis-(2-carboxyethyl)-5 (and 6)-carboxyfluorescein (BCECF). BCECF has previously been used to measure cytoplasmic pH of eukaryotic or prokaryotic cells (Tsujimoto *et al.*, 1988; Molenaar *et al.*, 1991; Futsaether *et al.*, 1993).

In this report we describe a method to load the alkaliphile with BCECF, measurement of the cytoplasmic pH from the fluorescence intensity of the BCECF and determination of the level of cytoplasmic pH homeostatic activity in response to the culture pH.

METHODS

Bacterial strains. *B. lentus* C-125 (FERM 7344; Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan) (Aono & Horikoshi, 1983) was used. A neutrophilic strain, *Bacillus subtilis* GSY1026 (Aono & Horikoshi, 1983), was used as a reference strain.

Media and growth conditions. The alkaliphilic bacterium was grown aerobically at 37 °C in a medium consisting of (per litre deionized water): K_2HPO_4 , 13.7 g; KH_2PO_4 , 5.9 g; citric acid, 0.34 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g; glucose, 10 g; peptone, 5 g; yeast extract, 1 g; and Na_2CO_3 , 10.6 g. The pH of this medium was about 10 (Aono, 1985). *B. subtilis* GSY1026 was grown in Luria broth at pH 7 (Miller, 1972). When it was necessary to keep the pH constant during growth, bacteria were grown in a vessel equipped with a pH-stat apparatus (Aono *et al.*, 1995).

Loading of cells with BCECF. (i) *B. lentus* C-125 was grown in 200 ml of the alkaline medium in which the pH was maintained at 10. Bacteria in stationary phase ($\text{OD}_{660} = 2$) were harvested by centrifugation (6000 g, 4 °C, 12 min). The

Abbreviations: BCECF, 2',7'-bis-(2-carboxyethyl)-5 (and 6)-carboxyfluorescein; BCECF-AM, acetoxymethyl ester of BCECF.

cells were washed with 0.1 M NaCl/0.1 M KCl/0.1% glucose/0.1 M CHES/NaOH (pH 9.1) and resuspended in 8 ml of the same buffer. A sample (80–160 µl) of 1 mM lipophilic acetoxymethyl ester of BCECF (BCECF-AM) dissolved in DMSO was added to the suspension, which was then gently stirred at 4 °C in the dark for 2.5 h. The cells were harvested by centrifugation, resuspended in 100 ml CHES buffer and incubated at 37 °C for 45 min to remove most of BCECF loaded into the cells. The cells were recovered by centrifugation and resuspended in buffer. The cell suspension was kept on ice until used.

(ii) *B. subtilis* GSY1026 was grown in the neutral medium, with pH maintained at 7.0. The cells were washed with 0.1 M NaCl/0.1 M KCl/0.1% glucose/0.1 M TES/NaOH (pH 7.0) and resuspended in the buffer. The cells were loaded with BCECF at pH 7.0, as described above. The cells loaded with BCECF were resuspended in TES buffer and kept on ice until used.

Measurement of fluorescence intensity of BCECF-loaded cells. The suspension of the BCECF-loaded cells was diluted 50-fold with 0.1 M NaCl/0.1 M KCl/0.1% glucose/0.1 M TES/NaOH (pH 6.0–8.6) or 0.1 M NaCl/0.1 M KCl/0.1% glucose/0.1 M glycine/NaOH (pH 8.0–12.0). The cells were incubated at 37 °C for 30 min.

A fluorescence excitation spectrum of intracellular BCECF was recorded using an F-2000 fluorescence spectrophotometer (Hitachi) with double monochromators. BCECF was excited at 430–520 nm and the emission was measured at 535 nm. The slit width for both the excitation and emission monochromators was 10 nm. Samples were stirred and maintained at 37 °C during the fluorescence measurement. Immediately after the fluorescence measurement, the extracellular pH of the suspension was measured with a glass electrode and the suspension was centrifuged at 6000 g for 5 min. Fluorescence intensity of extracellular BCECF was measured in the supernatant.

Estimation of cytoplasmic pH. Two parameters, the fluorescence intensity of BCECF at 510 nm (F_{510}) and a ratio of the intensities at 510 and 450 nm ($\text{Ratio}_{510/450}$), were used as measures of cytoplasmic pH (Molenaar *et al.*, 1991; Futsaether *et al.*, 1993). The ionophore gramicidin was used to relate these parameters to cytoplasmic pH for each batch of the cell suspension. Bacteria loaded with BCECF and washed at pH 9.1 as described above were resuspended in TES (pH 6.0–8.6) or glycine (pH 8.0–12.0) buffers (described above) containing 20 µM gramicidin. After the cell suspension ($\text{OD}_{660} = 2$) had been incubated at 37 °C for 30 min, the fluorescence intensity of the suspension was measured. Cytoplasmic pH was taken to be equal to the extracellular pH measured with a glass electrode. A calibration curve of F_{510} or $\text{Ratio}_{510/450}$ was constructed, using the method of least squares, in every experiment from which the cytoplasmic pH of the sample was estimated.

Materials. Gramicidin D and BCECF-AM were purchased from Sigma.

RESULTS

Loading BCECF into *B. lentus* C-125 cells using BCECF-AM

BCECF was loaded into *B. lentus* C-125 cells using the non-fluorescent and membrane-permeable derivative, BCECF-AM. Intracellular BCECF-AM is converted to

the strongly fluorescent and charged dye BCECF by cytoplasmic esterases (Futsaether *et al.*, 1993). C-125 cells did not take up BCECF when incubated with BCECF itself.

(i) Effect of incubation temperature. *B. lentus* C-125 cells were incubated in 0.1 M NaCl/0.1 M KCl/0.1% glucose/5 µM BCECF-AM/0.1 M Tricine/NaOH (pH 8.5) at temperatures between 0 and 30 °C for 2.5 h. Loading of BCECF into the cells was significantly dependent on the incubation temperature. Maximum loading occurred between 5 and 10 °C. Less than 20% of the maximum fluorescence intensity was observed at 0 °C or at temperatures higher than 20 °C. This may be due to an effect of temperature on permeation of BCECF-AM into the cells, enzymic hydrolysis of BCECF-AM to BCECF or leakage of BCECF from the cells. The incubation temperature was the most important factor in optimizing loading the cells with BCECF.

(ii) Incubation pH. We next examined the effect of the incubation pH on BCECF-AM during loading of the C-125 cells with BCECF. The cells grown at 37 °C and pH 10 were incubated in 0.1 M Good buffer containing 5 µM BCECF-AM, 0.1 M NaCl, 0.1 M KCl and 0.1% glucose at 4 °C for 2.5 h. The cells recovered by centrifugation were assayed for fluorescence intensity of BCECF. BCECF loading was optimal at pH 9 and was at least 30% less at pH 7–8 and pH 10.

(iii) Concentration of BCECF-AM. The cells of C-125 were incubated at 4 °C for 2.5 h in 0.1 M NaCl/0.1 M KCl/0.1% glucose/0.1 M Tricine/NaOH (pH 8.5) containing 2–40 µM BCECF-AM. The amount of BCECF accumulated by the cells was dependent on the BCECF-AM concentration. The BCECF accumulated reached a maximum when the cells were incubated with BCECF-AM at concentrations of 10 µM and higher. Half maximal accumulation was found at about 4 µM BCECF-AM.

Based upon these results, in subsequent experiments the C-125 cells were loaded with BCECF under the conditions described in Methods. Autolytic enzymes of *B. lentus* C-125 are derepressed by carbon starvation and are active at alkaline pH. The cells are stabilized in the presence of glucose even at alkaline pH (Aono & Sanada, 1994). Therefore, we added 0.1% glucose to the buffers to avoid the autolysis of the cells.

Leakage of BCECF from the cells

It has been reported that BCECF is excreted from *Lactococcus lactis* cells by ATP-driven extrusion systems (Molenaar *et al.*, 1992). The efflux of BCECF was stimulated upon energization of the cells by addition of lactose. The rate of BCECF leakage from *Propionibacterium acnes* was dependent on the cytoplasmic and external pH (Futsaether *et al.*, 1993). *P. acnes* appeared to excrete BCECF in response to ΔpH . However, BCECF excretion was not increased by addition of glucose to this organism. These reports suggest that micro-

Fig. 1. Leakage of BCECF from the cells. The C-125 cells grown at 37 °C and pH 10 were loaded with BCECF as described in Methods, and then resuspended in 0.1 M NaCl/0.1 M KCl/0.1 % glucose/0.1 M CHES/NaOH (pH 9.1). The suspension was incubated on ice (○) or at 37 °C (●). A portion of the suspension was periodically centrifuged. Fluorescence intensity (excitation at 505 nm and emission at 535 nm) of the supernatant diluted fourfold with 0.1 M CHES/NaOH (pH 9.7) was measured.

organisms differ in their retention of BCECF intracellularly. An excitation spectrum obtained from a cell suspension containing a high concentration of extracellular BCECF reflects not only the intracellular pH but also the extracellular pH. This effect could produce significant errors in the estimation of the cytoplasmic pH.

Leakage of BCECF from the C-125 cells was examined at pH 9.1 (Fig. 1). The leakage was quantified by measurement of fluorescence intensity of extracellular BCECF excited at 505 nm. Extracellular BCECF had an excitation maximum at 505 nm, which was slightly lower than that of the intracellular BCECF, found at 508–510 nm. The rate of BCECF leakage from C-125 was significantly dependent on the incubation temperature. At 37 °C, leakage was rapid during the first 20 min after resuspension. Intracellular BCECF at this time corresponded to about 50% of the amount originally loaded. Leakage was extremely slow on ice.

The C-125 cells loaded with BCECF and washed at 37 °C as described above were adapted to an alternative milieu pH and further leakage of the residual intracellular BCECF at 37 °C was examined during the adaptation. Although the cells had ceased to release BCECF by the end of the preceding wash (Fig. 1), the cells began to release residual BCECF at a slow, roughly constant rate in the fresh buffer. Approximately 10% of the intracellular BCECF had been excreted 30 min after resuspension. The BCECF leakage from the C-125 cells

was suppressed by gramicidin. The cells were incubated in the presence of glucose to avoid autolysis of the cells which occurs under carbon starvation and were therefore energized throughout the experiments. The organism seems to excrete intracellular BCECF by an energy-dependent extrusion system.

BCECF leakage from bacteria that had been washed once at 37 °C was slow. This slow rate was desirable when fluorescence intensity of the intracellular BCECF was being monitored because the proportion of extracellular BCECF would remain low. The fluorescence intensity measured could be corrected by the subtraction of that of a supernatant solution obtained from the BCECF-loaded cell suspension because of the slow rate of the BCECF leakage.

Calibration of the fluorescence determination of cytoplasmic pH

The fluorescence signal of intracellular BCECF must be calibrated by measurements using bacteria of known cytoplasmic pH. Ionophores, such as gramicidin and nigericin, have been used for this purpose to control bacterial cytoplasmic pH (Kitada *et al.*, 1989; Futsaether *et al.*, 1993; Hashimoto *et al.*, 1994; Molenaar *et al.*, 1991).

The fluorescence intensity of intracellular BCECF in the C-125 cells was measured in the presence of gramicidin. Gramicidin functions as an effective carrier for monovalent cations, such as H^+ , Na^+ and K^+ ions which were abundantly present in the buffers used. This ionophore causes the disappearance of a proton motive force gradient across the cytoplasmic membrane ($\Delta\mu H^+$). Accompanying the disappearance of $\Delta\mu H^+$, a pH gradient across the cytoplasmic membrane (ΔpH) becomes negligible (Kitada *et al.*, 1989).

Fluorescence intensity of intracellular BCECF increased or decreased rapidly in response to the extracellular pH after the addition of gramicidin to the cell suspension. Gramicidin did not cause leakage of BCECF. Therefore, the change of fluorescence signals based on alteration of cytoplasmic pH of the C-125 cells was due to flux of H^+ ions in response to extracellular pH (Fig. 2). The cytoplasmic pH shown in the figure was calculated as described below. The cells were adapted to the extracellular pH for 30 min with or without gramicidin. During this time, an equilibrium between the extracellular and cytoplasmic pH values was achieved. Theoretically, the cytoplasmic pH is slightly lower than the extracellular pH due to a membrane potential ($\Delta\psi$), when equilibrium across the membrane is established with gramicidin. The presence of a significant $\Delta\psi$ introduces some errors into the estimation of the cytoplasmic pH. However, we found similar cytoplasmic pH values by using the nigericin–potassium method to eliminate ΔpH (results not shown), indicating that the $\Delta\psi$ was not high enough to yield significant errors on measurement of the cytoplasmic pH of the cells when the pH equilibrium was accomplished.

Fig. 2. Changes in cytoplasmic pH of C-125 cells after treatment with gramicidin. The C-125 cells grown at pH 10 and loaded with BCECF were resuspended in TES-buffered 0.1 M NaCl/0.1 M KCl/0.1% glucose with pH values of 6.7 ($\square$), 8.2 ($\triangle$) or 8.8 ($\circ$). At 0 min (indicated by an arrow), 20 μ M gramicidin was added. The F_{510} of the intracellular BCECF was monitored and used to calculate cytoplasmic pH as described in Results.

Fig. 3. Excitation spectra of the C-125 cells loaded with BCECF and washed by incubation in 0.1 M NaCl/0.1 M KCl/0.1% glucose/0.1 M CHES/NaOH (pH 9.1) at 37 °C for 45 min. The cells were then incubated in 0.1 M TES or glycine buffer (pH 6.4–9.9) containing 0.1 M NaCl/0.1 M KCl/0.1% glucose/20 μ M gramicidin at 37 °C for 30 min. Fluorescence intensity (excitation at 430–520 nm and emission at 535 nm) of the suspension was recorded.

Fig. 3 shows a typical set of fluorescence excitation spectra of the C-125 cells loaded with BCECF and exposed to various pH values in the presence of gramicidin. The C-125 cells contained an unidentified fluorescent substance with an excitation maximum of

Fig. 4. Correlation between fluorescence signals of intracellular BCECF and cytoplasmic pH of C-125 cells. Parameters of Ratio_{510/450} ($\circ$) and F_{510} ($\bullet$) were calculated from the fluorescence excitation spectra shown in Fig. 3.

460 nm and, therefore, the profile of the excitation spectrum of the suspension varied according to the BCECF concentration in the cells. An isosbestic point was not clear in the spectra. The dye could not be used as a dual-excitation pH indicator under these conditions. When the cytoplasmic pH was fixed at the extracellular pH in the presence of gramicidin, two parameters of spectra (Ratio_{510/450} and F_{510} of BCECF) correlated with the cytoplasmic pH (Fig. 4). The Ratio_{510/450} gave good correlation with the cytoplasmic pH between 6.4 and 9.1. It was reported that a similar ratio (intensities at 505 and 450 nm) correlated with cytoplasmic pH (below pH 8) in *P. acnes* (Futsaether *et al.*, 1993). The F_{510} correlated with the pH from 7.6 to 9.9.

Measurement of cytoplasmic pH and the culture-pH-dependent cytoplasmic pH maintenance activity of C-125 cells

We used the two fluorescence calibration curves shown in Fig. 4 to measure cytoplasmic pH of C-125. By using the two parameters, the cytoplasmic pH was measured over the range of cytoplasmic pH 6–10. The calibration curves were constructed for each batch of the cell suspension, because each profile of the two calibration curves was somewhat dependent on the BCECF content in the cells, as described above.

B. lentus C-125 cells grown at a constant pH (7.0, 8.5 or 10.0) and loaded with BCECF were exposed to buffers in the pH range 6–12 (Fig. 5). The cytoplasmic pH of the cells grown at pH 10 was almost constant at a wide

Fig. 5. Cytoplasmic pH of C-125 cells as a function of the extracellular pH. *B. lentus* C-125 was grown at pH 7.0 ($\square$), 8.5 ($\triangle$) or 10.0 ($\circ$). *B. subtilis* GSY1026 (∇) was grown at pH 7.0 and used as a reference strain. The cells in the stationary phase of growth were loaded with BCECF and washed as described in Fig. 3. The cells were incubated for 30 min in 0.1 M TES or glycine buffer containing 0.1 M NaCl/0.1 M KCl/0.1 % glucose at various pH values. Measurement of fluorescence intensity of the intracellular BCECF is detailed in Methods. Cytoplasmic pH of the cells was calculated mainly from the Ratio_{510/450}. The pH above 9 was calculated from F_{510} .

range of extracellular pH values. Over the range pH 7.0–10.7 which was the physiological pH range for the organism, the cytoplasmic pH was maintained at 7.2–8.0 under the experimental conditions. At pH above 10.7, the organism lost its pH homeostatic activity. *B. lentus* C-125 does not grow at pH above 10.8 (Aono *et al.*, 1995).

The cells grown at pH 7.0 maintained neutral cytoplasmic pH (pH 7–8) only when exposed to an external pH below 9. Above 9, cytoplasmic pH increased much more than in bacteria grown at pH 10.0. The cytoplasmic pH of cells exposed to pH 10.5 was 9.3. This pH value was one unit higher than that of the cells grown at pH 10.0 and exposed to pH 10.5. Thus, the pH homeostatic activity of the cells grown at pH 7.0 was low, although higher than that of a neutrophilic micro-organism, *B. subtilis* GSY1026. The intracellular pH of C-125 grown at pH 7 became sensitive to extracellular pH above 8.5, whereas that of *B. subtilis* GSY1026 became sensitive when exposed to an extracellular pH above 8.2 (Fig. 5). *B. subtilis* GSY1026 does not grow at pH values above 8.5 (Aono *et al.*, 1996). The cytoplasmic pH of C-125 was about 7.2 at the extracellular pH of around 7.

When C-125 was grown at pH 8.5, the pH homeostatic activity was intermediate between those of the bacteria grown at pH 7.0 and 10.0. Cytoplasmic pH of the cells grown at pH 8.5 and exposed to pH below 10 was maintained neutral (pH 7–8). The cytoplasm became more alkaline when the cells were exposed to pH above 10. These results indicated that the culture pH determined the upper limit of the extracellular pH against which the cytoplasmic pH could be maintained neutral.

DISCUSSION

The cytoplasmic pH of another facultative alkaliphile, *Bacillus firmus* OF4, was reported to be 7.5–8.3 when the organism was grown at pH 7.5–10.5 (Sturr *et al.*, 1994). The cytoplasmic pH of C-125 measured by the fluorescence intensity of BCECF was similar to and slightly lower than the values reported for OF4. The cytoplasmic pH of C-125 grown at initial pH of 10.5 and exposed to pH 10.5 was previously reported to be 8.64 (Hashimoto *et al.*, 1994). This pH value was measured after prolonged incubation of the cells without any carbon source. We feel that this value was not physiologically significant. The value is likely to have been overestimated, probably due to autolysis or to de-energization caused by carbon starvation.

We have shown that the cytoplasmic pH of the alkaliphile can be measured by fluorescence intensity of BCECF loaded under the conditions described here. Cytoplasmic pH values of alkaliphiles have previously been determined from the distribution of weak acids or weak bases (Sugiyama *et al.*, 1985; Kitada *et al.*, 1989; Hashimoto *et al.*, 1994; Sturr *et al.*, 1994). This method assumes that only uncharged molecules of the electrolytes can pass through the biological membrane (Padan & Schuldiner, 1986). Alkylamines have been applied to alkaliphiles (inside acidic) exposed to high alkaline pH. pK_a values of these amines are in the range of 10–11. The distribution method using the amines is therefore most appropriate for determination of cytoplasmic pH in alkaliphiles exposed to pH below 9. Experimental errors would increase with increasing external pH. An amine with pK_a of 13–14 would be needed to determine the cytoplasmic pH of C-125 cells exposed to high alkaline pH, such as 11–12. Such an amine is not available. In addition, cytoplasmic pH is determined following equilibration of the electrolyte between the inside and outside of the cells. At a much higher pH than the pK_a of the amine used, few molecules are uncharged and permeable through the membrane. The rapid changes of cytoplasmic pH shown in Fig. 2 would be very difficult to follow by the distribution method.

The cytoplasmic pH has also been measured from fluorescence quenching of 9-aminoacridine (Tsuchiya & Takeda, 1979; Sugiyama *et al.*, 1985; Kitada *et al.*, 1994). This pigment is retained effectively in cells whose cytoplasmic pH is lower than the extracellular pH. C-125 grows at a relatively wide range of extracellular pH and its cytoplasmic pH is not always lower than the extracellular pH (Fig. 5). The pigment is thus not appropriate for measurement of cytoplasmic pH in C-125 exposed to neutral extracellular pH.

pH measurement on the basis of fluorescence intensity of intracellular BCECF can provide real-time information about the cytoplasmic pH. This method is useful for analysing the pH-homeostatic activity of alkaliphiles. However, a disadvantage of the method is the need to use stationary-phase bacteria which are incu-

bated at 4 °C and pH 9 to load BCECF and then washed at 37 °C to excrete excess BCECF (Fig. 1). This might prevent measurement of the cytoplasmic pH under normal physiological conditions. However, it was shown that the pH-homeostatic activity of C-125, measured in this study, was closely related to the growth pH. These results suggested that the physiologically normal cytoplasmic pH could be measured in spite of the unfavourable procedures.

It is well known that alkaliphiles growing in alkaline milieu maintain a neutral cytoplasmic pH (Sturr *et al.*, 1994; Krulwich, 1995). The facultative alkaliphile, C-125, developed this pH maintenance activity in response to the culture pH. The organism can grow in complex medium at pH 6.8–10.8 with the optimum pH for growth at 9 (Aono *et al.*, 1995). Probably due to some regulation of the pH-homeostatic activity level, the cytoplasm of the organism is always kept neutral (pH 7–8) regardless of the culture pH. Although the cytoplasmic pH measured in this study was not strictly that of the growing cells, the cytoplasmic pH values of the cells growing at pH 7.0, 8.5 and 10.0 were estimated to be 7.2, 7.7 and 8.0, respectively (Fig. 5).

The maintenance of pH is likely to be energy-dependent because the cytoplasmic pH of the C-125 cells exposed to alkaline pH was elevated by treatment with KCN or antimycin (results not shown). These chemicals inhibit respiratory activity of C-125. The respiratory activity of *B. lentus* C-125 is dependent on the culture pH (Aono *et al.*, 1996). The organism possesses $\Delta\psi$ - and Δ pH-dependent Na^+/H^+ antiport systems. The former antiporter activity increases with increasing culture pH (Kitada *et al.*, 1994). Further, anionic charges fixed in the cell walls also increase with increasing culture pH (Aono *et al.*, 1995). The cell-wall density of the charges is 2, 6 or 8 μmol (mg peptidoglycan) $^{-1}$ in the cells grown at pH 7.0, 8.5 or 10.0, respectively. These facts are consistent with the result that the cytoplasmic pH maintenance activity was developed in response to the culture pH. We presume that pH homeostasis of C-125 involves several functions of the organism.

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