Biochem. J. (1992) 285, 99–103 (Printed in Great Britain)

Instability of the protoplast membrane of facultative alkaliphilic *Bacillus* sp. C-125 at alkaline pH values below the pH optimum for growth

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Cell walls of facultative alkaliphilic *Bacillus* sp. C-125 consist of three polymers (peptidoglycan, teichuronopeptide and teichuronic acid). Protoplasts prepared from the strain with egg-white lysozyme regenerated cell walls at neutral pH, but not at pH above 8.5. The protoplasts were susceptible to lysis at alkaline pH. The protoplasts exposed to alkaline pH rapidly burst and lost ability to regenerate their cell walls. The alkali-instability was similar to that of protoplasts from neutrophilic *Bacillus subtilis* 168. The membrane vesicles were also labile at alkaline pH. The acidic wall components of strain C-125 may contribute to stabilization of the cytoplasmic membrane of cells growing at alkaline pH, probably by shielding the membrane from direct exposure to an alkaline environment.

INTRODUCTION

Previously, we classified several alkaliphilic strains of *Bacillus* into three groups on the basis of cell-wall components, cation requirement for growth and other physiological properties. The group 2 organisms require Na^+; strain C-125 belongs to group 2 (Aono & Horikoshi, 1983). This bacterium shows optimal growth at pH 9–10 and does not grow at pH 6.5 or below, when examined on agar media (Aono & Ohtani, 1990). Intracytoplasmic pH of strain C-125 grown at pH 10.5 is kept at approx. pH 8–9 (R. Aono & M. Ito, unpublished work). Therefore there is a pH difference between the inside and the outside of the cells. This pH difference must be caused and maintained by surface components and/or functions.

Cell walls of alkaliphilic *Bacillus* sp. C-125 are composed of peptidoglycan, teichuronic acid and teichuronopeptide. The peptidoglycan is of A1γ type. It is identical with that of neutrophilic *B. subtilis* in chemical structure (Aono *et al.*, 1984). The teichuronic acid is composed of galacturonic acid, glucuronic acid and *N*-acetyl-D-fucosamine (Aono & Uramoto, 1986). The teichuronopeptide is a complex of polyglucuronic acid and polyglutamic acid (Aono, 1985, 1987, 1989). Amounts of these acidic polymers are enhanced in the cell walls when strain C-125 is grown at alkaline pH (Aono, 1985). Mutants defective in the acidic polymers grow poorly at alkaline pH (Aono & Ohtani, 1990).

The present paper shows that protoplasts of alkaliphilic *Bacillus* sp. C-125 are unstable at alkaline pH at which the organism can thrive, and that the membrane vesicles exposed to alkaline pH liberate membrane components such as proteins and lipids.

MATERIALS AND METHODS

Organisms and cultivation conditions

The alkaliphilic *Bacillus* sp. strain C-125 and its streptomycin-resistant derivative strain C-125-002 (Thr^- Str^r) were grown in alkaline complex medium (pH 10.0) at 30 °C (Aono & Ohtani, 1990). The alkaline medium contained, per litre of deionized water: K_2HPO_4, 13.7 g; KH_2PO_4, 5.9 g; citric acid, 0.34 g; $MgSO_4,7H_2O$, 0.05 g; glucose, 5 g; peptone, 5 g; yeast extract, 0.5 g; Na_2CO_3, 10.6 g. Neutrophilic *Bacillus subtilis* Marburg168 GSY1026 was grown in LB broth (pH 7.0) as a reference strain (Aono & Horikoshi, 1983).

Preparation of protoplasts

Cells in the exponential phase of growth were harvested by centrifugation at 3000 *g* for 10 min at 4 °C. The cells were washed once with double-strength Penassay broth (Difco Laboratories, Detroit, MI, U.S.A.) containing 0.5 M-sucrose, 20 mM-$MgCl_2$, 20 mM-maleic acid and NaOH, pH 7.0 (neutral SMMP medium; Chang & Cohen, 1979). The cells were suspended in SMMP medium to a concentration of 15–20 A_{660} units/ml. Then 0.01 vol. of 1% (w/v) lysozyme solution was added to the suspension. Protoplast formation at 37 °C was monitored microscopically. The protoplasts were recovered by centrifugation at 1000 *g* for 30 min at 10 °C and washed twice with the SMMP medium.

Regeneration of cell walls by the protoplasts

Protoplasts were prepared from strain C-125-002 as described above. The protoplast suspension was spread on regeneration medium, which contained 5 g of yeast extract, 5 g of casamino acid, 20 g of glucose, 10 g of agar, 0.4 g of BSA, 0.1 g of streptomycin, 30 mM-$MgCl_2$, 1.25 mM-$CaCl_2$, 0.5 M-monosodium succinate and 30 mM-Tris per litre of deionized water. The pH was adjusted to 7.1 with NaOH, unless otherwise stated. Colony formation was carried out at 37 °C.

Preparation of cytoplasmic membrane

The protoplasts prepared from strain C-125 as described above were washed with 0.5 M-sucrose/20 mM-$MgCl_2$/20 mM-maleic acid/NaOH buffer (pH 7.0). The protoplasts were suspended in 50 mM-NaCl/2.5 mM-$MgCl_2$/25 mM-K_2HPO_4/HCl buffer (pH 7.0) in the presence of a small amount of DNAase I, and broken by gentle homogenization with a Potter–Elvehjem homogenizer. Unbroken protoplasts were removed from the suspension by centrifugation at 8000 *g* for 10 min at 4 °C. Membrane fraction was recovered from the supernatant by centrifugation at 100000 *g* for 45 min at 4 °C, and washed with the NaCl/$MgCl_2$/phosphate buffer.

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Table 1. Buffer composition used for HOPP medium

The following buffers were roughly adjusted to each pH value shown in the Table with NaOH before sterilization with an autoclave. HOPP media of various pH values were prepared by mixing one of the buffers with double-strength Penassay broth containing sucrose and $MgCl_2$. After mixing, each of the media HOPP-7 to -11 contained about 50 mM-Na^+ and -K^+.

	Buffer composition (mM)					
pH	KCl	K_2HPO_4	NaCl	$NaHCO_3$	Na_2CO_3	NaOH
7		50	100			
8		50	100		10	
9		50		50	25	
10	100				50	
11	100				25	75

Alkali-instability of protoplasts and cytoplasmic membrane

The composition of SMMP medium described above was modified as below. The basal medium used was high-osmotic-pressure Penassay medium, consisting of double-strength Penassay broth, 0.5 M-sucrose and 2.5 mM-$MgCl_2$ (HOPP medium). This HOPP medium was devised to contain about 50 mM each of Na^+ and K^+, and buffered with phosphate (around pH 7: HOPP-7 medium), phosphate plus carbonate (around pH 8 and 9: HOPP-8 and -9 media) or carbonate ions (around pH 10 and 11: HOPP-10 and -11 media) as shown in Table 1. Protoplast suspension in SMMP medium was diluted 20-fold with each of the buffered HOPP media prewarmed to 30 °C. The initial pH value of each incubation mixture was measured accurately immediately after addition of the protoplast suspension. The suspensions were incubated at 30 °C with or without gentle shaking. The shaking was carried out with a Monod shaker at 30 oscillations/min.

The same phosphate and/or carbonate buffers (Table 1) containing 2.5 mM-$MgCl_2$ were used for examination of stability of the cytoplasmic membrane. The membrane preparation was suspended in each of the buffers prewarmed to 30 °C. The suspensions were incubated at 30 °C without shaking, after the initial pH was measured.

Determination of protein

The concentration of protein in the HOPP media was determined by the method of Bradford (1976), with BSA as a reference standard. Protein in other samples was determined by the method of Lowry *et al.* (1951).

Determination of phospholipids

Samples were extracted by the method of Bligh & Dyer (Rouser & Fleischer, 1967) with chloroform and methanol. The extract was washed with water. Phosphorus content in the chloroform extract was determined by the molybdate method after the sample was ashed in the presence of $Mg(NO_3)_2$ (Ames, 1966).

RESULTS

pH-dependent regeneration of cell wall by protoplasts from facultative alkaliphilic *Bacillus* sp. C-125

Protoplasts prepared from strain C-125 regenerated their cell walls. The effect of pH on cell-wall regeneration was evaluated by using protoplasts prepared from strain C-125-002. The protoplasts were spread on the high-osmotic-pressure media containing streptomycin to avoid bacterial contamination, after

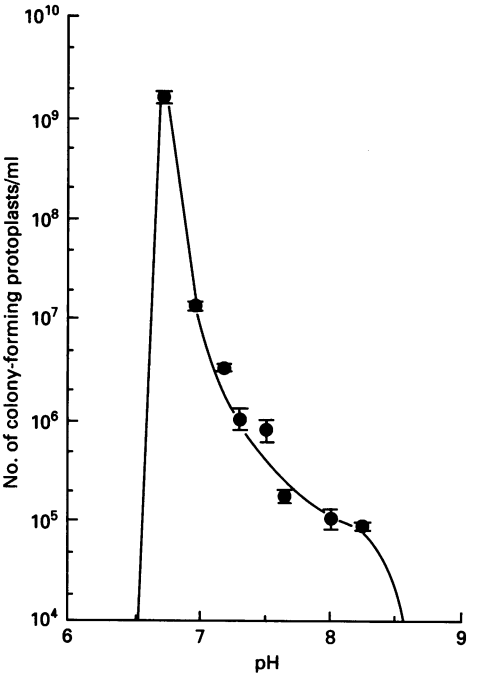

Fig. 1. Regeneration of cell walls by protoplasts prepared from a derivative of facultative alkaliphilic strain of *Bacillus* sp. C-125

Protoplasts were prepared from the strain C-125-002 (Thr^- Str^r) and spread on the regeneration medium containing streptomycin (0.1 mg/ml). The medium was adjusted to various pH values with HCl or NaOH. Immediately before use, the pH of the surface of the medium was measured with a flat type of glass electrode; these pH values are indicated in the Figure. The protoplasts were incubated at 37 °C for 4 days. Colonies formed on the media were counted every day. The graph shows the number of colonies found by 4 days in duplicate examinations.

the number of the protoplasts had been counted microscopically with a Thoma's haematocytometer.

Colony formation by the protoplasts was strikingly dependent on the pH of the regeneration medium. About 5% of the protoplasts regenerated at pH 6.7 (Fig. 1). Colony formation was not found at alkaline pH values above 8.5 or acidic pH values below 6.5. The acidic pH did not permit the cells of strain C-125 to grow. On the other hand, the alkaline pH values were suitable for growth of the organism. The number of colonies formed at pH 8.3 was extremely low, below 5×10^{-5} of that at pH 6.7. Protoplasts prepared from the wild strain C-125 gave similar results (not shown) for pH-dependent regeneration.

Instability of the protoplasts from alkaliphilic *Bacillus* sp. C-125 at alkaline pH

(i) Turbidimetric clarification of the protoplast suspension at alkaline pH. Fig. 2(*a*) shows the periodical decrease in turbidity of the protoplast suspensions prepared from alkaliphilic *Bacillus* sp. C-125 without shaking. The suspensions were clarified more rapidly at alkaline pH above 9.9 than at neutral pH. The turbidity decreased to about 63% or 27% at 3 min after exposure of the protoplasts at pH 9.9 or 10.7 respectively. Such rapid clarification of protoplast suspensions was more pronounced in neutrophilic *B. subtilis* GSY1026 at pH above 9.0 (Fig. 2*b*). The alkaline-pH-dependent clarification was also found in the protoplast suspensions, even when the suspensions were gently shaken to supply air (Fig. 2*c*).

(ii) Release of cytoplasmic material from the protoplasts at alkaline pH. The turbidity of a suspension of insoluble matter is generally altered by ionic strength or pH of the buffer used as

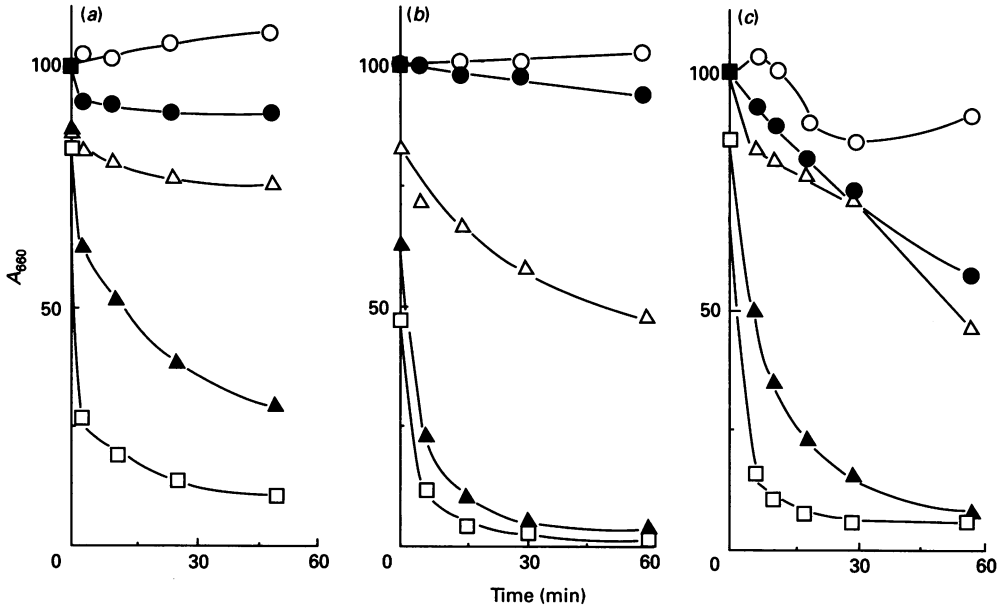

Fig. 2. Turbidimetric clarification of protoplast suspension

Protoplasts were prepared from alkaliphilic *Bacillus* sp. C-125 grown in alkaline medium (*a* and *c*) and from neutrophilic *Bacillus subtilis* GSY1026 grown in neutral medium (*b*) at 30 °C. The protoplasts were suspended in high-osmotic-pressure media which had been adjusted to pH 7.0 (○), 7.8 (●), 9.0 (△), 9.9 (▲) or 10.7 (□) and prewarmed to 30 °C. The pH values are those immediately after the protoplasts were suspended in the buffers. The initial A_{660} of the suspensions was about 0.6. The suspensions were incubated at 30 °C with (*c*) or without (*a* and *b*) shaking, and the A_{660} was periodically measured. The graph shows the A_{660} measured at times indicated as a percentage of initial A_{660}.

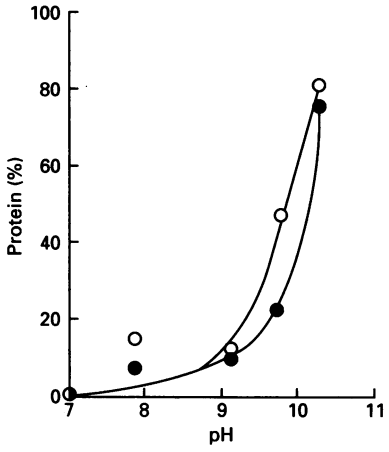

Fig. 3. Release of protein from the protoplasts exposed to alkaline pH

Protoplasts were prepared from alkaliphilic *Bacillus* sp. C-125 (●) or neutrophilic *Bacillus subtilis* GSY1026 (○) as described in legend of Fig. 2. The protoplasts were incubated at various pH values at 30 °C for 20 min. Initial A_{660} of the suspensions was about 1. The pH values shown are those immediately after the protoplast was suspended in the buffers. A sample of the suspension was withdrawn, diluted 10-fold with water and sonicated briefly to determine total cellular protein. The suspensions were centrifuged at 1000 *g* for 30 min at 10 °C. Protein in the supernatant was determined by the method of Bradford (1976), with BSA as a standard. The graph shows the amount of protein released from the protoplasts as a percentage of total cellular protein.

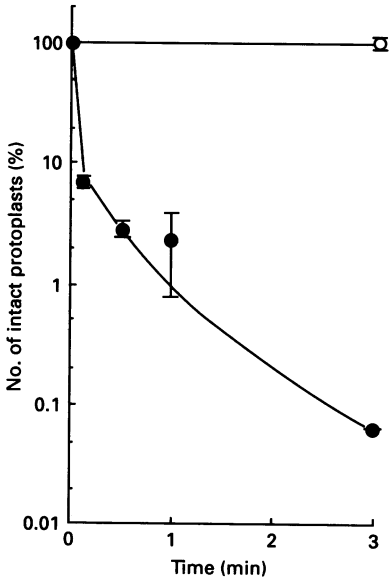

Fig. 4. Death of protoplasts exposed to alkaline pH

The protoplasts were prepared from strain C-125-002 and incubated in SMMP medium of pH 10.6 (●) or pH 7.2 (○) at 30 °C. Periodically the suspension was diluted with SMMP medium of pH 7.2 and spread on the neutral regeneration medium. Colonies formed at 37 °C by 4 days were counted. The examination was carried out in duplicate.

dispersant. We have therefore tested whether the clarification of the protoplast suspension observed at alkaline pH was caused by bursting of the protoplasts or not. Fig. 3 shows liberation of cellular protein from the protoplasts exposed to various pH values for a short period. The amount of protein released into the HOPP media was dependent on the pH of the suspension. At alkaline pH, the suspension became extremely viscous, probably owing to release of chromosomal DNA. These results indicated that cytoplasmic material was released from the protoplasts, and indicated that clarification of the protoplast suspension was caused by bursting of the protoplasts.

Alkaline-pH-dependent liberation of protein was also found in

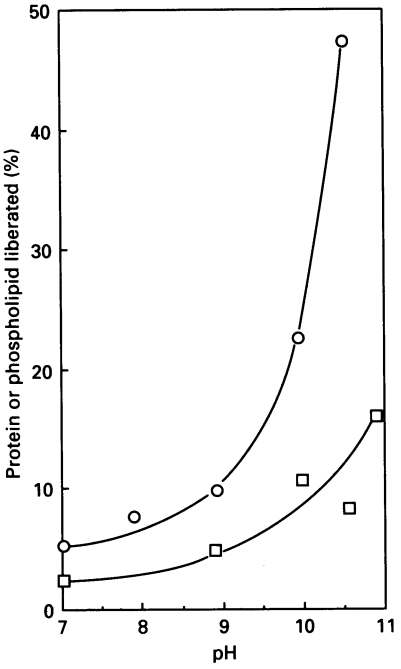

Fig. 5. Liberation of protein and phospholipid from membrane vesicles at alkaline pH

Cytoplasmic membrane fraction was prepared from protoplast of alkaliphilic *Bacillus* sp. C-125 by bursting in low-osmotic-pressure buffer. The membrane preparation was suspended at a concentration of 4 mg of protein/ml and exposed to various pH values at 30 °C for 30 min. The pH value in the Figure is that immediately after suspension of the membrane in the buffers. The suspension was centrifuged at 100000 *g* for 30 min at 4 °C, and the protein content of the supernatant was determined. Lipid in the supernatant was extracted with chloroform/methanol. The phosphorus content in the extract was determined by the molybdate method. Protein and phospholipid in the cytoplasmic membrane without incubation in the buffer were also determined. The graph shows percentages of the total protein (○) and phospholipid (□) liberated from the membrane fraction.

protoplasts prepared from neutrophilic *B. subtilis* GSY1026. The liberation was almost identical for the protoplasts of the two organisms. Liberation of protein was not found at pH 7–9. About 80% of the cellular protein, which might be mainly cytoplasmic proteins, was liberated from both protoplasts exposed to pH 10.2 within 20 min.

(iii) Death of intact protoplasts at alkaline pH. Cell walls were not regenerated by the protoplasts of the organism at alkaline pH, as described above (Fig. 1). Colony formation by the protoplasts exposed to alkaline pH was periodically examined in the regeneration medium of pH 7.1. The colony-forming ability decreased rapidly in the protoplasts exposed to high alkaline pH (Fig. 4). The number of colonies formed by protoplasts decreased to 0.08% of the initial number after 3 min incubation at pH 10.6. These results indicated that intact protoplasts capable of regeneration were rapidly killed at high alkaline pH at which the organism was able to grow.

Liberation of the constituents from membrane vesicles from alkaliphilic *Bacillus* sp. C-125 at alkaline pH

A membrane fraction was prepared from the protoplasts of strain C-125 by lowering the osmotic pressure. The membrane preparations were incubated at various pH values and then centrifuged at 10000 *g* for 30 min. Proteins and phospholipids that were constituents of the membrane were sedimented together with the membrane fraction exposed to pH 7–9. Membrane vesicles exposed to pH values above 10 liberated these constituents in the supernatant solution (Fig. 5). These results indicated that the membrane of the alkaliphilic organism was structurally labile at high alkaline pH, in common with membranes prepared from the usual neutrophilic micro-organisms.

DISCUSSION

Formation and maintenance of protoplasts from alkaliphilic *Bacillus* sp. C-125

Alkaliphilic *Bacillus* sp. C-125 is shaped into rod-form cells by a rigid layer composed of A1γ-type peptidoglycan, like those of neutrophilic *B. subtilis* GSY1026 and other alkaliphilic *Bacillus* spp. (Aono *et al.*, 1984). Treatment with lysozyme readily converted the rods in the exponential phase of growth into round protoplasts within 30 min under the conditions used in the present study. Typically, the preparation contained 3.2×10^{10} protoplasts and 1.0×10^{3} intact cells per ml, when the number of the protoplasts was counted with Thoma's haematocytometer and that of intact cells was counted as viable cells capable of growth on a low-osmotic-pressure medium. Among the protoplasts, at least 5% formed colonies on a high-osmolarity neutral medium. These results indicated that the preparation contained few intact cells on comparing the protoplasts capable of regeneration under the high osmotic pressure.

Regeneration of cell walls by protoplasts at neutral pH

Regeneration of cell walls by protoplasts of strain C-125 was highly dependent on regeneration pH (Fig. 1), leading to the following two possibilities. Firstly, protoplasts of the organism might be killed at alkaline pH. This possibility would imply that the protoplast was unstable at alkaline pH, even though nutrients and air were supplied for the protoplasts in the regeneration medium. This means that the organism requires its cell wall layer for stabilization of its cytoplasmic membrane or cytoplasm, or for full maintenance of biological functions provided by the membranes of protoplasts. The outer surface of the protoplast membrane would not be expected to have a barrier function against OH^- ions, irrespective of energy production.

If the protoplasts had not proved unstable at alkaline pH, the other possibility could be considered: synthesis of cell-wall components by the protoplasts might be extremely dependent on the external pH, although the protoplast itself might be stable. This possibility would imply that the outer surface of protoplasts was not maintained at neutral pH, even though the protoplasts were present in an environment which permitted the organism to produce energy. This would mean that functions on membrane losing cell walls are not enough to maintain the outer surface of protoplasts at neutral pH.

Instability of the protoplasts at alkaline pH

The clarification of a protoplast suspension and release of cytoplasmic components from protoplasts indicated that the protoplasts should be unstable at alkaline pH (Figs. 2 and 3). Instability of protoplasts may suggest that the protoplast membrane of the alkaliphile strain C-125 should be fragile at alkaline pH. However, these properties (decrease in turbidity and release of cytoplasmic materials) might be based on the physical or chemical nature of the membrane and not on biological activities in intact protoplasts.

On the other hand, there are various methods to evaluate biological activities of protoplasts, e.g. uptake of nutrients (Guffanti *et al.*, 1978; Kitada & Horikoshi, 1980), maintenance of intracellular pH (Krulwich *et al.*, 1982), respiration (Kitada *et al.*, 1983), ATP formation (Guffanti *et al.*, 1981). But these

properties of alkaliphiles have not been extensively measured at high alkaline pH, at which the properties must be described, probably because protoplasts or membrane vesicles are labile at that pH (Figs. 2 and 5). One of the most biologically meaningful evaluations is the measurement of the colony-forming ability of protoplasts by regeneration of cell walls (Figs. 1 and 4). The protoplasts of alkaliphilic *Bacillus* sp. C-125 regenerated cell walls and formed colonies only at neutral pH (Fig. 1). The intact protoplasts lost colony-forming ability immediately after exposure to alkaline pH (Fig. 4). Therefore, an instability of the protoplasts at alkaline pH was confirmed also by their biological properties. These results support the first possibility, that the protoplast membrane is unstable at alkaline pH, rather than the second possibility, pH-dependence of cell wall synthesis, mentioned above.

All the results concerning stability of protoplasts indicated that protoplasts were susceptible to alkaline pH at which the organism grew well, although the protoplasts were more stable than those of neutrophilic strain GSY1026. The instability of protoplasts from both strains is interesting, because strain C-125 grows at pH 11 and strain GSY1026 cannot grow at pH above 9 (Aono & Ohtani, 1990).

Putative function of cell wall layer for growth of the organism in an alkaline environment

A pH difference is present between the inside and the outside of cells growing in an alkaline environment. An Na^+–H^+ antiporter on the cell membrane seems to take part in creating the pH difference (Krulwich *et al.*, 1982). If intracellular pH could be maintained only by the porter, a region of the cell wall would be alkaline. Therefore one can suppose that the cytoplasmic membrane of the organism grown at alkaline pH would be exposed to alkaline pH. At least, the outer surface of the cytoplasmic membrane must be stable at alkaline pH. However, the results described here indicate that the protoplasts of the facultative alkaliphile C-125 are not stable at alkaline pH values (Figs. 1–4). It is likely that the outer surface of the protoplast membrane has no barrier capacity against a high concentration of OH^- ions. Also, the membrane vesicles were unstable at alkaline pH (Fig. 5). Provision of nutrients and air did not give the protoplast stability at alkaline pH (Figs. 1 and 2*c*).

Lysozyme digests peptidoglycan and removes non-peptidoglycan components away from the cells together with the peptidoglycan layer. Therefore the results described in the present paper indicate that the cell-wall components contribute to the ability of strain C-125 to grow in a high-alkaline environment (Aono & Horikoshi, 1983; Aono, 1985). The outer surface of the cytoplasmic membrane of intact cells is directly in contact with the cell wall, but not with an extracellular environment. Negative charges on the acidic non-peptidoglycan components of the cell wall of the organism should form an anionic environment around the cytoplasmic membrane. We propose here that the anionic environment in cell walls of the organism might be an electrochemical shelter for the alkali-unstable cytoplasmic membrane of the cells growing at alkaline pH, by attenuation of pH around the outer surface of the membrane to values below 9, at which the cytoplasmic membrane was relatively stable.

This work was partially supported by a grant for 'Biodesign Research Program' from RIKEN to R.A.

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Received 29 October 1991/13 January 1992; accepted 29 January 1992