ADAPTATION PROCESSES IN ALKALIPHILES WHEN CELL WALL ACIDITY IS ELEVATED

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Contents

Summary

The cell walls of alkaliphilic strains of *Bacillus* spp. consist of peptidoglycans and acidic polymers. The acidic polymers are linked to the peptidoglycans through acid labile linkages. The polymers found in the alkaliphiles are classified into three types: teichoic acids, teichuronic acids, and teichuronopeptides. Teichuronopeptides are formed by covalent combination of two acidic polymers, polyuronates and acidic polypeptides. Teichuronopeptides with molecular masses of approximately 21 kDa are distributed among the alkaliphilic isolates of *Bacillus* spp. although they are not found in neutrophilic strains. One half of the isolates produce teichuronopeptide, with each strain having a specific amino acid composition. The facultative alkaliphile strain C-125 produces teichuronic acid and teichuronopeptide in the cell walls. C-125 teichuronic acid is composed of glucuronic acid, galacturonic acid, and *N*-acetylfucosamine. C-125 teichuronopeptide is composed of poly-γ-L-glutamic acid and poly-α- and -β-1,4-glucuronic acid. A mutant defective in its synthesis of teichuronopeptide was constructed from C-125. This mutant showed sensitivity to high pH (alkaline conditions). The gene *tupA*, cloned from the C-125 chromosome, directs teichuronopeptide synthesis in the mutant. Upon the introduction of *tupA*, the mutant restores the teichuronopeptide synthesis and the alkaliphily. Anionic charges present in the cell walls aid intracellular pH homeostasis in the strain exposed to alkaline pH.

1. Introduction

Each microorganism thrives only in its intrinsically appropriate range of pH, and ceases to grow out of the pH range. Each microorganism has developed the ability to grow as an adaption to the environmental conditions of its habitat. Most of microorganisms that have been investigated eagerly, such as *Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisae*, have developed in moderate environments similar to those appropriate for humans. These microorganisms have developed without contact with alkaline environments, and thus have not needed to adapt to alkaline environments with pH above 9.

Certain microorganisms can thrive even at pH 9–11. These are called alkaliphiles or alkali-tolerants to emphasize their abilities to grow in alkaline conditions. Most microorganisms, which grow only at neutral pH, could be called neutrophiles. However, there is not a precise definition of alkaliphiles or alkali-tolerants. One of the definitions has been proposed as follows: alkaliphile, a microorganism which grows the most actively at pH above 9; and alkali-tolerant, a microorganism which can survive or grow at pH above 9 but grows the most actively in a range of pH 7–9. This definition is clear as a basic concept but experimentally complex when applied to each microorganism because the culture pH changes during growth. These different pH conditions for growth optima are not distinguished in this article. As a concept, alkaliphilic microorganisms are divided into two groups: ones that grow only at alkaline pH above 8 but not at pH 7; and ones that grow not only at alkaline pH but also at neutral pH. For convenience, the former is called an absolute alkaliphile and the latter a facultative alkaliphile.

Growth of neutrophiles is hampered in very alkaline environments. Probably several physiological functions are damaged in the neutrophilic cells that are exposed to high pH. One is alkalinization of the cytoplasm, which is readily caused by penetration of

hydroxyl ions into the cells or release of hydrogen ions from the cells. This alkalinization interferes with various intracellular metabolisms. The second is deterioration of the proton motive force on the cell membrane, which is inevitably caused by a pH gradient across the membrane (a high pH outside and a low pH inside). This deterioration would interfere with uptake of substrates and result in lowering of ATP synthesis. The third is structural disturbance of the membrane. The disturbance would result in decrease in barrier functions for substrates and ions, and destroy functional arrangement of membrane enzymes. These damages seem to seriously inhibit the growth of neutrophiles. Therefore, certain physiological functions resolving these problems are essential for alkaliphily of microorganisms. These functions are expected to act actively on the cell surface structures, including the cell membranes and cell walls, to protect the intracellular compartment from the alkalinization and the membrane from the disturbance.

Many alkaliphilic microorganisms which grow at pH 9–10 have been isolated, so far mainly for industrial applications of the alkaline enzymes produced by them. Predominant isolates are bacteria belonging to the genus of *Bacillus*, although alkaliphiles are distributed throughout the archaea, eukaryote, and prokaryote kingdoms. Alkaliphilic or alkali-tolerance mechanisms whereby alkaliphiles can thrive in alkaline conditions have been studied extensively for a few absolute or facultative alkaliphile strains of *Bacillus*.

2. Growth pH Ranges of Alkaliphilic Microorganisms

2.1. Diversity of Physiological Properties of Alkaliphilic Strains of *Bacillus* spp.

Many alkaliphilic microorganisms have been isolated from soils taken from several areas with various different environmental conditions. For the isolation, the alkaliphiles have been selected using alkaline complex media that are highly nutritive. One of the typical isolation media is composed of 1% Na_2CO_3, 1% glucose, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 2% (mass per volume) agar. Most of the alkaliphiles outgrown on the complex medium incubated at 37 °C are bacteria belonging to several species of the genus *Bacillus*. These alkaliphilic isolates of *Bacillus* spp. are different in their growth characteristics, such as the range of pH required for growth, and their requirements for vitamins and sodium ions for growth. Most of them can grow not only in the complex medium but also in an alkaline synthetic medium consisting of 10.6 g of Na_2CO_3, 13.7 g of K_2HPO_4, 5.9 g of KH_2PO_4, 5 g of glucose, 1 g of $(NH_4)_2SO_4$, 1 g of KNO_3, 0.34 g of citric acid, and 0.05 g of $MgSO_4 \cdot 7H_2O$ (per L of deionized water). The pH of this synthetic medium is about 10. By using 11.7 g of NaCl instead of Na_2CO_3, this medium can be used as a neutral synthetic medium, with pH 7, to culture facultative alkaliphiles of *Bacillus* spp. Some strains require vitamins to grow in the synthetic media.

Figure 1 shows typical features of growth of the isolates in an alkaline semi-synthetic medium with a composition similar to that described above. A low concentration of yeast extract (0.04%) was added to the medium for the purpose of a convenient vitamin supply. There are two types of isolates with regard to sodium requirements for growth. Approximately two-thirds of the *Bacillus* spp. isolates require a considerable level of

sodium ions to grow, irrespective of the culture pH. As an example, growth of *Bacillus* sp. strain C-125 is shown in panel A. The strain scarcely grew in the medium not containing sodium ions. The growth of the strain was fully developed by the addition of sodium ions at a level above 20 mmoles L^{-1}. Potassium ions could not be substituted for by sodium ions for the growth development of C-125. Some isolates do not clearly require sodium ions for growth. The growth rates of these organisms were improved by the addition of a considerable level of potassium ions. Strain M-29 grew despite the lack of addition of sodium ions (Figure 1B) although a small amount of sodium ions were supplied from the yeast extract added to the medium. The growth rate of strain M-29 was stimulated by the addition of 100–200 mmole L^{-1} potassium ions. Strain M-29 might require only a small amount of sodium ions. These differences might suggest that the alkaliphilic or alkali-tolerant mechanisms are somewhat different among the isolates.

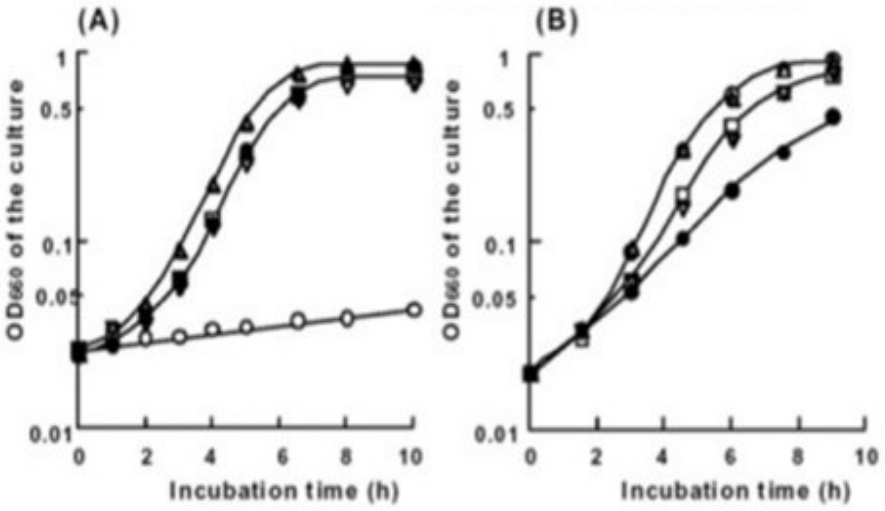

Figure 1. Cation-dependent growth of alkaliphilic strains of *Bacillus* spp.

2.2. Growth pH Ranges of Alkaliphilic Strains of Bacillus spp. and Their Alkaline pH-Sensitive Mutants

The growth pH ranges have been examined for several alkaliphiles by varying the initial culture pH. Figure 2 shows the growth of facultatively alkaliphilic strain C-125 in the complex medium of which initial pH was adjusted to various values. The medium contained 0.2 mole L^{-1} sodium and potassium ions. The culture pH was not controlled during the growth. Strain C-125 grew with pH ranging from 6.7 to 10.7. On the other hand, *Bacillus subtilis* GSY1026, a typical neutrophilic strain, grew at pH below 8.5.

Several types of mutants for which growth pH ranges were genetically altered have been constructed from alkaliphiles. Figure 2 shows growth of the three types of alkaline pH-sensitive mutants isolated from C-125. A mutant strain AS-409 was sensitive only to high pH. This mutant grows at pH 9.5, and thus has kept the alkaliphily as defined in the Introduction (Section 1). AS-350 was more sensitive to alkaline pH. It is interesting that though the upper limit of the growth pH range of AS-350 is similar to that of *B. subtilis*, this mutant has lost its alkaliphily. The lower limits of the growth pH ranges of

these two mutants are not changed from that of the parent strain C-125. Mutant AS-399 was highly sensitive to alkaline pH. The growth of this mutant was low even at pH 8. This mutant became sensitive also to neutral pH. Isolation of these different types of alkaline pH-sensitive mutants indicates that alkaliphily is controlled genetically by several physiological functions.

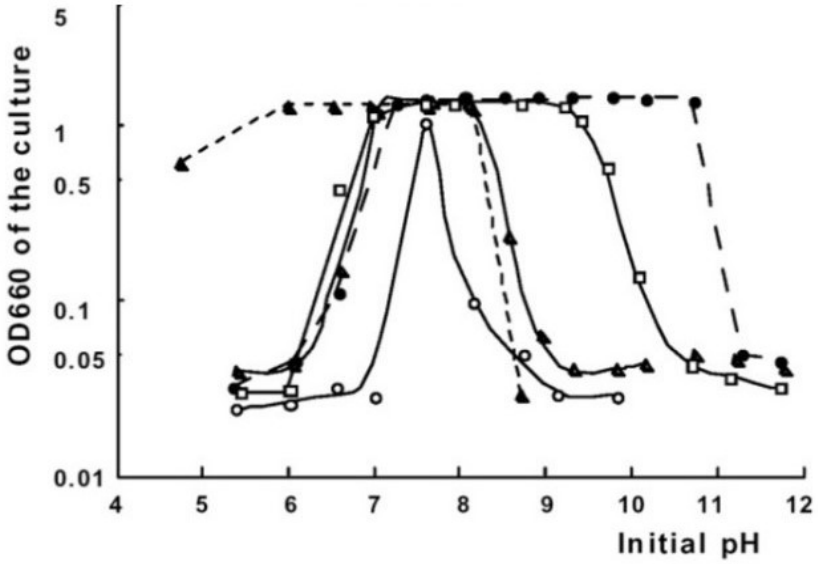

Figure 2. Initial pH-dependent growth of *Bacillus* sp. C-125 and its alkali-sensitive derivatives. Reproduced from Aono R., et al. (1992). *Biosci. Biotechnol. Biochem.* **56**, 842–844.

2.3. Growth pH Ranges of Protoplasts Prepared From Alkaliphilic Strains of *Bacillus* spp.

Egg white lysozyme hydrolyzes peptidoglycans of alkaliphilic strains of *Bacillus* spp. The viable cells are readily changed to protoplasts by the lysozyme treatment under hypertonic conditions. These protoplasts regenerate the cell walls and outgrow on an appropriate regeneration medium. The protoplasts prepared from four alkaliphilic strains of *Bacillus* spp. were outgrown on the regeneration medium consisting of 2% (mass per volume) glucose, 0.5% yeast extract, 0.5% casamino acids, 0.04% bovine serum albumin, 1% agar, 0.5 mole L^{-1} monosodium succinate, 30 mmole L^{-1} $MgCl_2$, 1.25 mmole L^{-1} $CaCl_2$, and 30 mmole L^{-1} Tris. The initial pH was adjusted to various values by the addition of HCl or NaOH. Colony formation by the protoplasts on the regeneration medium was monitored for four days at 37 °C. The outgrowth was dependent on the culture pH (Figure 3). No L-form cells were found in the colonies. Among the strains used, strain 2B-2 is a strict alkaliphile. The others are facultative alkaliphiles. Protoplasts derived from any strain did not outgrow at pH above 9, meaning that the intrinsic alkaliphily was lost in the protoplasts. In particular, the outgrowth of protoplasts of strains 2B-2 and C-125 was remarkably dependent on the culture pH. These protoplasts outgrew only around lower limits of the growth pH ranges of the cells. These results seem curious taking into consideration that the cells of these strains grow at alkaline pH above 10. It must be concluded that the cell walls enhance

the alkaliphily or alkali-tolerance of the cells although the magnitude of the aid is different among the strains.

The protoplasts of strain C-125 were incubated at pH 10.6 and their viability was monitored by measuring the frequency of the outgrowth on the neutral regeneration medium (Figure 4). The growth ability decreased rapidly in the protoplasts incubated at high pH, whereas growth was maintained in those incubated at neutral pH. The decrease found at alkaline pH is likely due to bursting of the protoplasts. Thus, C-125 protoplasts are unstable at high pH. This instability is similar to that of protoplasts prepared from *B. subtilis*. Therefore, the C-125 protoplasts are not only unable to grow at alkaline pH but also are killed in alkaline environments. This is a feature characteristic of neutrophiles. It is likely that at least the cell walls are involved in the alkaliphily of strain C-125.

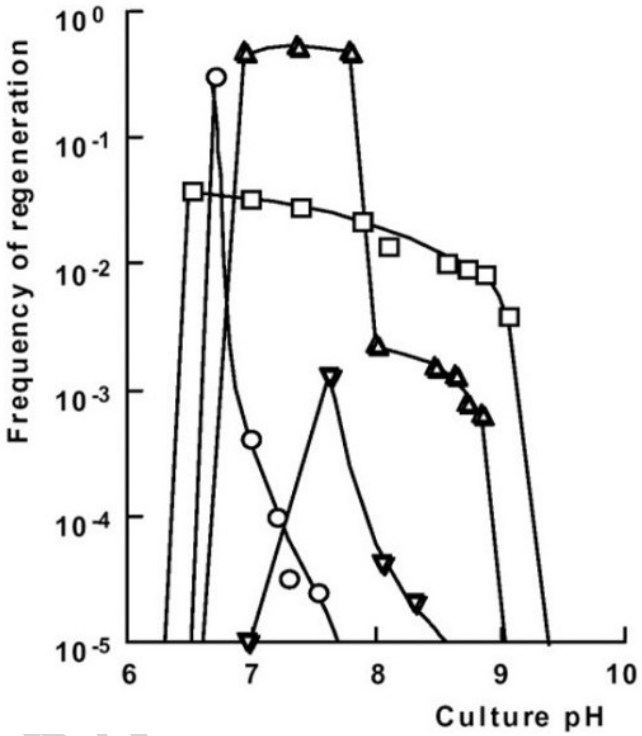

Figure 3. Colony formation by the protoplasts of alkaliphilic strains of *Bacillus* spp. Reproduced from Aono R. et al. (1993). *Biosci. Biotechnol. Biochem.* **57**, 1597–1598.

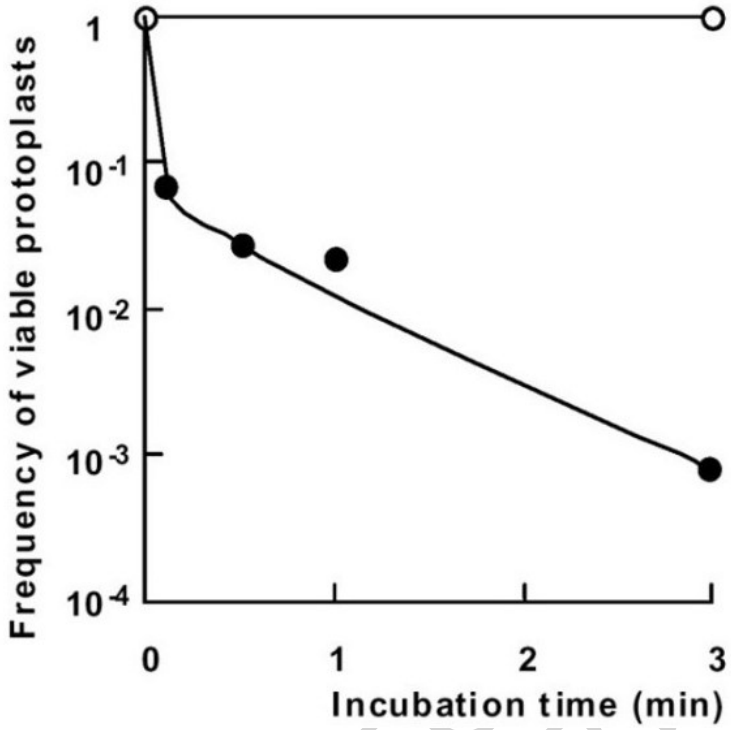

Figure 4. Death of the protoplasts exposed to alkaline pH
Reproduced from Aono R. et al. (1992). **Biochem J. 285**, 99–103.

3. Cell Surface Structure of Alkaliphilic Strains of Bacillus spp.

3.1. Structural Cell Wall Components of *Bacillus* spp.

The cytoplasms of alkaliphilic strains of *Bacillus* spp. are enveloped by cell membranes which are surrounded by cell walls, as is well known in neutrophilic strains. The cell walls provide rigidity and maintain the intrinsic shape of the bacterial cells. Generally, the cell walls are the outermost layers of the cells. In the alkaliphilic strains, the cell walls are in contact with the alkaline environment directly. Figure 5 shows the ultra cell surface structures of strain C-125 grown at pH 7 or 10. The cell walls of *Bacillus* spp. are composed of three main components: peptidoglycan, proteins, and polysaccharides or polyol compounds.

Peptidoglycan is composed of glycan strands substituted with short peptide chains. The glycan strands are generally composed of *N*-acetylglucosamine and *N*-acetylmuramic acid. The saccharides are rarely modified. The peptide moieties are linked to the *N*-acetylmuramic acid residues through peptide linkages. The peptides are greatly different in their amino acid compositions among bacteria belonging to different taxa. In the vast majority of strains of the genus *Bacillus*, the peptides are L-alanine-D-glutamic acid-*meso*-diaminopimelic acid-D-alanine. This type of peptide is called A1γ type. Adjacent peptide moieties are directly cross-linked between ω-amino group of diaminopimelic acid and γ-carboxyl group of D-alanine. Consequently, the peptidoglycans form mechanically rigid three-dimensional net-frame structures outside of the membrane. The

carboxyl group of glutamic acid or diaminopimelic acid is amidated depending on the species.

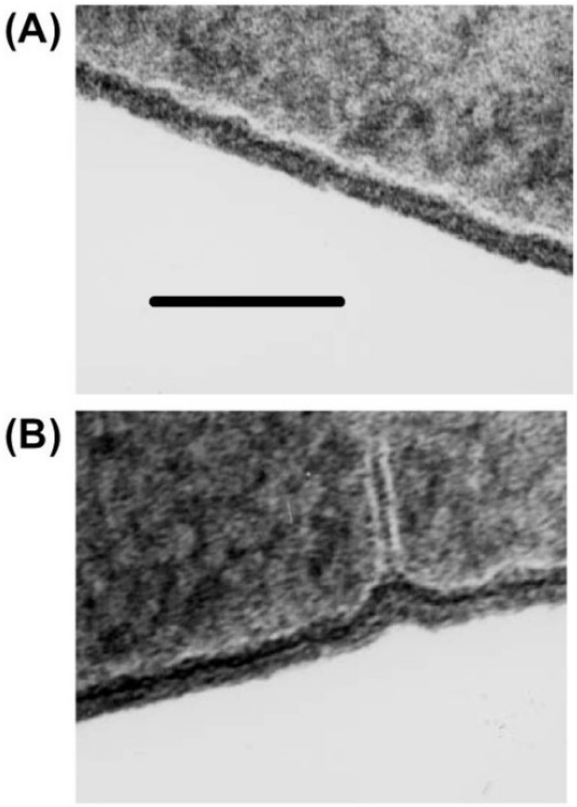

Figure 5. Electron micrographs of cell walls of strain C-125
Reproduced from Aono R. et al. (1995) *Microbiology* **141**, 2955–2964.

The cell walls contain several kinds of proteins. Proteins excreted from the cells are trapped transiently in the peptidoglycan layers. Enzymes involved in turnover of the peptidoglycans, such as autolytic enzymes, are functional in the cell wall layers. In some strains, surface-layer proteins also surround the cells. The cell walls of most Gram-positive bacteria possess polyol compounds and polysaccharides. These polymers are called teichoic acids and teichuronic acids, respectively. Chemical compositions of teichoic acids and teichuronic acids are greatly different among bacteria. Teichoic acids are composed of phosphoric acid and glycerol or ribitol. In several cases, these polyols are substituted with hexoses or hexosamines. Teichuronic acids are composed of hexosamines and hexuronic acids. It is known that these compounds replace one other in response to culture conditions.

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Bibliography

Aono R. (1985). Isolation and partial characterization of structural components of the walls of alkalophilic *Bacillus* strain C-125. *J. Gen. Microbiol.* **131**, 105–111. [This describes a method to isolate teichuronopeptide from alkaliphilic strains.]

Aono R. (1990). The poly-α- and -β-1,4-glucuronic acid moiety of teichuronopeptide from the cell wall of the alkalophilic *Bacillus* strain C-125. *Biochem. J.* **270**, 363–367. [Teichuronopeptide was named in this manuscript.]

Aono R. and Horikoshi K. (1983). Chemical composition of cell walls of alkalophilic strains of *Bacillus*. *J. Gen. Microbiol.* **129**, 1083–1087. [This represents a cell wall preparation from alkaliphilic strains of *Bacillus* spp.]

Aono R., Ito M., and Horikoshi K. (1992). Instability of the protoplast membrane of facultative alkaliphilic *Bacillus* sp. C-125 at alkaline pH values below the pH optimum for growth. [This discusses the alkali-instability of the membrane.]

Aono R., Ito M., and Horikoshi K. (1997). Measurement of cytoplasmic pH of the alkaliphile *Bacillus lentus* C-125 with a fluorescent pH probe. *Microbiology* **143**, 2531–2536. [This presents data about the cytoplasmic pH of an alkaliphilic strain.]

Aono R., Ito M., and Machida T. (1999). Contribution of the cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphile *Bacillus lentus* C-125. *J. Bacteriol.* **181**, 6600–6606. [This presents information on the *tupA* gene.]

Gilmour R., Messner P., Guffanti A.A., Kent R., Scheberl A., Kendrick N., and Krulwich T.A. (2000). Two-dimensional gel electrophoresis analyses of pH-dependent protein expression in facultatively alkaliphilic *Bacillus pseudomonas* OF4 lead to characterization of an S-layer protein with a role in alkaliphily. *J. Bacteriol.* **182**, 5969–5981. [This shows the presence of the S layer in an alkaliphile.]

Horikoshi K. (1999). *Alkaliphiles*, XXX pp. Tokyo: Kodansya. [This book presents a discussion on the various general aspects of alkaliphes.]

Kitada M., Hashimoto M., Kudo T., and Horikoshi K. (1994). Properties of two different Na^+/H^+ antiport systems in alkaliphilic *Bacillus* sp. strain C-125. *J. Bacteriol.* **176**, 6464–6469. [This article presents properties of C-125 Na^+/H^+ antiporters.]

Krulwich T.A., Ito M., Gilmour R., Hicks D.H., and Guffanti A.A. (1998). Energetics of alkaliphilic *Bacillus* species: physiology and molecules. *Advances in Microbial Physiology* **40**, 401–438. New York: Academic Press. [This discusses general aspects of the energetics of alkaliphilic *Bacillus* spp. in alkaline environments.]

Biographical Sketch

Rikizo Aono was born in Tokyo, Japan, in June 1948. He obtained a BSc in Agriculture in 1972and a PhD in 1977 from Tokyo University . He joined the academic staff at Riken Institute (Physical and Chemical Research Institute), Saitama, Japan, as a research assistant in 1977, and the staff at Yamanashi University, Yamanashi, Japan, as a research assistant in 1979. He was an associate professor at Yamanashi University from 1982 to 1988, and at Tokyo Institute of Technology from 1988 to 1999. He has been a professor of biological information at the Tokyo Institute since 1999.

His research activities have been in the field of microbiology in extreme environments. In particular, he has been concerned with adaptation mechanisms by which alkaliphiles grow in alkaline environments. He proposed that cell surface structures of alkaliphiles could display biological function, not only maintaining the cell shape but also developing alkaliphily of the organisms. In addition, he and his research team have investigated microbial resistance to harmful organic solvents. He has published 130 reviews and original articles in scientific journals or books.