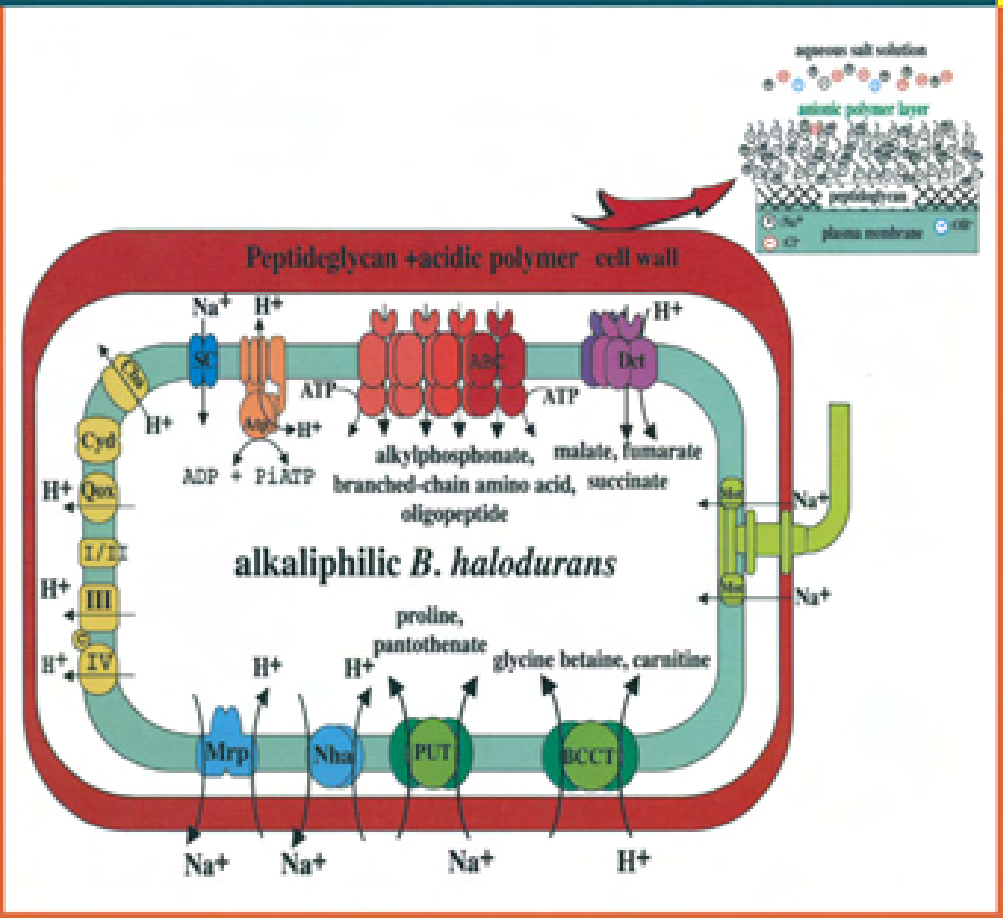
Koki Horikoshi



Alkaliphiles

Genetic Properties and Applications of Enzymes

Kodansha

Springer

Koki Horikoshi

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–Genetic Properties and Applications of Enzymes–

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Alkaliphiles

Genetic Properties and Applications of Enzymes

With 108 Figures and 60 Tables

Kodansha

Springer

Koki Horikoshi Professor Emeritus, Tokyo Institute of Technology and RIKEN Institute, Japan
Director General, Japan Agency for Marine-Earth Science and Technology(JAMSTEC)

ISBN 4-06-211513-1 Kodansha Ltd., Tokyo

ISBN-10 3-540-33372-X Springer Berlin Heidelberg New York
ISBN-13 3-540-33372-2 Springer Berlin Heidelberg New York

Library of Congress Control Number: 2006926727

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To my wife, Sachiko

Preface

> "If I have seen a little further it is by standing on the shoulders of Giants." Letter to Robert Hooke, Isaac Newton, February 5, 1676

In November 1956, I encountered an alkaliphilic bacterium, although not alkaliphilic in the true sense of the word. I was a graduate student in the Department of Agricultural Chemistry, University of Tokyo, working under the direction of Professor Kin-ichiro Sakaguchi. Autolysis of *Aspergillus oryzae* was the research theme for my doctoral thesis. The reason why Professor Sakaguchi asked me to study the autolysis of *Asp. oryzae* was a somewhat practical one. He thought the flavor and taste of Japanese *sake* came from an autolysate of *Asp. oryzae.* Every day, I cultured stock strains of *Asp. oryzae.* After one week of culture, all I had to do was taste the cultured fluid. (I used my *bero-meter, bero* meaning tongue in Japanese.) Naturally, I was entirely disappointed in microbiology itself.

One day in November, I found one cultivation flask in which mycelia of *Asp. oryzae* had completely disappeared. The night before, when I looked at the flasks, the mold was flourishing in all culture flasks. I still remember vivid pictures of bacteria thriving and moving. No mycelium could be seen under the microscope.

The microorganism isolated from that flask was *Bacillus circulans,* and strong endo-1,3-β-glucanase activity was detected in the culture fluid. This enzyme lyzed *Asp. oryzae.* It was the first time that mold cells had been found to be lyzed by bacteria, and these results were published in *Nature* (Horikoshi et al., 1958). However, this bacterium showed very poor growth in the absence of mycelia of *Asp. oryzae* and production of endo-1,3-β-glucanase was very low. Therefore, purification of endo-1,3-β-glucanase could be done only in culture fluid in the presence of mycelia of *Asp. oryzae.* I did not realize at the time that the culture fluid had alkaline pH value. A few years later, I attempted production of endo-1,3-β-glucanase in conventional media. I tested many culture media containing various nutrients. Addition of 0.5% sodium bicarbonate to conventional nutrient culture broth gave good growth and production of the enzyme. Autolysis of *Asp. oryzae* changed the culture medium from weakly acidic to alkaline pH. In this way I discovered that such a change in pH value accelerated bacterial growth and production of the enzyme (see p. 41).

In 1968, I visited Florence, Italy, and saw Renaissance buildings, which are so very different from Japanese architecture. Centuries earlier no Japanese could have imagined this Renaissance culture. Then suddenly I heard a voice whispering in my ear, "There could be a whole new world of microorganisms in different unexplored cultures." Memories of experiments on *B. circulans* done years ago flashed into my mind. Could there be an entirely unknown domain of microorganisms existing at alkaline pH? The acidic environment was being studied, probably because most food is acidic. However, very little work had been done in the alkaline region. Upon my return to Japan I prepared an alkaline medium containing 1% sodium carbonate, put small amounts of soil collected from various areas of the Institute of Physical and Chemical Research (RIKEN), Wako, Japan, into 30 test tubes and incubated them overnight at 37°C. To my surprise, microorganisms flourished in all test tubes. I isolated a great number of alkaliphilic microorganisms and purified many alkaline enzymes. The first paper concerning an alkaline protease was published in 1971.

Then, in 1972, I was talking with my father-in-law, Shigeo Hamada, about alkaliphilic microorganisms. He had been in London almost a century ago as a businessman and was curions about everything. He showed interest in alkaliphiles. These microorganisms were unique, required high alkalinity, and they could produce alkaline enzymes such as alkaline proteases, alkaline amylases, etc. As I was speaking, he said, "Koki, wait a minute, I have an interesting present for you." He brought out a sheet of old newspaper, *Nikkei Shimbun* dated June 11, 1958. A short column with one electron micrograph was like a punch to my head. I had not known this! The article stated:

> In Japan, since ancient times, indigo has been naturally reduced in the presence of sodium carbonate. Indigo from indigo leaves can be reduced by bacteria that grow under high alkaline conditions. Indigo reduction was controlled only by the skill of the craftsman. Takahara and his colleagues isolated the indigo reducing bacterium from a ball of indigo.

I then carefully checked scientific papers from *Chemical Abstracts* in the library of RIKEN. Only 16 scientific papers on alkaliphiles were discovered.* Alkaliphiles remained little more than interesting biological curiosities. No industrial application was attempted at all before 1968. I named these microorganisms that grow well in alkaline environments "alkaliphiles" and conducted systematic microbial physiological studies on them. It was very

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* Johnson, 1928; Downie and Cruickshank, 1928; Vedder, 1934; Jenkin, 1936; Bornside and Kallio, 1956; Chesbro and Evans, 1959; Kushner and Lisson, 1959; Takahara and Tanabe 1960; Chislett and Kushner, 1961; Shislett and Kushner, 196lb; Takahara et al., 1961 ; Takahara and Tanabe, 1962; Wiley and Stokes, 1962; Wiley and Stokes, 1963; Barghoorn and Tyler, 1965; Siegel and Giumarro, 1966.

surprising that these microorganisms, which are completely different from any previously reported, were widely distributed throughout the globe (even at the deepest point of the Mariana Trench in the Pacific Ocean) producing heretofore unknown substances. Here was a new alkaline world that was utterly different from the neutral world.

Over the past three decades my coworkers and I have focused on the enzymology, physiology, ecology, taxonomy, molecular biology and genetics of alkaliphilic microorganisms to establish a new microbiology of alkaliphilic microorganisms. A big question arises, "Why do alkaliphiles require alkaline environments?" The cell surface of alkaliphiles can keep the intracellular pH values about 7–8 in alkaline environments of pH 10–13. How the pH homeostasis is maintained is one of the most fascinating aspects of alkaliphiles. In order to understand this simple but difficult question, we carried out several basic experiments to establish gene recombination systems. Finally, after almost two years, the whole genome sequence of alkaliphilic *Bacillus halodurans* C-125 was completed. This was the second whole genome sequence of spore-forming bacteria thus far reported. This sequence work revealed interesting results. Many genes were horizontally transferred from different genera and different species as well. Small fragments of enzyme genes were inserted in opposite directions, or separated by insertion fragments. Therefore, these bacteria could not produce some enzymes. However, we still have not found the crucial gene(s) responsible for alkaliphily in the true meaning. Our results indicate that many gene products synergistically cooperate and exhibit alkaliphily (or adaptation to alkaline environments).

Industrial applications of these microorganisms have also been investigated extensively and some enzymes, such as alkaline proteases, alkaline amylases, alkaline cellulases and alkaline xylanases have been put to use on an industrial scale. Subsequently, many microbiologists have published numerous papers on alkaliphilic microorganisms in various fields. At the beginning of our studies, very few papers were presented, but now thousands of scientific papers and patents have been published. It is not clear which field our study of alkaliphiles will focus on next, but the author is convinced that alkaliphiles will provide much important information.

The author expresses sincere gratitude to his wife Sachiko for her invaluable help over the past 40 years.

Tokyo, Japan
April 2006

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Part II Enzymes of Alkaliphiles and Their Applications

Part I

Alkaliphiles and Their Genetic Properties

Alkaliphiles have been flourishing everywhere on our planet. Only a few microbiologists, however, have shown interest in this microbial domain.

There are large numbers of alkaliphiles in garden soil, although frequency is about 1/10 to 1/1000 of whole numbers of microorganisms depending on the environment in which they are collected. Why can they thrive in neutral environments as well as alkaline environments? It is because they can change their surroundings from neutral to alkaline pH value by producing basic compounds or by symbiosis. They have their own microcosm. They can also be isolated from excreta of living creatures because the inside of the digestive tract of animals is usually alkaline pH.

Microbiologists had ignored this large domain for a long time. The addition of 1% sodium carbonate opened up this vast unknown microbial domain. The time has come for microbiologists to reconsider conventional anthropocentric microbiology. Many problems, such as why alkaliphiles need alkaline environments remain to be solved. This question is the same as "why do neutrophiles require neutral environments for their growth?"

Other matters for investigation include how substrate molecules (including water) in nano-scale spaces react with enzymes or cell surfaces. What is the pH value in nano-scale volumes? Is it same as in macro-scale volumes?

The following sections describe the isolation, classification, physiology and genetics of alkaliphiles. However, the questions noted above have not yet been solved in their entirety.

1

Introduction

1.1 What Are Alkaliphiles?

There is no precise definition of what characterizes an alkaliphilic organism. As shown in Fig. 1.1, some microorganisms such as *Bacillus pseudofirmus* 2b-2, which are obligate alkaliphiles, cannot grow well at neutral pH values. There are many microbes which can thrive in the range of pH 7 to 10.5. These microbes are called facultative alkaliphiles and include, for example, *Bacillus halodurans* C-125. Furthermore, several microorganisms exhibit more than one pH optimum for growth depending on the growth conditions such as type of nutrients, metal ions and temperature. In the early stages of our study, alkaliphiles were thought to require only higher pH values for growth. In conventional classification experiments, the author noted a strange result. *Bacillus* sp. No. Ku-1, which was thought to be a facultative alkaliphilic strain, could grow in a nutrient broth containing 5% NaCl at pH 7.0, but could not grow in the absence of NaCl (Kurono and

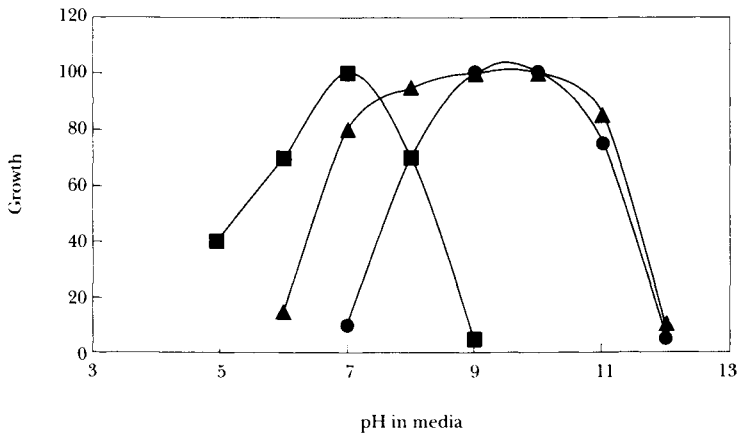


Fig. 1.1 pH dependence of microorganisms. The typical dependence of the growth of neutrophilic bacteria (*Bacillus subtilis*), obligate alkaliphilic bacteria (*Bacillus pseudofirmus* 2b-2) and facultative alkaliphilic bacteria (*Bacillus halodurans* C-125) are shown by solid squares, solid circles and solid triangles, respectively.

Horikoshi, 1973). This was the beginning of physiological studies of NaCl on the growth of alkaliphiles. Some organisms can appear to be either neutrophilic or alkaliphilic depending on the growth conditions in the presence or absence of sodium ions described above. Therefore, this monograph will use a much simpler and less strict definition. The term "alkaliphile" is applied only to microorganisms that grow optimally or very well at pH values above 9.0, but cannot grow or only grow slowly at neutral pH values of 6.5.

1.2 History of Alkaliphiles

The discovery of alkaliphiles is fairly recent. Only 16 scientific papers on the topic could be found when the author started experiments on alkaliphilic bacteria in 1968. The use of alkaliphilic microorganisms has a long history in Japan. Since ancient times (over 1,000 years ago), indigo, used to dye cloth, has been naturally reduced under alkaline conditions in the presence of slaked lime, wood ash and Japanese *sake*. Indigo from indigo leaves is reduced by particular bacteria that grow under these highly alkaline conditions in a traditional process called "indigo fermentation." The most important factor in this process is the control of the pH value. Formerly indigo reduction was controlled only by the skill of craftsmen, adjusting temperature, time and amounts of stepwise addition of the ingredients described above. Microbiological studies of the process, however, were not conducted until the rediscovery of these alkaliphiles by the author (Horikoshi, 1982). Alkaliphiles remained little more than interesting biological curiosities and no further industrial application was attempted.

Later the author and his colleagues isolated a great number of alkaliphilic microorganisms and purified many alkaline enzymes. The first paper concerning an alkaline protease was published in 1971 (Horikoshi, 1971a, b). Over the past three decades, his studies have focused on enzymology, physiology, ecology, taxonomy, molecular biology and genetics to establish a new microbiology of alkaliphilic microorganisms. Industrial applications of these microorganisms have also been investigated extensively and some enzymes, such as alkaline proteases, alkaline amylases, alkaline cellulases and xylanases, have been put to use on an industrial scale. Alkaliphiles have clearly evolved large amounts of information and ability in their genes to cope with particular environments, so their genes are a very valuable source of information waiting to be explored by the biotechnologist.

1.3 Why the Author Selected Alkaliphilic *Bacillus* Strains for Discussion in This Volume

Many alkaliphiles have been isolated from various sources. These include aerobic spore-formers, anaerobic non-spore-formers, halophiles, thermophiles including archaea, psychrophiles, piezophiles and others. However, in this work, the author focuses only on alkaliphilic *Bacillus* strains. This is because almost 2,000 scientific papers on alkaliphiles have been published since 1971, making it difficult to discuss all of them in a meaningful way. Moreover, recently, the whole genome sequences of three alkaliphilic *Bacillus* strains have determined. These data provide much useful information. And finally, the work of the author's group on alkaliphilic *Bacillus* strains paved the way to industrial applications of unique alkaline enzymes produced by alkaliphilic *Bacillus* strains.

2

Isolation, Distribution and Taxonomy of Alkaliphilic Microorganisms

2.1 Isolation

Alkaliphilic microorganisms have been isolated in alkaline media containing sodium carbonate, sodium bicarbonate and potassium carbonate. Sodium hydroxide is also used in large-scale fermenters using a pH control device. The recommended concentration of these compounds is about 0.5% to 2%, depending on the microorganisms used, and the pH of the medium held between about 8.5 and 11. Table 2.1 shows the standard media in the author's laboratory containing 1% sodium carbonate. It is most important that the sodium carbonate be sterilized separately; otherwise the microorganisms may show poor growth.

Isolation of the microorganisms is conducted by conventional means: a small amount of sample, such as soil, mud, sediments and feces is suspended in 1 ml of sterile water and one drop of the suspension is spread on a Petri dish containing Horikoshi-I or Horikoshi-II medium. The Petri dishes are incubated at 37°C for several days and the colonies that appear are isolated by the usual method. Enrichment culture is well known in the isolation of specific microorganisms, such as thermophiles and others. Some alkaliphiles grow very well under enrichment culture in alkaline conditions and are predominantly isolated. Very few slow growers are isolated by enrichment culture. Therefore, in the author's laboratory direct isolation of alkaliphiles from soil samples has been recommended. The isolated microorganisms are then kept at room temperature on slants or by conven-

Table 2.1 Basal media for alkaliphilic microorganisms

Ingredients	Horikoshi-I (g/l)	Horikoshi-II (g/l)
Glucose	10	-
Soluble starch	-	10
Polypeptone	5	5
Yeast extract	5	5
K_2HPO_4	1	1
$Mg_2SO_4 \cdot 7H_2O$	0.2	0.2
Na_2CO_3	10	10
Agar	20	20

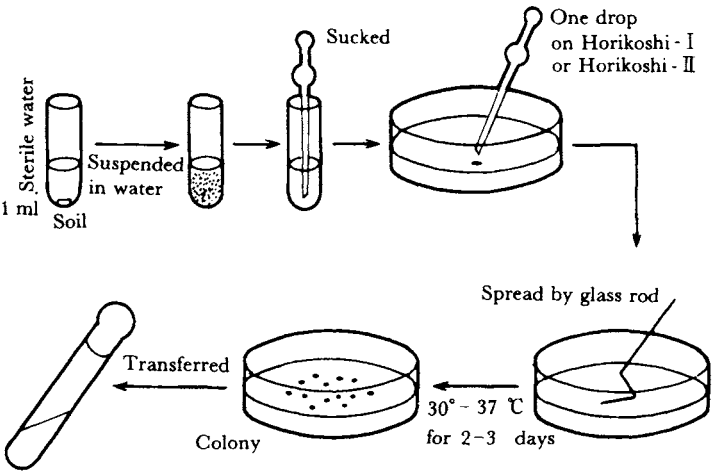


Fig. 2.1 Isolation of alkaliphilic microorganisms.
(Reproduced from K. Horikoshi and T. Akiba, *Alkalophilic Microorganisms: A New Microbial World*, p.10, Springer : Japan Scientific Societies Press (1982))

tional methods, such as by freeze drying or being maintained in liquid nitrogen (Fig. 2.1).

2.2 Distribution

There are many reports on alkaliphilic *Bacillus* strains isolated from soils of moderate environments as well as of extreme environments, such as alkaline lakes, deep sea, subground samples, etc.

2.2.1 Soil Samples

Alkaliphilic microorganisms are widely distributed throughout the world. They can be found in garden soil samples. In addition to bacteria, various kinds of microorganisms, including actinomycetes, fungi, yeast and phages, have been isolated.

Our studies indicate that there may be as many microorganisms in the alkaline pH region as in the neutral region. Alkaliphiles can be considered to be a new microbial world in nature which may be as large as the neutrophilic microbial world. The number of alkaliphilic bacteria found in soil was about 1/10 to 1/100 that of neutrophilic bacteria (Fig. 2.2). Alkaliphilic bacteria have very interesting properties, e.g, the ability to change the pH of their environment to suit their growth, as described in the author's previous volume (Horikoshi and Akiba 1982). Using alkaliphilic microorganisms the author and his colleagues established an alkaline fermentation process that is entirely different from conventional fermentation processes. Hundreds

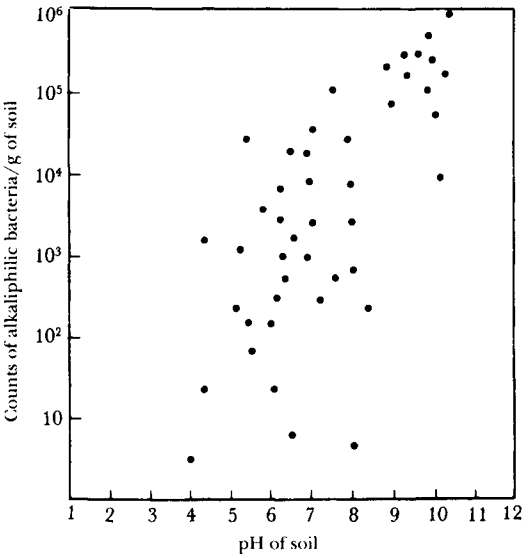


Fig. 2.2 Distribution of alkaliphilic bacteria in soil.
(Reproduced from K. Horikoshi and T. Akiba, *Alkalophilic Microorganisms: A New Microbial World*, p.11, Springer : Japan Scientific Societies Press (1982))

of new enzymes have been isolated by this process and their enzymatic properties investigated. Some of these enzymes have been produced on an industrial scale.

2.2.2 Deep Sea Samples

Deep sea sediment and deep sea water were also very good isolation sources for alkaliphilic *Bacillus* strains. On March 2, 1996, the 3-m long unmanned submersible *Kaiko* of Japan touched the bottom of the Challenger Deep in the Mariana Trench and successfully scooped out a mud sample, the first obtained at a depth of 10,897 m. Takami et al. (1997; 1999a) isolated thousands of microbes from mud samples collected from the Mariana Trench. The microbial flora found at a depth of 10,897 m was composed of actinomycetes, fungi, non-extremophilic bacteria, and various extremophilic bacteria such as alkaliphiles, thermophiles and psychrophiles. Phylogenetic analysis of Mariana isolates based on 16S rDNA sequences revealed that a wide range of taxa were represented as shown Table 2.2 and Fig. 2.3.

In order to explore the microbial diversity in various deep sea environments, Takami et al. attempted to isolate and characterize a number of bacteria from deep sea mud collected by the manned submersibles *Shinkais 2000* and *6500* (Fig. 2.4). They record considerable bacterial diversity and the occurrence of extremophilic bacteria at several deep sea sites located near the southern part of Japan. Hundreds of isolates of bacteria from

Table 2.2 Isolation of extremophilic bacteria from several deep sea sites

Category	Isolation conditions	Origin no.(depth)	Bacteria recovered (colonies g^{-1} dry sea mud)
Alkaliphile	pH 9.7 ± 0.3	163(1050 m)	$3.0–6.1 \times 10^2$
	25°C, 0.1 MPa	214(2759 m)	$0.2–2.3 \times 10^4$
		213(3400 m)	0.9×10^2
		M1(10897 m)†	$0.4–1.2 \times 10^3$
Thermophile	55°C	163(1050 m)	$0.8–2.3 \times 10^2$
	pH 7.3 ± 0.2, 0.1 MPa	214(2759 m)	$1.1–7.8 \times 10^2$
		213(3400 m)	$1.0–6.0 \times 10^2$
		M1(10897 m)†	$0.6–3.5 \times 10^3$
Psychrophile	4°C	163(1050 m)	$0.8–5.3 \times 10^2$
	pH 7.3 ± 0.2, 0.1 MPa	214(2759 m)	$1.4–7.8 \times 10^2$
		213(3400 m)	1.0×10^2
		M1(10897 m)†	2.0×10^2
Halophile	15% NaCl, 25°C	163(1050 m)	4.6×10^2
	pH 7.3 ± 0.2, 0.1 MPa	214(2759 m)	3.6×10^3
		213(3400 m)	0.9×10^2
		M1(10897 m)†	–
Acidophile	pH 3.7 ± 0.2	163(1050 m)	–
	25°C, 0.1 MPa	214(2759 m)	–
		213(3400 m)	–
		M1(10897 m)†	–
Nonextremophile	25°C, 0.1 MPa	163(1050 m)	$0.5–6.6 \times 10^3$
	pH 7.3 ± 0.2	214(2759 m)	$0.2–1.1 \times 10^5$
		213(3400 m)	$8.1–9.4 \times 10^2$
		M1(10897 m)†	$0.2–2.3 \times 10^5$

-, no growth obtained.
† From Takami et al.(1997)

deep sea mud samples were collected at various depths (1050–10897 m). Various types of bacteria such as alkaliphiles, thermophiles, psychrophiles and halophiles were recovered on agar plates at a frequency of 0.8×10^2 to 2.3×10^4/g of dry sea mud. No acidophiles were recovered. These extremophilic bacteria were widely distributed, being detected at each deep sea site, and the frequency of isolation of such extremophiles from the deep sea mud was not directly influenced by the depth of the sampling sites. Phylogenetic analysis of deep sea isolates based on 16S rDNA sequences revealed that a wide range of taxa were represented in the deep sea environments as shown in Fig. 2.3. Growth profiles under high hydrostatic pressure were determined for the deep sea isolates obtained in this study. No extremophilic strains isolated in this study showed growth at 100 MPa, although a few of the other isolates grew slightly at this hydrostatic pressure.

An extremely halotolerant and alkaliphilic bacterium was also isolated from deep sea sediment collected at a depth of 1050 m on the Iheya Ridge, Japan. The strain, designated HTE831 (JCM 11309, DSM 14371), was gram-positive, strictly aerobic, rod-shaped, motile by peritrichous flagella and spore-forming. Strain HTE831 grew at salinities of 0–21% (w/v) NaCl at pH 7.5 and 0–18% at pH 9.5. The optimum concentration of NaCl for

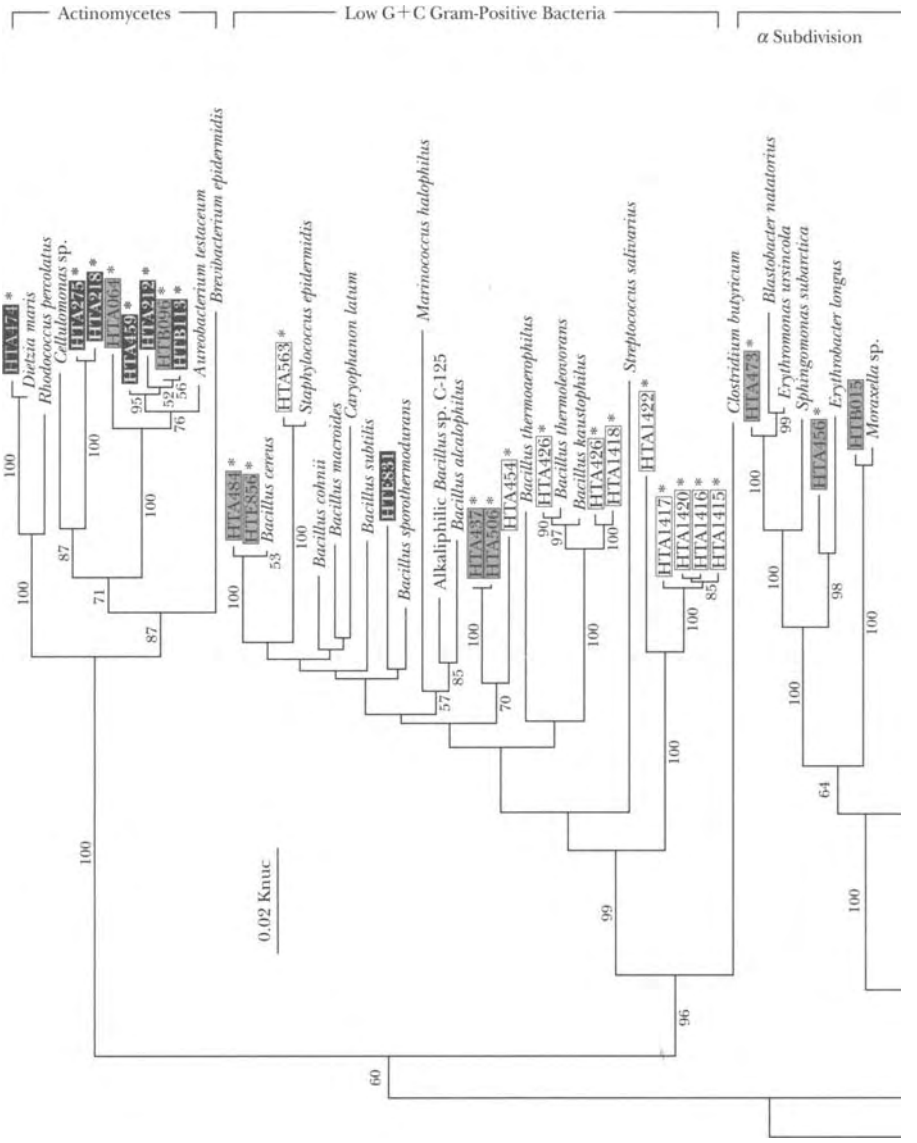
growth was 3% at both pH 7.5 and 9.5. The G + C content of its DNA was 35.8%. Low level (12–30%) of DNA-DNA relatedness between strain HTE831 and the species of these genera was found, indicating that HTE831 could not be classified as a member of a new species belonging to known genera. Based on phylogenetic analysis using 16S rDNA sequencing, chemotaxonomy and the physiology of strain HTE831, it is proposed that this organism is a member of a new species in a new genus, for which the name *Oceanobacillus iheyensis* is proposed. This strain has been studied its whole genome sequence to compare with *Bacillus halodurans* C-125 in many respects (Lu et al. 2001).

2.2.3 Alkaline Lakes

Many reports have been published after the author's rediscovery of alkaliphiles (see previous volumes (Horikoshi and Akiba 1982; 1993; Horikoshi 1999a)). Alkaliphilic bacteria were isolated from soil and water samples obtained from Ethiopian soda lakes in the Rift Valley area—Lake Shala, Lake Abijata, and Lake Arenguadi (Martins et al. 2001). Sixteen starch-hydrolyzing isolates were characterized, and subjected to 16S rRNA gene sequence analysis. All isolates except one were motile endospore-forming rods and found to be closely related to the *Bacillus* cluster, being grouped with *Bacillus pseudofirmus*, *B. cohnii*, *B. vedderi*, and *B. agaradhaerens*. The one exception had nonmotile coccoid cells and was closely related to *Nesterenkonia halobia*. The majority of the isolates showed optimal growth at 37°C and tolerated salinity of up to 10% (w/v) NaCl. Both extracellular and cell-bound amylase activity was detected among the isolates. The amylase activity of two isolates, related to *B. vedderi* and *B. cohnii*, was stimulated by ethylenediaminetetraacetic acid (EDTA) and inhibited in the presence of calcium ions. Pullulanase activity was expressed by isolates grouped with *B. vedderi* and most of the isolates clustered with *B. cohnii*; cyclodextrin glycosyltransferase was expressed by most of the *B. agaradhaerens*-related strains. Recently, using a metagenomic approach, Rees et al. (2003; 2004) isolated cellulase and esterase enzyme activities encoded by novel genes present in environmental DNA libraries which were made from DNA isolated directly from the Kenyan soda lakes, Lake Elmenteita and Crater Lake.

2.2.4 Others

A novel extreme alkaliphile was isolated from a mine water containment dam at 3.2 km below land surface in an ultra-deep gold mine near Carletonville, South Africa (Takai et al. 2001). The cells of this bacterium were straight to slightly curved rods, motile by flagella and formed endospores. Growth was observed over the temperature range 20–50°C (optimum 40°C; 45 min doubling time) and pH range 8.5–12.5 (optimum pH 10.0). The novel isolate described was a strictly anaerobic chemo-organ-


Actinomycetes
Low G+C Gram-Positive Bacteria
α Subdivision
HTA174 *
Dietzia maris
Rhodococcus percolatus
Cellulomonas sp.
HTA276 *
HTA218 *
HTA064 *
HTA459 *
HTA212 *
HTB000 *
HTB113 *
Aureobacterium testaceum
Brevibacterium epidermidis
HTA484 *
HTB356 *
Bacillus cereus
HTA563 *
Staphylococcus epidermidis
Bacillus cohnii
Bacillus macroides
Caryophanon latum
Bacillus subtilis
HTB283
Bacillus sporothermodurans
Marinococcus halophilus
Alkaliphilic Bacillus sp. C-125
Bacillus alcalophilus
HTA437 *
HTA506 *
HTA454 *
Bacillus thermoaerophilus
HTA426 *
Bacillus thermoleovorans
Bacillus kaustophilus
HTA436 *
HTA418 *
Streptococcus salivarius
HTA1422 *
HTA1417 *
HTA1420 *
HTA1416 *
HTA1415 *
Clostridium butyricum
HTA473 *
Blastobacter natatorius
Erythromonas ursincola
Sphingomonas subarctica
HTA456 *
Erythrobacter longus
HTB015
Moraxella sp.
0.02 Knuc

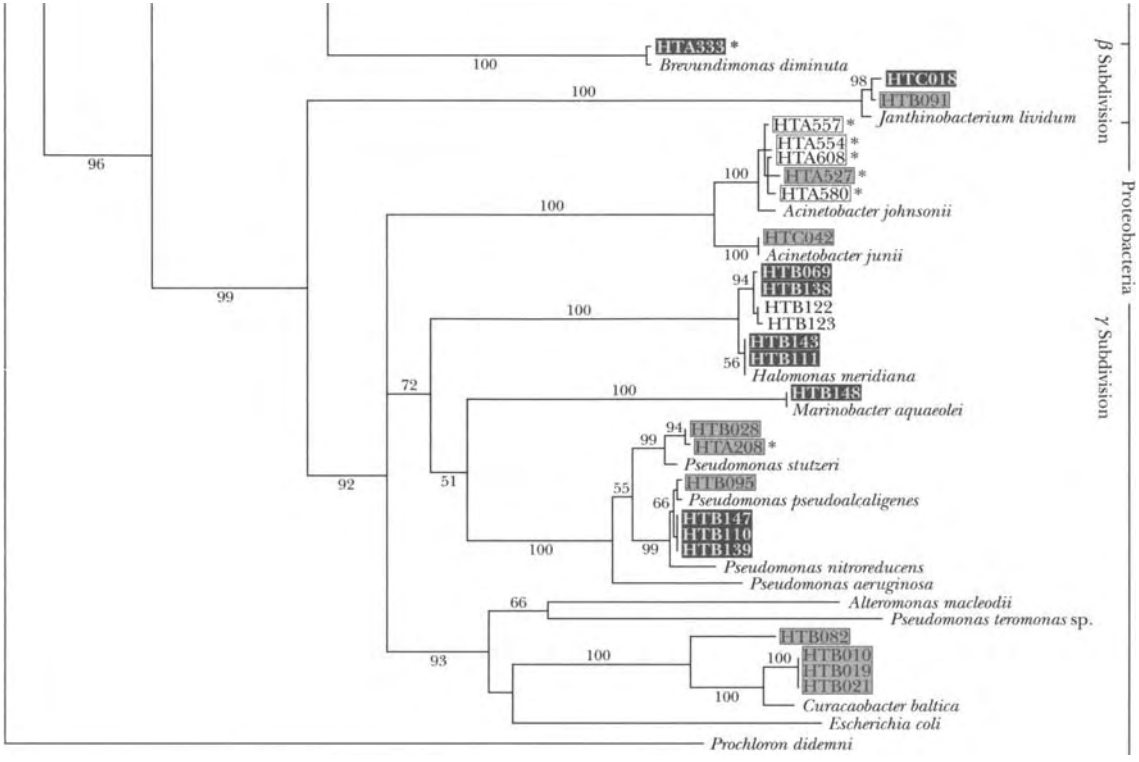


Fig. 2.3 Unrooted phylogenetic tree shows the relationship of bacteria isolated from the deep sea to reference organisms. The numbers indicate the percentages of bootstrap samples, derived from 10000 samples that supported the internal branches. Bootstrap probability values less than 50% were omitted from this figure. *Black box*, alkaliphile; *heavily shaded box*, halophile; *lightly shaded box*, neutrophile; *outlined box*, thermophile; *double-outline box*, psychrophile; *strain abbreviation in outline characters*, alkaliphilic halophile.*, Isolates from the Mariana Trench.
(Reproduced with permission from H.Takami et al., *FEMS Microbiol. Lett.*, **152**, 279(1997))

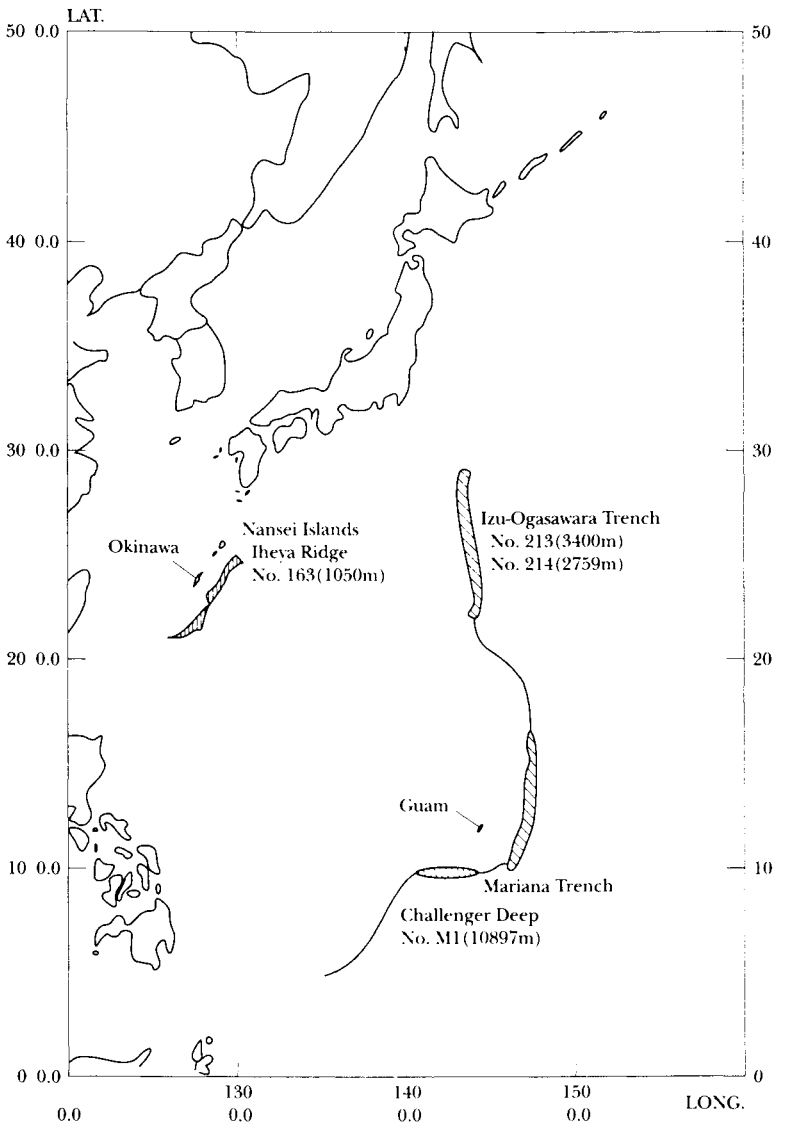


Fig. 2.4 Deep sea sites for collection of mud samples. Sediment sample no. 163 was collected from the Nankai Islands, Iheya Ridge, by the manned submersible *Shinkai 2000*. Sediment samples no. 213 and no. 214 were obtained from the Izu-Ogasawara Trench (30° 07. 05' N, 139° 58. 42' E and 29° 04. 2' N, 140° 43. 3' E), respectivey, by the manned submersible *Shinkai 6500*. Deep sea mud (*M1*) from the Challenger Deep region of the Mariana Trench was collected as described previously.
(Reproduced with permission from H. Takami et al., *FEMS Microbiol. Lett.*, **152**, 279(1997))

otroph capable of utilizing proteinaceous substrates such as yeast extract, peptone, tryptone and casein. Elemental sulfur, thiosulfate or fumarate, when included as accessory electron acceptors, improved growth. The G + C content of genomic DNA was 36.4 mol%. Phylogenetic analysis based on the 16S rDNA sequence indicated that the isolate is a member of cluster XI within the low G + C gram-positive bacteria, but only distantly related to previously described members. On the basis of physiological and molecular properties, the isolate represents a novel species, for which the name *Alkaliphilus transvaalensis* gen. nov., sp. nov. was proposed (type strain SAGM1T = JCM 10712T = ATCC 700919T).

A novel gram-positive facultatively alkaliphilic, sporulating, rod-shaped bacterium, designated WW3-SN6, has been isolated from the alkaline wash-waters derived from the preparation of edible olives (Ntougias et al. 2000; 2001). The bacterium is nonmotile, and flagella are not observed. It is oxidase positive and catalase negative. The facultative alkaliphile grows from pH 7.0 to 10.5, with a broad optimum from pH 8.0 to 9.0. It could grow in up to 15% (w/v) NaCl, and over the temperature range 4°C to 37°C with an optimum between 27°C and 32°C. Therefore, it is both halotolerant and psychrotolerant.

2.3 Taxonomy of Alkaliphiles

2.3.1 Phylogenetic Analysis of Alkaliphiles Based on 16S rDNA Analysis

Classic taxonomy of alkaliphiles before the introduction of 16S rDNA analysis has been described previously (Horikoshi and Akiba 1982; Hrikoshi and Grant 1991; 1998).

Nielsen et al. (1994) analyzed 16S rDNA of 14 alkaliphiles. They conducted comparative sequence analysis on about 1520 nucleotides, corresponding to 98% of the entire 16S rDNA of 14 alkaliphilic or alkalitolerant, gram-positive, aerobic, endospore-forming bacterial strains. *Bacillus alcalophilus* DSM 485T and *B. cohnii* DSM 6307T were included to represent the two validly described alkaliphiles assigned to the genus *Bacillus*. The majority of isolates (8 strains) clustered with *B. alcalophilus* DSM 485T form a distinct phylogenetic group (rRNA group 6) within the radiation of the genus *Bacillus* and related taxa. *Bacillus cohnii* DSM 6307T and two of the isolates, DSM 8719 and DSM 8723, were grouped with *B. fastidiosus* and *B. megaterium* and allocated to rRNA group 1. The remaining two strains, DSM 8720 and DSM 8721, show an equidistant relationship to both groups. Then, Nielsen et al. (1995) reported phenetic diversity of alkaliphilic *Bacillus* strains isolated from various sources and proposed nine new species. One hundred and nineteen strains of alkaliphilic and alkalitolerant, aerobic endospore-forming bacteria were examined for 47 physiological and bio-

chemical characteristics, and DNA base composition. Numerical analysis (S-J and S-SM/UPGMA clustering) revealed 11 clusters comprising three or more strains. Most of the phena were further characterized by analysis of carbohydrate utilization profiles using the API 50CH system, but strains of two taxa could not be cultured by this method. DNA reassociation studies showed that nine of the phena were homogeneous, but strains of phenon 4 and phenon 8 were each subdivided into two DNA hybridization groups. The strains could therefore be classified into 13 taxa plus a number of unassigned single-membered clusters. Two taxa were equated with *Bacillus cohnii* and *B. alcalophilus* and nine of the remainder were proposed as new species with the following names: *B. agaradhaerens* sp. nov., *B, clarkii* sp. nov., *B. clausii* sp. nov., *B. gibsonii* sp. nov., *B. halmapalus* sp. nov., *B. halodurans* comb. nov., *B. horikoshii* sp. nov., *B. pseudoalcalophilus* sp. nov. and *B. pseudofirmus* sp, nov. Two taxa were not sufficiently distinct to allow confident identification, so these have not been proposed as new species (Fig. 2.5). They described *Bacillus horikoshii* as follows: *Bacillus horikoshii* (ho.ri.ko' shi.i) sp. nov. ML gen.' n, horikoshii of Horikoshi, This description is taken from their study based on strains of DNA hybridization group 8b. The colonies are small, circular with an entire margin, shiny surface and a cream-white color. The cells are rod-shaped (0.6–0.7 × 2.0–4.0 μm) with ellipsoidal spores (0.5–0.7 × 0.7–1.2 μm) located subterminally in a sporangium, which may be slightly swollen. The strains of this species hydrolyze casein, hippurate, gelatin, pullulan and starch. Three of the four strains hydrolyze Tween 40 and 60. Growth is observed at pH 7.0, with an optimum at about pH 8.0. The strains grow between 10 and 40°C. Salt tolerance is moderate, with a maximum at 8–9% NaCl. The strains do not hydrolyze MUG or Tween 20, do not delaminate phenylalanine and do not reduce nitrate to nitrite. No growth is observed on ribose, D-xylose, L-arabinose, galactose, rhamnose, sorbitol, lactose, melibiose, melizitose, D-raffinose, sorbitol, lactose melibiose, melizitose, D-raffinose or D-tagatose. The G + C content of chromosomal DNA is between 41.1 and 42 mol%. Source: soil samples. Type strain is PN-121 (= DSM 8719).

The author's group has studied extensively the microbial properties including molecular biology of the alkaliphilic *Bacillus* sp. No. C-125 as one of the representative alkaliphiles that the author isolated. After characterization of strain C-125, Aono assigned the *Bacillus* sp. No. C-125 to the *Bacillus lentus* group 3 (Aono 1995).

Recently, Takami and Horikoshi (1999b) reidentified alkaliphilic *Bacillus* sp. C-125 as *Bacillus halodurans.* Alkaliphilic *Bacillus* sp. C-125 was taxonomically characterized by physiological and biochemical characteristics, 16S rDNA sequence similarity, and DNA-DNA hybridization analyses. *Bacillus* sp. strain C-125 was found to be aerobic, spore-forming, gram-positive, and motile. The morphology of living and non-living stained cells was observed by light microscopy and transmission electron microscopy, respectively. The cells were found to have numerous peritrichous flagella (Fig.

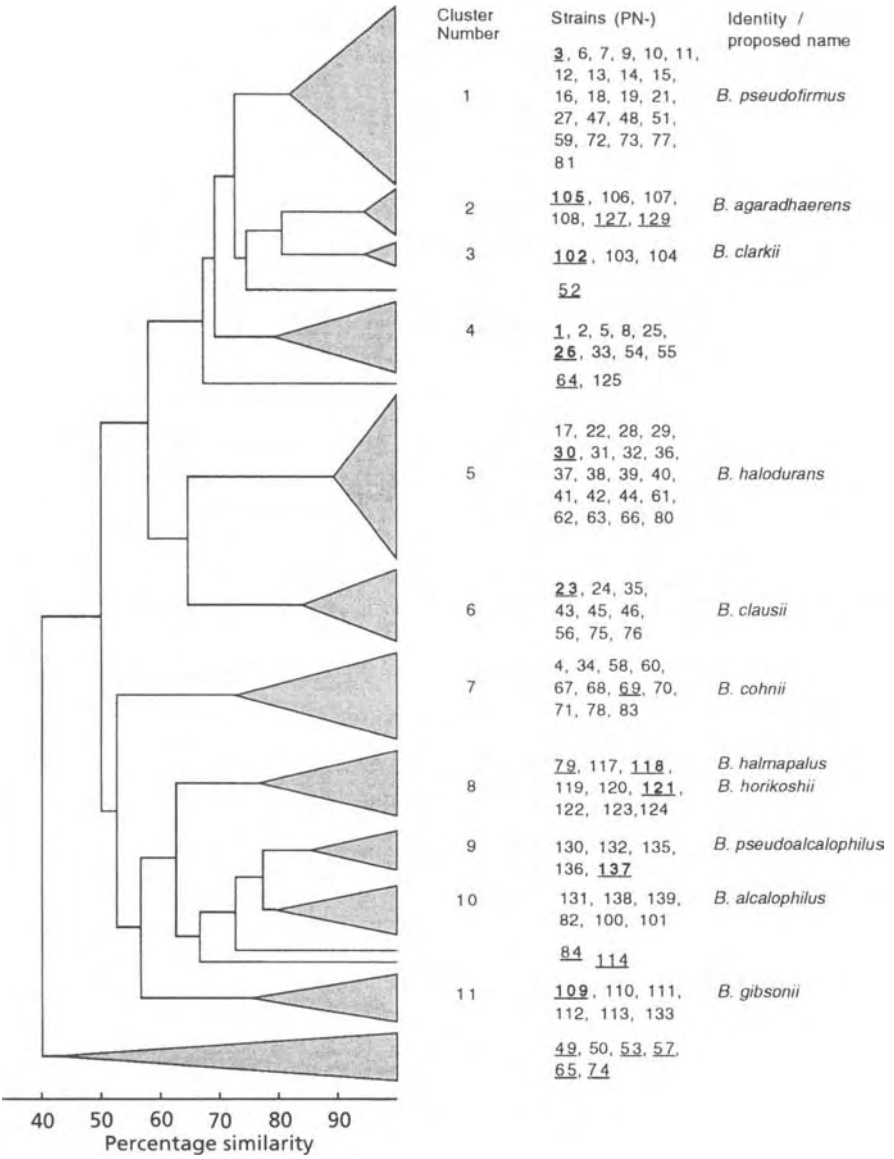


Fig. 2.5 Abbreviated dendrogram showing the allocation of strains to clusters based on the S_1/UPGMA analysis of the biochemical and physiological characteristics. Bold underlined strain numbers indicate those strains for which the full 16S rRNA sequence has been determined (Nielsen et al., 1994); underlined strain numbers indicate those for which a partial 16S rRNA sequence has been determined (unpublished).
(Reproduced with permission from Nielsen et al., *Microbiology-UK*, **141**, 1750 (1995))

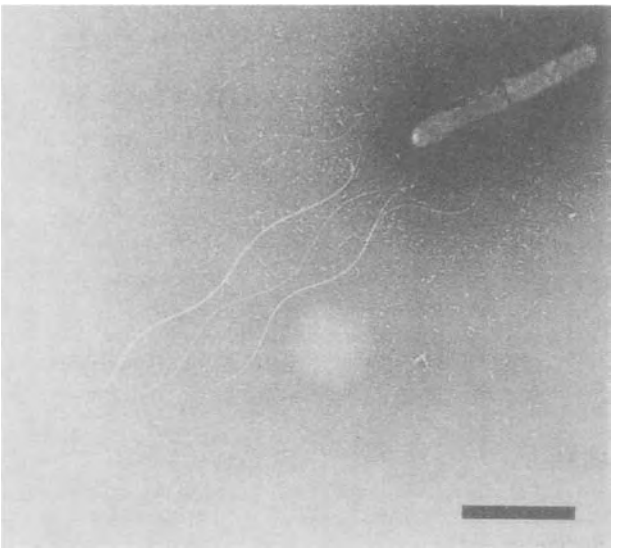


Fig. 2.6 Transmission electron micrograph of a negatively stained cell of strain C-125. The strain was grown aerobically at pH 9.5. For negative staining, one drop of culture was placed on a copper grid coated with 1% potassium phosphotungstic acid adjusted to pH 6.5 with potassium hydroxide. (bar = 2 mm)

2.6) and were actively motile when grown at alkaline pH. The characteristics of alkaliphilic *Bacillus* sp. C-125 are summarized in Table 2.3. The strain C-125 was found to grow at relatively high temperatures (up to 55°C) and in the presence of high concentrations of sodium chloride (up to 12%). Isoprenoid quinones were extracted from dried cells with chloroform-methanol (2:1) and purified by thin-layer chromatography. The purified isoprenoid quinones were analyzed by reverse-HPLC12) and the absorbance was measured at 270 nm using menaquinone as the standard. The major quinone in the strain C-125 was found to be menaquinone 7. Phylogenetic analysis of the strain C-125 based on comparison of 16S rDNA sequences showed that this strain is closely related to *Bacillus halodurans*. DNA-DNA hybridization analysis was done comparing C-125 and related *Bacillus* reference strains (Table 2.4). The highest level of DNA-DNA relatedness (86%) was found between the strain C-125 and *B. halodurans*. As shown in Fig. 2.7, those findings demonstrate that the strain C-125 is a member of the species *B. halodurans* C-125.

Then, *Bacillus* sp. AH-101 producing keratinase was also reidentified as *Bacillus halodurans* AH-101 (DSM497) by the same methods described above (Takami et al. 1999e).

Then, with a view toward verifying the original classification of alkaliphilic *Bacillus firmus* OF4, physiological and biochemical characteristics were more extensively catalogued than in the original studies, and this catalogue was supplemented with 16S rDNA sequence homology and more extensive DNA-DNA hybridization analyses (Takami and Krulwich 2000).

Table 2.3 Characteristics of alkaliphilic *Bacillus* sp. strain C-125

Characteristics	Strain C-125
Cell shape	Rods
Cell size	0.6–0.7 × 2.5–4.0 μm
Spore shape	oval
Sporangium swollen	–
Anaerobic growth	+
VP reaction	–
pH in VP broth	9.4
Growth at 40°C	+
at 50°C	+
at 55°C	+
Growth in medium pH 5.7	–
medium pH 7.0	+
Growth in NaCl 2%	+
NaCl 5%	+
NaCl 7%	+
NaCl 10%	+
NaCl 12%	+
NaCl 16%	–
Lysozyme broth	+
Acid from D-glucose	+
L-arabinose	–
D-xylose	W
D-mannitol	+
D-fructose	+
Hydrolysis of starch	+
gelatin	+
casein	+
Tween 80	–
Tween 60	+
Tween 40	+
Tween 20	+
Hydrolysis of hippurate	W
Use of citrate	+
propionate	+
NO_2 from NO_3	–
Indole production	–
Phenylalanine deaminase	+
Lecithinase	+
Major isoprenoid quinone	7

W : weak.

(Reproduced with permission from H. Takami et al., *Biosci. Biotechnol. Biochem.*, **63**, 943(1999))

Phylogenetic analysis of this alkaliphile based on a comparison of multiple 16S rDNA sequences from *Bacillus* species indicated that this strain is most closely related to *Bacillus pseudofirmus*. Consistently, in the DNA-DNA hybridization analysis of the alkaliphile and *Bacillus* reference strains, the highest level of DNA-DNA relatedness (96%) was found between the alkaliphile and the B. pseudofirmus type strain (DSM 8715(T)). The findings support the conclusion that this alkaliphile strain is more closely related to *B. pseudofirmus* OF4 than to *B. firmus*.

Table 2.4 DNA-DNA hybridization between *Bacillus* sp. C-125 and other related strains

Strain	%DNA-DNA hybridization with			
	Bacillus sp. C-125 (JCM 9153)	*Bacillus halodurans* (DSM 497^T)	*Bacillus alcalophilus* (DSM 485^T)	*Bacillus firmus* (DSM 12^T)
Bacillus sp. C-125 (JCM 9153)	100			
Bacillus halodurans (DSM 497^T)	86	100	46	47
Bacillus alcalophilus (DSM 485^T)	41	38	100	
Bacillus firmus (DSM 12^T)	< 30			100

T : Type strain.
(Reproduced with permission from H. Takami et al., *Biosci. Biotechnol. Biochem.*, **63**, 943(1999b))

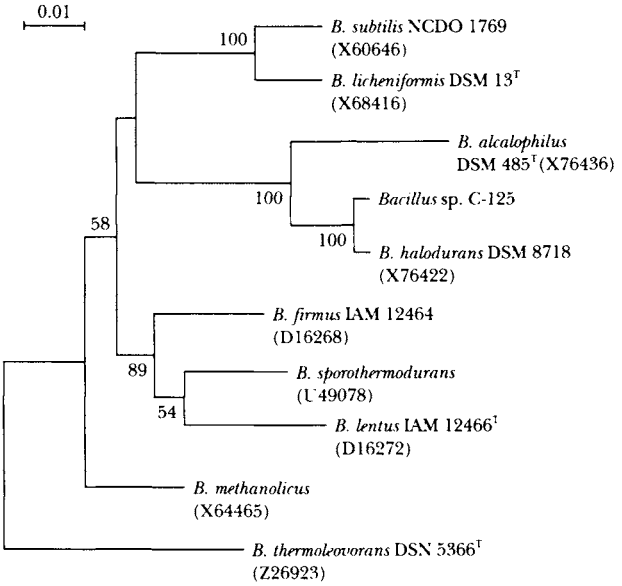


Fig. 2.7 Unrooted phylogenetic tree showing the relationship of strain C-125 to other alkaliphilic *Bacillus* strains. The numbers indicate the percentages of bootstrap values, derived from 1000 samples, that supported the internal branches. Bootstrap probability values less than 50% were omitted from this figure. Bar = 0.01 Knuc unit. T, Type strain ; ( ), accession number.
(Reproduced with permission from H. Takami et al., *Biosci. Biotechnol. Biochem.*, **63**, 943(1999b))

In 2005 Nogi et al. reported their extensive phylogenetic studies on alkaliphilic *Bacillus* strains. Unrooted phylogenetic tree of akaliphilic *Bacillus* strains isolate in the author's laboratory since 1968 is shown in Fig. 2.8.

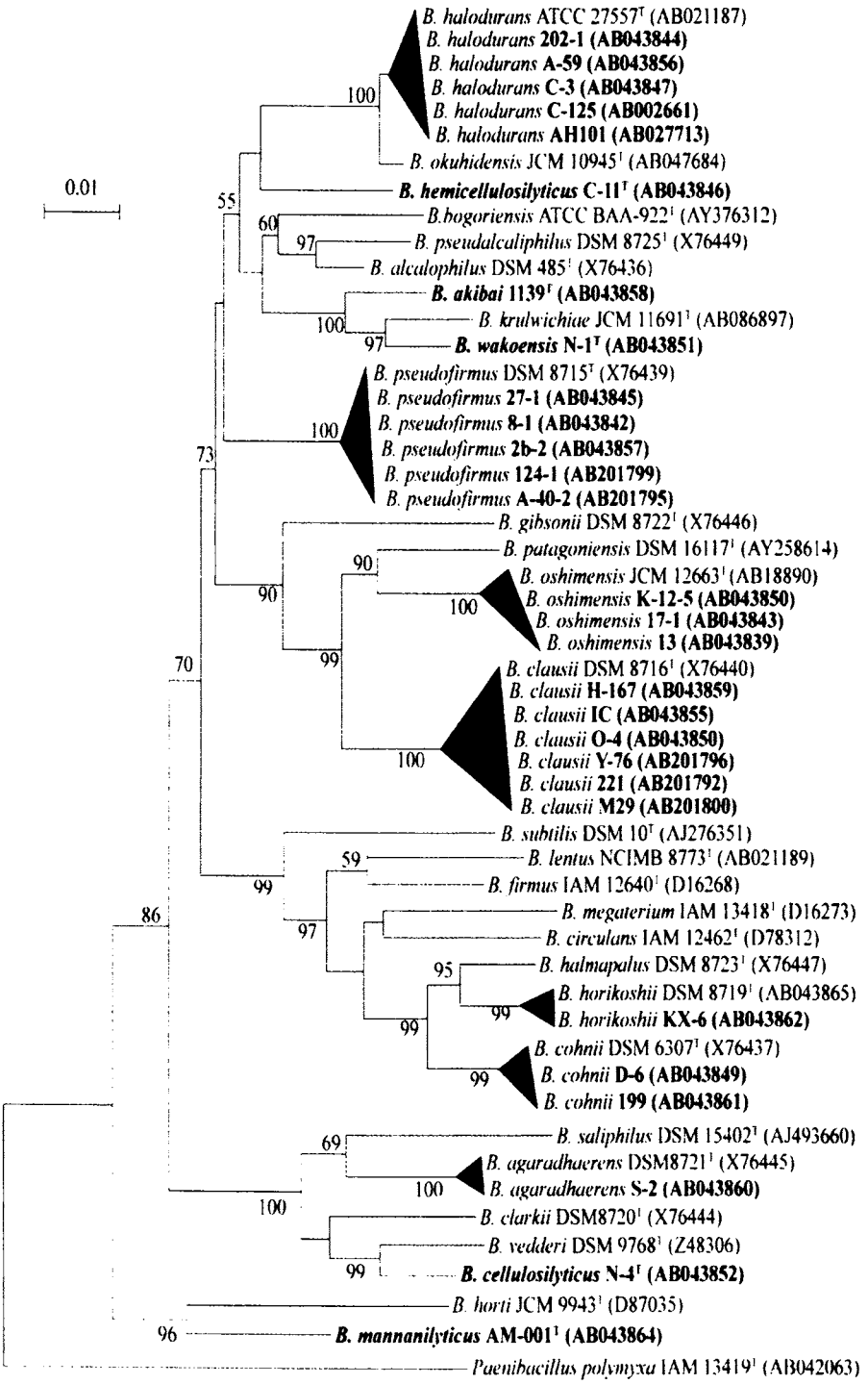


Fig. 2.8 Phylogenetic tree of alkaliphillic *Bacillus* strains. A number in parenthesis is the GenBank accession number of rRNA.
(Reproduced with permission from Y. Nogi et al., *Int. J. Syst. Erol. Microbiol*(2005))

3

Cell Structure

3.1 Flagella

Flagella-based motility is a major mode of locomotion for bacteria. Flagella are typically as long as or longer than the cell body, varying in length from one to many micrometers. Several alkaliphilic bacteria demonstrate vigorous motility at alkaline pH. Flagella exposed to alkaline environments and devices for movement may operate at alkaline pH.

3.1.1 Flagella Formation and Flagellin

Aono et al. (1992a) investigated pH-dependent flagella formation by facultative alkaliphilic *Bacillus halodurans* C-125. This strain grown at alkaline pH had peritrichous flagella and was highly motile. However, most of the cells grown initially at pH 7 were non-motile and possessed a few straight flagella. Sakamoto et al. (1992) cloned the flagellin protein gene (*hag* gene) of *B. halodurans* C-125 and expressed it in the *Escherichia coli* system. Sequencing this gene (Accession Number D10063) revealed that it encodes a protein of 272 amino acids (Mr 29995). The alkaliphilic *B. halodurans* C-125 flagellin shares high homology with other known flagellins, such as *B. subtilis* etc. However, the sequence of the *hag* gene shows very low homology with other flagellins so far tested. This result strongly suggests that codon usage of *B. halodurans* C-125 may be different from that of other bacteria (see also Table 7.3 in Chapter 7, Alkaline Proteases).

3.1.2 Flagellar Motor

Flagellar movement of neutrophilic bacteria is caused by H^+-driven motors. Cells of alkaliphilic *Bacillus* sp. No. YN-1 (Koyama et al. 1976a,b) in growth medium consisting of rich broth and NaCl showed vigorous motility between pH 8.5–11.5. *B. subtilis* showed motility between pH 6–8. The YN-1 cells were washed and resuspended in TG medium consisting of 25 mM Tris-HCl buffer (pH 9.0), 0.1 mM EDTA and 5 mM glucose; no translational swimming cells were observed. The addition of NaCl to the medium, however, caused quick recovery of swimming. The swimming speed increased with an increase of NaCl concentration up to 50 mM. Other cations such as

Table 3.1 Ion specificity of the motility of YN-1

Salts added	Swimming speed(μm/sec)
None	0
NaCl	16
$NaNO_3$	17
Na_2HPO_4	15
Na_2SO_4	18
NaSCN	18
Na-acetate	16
LiCl	0
KCl	0
NH_4Cl	0
RbCl	0
CsCl	0
$CaCl_2$	0
$MgCl_2$	0

YN-1 cells in TG medium (pH 9.0) were mixed with 15 mM of various salts, and the swimming speed was measured after 5 min incubation at 30°C.
(Reproduced with permission from M. Hirota, M. Kitada and Y. Imae, *FEBS Lett.*, **132**, 279(1981))

Li^+ and K^+ had no effect (Table 3.1). Hirota et al. (1981) reported on flagellar motors of alkaliphilic *B. pseudofirmus* No. 8-1 and *B. halodurans* C-125 powered by an electrochemical potential gradient of sodium ions, because the protein motive force is too low to drive flagellar motors in an alkaline environment. Such a Na^+ requirement is a unique property of alkaliphilic *Bacillus* strains. In order to clarify the role of Na^+ in motility, the effects of various ionophores, valinomycin, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), nigericin and monensin were studied (Sakamoto et al. 1992) as shown in Table 3.2. The results suggest that the membrane potential is a component of the energy source for the motility of alkaliphilic *Bacillus*. Monensin, which catalyzes Na^+/H^+ exchange, caused strong inhibition of motility, indicating that the flux of Na^+ by the chemical potential gradient of Na^+ is coupled with the motility of alkaliphilic *Bacillus* strains. Imae and Atsumi (1989) reported very interesting properties of flagellar motors. Bacterial flagellar motors are the reversible rotary engine which propels the cell by rotating a helical flagellar filament as a screw propeller. The motors are embedded in the cytoplasmic membrane, and the energy for rotation is supplied by the electrochemical potential of specific ions across the membrane. Thus the analysis of motor rotation at the molecular level is aligned to an understanding of how the living system converts chemical energy into two types: one being the H^+-driven type found in neutrophiles such as *Bacillus subtilis* and *Escherichia coli* and the other being the Na^+-driven type found in alkaliphilic *Bacillus* and marine *Vibrio*. Furthermore, Iwazawa et al (1993) studied the torque of the bacterial flagellar motor using a rotating

Table 3.2 Effect of ionophores on the motility of YN-1

Additions	Swimming speed (μm/sec)
None	22
Valinomycin (10 μM)	22
Valinomycin (10 μM) + KCl (60 mM)	0
KCl (60 mM)	22
CCCP (20 μM)	10
Nigericin (2 μM)	0
Monensin (6 μM)	6

Cells in TG medium (pH 9.0) containing 50 mM NaCl were mixed with ionophores as indicated, and the swimming speed was measured at 30°C within 1 min.
(Reproduced with permission from M. Hirota, M. Kitada and Y. Imae, *FEBS Lett.*, **132**, 279 (1981))

electric field. In this study the torque generated by the flagellar motor was measured in the tethered cells of a smooth-swimming *E. coli* strain using rotating electric fields to determine the relationship between the torque and speed over a wide range. By measuring the electric current applied to the sample cell and combining the data obtained at different viscosities, the torque of the flagellar motor was estimated up to 55 Hz, and also at negative rotation rates. By this method it was found that the torque of the flagellar motor decreases with rotation rate from negative through positive rate of rotation. In addition, the dependence of torque upon temperature was stronger than at the low speeds encountered in tethered cells.

3.1.3 Inhibitors for Flagellar Motors

These Na^+-driven flagellar motors were inhibited by amiloride (Sugiyama et al. 1988). The inhibition was rather specific and other biological functions such as pH homeostasis, ATP synthesis and membrane potential formation were not inhibited. From kinetic analysis of the data, it is evident that amiloride inhibits the rotation of the Na^+-driven flagellar motor by competing with Na^+ at the force-generating site of the motor. The Na^+ interacting site of the motors is somewhat similar to that of the Na^+ channels. Phenamil, an amiloride analogue, inhibited motor rotation without affecting cell growth (Atsumi et al. 1990; 1992). A concentration of 50 μM phenamil completely inhibited the motility of *Bacillus* RA-1 (Fig. 3.1), but showed no effect on the membrane potential, the intracellular pH, or Na^+-coupled amino acid transport, which was consistent with the fact that there was no effect on cell growth. As shown in Table 3.3, phenamil is a specific

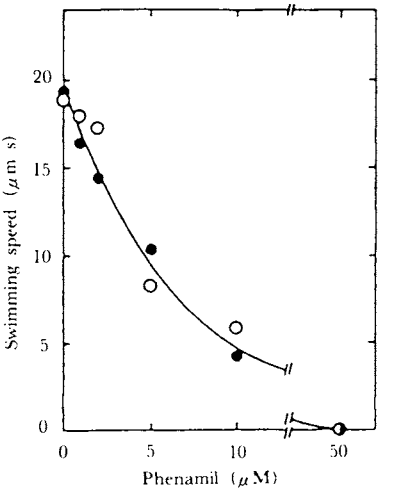


Fig. 3.1 Inhibition by phenamil of motility of RA-1 under conditions of growth. The swimming speeds of the cells were measured after incubation for 5 min (○) or 1 h (●) at 35°C.
(Reproduced with permission from T. Atsumi et al., *J. Bacteriol.*, **172**, 1636 (1990))

Table 3.3 Effect of phenamil on the motility of various bacterial species

Strain	Swimming speed (μm/s)[*1]		
	$-Na^+$	$+Na^+$	$+Na^+$ + phenamil
Motility tightly Na^+-dependent Alkaliphilic *Bacillus* strains :			
RAB (RA-1)[*2]	0	22	0
202-1	0	41	0
8-1	0	19	0
YN-1	0	28	6
YN-2000	0	21	6
Marine *Vibrio* sp., *V. alginolyticus*	0	69	1
Neutrophiles			
E. coli	28	29	33
B. subtilis	22	21	22
B. sphaericus	35	37	36

[*1] Motility was measured in TG medium. The pH of the medium was 9.5 for alkaliphiles and 7.5 for neutrophiles and the *Vibrio* sp. For alkaliphiles and neutrophiles, the medium was supplemented with 50 mM KCl ($-Na^+$) of 50 mM NaCl ($+Na^+$). For the *Vibrio* sp., the medium was supplemented with 400 mM KCl ($-Na^+$) or 400 mM NaCl ($+Na^+$). Phenamil was added to a final concentration of 100 μM.
[*2] Motility at pH 7.5 was also tightly Na^+ dependent and was completely inhibited by 100 μM phenamil.
(Reproduced with permission from T. Atsumi et al., *J. Bacteriol.*, **172**, 1638 (1990))

and potent inhibitor for the Na^+-driven flagellar motors not only in various strains of alkaliphilic *Bacillus* sp., but also in marine *Vibrio* sp.

In 1999, Kojima et al. (1999) reported that the rotation of the Na^+-driven flagellar motor is specifically and strongly inhibited by phenamil, an amiloride analogue. This was the first evidence that phenamil interacts directly with the Na^+-channel components (PomA and PomB) of the motor.

Recently, Sugiyama et al. (2004) analyzed the rotational characteristics of Na^+-driven flagellar motor in the presence and absence of coupling ion by the electrorotation method. The motor rotated spontaneously in the presence of Na^+, and the rotation accelerated or decelerated following the direction of the applied external torque. The spontaneous motor rotation was inhibited by the removal of external Na^+; however, the motor could be forcibly rotated by relatively small external torque applied by the electrorotation apparatus. The observed characteristic of the motor was completely different from that of ATP-driven motor systems, which form a rigor bond when their energy source, ATP, is absent. The internal resistance of the flagellar motor increased significantly when the coupling ion could not access the inside of the motor, suggesting that the interaction between the rotor and the stator is changed by the binding of the coupling ion to the internal sites of the motor.

Then, Ito et al. (2004) identified the stator-force generator that drives Na^+-dependent motility in alkaliphilic *Bacillus pseudofirmus* OF4 to be MotPS, MotAB-like proteins. These genes are downstream of the *ccpA* gene, which encodes a major regulator of carbon metabolism. *B. pseudofirmus* OF4 was only motile at pH values above 8. These DNA sequences did not show homology to those of *B. halodurans* C-125, but decoded amino acids sequences exhibited very high homology to those of *B. psuedofirmus* OF4. As shown in Fig. 3.2, disruption of *motPS* resulted in a nonmotile phenotype, and motility was restored by transformation with a multicopy plasmid containing the *motPS* genes. Purified and reconstituted MotPS from *B. pseudofirmus* OF4 catalyzed amiloride analogue-sensitive Na^+ translocation. In contrast to *B. pseudofirmus*, *Bacillus subtilis* contains both MotAB and MotPS systems. The role of the *motPS* genes from *B. subtilis* in several motility-based behaviors was tested in isogenic strains with intact *motAB* and *motPS* loci, only one of the two *mot* systems or neither *mot* system. *B. subtilis* MotPS (BsMotPS) supported Na^+-stimulated motility, chemotaxis on soft agar surfaces and biofilm formation, especially after selection of an up-motile variant. BsMotPS also supported motility in agar soft plugs immersed in liquid; motility was completely inhibited by an amiloride analogue. BsMotPS did not support surfactin-dependent swarming on higher concentration agar surfaces. These results indicate that BsMotPS contributes to biofilm formation and motility on soft agar, but not to swarming, in their laboratory strains of *B. subtilis* in which MotAB is the dominant stator-force generator. BsMotPS could potentially be dominant for motility in *B. subtilis* variants that arise in particular niches.

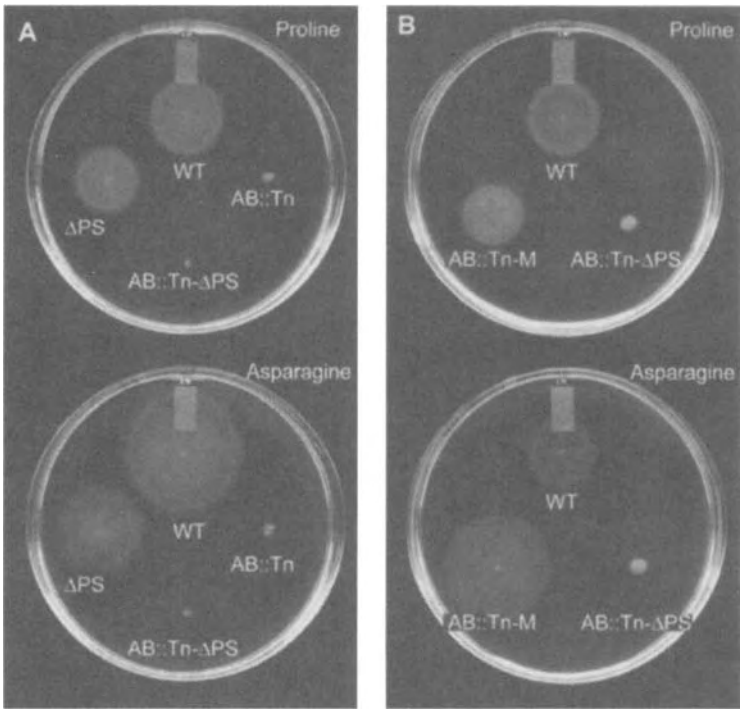


Fig. 3.2 The effect of *motAB* and *motPS* on chemotactic behavior of *B. subtillis*.
A. The indicated isogenic strains of *B. subtills* as they appear after 16 h of incubation on chemotaxis plates containing 1 mM added Na^+ and either 0.2 mM proline (top) or 0.3 mM asparagine (bottom).
B. Wild-type, AB:: Tn-M and AB:: Tn-ΔPS strains as they appear on chemotaxis plates containing 200 mM added Na^+ and either 0.2 mM proline (top) or 0.3 mM asparagine (bottom). After 24 h of incubation of the AB:: Tn-M and AB:: Tn-ΔPS strains, the wild-type strain was inoculated on to the plates, and incubation was continued for an additional 16 h. Thus, the image is after 40 h of incubation for the AB:: Tn-M and AB:: Tn-ΔPS stains and 16 h of incubation of the wild-type strain.
(Reproduced with permission from M. Ito et al., *Mol. Microbiol.*, **53**, 1035(2004))

Further work on alkaliphilic bacteria in this field is expected to yield more results of strong interest.

3.2 Cell Wall

3.2.1 Cell Wall of Neutrophilic Gram-positive Bacteria

The cell wall of bacteria not only provides shape, rigidity and gram-stainability but is also responsible for some biochemical reactions such as serological properties and phage absorption. The cell wall of gram-positive bacteria is usually thicker than that of gram-negative bacteria. The walls range from 15

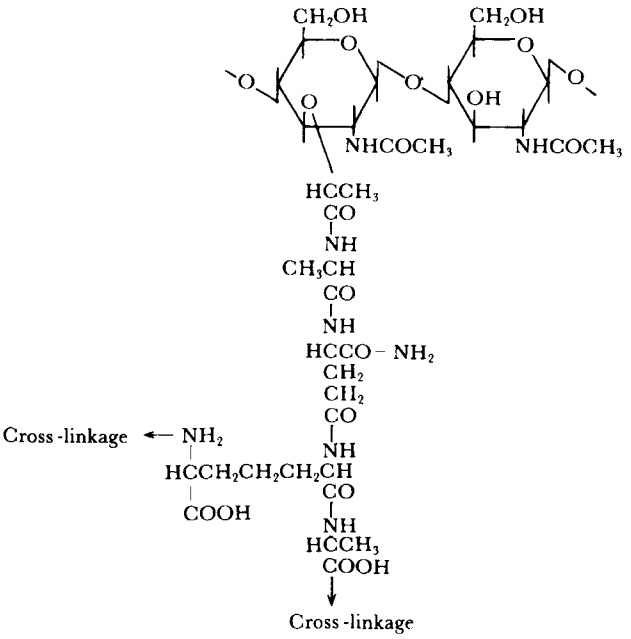


Fig. 3.3 Peptidoglycan of *Bacillus* species.

and 50 nm in thickness and are relatively featureless in thin section by electron microscopy. Growth conditions, age of culture and sporulation cause variations in thickness. The cell wall of gram-positive bacteria (*Bacillus* sp.) is composed of three main components: (1) peptidoglycan; (2) polysaccharides, teichoic acid or teichuronic acid; and (3) proteins. Biochemical experiments and a knowledge of the strength of these gram-positive walls suggest that there must be some ordered infrastructure within their matrices.

Peptidoglycan is a heteropolymer composed of glycan strands bound through two cross-linked peptide chains. The components of the glycan strands are *N*-acetylglucosamine and *N*-acetylmuramic acid, which are alternately linked by a β-1,4 bond. The peptide chains show great variation in amino acid composition among different genera and species of bacteria. Based on the anchoring point of the cross-linkage between the two peptide chains, the peptidoglycans have been classified into two main groups, A and B, each of which is further divided into several subgroups depending on the variation in the kind of interpeptide bridges and of the amino acids in position 3 of the peptide chain. The general structure of peptidoglycan of the genus *Bacillus* is shown in Fig. 3.3. A cross-linkage is formed between the ε-amino group of diaminopimelic acid in position 3 of a peptide chain and the carboxyl group of D-alanine in position 4 of another adjacent peptide chain. This type of peptidoglycan is classified as the Alγ-type, which is the most common structure of the genus *Bacillus*. L-Alanine is linked by its N-terminus to the carboxy group of muramic acid. The amino acid in posi-

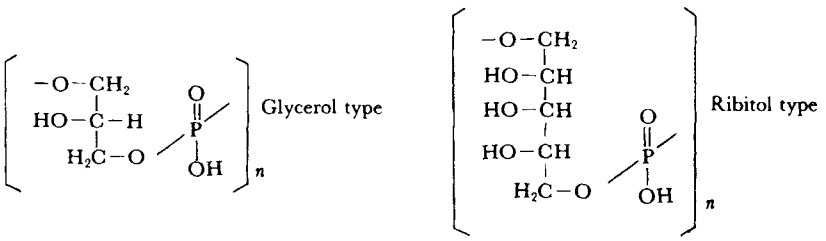


Fig. 3.4 Teichoic acid.

tion 2 is D-glutamic acid, which is linked by its γ-carboxyl group to meso-diaminopimelic acid in position 3, and position 4 is occupied by D-alanine. It is noted that amino acids with the D-configuration are present in the peptidoglycan, and these D- and L-amino acids occur in an alternating arrangement.

Teichoic acid is a polymer consisting of phosphoric acid and ribitol or glycerol to which D-glucose and D-alanine are attached (Fig. 3.4). The phosphoric acid and ribitol or glycerol are bound by ester linkage. Polysaccharide (teichuronic acid) contains glucose, galactose, mannose or rhamnose and their uronic acids derivatives. Teichoic acids and polysaccharides can be extracted by cold trichloroacetic acid (TCA) from wall fraction. Both components have serological activity and phage adsorption activity. Phosphate groups in the teichoic or in the teichuronic acid cause electron negative charge of the cell surface.

3.2.2 Cell Wall of Alkaliphilic *Bacillus* Strains

A. Composition of cell wall of alkaliphilic *Bacillus* strains

The cell wall is directly exposed to high alkaline environments, but the intracellular pH of alkaliphilic bacteria is neutral (pH 7–8.5), so the pH difference must be due to cell surface components. It has been observed that protoplasts of alkaliphilic *Bacillus* strains lose their stability against alkaline environments (Aono et al. 1992b). This suggests that the cell wall may play some role in protecting the cell from alkaline environments. The components of cell wall of several alkaliphilic *Bacillus* strains have been investigated by Aono and Horikoshi (1983), Ikura and Horikoshi (1983), and Aono et al. (1984) in comparison with those of neutrophilic *B. subtilis*. Table 3.4 summarizes the composition of the cell wall of alkaliphilic *Bacillus* strains isolated in the author's laboratory.

B. Composition of the peptidoglycans

As shown in Table 3.5, the peptidoglycans appeared to be similar in composition to those of *B subtilis*. Major constituents detected commonly in hydrolyzates of the peptidoglycans were glucosamine, muramic acid, D- and L-alanine, D-glutamic acid, *meso*-diaminopimelic acid and acetic acid.

Table 3.4 Composition of cell walls of alkaliphilic *Bacillus* strains

			Composition (μmol (mg cell wall)$^{-1}$)														
Group	Strain	Culture pH	Asp	Glu	L-Glu	Gly	Ala	DAP	Mur	GlcN	GalN	P	Glyc	Neutral sugars	Glc	Gal	Uronic acid
1	A-40	10		0.43			0.67	0.42	0.36	0.67	0.23	0.04		0.08			0.90
	2b-2	10		0.36			0.58	0.35	0.34	0.65	0.27	0.17		0.18	0.05		0.81
2	C-11	10		2.32	1.05		0.52	0.33	0.22	0.26	0.04	0.07		0.05			0.60
	C-125	7		1.75	1.08		0.88	0.52	0.48	0.46	0.04	0.03		0.04			0.46
		10		2.13	1.80		0.64	0.38	0.28	0.30	0.03	0.05		0.06			0.90
	Y-25	7		1.55	0.59		0.90	0.47	0.38	0.44	0.04	0.03		0.04			0.36
		10		1.97	0.86		0.54	0.34	0.29	0.32	0.03	0.05		0.06			0.79
	A-59	7	0.55	1.71	0.52		0.93	0.56	0.39	0.42	0.05	0.05		0.04			0.44
		10	0.71	1.82	0.70		0.61	0.38	0.27	0.30	0.04	0.06		0.07			0.62
	C-3	7	0.40	1.20	0.33	0.32	0.94	0.53	0.48	0.51	0.04	0.03		0.10	0.04		0.27
		10	0.72	1.50	0.54	0.52	0.80	0.39	0.36	0.37	0.06	0.06		0.22	0.08		0.49
3	C-59-2	10	0.21	0.26			0.35	0.24	0.18	0.45	0.05	1.65	0.56	0.96	0.45	0.49	0.11
	M-29	7		0.31			0.57	0.30	0.21	0.80	0.05	1.76	0.12	1.85	1.20	0.14	0.13
		10		0.33			0.59	0.31	0.30	1.01	0.05	1.70	0.13	1.73	1.21	0.11	0.12
	57-1	7	0.01	0.29			0.44	0.29	0.19	0.22	0.03	1.53	0.99	0.78		1.05	0.08
		10	0.01	0.30			0.45	0.30	0.21	0.25	0.05	1.82	1.23	0.67		1.08	0.08
	B. subtilis GSY 1026	7		0.54			0.80	0.49	0.48	0.50	0.19	1.77	0.83	1.34	1.02		0.11

The assay method for each compound is detailed in the text. Determination is uncorrected for destruction during acid hydrolysis. Each blank space represents not detected. The following abbreviations are used: Asp, aspartic acid ; Glu, glutamic acid ; Gly, glycine ; Ala, alanine ; DAP, diaminopimelic acid ; Mur, muramic acid ; GlcN, glucosamine ; GalN, galactosamine ; P, phosphorus ; Glyc, glycerol ; Glc, glucose ; Gal, galactose. (Reproduced from R. Aono and K. Horikoshi, *J. Gen. Microbiol.*, **129**, 1085 (1983))

Table 3.5 Composition of the peptidoglycan of alkaliphilic *Bacillus* strains[+]

Strain	Culture pH	*meso*-DAP content (μmol/mg)	Molar ratio to *meso*-DAP						Molar ratio of GlcN + Mur to acetic acid
			GlcN	Mur	D-Ala	D-Glu	L-Ala	NH_3	
A-40-2	10	0.94	0.91	0.98	0.86	0.94	0.64	0.09	0.83
2b-2	10	0.82	0.86	0.99	0.82	1.0	0.37	0.04	0.91
C-11	10	1.1	0.84	0.93	0.81	0.93	0.52	0.43	0.94
C-125	7	0.90	0.83	0.99	0.88	1.0	0.70	0.02	0.72
	10	0.93	0.87	1.0	1.0	1.1	0.54	0.01	0.80
Y-25	7	0.84	0.99	1.1	1.0	1.0	0.60	0.06	0.72
	10	0.93	0.81	0.97	0.95	1.0	0.54	0.02	1.1
A-59	7	0.83	0.91	1.1	1.0	1.1	0.62	0.03	0.84
	10	0.87	0.89	1.1	1.0	1.1	0.64	0.01	0.87
C-3	7	1.1	0.87	0.97	0.86	1.0	0.74	0.05	0.80
	10	0.99	0.96	0.84	0.80	1.1	0.60	0.04	0.74
C-59-2	10	0.94	0.91	0.90	0.75	1.0	0.75	0.08	1.0
M-29	7	0.84	0.91	1.1	1.0	1.0	0.81	0.16	0.88
	10	0.94	0.83	1.0	0.97	1.0	0.86	0.33	0.98
57-1	7	0.81	0.93	1.1	1.0	0.98	0.45	0.31	0.84
	10	0.87	1.1	1.1	0.93	1.1	0.62	0.49	1.2
B. subtilis GSY 1026	7	0.97	0.80	0.86	0.83	0.87	0.59	0.43	0.82

[+]Abbreviations: Ala, alanine ; GlcN, lucosamine ; Glu, glutamic acid ; *meso*-DAP, *meso*-diaminopimelic acid ; Mur, muramic acid.

Essentially, the composition of peptidoglycan was not changed whether the strain was cultured at pH 7 or 10. It was therefore concluded that all of the peptidoglycans of the alkaliphilic *Bacillus* strains so far examined are of the Alγ-type of peptidoglycan, which is found in the majority of the strains of the genus *Bacillus*.

C. Acidic polymers in the cell wall of alkaliphilic *B. halodurans* C-125

Most strains of group 2 can grow at neutral pH and require the presence of sodium ions. The same acidic amino acids and uronic acids are found in much smaller quantities in the walls prepared from bacteria grown at neutral pH. This indicates that the acidic components in the cell walls of the group 2 bacteria play a role in supporting growth at alkaline pH. One of the alkaliphilic *Bacillus* strains isolated in our laboratory, *B. halodurans* C-125, the whole genome sequence of which was determined by Takami et al. (2000), grows well at neutral pH. The chemical composition of its nonpeptidoglycan components was relatively simple compared with other group 2 strains (Aono 1985).

1. Teichuronic acid in the Nonpeptidoglycan

The nonpeptidoglycan components (TCA-soluble fraction) of alkaliphilic *B. halodurans* C-125 grown at alkaline or neutral pH contained two acidic structural polymer fractions (Fig. 3.5). The Al polymer of alkaliphilic *B. halodurans* C-125 was a teichuronic acid composed of glucuronic acid, galac-

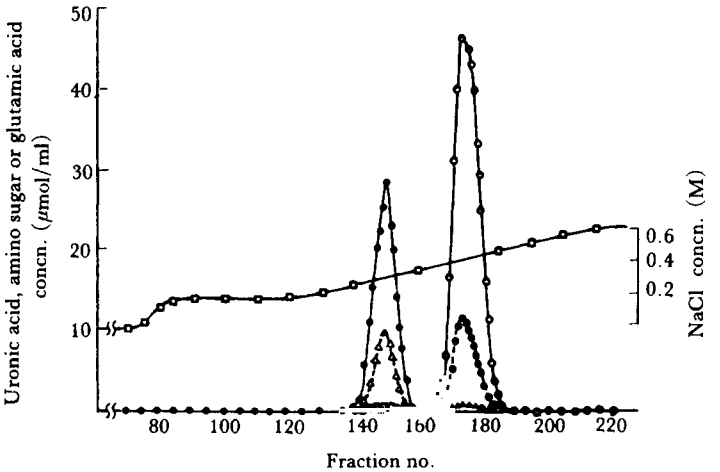


Fig. 3.5 DEAE-cellulose chromatography of the nondialyzable fraction of TCA extracts. The nondialyzable fraction of TCA extracts from the alkaline walls were dissolved in 50 mM-acetic acid/NaOH buffer (pH 5.0) and loaded on a column (2.5 × 85 cm) of DEAE-cellulose equilibrated with the same buffer. The column was eluted as described in the text. Fractions (12 ml) were collected and assayed for uronic acids (●), amino sugars (△), L-glutamic acid (○) and NaCl (□). Fractions 142–157 (fraction A1) and 168–186 (fraction A2) were pooled.
(Reproduced with permission from R. Aono, *J. Gen Microbiol.*, **131**, 108 (1985))

turonic acid and *N*-acetyl-D-fucosamine in a molar ratio of 1:1:1 (Aono et al. 1986). It is noteworthy that the amount of teichuronic acid is enhanced in the cell walls of *B. halodurans* C-125 grown at alkaline pH. This teichuronic acid amounted to 390 μg per mg peptidoglycan in the walls of the bacterium grown at an alkaline pH, 80 μg per mg at a neutral pH. The molecular weight of the teichuronic acid from the cells grown at alkaline pH was approximately 70,000 compared to a molecular weight of 48,000 at a neutral pH as estimated by gel chromatography.

2. Poly-γ-L-glutamic acid

The other fraction (A2) contained glucuronic acid and L-glutamic acid in a molar ratio of 1 : 5. This fraction was called "teichuronopeptide" by Aono (1989a; Aono and Ohtani 1990). In 1987, he isolated poly-γ-L-glutamic acid (plg; Mr about 43,000), but could not find glucuronic acid-glutamic acid copolymer (Aono 1987). Therefore, the acidic polymer found in the cell wall of *B. halodurans* was concluded to be a mixture of two kinds of polymers (poly-γ-L-glutamate and polyglucuronate). Then they made several mutants from wild *B. halodurans* C-125 (Aono et al. 1994; 1995). One of them, C-125-90 did not have plg in the cell walls and its growth in alkaline media was very poor. Aono et al. (1999) cloned *tupA* gene capable of restoring plg production and high alkali-tolerant growth (Fig. 3.6). Their results demonstrate that the acidic polymer plays a role in pH homeostasis in *B. halodurans* C-125.

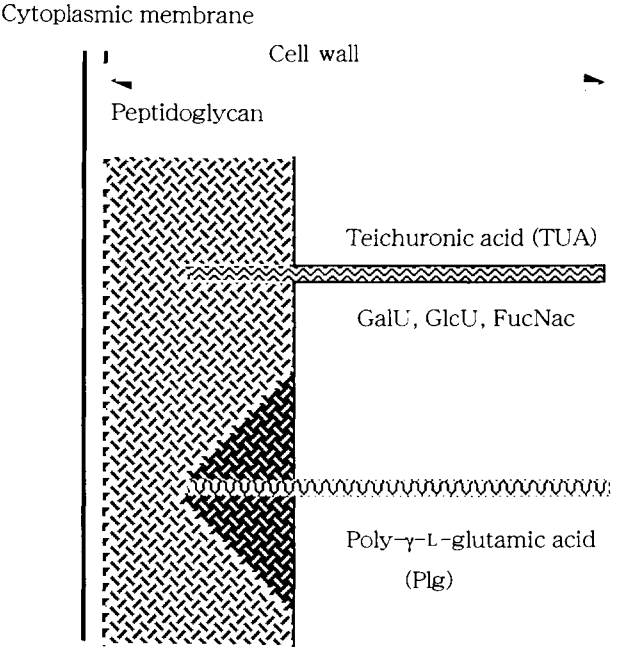


Fig. 3.6 Cell surface model of alkaliphilic *Bacillus halodurans* C-125.

Poly-γ-L-glutamate has been found widely in other bacteria of the genus *Bacillus*, e.g., *B. anthracis*, *B. mesentericus* and *B. subtilis*. These are capsular structures or extracellular mucilaginous material, and do not bind to the peptidoglycan layer of the cell wall. Recently, the whole genome sequence of *B. halodurans* C-125 was determined (Takami et al. 2000) and Ashiuchi and Misono (2002) found an operon of poly-γ-L-glutamate synthetase genes (Accession Numbers AB071407; AB071408; AB071409) activated by an operator in *tupA* gene fragment (Accession Number AB028644) (see section 6.2.3). These results show that the cells of *B. halodurans* C-125 are shaped by the Alγ-type of peptidoglycan, and the peptidoglycan is enclosed by at least two acidic polymers such as teichuronic acid and poly-γ-L-glutamatic acid with highly negative charges (Fig. 3.7). Donnan equilibrium in the bacterial cell wall was calculated to estimate the pH values inside the polymer layer of the cell walls when the outer aqueous solution is alkaline in nature. The fixed charge concentration in the polymer layer was estimated to be 2-5 mol/l from the data reported for gram-positive bacteria, particularly for an alkaliphilic bacterium *B. halodurans* C-125. According to Tsujii's calculation (Tsujii 2002) the pH values estimated to exist inside a polymer layer (cell wall) are more acidic than those of the surrounding environment by 1-1.5 U. Therefore, acidic polymers of poly-γ-L-glutamic acid and teichuronic acid are one of the important components in the cell wall of the alka-

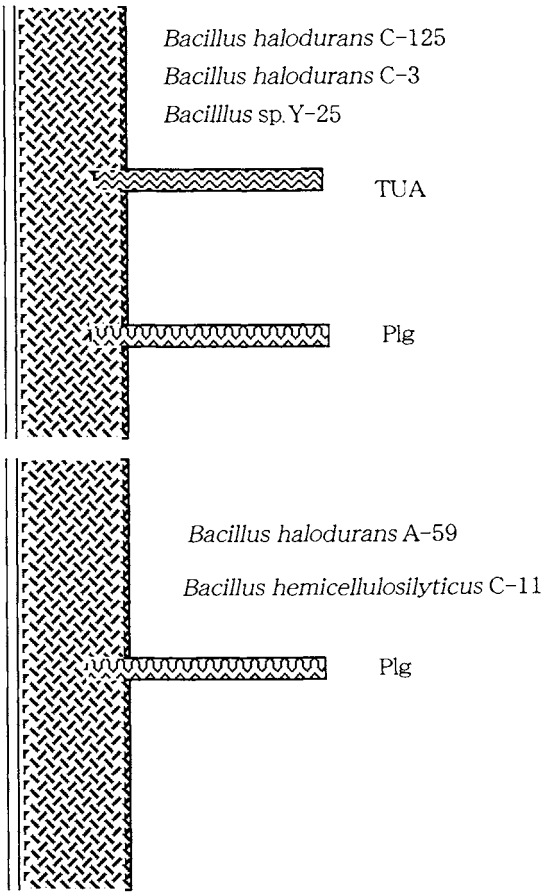


Fig. 3.7 Cell surface model of some alkaliphilic *Bacillus* strains. TUA, teichuronic acid; Plg, poly-γ-L-glutamic acid.

liphilic *B. halodurans* and contribute to the regulation of pH homeostasis in the cytoplasm.

D. Non-peptidoglycan of other bacteria

To obtain further information on group 2 cell walls of alkaliphilic *Bacillus* strains were prepared from four *B. halodurans* strains (A-59, C-3, C-11 and Y-25) and analyzed. Although several components with different compositions were detected, the cell walls commonly contained a polypeptide of acidic amino acids such as poly-γ-L-glutamatic acid. These results suggest that the substances were similar to one another in chemical structure (Fig. 3.7) (Aono et al. 1993a). As far as the author's group have tested, no γ-polyglutamate was detected in *B. pseudofirmus* group (2b-2 and A-40-2).

Gilmour et al. (2000) characterized an S-layer protein with a role in al-

kaliphily of *Bacillus pseudofirmus* OF4. The large majority of proteins of alkaliphilic *B. pseudofirmus* OF4 grown at pH 7.5 and 10.5 did not exhibit significant pH-dependent variation except a new surface layer protein (SlpA). They cloned the gene encoding SlpA and deposited it as AF242295. Although the prominence of some apparent breakdown products of SlpA in gels from pH 10.5-grown cells led to the discovery of the alkaliphile S-layer, the largest and major SlpA forms were present in large amounts in gels from pH 7.5-grown cells as well. SlpA RNA abundance was, moreover, unchanged by growth pH, and electrophoretic analysis of whole-cell extracts further indicated the absence of a 90-kDa band in the mutant. Logarithmically growing cells of the two strains exhibited no significant differences in growth rate, cytoplasmic pH regulation, starch utilization, motility, Na^+-dependent transport of α-aminoisobutyric acid, or H^+-dependent synthesis of ATP. However, the capacity for Na^+-dependent pH homeostasis was diminished in RG21 upon a sudden upward shift of external pH from 8.5 to 10.5. Therefore, the constitutive presence of SlpA enhances the capacity of the bacterium to adjust to high pH.

3.3 Cell Membrane

Cell membranes are critical cell structures. They function first and foremost as the cell's permeability barrier-the gatekeeper of substances that enter and leave the cell-but also as an anchor for many proteins and as the structural basis for ion gradient-mediated energy conservation reactions. The general architecture of all cell membranes is basically the same. Membranes of alkaliphilic *Bacillus* strains consist of proteins and lipids with hydrophilic external surfaces and hydrophobic internal matrix as well as those of neutrophiles. These form a lipid bilayer that can work concentrating, marinating dissolved substances in their cytoplasm, and keep the intracellular pH lower than the extracellular pH. The properties of lipids in the membranes are discussed in this section, although no systematic investigation has been reported. Few papers have been reported even though after 1990. In 1991, Dunkley et al. (1991) reported that facultative alkaliphiles lacked fatty acid desaturase activity and lost the ability to grow at near-neutral pH when supplemented with an unsaturated fatty acid. Two obligate alkaliphiles (*Bacillus alcalophilus* and *B. firmus* RAB) were found to have high levels of fatty acid desaturase, whereas two facultative alkaliphiles (*B. firmus* OF1 and OF4) had no detectable activity. The obligate strain outgrows the facultative strain in a chemostat at very high pH, whereas the converse is true at a pH 7.5, and the two strains grow equally well at pH 9.0. Thus the obligate strain is compromised at a near-neutral pH but is better adapted than a related facultative alkaliphile to an extremely alkaline pH. This was the first study on an enzyme of fatty acids in alkaliphiles.

Then the *B. pseudofirmus* OF4 cls gene was cloned and its gene product

was characterized (Guo et al. 1998). The gene that codes for cardiolipin (CL) synthase and an adjacent gene that codes for a MecA homologue in the alkaliphilic bacteria *B. pseudofirmus* OF4 have been cloned and sequenced (GenBank Accession Number U88888). The *cls* gene contains 1509 nucleotides, corresponding to a polypeptide of 57.9 kDa. The predicted amino acid sequence has 129 identities and 100 similarities with the *E. coli* CL synthase. Homologies were also noted with polypeptide sequences from putative *cls* genes from *B. subtilis* and *Pseudomonas putida.* Conserved histidine, tyrosine and serine residues may be a pair of the active site and participate in phosphatidyl group transfer. The *B. pseudofirmus* OF4 cls gene product was inserted into plasmid pET3 to form a recombinant plasmid pDG2, which overproduces CL synthase in *E. coli.* A membrane fraction containing the overproduced enzyme converts phosphatidylglycerol to CL and glycerol. The *B. firmus* enzyme is stimulated by potassium phosphate, inhibited by CL and phosphatidate. And it has a slightly higher pH optimum than the *E. coli* enzyme. Further role for alkaliphily was not discussed.

In 1999, the effect of growth pH on the phospholipid contents of the membranes from alkaliphilic bacteria was investigated by Enomoto and Koyama (1999). An aerobic alkaliphile *Bacillus* sp. YN-2000 and a facultatively anaerobic alkaliphile BL77/1 are able to grow over the wide pH range of 7-10.5. Net surface charge on the membranes from YN-2000 and BL77/1 were negative above pH 4, and the amounts were significantly increased when the bacteria were cultured at pH 10 as compared with those cultured at pH 7.5. Phospholipid contents of the membranes from both bacteria grown at pH 10 were much higher than those from the bacteria grown at pH 7.5, Phospholipids of the membranes from YN-2000 and BL77/1 were composed mainly of cardiolipin (CL), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). It is suggested that the increases by growth at pH 10 of negative charges on the membranes from the bacteria result mainly from the increases of acidic phospholipids such as CL and PG. Increases of phospholipid contents and/or negative charges on the membranes seem to contribute to the adaptation of YN-2000 and BL77/1 to an alkaline environment.

Although it is still premature to speculate relationships among pH, transport systems and lipids in alkaliphilic *Bacillus* strain membrane, lipids are probably an important component for maintaining pH homeostasis inside of cells.

4

Physiology

4.1 Growth Conditions

Alkaliphiles require alkaline environments and sodium ions not only for growth but also for sporulation and germination. Sodium ion-dependent uptake of nutrients has been reported in some alkaliphiles. Many alkaliphiles require various nutrients, such as polypeptone and yeast extracts, for their growth; several alkaliphilic *Bacillus* strains (*B. halodurans* C-125, A-59, C-3 and AH-101) can grow in simple minimal media containing glycerol, glutamic acid, citric acid, etc. One of the best strains for genetic analysis is alkaliphilic *B. halodurans* C-125 and its many mutants have been made by conventional mutation methods. Recently, whole genome sequence was determined and annotated (Takami et al. 2000).

4.1.1 pH Values of Culture Media

A. External pH values

Alkalinity in nature may be the result of the geology and climate of the area, of industrial processes, or promoted by biological activities. The most stable alkaline environments on earth are the soda lakes and soda deserts distributed throughout the world in tropical and sub-tropical areas. These environments are about pH 10–11.5. Many microorganisms have been isolated from these alkaline environments. More transient, localized alkaline environments due to animal excreta and such may exist in soils that are not alkaline overall. Therefore, many alkaliphilic bacteria can be isolated more commonly from soil (see Section 2.2). Some alkaliphilic bacteria can change external pH value to a pH suitable for growth and create their own world, as described in the following section.

Since 1968, Horikoshi and his coworkers have systematically studied alkaliphilic microorganisms (Horikoshi 1971a). They have paved the way in establishing a new microbiology, alkaliphilic microbiology, to use these microorganisms as microbial and genetic resources. These bacteria have an optimum pH value for growth of about 9 to 10.5 in Horikoshi-I medium. None of the alkaliphilic bacteria could grow at pH below 6.0 and all indicated optimum pH value for growth above 8. Therefore, bacteria with pH optima for growth in excess of pH 8, usually pH 9 to 11, are defined as alka-

liphilic bacteria. This property is the most characteristic feature of these bacteria.

B. Internal pH values

The facultative alkaliphile, *B. halodurans* C-125, grows between pH 6.8 and 10.8. Quantities of poly-γ-L-glutamic acid (plg) in the cell walls increase with increasing culture pH. In the range of 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. Aono et al. (1997) developed a new method to measure the cytoplasmic pH of the facultative alkaliphilic *Bacillus halodurans* 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 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. At pH above 10.7, the organism lost its pH homeostatic activity. *B. halodurans* C-125 does not grow at pH above 10.8. *B. subtilis* GSY1026 became sensitive when exposed to extracellular pH above 8.2 (Fig. 4.1). *B. subtilis* GSY1026 does not grow at pH values above 8.5.

The cytoplasmic pH maintenance activity of *B. halodurans* C-125 increased with increasing culture pH, indicating that the activity was regulated

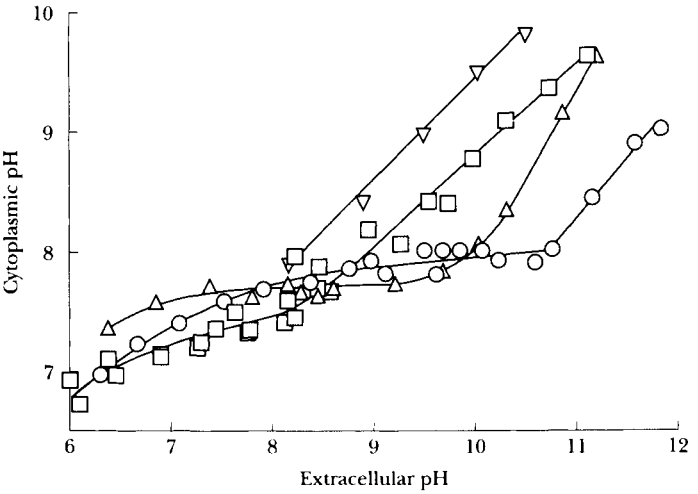


Fig. 4.1 Cytoplasmic pH of C-125 cells as a function of the extracellular pH. *B. halodurans* C-125 was grown at pH 7.0(□), 8.5(△) or 10.0(○). *B. subtillis* GSY1026(▽) was grown at pH 7.0 and used as a reference strain. The cells in the stationary phase of growth were loaded with BCECF. The cells were 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 the text. Cytoplasmic pH of the cells was calculated mainly from the $Ratio_{510/450}$. The pH above 9 was calculated from F_{510}.
(Reproduced with permission from R. Aono et al., *Microbiology*, **143**, 2531(1997))

in response to the culture pH. A mutant C-125-90 lacking plg could grow between pH 6.8 an 10.5, but did not grow at pH 10.8. At pH 10.5, the mutant exhibited poor growth rates, only 20% that of parental C-125. The mutant C-125-9 restores plg synthesis by the introduction of the gene "*tupA*" cloned from the parental strain and its alkaliphily. Further details are discussed in the Section 6.3.4.

C. External pH change during cell growth

Alkaliphilic bacteria can change their environment to a pH value suitable for growth. *Bacillus clausii* No.221, which is a good alkaline protease producer, can grow slowly at neutral pH, changing the pH of the culture broth. It was found that once the pH reached about 9, the bacteria began to grow rapidly and produced a large amount of the same alkaline protease. Ueyama and Horikoshi (unpublished data) isolated an alkaliphilic *Arthrobacter* sp. which utilizes an ε-caprolactam polymer. This microorganism was also capable of changing the broth pH to an optimum (Fig. 4.2). The author conducted another experiment, which has not been published. The following microorganisms were used in the experiment: 1) *Aspergillus oryzae*, 2) *Bacillus circulans* IAM 1165, which produces an enzyme that lyses *A. oryzae* at pH 7–8, 3) alkaliphilic *Bacillus pseudofirmus* A-57, which can grow in the range pH 8–10. These three strains were mix-cultured in Horikoshi-II medium of pH 5 in the absence of 1% Na_2CO_3. The cultivation was carried out for a week with continuous shaking at 30°C. *A. oryzae* grew in the medium first. After 3 days incubation, *A. oryzae* began to autolyze and the

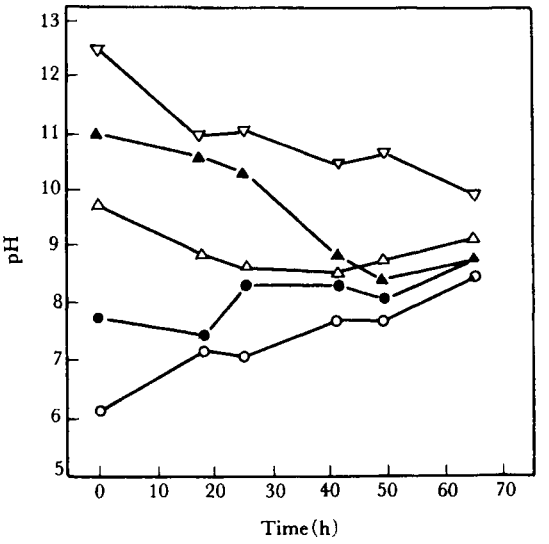


Fig. 4.2 Change in external pH values during cultivation of *Arthrobacter* sp.
(Reproduced from K. Horikoshi and T. Akiba, *Alkalophilic Micoroorganisms*, p.36, Springer: Japan Scientific Societies Press (1982))

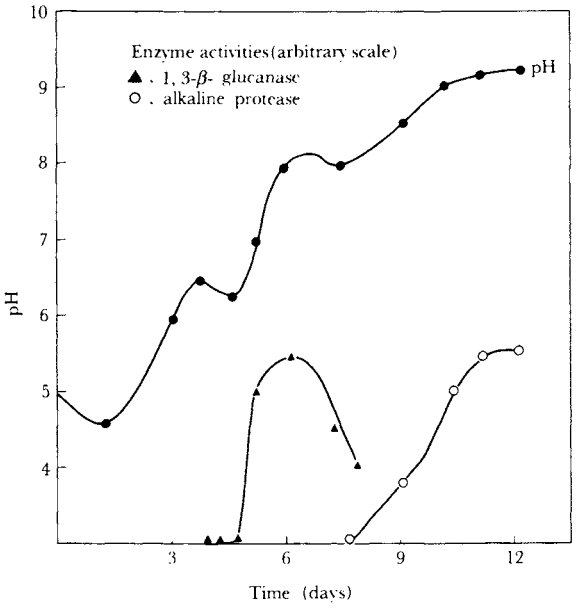


Fig. 4.3 pH and enzyme activities during mixed cultivation of *A. oryzae*, *B. circulans* and *B. pseudofirmus*. A-57.

pH of the broth increased to 6–7. Then *B. circulans* started to grow and produce the lytic enzyme which lysed *A. oryzae*. The pH of the broth gradually increased to 8. As a result, alkaliphilic *B. pseudofirmus* A-57 grew well and produced an alkaline protease (Fig. 4.3). This phenomenon is very interesting from the ecological point of view, because it may be the reason why alkaliphilic bacteria can create a microcosmos and live in acidic soil.

4.1.2 Sodium Ion

A. Growth

Another characteristic property of the alkaliphilic *Bacillus* strains is that for many of them sodium ions are an absolute requirement for growth and motility (see Section 3.1). The first finding of the sodium requirement was described by Horikoshi and Kurono in a patent application for the extracellular production of catalase from alkaliphilic *Bacillus* sp. No. Ku-1 (FERM: No. 693). The strain isolated could not grow in nutrient broth in the absence of NaCl, but the addition of NaCl (1–5%) induced good growth in the nutrient broth, even though the pH value of the broth was not changed. In 1973, two scientific papers were published (Boyer et al. 1973; Kurono and Horikoshi 1973), but the sodium requirement was cited as only one of the cultural characteristics. No one was interested in sodium ions until Kitada and Horikoshi's paper was published (Kitada 1977). Amino acid uptake into the cells as a function of NaCl was exhibited and they concluded

that the presence of NaCl plays an important role in the active transport mechanism of amino acid into the cells. This section discusses the effects of sodium ions on growth, sporulation, germination and flagellar motors. In some alkaliphilic *Bacillus* strains, K^+ ions can be substituted for Na^+ ions.

B. Sporulation

Sporulation and germination are typical differentiation processes of bacteria. *B. subtilis* has been studied biochemically and genetically for many years. Several types of evidence suggest that a specific change in transcription level causes dramatic differentiation. In particular, sigma or sigmalike subunits are responsible for the specificity of transcription in the differentiation process.

A sodium requirement was also observed in the differentiation process, spore formation and germination. However, only one report on sporulation of an alkaliphilic *Bacillus* strain has been published. Kudo and Horikoshi (1979) isolated alkaliphilic *Bacillus pseudofirmus* No. 2b-2 from soil. This strain showed excellent spore yield in alkaline Schaeffer medium. The optimum pH for sporulation is close to that for growth, but the range is narrow. The optimum temperature for sporulation was almost the same as that for growth (34–37°C). Growth at 45°C was faster than at 34°C. Table 4.1 shows the effect of NaCl concentration on sporulation. The optimum concentration was similar to that for growth, but the range was narrow.

Table 4.1 Effect of NaCl concentration on sporulation of *Bacillus* sp. No. 2b-2

NaCl concentration (M)	Final OD_{610}†	Growth rate	Percent of sporulation
0	2.9	54	0
0.02	3.7	46	30–40
0.2	4.7	44	70
0.4	4.0	44	30–40
1	2.9	46	1

† The final OD and the sporulation rate were measured after 47 h of incubation at 37°C.
(Reproduced from T. Kudo and K. Horikoshi, *Agric. Biol. Chem.*, **43**, 2613 (1979))

C. Germination

Kudo and Horikoshi (Kudo 1983a, b) reported germination of alkaliphilic *B. pseudofirmus* No. 2b-2. In 0.2 M NaCl solution spores of *B. pseudofirmus* No. 2b-2 germinated very well in the presence of both L-alanine and inosine, but germinated poorly in the presence of only L-alanine. However, no germination was observed in the absence of NaCl (Table 4.2). The optimum temperature for germination was about 37°C, and germination occurred in the range of pH 8.5 to 11.1. The optimum pH for germination was around 10.0 (Fig. 4.4). The optimum concentration of NaCl for the germination was 0.1 to 0.5 M. Other cations such as K^+, NH_4^+, Rb^+, Cs^+ and Ca^{2+} did not show this stimulating effect. Only Li^+ showed weak stimu-

Table 4.2 Germination of *Bacillus* sp. No. 2b-2 spores

Compound added[1]			Absorbance reduction (%)[2]
Alanine	+ Inosine	+ NaCl	46
Alanine		+ NaCl	10
	Inosine	+ NaCl	3
Alanine	+ Inosine		0
		NaCl	0

[1]The final concentration of all germinants was 0.4 mM and the final concentration of NaCl was 0.2 M.

[2]Germination is expressed as the optical density reduced (%) after 60 min at 37 °C.

All experiments were conducted in 0.1 M 2-amino-2-methyl-1,3-propanediol (AMPD) buffer (pH 9.7).

(Reproduced from T. Kudo and K. Horikoshi, *Agric. Biol. Chem.*, **47**, 666 (1983))

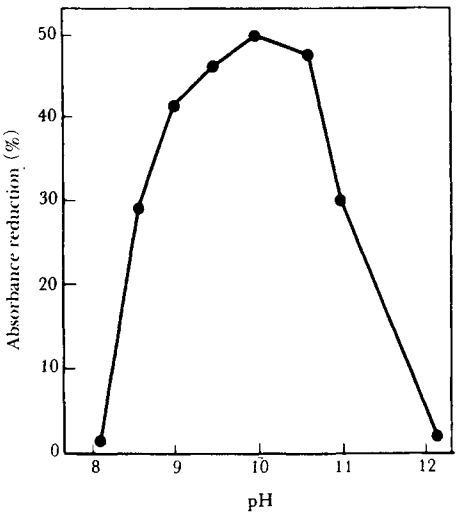


Fig. 4.4 Effect of pH on germination of spores of *Bacillus* sp. No. 2b-2.
(Reproduced from T. Kudo and K. Horikoshi, *Agric. Biol. Chem.*, **47**, 666 (1983))

lation. In the absence of Na^+, loss of heat resistance, acquisition of stainability and decrease in absorbance were not observed at all even in the presence of germinants such as L-alanine and inosine. However, when Na^+ was added to the medium at the times indicated by arrows the absorbance decreased immediately (Fig. 4.5).

About two decades after of Kudo's paper, Krulwich's group reported that Orf9 was responsible for cation transport (Wei et al. 2003). A putative transport protein (Orf9) of alkaliphilic *B. pseudofirmus* OF4 belongs to a transporter family (CPA-2) of diverse K^+ efflux proteins. Orf9 greatly increased the concentration of K^+ required for growth of a K^+ uptake mutant of *E. coli*. Nonpolar deletion mutants in the *orf9* locus (Accession Number U89914.2) of the chromosome were isolated. During extensive genetic studies of the mutants, they found endospore formation in amino

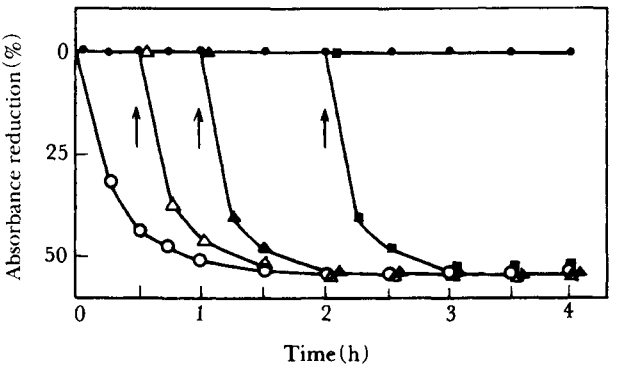


Fig. 4.5 Effect of Na^+ on decrease in absorbance. NaCl was added at the times indicated by arrows to the germination medium (pH 9.7, without NaCl).
(Reproduced from T. Kudo and K. Horikoshi, *Agric. Biol. Chem.*, **47**, 667 (1983))

acid-rich medium to be significantly defective and germination modestly defective in the *orf9* and *orf7-orf10* deletion mutants.

Sporulation agar plates containing L-glutamate were incubated at 30°C for six days. Spores were harvested from the surface and washed in ice-cold deionized water. For the wild type, washed-spore preparations were about 95% phase-bright spores. Spores were stored at −20°C in deionized water. Spore germination was measured by the change in optical density (OD) of samples at 490 nm after being heat activated in water at 70°C for 30 min. The germination was then carried out in 125 mM 2-amino-2-methyl-1-propanol (AMP) buffer, pH 10.0, with appropriate germinants (200 mM NaCl and either 10 mM inosine, or 10 mM L-alanine with 5 g of O-carbamyl-D-serine). A 50% loss in OD corresponded to 100% germination of the *B. pseudofirmus* OF4 wild-type spores. There was no dramatic indication of the involvement of the *orf9* gene locus. Some modest differences in germination properties were observed, however, which may still reflect undetected differences between the wild-type and mutant spores rather than a direct relationship between the mutations and germination. Incubation of wild-type spores in AMP buffer at 30°C with either NaCl or KCl, at 200 mM, but not in their absence, resulted in slow germination after a significant lag, as measured by the reduction in OD at 490 nm. NaCl was significantly more efficacious than KCl in supporting germination and was used for subsequent studies. pH 10 was used routinely after it was found that germination of wild-type spores was comparable at pH 10 to 11.9 but was barely detectable at pH 9 and below. As shown in Fig. 4.6A, germination of wild-type spores in the presence of 200 mM NaCl was significantly enhanced by the addition of either L-alanine or inosine. Although not shown, neither of the organic germinants resulted in any germination when added to AMP buffer in the absence of NaCl or KCl. As shown in Fig. 4.6B and C, the germination response of the *orf9* mutant strains was less than that of the wild type with NaCl and either L-alanine or inosine. Although it is premature to discuss

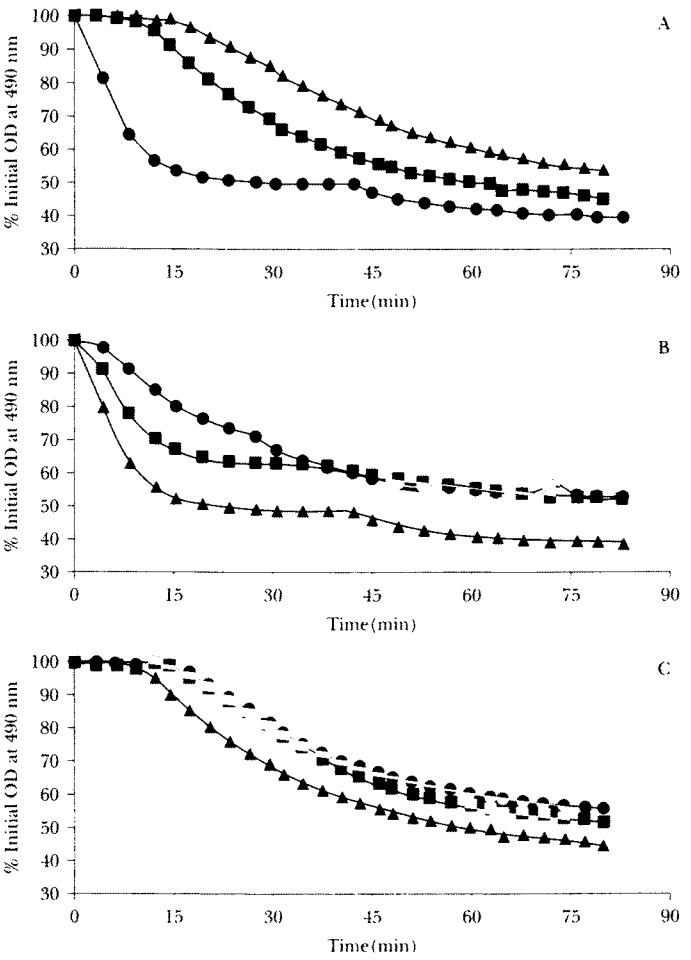


Fig. 4.6 Germination of spores from *B. pseudofirmus* OF4 wild type and *orf9* mutant strains. (A) Germination of *B. pseudofirmus* OF4 wild type in 200 mM NaCl(triangles), 200 mM NaCl plus 10 mM L-alanine(squares), and 200 mM NaCl plus 10 mM inosine(circles). (B) Germination in 200 mM NaCl and 10 mM inosine of *B. pseudofirmus* OF4 wild type(triangles), *orf7-orf10* deletion mutant(squares), and *orf9* mutant(circles). (C) Germination in 200 mM NaCl and 10 mM L-alanine of *B. pseudofirmus* OF4 wild type(triangles), *orf7-orf10* deletion mutant(squares), and *orf9* mutant(circles). (Reproduced with permission from Y. Wei et al., *J. Bacteriol.*, **185**, 5133(2003))

the step stimulated by Na^+ and K^+, these ions stimulate the uptake of the germinants into the spores.

4.1.3 Temperature and Nutrition

High temperature for growth is not a characteristic property of alkaliphiles. The highest temperature for bacteria so far reported is 57°C for *Bacillus*

Table 4.3 Media for *Bacillus halodurans* C-125

Horikoshi minimal medium	Glutamate	0.2%
	Glycerol	0.5%
	K_2HPO_4	1.4%
	KH_2PO_4	0.4%
	$MgSO_4 \cdot 7H_2O$	0.02%
	Na_2CO_3	1.0%
	w/v	
Aono and Horikoshi synthetic medium	Citric acid	0.034%
	Glucose	0.5%
	Ammonium sulfate	0.1%
	KNO_3	0.1%
	K_2HPO_4	1.37%
	KH_2PO_4	0.59%
	$MgSO_4 \cdot 7H_2O$	0.05%
	Na_2CO_3	1.0%
	w/v	
Modified DM-3 protoplast regeneration medium	Agar	1.0%
	Sodium succinate	0.5 M
	Casamino acid(Difco)	0.5%
	Yeast extract(Difco)	0.5%
	K_2HPO_4	0.35%
	KH_2PO_4	0.15%
	Glucose	2.0%
	$MgCl_2$	0.1 M
	$CaCl_2$	1.25 mM
	Bovine serum albumin	0.04%
	Chloramphenicol	2.5 mg/ml
	pH	7.3–6.8

clausii No. 221 (ATCC 21522), which is an alkaline protease producer. Kimura and Horikoshi (1988) isolated several psychrophilic alkaliphiles. One of them, strain 207, which is an aerobic coccus 0.8–1.2 μm in diameter, can grow at temperatures of −5 to 39°C at pH 8.5. The optimum pH value for growth changed from 9.5 at 10°C to 9.0 at 20°C. But almost all alkaliphilic bacteria showed optimum growth at 25–45°C.

No precise experiment has been reported on nutrient requirements. During the development of new host-vector systems, Aono investigated more than 20 strains of alkaliphilic *Bacillus* strains and demonstrated that vitamins such as biotin, thiamine and niacin are required for some alkaliphilic *Bacillus* strains. Kudo et al. (1990) found that some alkaliphilic *Bacillus* strains could grow on minimal media; alkaliphilic *B. halodurans* C-125 (FERM No. 7344) grows well on a minimal medium containing glutamate and glycerol, as shown in Table 4.3.

4.2 Mutants, Antiporters and Alkaliphily

How alkaliphiles adapt to their alkaline environments is one of the most in-

teresting and challenging topics facing microbiologists. The followings are the first reports of a DNA fragment responsible for the alkaliphily of alkaliphilic microorganisms. These fragments (in early works they were called pALK, then Mrp by Krulwich et al., and Sha by Kosono et al.) were first discovered in work on alkaliphilic *Bacillus halodurans* C-125. Breakthrough studies in alkaliphilic *B. halodurans* C-125 by the present author's group ultimately led to the discovery of a novel gene locus that encodes the crucial alkaliphile Na^+/H^+ antiporter for pH homeostasis (Kudo et al. 1990; Hashimoto et al. 1994).

4.2.1 Isolation and Properties of Alkali-sensitive Mutants

Two series of experiments have been conducted by the author's colleagues. Kudo et al. (1990) focused on the cell membrane of *B. halodurans* C-125, mainly on H^+/Na^+ antiporters that regulate intracellular pH values. Aono et al. (1992b) analyzed the cell walls of *B. halodurans* C-125 and found that cell walls as well as the cell membrane are responsible for pH homeostasis (see the Section 3.2).

4.2.2 Antiporter (pALK) Mutants

A. Isolation of alkali-sensitive mutants

Kudo et al. (1990) reported alkali-sensitive mutants of alkaliphilic *B. halodurans* C-125 by mutagenesis. This strain was selected for the following study since it grows well in Horikoshi minimal medium over the pH range of 7 to 11.5 at 37°C. Alkaliphilic *B. halodurans* C-125 (Trp^- Ura^- Cm^s) was treated with nitrosoguanidine in 25 mM glycine-NaOH-NaCl buffer of pH 8.5 and plated on Horikoshi-II medium (pH 7.5) containing 5g/l NaCl instead of sodium carbonate. Colonies that appeared on the plates were transferred onto Horikoshi-II medium (pH 10.3). After 16 h of incubation at 37°C, seven alkali-sensitive mutants that could not grow at pH 10.5 but grew well at pH 7.5 were obtained from 7×10^4 colonies. Two mutants, Nos. 18224 and 38154 which showed different properties from each other, were selected (Table 4.4).

One of the mutants, No. 38154, was unable to sustain low internal pH in the presence of either Na_2CO_3 or K_2CO_3. The internal pH was 10.4, which was the same as that for all strains in the presence of K_2CO_3. Although the other mutant, No. 18224, cannot grow at pH > 9, the internal pH of the mutant was 8.7 in the presence of Na_2CO_3. This value is close to that of the parent strain (pH 8.6). Mutant No. 18224 cannot maintain a low internal pH in the presence of K_2CO_3. It is suggested that mutant No. 38154 was defective in the regulation of internal pH, whereas mutant No. 18224 apparently showed normal regulation of internal pH values: Na^+ ion is also assumed to play an important role in pH homeostasis.

Table 4.4 Alkali-sensitive mutants of alkaliphilic *Bacillus halodurans* C-125

Strain No.	Growth capable pH	Internal pH[1]	Cell morphology
12797	< 8.5	8.55	Curling
13797	< 8.5	8.64	Curling, elongation
18224	< 8.5	8.72	Elongation
19363	< 8.5	8.92	Elongation
47629	< 8.5	8.64	Curling
38154	< 8.5	10.40	Normal
C-125	< 11.0	8.64	Normal

[1] Measured by incorporation of ^{14}C-methylamine into cells at pH 10.5.
(Reproduced with permission from M. Hashimoto et al., *Biosci. Biotechnol. Biochem.*, **58**, 2090 (1994))

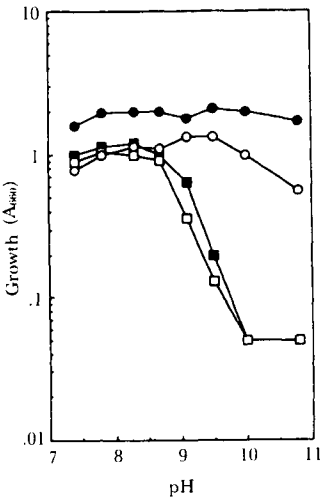


Fig. 4.7 Effect of pH on growth of alkali-sensitive mutant 38154 and its derivatives. pH was adjusted by the addition of NaOH, Na_2CO_3, $NaHCO_3$, or NaCl. Overnight cultures were added to fresh Horikoshi-II medium at an absorbance at 660 nm (A_{660}) of 0.01. Each point represents cell growth after 7h at 37°C. Parental strain C-125 (●), mutant 38154 (■), mutant 38154 carrying pHW1 (□), and mutant 38154 carrying pALK2 (○) were cultivated.
(Reproduced with permission from T. Kudo et al., *J. Bacteriol.*, **172**, 7283 (1990))

B. Molecular cloning of DNA fragments conferring alkaliphily

The DNA fragment of the parental *B. halodurans* C-125 that restores alkaliphily to the alkali-sensitive mutant strain No. 38154 was found in a 2.0-kb DNA fragment, which was cloned in a recombinant plasmid pALK2 (Fig. 4.7). Another recombinant plasmid, pALK1, also restored alkaliphily to another alkali-sensitive mutant strain, No. 18824.

Nucleotide sequence analysis and restriction mapping indicated about

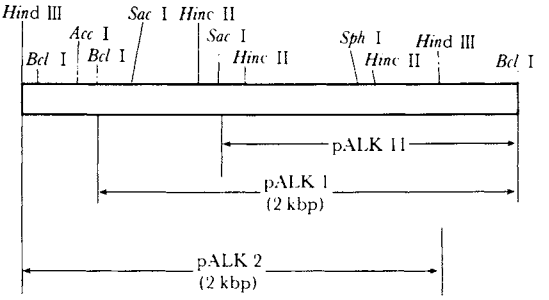


Fig. 4.8 Restriction maps of plasmids pALK1, pALK11, and pALK2. Restriction sites and their coordinates are indicated.
(Reproduced with permission from T. Kudo et al., *J. Bacteriol.*, **172**, 7283 (1990))

Table 4.5 Intracellular pH of mutants 18224 and 38154 and of transformants*

Strain	Intracellular pH
C-125	8.64
18224	8.72
38154	10.40
38154 (pALK2)	8.80

* Cells were grown to stationary phase at pH 8, washed with 20 mM Tris buffer (pH 8), and suspended in 100 mM carbonate buffer (pH 10.4) prepared with Na_2CO_3. The cell suspensions were incubated for 10 min at 25 °C with vigorous aeration in the presence of 2.2 μM ^{14}C-methylamine. Samples were removed after 10 min and filtered for determination of ΔpH. Control experiments in which 20 μM gramicidin was included in the incubation mixture were conducted for each assay. Assuming that ΔpH = 0 in the presence of gramicidin, internal pH was calculated from the difference in the presence and absence of gramicidin.
(Reproduced with permission from T. Kudo et al., *J. Bacteriol.*, **172**, 7283 (1990))

1.6 kb of common sequence between pALK1 and pALK2 (Fig. 4.8). Plasmid pALK1 conferred alkaliphily on mutant 18224 but not on mutant 31154. Mutant 38154, which could not regulate its internal pH value in alkaline media, recovered pH homeostasis after the introduction of pALK2 (Table 4.5) but not after the introduction of pALK1. These results indicate that there are at least two factors involved in alkaliphily located in a closed linked region of chromosomal DNA, although the precise functions of these two DNA fragments are not known yet. These results strongly suggest that at least two genetic loci that are required for alkaliphily and that they are clustered on the chromosome.

Furthermore, nucleotide sequence analysis showed that pALK2 contained parts of two ORFs (ORF1 and ORF3) and one complete ORF (ORF2) (Fig. 4.9). To identify the region in pALK2 responsible for complementation of the defect in mutant 38154, three deleted derivatives of pALK2 were constructed and each was tested for the capacity to restore alkaliphilic growth in mutant 38154 (Hamamoto et al. 1994). The ΔΨ-depen-

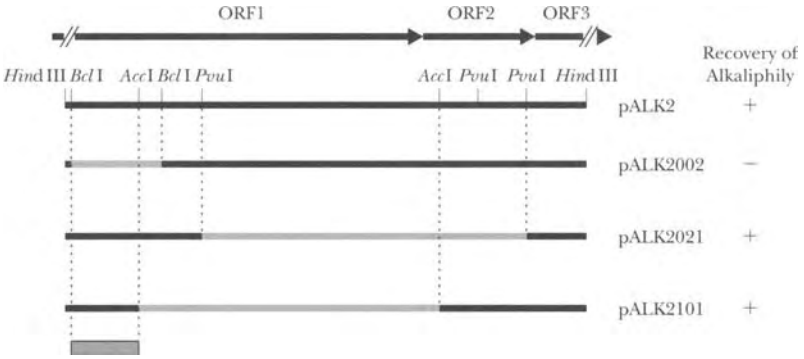


Fig. 4.9 Restriction maps of a DNA fragment from strain C-125 responsible for the recovery of alkaliphily in the 38154 mutant. A 2.0-kb *Hin*d III fragment cloned in pALK2 is shown together with the open reading frames(*arrows*) located in the fragment. The subcloned parts of the *Hin*d III fragment are indicated by the thick line. The regions shown by dots were deleted in subcloning parts of the insert from pALK2. The effectiveness of each plasmid in restoring alkaliphily, as determined by testing transformants for the ability to grow in Horikoshi-II liquid medium (pH 10.0) at 37°C, is shown on the right. The DNA fragment that appears to be responsible for the recovery of alkaliphily is shown by a shaded box.
(Reproduced with permission from T. Hamamoto et al., *Mol. Microbiol.*, **14**, 939(1994))

dent Na^+/H^+ antiporter activity expressed by each of these types of transformants was then examined by measuring H^+ influx into right-side-out membrane vesicles. Consistent with these growth phenotypes, imposing an artificial $\Delta\Psi$ resulted in intravesicular acidification in the case of the 38154 (pALK2021) and 38154 (pALK2101) transformants, whereas Na^+-loaded vesicles derived from the 38154 (pALK2002) transformant exhibited no H^+ influx. These results indicate that the mutation site in the genome of mutant 38154 is in the DNA region between the *Bcl*I and *Acc*I sites in ORF1 (Fig. 4.9), and that the ORF1 product is responsible for the Na^+/H^+ antiport activity that is important for the alkaliphily of the parental strain.

To identify the mutation site in the genome of mutant 38154, the nucleotide sequence of the corresponding DNA fragment was determined. Direct sequencing revealed a substitution mutation from G to A that results in a single amino acid change in the 393rd residue in the ORF1 product (Fig. 4.10). A 5.1-kb DNA fragment containing ORF1 to ORF4 was then cloned, and a restriction map of the entire cloned fragment is shown in Fig. 4.8. Another alkali-sensitive mutant, strain 18224, was found to have a mutation resulting in an amino acid substitution in the 82nd residue in the ORF3 product (Seto et al. 1995). Mutant 18224 still retains the ability to control the internal pH, although it shows alkali-sensitive growth (Hashimoto et al. 1994). It appears that ORF3 is not involved in Na^+/H^+ antiport itself but it may be involved in a regulatory process or some other function associated with ion transport. This is the first report of a DNA

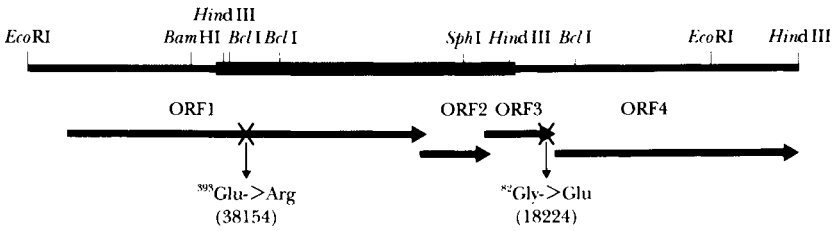


Fig. 4.10 Positions of amino acid substitutions responsible for the alkali sensitivity of mutants 38154 and 18224. A 5.1-kb *Eco* RI-*Hind* III fragment from strain C-125 is shown together with ORF1 to ORF4(arrows) located in the fragment. A *Hind* III fragment cloned in pALK2, which restores the alkaliphily of the mutant 38154, is indicated by a thick line. Amino acid substitutions from ^{393}Gly to Arg in the ORF1 product and from ^{82}Gly to Glu in the ORF3 product are responsible for the alkali sensitivity of mutants 38154 and 18224, respectively.
(Reproduced with permission from T. Hamamoto et al., *Mol. Microbiol.*, **14**, 939(1994) and Y. Seto et al., *Biosci. Biotechnol. Biochem.*, **59**, 1364(1995))

fragment responsible for an Na^+/H^+ antiporter system in the alkaliphily of alkaliphilic microorganisms.

C. Other DNA fragments conferring alkaliphily in B. halodurans C-125

Aono et al. (1992c) analyzed the acidic components constructing the cell wall of facultative alkaliphile *B. halodurans* C-125. Cell wall-defective mutants were previously isolated from the parental strain. During the course of studies, several alkali-hypersensitive mutants, whose cell walls did not appear to be defective were isolated. A threonine auxotrophic C-125-001 was first isolated from wild strain C-125 treated with 1% ethylmethanesulfonate (EMS) in Aono's alkaline synthetic medium (see Table 4.3). A conventional penicillin-screening method was used for the C-125-001 cells mutagenized with 1% EMS. The alkali-sensitive mutants that grew on a neutral complex medium (pH 7.2) but not on an alkaline medium (pH 10.3) were selected by the replica method. About 0.5% of the colonies grown at pH 7.2 showed somewhat poorer growth at pH 10.3 than the parent strain. Our collection of mutants isolated while looking for cell wall-defective mutants contained three types of alkali-sensitive mutants in pH-dependent growth. Among these, a type 1 mutant (AS-399) was duly alkali-sensitive. The upper limit of pH for growth was about 10, and that of the parent strain C-125 was about 11. The pH-dependent growth of these mutants appeared to be similar to those of mutants 18224 and 38154. Type 2 mutants (AS-187, -292 and -350) were more sensitive than mutant AS-399 (Fig. 4.11). The upper limits were almost identical to that of neutrophilic *Bacillus subtilis* GSY1026 used as a reference strain. A type 3 mutant (AS-409) was extremely alkali-sensitive and grew only at around pH 7.5. Phenotypes of these mutants may become neutrophilic. However, none of the mutants in this study grew at pH below 6.5. The lower limit of pH for growth of the mutants was identical to that of

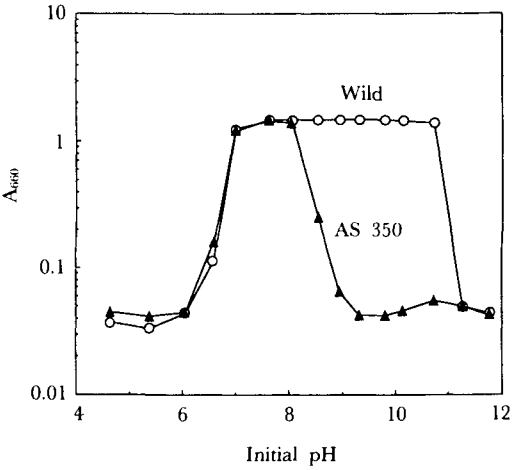


Fig. 4.11 pH-dependent growth of C-125 wild strain and alkali-sensitive mutant AS-350. Medium was adjusted to various pHs indicated in the figure by addition of NaCl, $NaHCO_3$, Na_2CO_3, and NaOH. Both strains were grown in neutral medium (pH 7.3) at 37 °C for 12 h. Each culture was diluted 100-fold with fresh media of various pH. All the subcultures were aerobically incubated at 37 °C. Absorbance at 660 nm of each culture was recorded after 8 h.

the parent strain. Therefore, these mutants did not phenotypically become neutrophiles. Measurement of 9-aminoacridine incorporation in cells of the mutants indicated that the mutants were defective in the regulation of internal pH in an alkaline environment.

A gene that complemented the mutation in AS-350 and restored the alkaliphilic growth of the mutant was cloned from the parent strain (Aono et al. 1993c). Of approximately 1,000 chloramphenicol-resistant transformants, two grew on the alkaline medium. Plasmids harbored by these two transformants were found to be the same in size and restriction map. The plasmid, designated pAG10, grew in the alkaline medium as well as the parent strain C-125 (Fig. 4.12). The upper and lower limits of pH for growth of AS-350 carrying pHG10 were the same as those of the parent strain. The plasmid pAG10 restored only mutant AS-350 to alkaliphily. The pH range for growth of AS-350 carrying pAG10 was not expanded in comparison to that of the parent strain. Other type 2 mutants (AS-187 and -292) were also restored to alkaliphilic growth with pAG10. On the other hand, a type 1 mutant did not change to alkaliphilic. A type 3 mutant (AS-409) was partially restored to alkaliphily with the plasmid. The plasmid pAG10 contained an approximately 2.7-kb foreign DNA in the *Bcl*I side of the vector pHW1. Various deletion plasmids were prepared. As shown in Fig. 4.13, the mutation in the AS-350 should be complemented with a 1.0-kb *Dra*I-*Xba*I region of the plasmid, but there is no homology between pAG10 and pALK1 or pALK2. These results indicate that all the genes responsible for alkaliphily are not clustered on the same loci.

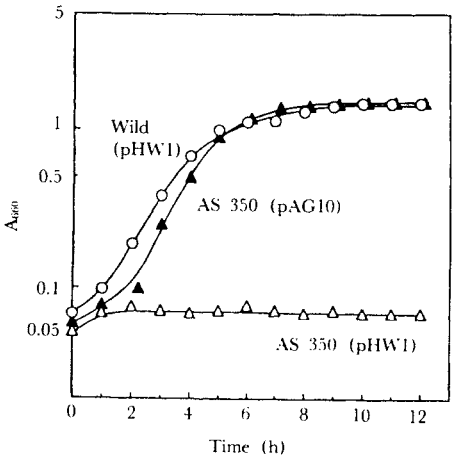


Fig. 4.12 Restoration of alkaliphily in mutant AS-350 transformed with pAG10. C-125 wild strain carrying pHW1, mutant AS-350 carrying pHW1 or pAG10 was aerobically grown in alkaline medium (pH 10.3) containing chloramphenicol (10 μg/ml) at 37°C. Absorbance at 660 nm (A_{660}) was measured periodically.

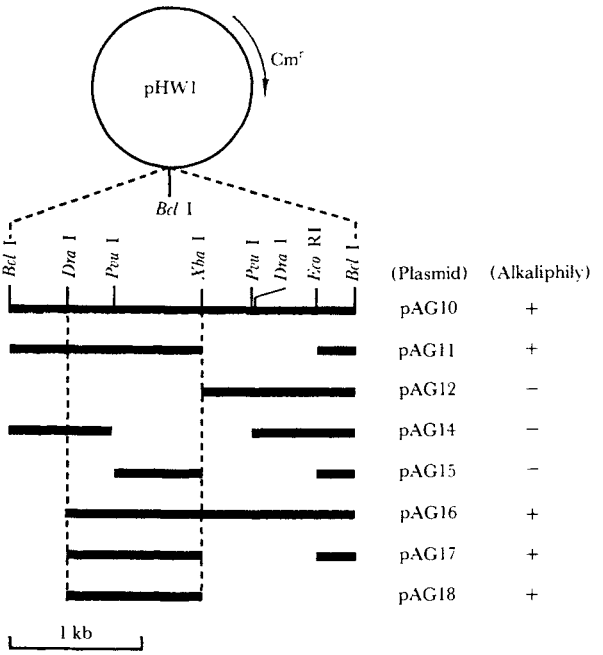


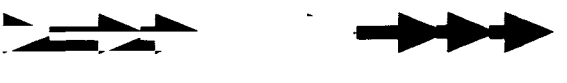
Fig. 4.13 Deletion derivatives from pAG10. Each plasmid from pAG11 to 18 is a deletion derivative from pAG10. Mutant AS-350 carrying each plasmid was examined on the alkaline agar plate (pH 10.3) for whether alkaliphily would be restored (+) or not (−). Cm^r, chloramphenicol-resistant gene.

4.2.3 Ubiquitous Distribution of *Mrp* Operons

After whole DNA sequence analysis, direct evidence for a *pALK/mrp/sha* operon has been shown in *B. subtilis*, *B. halodurans* C-125, *B. pseudofirmus* OF4, *Oceanobacillus iheyensis*, *B. clausii* etc., as shown in Fig. 4.14.

The functional studies conducted to date on individual pALK/Mrp/Sha systems include limited assays of cation and proton fluxes in whole cells and in everted membrane vesicles. To avoid confusion, the author use Mrp system for the pALK/Mrp/Sha system. Information from these assays is supplemented by inferences drawn from physiological experiments on Na^+- or K^+-sensitivity, Na^+ exclusion from whole cells or the cation-dependence of Mrp-dependent pH homeostasis. Mrp-dependent antiporters in alkaliphilic *Bacillus* and in *B. subtilis* can be energized by an imposed transmembrane potential (Hamamoto et al. 1994; Ito et al. 1999; 2000) as well as by an imposed transmembrane ΔpH (Hiramatsu et al. 1998; Ito et al. 2001), i.e. Mrp-dependent monovalent cation/proton antiporters behave as expected for a secondary antiporter energized by the Δp. The energization by an imposed transmembrane electrical potential is consistent with an electrogenic monovalent cation/proton exchange that involves net movement of charge during each antiporter turnover, e.g., a Na^+/H^+ antiporter for which the stoichiometry of H^+: Na^+ transported per turnover is more than 1 (Swartz et al., 2005). An electrogenic Na^+/H^+ antiporter can utilize the energy of the transmembrane potential component of the Δp, the ΔΨ (inside negative in whole cells or right-side-out membrane vesicles) that is a useful feature for antiporters supporting alkaline pH homeostasis. No detailed biochemical characterization of a Mrp antiporter has yet been conducted in membrane vesicles, e.g. measuring kinetic parameters, nor are there yet any data reported for a purified, reconstituted Mrp system.

As described in the previous section, the first role established for a Mrp antiporter was in cytoplasmic pH homeostasis of alkaliphilic *B. halodurans* C-125 at highly alkaline external pH, studies that also demonstrated a Mrp role in Na^+-resistance (T. Hamamoto et al. 1994; M. Kitada et al. 2000;



	MapA	*B*	*C*	*D*	*E*	*F*	*G*
B. halodurans	BH 1319	BH1318	BH1317	BH1316	BH1315	BH1314	BH1313
	Palk ORF1	ORF2	ORF3	ORF4	ORF5	ORF6	ORF7
B. pseudofirmus	*mrpA*	*mrpB*	*mrpC*	*mrpD*	*mrpE*	*mrpF*	*mrpG*
Oceanobacillus iheyensis							
	OB3109	OB3108	OB3107	OB3106	OB3105	OB3104	OB3103
B. clausii	ABC1635	ABC 1634	ABC1633	ABC1632	ABC1631	ABC1630	ABC1629

Fig. 4.14 *pALK/mrp/sha* Operon in alkaliphilic *B. halodurans*, *B. pseudofirmus*, *B. clausii*, *Oceanobacillus iheyensis* and *B. subtilis* subsp. *subtilli 168.*

Krulwich et al. 2001). The Na^+/H^+ antiporter activity is attributed to MrpA since the point mutation that leads to a non-alkaliphilic phenotype was in mrpA. The finding that a mutant in *MrpC* is also non-alkaliphilic (Seto et al. 1995) raises the possibility that additional *mrp* genes play critical roles even if MrpA contains the cation and proton translocation pathways. A requirement for multiple *mrp* genes in the extreme alkaliphiles is consistent with our inability to recover mutants of genetically accessible *B. pseudofirmus* OF4 when attempts were made to disrupt any one of several *mrp* genes. The major Na^+/H^+ antiporter of alkaliphilic *Bacillus* species may be required throughout their pH range. The Na^+-specific monovalent cataion/proteon antiporter is necessary for the alkaliphile to maintain a cytoplasmic pH of 8.2 to 9.5 at external pH values from 10.5 to 11.2.

In neutrophilic *B. subtilis*, the Mrp system has a role in Na^+-resistance and in both Na^+-and K^+-dependent alkaline pH homeostasis (Ito et al. 1999). Recently, Kosono et al. (2004) reported that a *mrpA/shaA* mutant of *B. subtilis* changes its use of the diverse ECF (extracytoplasmic function), σW and σ-dependent transcription in transition phase this is consistent with complex stress reaction. In contrast to the central role of Mrp in pH homeostasis of alkaliphilic *Bacillus* species, Mrp is not the dominant antiporter in this process in *B. subtilis*; that role belongs to the multifunctional (tetracycline-divalent metal)$^+$ $(Na^+)(K^+)/H^+$ antiporter Tet^+ (Ito et al. 1999). On the other hand, the *B. subtilis* Mrp system plays a dominant role in Na^+-resistance in this organisms, as indicated by the Na^+-sensitive phenotypes of *mrp/sha* mutants. This result suggests that *mrp* is the essential gene of *B. subtilis* as examined on LB medium even though a *mrp* null strain is viable (Ito et al. 2000). As Swartz reviewed (2005), it will be of interest to compare the activity versus pH profile of alkaliphile and *B. subtilis* Mrp in many respects.

The other transport substrate for a Mrp system has been mentioned, i.e. a capacity for cholate efflux by the *B. subtilis* Mrp system (Ito et al. 1999; 2001). A *mrp* null strain of *B. subtilis*, from which the entire *mrp* operon is deleted, exhibits significantly reduced resistance to growth inhibition by the addition of cholate that is complemented by the introduction of the *mrpF* gene into the chromosomal *amyE* locus under the control of an IPTG-inducible promoter (Ito et al. 2000). Reduced cholate efflux was observed in starved whole cells of the mutant relative to the wild type. This defect is complemented significantly by the re-introduction of mrpF. Homology has been noted between MrpF and Na^+-coupled bile transporters (Ito et al. 1999) and between MrpF and a region of voltage-gated Na^+ channels (Mathiesen et al. 2003). However, no crucial experimental data for MrpF-mediated coupling between Na^+ and cholate fluxes has been found (Ito et al. 1999).

According to Swartz's review paper (2005), it has been considered that physiological role in alkali-, Na^+- and K^+-resistance may secondarily impact processes such as sporulation and nitrogen fixation in particular species.

The Mrp antiporter system may have a substrate or activity in addition to the primary process/es, although still no clear result has been found yet.

4.2.4 Respiration-dependent ATP Synthesis

Alkaliphiles maintain a cytoplasmic pH lower than that of the outside pH. The proton motive force is about −50 mV at pH 10.5, which is the optimum pH value for many alkaliphiles, and this value is not sufficient to thrive in an alkaline milieu. Therefore, alkaliphiles must develop strategies for energy conservation. One possibility is to change the coupling ion, such as Na^+-coupled ATPase. Krulwich and her colleagues reported, however, that respiratory chains that pump H^+ ion outward and H^+-coupled ATP synthases are almost the same as these of neutrophilic microorganisms. Another possibility is that protons produced by electron transfer systems are not released into free solution but directly coupled to ATP synthase. Recently, Wang et al. (2004) showed that ATP synthesis at pH 10.5 in *B. pseudofirmus* OF4 depended upon alkaliphile-specific feature in the proton pathway through *a*- and *c*-subunits ATP synthase. They introduced site-directed changes in the *a*- and *c* subunits of the ATP synthase corresponding to the consensus sequence for non-alkaliphilic *Bacillus*. Five of the six single mutants assembled an active ATPase/ATPsynthase, and four of these mutants exhibited a specific defect in non-fermentative growth at high pH values. Most of these mutants lost the ability to generate the high phosphorylation potentials at low bulk Δp that are characteristic of alkaliphiles. These results strongly indicate that there are still many problems to be solved carefully. Further discussion on alkaliphily is conducted in a later section.

4.3 Intracellular Enzymes

When the author began to study the physiology of alkaliphiles in the 1970' s the properties of intracellular enzymes of alkaliphilic microorganisms were very attractive. How did extreme environments affect intracellular enzymes? Were there critical differences between intracellular enzymes of neutrophiles and alkaliphiles? Many intracellular enzymes were isolated and purified, and their properties investigated. Some enzymes exhibited relatively higher pH optima than neutrophilic bacteria. However, there appeared to be nothing exceptional in intracellular enzymes. Cell-free protein synthesis systems showed maximal activity at between pH 8 and 8.5, but this was only 0.5 pH unit higher than that observed for neutrophilic *B. subtilis*. This is apparently due to the ability of alkaliphiles to regulate intracellular pH towards neutrality.

4.3.1 α- Galactosidases

The first work on these enzymes was done by Akiba and Horikoshi (1976a,b; 1978). Two kinds of α-galactosidase-producing bacteria, *Micrococcus* sp. No. 31-2 and *Bacillus* sp. No. 7-5, were isolated from soil on a modified Horikoshi-I medium (1% raffinose was substituted for glucose). Alkaliphilic *Micrococcus* sp. No. 31-2 induced a cytoplasmic α-galactosidase while alkaliphilic *Bacillus* sp. No. 7-5 produced an extracellular α-galactosidase constitutively. The properties of these enzymes resembled each other, as shown in Table 4.6. The optimum pH range of these enzymes was higher than that of yeast, mold and plant seeds.

Table 4.6 Comparison of the enzymatic properties of α-galactosidase between alkaliphilic and neutrophilic microorganisms

Property	*Micrococcus* sp. No. 31-2	*Bacillus* sp. No. 7-5	*Mortierella vinacea*
Molecular weight	367,000	312,000	
Optimum pH	7.5	6.5	4.0–6.0
pH stability range	7.5–8.0	6.0–8.5	7.0–11.0
Optimum temperature, ℃	40	40	
Km ONPG †, mM	0.47	1.0	0.36 ± 0.014
Meliboise, mM	1.5	7.9	0.39 ± 0.029
Raffinose, mM	12.6	24.1	1.83 ± 0.13

† *o*-Nitrophenyl-α-D-galactoside.
(Reproduced from K. Horikoshi and T. Akiba, *Alkalophilic Microorganisms*, p.55, Springer: Japan Scientific Societies Press (1982))

4.3.2 β-Galactosidases

β-Galactosidase was produced by alkaliphilic *B. halodurans* C-125 not only in alkaline medium but also in neutral medium. However, the induction of the enzymes was much faster at pH 10.2 than at pH 7.2. The enzymes produced in media of different pH values possessed the same enzymatic properties (Ikura et al. 1979a, b; 1988). The molecular weight of the enzyme was about 185,000. The enzyme was most active at pH 6.5 and stable over the pH range 5.5 to 9.0.

Choi et al. (1995) extracted a β-galactosidase from alkaliphilic and thermophilic *Bacillus* sp. TA-11. The enzyme was purified 20-fold from the crude extract by ion exchange and gel-filtration chromatography. The molecular mass of the native enzyme was estimated to be 200 kDa. SDS/PAGE revealed three protein bands of 62, 40 and 34 kDa. Maximum enzyme activity was observed at pH 6.0 and 40℃, but the enzyme was stable over the pH range of 6–12 and below 55℃. These results strongly suggest that the enzymatic properties of β-galactosidases are essentially similar to those of neutrophilic *Bacillus* species.

4.3.3 RNA Polymerases

Many alkaliphilic *Bacillus* strains have been isolated in the author's laboratory, but no information on the differentiation of these bacteria was obtained. Kudo and Horikoshi (1978a, b) studied the RNA polymerase from vegetative cells and spores of alkaliphilic *B. halodurans* 2b-2, which is the best spore former, to ascertain whether any changes occurred during the differentiation process. The molecular weight of $\beta\beta'$, σ and a was 165,000, 97,000(?) and 42,000, respectively. These subunits were essentially identical to those of *B. subtilis*. The σ factor was the same as σ-43 in *B. subtilis*. Another σ factor was also found in *Bacillus* sp. No. 38-2, which corresponds to σ-30.

4.3.4 Protein Synthesizing System

Only one paper has been published on a cell-free protein-synthesizing system using alkaliphilic *B. halodurans* A-59 and C-125 (Ikura et al. 1978). Microorganisms grown in Horikoshi-I medium were collected by centrifugation (6,000 × g) and washed twice with 0.01 M Tris-HCl buffer (pH 7.5) containing 0.01 M $MgCl_2$, 6 mM 2-mercaptoethanol and 0.06 M KCl (TMM buffer). The enzymes were extracted with TMM buffer equivalent to twice the volume of cells ground with two to three times their weight in alumina in a prechilled mortar. The extract was centrifuged twice at 30,000 × g for 30 min. The upper two-thirds of the supernatant was dialyzed against TMM buffer for 3 h. From this supernatant fluid, ribosomes were precipitated by centrifugation at 105,000 × g for 2 h, washed once with TMM buffer, and finally resuspended in TMM buffer. The supernatant was centrifuged at 105,000 × g for 2.5 h and the upper two-thirds used as the S-100 fraction. tRNA was prepared from the cells by the conventional method.

The optimum pH for protein synthesis directed by poly-U or endogenous mRNA was about 8.5, which was about 0.5 higher than that of *B. subtilis* (Fig. 4.15). As the reference, the protein synthesizing system of *B. subtilis* was tested under the same condition. To confirm this result, protein-synthesis directed by endogenous mRNA was performed and the optimum pH was also slightly higher than that of *B. subtilis* (about pH 8.5).

Ribosomes of alkaliphilic *B. halodurans* A-59 and C-125 were of the 70S type, and no difference was observed in their thermal denaturation curves. Phenylalanyl-tRNA synthetase activity at different pH values also indicated no remarkable difference between alkaliphilic *Bacillus* and *B. subtilis* Marburg 168. Phenylalanine incorporation was tested in a series of homogeneous and heterogeneous combinations. The results are shown in Table 4.7. In the systems containing alkaliphilic *Bacillus* ribosomes or S-100, phenylalanine incorporation at pH 8.4 was higher than at pH 7.5, although the activity of heterogeneous systems was lower than that of homogenous systems. In conclusion, alkaliphilic *B. halodurans* A-59 grows well under

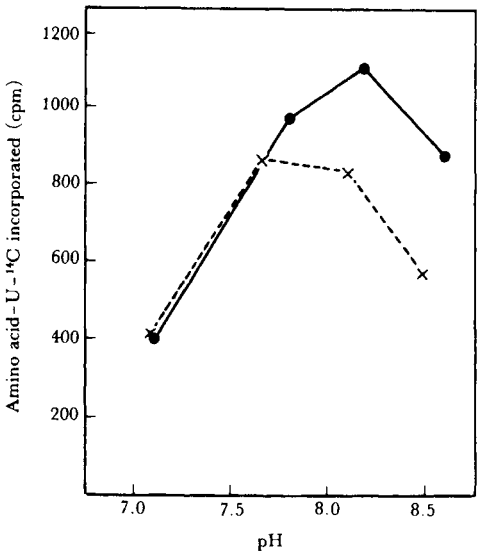


Fig. 4.15 Effect of pH on incorporation of amino acid mixture into protein directed by endogenous mRNA. *Bacillus halodurans* A-59 system contained 0.2 mg ribosomal protein and 0.35 mg S-100. The *Bacillus subtilis* system contained 0.32 mg of ribosomal protein and 0.45 mg S-100. Zero time values were subtracted. Other conditions are described in the text. ●—●, *B. halodurans* A-59, ×---×, *B. subtilis*. (Reproduced from Y. Ikura and K. Horikoshi, *Agric. Biol. Chem.*, **42**, 755 (1978))

Table 4.7 Phenylalanine incorporation in homogeneous and heterogeneous systems

Ribosomes	S-100	Phenylalanine incorporated (nmol)		Ratio between pH 8.4 to 7.5
		pH 7.5	pH 8.4	
Bacillus halodurans A-59	*B. halodurans* A-59	5.82	9.37	1.61
—	*B. halodurans* C-125	6.12	8.65	1.41
—	*B. subtilis*	5.84	6.37	1.09
B. halodurans C-125	*B. halodurans* A-59	5.17	6.62	1.28
—	*B. halodurans* C-125	5.32	8.12	1.53
—	*B. subtilis*	5.63	6.46	1.15
B. subtilis	*B. halodurans* A-59	6.31	7.15	1.13
—	*B. halodurans* C-125	6.71	8.34	1.24
—	*B. subtilis*	9.65	6.59	0.68

The reaction mixture contained 0.2 mg ribosomal protein and 0.3 mg of S-100 protein; the other conditions are described in the text. Blanks (− poly U) are subtracted.
(Reproduced from Y. Ikura and K. Horikoshi, *Agric. Biol. Chem.*, **42**, 756 (1978))

highly alkaline conditions, but the protein-synthesizing mechanism is essentially the same as that of *B. subtilis.*

No direct method has been established for determining the internal pH. The pH optimum of the protein synthesizing mechanism strongly suggests that the internal pH value may be 8 to 8.5, not 10. Heterogeneous combination also supports this possibility. The results clearly indicate that the differences between alkaliphilic *Bacillus* and neutrophilic *Bacillus* exist in the cell surface and not within the cells.

5

Molecular Biology

At the early stage of the author's works, no genetic studies of alkaliphiles were carried out. No genetically manipulated alkaliphiles, of course, could be investigated. Only *E. coli* and *B. subtilis* systems had been developed before the author's works on gene engineering. Thus, his colleagues had to start selecting parental strains of alkaliphilic *Bacillus* strains from hundreds of stock cultures in the author's laboratory. Although the optimum pH value for growth depend on media used, the stock cultures can be roughly divided into two groups. One group of alkaliphilic *Bacillus* strains can grow in the range of pH 6.5 to 10.8 as shown in Fig. 5.1, this group belongs to *Bacillus halodurans*. The other group can not grow at neutral pH values and grow well in the pH range of 7.5 to 11. The author decided to use the *B. halodurans* group and to make deletion mutants that could not grow at pH 10 and tried to isolate the DNA fragment/s responsible for alkaliphily. The following genetic works were conducted mainly using *B. halodurans* as the parental strain.

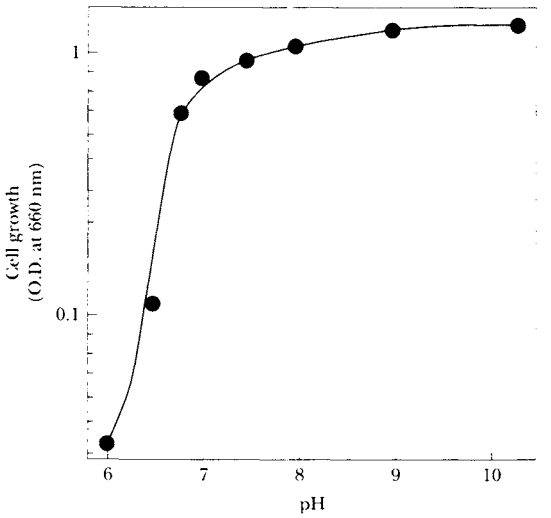


Fig. 5.1 Effects of pH on growth of the parental strain (C-125). pHs were adjusted by the addition of $NaHCO_3$ or Na_2CO_3. Overnight cultures were added to fresh alkaline medium at an optical density at 660 nm of 0.02. Each point represents cell growth after 8 h at 37°C.

Apart from the isolation of mutants, little is known about the genetics and molecular biology of alkaliphiles. No detailed genetic map has been constructed except for an incomplete map of *Bacillus halodurans* C-125 (Sutherland et al. 1993). It is perhaps inevitable that the techniques of molecular biology, developed using other organisms, have been applied to alkaliphiles (mainly bacilli) and a significant number of genes from alkaliphiles have been cloned into alkaliphilic or neutrophilic hosts. This may have advantages such as increased levels of expression of the required enzyme in the host, alterations in the properties of the enzyme and excretion of normal intracellular enzymes into the medium.

5.1 Alkaliphilic Microorganisms as DNA Sources

5.1.1 Secretion Vector

The author and his coworkers have tried to produce *Escherichia coli* strains which could secrete gene products from the cells. *E. coli* is widely used in genetic engineering experiments because it has been extensively studied and much is known about its genetics and biochemistry. Unfortunately, with the exception of a few proteins such as colicin, cloacin and hemolysin , *E. coli* does not secrete gene products from the cell. If *E. coli* could be modified to secrete recombinant DNA products, it would be of considerable interest from an industrial point of view.

One of the most important processes in the fermentation industry is the extracellular production of proteins. The reasons are as follows: (1) If gene products remain in the cells, the products cannot exceed the maximum volume of cells. Also, the process of secretion allows for production from continuous culture, and substances which have an inhibitory effect to microbial metabolism may be produced as extracellular products from cells. (2) Usually, the number of proteins secreted from a cell is not so large, so that purification processes are relatively simple and can be used in fermentation industries.

A. Cell surface of bacteria

Bacteria are classified into two groups, gram-positive and gram-negative, according to the nature of the cell surface. Gram-positive bacteria have a plasma membrane and a peptidoglycan layer in the cell surface, while gram-negative bacteria have a plasma membrane (inner membrane), peptidoglycan and an outer membrane (Fig. 5.2). The plasma membrane is a phospholipid bilayer containing approximately 300 different proteins, many of which are involved in respiration, electron transport, nutrient uptake and membrane biogenesis. The outer membrane in gram-negative bacteria is a glycolipid-phospholipid bilayer, with the polysaccharide groups of the glycolipid being exposed on the outer surface of the cell. The periplasm con-

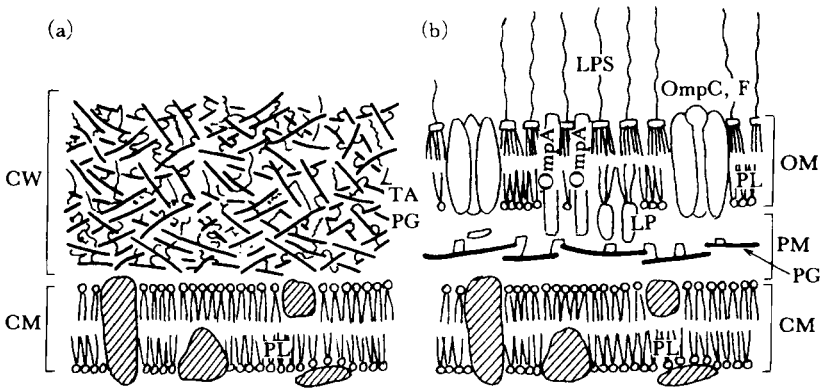


Fig. 5.2 Cell surfaces. (a) Gram-positive bacteria, (b) Gram-negative bacteria. CM, Cytoplasmic membrane; OM, Outer membrane; PM, Periplasm; PG, Peptidoglycan; TA, Teichoic acid; LPS, Lipopolysaccharide; PL, Phospholipid.

tains approximately 100 proteins involved in nutrient uptake and catabolism. Protein molecules have signal peptides which allow for their secretion into either the culture medium in the case of gram-positive bacteria or into the periplasmic space in the case of gram-negative bacteria, In the case of gram-negative bacteria such as *E. coli*, the outer membrane acts as a barrier, and the protein molecules are trapped in the periplasmic space.

B. Changing *E. coli* to become permeable

A penicillinase gene from alkaliphilic *Bacillus* sp. No. 170 was cloned in *E. coli* HB101 by using pMB9. Plasmid-borne penicillinase was found to be produced in the culture medium (Kudo et al. 1983c), as shown in Fig. 5.3. The alkaliphilic *Bacillus* sp. No. 170, a penicillinase producer, was digested with *Hin*dIII or *Eco*RI restriction enzymes and shotgun cloned in pMB9 by conventional means. Two plasmids, pEAP1 and pEAP2, were obtained from the transformants. The cleavage maps of pEAP1 and pEAP2 are shown in Fig. 5.4. The 2.4-kb *Hin*dIII fragment containing the penicillinase gene was located in the middle of the 4.5-kb *Eco*RI fragment. The plasmid-encoded penicillinase was immunologically crossed with penicillinase III of alkaliphilic *Bacillus* sp. No. 170. *E. coli* carrying plasmids were aerobically grown in LB-broth for 20 h at 37°C and enzymatic activities determined. Most of the penicillinase produced by these cells was detected in the culture medium. Less than 15% of the total activity was observed in the periplasmic and cellular fractions. However, almost all the β-lactamase produced by *E. coli* HB101 (pBR322) was trapped in the periplasmic space. *E. coli* HB101 carrying pEAP2 was inoculated into 500-ml flasks containing 100 ml of LB-broth with 0.2% glycerol and cultured at 37°C. As shown in Fig. 5.3, the bacteria reached maximum cell concentration at 16 h, and no lysis of the cells was

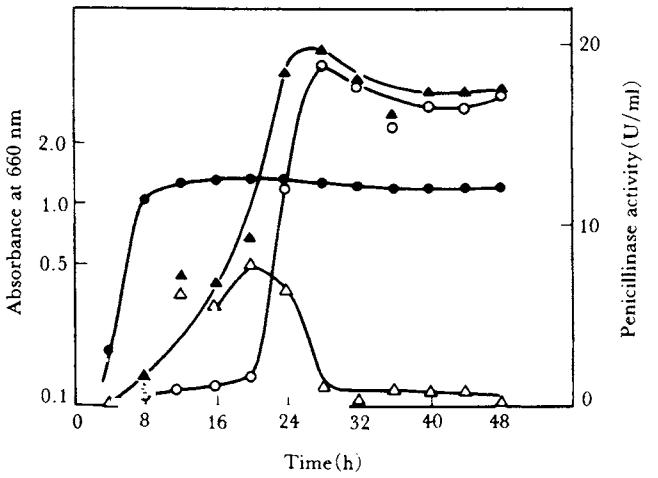


Fig. 5.3 Bacterial growth and penicillinase production by *Escherichia coli* HB101 (pEAP2). *E. coli* HB101 (pEAP2) was inoculated into LB-broth containing 0.2% glycerol and cultured at 37°C on a rotary shaker. Bacterial growth (absorbance at 660 nm, ●) and penicillinase activities (extracellular, ○; intracellular, △; and total, ▲) were determined.

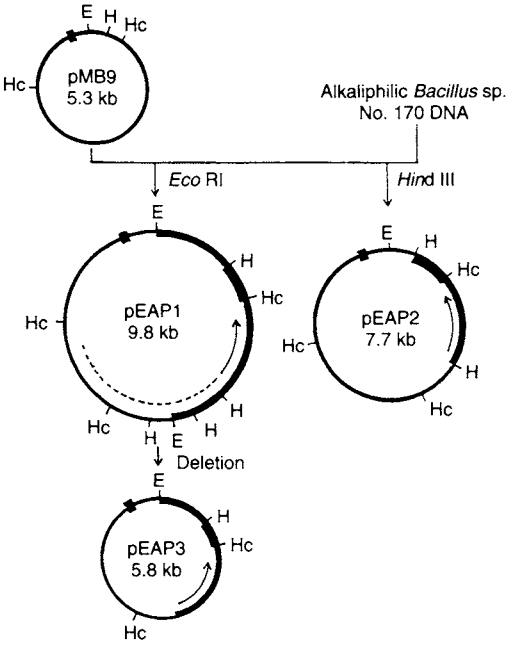


Fig. 5.4 Construction of plasmids pEAP1, pEAP2 and pEAP3 containing the *Bacillus* penicillinase gene. (——), vector DNA; (▬▬), *Bacillus* DNA. Arrow denotes direction of transcription of the *Bacillus* penicillinase gene. E, *Eco* RI; H, *Hind* III; Hc, *Hinc* II. (Reproduced with permission from T. Kobayashi et al., *J. Bacteriol.*, **166**, 729 (1986))

Table 5.1 Distribution of enzymes in *E. coli* HB101 carrying plasmids

Plasmids		Extracellular	Periplasmic	Cellular	Total
None	Protein	0.04(4%)	0.07(6%)	0.97(90%)	1.08(100%)
	APase	0.02(2%)	1.05(79%)	0.26(20%)	1.33(100%)
	β-gal	0.03(2%)	0.00(0%)	1.20(98%)	1.23(100%)
pMB9	Protein	0.01(1%)	0.03(4%)	0.75(95%)	0.79(100%)
	APase	0.01(2%)	0.32(67%)	0.15(31%)	0.48(100%)
	β-gal	0.00(0%)	0.00(0%)	0.80(100%)	0.80(100%)
pEAP2	Protein	0.18(21%)	0.12(14%)	0.55(65%)	0.85(100%)
	PCase	10.70(83%)	0.40(3%)	1.80(14%)	12.90(100%)
	APase	0.29(58%)	0.15(30%)	0.06(12%)	0.50(100%)
	β-gal	0.09(10%)	0.03(3%)	0.78(87%)	0.90(100%)

E. coli strains were aerobically grown in the LB-broth for 20 h at 37 °C. Enzymatic activities of alkaline phosphatase (APase) and β-galactosidase (β-gal) are expressed as absorbance at 420 nm. Penicillinase (PCase) activity is expressed as units per milliliter of broth. Protein concentration is expressed as milligrams in 1 ml of broth.
(Reproduced from T. Kudo, C. Kato and K. Horikoshi, *J. Bacteriol.*, **156**, 951 (1983))

observed up to 48 h (viable counts 3-2 × 10^9). The extracellular penicillinase activity increased at about 24 h and reached maximum at 28 h. No intracellular penicillinase was observed after 28-h cultivation. The distribution of β-galactosidase and alkaline phosphatase activities were also investigated (Table 5.1). Essentially, neither protein nor enzymatic activity was detected in the culture broth of *E. coli* HB101 or *E. coli* HB101 (pMB9). About two thirds of the alkaline phosphatase was observed in the periplasmic space and almost all of the β-galactosidase was in the cellular fraction. On the other hand, in *E. coli* HB101 (pEA2) it is striking that 21% of the total protein, 58% of the alkaline phosphatase and 83% of the penicillinase were found in the culture broth. About 87% of the activity of β-galactosidase, a typical intracellular enzyme, was detected in the cellular fraction and not in the periplasmic fraction. These results suggest that the outer membrane of *E. coli* was changed by the introduction of pEAP2 into the cells, because a periplasmic enzyme, alkaline phosphatase, was released from the periplasmic space.

The fragment contains the penicillinase gene and an open reading frame of the *kil* gene with a promoter. Our results showed that the activation of the *kil* gene of pMB9 by a promoter (*Ex* promoter) in the inserted fragment increased outer membrane permeability (Kobayashi et al. 1986). Insertional mutation in the −35 region of the *Ex* promoter inhibited secretion. High resolution S1 nuclease mapping indicated that the *kil* gene was activated by the *Ex* promoter, which had a TATTAT sequence at −10 and TTGATA at −35 (Fig. 5.5). The penicillinase itself was not responsible for this secretion.

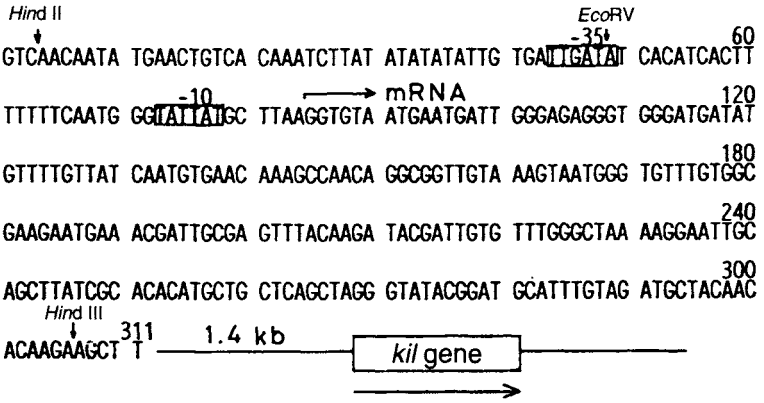


Fig. 5.5 Nucleotide sequence of the *Ex* promoter isolated from alkaliphilic *Bacillus* sp. 170. The putative −35 and −10 are boxed. The location and direction of the *kil* gene are indicated. The space between the *Hind* III site and the *kil* gene is approximately 260 kb in pEAP37.
(Reproduced with permission from T. Kobayashi et al., *J. Bacteriol.*, **166**, 730 (1986))

C. Role of the *kil* gene

Penicillinase was not responsible for protein secretion. The Ex-promoter is not very strong, being almost the same as the tetracycline promoter of pMB9. The *kil* gene and *Ex* promoter are necessary for the excretion of penicillinase from the cells. The *kil* gene of ColE1 is required for mitomycin-induced lethality and release of colicin from the cells. However, under our experimental conditions, the *kil* gene did not kill the *E. coli* cells but instead made the outer membrane permeable. Aono (1988a) succeeded in isolating the *kil* gene proteins from the *E. coli* cell envelope. One of the peptides (Mr 4800), which was probably a precursor peptide, was detected in the inner-membrane fraction from the organism when envelope proteins were subjected to differential solubilization. The other (Mr 3500), which was a mature peptide, was detected in the outer-membrane fraction of the organism. The mature peptide was only detected in the envelope of cells releasing the penicillinase transiently accumulated in the periplasm into the culture medium. As shown in Fig. 5.6, the *kil* gene exhibits partial homology with the gene for Braun's lipoprotein detected in the outer membrane of *E. coli*, not only at the level of the DNA sequence but also at the level of the amino acid sequence. Therefore, the product of the *kil* gene may act as a perturbator of the cell membrane.

Aono (1988b; 1989b) found that the extracellular production of alkaliphilic *Bacillus* penicillinase by *E. coli* HB101 carrying pEAP31 was affected by several parameters such as concentration of carbohydrates and NaCl, pH values of culture broth, culture temperature and aerobic conditions. He noted that the most critical condition is culture temperature, and no secretion was observed at temperatures lower than 26°C. Cultivation at various temperatures (22 to 32°C) revealed that the penicillinase accumulated in

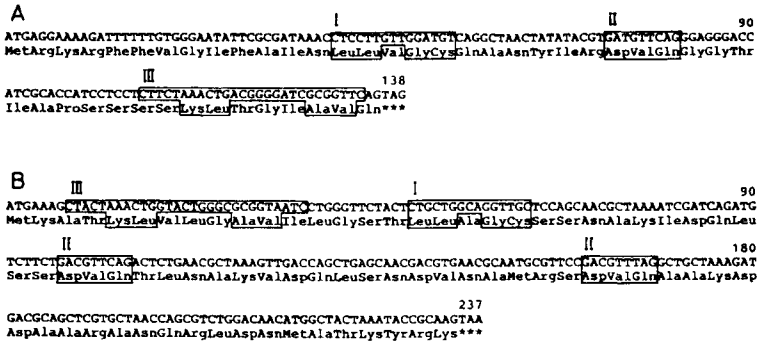


Fig. 5.6 Comparison of the nucleotide and the derived amino acid sequences of the *kil* gene (A) and the lipoprotein gene (B). Homologous regions I, II, and III, are boxed. (Reproduced with permission from T. Kobayashi et al., *J. Bacteriol.*, **166**, 731 (1986))

Table 5.2 Distribution of enzymes in *E. coli* HB101 carrying pEAP 31 at various temperatures

Cultivation Temper -ature	Total PCase (U/ml)	Distribution of PCase(%)				Release from cells			Growth
		Extra	Intra	Peri	Cyto	APase (%)	β-gal (%)	Protein (μg/ml)	A_{660}
16°C	73	1	99	87	12	1	ND	36	5.3
20	174	1	99	87	12	2	ND	36	5.7
22	181	2	98			2	ND		5.5
24	206	5	95	88	7	4	2	51	5.7
26	206	17	83			11	ND		5.6
28	183	66	34	31	4	42	1	186	4.4
30	155	81	19	17	2	55	14	339	3.4
32	148	95	5	3	2	85	26	468	2.3
36	102	94	6	4	2	73	37	470	2.3
38	88	75	25	20	5	66	11	250	3.0
40	55	85	15	10	5	67	7	273	3.2
43	< 1					43	ND	44	2.6

Extra, Extracellular fraction; Intra, Intracellular fraction; Per, Periplasmic fraction; Cyto, Cytoplasmic fraction.
(Reproduced with permission from R. Aono, *Appl. Microbiol. Biotechnol.*, **31**, 397(1988))

the periplasmic space of *E. coli* at 22°C was released from the cells at 32°C (Table 5.2). The following conditions for the production of extracellular penicillinase by *E. coli* HB101 carrying pEAP31 were recommended. The organism should be inoculated in 200 to 300 ml of LG-broth (10 g Bactotryptone (Difco), 5 g Bacto yeast extract (Difco), 2 g glycerol, 1 g glucose and 10 g NaCl in 1000 ml of deionized water) in a 500-ml volume cultivation flask and then shaken at 30°C on a high speed reciprocal shaker (172 oscillation/min with 3.2 cm strokes). The selection of cultivation conditions is the most important factor in producing extracellular enzymes. *E. coli* HB101 carrying pEAP31 grown at 30°C released the outer membrane proteins, lipopolysaccharide and phosphatidylethanolamine as well as peni-

cillinase into the culture medium. Aono analyzed the fatty acid content in *E. coli* HB101 carrying or not carrying pEAP31 but observed no difference. Recently, Aono reported that the penicillinase that accumulated in particular subcellular fractions of *E. coli* grown under different conditions was purified and characterized. Periplasmic or extracellular penicillinase (24 kDa) was a mature protein, indicating that the putative precursor (27 kDa) was processed at the correct amino acid residue, probably by signal peptidase I. Cytoplasmic penicillinase contained two unusual proteins (25 kDa) produced by proteolytic cleavage of the precursor within its signal sequence (Aono 1992).

D. Extracellular production of microbial enzymes from *E. coli*

An excretion vector, pEAP37, was constructed by Kobayashi et al. (1986) as shown in Fig. 5.7. Several enzyme genes of alkaliphilic *Bacillus* strains were

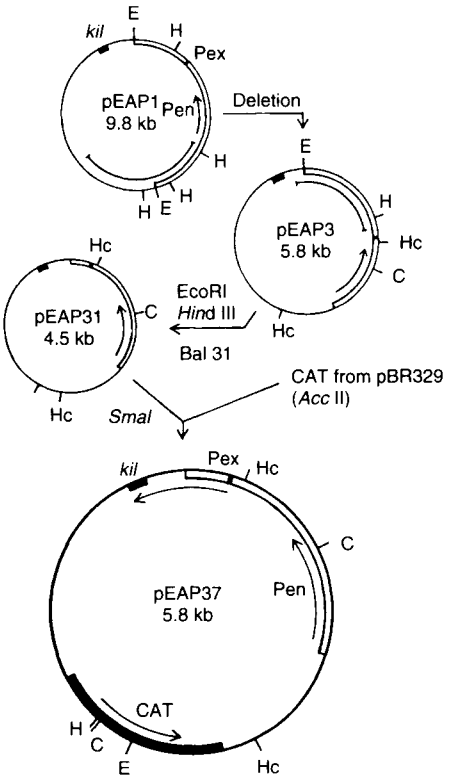


Fig. 5.7 Construction of pEAP37. The thin line represents the DNA region derived from pMB9. The open bar represents the DNA fragment containing the penicillinase gene from an alkaliphilic *Bacillus* sp. and the black bar represents the DNA fragment containing the chloramphenicol-resistant gene (CAT) derived from pBR329. The location of the *kil* gene and *Ex* promoter are indicated by a black box. The dotted lines in pEAP1 and pEAP3 indicate the deletion regions. Abbreviations: C, *Cla* I; S, *Sma* I. (Reproduced with permission from C. Kato et al., *FEMS Microbiol. Lett.*, **36**, 32 (1986))

Table 5.3 Distribution of the xylanases and cellulases in *E. coli*

Plasmid	Extracellular fraction	Periplasmic fraction	Cellular fraction	Total activity
pAX2	23 (4.8)	147 (30.4)	315 (64.8)	485
p7AX2	135 (62.1)	8 (3.6)	74 (34.3)	217
pNK1	12 (9.2)	60 (44.0)	64 (46.8)	136
p7NK1	282 (66.7)	92 (21.8)	49 (11.5)	423
pFK1	7 (6.1)	88 (74.3)	23 (19.6)	118
p7FK1	93 (60.2)	16 (10.6)	45 (29.2)	154
pAX1	10 (1.6)	290 (47.9)	350 (50.5)	605
pXP102	120 (88.8)	10 (7.4)	5 (3.8)	135

Note: The activities (units) of the enzymes are presented in each column. The percentages of the total activities are shown in parentheses.
(Reproduced with permission from C. Kato et al., *FEMS Microbiol. Lett.*, **36**, 33 (1986))

expressed in *E. coli* and secreted into the culture broth using the secretion vector pEAP37.

(1) p7AX2 (Kato et al. 1986): The plasmid pAX1 (Kudo et al. 1985a) was digested with *Bgl* II and a 4.0-kb DNA fragment containing the xylanase gene (*xyl-L*) was isolated. This fragment with a *Bam*HI linker was inserted into the *Hinc*II site of pEAP37. p7AX2 was isolated from the Cm-resistant, Ap-sensitive and xylanase-producing transformants. Another plasmid pAX2 was constructed by the insertion of the 4.0-kb DNA fragment with *Bam*HI linker into the *Bam*HI site of pBR322. (2) p7NK1 and p7FK1: Plasmids pNK1 (Sashihara et al. 1984) and pFK1 (Horikoshi et al. 1984; Fukumori et al. 1985; 1986a, b) containing cellulase genes of different alkaliphilic *Bacillus* strains have been found in our laboratory. A 2.0-kbp Hind III fragment from pNK1 and a 3.0-kb *Hin*dIII-*Hinc*II fragment from pFK1, each containing cellulase genes, were isolated. These fragments were filled in and ligated with *Hinc*II-digested pEAP37. Plasmids p7NK1 and p7FK1 were constructed.

E. coli HB101 carrying each constructed plasmid was cultured aerobically in LB-broth at 37°C. After 24 h, the cells were harvested by centrifugation and fractionated into extracellular, periplasmic and cellular fractions. Most of the xylanase and cellulase activities were detected in the extracellular fractions (Table 5.3). In control experiments using *E. coli* carrying pBR322 with inserts coding for xylanase and cellulase, the enzymatic activities remained in the periplasmic and cellular fractions.

(2) Georganta et al. (1991) investigated the expression level of CGTase gene of alkaliphilic *Bacillus* No. 38-2 in various hosts. *E. coli* carrying pEAP85-CGT excreted 70% of total CGTase (500 U/ml) into the culture broth. This amount was higher than that produced by the parent strain. *Bacillus subtilis* carrying pGK-1 produced a very low level of CGTase (less than 0.1 U). These results were only from laboratory scale experiments and a stock strain was used.

E. Extracellular production of human proteins

1. Human growth hormone (hGH) (Kato et al. 1987)

A *Dra*I fragment containing the promoter and the structure gene isolated from pEAP2 was digested by RsaI and connected with a *Hin*dIII linker. The fragment (PS fragment) thus obtained contained the promoter and the whole signal peptide sequence of penicillinase. This fragment was ligated with hGH gene, inserted into the *Hin*dIII site of pBR322 and introduced into *E. coli*. The transformant (Ap^r, Cm^s) was cultured in LB-broth for 24 h at 37°C. The distribution of the hGH produced was analyzed by radioimmunoassay. More than 90% of the hGH was detected in the periplasmic space of *E. coli*. The fragment having the PS fragment and hGH gene was inserted into the *Hin*cII site of the secretion vector pEAP37 and introduced into *E. coli*. The transformant was cultured by the method described above. About 60–70% of total hGH activity was detected in the culture broth. Western blotting indicated that the protein produced by the plasmid was immunologically the same as that of the authentic hGH sample. The yield of hGH in the culture broth was about 60–70 mg/l.

2. Production of the Fc fragment in the culture broth

The Fc fragment (Fig. 5.8) is a protein fraction crystallized from the papain hydrolyzate of immunoglobulin. Its molecular weight is 50,000 and it has two intramolecular disulfide bonds. The Fc fragment has several biological activities. The DNA fragment of the Fc structure gene was ligated with a DNA fragment coding the penicillinase signal peptide isolated from alkaliphilic *Bacillus* sp. No. 170 and inserted into the secretion vector. After plasmid was introduced into *E. coli* HB101 and cultured in the LB-broth for 24 h, more than 60% of the gene products was detected in the culture broth (Fig. 5.9). Without the signal sequence, no gene product was detected in either the periplasmic space or the culture broth In conclusion the author and Kato believe that the secretion vector pEAP37 may prove to be useful

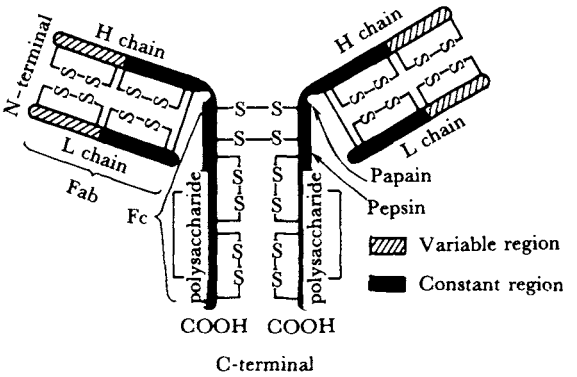


Fig. 5.8 Human immunoglobulin.

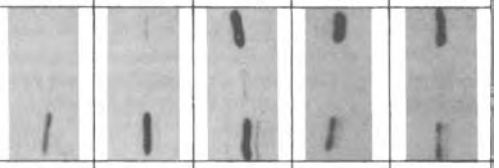
Time(h)		6	12	18	24	30
hIgG-Fc	E					
	P					
	C					
Alkaline phosphatase (%)	E	0	0	36	83	82
	P	73	38	50	4	4
	C	27	62	14	13	14
β-galactosidase (%)	E	0	0	2	13	17
	P	3	0	2	3	9
	C	97	100	96	84	74

Fig. 5.9 Production of hIgG-Fc in culture broth.

industrially for extracellular production of proteins from genetically modified *E. coli.*

5.1.2 Promoters

Promoters of *Bacillus* strains have been studied from the industrial point of view for the hyperproduction of extracellular enzymes, such as alkaline proteases and alkaline cellulases.

A. *Ex* promoter of alkaliphilic *Bacillus* sp. No. 170

As described in section 5.1.1C, Kobayashi et al. (1986) isolated the *Ex* promoter, which had a TATTAT sequence at −10 and TTGATA at −35 (Fig. 5.5). This promoter is not so strong, but it was the first finding of a promoter of alkaliphiles. In order to develop a more useful system for extracellular protein production from *E. coli*, Murakami et al. (1989) constructed new excretion vectors, pEAP82-1, 82-2, and 82-3, which are derivatives of the secretion vector pEAP37. These vectors have, respectively, a single, double and triple *Ex* promoter upstream from the penicillinase structural gene. *E. coli* HB101 carrying pEAP82-2 or 82-3 produced, respectively, about two or three times as much penicillinase protein than that produced by *E. coli* carrying pEAP82-1, and 70% to 80% of the protein was excreted into the culture medium. The *E. coli* carrying pEAP82-3 was cultivated at various temperatures and the optimum for extracellular penicillinase production was found to be 30–33°C. Using this multipromoter excretion system, the amount of extracellular production of human growth hormone increased several fold as observed with penicillinase excretion.

B. Promoter of *Bacillus* sp. KSM-635

During studies of industrial production of alkaline cellulase for laundry detergent, Ozaki et al. (1990) isolated a gene for alkaline cellulase from the alkaliphilic *Bacillus* sp. KSM-635. There was an open reading frame (ORF) of 2823 bp, which encoded 941 amino acid residues. It was noteworthy that two putative ribosome binding sites and a sigma 43 type, promoter-like sequence were found upstream from an initiation codon in the ORF. Such a multiple promoter system can produce a large amount of alkaline cellulase in an industrial scale plant.

C. A conditionally regulated DNA fragment

Alkaliphilic *Bacillus* strains sporulate well at pH 10, which is also the optimum pH for germination of the spores. Almost all extracellular enzymes are fully produced at the stationary phase. For alkaline cyclodextrin glycosyltransferase production, the alkaliphilic *Bacillus* strain No. 38-2 must be grown for at least 48–72 h. Why are these extracellular enzymes produced at particular times? One approach for analyzing the regulation mechanisms of production of extracellular enzyme is to clone elements that control developmental gene expression using expression probe vectors. One of these expression vectors is pGR71 (Goldfarb et al. 1982). In *B. subtilis*, expression of the chloramphenicol acetyltransferase (CAT) gene carried by pGR71 requires insertion of *Bacillus* promoters and ribosomal binding sites into the *Hin*dIII site immediately upstream from the CAT gene. In order to screen for insertionally activated and developmentally induced gene expression, Kudo et al. (1985b) cloned fragments and examined their CAT activities during the vegetative and sporulation phases of growth. Fragments which were controlled by temperature, pH or NaCl concentration in the pGR71 CAT expression system were also isolated.

D. Media and strains used

The following media were used throughout the experiments: Medium 2 × SSG (modified Schaeffer medium) containing 16 g of nutrient broth (Difco Laboratories), 0.5 g of $MgSO_4 \cdot 7H_2O$ and 2 g of KCl; after autoclaving, $Ca(NO_3)_2$, $MnCl_2$, $FeSO_4$ and glucose were added to concentrations of 1 mM, 0.1 mM, 1 μM and 0.1%, respectively. Alkaliphilic *Bacillus* No. 38-2 (ATCC 21783) was grown aerobically at 37°C to early log phase in Horikoshi-II medium for DNA extraction. Other strains and plasmids used are listed in Table 5.4.

E. Construction of recombinant plasmids

Chromosomal DNA of alkaliphilic *Bacillus* No. 38-2 and plasmid DNAs were completely digested with *Hin*dIII at 37°C. After digestion, 1 μg of pGR71 and 3 μg of chromosomal DNA were ligated with T4 ligase overnight at room temperature. Competent *B. subtilis* 1012 cells were transformed with the ligation mixture described above. After 90 min of incubation to allow

Table 5.4 Strains and vectors used

Strain or plasmid	Genotype or property	Source
Alkaliphilic *Bacillus* sp. No. 38-2	Produces an alkaline amylase	Author's laboratory
B. subtilis		
NIG1121	*met his spo*	Y. Sadaie
NIG1131	*met his spo OA*34	Y. Sadaie
NIG1132	*met his spo OB*36	Y. Sadaie
NIG1133	*met his spo OC*7	Y. Sadaie
NIG1134	*met his spo OD*8	Y. Sadaie
NIG1139	*met his spo OE*81	Y. Sadaie
NIG1140	*met his spo OF*221	Y. Sadaie
NIG1135	*met his spo OG*14	Y. Sadaie
NIG1136	*met his spo OH*17	Y. Sadaie
NIG1137	*met his spo OJ*87	Y. Sadaie
NIG1138	*met his spo OK*141	Y. Sadaie
IS17	*trp C2 phe* A1 *spo OE*11	BGSC[†]
IS22	*trp C2 rpo B2 spo OH*17	BGSC
IS24	*trp C2 phe* A1 *spo OH*81	BGSC
1012	*lec* A8 *met B5 hsr M*1	H. Honda
pGR71	Km[r]	D.S. Goldfarb

[†] BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus.

Bacterial strains and vectors	Genotypes or properties
Alkaliphilic *Bacillus* No. 221	Produces an alkaline protease
Alkaliphilic *Bacillus* No. 38-1	Produces an alkaline amylase
E. coli CV512	*leu* A371
E. coli HB101	*leu* B, *pro, thi, rec* A, *hsd* R, *hsd* M, *str* A
E. coli CV520	*leu* C171
E. coli CV526	*leu* D101
B. subtilis 1012	*leu* A8, *met* B5
B. subtilis GU134	*leu* B6, *trp* C2
B. subtilis GU741	*leu* C7, *trp* C2
B. subtilis GU229	*ilv* B2, *trp* C2
B. subtilis DB-1	Wild type strain
Cl. butyricum M588	Used as gastrointestinal tonic
Plasmid pBR322	Amp[r], Tc[r]
Plasmid pGR71	Km[r]

expression of the pGR71 kanamycin resistance the cells were plated onto LB plates containing kanamycin (20 μg/ml). To analyze the chloramphenicol acetyltransferase (CAT) activity profile, *B. subtilis* carrying the pGR71 derivatives was cultivated in the 2 × SSG sporulation medium containing 10 μg/ml kanamycin. The culture was sampled at various stages of growth and sporulation, and the cell extracts were prepared by sonication for CAT en-

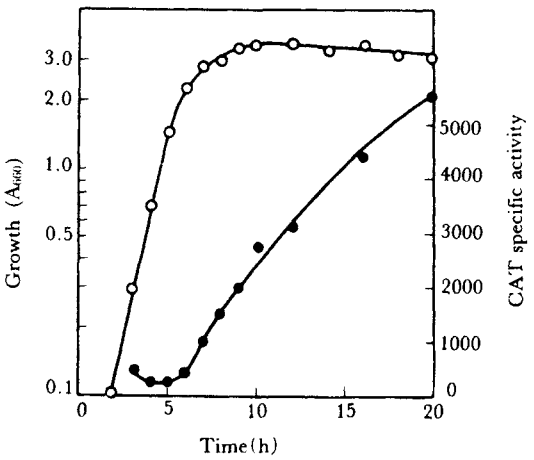


Fig. 5.10 CAT activity as a function of growth and sporulation in *B. subtilis* 1012 harboring plasmid pGR71-5. Symbols: ○, cell growth; ●, CAT specific activity. One unit of CAT specific activity was defined as 1 nmol of DTNB reduced per min per mg of total soluble protein. A_{660}, absorbance at 660nm.
(Reproduced from T. Kudo, A. Ohkoshi and K. Horikoshi, *J. Bacteriol.*, **161**, 159 (1985b))

zyme activity. As shown in Fig. 5.10, the CAT activity profile of pGR71-5 indicated that CAT expression from the plasmid was induced after the cessation of vegetative growth and that CAT specific activity increased until late in sporulation. The expression of CAT activity of pGR71-5 was strongly suppressed by the addition of glucose in the 2 × SSG medium. This expression was dramatically decreased in *spoOE* and *spoOH* mutants, which had a very low level of CAT activity, as shown in Fig. 5.11. This loss of activity was a function of the host cell genotype and not due to plasmid rearrangement. When pGR71-5 was isolated from *spoOE* or *spoOH* strains carrying the plasmid and reintroduced into the *spo*$^+$ strains, the high CAT activity characteristic of the original pGR71-5 isolate was restored. The nucleotide sequence of the entire 50-bp fragment was determined and the site of regulated transcription initiation located by high resolution S1 nuclease mapping of the *in vivo* transcript (Fig. 5.11). From the results, the promoter sequences were deduced to be 5'CGAATCATGA3' at −10 and 5' AGGAATC3' at −35. This transcript was not detected in either *spoOE* or *spoOH* mutants, indicating that these gene products control the developmentally regulated CAT expression at the transcription level.

In conclusion, the *Hin*dIII fragment of pGR71-5 was developmentally regulated in the sporulation medium of *B. subtilis* and controlled by the *spoOE* and *spoOH* loci. The authors reported that the promoter obtained from alkaliphilic *Bacillus* resembled the promoters recognized by SigH (sigma-30) of *B. subtilis* having 5'GAATTNNT3' at −10 and 5'GCAGGANTT3' at −35 and that this promoter was under glucose and *spoO* control in a heterologous system.

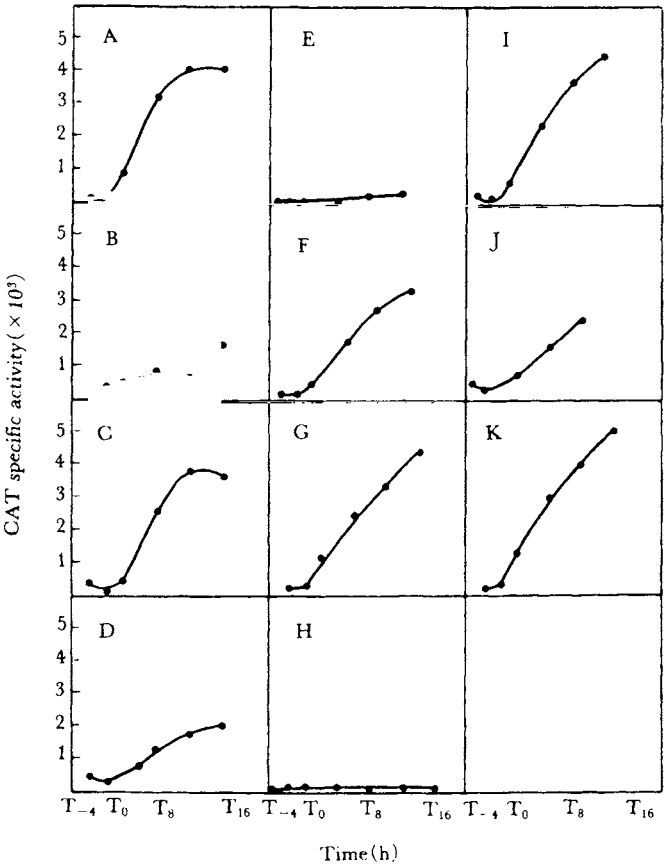


Fig. 5.11 Effect of various *spoO* mutations on CAT expression. *B. subtilis* cells carrying pGR71-5 were inoculated into 500 ml of medium 2 × SSG and cultured at 37°C on a rotary shaker. CAT activity was measured at different times during the growth and sporulation phases. (A) Strain NIG1131 (*spoOA*). (B) Strain NIG1132 (*spoOB*). (C) Strain NIG1133 (*spoOC*). (D) Strain NIG1134 (*spoOD*). (E) Strain NIG1139 (*spoOE*). (F) Strain NIG1140 (*spoOF*). (G) Strain NIG1135 (*spoOG*). (H) Strain NIG1136 (*spoOH*). (I) Strain NIG1137 (*spoOJ*). (J) Strain NIG1138 (*spoOK*). (K) Strain NIG1121 (*spo*$^+$).
(Reproduced from T. Kudo, A. Ohkoshi and K. Horikoshi, *J. Bacteriol.*, **161**, 160 (1985b))

5.2 Host-Vector Systems of *Bacillus halodurans* C-125

There is no genetic information on alkaliphiles before 1983. The first recombinant DNA experiment in this field was the cloning and expression of leucine genes of alkaliphilic *Bacillus* strains in *E. coli*. Since then, our interest has focused on genetic engineering of alkaliphilic *Bacillus* strains to study alkaliphily. One of the characteristic properties of alkaliphiles is pH homeostasis in the cell. Intracellular pH values are maintained around 7–8,

although pH values of the culture media are 9.5–11.0 as described in section 4.1.1B. This pH difference is caused by the cell surface. In order to analyze the genetic properties of alkaliphilic *Bacillus* strains we had to make host-vector systems. The authors group thought that if suitable host-vector systems of alkaliphilic *Bacillus* strains are established, more information on alkaliphily will be obtained.

The author and his coworkers carried out tedious experiments, on the longevity of alkaliphiles in nature, toxicity and other topics to receive approval from the Japanese government since at that time a special permit was required to start new gene engineering projects, except for those on *E. coli* and *B. subtilis*. Three years passed before recombinant DNA experiments on alkaliphilic *Bacillus* strains could begin. The first task was the construction of host-vector systems for which we mapped out a strategy. Host *Bacillus* strains must have the following properties: good growth in synthetic media, good protoplast formation and regeneration, and high frequency of transformation. If possible, the host should be *rec* minus. Vectors must be easy to introduce into hosts and be genetically stable.

5.2.1 Selection of Host Strains

The following alkaliphilic strains were used in this study. *B. pseudofirmus* 2b-2 is a strict alkaliphile that belongs to group 1. *B. halodurans* A-59 and C-125 are facultative alkaliphiles and belong to group 2. *Bacillus clausii* M-29 is a facultative alkaliphile belonging to group 3. All of them grow very well at pH 10.3 after 18 h incubation at 37°C either in liquid or on solid media.

Strain Nos. 2b-2 and M-29 could not grow well in Horikoshi-I or -II medium in the absence of yeast extract. On the other hand, *B. halodurans* A-59 and C-125 grew well in the absence of yeast extract. Kudo et al. (1990) made a synthetic medium containing 0.2% glutamate and 0.5% glycerol for these strains. In 1992, Aono et al. developed a novel minimal medium containing citric acid, glucose and ammonium sulfate for No. C-125 (1992b) Alkaliphilic *B. halodurans* A-59 (ATCC21591) and C-125 (JCM9153) can grow well in both media in a range of pH 7.5 to 11.

5.2.2 Stability of Antibiotics in Horikoshi-I Medium

In the field of microbial genetics, many antibiotics have been used as resistant markers in order to concentrate or screen mutants having antibiotic-resistant genes. However, under alkaline environments the several antibiotics so far tested are very unstable and decompose after 2–3 days incubation at 37°C. Stability of the five most popular antibiotics in microbial genetics was tested in media of varying pH values (Usami et al. 1990). From the author's stock cultures which can grow in the range of pH 7.0 to 11.0, seven strains, including *B. halodurans* A-59 (ATCC21591) and No. C-125 (JCM9153), were tested. One of them, alkaliphilic *B. rikeni* 13 (ATCC 31006), which is a pro-

Table 5.5 Growth at various pH values in the presence of antibiotics

Antibiotic	pH values			
	7.6	8.5	9.5	10.2
Tetracyline				
50 μg/ml	–	–		+
100	–	–	+	+
250	–	–	+	+
Ampicillin				
50 mg/μl	+	+	+	+
100	+	+	+	+
250	–	–	+	+
Chloramphenicol				
20mg/μl	–	–	–	–
40	–	–	–	–
100	–	–	–	–
200	–	–	–	–
Kanamycin				
40	–	+	+	+
100	–	+	+	+
200	–	+	+	+

Symbols : – no growth, + growth. Media used : nutrient agar for pH 7.6, others were modified Horikoshi-I medium. Growth was observed after 3 days incubation at 37°C. (Reproduced with permission from R. Usami et al., *Starch*, **46**, 230(1990))

ducer of alkaline amylase (see Chapter 8), is shown in Table 5.5 as an example. Our results indicated that chloramphenicol could be used as a resistance marker in the field of genetics of alkaliphilic *Bacillus* strains. Neither tetracycline nor ampicillin could be used. Nutrient requirements and chloramphenicol-resistance are useful markers for the alkaliphilic hosts so far tested.

5.2.3 Preparation of Mutants

Mutants of alkaliphilic strains can be readily obtained by conventional mutagenesis at pH 7.5–8, e.g., with nitrosoguanidine or ethylmethanesulfonate, or spontaneous mutagenesis (Aono and Ohtani 1990; Kudo et al. 1990). Auxotrophic mutants were concentrated by the penicillin-screening method (1–3 mg/ml) at pH 8, then selected by the replica method using a synthetic alkaline medium (Table 2.1). A spontaneous streptomycin-resistant mutant was isolated from one of the auxotrophic mutants on a neutral complex medium containing streptomycin (1 mg/l).

5.2.4 Preparation of Stable Protoplasts, Transformation and Regeneration

Alkaliphilic *Bacillus* strains are shaped in the form of rods by rigid layers of the Alγ-type of peptidoglycan, which can be readily hydrolyzed with egg white lysozyme. The first protoplast transformation of alkaliphilic *Bacillus halodurans* A-59 by plasmid DNA was reported by Usami et al. (1990) An overnight culture of the host strain, alkaliphilic *B. halodurans* A-59 (leu^-, Cm^s) was diluted 20-fold with 50 ml of Horikoshi-II medium and grown at 37°C with shaking at an early log phase of growth (A660 = 0.4). The cells were harvested and resuspended in 5 ml of ASMMP buffer. Lysozyme was added to a final concentration of 500 μg/ml and the suspension was incubated at 37°C with gentle shaking. ASMM buffer consisted of 0.54 M sucrose, 0.02 M maleate and 0.02 M $MgCl_2$ (pH 7.2). ASMMP medium was prepared by mixing equal volumes of 4x strength Penassay broth (Difco) and 2 × strength ASMM. Protoplasts were collected by centrifugation at 2,600 × g for 20 min, washed once with ASMMP buffer, and resuspended in 5 ml of the same buffer. Plasmid pHW1 (0.1–1 μg) was mixed with 0.5 ml of the protoplast suspension, after which 1.5 ml of 30% polyethylene glycol (PEG) 8000 solution (Fig. 5.12) was added. After 5 to 10 min incubation, 5 ml of ASMMP buffer was added, and the protoplasts were collected by centrifugation. The collected protoplasts were resuspended in I ml of ASMMP containing 0.3% NaCl and incubated for 15 h at 30°C. Transformants were selected on a modified DM-3 regeneration plate containing 20 μg/ml of chloramphenicol. The modified DM-3 regeneration medium (pH 7.3) consisted of 0.8% agar, 13.5% sodium succinate, 0.5% casamino acid (Difco), 0.5% yeast extract (Difco), 0.35% K_2HPO_4, 0.15% KH_2PO_4, 0.5% glucose, 0.41% $MgCl_2$, 0.05% filter-sterilized bovine serum albumin and 1% NaCl. Under optimized transformation conditions, the transformation efficiency was approximately 2×10^6 transformants per milligram of plasmid DNA (Fig. 5.13). The use of a slightly higher pH for buffer and medium and a lower concentration of PEG with a longer incubation period resulted in higher frequency of transformation.

A modified procedure for *B. halodurans* C-125 was reported by Kudo et al. (1990). Protoplasts were prepared in ASMMP buffer, consisting of 0.5 M sucrose, 0.02 M maleate, and 0.02 M $MgCl_2$ in double strength Penassay broth (pH 7.5), and polyethylene glycol #8000 (final concentration, 22.5%) was used to introduce the plasmid DNA into the protoplasts. Modified DM-3 protoplast regeneration medium (pH 7.3) described above was used. Under these conditions, the transformation efficiency was approximately 5×10^6 transformants per milligram of plasmid DNA.

Aono et al. (1992b; 1993b) extensively studied the stability of the protoplast membrane of *B. halodurans* C-125 at varying pH values and reported an improved method for the regeneration of protoplasts. Protoplasts prepared from the strain with lysozyme regenerated cell walls at neutral pH,

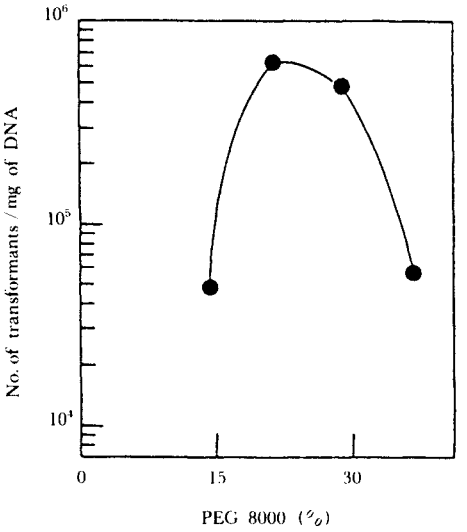


Fig. 5.12 Effect of concentration of PEG8000 on transformation efficiency. The concentration indicated in the figure is the final concentration in the reaction mixture. pHWI DNA prepared from *Bacillus* sp. A-59 was used as the donor plasmid. (Reproduced with permission from R. Usami et al., *Starch/Stärke*, **46**, 231 (1990))

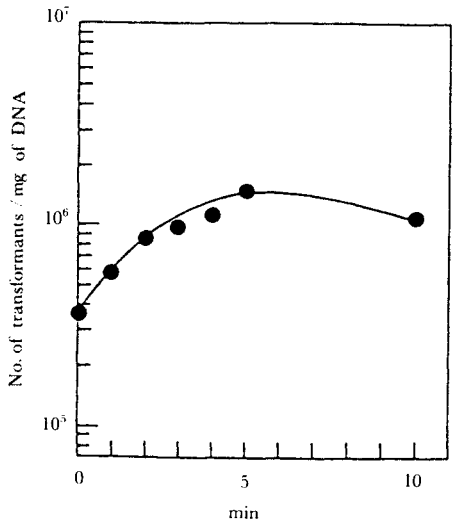


Fig. 5.13 Effect of incubation time with PEG on transformation frequency. The final concentration of PEG used for transformation was 22.5%. (Reproduced with permission from R. Usami, *Starch/Stärke*, **46**, 232 (1990))

but not at pH above 8.5. As shown in Fig. 5.14, cellular protein was released from the protoplasts exposed to various pH values for a short period. The amount of protein released into the medium was dependent on the pH of

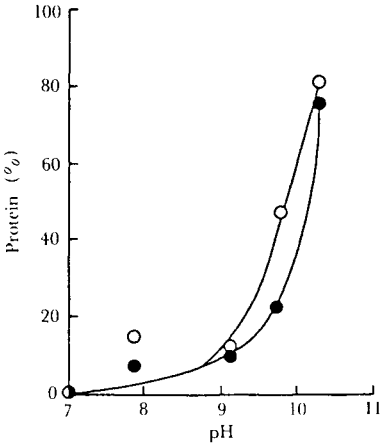


Fig. 5.14 Release of protein from protoplasts exposed to alkaline pH. Protoplasts were prepared from alkaliphilic *Bacillus* sp. C-125 (●) or neutrophilic *Bacillus subtilis* GSY1026 (○). The protoplasts were incubated at various pH values at 30°C for 20 min. Initial A_{660} of the suspensions was about 1. The pH values shown are those immediately after the protoplast was suspended in the buffer.
(Reproduced from R. Aono, M. Ito and K. Horikoshi, *Biochem. J.*, **285**, 101 (1992))

the suspension. Alkaline pH-dependent liberation of protein was also found in the 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 was liberated from both protoplasts exposed to pH 10.2 within 20 min. The colony-forming ability decreased rapidly in the protoplasts exposed to high alkaline pH. The number of colonies formed by protoplasts decreased to 0.08% of the initial number after only 3 min incubation at pH 10.6. These results indicated that the protoplasts exposed to alkaline pH rapidly burst and lost the ability to regenerate cell walls (Fig. 5.15). The alkali instability was similar to that of protoplasts from neutrophilic *B. subtilis* 168. The membrane vesicles were also labile at alkaline pH. The acidic wall components of *B. halodurans* 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.

Furthermore, regeneration of protoplasts from the four alkaliphilic strains of *Bacillus* sp. (*B. pseudofirmus* 2b-2, *B. halodurans* C-125 and A-59 and *B. clausii* M-29) was also studied. These alkaliphilic strains are shaped like rods by rigid layers of the Alγ type of peptidoglycan. Their cell walls contain acidic nonpeptidoglycan components as major structural components. Distinct types of the acidic components are found in the cell walls according to group: group 1 strains contain teichuronic acid; group 2 strains contain teichuronic acid and teichuronopeptide; group 3 strains contain teichoic acid. The peptidoglycan layers in these cell walls can be readily digested with hen egg white lysozyme. These strains were aerobically grown at 37°C

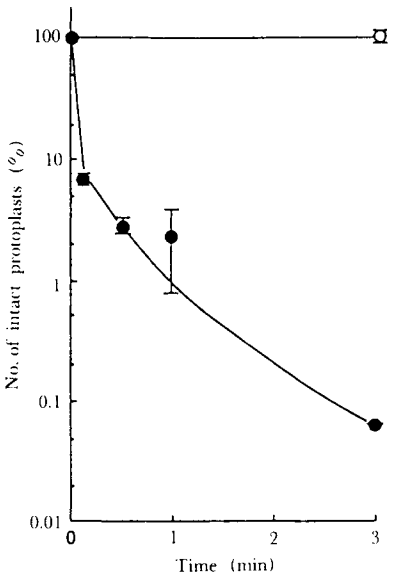


Fig. 5.15 Death of protoplasts exposed to alkaline pH. The protoplasts were prepared from strain C-125-002 and incubated in SMMP medium of pH 10.6 (●) or pH 7.2 (○) at 30°C. Periodically the suspension was diluted with SMMP medium of pH 7.2 and spread on the neutral regeneration medium. Colonies formed at 37°C by 4 days were counted. The examination was carried out in duplicate.
(Reproduced from R. Aono, M. Ito and K. Horikoshi, *Biochem. J.*, **285**, 101 (1992))

in the following alkaline medium: glucose, 0.2%; polypeptone, 0.5%; yeast extract, 0.5%; citric acid, 0.034%; K_2HPO_4, 1.37%; KH_2PO_4, 0.59%; $MgSO_4 \cdot 7H_2O$, 0.005%; Na_2CO_3, 1.06%(w/v). Cells in the exponential phase of growth were collected by centrifugation and washed once with double-strength Penassay broth (Difco Laboratories) containing 0.5 M sucrose, 20 mM $MgCl_2$, and 20 mM maleic acid, pH 7.0 (SMMP medium) and resuspended in the SMMP medium to a concentration of A_{660} 15–20. One-hundredth volume of 1% lysozyme solution was added to the cell suspension described above. The protoplasts were collected by centrifugation at 1,000 × g for 30 min at 10°C and washed twice with the SMMP medium.

The protoplasts of the alkaliphilic strains were spread onto a regeneration medium containing 5 g of yeast extract, 5 g of casamino acid, 20 g of glucose, 30 mM $MgCl_2$, 1.25 mM $CaCl_2$, 0.5 M monosodium succinate, 4 g of bovine serum albumin, 10 g of agar and 30 mM Tris in 1 liter of deionized water. The pH of the medium was adjusted with NaOH or HCl. After incubation of the protoplasts at 37°C for 2–4 days, colonies formed on the regeneration medium. The morphology of the cells grown on the hypertonic medium was bacilli form. No L-form cells were found in the colonies.

The protoplast from *B. halodurans* A-59 showed the highest regeneration frequency among the four strains tested, as shown in Fig. 5.16. The

colony formation was extremely low at alkaline pH. The *B. halodurans* C-125 was strikingly dependent on the regeneration pH: At pH 6.7, 30% of the protoplasts were regenerated, but only 0.04% of the protoplasts formed colonies at pH 7.0. Once the protoplasts were regenerated, optimum pH for growth was pH 9 to 10 exhibiting characteristics of alkaliphilic *Bacillus* strains. *B. pseudofirmus* 2b-2 was the most alkaliphilic among the strains tested. The strain scarcely grew at pH 7.5 by 24 h. After 3 days, small colonies were found at this pH. One the other hand, frequency of colony formation of the protoplasts was the highest at pH 7.6, although the colonies were minute. The efficiency was 0.1% and extremely low compared to those of protoplasts from other strains so far tested. *B. clausii* M-29 was the most tolerant of neutral pH, and formed small colonies even at pH 6.6 by 24 h. The protoplasts from the strain formed colonies in a broad range of regeneration pH (Fig. 5.16). About 4% of the protoplasts from the strain M-29

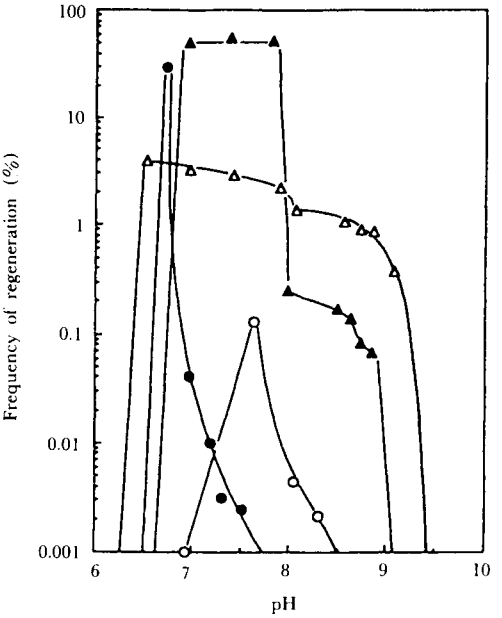


Fig. 5.16 Colony formation by protoplasts prepared from alkaliphilic strains of *Bacillus* spp. Protoplasts were prepared as described in the text from the alkaliphilic strain 2B-2 (○), A-59 (▲), C-125 (●), or M-29 (△). Protoplasts in the suspension were microscopically counted with a Thomas hematocytometer. The protoplast suspension was spread on the regeneration medium adjusted to various pHs with NaOH or HCl. Resulting pH of the surface of the medium was measured with a flat type of commercial glass electrode immediately before use. These pHs are indicated in the figure. The protoplasts were incubated at 37°C for 4 days. Colonies formed on the regeneration medium were counted every day. The graph shows the ratio of the number of colonies found by 4 days to that of the protoplasts spread on the medium in duplicate examinations.
(Reproduced from R. Aono, M. Ito and K. Horikoshi, *Biosci. Biotechnol. Biochem.*, **57**, 1597 (1993))

formed colonies at pH 6.5, and 0.4% did even at pH 9.1. Colony formation was extremely low at pH above 9.8. The frequency of colony formation at pH 9.8 was estimated to be below 0.002%. Thus, the frequency of colony formation was dependent on the regeneration pH and the highest at 6.5, although the cells of strain M-29 showed optimum pH for growth at 9.5.

These results clearly indicated that the protoplasts of the alkaliphilic strains of *Bacillus* effectively formed colonies and regenerated their cell walls at neutral pH. The efficiency was somewhat lowered at the alkaline pH which was optimum for growth of each strain, although the pH-dependence was differed among strains. The protoplasts are probably much more susceptible to alkaline pH than the intact cells generating the protoplasts. The protoplasts from strain 2b-2, A-59, or C-125 belonging to groups 1 or 2 of alkaliphilic *Bacillus* were especially susceptible to alkaline pH. The cell walls of these alkaliphilic strains are characterized by the nature of the anionic constituents found in these cell walls. These anionic compounds may contribute to the ability of alkaliphilic organisms to thrive in an alkaline environment.

5.2.5 Cell Fusion of Alkaliphiles

A procedure for genetic recombination after cell fusion of protoplasts from the facultative alkaliphile *B. halodurans* C-125 was developed by Aono et al. (1994). The strains used in this study are listed in Table 5.6. All mutant strains are derivatives of an alkaliphilic strain of *B. halodurans* C-125. Strain C-125-11, a mutant lacking teichuronic acid (TUA), was obtained by treating a threonine-requiring and streptomycin-resistant derivative of parental C-125 with ethylmethanesulphonate (Aono and Ohtani 1990). Strain C-125-90 obtained from C-125-11 lacks TUA and the polyglutamic acid moiety of TUP. Strain C-125-F19 (methionine-requiring and nalidixic acid resistant) was constructed by protoplast fusion between C-125-90 and the wild-type strain. Protoplasts were prepared from two auxotrophic and antibiotic-resistant strains (Met^- Nal^r and Thr^- Str^r, respectively) of the facultative alkaliphile *B. halodurans* C-125 by treatment with lysozyme. Equal volumes of two parental protoplasts (C-125-073 and C-125-90) were mixed and collected by centrifugation followed by the addition of polyethylene glycol (#4000). The protoplasts fused effectively in the presence of polyethylene glycol. Fusants obtained between two parental protoplasts were regenerated on solid medium of the modified DM-3 (Table 2.1) containing the two antibiotics nalidixic acid (Nal) and streptomycin (Str) after 5 days incubation at 37°C. Parental protoplasts were regenerated at high frequency (43–97%) on non-selective medium but not on selective medium. The Nal^r Str^r fusants had the form of bacilli. Met and Thr markers segregated among the fusants with a predominantly Met^+ Thr^+ phenotype. The exfusants restore the parental ploidy. From these fusants, Aono et al. (1994) isolated one fusant C-125-F19 containing TUA but not containing the polyglutamic-acid

Table 5.6 Bacterial strains used

Strain	Phenotype	Reference
C-125	Wild-type	Aono & Horikoshi(1983)
C-125-11	Thr$^-$ Str^r TUA$^-$	Aono & Ohtani(1990)
C-125-90	Thr$^-$ Str^r TUA$^-$ TUP-Glu$^-$	Aono & Ohtani(1990)
C-125-F19	Met$^-$ Str^r Nal^r TUP-Glu$^-$	Ito et al.(1994)

Abbreviations: Thr$^-$, threonine requrtement; Met$^-$, methionine requirement; Str^r, streptomycin resistnace; Nal^r, nalidixic-acid resistance; TUA$^-$, loss of TUA; TUP-Glu$^-$, loss of poly(glutamic acied) chain in TUP.
(Reproduced with permission from R. Aono, et al., *Microbiology*, **140**, 3085(1994))

Table 5.7 Culture-pH dependence of the non-peptidoglycan components of cell walls

Strain	Culture pH	Amounts relative to DAP(μmol/μmol)			
		L-Glu	UA	FucN	Total anionic compounds
C-125	6.8	1.50	0.51	0.08	2.0
	7.0	2.10	0.69	0.12	2.8
	8.0	2.90	1.70	0.36	4.6
	9.0	3.70	1.90	0.49	5.6
	10.0	4.10	2.10	0.49	6.2
	10.5	4.40	2.40	0.46	6.8
C-125-11	6.8	3.40	0.74	ND	4.1
	7.0	3.40	0.79	ND	4.2
	8.0	4.10	0.90	ND	5.0
	9.0	4.30	0.91	ND	5.2
	10.0	4.50	1.00	ND	5.5
	10.5	4.60	1.10	ND	5.7
C-125-F19	6.8	0.47	1.20	0.19	1.7
	7.0	0.53	1.30	0.07	1.8
	8.0	0.50	2.20	0.19	2.7
	9.0	0.53	2.30	0.15	2.8
	10.0	0.80	2.90	0.23	3.7
C-125-90	6.8	0.46	1.10	ND	1.6
	7.0	0.51	1.30	ND	1.8
	8.0	0.65	1.80	ND	2.4
	9.0	0.92	1.80	ND	2.7
	10.5	1.75	2.10	ND	3.8

The cell walls were prepared from bacteria grown at the constant pH indicated and assayed for constituents of TUA, TUP and peptidoglycan. The L-glutamic acid(L-Glu), uronic acids(UA) and fucosamine(FucN) contents relative to the DAP content are shown. ND, Not detected.
(Reproduced with permission from R. Aono, et al., *Microbiology*, **140**, 3085(1994))

chain of TUP. Aono et al. concluded that increased levels of acidic polymers in the cell walls of *B. halodurans* C-125 may be a necessary adaptation for growth at elevated pH (Table 5.7).

5.2.6 Selection of Vectors

A. Screening of Plasmids from Alkaliphiles

No information on plasmids in alkaliphilic microorganisms had been reported prior to 1983. Plasmids of alkaliphilic bacteria are required to make new host-vector systems. Plasmid screening was performed using a rapid-isolation method with about 200 *Bacillus* strains from our stock culture collection of alkaliphilic bacteria. Plasmids were found in two strains of alkaliphilic *Bacillus* sp. No. H331 and *B. rikeni* 13 (Usami et al. 1983). In *Bacillus* sp. No. H331 two plasmids, pAB3311 (16 kb) and pAB3312 (6 kb), were observed in an electron micrograph. The molecular size of pAB13 in *B. rikeni* 13 was estimated to be 7.9 kb from the molecular size of the fragments produced by restriction endonucleases and from the electron micrograph. The restriction map of pAB13 is shown in Fig. 5.17. Although these plasmids are still cryptic, the estimated molecular sizes, stabilities and restriction maps indicate that pAB13 is a most suitable candidate for use as a vector.

Further work on this is not in progress because a plasmid vector pHW1 constructed for *Bacillus subtilis* is available for alkaliphilic *B. halodurans* C-125 and *B. halodurans* A-59. The plasmid pHW1 contains a chloramphenicol acetyltransferase gene as a marker. This antibiotic is the only one found to be stable under alkaline conditions (Horinouchi and Weisblum 1982).

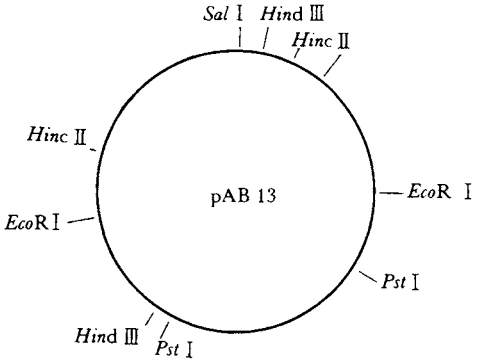


Fig. 5.17 Restriction map of pAB13.
(Reproduced with permission from R. Usami et al., *Agric. Biol. Chem.*, **47**, 2102 (1983))

B. Phage vectors

Horikoshi and Yonezawa (1978) isolated a phage from nature which infects alkaliphilic *Bacillus* sp. Y-25. The phage was assayed for plaque-forming units by the double-layer method. Turbid plaques (1–2 μm in diameter) were observed after 24 h of incubation at 30°C. An electron micrograph of the phage showed that the phage has an icosahedral head of about 100 nm in diameter attached to a tail 210 nm long and 8 to 10 nm wide. The phage grew well at pH 9 to 11, the highest titer being observed at pH 10.5. Since

then, one paper reported on alkaliphilic phages (Jarrell et al. 1997). Conventional phages of *Bacillus subtilis* infect *B. halodurans* C-125 or A-59 under neutral conditions such as pH 7–7.5. Therefore, the author's group has stopped work to develop a phage vector.

5.2.7 Summary and Conclusion

Two hosts and one vector, pHW1, have been discussed in this section. Transformation efficiency of both hosts is approximately 10^6. Regeneration of protoplasts depends on the pH value of the regeneration medium. DM-3 of pH 6.7–7.3 must be used in the case of *B. halodurans* C-125. *B. halodurans* A-59 regenerates the cell wall in the pH range of 7 to 8. However, this strain flocculated in conventional alkaline or neutral liquid media during shaking culture. As a result, the author's group used alkaliphilic *B. halodurans* C-125 as the host in the following genetic experiment which paved the way to the recent analysis of whole genome sequences.

5.3 Host Vector System of *Bacillus pseudofirmus* OF4

5.3.1 Bacterial Strains and Plasmids

After the host vector system of *B. halodurans* C-125 was established, Ito developed another host vector system of *B. pseudofirmus* OF4 in Krulwich's laboratory (Ito et al. 1997). *B. pseudofirmus* OF4811M, a streptomycin-resistant (Sm^r) methionine auxotroph, was grown either in a medium of pH 7.5 or in a medium of pH 10.5 with malate as the carbon source (Clejan et al. 1989). The mutant strain of *B. pseudofirmus* OF4811M, DA78, that can not grow at pH 10.5 and is completely unable to acidify its cytoplasm relative to the medium during a pH shift from 8.5 to 10.5 was grown on a rich medium at pH 8.5 (Krulwich et al. 1996). The *B. subtilis* wild-type strain BD99 and *tetA*(L) deletion strain JC112 were obtained. *E. coli* EP432, with Na^+/H^+ antiporter genes *nhaA* and *nhaB* disrupted, was grown in LBK medium supplemented with NaCl as indicated. The *E. coli-B. subtilis* shuttle plasmid pYH56 (ColE1 *rep* pUB110 *rep*, conferring kanamycin resistance Km^r in gram-positive bacteria and ampicillin resistance (Ap^r) in gram-negative bacteria) and pBK15 (Ap^r in *E. coli* and chloramphenicol resistance (Cm^r to 5 μg/ml) in *B. pseudofirmus* OF4811M) were used as vectors. The plasmid pJB10, which contained a truncated version of *nhaC*, was isolated from the pGEM3Zf(+) library of *B. pseudofirmus* OF4 DNA. Plasmid pJX5, containing most of *nhaC* and an additional downstream sequence, was used to extend the sequence downstream of *nhaC* (Ivey et al. 1991). The plasmid pG1host4 (pGK12 *rep*, conferring erythromycin resistance Erm^r in gram-positive and -negative bacteria was obtained from Appligene, Inc., Pleasanton, CA.

5.3.2 Preparation and Transformation of Protoplasts

Protoplasts were prepared from *B. pseudofirmus* OF4811M using the protocol described previously for *B. halodurans* C-125 and spread on modified DM-3 medium (Aono et al. 1992b). In initial experiments the medium was adjusted to various pH values with NaOH or HCl. The protoplasts were incubated at 37°C for 3 days, after which the number of colonies was recorded. Colony formation by the protoplasts was strikingly dependent upon the pH of the regeneration medium. About 30% of the protoplasts regenerated at pH 7.0, whereas lower numbers of colonies were found as the pH was increased during regeneration, and no regeneration occurred at pH 6.5. Although optimal regeneration of cell walls occurred at pH 7.0, the colonies formed at this pH were tiny in comparison to those formed at slightly higher pH values, consistent with earlier observations on the effect of pH on the growth of *B. pseudofirmus* OF4 (Sturr et al. 1994). Thus, regeneration was routinely carried out at pH 7.5. Under these conditions, the transformation efficiency was approximately 10^6 transformants per milligram of plasmid DNA. Protoplasts were prepared in SMMP medium, consisting of 0.5 M sucrose, 0.02 M maleic acid, and 0.02 M $MgCl_2$ in double-strength Penassay broth (pH 7.5) (Aono et al. 1992b). Polyethylene glycol 8000 (final concentration, 22.5%) was used to introduce the plasmid DNA into the protoplasts. Kanamycin (200 μg/ml) was added to the modified DM-3 medium for selection of transformants.

5.3.3 Construction of *nhaC*-deleted *B. pseudofirmus* OF4 (N13)

PCR was performed on purified *B. pseudofirmus* OF4 chromosomal DNA using the same two oligonucleotide primers that were employed to prepare the insert for pNhaC. The purified PCR product was first ligated into *Hinc*II-digested pGEM3Zf(+) (Ap^r; Promega). The recombinant plasmid was selected by blue/white screening in *E. coli* DH5a. After isolation, the *nhaC* fragment was released by *Bam*HI-*Pst*I treatment and ligated to the temperature-sensitive plasmid pG1host4 cut with the same endonuclease. After isolation, the plasmid was digested with *Sca*I and a 772-bp *Sca*I/*Sca*I fragment was removed from the middle of *nhaC* (Accession Number U61539). A gene encoding Cm^r was ligated to this linear plasmid, resulting in a recombinant plasmid containing a fragment of *nhaC* replaced by the Cm^r gene (D*nhaC*::Cm^r). After isolation, the recombinant plasmid was introduced into *B. pseudofirmus* OF4 by protoplast transformation, and cell walls were regenerated at 28°C. Transformed cells were resistant to both erythromycin (1 mg/ml) and chloramphenicol (3 mg/ml). Gene replacement was achieved by the approach of Biswas et al. (1993). For isolation of single-crossover integrants, transformants growing at 28°C were diluted and plated onto complex medium containing erythromycin and incubated at 39°C. Of a total of 1.1×10^8 colonies, 1.3×10^5, or 0.12%, were Erm^r at 39°C. The

Table 5.8 Internal pHs of *B.pseudofirmus* OF4811M/pYH56, N13/pYH56, and N13/pNhaC after a shift of the outside pH from 8.5 to 10.6

pH of medium and strain [†1]	Cytoplasmic pH at Na^+ concn [†2] of :		
	50 mM	5 mM	1 mM
10.5			
B. pseudofirmus OF4811M/pYH56	8.50 ± 0.04	8.71 ± 0.08	9.17 ± 0.10
N13/pYH56	8.55 ± 0.07	8.97 ± 0.09	9.50 ± 0.08
N13/pNhaC	8.54 ± 0.09	8.74 ± 0.08	8.83 ± 0.04
7.5			
B. pseudofirmus OF4811M/pYH56	8.60 ± 0.03	8.96 ± 0.07	ND [†3]
N13/pYH56	8.76 ± 0.09	9.46 ± 0.10	ND
N13/pNhaC	8.65 ± 0.08	8.93 ± 0.11	ND

[†1] Strains were grown in buffer at either pH 10.5 or pH 7.5 as described in the text.
[†2] Na^+ concentration indicates the amount of Na^+ present in the pH shift buffer.
[†3] ND, not determined.
(Reproduced with permission from M. Ito et al., *J. Bacteriol.*, **179**, 3651(1997))

single-crossover event, resulting in integration of the entire plasmid in the chromosome, was confirmed by PCR analysis. For the double-crossover event that completes the gene replacement, a single-crossover integrant was grown overnight at 28°C in complex broth containing chloramphenicol. Cells were then diluted and plated onto medium of the same composition and incubated at 39°C. Double-crossover candidates were identified on the basis of Cm^r and Erm^s; 8 candidates were found among 200 total colonies. Both Southern hybridization and PCR analyses confirmed that the gene replacement event had taken place in these candidates. The *nhaC* deletion strain (N13) has been used in further studies (Ito et al. 1997). Table 5.8 from their paper shows internal pHs of *B. pseudofirmus* OF4811M/pYH56, N13/pYH56 and N13/pNhaC after a shift of the outside pH from 8.5 to 10.6.

6

Whole Genome Sequences of Alkaliphilic *Bacillus* Strains

6.1 Introduction

How alkaliphiles adapt to their alkaline environments is one of the most interesting and challenging topics facing microbiologists. In 1992, to aid our understanding of alkaliphiles, the author and his colleagues decided to construct a physical map for the alkaliphilic *Bacillus halodurans* C-125 (JCM9153) chromosome. This strain was chosen as a model alkaliphile since 1) it exhibits good growth between pH 7.0 and 11, and requires sodium ions for growth; 2) it grows well on minimal medium, allowing cloning of genes by a selective marker and complementation approach; 3) an efficient transformation system for cloning is available; 4) several genes have been cloned from this strain and used as markers on its chromosome map; and 5) this strain is not closely related to *Bacillus pseudofirmus* OF4 of Krulwich's group. Further taxonomical details of *B. halodurans* C-125 are shown in Section 2.3.

Sutherland et al. (1993) reported tentative physical map of *B. halodurans* C-125, although several parts of the physical map were incomplete. After seven years, Takami et al. (2000) reported the complete whole genome sequence of *B. halodurans* C-125 that was the second whole genome sequence of the *Bacillus* chromosome.

A great number of ubiquitous *Bacillus* strains have been isolated from various terrestrial and deep-sea environments even, from the Mariana Trench at the depth of 10897 m. Some of those *Bacillus* strains possess various capabilities to adapt to extreme environments such as high and low temperature, high and low pH, high salinity and high pressure (Takami et al. 1997; 1999a). From these deep-sea isolates, many extremophilic bacteria such as alkaliphiles, halophiles and thermophiles were found. These were expected to thrive in an extreme environment utterly different from the *in situ* conditions of high hydrostatic pressure and low temperature in the deep-sea sampling sites.

6.2 Alkaliphilic *Bacillus* Species

The author's microbial genome research group has determined the com-

plete genomic sequences of two alkaliphilic *Bacillus* species, *Bacillus halodurans* C-125 and extremely halotolerant and alkaliphilic *Oceanobacillus iheyensis* HTE831 isolated from deep-sea sediment collected at a depth of 1050m to elucidate the mechanisms of adaptation to alkaline or hypersaline environments and the substantial difference between terrestrial and benthic life in the deep sea. These sequences from two alkaliphilic *Bacillus* related species highlighted common genes involved in the alkaliphilic phenotype. Alkaliphilic *B. halodurans* C-125 (JCM9153) was isolated from the soil sample in 1970 and characterized as a β-galactosidase (Ikura et al. 1979a; 1979b) and xylanase producer (Honda et al. 1985a, b, c; 1986). It is the most thoroughly characterized strain, physiologically, biochemically and genetically, among those in the author's collection of alkaliphilic *Bacillus* isolates (Horikoshi 1999a, b). As described above, the facultative alkaliphilic *B. halodurans* C-125 can grow at pH 7 to 11 in the presence of sodium ions (1–2%) in the medium. Over the past three decades, Horikoshi's colleagues have focused on the enzymology, physiology, and molecular genetics of alkaliphilic microorganisms.

6.2.1 Construction of the Physical Map of the *B. halodurans* C-125 Chromosome

As the first step in genome analysis, Takami et al. (1999a) attempted to construct a physical map of the chromosome of the strain C-125, which has a size of 4.2 Mb. The enzymatic digestion of the *B. halodurans* C-125 chromosome with *Asc*I or *Sse*8387I resulted in 20 resolvable fragments. A linking clone contains DNA which overlaps two adjacent restriction fragments, and it can be used as a hybridization probe to establish the identity of these two contiguous fragments. Isolation of *Asc*I- and *Sse*8387I-linking clones was attempted in order to join adjacent fragments generated by *Asc*I or *Sse*8387I digestion in mapping the chromosome. Linking clone libraries were constructed using *Hind*III or *Eco*RI or *Eco*RV as the R enzyme. Plasmid DNA was isolated from individual clones and screened for the presence of two *Asc*I or *Sse*8387I sites and two R enzyme sites. Seventeen possible *Asc*I-linking clones (A-Q) or *Sse*8387I-linking clones (a-q) were identified and screened for authenticity by Southern blot analysis of R enzyme-digested *Bacillus halodurans* C-125 chromosomal DNA (Table 6.1). All *Asc*I- and *Sse*8387I-linking clones also listed in Table 6.1 were used as hybridization probes in Southern blot analyses of *Asc*I and *Sse*8387I digests of C-125 chromosomal DNA in PFGE gels. In most cases, the linking clones for a particular enzyme hybridized to two different fragments obtained after digestion with the enzyme. Each *Asc*I or *Sse*8387I contiguous fragment was joined by means of the hybridization and cross-hybridization patterns obtained upon analysis of chromosomal DNA digested with *Sse*8387I or *Asc*I when hybridized to *Sse*8387I- or *Asc*I-linking clones. The DNA probes listed in Table 6.1 were used for mapping of genetic loci on the physical map. In addition, the

Table 6.1 DNA probes used for construction of the physical map

Probe	*Size*	*Characteristics*
*Asc*I-A	1.1	C-125 *Asc* I-linking clone
*Asc*I-B	1.5	C-125 *Asc* I-linking clone
*Asc*I-C	1.6	C-125 *Asc* I-linking clone
*Asc*I-D	1.7	C-125 *Asc* I-linking clone
*Asc*I-E	2.5	C-125 *Asc* I-linking clone
*Asc*I-F	3.6	C-125 *Asc* I-linking clone
*Asc*I-G	2.4	C-125 *Asc* I-linking clone
*Asc*I-H	6.9	C-125 *Asc* I-linking clone
*Asc*I-I	3.9	C-125 *Asc* I-linking clone
*Asc*I-J	0.6	C-125 *Asc* I-linking clone
*Asc*I-K	0.6	C-125 *Asc* I-linking clone
*Asc*I-L	6.5	C-125 *Asc* I-linking clone
*Asc*I-M	5.7	C-125 *Asc* I-linking clone
*Asc*I-N	4.2	C-125 *Asc* I-linking clone
*Asc*I-O	2.8	C-125 *Asc* I-linking clone
*Asc*I-P	2.2	C-125 *Asc* I-linking clone
*Asc*I-Q	3.5	C-125 *Asc* I-linking clone
*Sse*8387I-a	0.5	C-125 *Sse*8387I-linking clone
*Sse*8387I-b	2.2	C-125 *Sse*8387I-linking clone
*Sse*8387I-c	4.5	C-125 *Sse*8387I-linking clone
*Sse*8387I-d	2.4	C-125 *Sse*8387I-linking clone
*Sse*8387I-e	3.2	C-125 *Sse*8387I-linking clone
*Sse*8387I-f	6.3	C-125 *Sse*8387I-linking clone
*Sse*8387I-g	1.5	C-125 *Sse*8387I-linking clone
*Sse*8387I-h	1.3	C-125 *Sse*8387I-linking clone
*Sse*8387I-i	2.0	C-125 *Sse*8387I-linking clone
*Sse*8387I-j	4.0	C-125 *Sse*8387I-linking clone
*Sse*8387I-k	2.8	C-125 *Sse*8387I-linking clone
*Sse*8387I-l	2.2	C-125 *Sse*8387I-linking clone
*Sse*8387I-m	4.0	C-125 *Sse*8387I-linking clone
*Sse*8387I-n	4.0	C-125 *Sse*8387I-linking clone
*Sse*8387I-o	2.1	C-125 *Sse*8387I-linking clone
*Sse*8387I-p	8.0	C-125 *Sse*8387I-linking clone
*Sse*8387I-q	6.0	C-125 *Sse*8387I-linking clone
C-125*gyrB*	0.4	DNA gyrase subunit B
C-125*rpoA*	1.0	RNA polymerase α subunit
C-125*sigA*	1.1	RNA polymerase major sigma factor(σ^A)
C-125*secY*	1.3	preprotein translocase subunit
C-125*hag*	0.5	flagellin
C-125*pALK*	2.4	alkaline resistance
C-125*xyl*(A)	1.8	alkaline xylanase
C-125*groEL*	1.5	heat-shock protein (chaperonin)
C-125*recA*	0.8	homologous recombination and DNA repair
B. subtilis trpS	1.0	*B. subtilis* tryptophanyl-tRNA synthetase

ORFs identified and annotated in the linking clones were also used for mapping. The assigned positions of the genes on the physical map of *B. halodurans* C-125 were compared with those on the genetic and physical maps of *B. subtilis*. DNA probes for *secY* and *rpoA* from strain C-125 hybridized to the same fragments 4A and 3S'. The genetic loci on the physical map of strain C-125 were within approximately 12° of their positions on the *B. sub-*

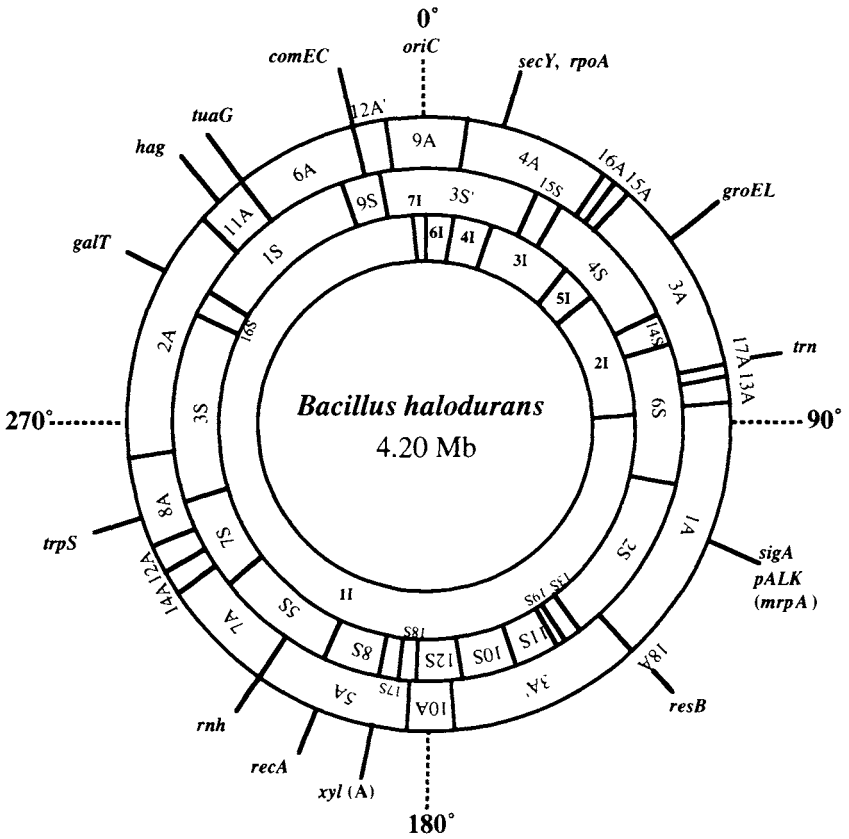


Fig. 6.1 *AscI*/*Sse*8387I/I-*CeuI* physical and genetic map of the chromosome of *Bacillus* sp. strain C-125. Outer, middle and inner circles show the *Asc*I-, Sse83871-and I-*Ceu*I physical map, respectively. The locations of several housekeeping genes are indicated on the map. The dashed line indicates the approximate position of the gene.

tilis physical map (Fig. 6.1). It was also found that probes for *groEL* and *hag* hybridized to 3A and 4S, and 11A and 1S, respectively. Their positions on the map were also close to those of *B. subtilis* (55° for *groEL* and 310 for *hag*) as shown in Fig. 6.1. On the other hand, it appeared that genes such as *trpS*, *appA*, *rnh* and *recA* have divergent map positions, being located at 103 to 150° on the *B. subtilis* genetic map and approximately 200 to 250° on the map of *B. halodurans* C-125. The map positions of three genes, *sigA*, *pALK* (*mrp*) and *resB*, located at 100 to 140° also differ from the positions 206 to 277° on the *B. subtilis* map. Therefore, it appears that the region around 200 to 250° on the *B. halodurans* C-125 map may correspond to the region around 100 to 140° on the genetic map of *B. subtilis*.

6.2.2 Sequencing and Assembly of the Whole Genome Shotgun Library

A 20 μg aliquot of chromosomal DNA was sonicated for 5–25 sec with a Bioruptor UCD-200TM (Tosho Denki Co., Japan). The sonicated DNA fragments were blunt-ended using a DNA blunting kit (Takara Shuzo, Kyoto, Japan) and fractionated by 1% agarose gel electrophoresis. DNA fragments 1–2 kb in length were excised from the gel and eluted by the freeze-squeeze method. The DNA recovered was ligated to the *Sma*I site of pUC18, which had been previously treated with BAP, and introduced into competent XL1-Blue cells by the standard method. Transformants with a frequency of $5–6 \times 10^5$/μg DNA were cultivated in LB liquid medium at 30°C and 1 μl of culture broth was used for template DNA. The insert in the plasmid, amplified using a standard PCR method, was used for sequencing. The genome of alkaliphilic *B. halodurans* C-125 was basically sequenced by the whole genome random sequencing method as described. The DNA fragment inserted into pUC18 was amplified by PCR using M13-20 and reverse primers. PCR fragments, treated with exonuclease I and shrimp alkaline phosphatase (Amersham, OH, USA) to eliminate excess primers in the PCR reaction mixture, were used for sequencing analysis as template DNA. Sequencing was performed with an ABI Prism 377 DNA sequencer using a Taq Dye Terminator Cycle Sequencing Kit (Perkin Elmer, CT, USA). DNA sequences determined by means of the ABI sequencer were assembled into contigs using Phrap with default parameters and without quality scores. At a statistical coverage of 7.1-fold, the assembly using Phrap yielded 656 contigs. Sequences were obtained from both ends of 2000 randomly chosen clones from a library. These sequences were then assembled with consensus sequences derived from the contigs of random phase sequences using Phrap. Gaps between contigs were closed by shotgun sequencing of clones which bridged the contigs of random phase sequences. The final gaps were closed by direct sequencing of the products amplified by long accurate PCR with a LA PCR Kit v.2 (Takara Shuzo). The sequence has been deposited in the DDBJ/EMBL/GenBank with the accession numbers AP001507-AP001520.

6.2.3 Annotation

The predicted protein coding regions were initially defined by searching for open reading frames (ORFs) longer than 100 codons using the Genome Gambler program. Coding potential analysis of the entire genome was performed with the GeneHacker Plus program using hidden Markov models trained with a set of *B. halodurans* C-125 ORFs longer than 300 nt. This program evaluates quality of the Shine-Dalgano sequence (SD) and codon usage for a series of two amino acids. The SD sequence was complementary to one found at the 3'-end of 16S rRNA. The SD sequence (UCUUUCCUC-

CACUAG...) of alkaliphilic *B. halodurans* C-125 is the same as that of *B. subtilis*. Searches of the protein databases for amino acid similarities were performed using BLAST2 sequence analysis tools with subsequent comparison of protein coding sequences (CDSs) showing significant homology ($>10^{-5}$ significance) performed using the Lipman-Pearson algorithm. Significant similarity was defined as at least 30% identity observed over 60% of the CDS, although those CDSs showing <30% identity over> 60% of the protein were also included. A search for paralogous gene families, such as factors, ATPases, antiporters and ATP-binding cassette (ABC) transporters, in the *B. halodurans* C-125 genome was performed with stepwise BLAST2P, identifying pairwise matches above $P \leqq 10^{-5}$–10^{-80} over 50% of the query search length, and subsequently by single linkage clustering of these matches into multigene families.

6.2.4 General Feature of the *B. halodurans* C-125 Genome

The genome of *B. halodurans* is a single circular chromosome (Takami et al. 1999a) consisting of 4202352 base pairs bp with an average G+C content of 43.7% (Table 6.2). The G+C content of DNA in the coding regions and non-coding regions is 44.4% and 39.8%, respectively. Based on analysis of

Table 6.2 General features of *B. halodurans* and *O. iheyensis*

General features	*B. halodurans*	*O. iheyensis*	*B. subtilis*
Size(base pairs)	4,202,352	3,630,528	4,214,630
G+C content(mol%)			
Total genome	43.7	35.7	43.5
Coding region		36.1	
Noncoding region		31.8	
Protein coding sequences			
Predicted number	4,066	3,496	4,106
Average length(bp)	879	883	896
Percent of coding region	85	85	87
Initiation codon			
Percent of AUG	78	79	78
Percent of GUG	12	8	9
Percent of UUG	10	12	13
RNA elements			
Percent of stable RNA	1.02	1.04	1.27
Number of *rrn* operons	8	7	10
Number of tRNA	78	69	86
Insertion elements			
Prophage	0	0	10
Transposase of IS	93	14	0
IS*L3* family	43	9	0
IS*200*/IS*605* family	8	2	0
IS*30* family	3	1	0
IS*1272*/IS*660* family	5	1	0
IS*3* family	5	1	0
Recombinase of group II intron	5	5	0

the G+C ratio and G−C skew (G−C/G+C), it was estimated that the site of termination of replication (*terC*) is nearly 2.2–2.3 Mb (193°) from the origin. However, the gene encoding the replication termination protein (*rtp*) in the genome of *B. halodurans* C-125 could not be found. Several A+T rich and G+C rich islands are likely to reveal the signature of transposons or other inserted elements (Fig. 6.2). Takami et al. (2000) identified 4,066

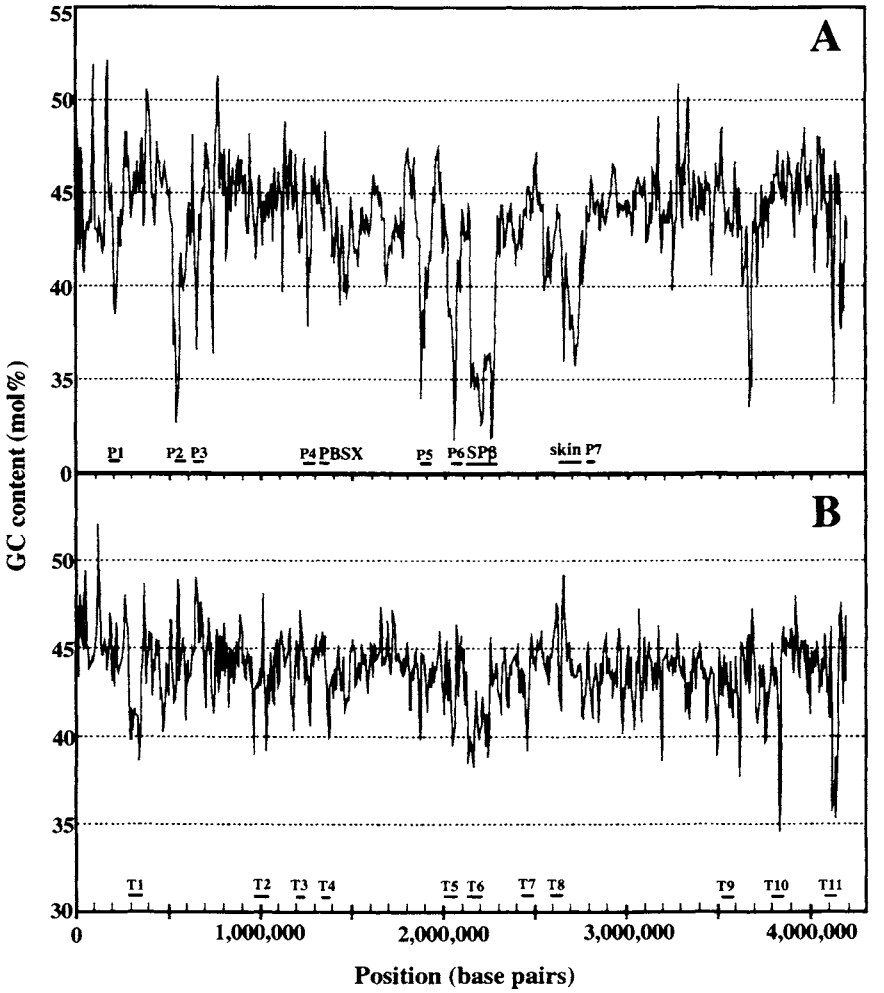


Fig. 6.2 Comparison of G+C profiles between the genomes of *B. halodurans* and *B. subtilis*. Distribution of A+T-rich islands along the chromosome, in sliding windows of 10,000 nucleotides, with a step of 5,000 nucleotides. Known phages(PBSX, Spβ and skin) are indicated by their names and prophage-like elements in the *B. subtilis* genome are numbered from P1 to P7. The A+T-rich or G+C-rich regions containing transposases in the *B. halodurans* genome are also indicated from T1 to T11. A: *B. subtilis*, B: *B. halodurans*.
(Reproduced with permission from H. Takami et al., *Nucleic Acids Research*, **28**, 4326 Oxford Univ. Press(2000))

protein coding sequences (CDSs) (Table 6.2), and 78% of the genes started with ATG, 10% with TTG and 12% with GTG, as compared with 87%, 13% and 9%, respectively, for the case of *B. subtilis* (Table 6.2). The average size of the predicted proteins in *B. halodurans* C-125 is 32,841 daltons, ranging from 1,188 to 199,106 daltons. Predicted protein sequences were compared with sequences in a non redundant protein database and biological roles were assigned to 2141 (52.7%) of them. In this database search, 1182 predicted coding sequences (29.1%) were identified as conserved proteins of unknown function in comparison with proteins from other organisms including *B. subtilis*, and for 743 (18.3%) there was no database match (Table 6.2). Among all the CDSs found in the *B. halodurans* genome, 2310 (56.8%) were widely conserved in organisms including *B. subtilis* and 355 (8.7%) of the CDSs matched the sequences of proteins found only in *B. subtilis* (Fig. 6.3). The ratio of proteins conserved through various organisms including *B. subtilis*, among functionally assigned CDSs (2141) and among CDSs (1182) matched with hypothetical proteins from other organisms was 80.5% and 49.7%, respectively. Of 1183 CDSs, 23.8% matched hypothetical proteins found only in the *B. subtilis* database, showing relatively high similarity values (Fig. 6.3).

One hundred twelve CDSs in the *B. halodurans* genome showed significant similarity to transposase or recombinase from various species such as *Anabena* sp. *Rhodobacter capsulatus*, *Lactococcus lactis*, *Enterococcus faecium*, *Clostridium beijerinckii*, *Staphylococcus aureus* and *Yersinia pseudotuberculosis*, in-

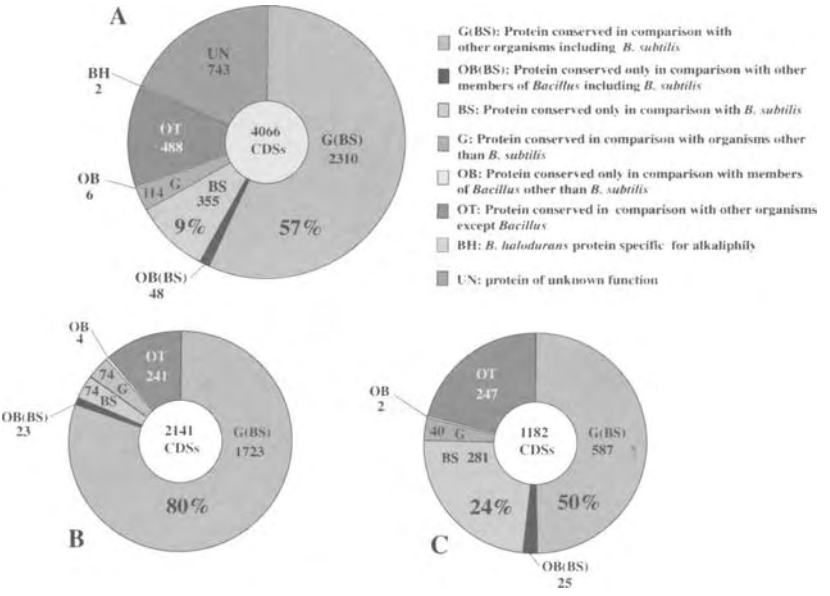


Fig. 6.3 Summary of conserved CDSs identified in the genome of *B. halodurans* C-125. (A)All CDSs; (B)CDSs with a known functional role; (C)CDSs of unknown function. (Reproduced with permission from H. Takami et al., *Nucleic Acids Research*, **28**, 4327, Oxford Univ. Press(2000))

dicating that they have played an important evolutionary role in horizontal gene transfer and also in the internal rearrangement of the genome. These CDSs were categorized into 27 groups by similarity pattern and genes are widely spread throughout the genome. In other bacterial genome, it has been reported that *Synecosystis* sp. PC6803, *E. coli* MG1655, *Mycobacterium tuberculosis*, *Deinicoccus radiodurans*, and *Lactococcus lactis* contain many transposase genes like *B. halodurans* C-125. On the other hand, no prophage, which seems to be active although several phage-related proteins was identified in the *B. halodurans* C-125 genome.

When Takami and Horikoshi started the sequencing studies, they thought that *Bacillus halodurans* C-125 should be similar to *B. subtilis* in terms of genome size, G+C content of genomic DNA, and physiological properties used for taxonomical identification except for the alkaliphilic phenotype. Also, the phylogenetic placement of *Bacillus halodurans* C-125 based on 16S rDNA sequence analysis indicates that this organism is more

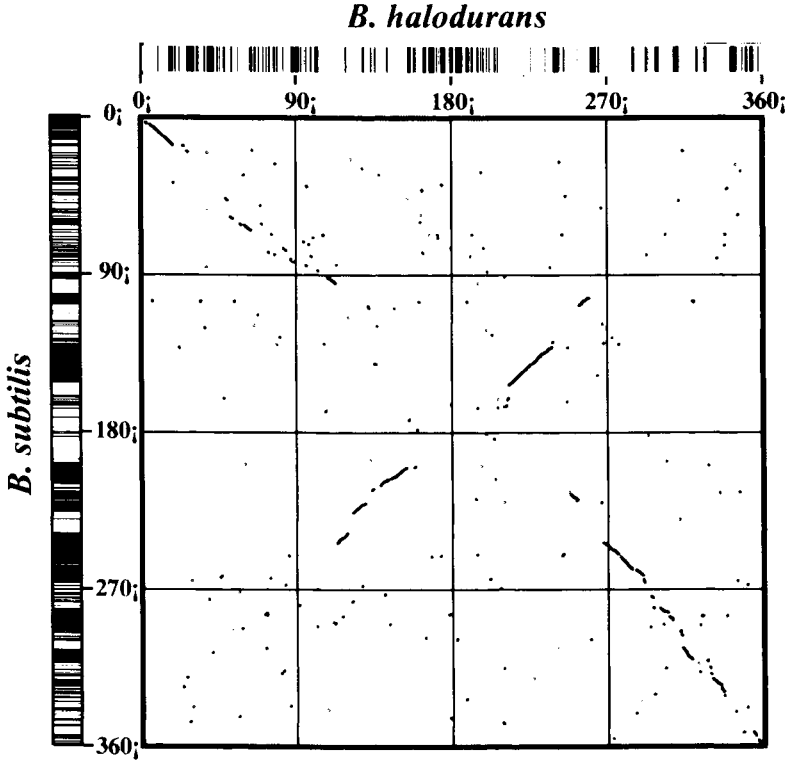


Fig. 6.4 Structural analysis of the *B. halodurans* and *B. subtilis* genomes. Distribution of orthologues between the two *Bacillus* species. The dots were plotted at the positions where the gene for an orthologous protein exists when comparing the two genomes. The columns appearing like a bar code clearly show the positions of the genes for orthologous proteins in *B. halodurans* and *B. subtilis*.
(Reproduced with permission from H. Takami et al., *Nucleic Acids Research*, **28**, 4328 Oxford Univ. Press(2000))

closely related to *B. subtilis* than to other members of the genus *Bacillus*. Therefore, the question arises, how does the genome structure differ between two *Bacillus* strains which have similar properties, except for alkaliphily. As the first step to answering this question, Takami et al. analyzed the genome structure at the level of orthologous proteins comparing the *B. halodurans* and *B. subtilis* genomes continuously from the replication origin region (*oriC*). Fig. 6.4 shows the distribution of orthologous proteins, comparing *B. halodurans* and *B. subtilis*, and the dot patterns in these figures resemble each other. About 1500 genes, some of which constitute operons, categorized mainly as genes associated with the functions noted below, are well conserved in the region common to *B. halodurans* and *B. subtilis*: mobility and chemotaxis, protein secretion, cell division, main glycolytic pathways, TCA cycle, metabolism of nucleotides and nucleic acids, metabolism of coenzymes and prosthetic groups, DNA replication, RNA modification, ribosomal proteins, aminoacyl-tRNA synthetases, protein folding, etc. On the other hand, the region around 112°–153° in the *B. halodurans* genome corresponds to the region around 212°–240° in the *B. subtilis* genome, as suggested in a previous paper (Takami et al. 1999c, d). Further information on each gene is provided below.

6.3 Further Works on Genome Analysis of Alkaliphilic *Bacillus halodurans*

6.3.1 Origin of Replication

There are 14 CDSs in the *oriC* region of chromosome of *B. halodurans*. The organization of the CDSs in the region is basically similar to those of other bacteria. In particular, the region from *gidB* to *gyrA* (BH 4060–4066 and BH1–BH7) was found to be the same as that of *B. subtilis*. On the other hand, it was found that there are 10 CDSs (BH8–BH18), including three CDSs previously identified in the 13.3 kb of *oriC* region (Takami et al. 1999d) between *gyrB* and the *rrnA* operon corresponding to the *rrnO* operon in the *B. subtilis* genome although there is no CDS between *gyrA* and *rrnO* in *B. subtilis*. Of these 10 CDSs, only one CDS (BH8) was found to have a homologue in another organism, interestingly, not in the genus *Bacillus*, and others were unique to the *B. halodurans* genome (Takami et al. 2000).

6.3.2 Transcription and Translation

Genes encoding the three subunits (a, b, b') of the core RNA polymerase have been identified in *B. halodurans* along with the genes for twenty sigma factors. Sigma factors belonging to the s^{70} family (s^{A}, s^{B}, s^{D}, s^{E}, s^{F}, s^{G}, s^{H}, and s^{K}) are required for sporulation and s^{L} is well conserved between *B. halodu-*

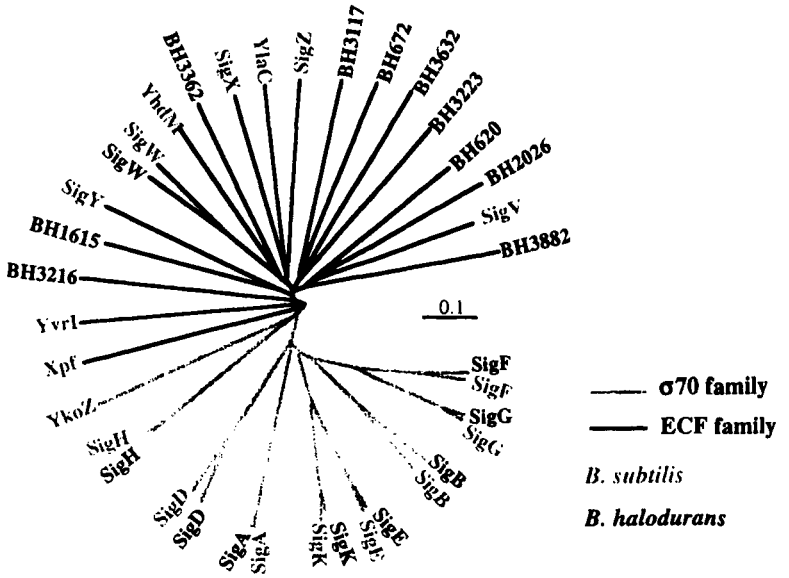


Fig. 6.5 Dendrograms of members of sigma factors. These unrooted distance dendrograms were generated from the multiple sequence alignment of each paralogue and orthologue between the *B. halodurans* and *B. subtilis* genomes. Bar = 0.1 Knuc unit. (Reproduced with permission from H. Takami et al., *Nucleic Acids Research*, **28**, 4317, Oxford Univ. Press(2000))

rans and *B. subtilis*. As shown in Fig. 6.5, out of eleven sigma factors identified in *B. halodurans*, belonging to the extracytoplasmic function (ECF) family, s^W is also found in *B. subtilis* but the other ten (BH640, BH672, BH1615, BH2026, BH3117, BH3216, BH3223, BH3380, BH3632 and BH3882) are unique to *B. halodurans* (Takami et al. 2000). These unique sigma factors may play a role in the special physiological mechanisms by which *B. halodurans* is able to live in an alkaline environment, because it is well known that ECF sigma factors are present in a wide variety of bacteria and serve to control the uptake or secretion of specific molecules or ions and to control responses to a variety of extracellular stress signals.

Seventy-nine tRNA species, organized into 11 clusters involving 71 genes plus 8 single genes, were identified (Table 6.2). Of the 11 clusters, 6 were organized in association with ribosomal RNA (rRNA) operons. Eight rRNA operons are present in the C-125 genome and their organisation is the same as that in *B. subtilis* (tRNA-16S-23S-5S, 16S-tRNA-23S-5S, and 16S-23S-5S-tRNA). With respect to tRNA synthetases, the C-125 genome lacks the glutaminyl-tRNA synthetase gene (*glnS*), one of two threonyl-tRNA synthetase gene (*thrZ*), and one of two tyrosyl-tRNA synthetase genes (*tyrS*). The *B. subtilis* genome has all of these tRNA synthetase genes except for the glutaminyl-tRNA gene. It is likely that glutamyl-tRNA synthetase aminoacylates $tRNA^{Gln}$ with glutamate followed by transamidation by Glu-tRNA amidotransferase in both *Bacillus* species.

6.3.3 Competence and Sporulation

Of 20 genes related to competence in *B. subtilis*, thirteen genes (*cinA*, *comC*, *comEA*, *comEB*, *comEC*, *comER*, *comFA*, *comFC*, *comGA*, *comGB*, *comGC*, *comGD*, and *mecA*) mainly expressed in the late stage of competence were identified in the *B. halodurans* genome, but Takami et al. (2000) could not find any of the genes expressed in the early stage of competence. Among six genes whose products are known to serve as components of DNA transport machinery, only three genes (*comGB*, *comGC*, and *comGD*), but not the others well conserved in *B. subtilis*, were identified in *B. halodurans* C-125. Actually, competence has not been demonstrated in C-125 experimentally, although Takami et al. attempted to use standard and modified methods by changing some conditions such as pH, temperature and medium for transformation. It has become clear that this is due to lack of some of the necessary genes, especially those expressed in the early stage such as *comS*, *srfA*, and *rapC*. Only 68 genes related to sporulation were identified in the C-125 genome, in contrast with 138 genes found in the *B. subtilis* genome. Although the minimum set of genes for sporulation was well conserved, the same as in the case of *B. subtilis*, the C-125 genome lacks some genes encoding key regulatory proteins (the response regulator for aspartate phosphatase and the phosphatase regulator) and the spore coat protein for sporulation conserved in the *B. subtilis* genome. In particular, the *rap* (*rapA-K*) and *phr* (*phrA*, *phrC*, *phrE-G*, *phrI*, and *phrK*) genes were not found in the C-125 genome, suggesting that C-125 may have another type(s) of regulatory genes for control of sporulation in a manner the same as or different from that in *B. subtilis*, because sporulation has been observed in *B. halodurans*.

6.3.4 Cell Walls

The peptidoglycan of alkaliphilic *B. halodurans* C-125 appears to be similar to that of neutrophilic *B. subtilis*. However, the cell wall components in C-125 are characterized by an excess of hexosamines and amino acids compared to that of *B. subtilis*. Glucosamine, muramic acid, D- and L-alanine, D-glutamic acid, meso-diaminopimelic acid and acetic acid were found in cell wall hydrolysates (Horikoshi 1999a). Although some variation was found in the amide content of the peptidoglycan isolated from alkaliphilic *Bacillus halodurans* C-125, the pattern of variation was similar to that known to occur in *B. subtilis*. All genes related to peptidoglycan biosynthesis such as *mraY*, *murC-G*, *cwlA*, *ddlA* and *glnA* confirmed to be present in the *B. subtilis* genome, were also conserved in the C-125 genome. A bacitracin-resistance gene found in the *B. subtilis* genome is duplicated in the C-125 genome (BH474 and BH1538). On the other hand, although *tagH* and *tagG* genes were identified in *B. halodurans* C-125, 13 other genes for teichoic acid biosynthesis found in *B. subtilis* (*dltA-E*, *ggaA*, *ggaB*, *tagA-C*, *tagE*, *tagF* and *tagO*) are missing in the *B. halodurans* genome. *B. halodurans* C-125 also

lacks six genes (*tuaB-tuaF* and *tuaH*) for teichuronic acid biosynthesis, all except *tuaA* and *tuaG*, in comparison with those of *B. subtilis* (Takami et al. 2000). In addition to peptidoglycan, the cell wall of alkaliphilic *B. halodurans* is known to contain certain acidic polymers, such as galacturonic acid, glutamic acid, aspartic acid and phosphoric acid. The negative charges on acidic nonpeptidoglycan components may give the cell surface the ability to absorb sodium and hydronium ions and to repel hydroxide ions, and, as a consequence, may contribute to allowing the cells to grow in alkaline environments. A mutant defective in poly-γ-L-glutamic acid synthesis (Accession Numbers AB071407-AB071409) which was called *tupA* by Aono et al. (1999) grows very slowly at alkaline pH. Further details are discussed in Section 3.2.2. Takami et al.'s study (2000) has made it clear that *B. halodurans* C-125 has no paralogue of poly-γ-L-glutamic acid synthesis gene (*plg* 1-3) in the genome and the orthologue of these genes have not been found in other microorganisms except that of *Oceanobacillus iheyensis* HTE831 (Fig. 6.6)

6.3.5 Membrane Transport and Energy Generation

B. halodurans C-125 requires Na^+ for growth under alkaline conditions. The presence of sodium ions in the surrounding environment has been proved to be essential for effective solute transport through the cytoplasmic membrane of C-125 cells. According to the chemiosmotic theory, a protonmotive force is generated across the cytoplasmic membrane by the electron transport chain or by extrusion of H^+ derived from ATP metabolism through the action of ATPase. Takami et al. (2000) identified four types of ATPases (preprotein translocase subunit, class III heat-shock ATP-dependent protease, heavy metal-transporting ATPase and cation-transporting ATPase). These ATPases are well conserved between *B. halodurans* and *B. subtilis.*

Through a series of analyses such as a BLAST2 search, clustering analysis by the single linkage method examining all CDSs identified in the *B. halodurans* C-125 and *B. subtilis* genomes (8166 CDSs), and multiple alignment, 18 CDSs were grouped into the category of antiporter- and transporter-related protein genes in the C-125 genome. In this analysis, five CDSs were found to be candidates for Na^+/H^+ antiporter genes (BH1316, BH1319, BH2844, BH2964 and BH3946). However, no gene encoding antibiotic-resistance proteins in the C-125 were found, whereas the *B. subtilis* genome has nine different ones. Eleven genes for multidrug-resistant proteins were identified in the C-125 genome, 6 fewer than in *B. subtilis.* A non-alkaliphilic mutant strain (mutant 38154) derived from *B. halodurans* C-125, which is useful as a host for cloning genes related to alkaliphily has been isolated and characterized (Kudo et al. 1990). A 3.7-kb DNA fragment (pALK fragment) from the parent strain restored the growth of mutant 38154 under alkaline pH conditions. This fragment was found to contain CDS BH1319 which is one of the Na^+/H^+ antiporter genes in *B.*

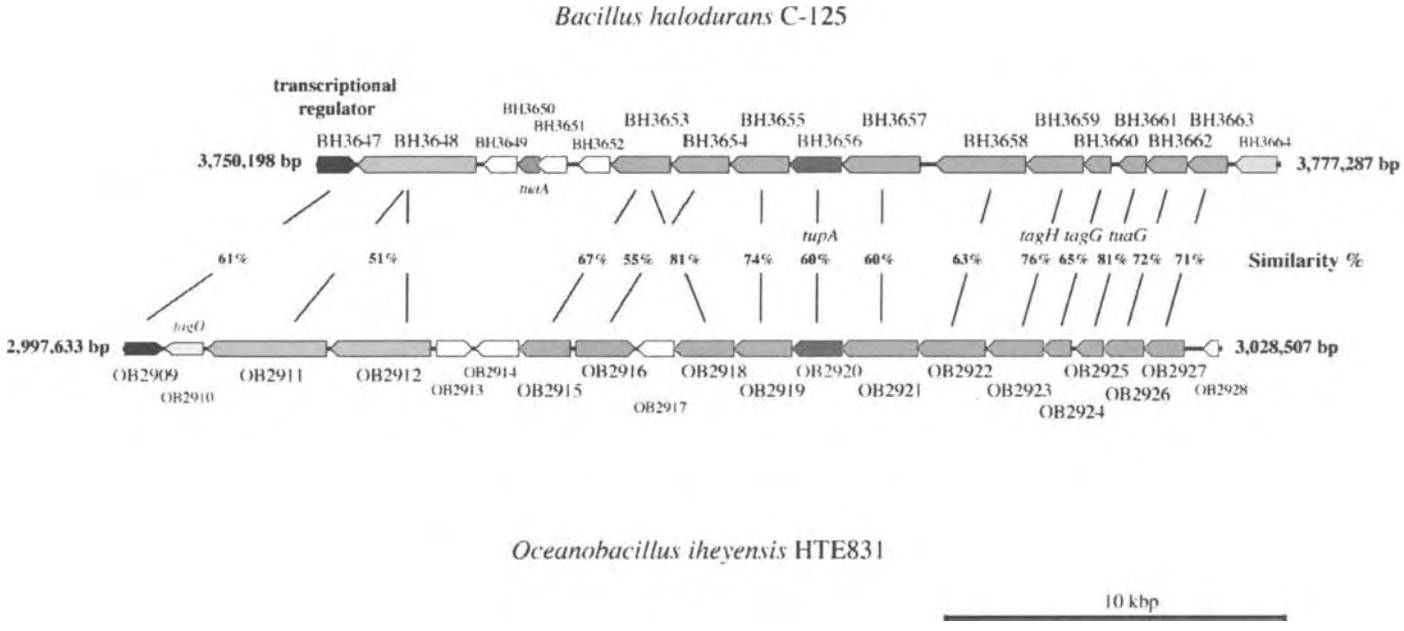


Fig. 6.6 Poly-γ-L-glutamic acid synthesis operons in *Bacillus halodurans* C-125 and *Oceanoacillus iheyensis* HTE831. BH3653, *plg 2*; BH3654, *plg 3* and BH3655, *plg 1*.

halodurans. The transformant was able to maintain an intracellular pH lower than the external pH and the cells expressed an electrogenic Na^+/H^+ antiporter driven only by $\Delta\Psi$ (membrane potential, interior negative) (Horikoshi 1999a). *B. subtilis* has an orthologue (*mprA*) of BH1319 and it has been reported that a *mprA*-deficient mutant of *B. subtilis* showed a sodium-sensitive phenotype (Kosono et al. 2000). On the other hand, a mutant of strain C-125 with a mutation in BH1317 adjacent to BH1319 has been isolated and it showed an alkali-sensitive phenotype, although whether the Na^+/H^+ antiporter encoded by BH1317 is active in this mutant has not yet been confirmed experimentally. In addition, it has been reported that BH2819, the function of which is unknown and which is unique to the C-125 genome, is also related to the alkaliphilic phenotype (Aono et al. 1992c).

B. halodurans C-125 has a respiratory electron transport chain, and the basic gene set for it is conserved as compared with *B. subtilis*, but the gene for cytochrome *bd* oxidase (BH3974 and BH3975) is duplicated in the C-125 genome. It is also clear that two genes for *bo3*-type cytochrome c oxidase (BH739 and BH740) not seen in *B. subtilis* are present in the C-125 genome. The C-125 genome has a F_1F_0-ATP synthase operon. The gene order in this operon (e subunit-b subunit-g subunit-a subunit-d subunit-subunit b-subunit c-subunit a) is identical to that seen in *B. subtilis*. In addition to the F_1F_0-ATP synthase operon, the operon for a Na^+-transporting ATP synthase and the operon for a flagellar-specific ATP synthase are also conserved between *B. halodurans* and *B. subtilis*.

6.3.6 ABC Transporters

Members of the superfamily of adenosine triphosphate (ATP)-binding-cassette (ABC) transport systems couple the hydrolysis of ATP to the translocation of solutes across a biological membrane. As in the case of *B. subtilis*, ABC transporter genes are the most frequent class of protein-coding genes found in the *B. halodurans* genome. They must be extremely important in gram-positive bacteria such as *Bacillus*, because these bacteria have an envelope consisting of a single membrane. ABC transporters allow such bacteria to escape the toxic action of many compounds. Through the series of analyses described above, 75 genes coding for ABC transporter/ATP-binding proteins were identified in the *B. halodurans* genome (Fig. 6.7). In this analysis, 67 CDSs were grouped in the category of ATP-binding protein genes, although 71 ATP-binding protein genes have been identified in the *B. subtilis* genome. They found that *B. halodurans* has eight more oligopeptide ATP-binding proteins, but four fewer amino acid ATP-binding proteins, as compared with *B. subtilis* (Fig. 6.7). No other substantial difference between *B. halodurans* and *B. subtilis* in terms of the other ATP-binding proteins could be found, although it should be noted that the specificity of some of these proteins is not known. The genes for oligopeptide ATP-bind-

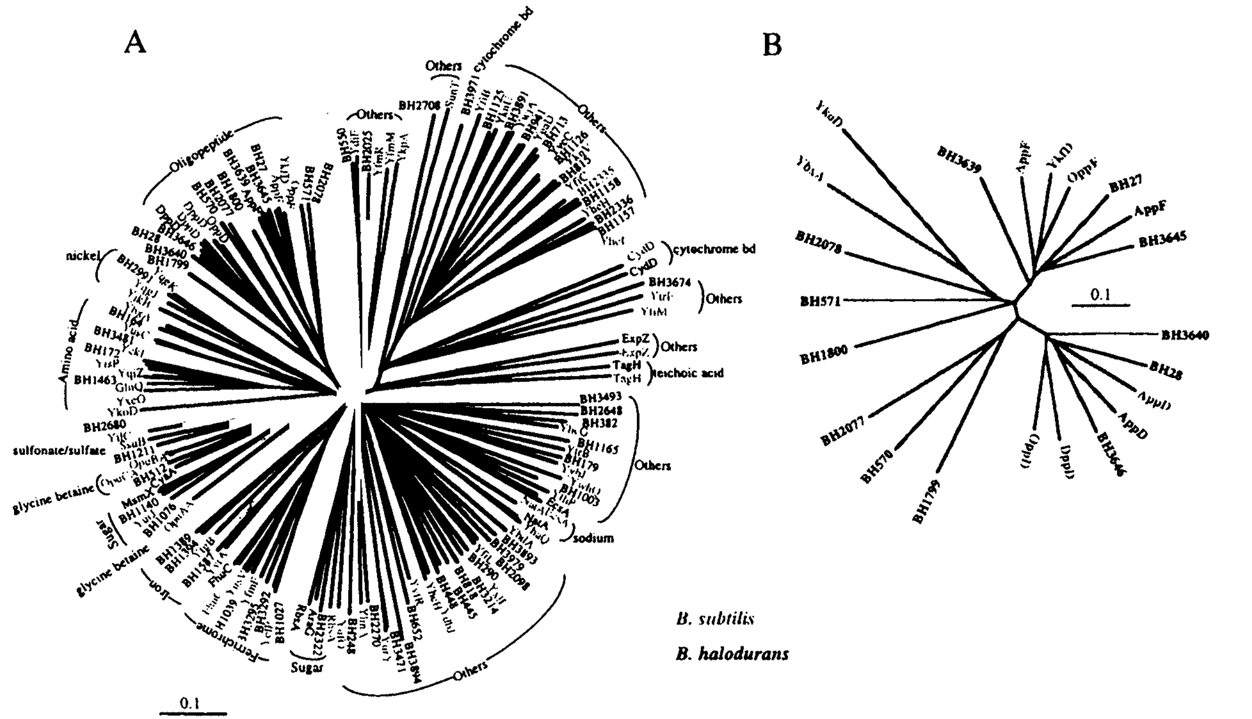


Fig. 6.7 Dendrograms of members of the ABC transporter family of *B. halodurans* and *B. subtilis*. A, ATP-binding protein family; B, oligopeptide ATP-binding protein family. These unrooted dendrograms were generated in the same manner as those in Fig. 6.5. Bar = 0.1 Knuc unit.

ing proteins (BH27, BH28, BH570, BH571, BH1799, BH1800, BH2077, BH2078, BH3639, BH3640, BH3645, BH3646, AppD and AppF) are distributed throughout the C-125 genome (Takami et al. 2000). And, they speculated that these genes may contribute to survival under highly alkaline conditions, although there is no direct evidence to support this. On the other hand, 43 CDSs were identified as ABC transporter/permeases in the *B. halodurans* genome. Surprisingly, *B. halodurans* has only one amino acid permease in contrast with the twelve present in the *B. subtilis* genome. In addition, it is clear that *B. halodurans* lacks the sodium permease gene present in *B. subtilis*, whereas *B. subtilis* lacks the nickel permease gene present in *B. halodurans*.

6.3.7 IS Elements of *B. halodurans* C-125

Takami et al. (2001) conducted work on the identification and distribution of new insertion sequences in the genome of alkaliphilic *B. halodurans* C-125. As shown in Tables 6.2, fifteen kinds of new insertion sequences (ISs), IS641 to IS643, IS650 to IS658, IS660, IS662 and IS663, and a group II intron (Bh.Int) were identified in the 4,202,352-bp genome of alkaliphilic *B. halodurans* C-125. Of 120 ISs identified in the C-125 genome, 29 were truncated, indicating the occurrence of internal rearrangements of the genome. The ISs other than IS650, IS653, IS660 and IS663 generated a 2- to 9-bp duplication of the target site sequence, and the ISs other than IS650, IS653 and IS657 carry 14 - to 64 -bp inverted repeats. Sequence analysis revealed that six kinds of ISs (IS642, IS643, IS654, IS655, IS657, and IS658) belong to a separate IS family (IS630, IS21, IS256, IS3, IS200/IS605, and IS30, respectively) as new members. Also, IS651 and IS652 were also characterized as new members of the ISL3 family. Significant similarity was found between the transposase (Tpase) sequences between IS650 and IS653 (78.2%), IS651 and IS652 (56.3%), IS656 and IS662 (71.0%), and IS660 and IS663 (44.5%), but the others showed no similarity to one another. Tpases in 28 members of IS651 in the C-125 genome were found to have become diversified. Most of the IS elements widely distributed throughout the genome were inserted in noncoding regions, although some genes, such as those coding for an ATP-binding cassette transporter/permease, a response regulator and L-indole 2-dehydrogenase, have mutated through the insertion of IS elements. It is evident, however, that not all IS elements have transposed and caused rearrangements of the genome in the past 17 years during which strain C-125 was subcultured under neutral and alkaline conditions. Takami et al. (2004) reported that insertion sequences identified in *B. halodurans* were widely distributed among bacilli and that a new transposon was disseminated in alkaliphilic bacilli, as shown in the following section.

6.3.8 Was the *B. halodurans* Genome Changed by Hundreds Transfers on Slants over 17 Years?

B. halodurans C-125 was isolated from a soil sample using an alkaline culture medium in 1970 (Horikoshi 1999a) and kept as spores on a plate until 1977. This strain was characterized as a β-galactosidase producer and identified as a member of the genus *Bacillus* in 1977 (Ikura et al. 1978), after being subcultured several times for experiments (Ikura et al. 1979). Thereafter, the strain was kept as spores on a plate again until 1983. In the autumn of 1983, the strain was used in a study involving screening for xylanase production and it was then lyophilized in an ampule and stored for the purposes of obtaining a patent for alkaline xylanase on November 13, 1983 (Honda et al. 1985a, b, c). Thereafter, over the next two decades, it was often used in various experiments as an enzyme producer and as a standard strain for studies on the mechanisms of adaptation to alkaline environments. Now the author's colleagues are very intrigued by the question of what kind of changes mediated by IS elements occurred in the genome comparing the current strain (C-125-00) and the strain lyophilized in 1983 (C-125-83), because strain C-125 has been transferred alternatively under alkaline and neutral conditions during the past two decades. Therefore, they examined the pattern of amplification of IS elements from the genome, comparing the two strains C-125-83 and C-125-00 by PCR using the primer sets. All IS elements identified were amplified from the C-125-83 genome and the C-125-00 genome (Fig. 6.8), showing exactly the same amplification pattern in both cases, suggesting that no IS element except for indigenous ones in the C-125-83 genome had transposed in the genome over the past 17 years.

Takami et al. designed appropriate site-specific primer sets for IS elements localized in each position in the genome and compared each PCR fragment from the genomes of the two strains (C-125-83 and C-125-00) to investigate what kind of internal rearrangement in the genome had occurred through the action of IS elements. In addition, the patterns of digestion of chromosomal DNA from these two strains with various restriction endonucleases were also compared to check whether any changes had occurred in the genome during the 17-year period of subculture. All IS elements located in non-coding regions of the genome were amplified by PCR with exactly the same pattern between strains C-125-83 and C-125-00 for comparison of the DNA fragments amplified from the two strains using primers specific for each of eleven IS element members (IS*641*-03, IS*643*-01, IS*651*-10, IS*652*-02, IS*653*-01, IS*654*-03, IS*655*-04, IS*656*-02, IS*657*-05, IS*660*-05 and Bh.Int-03). Also, the amplification patterns of twelve IS elements inserted in CDSs (IS*652*-14, IS*655*-03, IS*655*-05, IS*656*-01, IS*656*-03, IS*651*-08, IS*651*-17, IS*653*-05, IS*657*-01, IS*658*-01, IS*658*-03 and IS*658*-04) were the same between strains C-125-83 and C-125-00. Furthermore, there was no difference between the two strains in terms of the pattern of digestion of

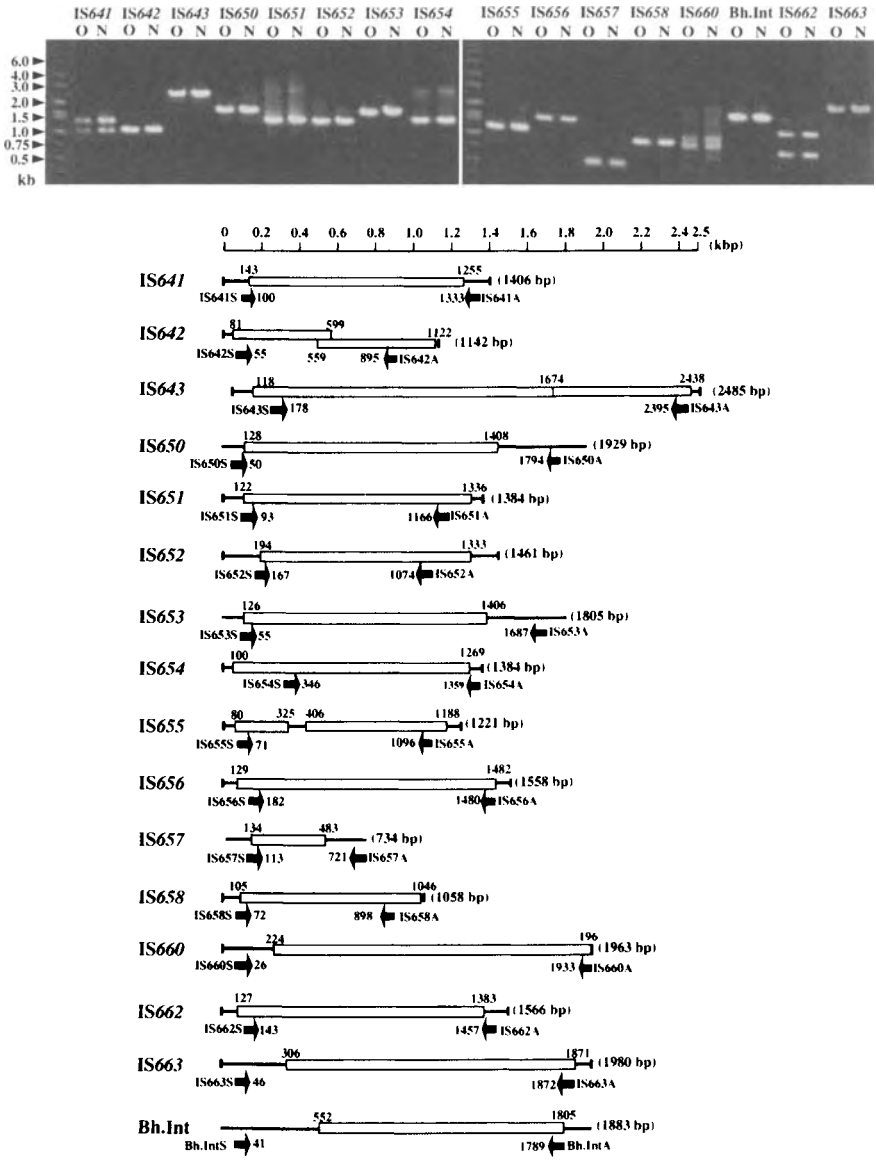


Fig. 6.8 Comparison of the IS elements amplified by PCR from the chromosomes of the strains C-125-83 and C-125-00. The primer sets used are shown by short solid arrows. O, strain C-125-83; N, strain C-125-00.

chromosomal DNA comparing fragments in the high molecular size range from 48.5 to 533.5 kb (*Asc*I and I-*Ceu*I) or comparing fragments in the low molecular size range from 9.42 to 97 kb (*Pac*I, *Sma*I, *Bss*HII, and *Swa*I) (Fig. 6.9). These results demonstrate that insertion of IS elements into CDSs and non-coding regions in the genome occurred at least before 1983 and pre-

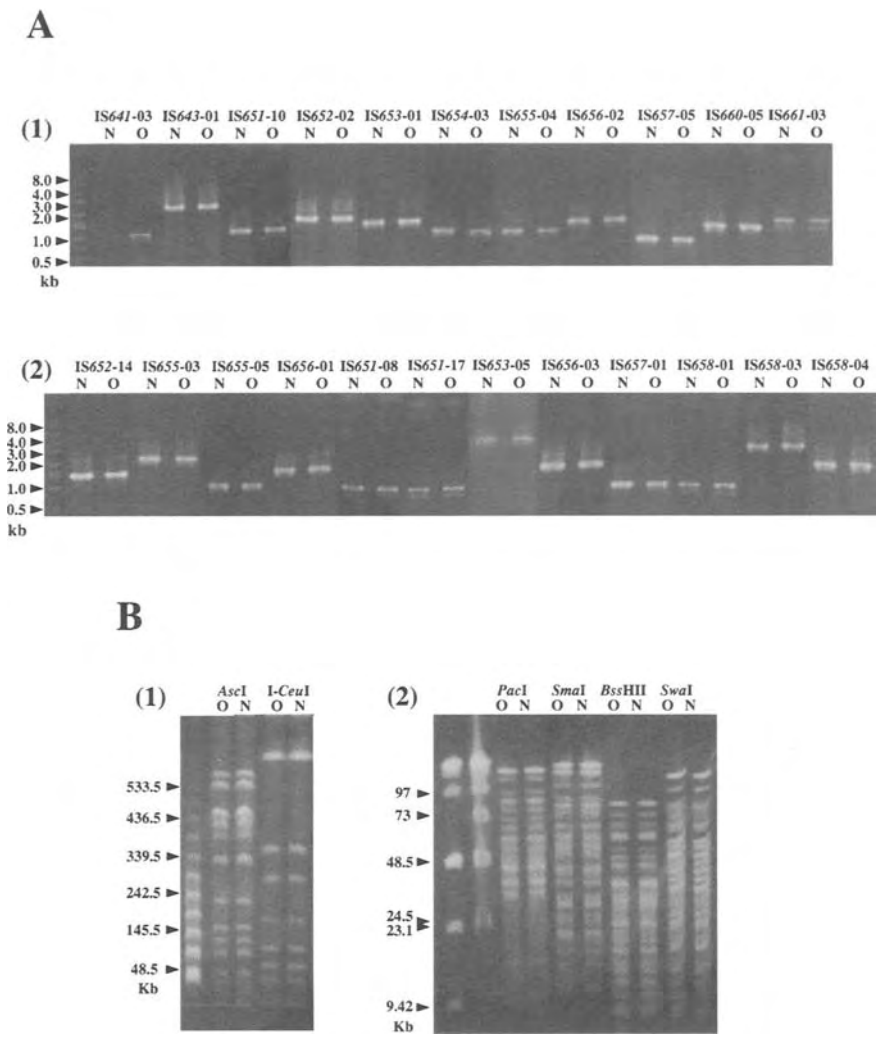


Fig. 6.9 Analysis of the change in the C-125 genome by subculture for 17 years. A: Amplification pattern of the DNA fragments by PCR with the following site specific primer sets around each IS element in the genome. (1) IS elements inserted in noncoding region. (2) IS elements inserted in CDSs. O: strain C-125-83. N: strain C-125-00. B: Digestion pattern of the genomes with various restriction endonucleases. (1) High range separation (48.5–533.5 kb), (2) Low range separation (9.42–97 kb). O: strain C-125-83. N: strain C-125-00.

sumably before 1970 when *B. halodurans* C-125 was isolated because this strain had been kept as spores on a plate as mentioned above.

The *B. halodurans* genome contains 120 IS elements and 91 of them still in the intact form seem to have the potential to transpose themselves into the genome of their host or the genome of another strain. However, there

is no sign of transposition of IS in the genome of strain C-125 during the past 17-year period of subculture in the laboratory with the cells grown in Horikoshi-II medium under neutral or alkaline conditions. This indicates that the genome of *Bacillus halodurans* C-125 is quite stable. Therefore, it is of interest to determine when the IS elements jump in the genome and what triggers their transposition. As noted above, the author's group had a specific interest in how the behavior of ISs and internal rearrangement in the genome affects enzyme productivity and the stability of enzyme production, especially when systematic breeding of the strain is attempted for industrial applications. As the first step to answer the above questions, they are now looking for the trigger of transposition of IS elements.

6.3.9 Other Genes Found in the Genome of *B. halodurans* C-125

A. Oxygen Sensor

Hou et al. (2001) isolated a globin-coupled oxygen sensor. They discovered heme-containing signal transducers from the archaeon *Halobacterium salinarum* (HemAT-Hs) and the gram-positive bacterium *B. subtilis* (HemAT-Bs). These proteins bind diatomic oxygen and trigger aerotactic responses. It was identified that HemAT oxygen-sensing domains contain a globin-coupled sensor (GCS) motif, which exists as a two-domain transducer, having no similarity to the PAS domain (period circadian protein, Ah receptor nuclear translocator protein, single-minded protein) superfamily transducers. Using the GCS motif, Hou et al. predicted that a 439-amino-acid protein annotated as a methyl-accepting chemotaxis protein (MCP) in the facultatively alkaliphilic bacterium *B. halodurans* C-125 is a globin-coupled oxygen sensor. Hou et al. cloned, expressed, and purified GCS(Bh) and performed spectral analysis. GCS(Bh) binds heme and shows myoglobin-like spectra (Fig. 6.10 and 6.11). This suggests that GCS(Bh) acts as an oxygen sensor and transmits a conformational signal through a linked signaling domain to trigger an aerotactic response in *B. halodurans* C-125.

B. Voltage-gated Ion Channels

Alkaliphilic microorganisms have a sophisticated pH-homeostatic mechanism. Inorganic ion transport is one of the most important candidate to solve this topic of scientific interest.

The first discovery of a prokaryote voltage-gated Ca^{2+} channel with only one 6TM motif per subunit was done by Durell and Guy (2001). Until their work, voltage-gated Ca^{2+} channel proteins have been found only in eukaryotes. A gene recently discovered in the eubacterium *B. halodurans* C-125 codes (BH1501) for a protein closely related to eukaryotic Ca^{2+} channels, but has only one six-transmembrane-segment (6TM) motif, instead of four, in its pore-forming subunit. This is supported by the comparison of consen-

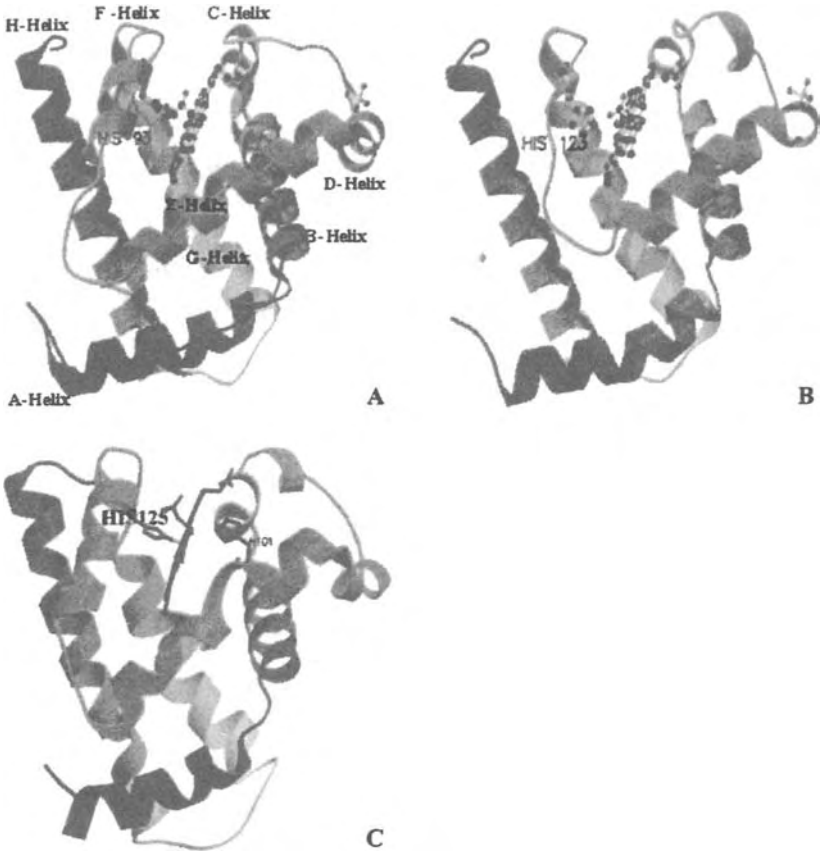


Fig. 6.10 Homology models of the heme-binding domains of GCS_{Bh} and HemAT-*Bs* compared to the known structure of sperm-whale myoglobin (SWMb). The homology models are of putative heme-binding domains of GCS_{Bh180}(C) and $HemAT\text{-}Bs_{176}$(B), and the crystal structure is of the heme-binding domain of SWMb(A).
(Reproduced with permission from S. B. Hou et al., *Extremophiles*, **5**, 351(2001))

sus sequences, which, along with the patterns of residue conservation, indicates a similar structure in the membrane to voltage-gated K^+ channels. From this result, they hypothesized that Ca^{2+} channels originally evolved in bacteria, and that the specific eubacteria protein highlighted here is an ideal candidate for structure determination efforts.

Then, Ren et al. (2001) reported a prokaryotic voltage-gated sodium channel. The pore-forming subunits of canonical voltage-gated sodium and calcium channels are encoded by four repeated domains of six-transmembrane (6TM) segments. They expressed and characterized a bacterial ion channel (NaChBac) from *B. halodurans* C-125 that is encoded by one 6TM segment. The sequence, especially in the pore region, is similar to that of voltage-gated calcium channels. The expressed channel was activated by

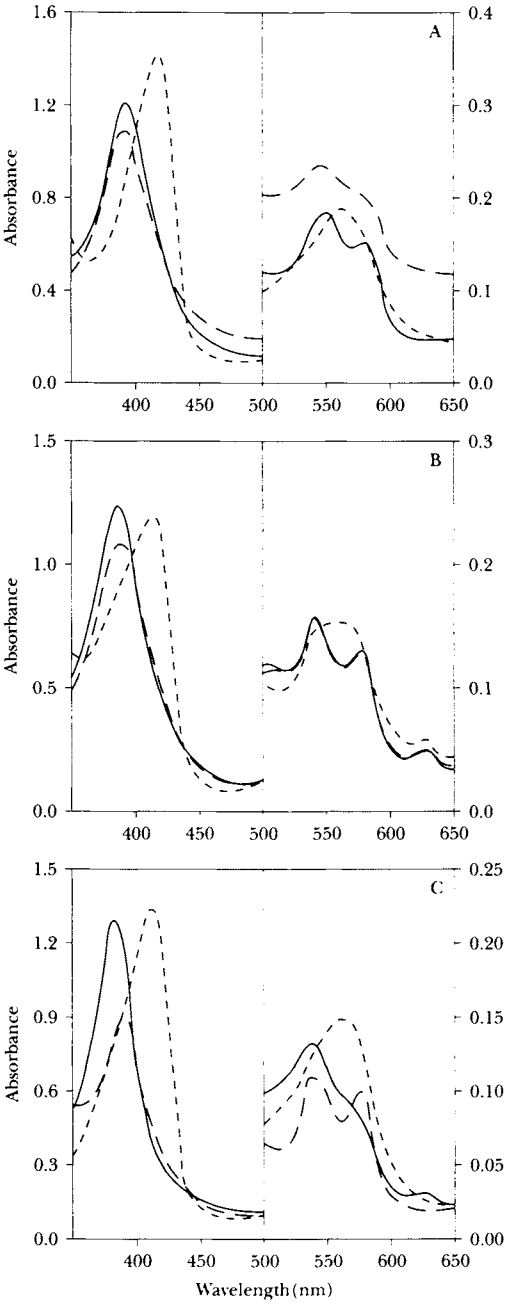


Fig. 6.11 Absorption spectra of oxygenated (*solid lines*), deoxygenated (*dashed lines*) and re-oxygenated (*dot-dash lines*) truncated His-tagged GCS_{Bh} for A GCS_{Bh180}; B GCS_{Bh190}; and C GCS_{Bh200}. Spectra were measured in 200 mM NaCl, 50 mM Na_2HPO_4, pH 8.0. Deoxygenated samples were prepared by the addition of sodium dithionite to the protein solutions.
(Reproduced with permission from S. B. Hou et al., *Extremophiles*, **5**, 351(2001))

voltage and blocked by calcium channel blockers. However, the channel was selective for sodium. The identification of NaChBac as a functionally expressed bacterial voltage-sensitive ion-selective channel provides insight into both voltage-dependent activation and divalent cation selectivity. Following the above paper, Yue et al. (2002) published a report on the cation selectivity filter of the bacterial sodium channel, NaChBac, because *B. halodurans* C-125 voltage-gated sodium-selective channel (NaChBac) is an ideal candidate for high resolution structural studies. It can be expressed in mammalian cells and its functional properties studied in detail. It has the added advantage of being a single six-transmembrane (6TM) orthologue of a single repeat of mammalian voltage-gated Ca^{2+} (Ca(V)) and Na^{+} (Na(V)) channels. It was found that six amino acids in the pore domain (LESWAS) participate in the selectivity filter. Replacing the amino acid residues adjacent to glutamatic acid (E) by a negatively charged aspartate (D; LEDWAS) converted the Na^{+}-selective NaChBac to a Ca^{2+}- and Na^{+}-permeant channel. When additional aspartates were incorporated (LDDWAD), the mutant channel resulted in a highly expressing voltage-gated Ca^{2+}-selective conductance.

Recently, Chahine et al. (2004) reported the role of argine residues on the S4 segment of *B. halodurans* C-125 Na^{+} channel in voltage-sensing. The one-domain voltage-gated sodium channel of *B. halodurans* (NaChBac) is composed of six transmembrane segments (S1-S6) comprising a pore-forming region flanked by segments S5 and S6 and a voltage-sensing element composed of segment S4. To investigate the role of the S4 segment in NaChBac channel activation, the cysteine mutagenesis approach was used where the positive charges of single and multiple arginine (R) residues of the S4 segment were replaced by the neutrally charged amino acid cysteine (C). To determine whether it was the arginine residue itself or its positive charge that was involved in channel activation, arginine to lysine (R to K) mutations were constructed. Wild-type (WT) and mutant NaChBac channels were expressed in tsA201 cells and Na^{+} currents were recorded using the whole-cell configuration of the patch-clamp technique. The current/voltage (*I-V*) and conductance/voltage (*G-V*) relationships steady-state inactivation (h_{∞}) and recovery from inactivation were evaluated to determine the effects of the S4 mutations on the biophysical properties of the NaChBac channel. R to C on the S4 segment resulted in a slowing of both activation and inactivation kinetics. Charge neutralization of arginine residues mostly resulted in a shift toward more positive potentials of *G-V* and (h_{∞}) curves. The *G-V* curve shifts were associated with a decrease in slope, a fact which may reflect a decrease in the gating charge involved in channel activation. Single neutralization of R114, R117, or R120 by C resulted in a very slow recovery from inactivation. Double neutralization of R111 and R129 confirmed the role of R111 in activation and suggested that R129 is most probably not part of the voltage sensor. Most of the R to K mutants retained WT-like current kinetics but exhibited an intermediate *G-*

V curve, a steady-state inactivation shifted to more hyperpolarized potentials, and intermediate time constants of recovery from inactivation. This indicates that R, at several positions, plays an important role in channel activation. The data are consistent with the notion that the S4 is most probably the voltage sensor of the NaChBac channel and that both positive charges and the nature of the arginine residues are essential for channel activation.

C. Enzyme Genes

Many enzymes have been expressed and characterized from the author's annotation data. Nishimoto et al. (2002b) made chimeric xylanases by using *XynA* of *B. halodurans* C-125 and XylB of *Clostriudium stercorarium* F9 and purified. The respective pH and temperature stabilities of the purified enzymes were observed from pH 5.6 to 11.6 and up to 45°C. These enzymes were slightly less stable than the parental xylanases. Then, Nishimoto et al. studied on pH-activity relationship of XylA using aryl-xylobiosides.

In 2004, Zhuang et al. (2004) expressed the BH1999, and the product was identified as gentisyl-coenzyme A thioesterase. Gentisyl-CoA thioesterase shares the backbone fold and the use of an active site aspartate residue to mediate catalysis with the 4-hydroxybenzoyl-CoA thioesterase of the hotdog fold enzyme superfamily. A comparative study of these two enzymes showed that they differ greatly in the rate contribution made by the catalytic aspartate, in the pH dependence of catalysis and in substrate specificity (Fig. 6.12).

Fujita et al. (2004) characterized an endo-β-*N*-acetylglucosaminidase from alkaliphilic *B. halodurans* C-125. The genome sequencing project on

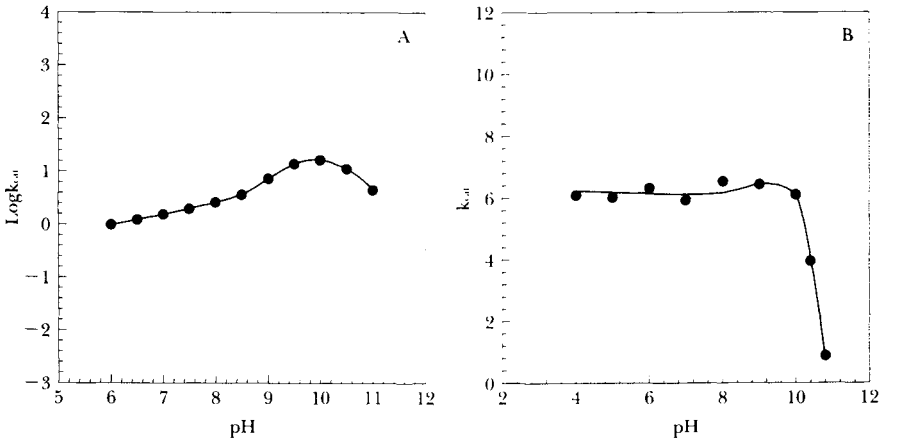


Fig. 6.12 (A) Kinetic pH rate profile: log k_{cat} (per second) versus pH rate for wild-type *B. halodurans* gentisyl-CoA thioesterase measured at 25°C. For details see text. (B) The stability pH profile: k_{cat} (per second) of wild-type *B. halodurans* gentisyl-CoA thioesterase incubated for 2 min in the buffers used in A and then assayed at 25°C in 50 mM CAPSO and CAPS (pH 10) −0.2 M KCl buffer.
(Reproduced with permission from Z. Zhuang et al., *J. Bacteriol.*, **186**, 393(2004))

B. halodurans C-125 revealed a putative endo-β-*N*-acetylglucosaminidase (Endo-BH), which consists of a signal peptide of 24 amino acids, a catalytic region of 634 amino acids exhibiting 50.1% identity with the endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A), and a C-terminal tail of 220 amino acids. Transformed *E. coli* cells carrying the Endo-BH gene exhibited endo-β-*N*-acetylglucosaminidase activity. Recombinant Endo-BH hydrolyzed high-mannose type oligosaccharides and hybrid type oligosaccharides and showed transglycosylation activity. On deletion of 219 C-terminal amino acid residues of Endo-BH, the wild type level of activity was retained, whereas with deletions of the Endo-A homologue domain, the proteins were expressed as inclusion bodies and these activities were reduced. These results suggest that the enzymatic properties of Endo-BH are similar to those of Endo-A, and that the C-terminal tail does not affect the enzyme activity. Although the C-terminal tail region is not essential for enzyme activity, the sequence is also conserved among endo-β-*N*-acetylglucosaminidases of various origins.

Ruijssenaars and Hartmans (2004) cloned a multicopper oxidase exhibiting alkaline laccase activity. The gene product of open reading frame BH2082 from *Bacillus halodurans* C-125 was identified as a multicopper oxidase with potential laccase activity. A homologue of this gene, lbh1, was obtained from a *B. halodurans* C-125 isolate from the author's culture collection. The encoded gene product was expressed in *E. coli* and showed laccase-like activity, oxidizing 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), 2,6-dimethoxyphenol and syringaldazine (SGZ). The pH optimum of Lbh1 with SGZ is 7.5–8 (at 45°C) and the laccase activity is stimulated rather than inhibited by chloride. These unusual properties make Lbh1 an interesting biocatalyst in applications for which classical laccases are unsuited, such as biobleaching of kraft pulp for paper production.

6.4 Whole Genome Sequence of *Bacillus clausii* KSM-K16

Alkaline protease M-protease (see Section 7.4) producer *B. clausii* KSM-16 was isolated by Hakamada(Hakamada et al. 1994) of Kao Corporation and industrialized. Quite recently whole genome sequence was completed and deposited as accession number AP006627 in GenBank. Total Bases are 4303871 bp, further details, however, have not yet been published. According to author's search, no poly-γ-L-glutamic acid synthesis genes discovered in the genomes of *B. halodurans* C-125 and *Ob. Iheyensis* HTE831 were detected.

6.5 *Oceanobacillus iheyensis* HTE831

In order to understand adaptation mechanisms under alkaline and high salt

conditions, Takami et al. (1999a) isolated a new strain HTE831 from deep-sea mud collected at a depth of 1050 m on the Iheya Ridge, Japan. This was an extremely halotolerant and facultatively alkaliphilic bacterium. They proposed that the new genus, *Oceanobacillus* should be created for the deep-sea isolate HTE831T and described a new species within the genus *Oceanobacillus* called *Ob. iheyensis* on the basis of the following results.

Description of *Oceanobacillus* (Lu et al. 2001) gen. nov.

Oceanobacillus (o.ce.a.no.ba.cillus. L. n. *oceanus*, the ocean; L. dim. n. *bacillus*, a small rod; M.L. masc. *Oceanobacillus*, the ocean bacillus/rod).Gram-positive, motile by peritrichous flagella, spore-forming rods. Endospores are subterminal or terminal and slightly swell the sporangia. Obligately aerobic, facultatively alkaliphilic and extremely halotolerant. Cells are 0.6–0.8 μ × 2.5–3.5 μ. Endospores are ellipsoid. Colonies are creamy white. Grows at 0–21% (w/v) NaCl, with optimum growth at 3% NaCl. The pH range for growth is 6.5–10 (optimum 7.0–9.5). Grows on glucose, mannose, maltose and turanose. Hydrolyzes gelatin, casein, Tween 40 and Tween 60. Does not hydrolyze starch. The following four substrates are assimilated: glucose, maltose, mannose and turanose. Acid production occurred from glucose, mannose, glycerol, fructose, and maltose, but not from xylose, rhamnose, glucitol, trehalose, galactose, lactose and melibiose. Catalase-positive, oxidase-variable, DNase-negative and urease-negative. VP reaction, indol and H_2S production, and use of citrate are negative. Aminopeptidase and KOH tests are negative. Nitrate reduction to nitrite is negative. Cells are resistant to erythromycin, nalidixic acid and spectinomycin but susceptible to ampicillin, gentamycin, kanamycin, tetracycline, bacitracin, carbenicillin, chloramphenicol, novobiocin, penicillin G and rifampicin. The genome size is about 3.6 Mb. The G+C content of the type species is 35.8%. 16S rDNA sequence exhibits 90.5–94.4% homology with those from *B. halodenitrificans* and the members of the genera *Halobacillus*, *Gracilibacillus*, *Virgibacillus*, and *Salibacillus*. Based on 16S rRNA gene sequencing analysis, the genus *Oceanobacillus* does not form a branch with other genera such as *Halobacillus*, *Gracilibacillus*, *Virgibacillus* and *Salibacillus*. The major cellular fatty acids are anteiso-15:0, iso-15:0 and iso-14:0. The main quinone is menaquinone.

6.5.1 Estimation of Genome Size of *Ob. iheyensis* HTE831 Chromosome

Chromosomal DNA of HTE831 for PFGE was prepared in agarose plugs by the method previously described (Takami et al. 1999a). Agarose blocks containing the chromosomal DNA were washed twice in 50 ml of 0.1 TE-buffer then equilibrated with the corresponding restriction buffer at 4°C for 1 h. DNA was digested with 100–200 units of *Apa*I or *Sse*8387I (Takara Shuzo, Otsu, Japan) at 37°C overnight in 500 μl of the restriction buffer recommended by the manufacturer. PFGE in 1% PFC agarose was performed

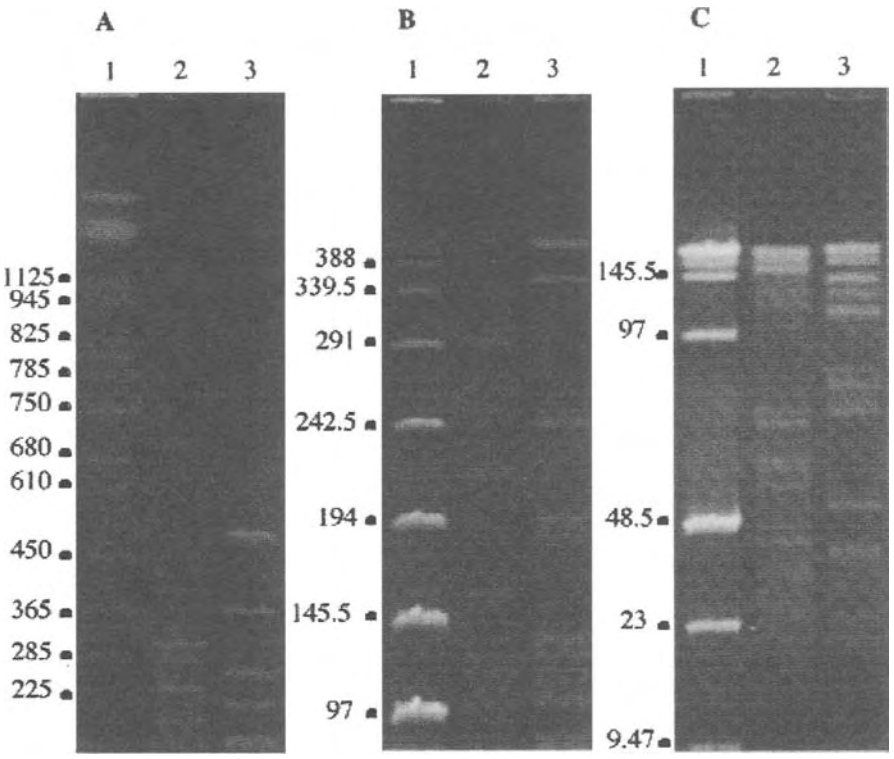


Fig. 6.13 Digestion patterns of the chromosomal DNA of strain HTE831 obtained with *Sse* 83871 and *Apa*I. A: Separation of fragments ranging in size from 200 to 1000 kb. B: Separation of fragments ranging in size from 100 to 400 kb. C: Separation of fragments ranging in size from 5 to 75 kb. Lanes: 1, Molecular size marker; 2, complete *Apa*I digestion; 3, complete *Sse* 83871 digestion.
(Reproduced with permission from J. Lu et al., *FEMS Microbiol. Lett.*, **205**, 291(2001))

by the method previously described (Takami et al. 1999c; Nakasone et al. 2000). Restriction endonucleases that recognize an 8-bp sequence were tested for their ability to digest the chromosome of strain HTE831. *Apa*I (5'-GGGCC/C-3') and *Sse* 8387I (5'-CCTGCA/GG-3') generated 37 and 25 resolvable fragments, respectively (Fig. 6.13). The sizes of these fragments were determined by comparison with size standards on a series of PFGE gels. The mean total size of the genome of HTE831, estimated by totaling the *Apa*I or *Sse* 8387I fragments, was 3.6 Mb (Lu et al. 2001).

6.5.2 Shotgun Sequencing of the Whole Genome of *Ob. iheyensis*

The genome of *Ob. iheyensis* HTE831 was sequenced by the whole genome

random sequencing method which was essentially the same as the method described above (Takami et al. 2000). DNA sequences determined by means of MegaBace1000 (Amersham Biosciences Inc., NJ, USA) and ABI PRISM377 DNA sequencer (Perkin Elmer, CT., USA) were assembled into contigs using Phrap with default parameters. At a statistical coverage of 6- to 7-fold, the assembly using Phrap yielded several hundred contigs. Two thousand sequences were obtained from the reverse end of shotgun clones and both ends of genomic libraries ranging from 4–6 kb. These sequences were assembled with consensus sequences derived from the contigs of random-phase sequences using Phrap. Then the contigs were reduced to about 100 by this step. In addition, 1000 to 2000 sequences from both ends of the 20-kb insert in l phage clones were also assembled to bridge the remaining contigs. Gaps between contigs were closed by shotgun sequencing of large fragments, bridging the contigs of random-phase sequences. The final gaps were closed by direct sequencing of the products amplified by long accurate PCR as described previously (Takami et al. 2000). In the case of the *Ob. iheyensis* genome, the assembly using Phrap yielded 330 contigs at a statistical coverage of 5.9-fold. Two thousand sequences were obtained from the reverse end of shotgun clones and both ends of genomic libraries ranging from 4–6 kb.

6.5.3 General Features of the *Ob. iheyensis* Genome

The genome of *Ob. iheyensis* is a single circular chromosome (Takami et al. 2002) consisting of 3 630 528 bp with an average G+C content of 35.7% (Table 6.2). The G+C content of DNA in the coding region and non-coding region is 36.1 and 31.8%, respectively. Based on analysis of the G+Cratio and G−C skew (G−C/G+C), it was estimated that the site of termination of replication (*terC*) is nearly 1.77–1.78 Mb (176°) from the replication origin. They identified 3496 CDSs, on average 883 nt in size, using a coding region analysis program. Coding sequences cover 85% of the chromosome. It was found that 79.5% of the genes started with ATG, 7.8% with GTG and 12.7% with TTG. These values are quite similar to those of *B. subtilis* and *B. halodurans*, whose whole genomic sequences have been completely defined previously. The average size of the predicted proteins in *Ob. iheyensis* is 32.804 kDa, ranging from 2.714 to 268.876 kDa. Predicted protein sequences were compared with sequences in nonredundant protein databases and putative biological roles were assigned to 1972 (56.4%) of them. In this database search, 1069 proteins deduced from CDSs (30.6%) were identified as conserved proteins of unknown function in comparison with proteins from other organisms, and for 456 (13%) there was no database match. Sixty-nine tRNA species, organized into 10 clusters involving 63 genes plus 6 single genes, were identified. Furthermore, poly-γ-L-glutamic acid synthesis genes (*plg* 1–3) was found in the genome that may be responsible for alkaliphily (Fig. 6.6). Of the 10 tRNA clusters, 5 were organized in

association with rRNA operons. Seven rRNA operons are present in the HTE831 genome and in two cases their organization is the same as that of *B. subtilis* and *B. halodurans* (16S-23-S-5S, 16S-23S-5S-tRNA and tRNA-16S-23S-5S), but another gene order is also present (16S-tRNA-23S-5S-tRNA).

The *Ob. iheyensis* genome possesses 21 genes encoding putative transposases or recombinases with similarity to sequences present in the genomes of *B. halodurans*, *Marinococcus halophilus* or *Enterococcus faecium*. However, the variety and number of these are much less than the 112 that were categorized into 27 groups found in the *B. halodurans* genome. In a series of analyses of the genes encoding transposases or recombinases, Takami et al. (2002) defined a gene divided into several pieces as one gene because the transposable elements are often mutagenized by deletions, insertions and substitutions of nucleotides. Fourteen of those genes showed significant similarity to transposases of IS elements present in *B. halodurans*, categorized into the ISL3, IS200/IS605, IS30 and IS1272/IS660 families, and five were also similar to recombinases of the group II intron found in *B. halodurans* (Table 6.2). Although these putative genes encoding transposases and recombinases present in the *Ob. iheyensis* genome were not identified in *B. subtilis* 168, the transposase genes of IS elements belonging to the IS200/IS605 and IS1272/IS660 families and, especially, the recombinase gene of the group II intron were widespread among other species of *Bacillus* and related genera as determined by Southern blot hybridization. The genome also contains 27 putative phage-associated genes, which are similar to those of *B. subtilis*, *Staphylococcus aureus*, *Lactococcus lactis*, *Clostridium acetobutylicum* and *Streptococcus species*, although the *Ob. iheyensis* genome contains no intact prophage such as Spb, PBSX or skin found in the genome. The sequence has been deposited in DDBJ/EMBL/GenBank with the accession numbers AP004593-AP004605.

6.5.4 IS Elements Found in *Ob. Iheyensis*

During whole genome analysis of *Ob. Iheyensis* HTE831, Takaki et al. (2004) found new insertion sequences in the genome of the extremely halotolerant and alkaliphilic *Ob. iheyensis* HTE831.

Six kinds of new insertion sequences (ISs), IS667 to IS672, a group 11 intron (Oi.Int), and an incomplete transposon (Tn8521oi) were identified in the 3,630,528-bp genome of the extremely halotolerant and alkaliphilic *Ob. iheyensis* HTE831. Of 19 ISs identified in the HTE831 genome, seven were truncated, indicating the occurrence of internal rearrangement of the genome. All ISs except IS669 generated a 4- to 8-bp duplication of the target site sequence, and these ISs carried 23- to 28-bp inverted repeats (IRs). Sequence analysis revealed that four ISs (IS669, IS670, IS671 and IS672) were newly identified as belonging to separate IS families (IS200/IS605, IS30, IS5 and IS3, respectively). IS667 and IS668 were also characterized as new members of the ISL3 family. Tn8521oi, which belongs to the Tn3 fami-

ly as a new member, generated a 5-bp duplication of the target site sequence and carried complete 38-bp IRs. Of the eight protein-coding sequences (CDSs) identified in Tn8521oi, three CDSs (OB481, OB482 and OB483) formed a ger gene cluster, and two other paralogous gene clusters were found in the HTE831 genome. Most of the ISs and the group II intron widely distributed throughout the genome were inserted in noncoding regions, while two ISs (IS667-08 and IS668-02) and Oi.Int-04 were inserted in the coding regions.

6.6 Future of Whole Genomes of Two Alkaliphiles

Alkaliphilic *B. halodurans* C-125 is the second *Bacillus* species whose whole genomic sequence has been completely defined. The whole genomic sequence of *Oceanobacillus iheyensis* HTE831 has just been determined as a second alkaliphilic *Bacillus*-related species. The genomic sequences of the two bacilli should facilitate the study of model transport systems including alkaliphily of alkaliphilic bacteria. Comparative genomics among several species showing different adaptable capability in species of the genus *Bacillus* and other closely related genera will provide a better understanding of the features of the ancestral *Bacillus* species prior to its diversification. The genome sequence may also provide important clues to understanding the mechanisms of adaptation to extreme environments and stress response in the different levels through analyses for metabolic and regulatory networks.

Part II

Enzymes of Alkaliphiles and Their Applications

Many microbiologists and enzymologists have spent time and effort to find alkaline amylases and alkaline cellulases in traditional ways. These alkaline enzymes, however, could not be isolated from conventional neutrophiles.

The addition of 1% sodium carbonate to culture medium provided a success means for isolating alkaline enzymes from alkaliphiles. Such a simple cultivation became the trigger for the production of new enzymes. Numerous scientific papers are now being published on alkaline enzymes, including enzyme genes.

Such new enzymes have paved the way to new applications in many fields. Cyclodextrin production, laundry detergents containing cellulases and/or protease, biological bleaching of craft pulp by xylanases are some typical industrial applications.

The amino acid sequences and DNA sequences have been determined for many alkaline enzymes. It became possible to produce some enzymes produced in industrial scale plants after genetic modifications. Many microbiologists are seriously studying the type of structure of enzymes that can provide alkaliphily. Of course, it is not possible to produce sufficient amounts of alkaliphilic enzymes without the help of microorganisms.

Experience has taught the author the following:

Microorganisms can do anything you want. You just have to figure out how.

7

Alkaline Proteases

Studies of alkaliphiles have led to the discovery of many types of enzymes that exhibit interesting properties. The first report concerning an alkaline enzyme was published by the present author in 1971 and described an alkaline protease produced by *Bacillus clausii* 221. Since that time hundreds of new enzymes have been isolated and purified in many laboratories. Some of these have been produced on an industrial scale and commercialized.

7.1 Early Works on Alkaline Proteases before 1994

In 1971, Horikoshi (1971a) reported the production of an extracellular alkaline serine protease from alkaliphilic *Bacillus clausii* 221. This strain, isolated from soil, produced large amounts of alkaline protease that differed from the subtilisin group. As shown in Fig. 7.1, the optimum pH of the purified enzyme was 11.5 with 75% of the activity maintained at pH 13.0. The

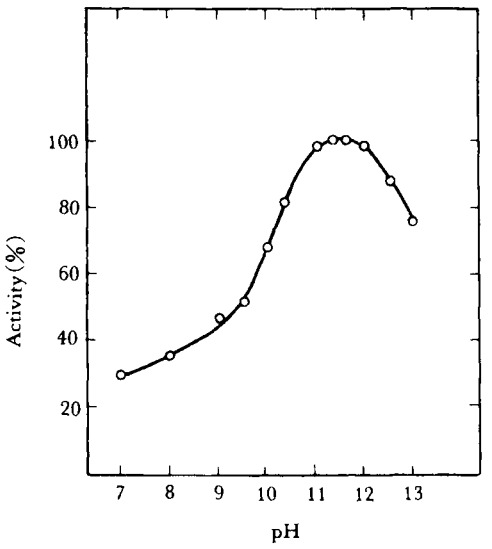


Fig. 7.1 Effect of pH on enzyme activity.
(Reproduced from K. Horikoshi, *Agric. Biol. Chem.*, **35**, 1410 (1971))

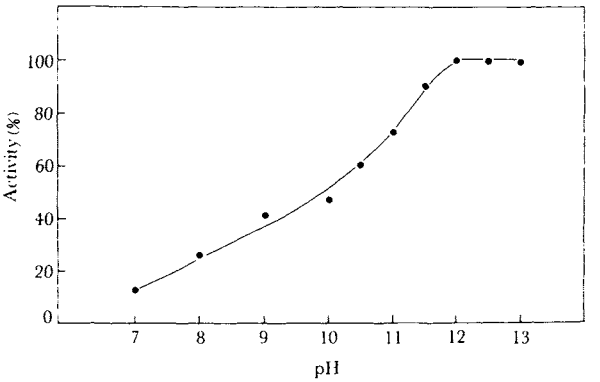


Fig. 7.2 Effect of pH on enzyme activity.
(Reproduced with permission from H. Takami *et al.*, *Appl. Microbiol. Biotechnol.*, **38**, 103 (1992))

enzyme was completely inhibited by diisopropylphosphate or 6M urea, but not by ethylenediamine tetraacetic acid or *p*-chloromercuribenzoate. The molecular weight of the enzyme was 30,000, which is slightly higher than that of other alkaline proteases. The addition of 5 mM calcium ions revealed a 70% increase in activity at the optimum temperature (60°C). Twenty years later, Takami et al. (1992) cloned this enzyme gene and determined the sequence (Accession number S48754). Subsequently, two *Bacillus* species, AB42 and PB12, which also produced alkaline protease, were reported (Aunstrup et al. 1972). They exhibited a broad pH range of 9.0–12.0, and the temperature optimum of the strains was 60°C for AB42 and 50°C for PB12. Since these reports, many alkaline proteases have been isolated from alkaliphilic microorganisms (Tsai et al. 1983, 1984, 1986; Nomoto et al. 1984a; Tsuchida et al. 1986; Fujiwara et al. 1987). Fujiwara et al. (1993) purified thermostable alkaline protease from a thermophilic alkaliphilic *Bacillus* sp. B18. The optimum pH and temperature for the hydrolysis of casein were pH 12–13 (Fig. 7.2) and 85°C, both of which are higher than those of alkaline proteases. Takami et al. (1989) isolated a new alkaline protease from alkaliphilic *Bacillus* sp. No. AH-101. The enzyme was most active toward casein at pH 12–13 and stable under 10 min incubation at 60°C and pH 5–13. The optimum temperature was about 80°C in the presence of 5 mM calcium ions. The alkaline protease showed a higher hydrolyzing activity against insoluble fibrous natural proteins such as elastin and keratin in comparison with subtilisins and proteinase K (Takami et al. 1990; 1992) (Accession number D13158). Takami et al. (1999e) reidentified alkaliphilic *Bacillus* sp AH-101 as *Bacillus halodurans* No. AH-101.

Takami et al. (1992) reported that the gene encoding an alkaline serine protease from alkaliphilic *Bacillus clausii* 221 was cloned in *Escherichia coli* and expressed in *Bacillus* subtilis. An open reading frame of 1,140 bases, identified as the protease gene, proceeded by a putative SD sequence (AG-

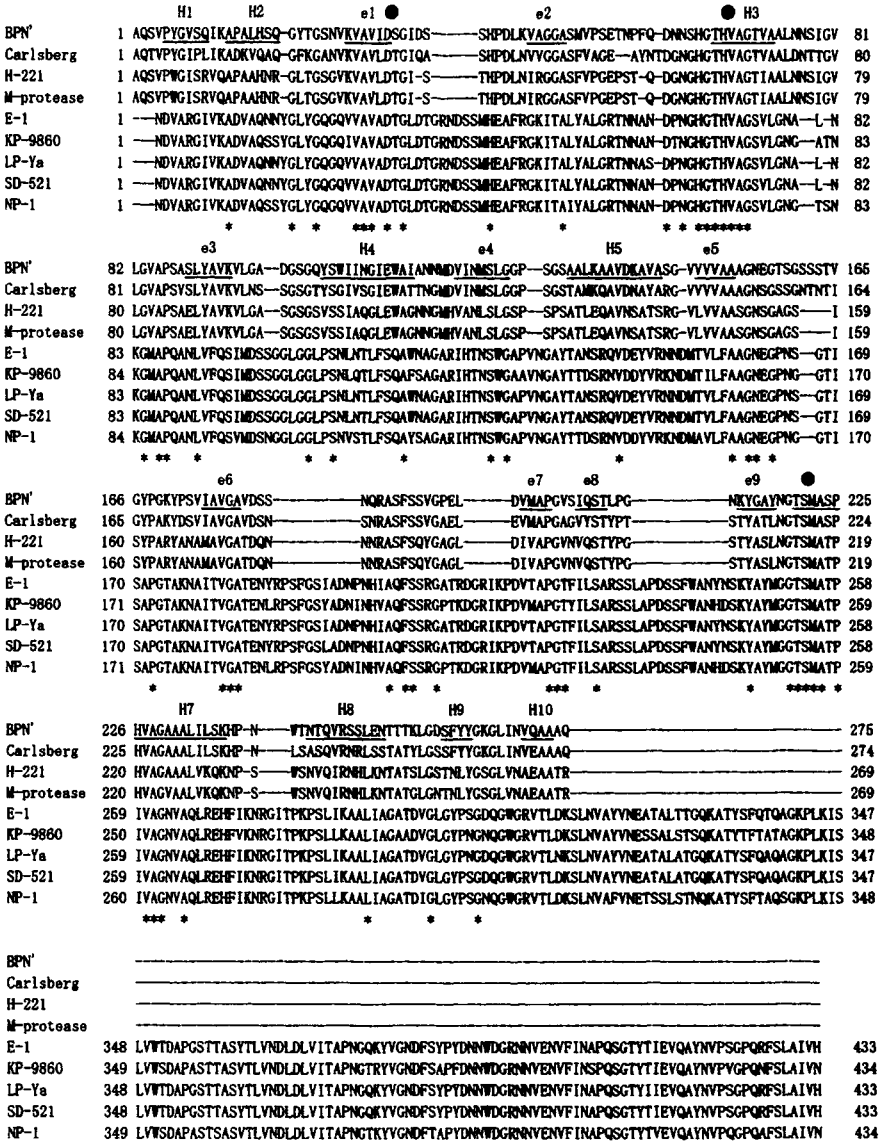


Fig. 7.3 Multiple sequence alignment among the five oxidatively stable proteases and other subtilisins. Each amino acid sequence is numbered from the N-terminal residue of the mature enzyme and is indicated by the single-letter codes. A common catalytic triad of the three amino acids, Asp (electrophile), His (base), and Ser (nucleophile), is shown by solid circles. Residues identical in all the sequences are marked with asterisks. Above the alignment, the sequences of structural elements determined by X-ray crystallographic analysis of BPN' (26) are given. Underlines mark the positions of α-helices (H1-H10) and β-strands (e1-e9).

GAGG) with a spacing of 7 bases. The deduced amino acid sequence had a pre-pre-peptide of 111 residues followed by the mature protease comprising 269 residues (Accession number S48754). The deduced amino acid sequence of the alkaline protease from *Bacillus clausii* 221 had higher homology to the protease from other alkaliphilic bacilli (see Fig. 7.3).

Han and Damodaran (1998) reported that an extracellular endopeptidase from a strain of *Bacillus pumilus* displaying high stability in 10% (w/v) sodium dodecyl sulfate and 8 M urea was purified and characterized. Some of the enzymes described above are now commercially available as detergent additives.

7.2 Isolation of Alkaline Proteases for Laundry Detergent Additives

Kobayashi et al. (1995) isolated M-protease that was suitable for use in detergents from alkaliphilic *Bacillus* sp. KSM-K16. The M-protease was purified to homogeneity from the culture broth by column chromatographies. The N-terminal amino acid sequence was Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile-Ser-Arg-Val-Gln-Ala-Pro-Ala-Ala-His-Asn-Arg-Gly-Leu-Thr-Gly. The molecular mass of the protease was 28 kDa, and its isoelectric point was close to pH 10.6. Maximum activity toward casein was observed at 55°C, as shown in Fig. 7.4. The activity was inhibited by phenylmethylsulfonyl fluoride and chymostatin. The enzyme was very stable in long-term incubation with liquid detergents at 40°C. This protease exhibited good properties as a laundry detergent additive except for oxidant resistance. The gene responsible for the M protein was cloned and sequenced. The accession number is Q99405.

Then, Shirai et al. (1997) studied the crystal structure of the M-protease of alkaliphilic *Bacillus* sp. KSM-K16 by X-ray analysis to better understand the alkaline adaptation mechanism of the enzyme. This analysis revealed a decrease in the number of negatively charged amino acids (aspartic acid and glutamic acid) and lysine residues, and an increase in arginine and neutral hydrophilic amino acids (histidine, asparagine and glutamine) residues during the course of adaptation.

A new intracellular alkaline serine protease from alkaliphilic *Bacillus* sp. NKS-21 was cloned, sequenced and characterized by Yamagata and Ichishima (1995a). It encoded an intracellular serine protease (ISP-1) in which there was no signal sequence. Its molecular weight was 34,624. The protease showed about 50% homology with those of intracellular serine proteases (ISP-1) from *Bacillus subtilis*, *B. polymyxa* and alkaliphilic *B. clausii* 221. The cloned intracellular protease was expressed in *E. coli*, and showed stability under alkaline condition at pH 10 and tolerance to surfactants.

Yamagata et al. (1995b), then reported that the gene for an extracellular alkaline serine protease (ALP I) from *Bacillus* sp. NKS-21 had been cloned and its nucleotide sequence determined. The gene contained an

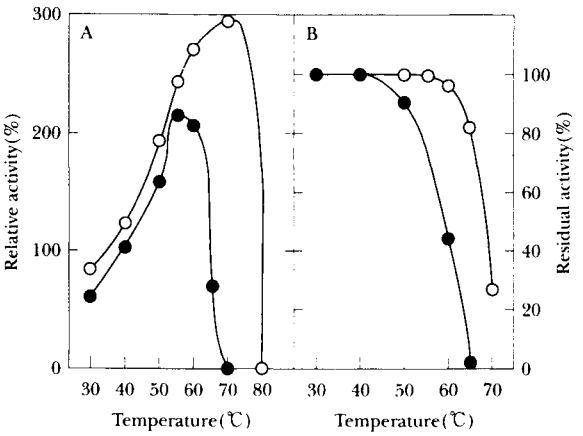


Fig. 7.4 Effects of temperature. **A** Effects of temperature on the enzymatic activity. M-protease (0.25 μg) was added to 1.0%(w/v) casein in 50 mM borate/NaOH buffer (pH 10.0). After a 10-min reaction at various temperatures, each reaction was stopped by addition of trichloroacetic acid. The enzymatic activities are expressed as percentages of the activity at 40°C in the absence of $CaCl_2$: ○ activity in the presence of 5 mM $CaCl_2$, ● that in the absence of $CaCl_2$. **B** Effects of temperature on the stability of the enzyme. M protease (3.0 μg) was preincubated for 10 min at various temperatures in 20 mM borate/NaOH buffer (pH 9.0). Heating of the enzyme was stopped by cooling in an ice bath. Aliquots suitably diluted with 50 mM borate/NaOH buffer (pH 10.0) were used for measurement of the residual activity at 40°C for 10 min. The enzymatic activity, after heating at 30°C for 10 min, was taken as 100%: ○ residual activity in the presence of 5 mM $CaCl_2$, ● that in the absence of $CaCl_2$. (Reproduced with permission from T. Kobayashi et al., *Appl. Microbiol. Biotechnol.*, **43**, 473(1995))

open reading frame of 1125 bp, encoding a primary product of 374 amino acids. The mature protease, composed of 272 amino acids, was preceded by a putative signal sequence of 37 amino acids and a pro-sequence of 65 amino acids (Q45523).

Gessesse (1997), Gessesse and Gashe (1997) isolated an alkaliphilic *Bacillus* sp AR-009 producing a high level of alkaline protease from an alkaline soda lake in Ethiopia. The optimum temperature and pH for activity were 65°C and 9.5–11.5 respectively. Above 50°C, Ca^{2+} was required for enzyme activity and stability. Banerjee et al. (1999) isolated an alkaline protease from a facultatively thermophilic and alkaliphilic strain of *Bacillus brevis*. The enzyme from a shake flask culture displayed maximum activity at pH 10.5 and 37°C. However, the enzyme required Ca^{2+} ion to maintain its stability.

An alkaliphilic hyperproducer of alkaline protease, *Bacillus* sp. NG312, was isolated, and the enzyme showed maximum activity at pH 11.0 and 60°C by Singh et al. (1999). The temperature optimum was increased at 10°C in the presence of Ca^{2+}. These enzymes are unsuitable for use as laundry detergent additives, because builders of laundry detergents contain additives to reduce Ca^{2+} concentration.

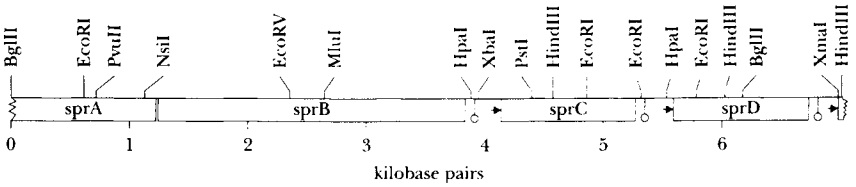


Fig. 7.5 Restriction endonuclease map of the DNA fragment carrying the four serine protease genes cloned from *Bacillus* sp. strain LG12. , putative terminator; ►, promoter.

Schmidt et al. (1995)analyzed a series of serine protease genes of alkaliphilic *Bacillus* sp strain LG12. As shown in Fig. 7.5, four tandem subtilisin-like protease genes were found on a 6,854-bp DNA fragment. The two downstream genes (*sprC* and *sprD*) appear to be transcribed independently, while the two upstream genes (*sprA* and *sprB*) seem to be part of the same transcript. Yeh et al. (1997)improved the translational efficiency of an alkaline protease *YaB* gene with different initiation codons in *Bacillus subtilis* and alkaliphilic *Bacillus* YaB.

Martin et al. (1997) found that the solution structure of serine protease PB92 from *Bacillus alcalophilus* presents a rigid fold with a flexible substrate-binding site. The solution structure of the serine protease PB92 presents a well defined global fold, which is rigid with the exception of a restricted number of sites.

More than 50 other reports have been published. However, the properties of the enzymes described are almost the same as those described above.

7.3 Isolation of Detergents and H_2O_2 Resistant Alkaline Proteases

Alkaline proteases are used extensively in detergents, the food industry, and leather tanning. Enzymes produced commercially are derived only from microorganisms, and the microorganisms must be able to produce a high enzyme yield from low-cost substrates. The success of alkaline proteases in detergents is depend on whether the enzymes have the following properties 1) a wide pH activity range, 2) stability under high alkaline conditions, 3) high activity and stability in the presence of surfactants, 4) high stability in the presence of builders such as chelating reagents and bleaching agents, 5) high activity over a wide temperature range, 6) long shelf-life, and 7) low production cost. Although many enzymes have been reported, the alkaline proteases described above, however, have several weak points in their enzymatic properties, e.g., they are sensitive in the presence of oxidants and chelating agents. These disadvantages have been overcome by the isolation of new *Bacillus* strains by the author's group. Details are given below.

7.3.1 E-1 Enzyme of *Bacillus cohnii* D-6

In 1972, Yoshida and Horikoshi discovered a very stable alkaline protease in the presence of detergents containing high concentration of perborate in the absence of calcium ion, (Japanese patent: JP 740710). *Bacillus* sp. No. D-6 (FERM No. 1592, later designated *Bacillus cohnii* D-6, FERM P-1592) produced an alkaline protease, E-1, that was more stable in the presence of detergent additives at 60°C than *Bacillus clausii* 221 protease. Several properties are presented in Table 7.1. If the enzyme productivity could be increased, the E-1 enzyme would be the best enzyme for detergent additives. After our discovery, many researchers tried to industrialize it, but without success. Almost thirty years later, Saeki and our colleagues (2000) dramatically increased the productivity to more than 10 grams/liter using gene technology.

The author's stock culture of *Bacillus cohnii* D-6 was grown in a liquid medium (pH 9.0) composed of 0.5% glucose, 0.2% Polypepton S (Nippon Pharmaceutical), 0.05% yeast extract (Difco), 0.1% KH_2PO_4 and 0.26% Na_2CO_3 (autoclaved separately). The protease activity in the presence H_2O_2 was examined by the method described in Fig. 7.6.

Table 7.1 Properties of an alkaline protease from *Bacillus* sp. No. D-6

Property	Protease
	E-1
Optimum pH	10.5
Stable pH(60°C, 10 min)	5–12
Stable temperature, °C(at pH 9)	
$-Ca^{2+}$	75
$+Ca^{2+}$ (10 mM)	75
Molecular weight, $\times 10^4$	2–3
Isoelectric point	10
Active center	Ser
Stability in detergent(DBS†: 0.2%, 50°C, 60 min)	60

† DBS: Dodecyl benzenesulfonate

A. Purification of enzymes

The E-1 enzyme was purified to homogeneity by the following procedure. The protease fraction was precipitated at 90% saturation of ammonium sulfate from the culture broth. The precipitate was dissolved in a small volume of 10 mM Tris-HCl buffer (pH 7.5) plus 2 mM $CaCl_2$, and dialyzed overnight against the same buffer. The dialyzate was applied on a column of DEAE Bio-GelA (Bio-Rad) that had been equilibrated with the same buffer. The column was washed with the buffer, and non-adsorbed active fractions were concentrated by ultrafiltration. The concentrate was dialyzed overnight against a large volume of 10 mM Tris-HCl buffer (pH 7.5) plus 2 mM $CaCl_2$. The dialyzate was then placed on a column of SP-Toyopearl

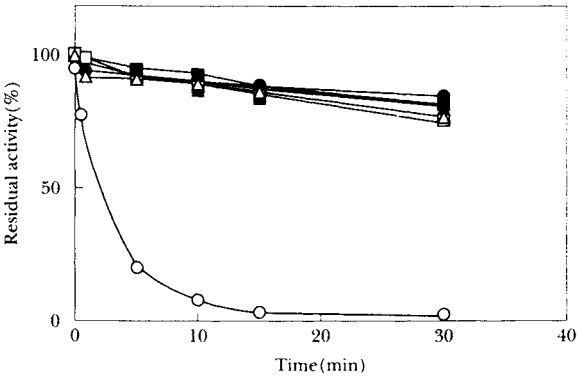


Fig. 7.6 Rates of oxidative inactivation by H_2O_2 of the five proteases. Each enzyme was incubated in the presence of 50 mM H_2O_2 at 30°C and at pH 10 in 5.0 ml of 20 mM Britton-Robinson buffer. Timed samples (0.5 ml) were withdrawn and mixed with 50 µl of catalase solution (20 milliunits/ml) to remove residual H_2O_2. The residual activities of E-1 (●), KP-9860 (■), LP-Ya ( ), SD-521 (▲), and NP-1 (△), including M-protease (○), were measured by incubation at 30°C for 10 min with Ala-Ala-Pro-Leu-pNA as substrate.
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550W (Tosoh) equilibrated with the same buffer, and the protease was eluted using a gradient of 0–50 mM NaCl in the buffer. The active fractions were collected, concentrated by ultrafiltration and used as the final preparation of purified enzyme.

B. Properties of the purified E-1 enzyme

The purified E-1 had a molecular mass of about 43 kDa estimated by SDS-PAGE and a specific activity of 115 units/mg protein at pH 10.5 in 50 mM borate buffer. Isoelectrofocusing analysis indicated the pI value of the enzyme to be pH 9.7–9.9. The N-terminal amino acid sequence determined was Asn-Asp-Val-Ala-Arg-Gly-Ile-Val-Lys-Ala-Asp-Val-Ala-Gln. In the absence of calcium ions, caseinolytic activity of E-1 at 35°C was observed in a wide pH range of 6–12, with optimum pH around 10–11 in 20 mM glycine-NaOH buffer. E-1 was stable at pH 6–12 but unstable below pH 5 and above pH 13 after a 24 h incubation at 25°C. The optimal temperature for activity at pH 10 was 65°C. The enzyme was stable up to 65°C after 30 min incubation at pH 10 in the presence of 5 mM $CaCl_2$. Phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (1 mM each) inhibited the enzyme activity by 98 and 92%, respectively. Chelating agents, such as *o*-phenanthroline, EDTA, and EGTA (5 mM each) did not inhibit the enzyme activity at all. The relative ratio of activity toward Ala-Ala-Pro-Leu-pNA, Ala-Ala-Pro-Phe-pNA, Ala-Pro-Ala-pNA and Ala-Ala-Ala-pNA was approximately 100:10:7:1. In contrast with E-1, typical subtilisins such as M-protease (Kobayashi et al. 1995), *Bacillus clausii* 221 enzyme, and BPN' hydrolyzed Ala-Ala-Pro-Phe-

pNA two to eight times faster than Ala-Ala-Pro-Leu-pNA. The most striking property of the E-1 enzyme is its strong resistance to oxidants such as H_2O_2, as well as to chelating reagents such as EDTA and EGTA. As shown in Fig. 7.6, M-protease used as the control was completely inactivated by H_2O_2 under the same conditions.

C. Cloning of E-1 protease gene

The complete protease gene was cloned by the cassette-ligation-mediated PCR method with a Takara LA PCR *in vitro* cloning kit according to the manufacturer's instructions. The nucleotide sequence for E-1 was submitted to the DDBJ data bank with Accession Number AB046402 (Saeki et al. 2000).

The gene contains a single open reading frame (ORF), which begins with an ATG initiation codon at nucleotide 406 and ends with a TAA termination codon at 2311 in the 2478-bp nucleotide sequence determined. Upstream of the ORF, the putative ribosome binding sequence 5'-GAGGAG-3' is found, separated by 10 bp from the initiation codon. There is a promoter-like sequence, with 5'-TTTAGA-3' as the potential −35 region and 5'-TACATT-3' as the potential −10 region, separated by 17 bp. A long inverted-repeat sequence (−98.9 kJ/mol) from nucleotide 2323 to 2366 is found 9 bp downstream from the termination codon of the ORF. The ORF in the nucleotide sequence encodes 635 amino acids with a possible 202-amino acid pre-pro-peptide.

Subsequently, *Bacillus* KSM-9860, *Bacillus* LP-Ya, *Bacillus* SD-521 and *Bacillus* NP-1 were isolated and the alkaline proteases of these alkaliphiles were purified by the conventional manner

Their enzymatic properties were almost the same as those of the E-1 enzyme. Furthermore, for better understanding the oxidant resistance of E-1, Saeki et al. (2002) analyzed sequences the alkaline protease genes of KSM-9860 (Accession Numbers AB046403), of LP-Ya (Accession Numbers AB046404), of SD-521 (Accession Numbers AB046406) and of NP-1 (Accession Numbers AB046406). As shown in Fig. 7.3, the deduced amino acid sequence Asn-Asp-Val-Ala-Arg-Gly-Ile-Val-Lys-Ala-Asp-Val-Ala is common to the internal sequences and coincides with the N-terminal sequence of mature E-1. The deduced amino acid sequences of KP-9860, LP-Ya, SD-521 and NP-1 showed very high homology to that of E-1 with 88.2, 98.6, 99.3 and 88.2% identity, respectively. However, the five enzymes exhibited very limited homology to those of true subtilisins, such as subtilisins Carlsberg with 26% and BPN' with 24% identity, high-alkaline proteases, such as *Bacillus clausii* 221 and M-protease with 25% identity for each. This low sequence homology is due to many insertions between the α-helixes and β-strands in BPN', Carlsberg, *Bacillus clausii* 221 and M-protease.

Evolutionary distances among the proteases were computed, and a phylogenetic tree was constructed by a neighbor-joining algorithm, as shown in Fig. 7.7. The 29 proteases were unequivocally grouped into four clusters

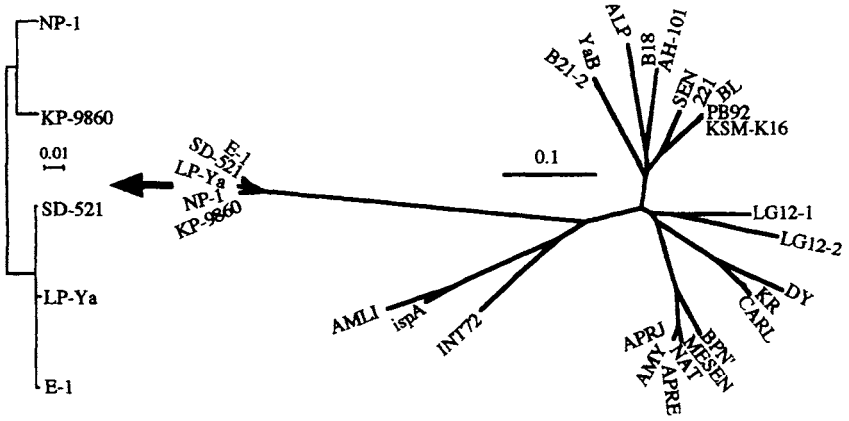

Fig. 7.7 Unrooted phylogenetic tree of subtilisins inferred from the amino acid sequence alignment of the conserved regions around active sites. Conserved sequence segments around the catalytic triads (Asp32, His64, and Ser221 in BPN' numbering) of the five oxidatively stable proteases were aligned manually with those of other subtilisins of known sequence. Bar is Knuc unit. Sources of sequences aligned: Q45521, *Bacillus* sp. strain B18'; D13158, *Bacillus* sp. strain AH-101; Q45523, *Bacillus* sp. strain NKS-21; P41362, *Bacillus* sp. strain 221; P27693, *Bacillus alkalophilus* PB92 (ATCC31408); Q99405, *Bacillus* sp. strain KSM-K16; P29599, *Bacillus lentus*; AB005792, *Bacillus* sp. strain B21-2; P20724, *Bacillus* sp. strain YaB; Q45522, *Bacillus* sp. strain G-825-6; Q45466 and Q45467, *Bacillus* sp. strain LG12; P007780, *Bacillus licheniformis*; Q53521, *B. licheniformis* PWD-1 (ATCC 53757); P00781, *Bacillus subtilis* DY; P29142, *Bacillus stearothermophilus* NCIMB10278; P00783, *B. subtilis* var. *amylosachariticus*; P04189, *B. subtilis* 1168; P35835, *B. subtilis* (*natto*) NC2-1; P07518, *Bacillus mesentericus*; P00781, *Bacillus amyloliquefaciens*; P11018, *B. subtilis* IFO3013; Q998944, *B. amyloliquefaciens*, P29139, *Bacillus polymyxa* 72.
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based on amino acid sequence analysis. The two major clusters are the groups of true subtilisins and highly alkaline proteases. Each cluster was composed of 10 or 11 members, and some subclasses were constituted in the clusters. The third cluster is composed of E-1, KP-9860, LP-Ya, SD-521 and NP-1.

7.3.2 KP43 Enzyme from *Bacillus halmapalus* KSM-KP43

In 2002, Saeki et al. (2002)isolated *Bacillus* KSM-KP43 (FERM BP-6532) from a soil sample collected in Haga, Tochigi Prefecture, Japan. This strain also produces a serine protease having high stability in the presence of oxidation reagents such as H_2O_2. This property is required for use as bleach-based detergent additives.

As shown in Table 7.2, KSM-KP43 is a gram-positive, strictly aerobic, motile, sporulating, rod-shaped (0.4–0.9 × 2.6–4.2 μm) with peritrichous flagella. Spores were ellipsoidal and located centrally to paracentrally, not swelling the young sporangium. The isolate grew in nutrient broth at pH

Table 7.2 Phenotypic properties of KSM-KP43

Property	Reaction of strain		Property	Reaction of strain	
	KSM-KP43	*Bacillus halmapalus*[a]		KSM-KP43	*Bacillus halmapalus*[a]
From	Rod	Rod	Growth at 10°C	+	+
Mobility	+	+	Growth at 20°C	+	+
Flagella	Peritrichous	Peritrichous	Growth at 40°C	+	+
Spore	+	+	Growth at 45°C	−	−
	(central to paracentral)	(central)	Growth at pH 7	+	+
Gram stain	Positive	Positive	Growth at pH 8	+	+
Catalase	+		Growth at pH 9	+	+
Oxidase	+		Growth at pH 10	+	+
NO_3 to NO_2	−	−	Growth at 5% NaCl	+	−
Substrate utilized:			Growth at 7% NaCl	+	−
D-Glucose	+		Growth at 9% NaCl	+	−
D-Mannose	+	+	Hydrolysis of starch	+	+
Fructose	+		Hydrolysis of gelatin	+	+
Galactose	−	−	Hydrolysis of escculin	+	
Maltose	+		Hydrolysis of pullulan		+
Sucrose	+		Hydrolysis of Tweens	+	−
Lactose	−	−	20, 40, and 60		
L-Arabinose	−	−	β-Glucuronidase	−	−
D-Xylose	−	−	Arginine hydrolase	−	
Inositol	−	−	Lysine hydrolase	−	
D-Sorbitol	+	−	Urease	−	
D-Mannitol	+		Utilization of citrate	−	
Glycerol	−	−	H_2S production	−	
Melibiose	+	−	Indole production	−	
D-Raffinose	−	−	Acetoin production	−	
Rhamnose	+	−			
Trehalose	−				

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between 6.8 and 10 with an optimum around pH 9 and with up to 9% NaCl (w/v) at pH 7. The temperature range for growth was 10°C to 40°C with an optimum around 30°C. KSM-KP43 was positive for utilization of D-glucose, D-mannose, fructose, maltose, sucrose, lactose, melibiose, D-sorbitol, D-mannitol, trehalose and *N*-acetylglucosamine, for hydrolysis of starch, gelatin, Tweens 20, 40 and 60, and enzyme tests for catalase and oxidase. It was negative for utilization of L-arabinose. D-xylose, galactose, inositol glycerol and D-raffinose, formation of indole, H_2S and acetoin, utilization of citric acid, reduction of NO_3 to NO_2, and activities of β-glucuronidase, urease, lysine hydrolase and arginine hydrolase. The 16S rDNA sequence of KSM-KP43 exhibited the closest match (98.8% homology) with that of *Bacillus halmapalus*.

The G+C content of the KSM-KP43 genomic DNA was 41.6 mol%, whereas that of *B. halmapalus* was 36.8 mol%. And it was found that the DNA-DNA hybridization of KSM-KP43 with *B. halmapalus* revealed a low association (less than 25%). Furthermore, there were also differences in cel-

lular fatty acid composition between the two organisms. Therefore, KSM-KP43 strain may be a novel species of alkaliphilic *Bacillus*. Further taxonomic studies were not reported.

A. Purification and properties of the KP43 enzyme

The KP43 enzyme was purified by the same method described above and had a molecular mass of 43 kDa. The pI value of the enzyme was pH 9.7–9.9. The *N*-terminal amino acid sequence determined was Asn-Asp-Val-Ala-Arg-Gly-Ile-Val-Lys-Ala-Asp-Val-Ala-Gln-Ser-Ser-Tyr-Gly-Leu-Tyr-Gly. In

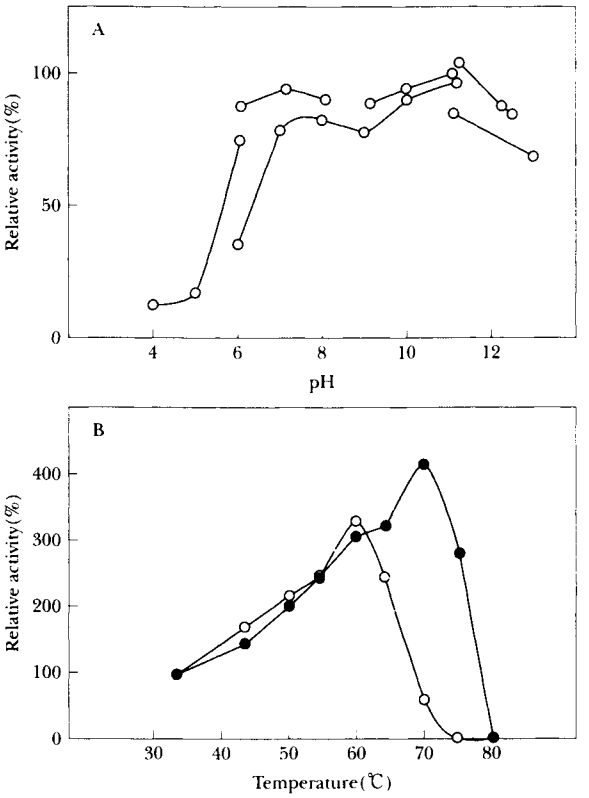

Fig. 7.8 Effects of pH and temperature on the activity of KP43. **A** Effect of pH on activity. Acetate buffer (pH 4–6), sodium phosphate buffer (pH 6–8), Tris-HCl buffer (pH 7–9), glycine-NaOH buffer (pH 8–11), borate buffer (pH 9–11), and KCl-NaOH buffer (pH 12–13) were used. Assays were done at 35℃ for 10 min in the indicated buffers at 20 mM with casein as substrate. The values are shown as percentages of the maximum specific activity of KP43 observed at pH 11 in glycine-NaOH buffer, which is taken as 100%. **B** Effect of temperature on activity. The reactions were done at the indicated temperatures for 5 min and at pH 10 in 50 mM glycine-NaOH buffer in the absence (*open circles*) or presence (*solid circles*) of 5 mM $CaCl_2$. The values are shown as percentages of the activity observed at 35℃ in the absence of $CaCl_2$, which is taken as 100%.
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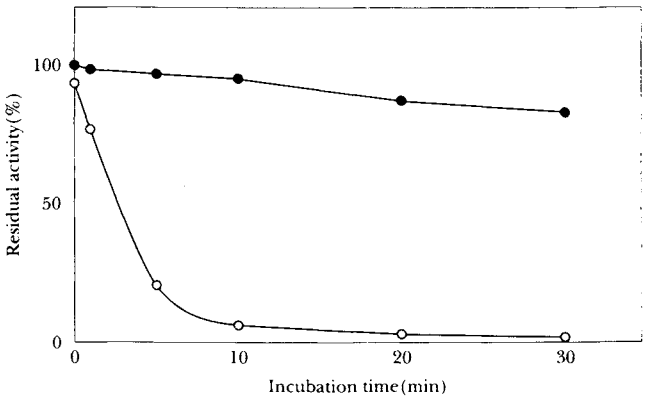

Fig. 7.9 Rates of oxidative inactivation by H_2O_2 of KP43. The enzyme was incubated in the presence of 50 mM H_2O_2 at 30°C and at pH 10 in 5.0 ml of 20 mM Britton-Robinson buffer. Samples (0.5ml) were with-drawn at appropriate time intervals and mixed with 50 µl of catalase solution (20 mU/ml) to remove residual H_2O_2. The residual activities of KP43 (*solid circles*), including a highly alkaline enzyme, M-protease (Kobayashi et al. 1995), as reference (*open cercles*), were measured by incubation at 30°C for 10 min with 5 mM Ala-Ala-Pro-Leu-pNA as the substrate.
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the absence of calcium ions, caseinolytic activity of KP43 at 35°C was observed at a pH range of 4–13, with an optimum pH at 11–12 in glycine-NaOH buffer (Fig. 7.8A). KP43 was stable at pH 6–12 but unstable below pH 5 and above pH 13 after 24-h incubation at 25°C. The optimal temperature for activity at pH 10 was 60°C in the absence and 70°C in the presence of 5 mM $CaCl_2$ (Fig. 7.8B). The enzyme that is serine protease was stable up to 55°C in the absence of 5 mM $CaCl_2$ and to 65°C in the presence of 5 mM $CaCl_2$ after 30-min incubation at pH 10. The striking feature of KP43 is also its strong resistance to H_2O_2 (Fig. 7.9), as has been reported for E-1 (Saeki et al. 2000).

B. Cloning of the gene for the KP43 enzyme

The gene encoding the enzyme was cloned using a mixed primer designed from the N-terminal amino acid sequence (Met-Phe-Asp-Ser-Ala-Pro-Phe-Ile-Gly-Ala-Asn-Asp-Ala-Trp-Asp-Leu-Gly-Phe-Tyr-Gly) of the purified enzyme. The nucleotide sequence of the gene (accession number AB051423; AB096094) consisted of a 2427-bp open reading frame (ORF) that encoded a putative pre-pro-peptide (152 amino acids) and a mature enzyme (656 amino acids; 68,506 Da). The deduced amino acid sequence of the enzyme exhibited very high homology to that of the E-1 enzyme, although the nucleotide sequence KP43 gene showed lower homology to the E-1 protease gene. This result reflects a difference in codon usage between *Bacillus halmapalus* KP43 and *Bacillus cohnii* D-6 as shown in Table 7.3.

Table 7.3 Codon usage in alkaline protease E-1 and KP43

Sequence name number of codon		> E-1 AB046402 433		> KP43 AB051423 435	
AA	Codon	Number	(%)	Number	(%)
Phe	TTT	9	69.2	5	38.5
	TTC	4	30.8	8	61.5
Leu	TTA	19	70.4	4	14.3
	TTG	0	0.0	5	17.9
	CTT	4	14.8	8	28.6
	CTC	0	0.0	1	3.6
	CTA	4	14.8	5	17.9
	CTG	0	0.0	5	17.9
Ile	ATT	10	50.0	8	40.0
	ATC	4	20.0	10	50.0
	ATA	6	30.0	2	10.0
Met	ATG	6	100.0	7	100.0
Val	GTT	11	36.7	5	16.1
	GTC	4	13.3	6	19.4
	GTA	13	43.3	9	29.0
	GTG	2	6.7	11	35.5
Cys	TGT	0	0.0	0	0.0
	TGC	0	0.0	0	0.0
Ser	TCT	12	35.3	8	20.0
	TCC	1	2.9	12	30.0
	TCA	4	11.8	2	5.0
	TCG	7	20.6	5	12.5
	AGT	7	20.6	5	12.5
	AGC	3	8.8	8	20.0
Pro	CCT	8	36.4	5	25.0
	CCC	0	0.0	0	0.0
	CCA	10	45.5	7	35.0
	CCG	4	18.2	8	40.0
Thr	ACT	7	21.2	6	17.1
	ACC	1	3.0	5	14.3
	ACA	15	45.5	14	40.0
	ACG	10	30.3	10	28.6
Ala	GCT	19	37.3	14	28.0
	GCC	2	3.9	9	18.0
	GCA	18	35.3	17	34.0
	GCG	12	23.5	10	20.0
Tyr	TAT	14	77.8	9	56.3
	TAC	4	22.2	7	43.8
Trp	TGG	6	100.0	5	100.0
His	CAT	7	100.0	7	100.0
	CAC	0	0.0	0	0.0
Gln	CAA	16	94.1	7	46.7
	CAG	1	5.9	8	53.3
Asn	AAT	26	63.4	23	60.5
	AAC	15	36.6	15	39.5
Lys	AAA	13	92.9	12	80.0
	AAG	1	7.1	3	20.0
Asp	GAT	18	81.8	17	70.8
	GAC	4	18.2	7	29.2
Glu	GAA	4	50.0	4	57.1
	GAG	4	50.0	3	42.9
Arg	CGT	4	23.5	3	20.0
	CGC	3	17.6	5	33.3
	CGA	3	17.6	1	6.7
	CGG	0	0.0	2	13.3
	AGA	7	41.2	4	26.7
	AGG	0	0.0	0	0.0
Gly	GGT	13	27.7	8	16.7
	GGC	4	8.5	10	20.8
	GGA	23	48.9	22	45.8
	GGG	7	14.9	8	16.7
Stop	TAA	1	100.0	1	100.0
	TAG	0	0.0	0	0.0
	TGA	0	0.0	0	0.0

C. Effects of chemical reagents

Chemical reagents were incubated with suitably diluted protease at 20°C for 10 min in 50 mM Tris-HCl buffer (pH 7.0), and the residual activity was measured at pH 10.5. Phenyl-methylsulfonyl fluoride (PMSF:1 mM) strongly inhibited enzyme activity by 97.4%. EDTA, EGTA (10 mM each), 2-mercaptoethanol, monoiodoacetate, N-ethylmaleimide, and 5,5'dithiobis-(2-nitrobenzoic acid) (5 mM each), and *p*-chloromercuribenzoate (0.1 mM) did not inhibit enzyme activity. On the other hand, N-bromosuccinimide (0.5 mM) and 2-hydroxy-5-nitrobenzyl-bromide (5 mM) strongly inhibited enzyme activity by 98% and 96%, respectively. Hydrogen peroxide (100 mM) did not inhibit its enzyme activity when incubated at 20°C for 10 min in 50 mM borate buffer (pH 10.5).

The KP43 enzyme is stable in builders containing high concentrations of perborate as bleaching agents. And in 2004 it began to be produced in industrial-scale plants as the best laundry detergent additive enzyme and made commercially available.

7.4 Phylogenetic Tree of Alkaline Proteases

An unrooted phylogenetic tree of subtilisins including true subtilisins, high-alkaline proteases such as M-protease (Hakamada et al. 1994), intracellular proteases, and oxidant stable proteases (Saeki et al. 2000a, b) is shown in Fig. 7.10. As expected, high molecular mass proteases such as Vpr, and Vpr-like proteases form a clearly distinct clan from clans of high alkaline proteases and oxidant-stable proteases.

7.5 Dehairing

Alkaline enzymes have been used in the hide-dehairing process, where dehairing is carried out at pH values between 8 and 10. These enzymes are commercially available from several companies.

An extracellular alkaline serine protease called DHAP and produced by a *Bacillus pumilus* strain demonstrates significant dehairing function. This protease is purified by hydrophobic interaction chromatography, ion exchange, and gel filtration. DHAP had a pI of 9.0 and a molecular weight of approximately 32,000 Daltons. It shows maximal activity at pH 10 and temperature of 55°C; the enzyme activity can be completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphates (DFP). The first 20 amino acid residues of the purified DHAP have been determined to have the sequence AQTVPYGIPQIKAPAVHAQG. This sequence demonstrates high homology with protease from another *B. pumilus* strain (Huang et al. 2003).

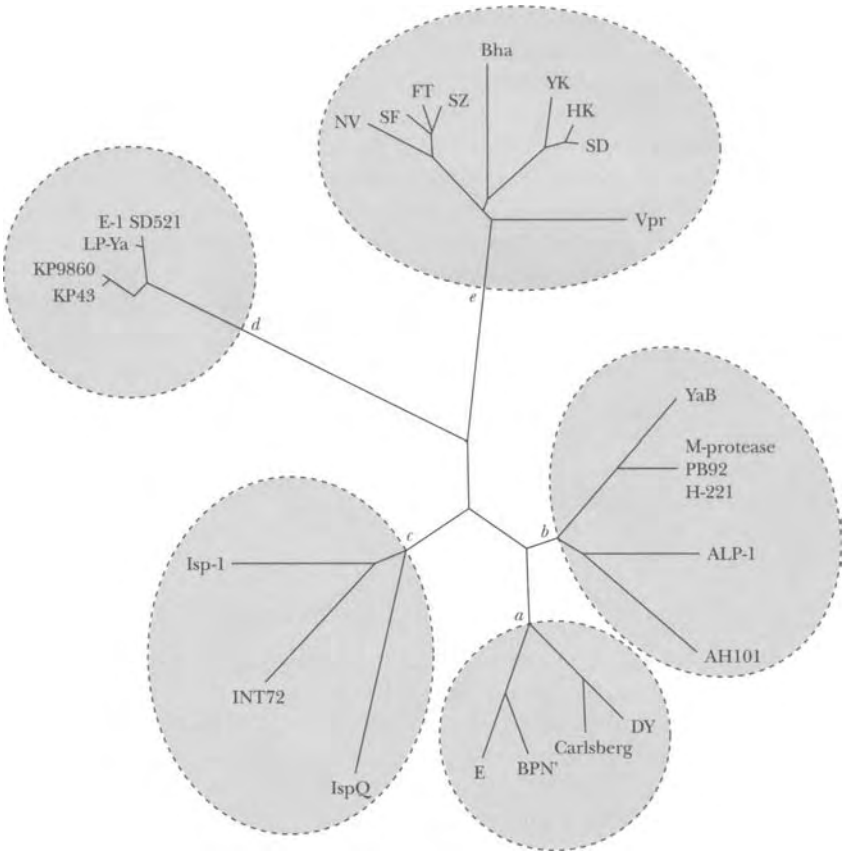

Fig. 7.10 Phylogenetic tree of subtilisins. A phylogenetic tree was inferred by the bootstrapping neighbor-joining method (Saitou and Nei 1987) in the ClustalX program. *Bar* represents *Knuc* unit. A Unrooted phylogenetic tree of subtilisins. *Group a* True subtilisins, *group b* high-alkaline proteases, *group c* intracellular proteases, *group d* oxidatively stable alkaline proteases (OSPs) (Saeki et al. 2002), *group e* HMSs. Sources of sequences aligned: subtilisin E (P04189) from *Bacillus subtilis* 168; BPN' (Q44684) from *B. amyloliquefaciens*; Carlsberg (P00780) from *B. licheniformis* ; DY (P00781) from *B. subtilis* DY ; YaB (P20724) from *Bacillus* sp. YaB; M-protease (Q99405) from *Bacillus* sp. KSM-K16; PB92 (P27693) from *B. alcalophilus* PB92; H-221 (P41362) from *Bacillus clausii* 221; ALP-1 (Q45523) from *Bacillus* sp. strain NKS-21, AH101 (D13158) from *Bacillus halodurans* AH-101; Isp-1 (P08750) from *B. subtilis* IFO3013; INT72 (P29139) from *B. polymyxa* 72; IspQ (Q45621) from *Bacillus* sp. strain NKS-21; SD-521 (AB046405) from *Bacillus* sp. strain SD-521; E-1 (AB046402) from *Bacillus* sp. strain D-6; LP-Ya (AB046404) from *Bacillus* sp. strain Y; KP9860 (AB046403) from *Bacillus* sp. strain KSM-KP9860; KP43 (AB051423) from *Bacillus* sp. strain KSM-KP43; NP-1 (AB046406) from *Bacillus* sp. strain NCIB12289.
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7.6 Others

An interesting application of alkaline protease was developed by Fujiwara et al. (Fujiwara et al. 1987; 1991; Ishikawa et al. 1993). They reported that the alkaline protease isolated was used to decompose the gelatinous coating of X-ray films, from which silver was recovered. Protease B18' had a higher optimum pH and temperature of around 13.0 and 85°C. The enzyme was most active toward gelatin on film at pH 10. In 1999, Masui et al. (1999) reported enzymatic decomposition of gelatin layers on X-ray films and repeated utilization of the enzyme for potential industrialization was investigated using thermostable alkaline protease from the alkaliphilic *Bacillus* sp. B21-2. The decomposition of gelatin layers at 50°C with the mutant enzyme (Ala187 was replaced by Pro) was higher than the decomposition using wild-type and other mutant enzymes. In a experiment repeated every 60 min (20 U ml(−1), 50°C), the mutant enzyme could be used satisfactorily five times while the wild-type enzyme could only be three times.

Cheng et al. (1995) reported a keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. This enzyme was stable from pH 5 to 12. The optimal reaction pHs for feather powder and casein were 8.5 and 10.5 to 11.5, respectively. Zaghloul et al. (1998) also reported isolation, identification, and keratinolytic activity of several feather-degrading bacteria isolated from Egyptian soil. These isolates degrade chicken feather.

8

Starch-degrading Enzymes

Amylose is a linear polysaccharide that is formed by α-1,4-linked α-D-glucose units. Amylopectin is also a polysaccharide of α-D-glucose monomers, but it contains α-,6-branching points. α-Amylases catalyze the hydrolysis of the α-1,4-glucosidic linkages of starch. It randomly cleaves the internal bonds in amylose and amylopectin. Cyclodextrin glucanotransferases, maltogenic α-amylases and maltotetraose-forming amylases catalyze a similar reaction. Pullulanase and isoamylase hydrolyze the α-1,6-glucosidic linkages. Neopullualanase and amylopullulanase hydrolyze both the α-1,4-glucosidic linkages and the α-1,6-glucosidic linkages of starch. According to its sequence and active site topology, α-amylase is grouped into family 13.

However, no report concerning optimum activity in the alkaline pH range were published prior to 1971 despite extensive study by many researchers. One noted Japanese enzymologist, Prof. Juichiro Fukumoto of Osaka City University, who investigated amylase, used to say, "There are no alkaline amylases in nature, because I spent over 30 years looking but was not able to discover any."

The present author attempted to isolate alkaliphilic microorganisms producing alkaline amylases, and in 1971 an alkaline amylase was produced in Horikoshi-II medium by cultivating alkaliphilic *Bacillus* sp. No. A-40-2 (Horikoshi 1971b). Several types of alkaline starch-degrading enzymes were subsequently discovered by cultivating alkaliphilic microorganisms (Boyer et al. 1972; Yamamoto et al. 1972). No alkaline amylases produced by neutrophilic microorganisms have so far been reported.

8.1 α-Amylases

8.1.1 *Bacillus pseudofirmus* A-40-2 Amylase

Production of the alkaline amylase was first achieved in an alkaliphilic *Bacillus pseudofirmus* A-40-2 (ATCC21592), that was selected from about 300 colonies of bacteria grown in Horikoshi-II medium (Horikoshi 1971b). The isolated strain was an aerobic, sporeforming, gram-positive, motile, rod-shaped bacterium with peritrichous flagella. It was clear that the bacterium belonged to the genus *Bacillus* (Table 8.1). Although the morphological,

Table 8.1 Morphological, cultural and biochemical characteristics of *B. pseudofirmus* A-40-2

1. Morphological characteristics			
Form	Rods		
Size	0.5–0.6 μm × 2–2.5 μm		
Motility	Motile		
Gram strain	Positive		
Sporangia	Slightly swollen		
Spores	0.9–1.0 μm × 1.2–1.5 μm ; oval ; central		
2. Cultural characteristics			
		Growth at	
		pH 7	pH 10.3
Nutrient broth		–	+
Nutrient agar slant		–	+
Glucose-nutrient broth		–	+ +
Glucose-nutrient agar slant		–	+ +
Potato		–	+ +
Horikoshi-I medium		–	+ +
Horikoshi-II medium		–	+ +
Glucose-nitrate agar slant		–	–
Glucose-asparagine agar slant		–	–
Anaerobic growth in glucose broth		–	–
Anaerobic production of gas from nitrate		–	–
Horikoshi-I medium containing 5% NaCl		–	+ +
3. Biochemical characteristics			
Hydrolysis of gelatin and casein		Positive	
Hydrolysis of starch		Positive	
Utilization of citrate		Utilized	
Utilization of ammonium salts		Utilized	
Reduction of nitrate to nitrite		Reduced	
Voges-Proskauer test		Positive	
4. pH and temperature			
pH for growth in Horikoshi-I medium †		pH 7.5 to pH 11	
Temperature for growth in Horikoshi-I medium		up to 45°C at pH 10.3	

† pH in Horikoshi-I medium was adjusted by adding HCl or NaOH.
– indicates no growth ; +, normal growth ; + +, abundant growth.
(Reproduced from Horikoshi, *Agric. Biol. Chem.*, **35**, 1785 (1971))

cultural and biochemical characteristics of the strain resembled those of *Bacillus subtilis*, the special feature of the bacteria was that growth was very good in alkaline media, and the optimal pH for growth was about 10. No growth was detected in neutral media.

Bacillus pseudofirmus A-40-2 was grown aerobically at 37°C in Horikoshi-II medium for three days. The alkaline amylase in the culture fluid was purified by a DEAE-cellulose column and a hydroxyl apatite column followed by gel filtration. The molecular weight was estimated to be about 70,000 by gel filtration method. The enzyme is most active at pH 10.0–10.5 and retains

50% of its activity between pH 9.0 and 11.5. The enzyme is not inhibited by 10 mM EDTA at 30°C, and completely inactivated by 8 M urea. However, about 95% of the activity is recovered upon removal of urea by dialysis. The enzyme can hydrolyze 70% of starch to yield glucose, maltose and maltotriose. Therefore, the enzyme is a type of saccharifying α-amylase.

8.1.2 *Bacillus* Strain NRRL B-3881 Amylase

After Horikoshi's paper (Horikoshi 1971b), Boyer and Ingle (Boyer et al. 1972) and Boyer et al. (1973) reported alkaline amylase in the strain NRRL B-3881. This was the second report of an alkaline amylase. The B-3881 amylase showed optimum pH for enzyme action at 9.2. A-40-2 amylase retains 50% of its activity between pH 9.0 and 11.5, and B-3881 enzyme retains the same activity between pH 7.0 and 10.5. Both amylases are relatively more stable against EDTA than either *Bacillus amyloliquefaciens* or *B. subtilis* amylase. The enzyme yields maltose, maltotriose and small amounts of glucose and maltotetraose, all of which have a β-configuration. The properties of these amylases are given in Table 8.2.

8.1.3 *Bacillus halodurans* A-59 Amylase

Alkaliphilic *Bacillus halodurans* A-59 (ATCC21591) was isolated from soil by using Horikoshi-II medium. The properties of the strain are almost the same as those of *Bacillus subtilis.* It can grow either in neutral media or in alkaline media. This differs from *Bacillus pseudofirmus* A-40-2. The alkaline amylase was purified by conventional methods, such as DEAE-cellulose columns and gel filtration. The purified enzyme has a molecular weight of 50,000. Other properties were not significantly different from the alkaline amylase of alkaliphilic *B. pseudofirmus* A-40-2 (Table 8.2).

Table 8.2 Properties of alkaline amylases from alkaliphilic *Bacillus* sp. strains No. A-40-2, No. A-59 and NRRL B-3881

Properties	Amylases		
	A-40-2 †1	A-59 †2	B-3881
Optimum pH	10.5	10.5	9.2
Optimum temperature (°C)	55	50	50
Molecular weight	70,000	50,000	
Hydrolysis product from starch	Glc > Mal > M_3	Glc > Mal > M_3	Mal > M_3 > Glc
Inhibition by EDTA	Not inhibited	Not inhibited	Partially inhibited
Type	Saccharifying α-amylase	Saccharifying α-amylase	Saccharifying α-amylase

Abbreviations : Glc, glucose ; Mal, maltose ; M_3, maltotriose.
†1 *B. pseudofirmus* A-40-2.
†2 *B. halodurans* A-59.
(Reproduced with permission from E. M. Boyer and M. B. Ingle, *J. Bacteriol.*, **110**, 992(1972))

Another alkaliphilic *Bacillus* sp., NCIB 11203, which was isolated from soil, was found to produce several enzymes in culture broth; these included alkaline amylase, alkaline protease and alkaline phosphatase (Mctigue et al. 1994; 1995). Essentially, the enzymes are similar to those in the previous report on alkaliphilic *B. halodurans* A-59.

8.1.4 Amylases of Other Alkaliphiles

Kim et al. (1995) isolated a maltotetraose-forming alkaline α-amylase from an alkaliphilic *Bacillus* strain, GM8901. An alkaliphilic bacterium, *Bacillus* sp. strain GM8901, grown at pH 10.5 and 50°C, produced five alkaline amylases in culture broth. At an early stage of the bacterial growth, amylase I (Amyl I) was produced initially and then, as cultivation progressed, four alkaline amylases, Amyl II, Amyl III, Amyl IV, and Amyl V, were produced. A serine protease present in the culture medium was believed to be involved in Amyl I degradation. Amyl I had an extremely high optimal pH of 11.0 to 12.0 and was stable in a broad pH range of 6.0 to 13.0. Amyl I had an optimal temperature of 60°C and was stable to 50°C. Thermostability increased in the presence of Ca^{2+} and soluble starch.

A raw starch-degrading alkaline amylase of *Bacillus* sp. IMD 370 was also isolated by Kelly et al. (1995). The amylase of *Bacillus* sp. IMD 370 is the first report of an alkaline amylase with the ability to digest raw starch, and the enzyme digested raw corn starch to glucose, maltose, maltotriose and maltotetraose. The maximum pH for raw starch hydrolysis was pH 8.0, as opposed to pH 10.0 for soluble starch hydrolysis. It is of interest that degradation of raw starch was stimulated sixfold in the presence of β-cyclodextrin (17.5 mM).

Lin et al. (1998) then isolated a raw starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. The amylase was purified from culture supernatant of *Bacillus* sp. TS-23. The molecular mass of the purified amylase was estimated at 42 kDa by electrophoresis. The enzyme had a p*I* of 4.2. The optimal pH and temperature for activity were 9.0 and 70°C respectively. The thermoactivity of the purified enzyme was enhanced in the presence of 5 mM Ca^{2+}; under this condition, enzyme activity could be measured at a temperature of 90°C. The enzyme preferentially hydrolyzed high molecular-mass substrates with a α-1,4-glucosidic bond except glycogen. The raw starches were partly degraded by the purified amylase to yield predominantly oligosaccharides with degrees of polymerization 3, 4 and 5.

Lo et al. (2001a, b) purified TS-23 α-amylase (Accession Number U22045) to the homogeneous state from the culture medium of recombinant *E. coli.* The enzyme was stimulated by Mn^{2+} Co^{2+} and Fe^{2+} ions but was strongly inhibited by Hg^{2+} and Cu^{2+} and by the well-characterized inhibitors, diethylpyrocarbonate and N-bromosuccinimide. The enzyme was active in the presence of 8% sodium dodecyl sulphate (SDS). *Bacillus* sp. TS-

23 α-amylase was stable when it was preincubated with 6% SDS for up to 1 h at 30℃, while inactivation was observed at 60℃. Under optimal conditions, this enzyme was able to attack the α-1,4 linkages in soluble starch, amylose, amylopectin and glycogen to generate maltopentaose as the major end product.

Recently, Lin et al. (2003) reported replacement of methionine 208 in a truncated *Bacillus* sp TS-23 α-amylase with leucine enhanced its resistance to hydrogen peroxide. Wild-type enzyme was sensitive to chemical oxidation, but Met208Leu was stable even in the presence of 500 mM H_2O_2.

8.2 Maltohexaose-producing Enzymes

From the beginning of the 1970s, many bacterial strains which produce amylases catalyzing the degradation of starch to malto-oligosaccharides (Gn-amylase) have been isolated and the enzymes characterized.

8.2.1 G6-amylase of *Bacillus* sp. No. 707

In order to obtain hyperproducers of enzymes for industrial applications, an alkaliphilic bacterium, *Bacillus* sp. No. 707, a producer of Gn-amylase, was isolated from soil and the gene for maltohexaose-producing amylase, G6-amylase, was cloned (Kimura et al. 1988). An alkaliphilic bacterium, *Bacillus* sp. No. 707, produces at least five Gn-amylase components in Horikoshi-II medium. The gene for G6-amylase of the chromosomal DNA of *Bacillus* sp. No. 707 was cloned and expressed in *E. coli* and *B. subtilis*. The major hydrolysis product from soluble starch by the enzyme from *Bacillus* sp. No. 707 was G4. However, the product of the enzyme from transformed *B. subtilis* (pTUB81 2) and *E. coli* (pTUE306) was G6 and the content of G6 in the hydrolyzate was approximately 50–60%.

The nucleotide sequence of the gene for the G6-producing enzyme (Accession Number M18862) was determined to pinpoint the location of the enzyme gene (Tsukamoto et al. 1988). The deduced amino acid sequence of the extracellular mature enzyme was more than 60% homologous to those of the liquefying type α-amylases but not to those of the saccharifying type α-amylases. This suggests that the enzyme gene is derived from a common ancestor gene of other liquefying α-amylases. However, the signal peptide and N-terminal, which are essentially not responsible for the enzyme activity, are completely different from those of the other α-amylases.

8.2.2 G6-amylase of *Bacillus halodurans* H-167 Enzyme

Independently, Hayashi et al. (1988 a,b) isolated alkaliphilic *Bacillus halodurans* H-167 producing maltohexaose-forming enzymes in their culture broths.

Table 8.3 Morphological and biochemical properties of the isolated bacterium, strain H-167

Cells	Rods (2–3 μm × 0.4–0.7 μm)
Motility	Motile
Spores	Central
Sporangia	Swollen
Gram stain	Variable
pH for growth	pH 7–12
Temperature for growth	15–52°C
Catalase test	+
Oxidase test	+
Reduction of nitrate	+
V-P test	–
Utilization of citrate	–
Urease test	–
Production of indole	–
Growth in NaCl	Up to 12%
GC content	46.3%

Acid formation from carbohydrates

	Arabinose	Glucose	Xylose	Mannitol
Aerobic	+	+	+	–
Anaerobic	+	+	+	+

(Reproduced with permission from T. Hayashi et al., *Agric. Biol. Chem.*, **52**, 445 (1988))

The strain (Table 8.3) produced three α-amylases which yielded maltohexaose as the main product from starch. The optimum culture conditions for enzyme production were: initial medium, pH 9.4; culture temperature, 37°C; and 50–60 h cultivation under aerobic conditions. The enzymes (H-I-1, H-I-2, and H-II) were separated completely and purified to homogeneity. The optimum and stable pH values of amylase H-I-1 are shown in Fig. 8.1. No distinct differences were found either in the pH activity curves or pH stability curves among H-I-1, H-I-2, and H-II. All enzymes were most active at pH 10.5 and stable in the range pH 7.5–12.0 on standing at 50°C. Other properties are shown in Table 8.4, and all enzymes produced maltohexaose in the early stage of hydrolysis, as shown in Fig. 8.2. The maximum yield was about 25% to 30%.

Shirokizawa et al. (1989; 1990) cloned the gene of G6-amylases of *B. halodurans* H-167 and expressed it in the *E. coli* system. A plasmid (pSB404) having an insert of approximately 3.0 kb DNA, which was responsible for G6-enzyme, was isolated.

HPLC analysis showed that the component of the hydrolysis products with the amylase of *E. coli* HB101 (pSB404) maltohexaose was formed as the main product from starch, and minor peaks of maltotetraose and maltose were also observed. The pH activity profile of the plasmid-borne amylase was essentially the same as that of the amylases from *B. halodurans* H-167 (Fig. 8.3). After SDS-PAGE, three bands having amylase activity were detected for the supernatant of *E. coli* HB101 (pSB404). The molecular

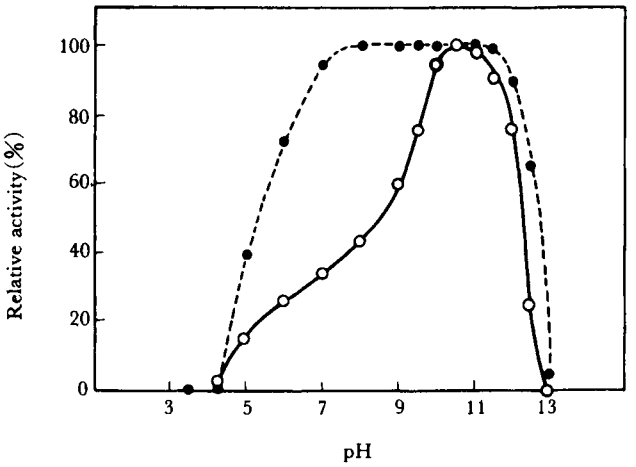

Fig. 8.1 Effect of pH on activity and stability of the amylase H-I-1. Each pH was obtained with the following buffer system: acetate buffer, pH 3.5–6.0; Tris-HCl buffer, pH 7.0–8.0; glycine-NaOH buffer, pH 9.0–11.5; KCl-NaOH buffer, pH 12.0–13.0. —○— pH activity curve; . ····●···· pH stability curve.
(Reproduced with permission from T. Hayashi et al., *Appl. Microbiol. Biotechnol.*, **28**, 283 (1988))

Table 8.4 Properties of three amylases (H-I-1, H-I-2, and H-II) from alkaliphilic *Bacillus* H-167

Property	H-I-1	H-II-2	H-II
Molecular weight	73,000	59,000	80,000
Isoelectric point	4.1	3.5	4.3
Optimum pH	10.5	10.5	10.5
Optimum temperature	60°C	60°C	60°C
pH stability	7–12	7–12	7–12
Heat stability	55°C	55°C	55°C
K_m for amylose (DP 17)	0.35 mM	0.43 mM	0.40 mM
Inhibitors		Hg^{2+}, Zn^{2+}, Pb^{2+}, Co^{2+}, Ni^{2+}	

(Reproduced with permission from T. Hayashi et al., *Appl. Microbiol. Biotechnol.*, **28**, 284 (1988))

weights were about 90,000, 73,000 and 60,000. The 3.0 kb DNA fragment inserted in pSB404 is clearly too short to encode the three amylase genes. Multiple enzymes are usually generated through the following mechanisms: (i) multiple genes, (ii) proteolytic processing of enzymes inside the cell or (iii) multiple transcription of a gene. The cultivation of *B. halodurans* H-167 in the presence of an inhibitor (antpain, 1 mg/ml) indicated that the 73-kDa and 60-kDa amylases were the products of proteolytic degradation of the 90-kDa amylase. Thus mechanism (ii) is the most likely for the formation of multiform G6-amylases. Recently, Takami et al. (2000) determined the entire DNA sequence of *B. halodurans* C-125, and its G6-formine en-

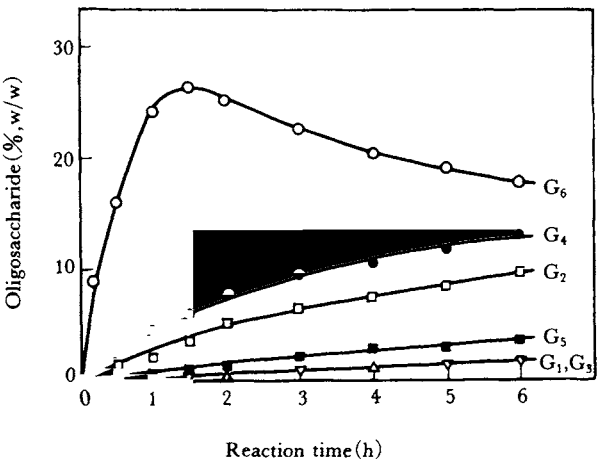

Fig. 8.2 Time course of hydrolysis of starch by enzyme H-I-1. One ml of 5% soluble starch in 20 mM glycine-NaOH buffer (pH 10.5) was incubated at 50°C with 1.0 U of purified enzyme H-I-1. Symbols: —▽—, glucose (G_1); — —, maltose (G_2);—△—, maltotriose (G_3); —●—, maltotetraose (G_4); —■—, maltopentaose (G_5); —○—, maltohexaose (G_6).
(Reproduced with permission from T. Hayashi et al., *Agric. Biol. Chem.*, **52**, 447 (1988))

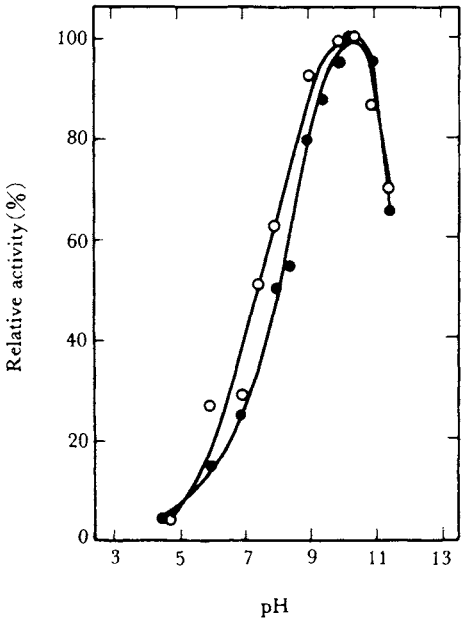

Fig. 8.3 pH activity of the amylases. ○: *Bacillus*; ●: plasmid-borne.
(Reproduced with permission from T. Hayashi et al., *Agric. Biol. Chem.*, **52**, 447(1988))

zyme exhibits very high homology to that of *B. halodurans* H-167, although several microbial properties of these strains are different. Furthermore, no homology was exhibited between the DNA sequence of the maltohexaose-forming enzyme of *Bacillus* sp. No. 707 and ours except for the A, B, and C domains found in amylases. These results indicate that our G6-forming amylase is different from that previously reported (Kimura et al. 1988).

8.3 Pullulan-degrading Enzymes

Pullulan, a polysaccharide originally produced by the yeast *Pullularia pullulans*, is composed of linear chains of maltotriose units attached by an α-1,6-linkage. Pullulanase, which causes cleavage of the α-1,6-links of pullulan, is distributed in several bacteria.

The demand for liquefying α-amylase for use in laundry and automatic dishwashing detergents has been growing for several years. Detergent enzymes must be able to function in washing machines or dishwashers under conditions that are very unfavorable for the stability of the enzyme. The pH is high alkaline in washing conditions. The high temperature (55–60°C) in a dishwasher requires thermostable enzymes. Enzymes are preferentially resistant to various detergent ingredients, such as sufactants, chelators and oxidants. However, most bacterial liquefying α-amylases, such as *Bacillus amyloliquefaciens* (BAA) and *Geobacillus stearothermophilus* (BSA), including *Bacillus licheniformis* (BLA), have optimal pH of between 5 and 7.5. Therefore, they are not practically suitable for use in laundry and dishwashing detergents with high alkalinity.

8.3.1 Pullulanase of *Bacillus halodurans* 202-1

In 1975, while screening alkaline amylases from alkaliphilic *Bacillus* strains (Nakamura et al. 1975), discovered that an alkaliphilic bacterium, *Bacillus halodurans* 202-1, produced an extracellular pullulanase in an alkaline medium of pH 10 (1975). The isolate did not grow at neutral pH. The pullulanase from strain No. 202-1 was purified 290-fold by DEAE cellulose adsorption at pH 8.0, precipitated with 75% saturated acetone and with 80% saturated $(NH_4)_2SO_4$, column chromatography on DEAE cellulose, and gel filtration on a Sephadex G-200. The purity of the enzyme was confirmed by disc gel electrophoresis. The molecular weight of the alkaline pullulanase was 92,000, and the isoelectric point below 2.5. The enzyme has an optimum pH at 8.5–9.0 and is stable for 24 h at pH 6.5–11.0 at 4°C. It is most active at 55°C, and is stable up to 50°C for 15 min in the absence of substrate. The enzyme is inhibited by Hg^{2+} and Zn^{2+}, but not by sulfhydryl reagents or by chelating agents such as EDTA. The hydrolysis of amylopectin (from rice) and glycogen (from oysters) by alkaline pullulanase followed by γ-amylase resulted in complete degradation of these substrates, while hydrolysis

by γ-amylase alone resulted in partial degradation. This was the first finding of an alkaline pullulan-degrading enzyme, and paved the way for the discovery of a new alkaline pullulanase for industrial applications such as detergent additives.

8.3.2 Pullulanase of Alkaliphiles Isolated by Kao Corporation's Group

In screening alkaline cellulases for detergent additives, Ara et al. (1992) and Igarashi et al. (1992) isolated a novel alkaline pullulanase from alkaliphilic *Bacillus* sp. KSM-1876, which was identified as a relative of *Bacillus circulans*. The enzyme had an optimum pH for enzyme action of around 10.0–10.5, the highest pH for optimum pullulanase activity. This enzyme is a good candidate for use as detergent additives in dish washers, especially to remove starch from dishes in the presence of detergents. The gene (Accession Number AB049812) was isolated from KSM-1876. However, the plasmid-borne enzyme was not thermostable and not alkaline pullulanase, having an optimum pH of 7.5. The enzyme hydrolyzed pullulan 3.0-fold faster than soluble starch, but hydrolyzed amylose and amylopectin less efficiently. Therefore, the gene cloned was not the major alkaline pullulanase gene, but a minor one.

Ara et al. isolated new alkaliphilic *Bacillus* sp. KSM-1378 and reported two independent active sites for the α-1,4 and α-1,6 hydrolytic reactions (1995; 1996; Hatada et al. 1996). Alkaliphilic *Bacillus* sp. KSM-1378 produces an alkaline amylopullulanase that hydrolyzes both α-1,4 linkages in amylose, amylopectin and glycogen, and α-1,6 linkages in pullulan. The enzymatic properties, improvement in thermostability and oxidative stabilization of AmyK (Hagihara et al. 2001a, b), were extensively studied and reported.

A. Enzymatic properties of AmyK

The homogeneous preparation of AmyK had a specific activity of 5,000 units (U)/mg at 50°C and at pH 8.5 in 50 mM Tris-HCl buffer. The molecular mass of the purified enzyme was approximately 53 kDa by SDS-PAGE. The isoelectric point was around pH 9. When AmyK was preincubated with 10 mM EDTA or EGTA, its activity decreased to 10% or 9% of the initial activity, respectively. AmyK was resistant to incubation at 40°C for 1 h with various sufactants, such as SDS, polyoxyethylene alkyl ether, sodium α-sulfonated fatty acid ester, linear alkylbenaene sulfonate and alkyl glucoside (each added at 0.1% w/v). Moreover, the enzyme is unique in that at the early stage of hydrolysis of soluble starch, the enzyme produced G5 to G9 in quantities much larger than G2 and G3. G1 and G4 formed at this stage were trace. On further incubation, G3 and G5 increased while G6 to G8 decreased. These results indicate that AmyK is classified as a liquefying coamylase.

As shown in Fig. 8.4, the maximum activity was observed around pH 8.0–9.0 The enzyme was stable over a range between pH 6 and pH 10.

The optimal temperature for activity was around 55°C at pH 8.5 in 50 mM Tris-HCl buffer (Fig. 8.5a), while that of BLA was around 80°C. At 80°C, the specific activities of BLA and AmyK were almost equal, and they were approximately 25% of the maximum activity of AmyK observed at 55°C. The time course of the thermal inactivation of AmyK was followed at various temperatures and at pH 8.5 in 20 mM Tris-HCl buffer (Fig. 8.5b). The enzyme retained its full activity after 60 min of incubation at 45°C, but only 32% and 30% of original activity remained after 60 min of incubation at 50°C and 60°C, respectively. However, in the presence of 0.1 mM $CaCl_2$, nearly 100% and 65% of the original activity remained at 50°C and 60°C, respectively. The enzymatic activity was eliminated after heating at 70°C for 60 min even in the presence of 0.1 mM Ca^{2+} ions. In contrast, BLA was quite stable to incubation at 70°C at least up to 60 min under their test conditions. Deduced amino acid sequence of AmyK showed moderate homology to those of α-amylases from *Bacillus licheniformis* (Hagihara et al. 2001b). The entire gene for AmyK was cloned and sequenced (Accession Number AB008763). It contained an open reading frame of 1545 bp encoding 515 amino acids including a putative signal peptide of 31 amino acids. The molecular mass of the mature enzyme was 55,391 Da. The deduced amino se-

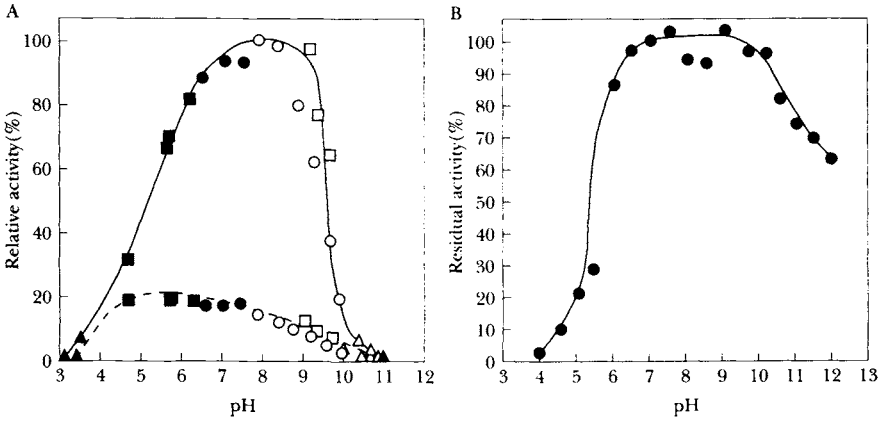

Fig. 8.4 Effect of pH on the activity and stability of AmyK. **A**. The pH-activity curves of purified AmyK and BLA (each at 0.2 U/ml) are shown by solid and dotted lines, respectively. The buffers used (50 mM each) were as follows: glycine-HCl, pH 3.0–3.5 (▲); acetate, pH 4.0–6.0 (■); Tris-HCl, pH 6.5–8.5 (●); glycine-NaOH, pH 9.0–10.5 (□); glycine-NaCl-NaOH, pH 8.0–10.5 (○); carbonate, pH 10.0–11.0 (△). The values are shown as percentages of the maximum specific acitivity of AmyK observed at pH 8.0–8.5, which is taken as 100%. **B**. To assess the pH stability of AmyK, the enzyme (2.0 U/ml) was preincubated at the indicated pH in 10 mM Britton-Robinson buffer and at 40°C for 30min, and then samples (0.1ml) were used for the measurements of the residual activity under the standard conditions of enzymatic assay. The values are shown as percentages of the original activity, which is taken as 100%.
(Reproduced from S. Ito and K. Horikoshi, *J. Biol. Macromol.*, **4**, 3(2004))

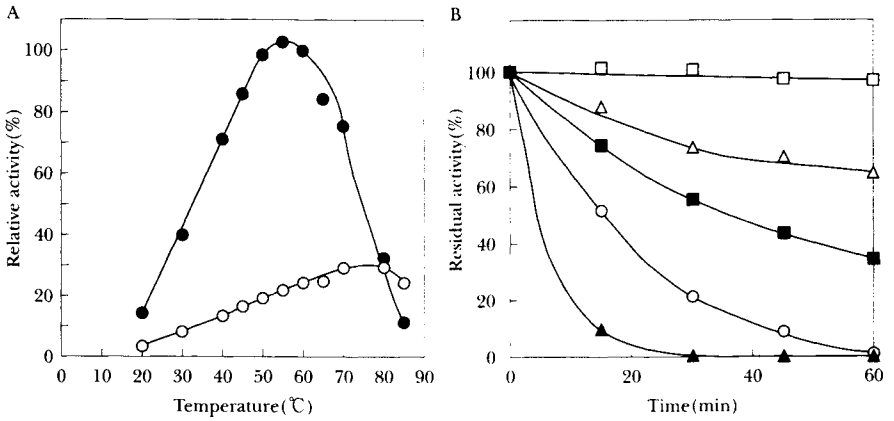

Fig. 8.5 Effect of temperature on the activity and stability of AmyK. A. The temperature-activity curves of purified AmyK (●) and BLA(○)(each at 0.18 U/ml) are shown. The reactions were done at the indicated temperatures for 10 min and at pH 8.5 in 50 mM Tris-HCl buffer. The values are shown as percentages of the maximum activity of AmyK observed at 55℃, which is taken as 100%. B. To assess the thermostability of AmyK, the enzyme (1.8 U/ml) was heated at the indicated temperatures in the presence at 50℃(□), 60℃(△), and 70℃(○)), or in the absence at 50℃(■) and 60℃(▲)of 0.1 mM Ca^{2+} ions in 50 mM Tris-HCl buffer(pH 8.5). Samples (0.1 ml) were used for the determination of the residual activity under the standared conditions of enzyme assay.
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quence of AmyK did not exhibit high homology to those of saccharifying α-amylases reported so far. Four conserved regions, which are necessary for the catalyiic activity of cc-amylases were conserved in AmyK as Aspl02 to Hisl07 (region I), Gly232 to His240 (region II), Glu266 to Lys269 (region m), and Phe328 to Asp333 (regions IV). They form the active center, the substrate binding sites, and the Ca^{2+} binding sites. In order to increase production of the enzyme, they used an excretion vector pHSP64 with *B. subtilis* as the host. The transformed *B. subtilis* produced the AmyK activity, corresponding to approximately 1.0 g protein/liter of an optimized liquid culture.

B. Improvement of the thermostability of AmyK

It is very interesting that the original amino acid sequence of AmyK conserves the corresponding amino acid residues at amino acids 135 (Tyr) and 214 (Ile), respectively. Van der Laan (1991) and Aehle et al. (1995) showed that two substitutions, Val128Glu/His133Tyr or His133Tyr/Asnl88Asp, improved the thermostability of BLA in the presence of excess $CaCl_2$ (1.5 mM). Again, the unique amino acid sequence of AmyK is emphasized in that the substituted residues in BLA are conserved as Tyrl35 and Glul30 in the enzyme (although the substituted Aspl88 corresponds to lle193). Therefore, there may be another unidentified structural element(s) respon-

sible for the thermostabilization of AmyK, if its thermostability could be further improved as high as that of BLA or the engineered BAA.

It was also reported that an increase in hydrophobicity by changing amino acid residues enhanced the thermostability of this enzyme. Machius et al. (1998) suggested that the loop containing the Arg-Gly residues in BM has two more amino acid residues than BLA and that this could cause increased mobility of this region and decreased thermostability of the whole protein. In fact, the amino acid sequences of AmyK, BAA and *Geobacillus stearothermophilus* (BSA) conserved the corresponding Arg-Gly residues at the respective amino acid positions. However, substituted Ala269 in *Bacillus macerans* (BM) corresponds to Ala274 for AmyK and Ala269 for BLA in their original amino acid sequences.

Therefore, Igarashi et al. (1998) deleted Arg181-Gly182 residues from the AmyK molecule by site-directed autogenesis. The dipeptide-deleted mutant protein (dRG) became much more thermostable, as shown in Fig 8.6.

Furthermore, double mutation with Arg124Pro and dRG made the enzyme much more thermostable as shown in Fig. 8.7. This was the first report that thermostability of α-amylase was improved by enhanced calcium binding to the enzyme molecule.

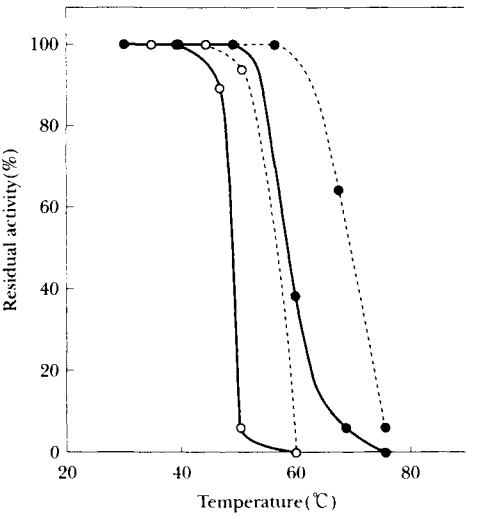

Fig. 8.6 Comparison of the thermostability of AmyK and dRG. To assess the thermostability of AmyK and dRg, the enzymes (2.0 U/ml) were individually heated for 2 h at the indicated temperatures in the absence or presence of 2.0 mM $CaCl_2$ in 50 mM Tris-HCl buffer (pH 8.5). Samples were transferred after 2 h to wet ice (0℃) and the residual activity in them (0.1-ml aliquot) was assayed by measuring the rate of soluble starch hydrolysis under the standard conditions of the assay. The residual activities of AmyK(○) and dRG(●) in the absence (solid lines) or presence of $CaCl_2$ (dotted lines) are expressed as percentages of the respective original activities, which are each taken as 100%.
(Reproduced with permission from K. Igarashi et al., *Biochem. Biophys. Res. Commun.*, **248**, 372(1998))

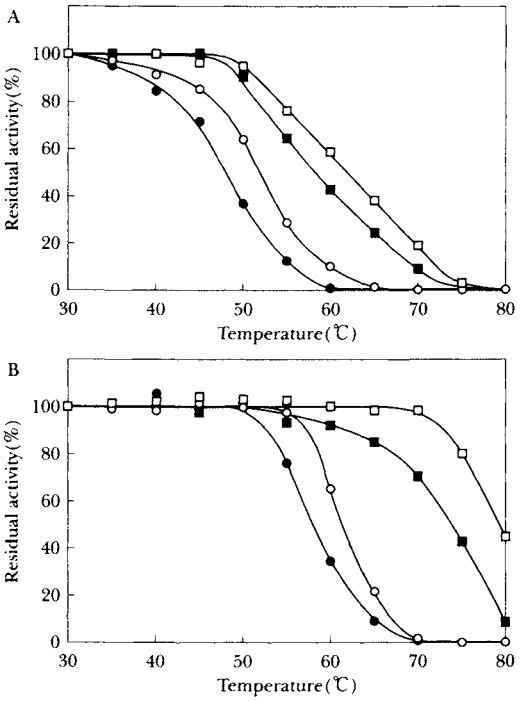

Fig. 8.7 Effects of temperature on the stability of wild-type AmyK(●), R124P(○), dRG(■), and dRG-R124P(□). Each enzyme (0.04 μg/ml) was heated for 10 min at different temperatures in 10 mM Tris-HCl buffer(pH 8.5) without (A) and with (B) 0.1 mM $CaCl_2$, and assayed for the activity remaining. The activity of each enzyme after treatment at 30℃ is taken as 100%.
(Reproduced with permission from K. Igarashi et al., *Biosci. Biotechnol., Biochem.*, **63**, 1535(1999))

C. Stabilization of AmyK in the presence of H_2O_2

In the AmyK molecule, there are 10 Met residues at positions 9, 10, 105, 116, 202, 208, 261, 309, 382 and 430, as deduced from its nucleotide sequence. The chemical oxidant-resistant AmyK was made mainly by replacing Met202 with non-oxidizable amino acid residues, such as Thr, Leu. Ile, Ser and Ala. The mutated enzymes exhibited strong stability in 10 mM Tris-HCl buffer (pH 8.5) containing 2% H_2O_2 and 2 mM $CaCl_2$ at 30℃ for up to 60 min (Fig. 8.8).

Recently, Ca^{2+}-free α-amylase, AmyK38 (Accession Number AB051102) was discovered from an alkaliphilic *Bacillus* sp. KSM-K38 that is highly resistant to chelating reagents and chemical oxidants and requires Na^+ for activity. A methionine residue, which is conserved and susceptible to chemical oxidation, was replaced with leucine in AmyK38 (Hagihara et al. 2001a,b). Moreover, many conserved residues that are crucial ligands for Ca^{2+} were replaced with other amino acids, thereby leading to loss of the Ca^{2+} coordination of geometries. By building a molecular model, they showed the cal-

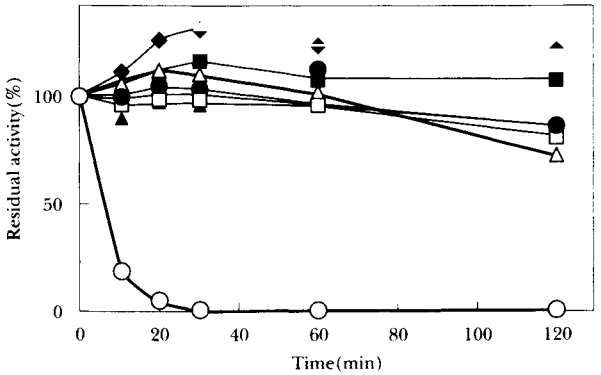

Fig. 8.8 Rates of oxidative inactivation by H_2O_2 of the wild-type and mutant enzymes. Each enzyme was incubated in the presence of 0.5 M H_2O_2 at 30°C and at pH 8.9 in 50 mM Tris-HCl buffer. Timed samples were withdrawn and mixed with 140 μg/ml catalase to quench excess H_2O_2, and the residual activities were measured under the standard conditions of enzyme assay. The original activity of each enzyme is taken as 100%. The wild-type and mutant enzymes retained full activities during the incubation without H_2O_2 up to 2 h. Symbols used are: ○, wild-type AmyK; ◆, Met202Ala; ▲, Met202Ser; ■, Met202Val, □, Met202Leu; ●, Met202Ile; △, met202Thr.
(Reproduced with permission from H. Hagihara et al., *Eur. J. Biochem.*, **268**, 3974(2001))

cium-independent, oxidatively stable active-site topology and structural integrity of AmyK-38.

As a result, their experiments clearly indicated that the deletion of Arg181-Gly182 or Thr183-Gly184 on a loop of domain B in AmyK caused enhanced thermostability and chelator resistance, and that the enzyme can also be thermostabilized by replacing proline for Arg124 on a loop region in domain B. The double mutant enzyme is suitable for practical use at high temperatures and high chelator concentrations under high alkalinity. α-Amylase is very promising for use not only in the starch and food industries but also in the detergent industry.

8.3.3 Other Alkaline Pullulanases

For industrial applications two highly alkaliphilic bacteria and potent producers of alkaline pullulanase were isolated from Korean soil (Kim et al. 1993a,b). The two isolates, identified as *Bacillus* sp. S-1 and *Micrococcus* sp. Y-1, grow on starch under alkaline conditions and effectively secrete extracellular pullulanases. The two isolates were extremely alkaliphilic since bacterial growth and enzyme production occurred at pH values ranging from pH 6.0 to 12.0 for *Micrococcus* sp. Y-1 and pH 6.0 to 10.0 for *Bacillus* sp. S-1. Both strains secrete enzymes that possess amylolytic and pullulanolytic activities. Extracellular crude enzymes of both isolates gave maltotriose as the major product formed from soluble starch and pullulan hydrolysis. The ex-

tracellular enzymes of both bacteria were alkaliphilic and moderately thermoactive; optimal activity was detected at pH 8.0–10.0 and between 50 and 60°C. Even at pH 12.0, 65% of original Y-1 pullulanase activity and 10% of S-1 pullulanase activity was retained. The purified enzyme S-1 had a molecular mass of about 140 kDa under denatured and natural conditions. The pI was 5.5. Kim and Kim (1995) then analyzed substrate specificity of a bifunctional pullulanase of alkaliphilic *Bacillus circulans* F-2. *Bacillus circulans* F-2 amylase-pullulanase enzyme (APE) displayed dual activity with respect to glycosidic bond cleavage. The enzyme was active on α-1,6 bonds in pullulan, amylopectin and glycogen, while it showed α-1,4 activity against maltooligosaccharides, amylose, amylopectin and soluble starch, but not pullulan. Kinetic analysis of the purified enzyme in a system which contained both pullulan and amylose as two competing substrates was used to distinguish the dual specificity of the enzyme from the single-substrate specificity known for pullulanases and α-amylases. Enzyme activities were inhibited by some metal ions, and by metal-chelating agents with a different mode. The enzyme-inhibitory results of amylase and pullulanase with Hg^{2+} and Co^{2+} ions were different, indicating that the activation mechanisms of the two enzyme activities are different. Cyclomaltoheptaose inhibited both α-amylase and pullulanase activities. The Accession Number of F2 is M16657. When both amylose and pullulan were simultaneously present, the observed rate of product formation closely fitted a kinetic model in which the two substrates were hydrolyzed at different active sites. These results suggest that the F-2 enzyme is a kind of amylopullulanase which possess both α-1,6 and α-1,4 cleavage activities at the same active site.

Lee et al. isolated a thermophilic and alkaliphilic *Bacillus* sp. strain, XAL601, from soil (1994). It produces a thermostable and alkaline-stable enzyme with both α-amylase and pullulanase activities. The α-amylase-pullulanase gene (*aapT*) from this *Bacillus* strain was cloned and its nucleotide sequence determined: A very large open reading frame composed of 6,096 bases, which encodes 2,032 amino acid residues with an M(r) of 224,992, was found (Accession Number D24467). The deduced amino acid sequence revealed that the four highly conserved regions that are common among amylolytic enzymes were well conserved. These include an active center and common substrate-binding sites of various amylases. In the C-terminal region, a six-amino-acid sequence (Gly-Ser-Gly-Thr-Thr-Pro) is repeated 12 times. The *aapT* gene was then subcloned in *E. coli* and over expressed under the control of the *lac* promoter. Purification of AapT from this recombinant *E. coli* was performed, and it was shown that the aapT gene product exhibits both α-amylase and pullulanase activities in one active site. The optimum temperature and pH for enzyme activity were found to be 70°C and pH 9, respectively. Furthermore, AapT was found to adsorb strongly to crystalline cellulose (Avicel) and raw corn starch. Final hydrolyzed products from soluble starch ranged from maltose (G2) to maltotetraose (G4). Only maltotriose (G3) was produced from pullulan. The

enzyme also hydrolyzes raw starch under a broad range of conditions (60 to 70°C and pH 8 to 9).

8.4 Cyclodextrin (CD) Forming Enzymes

The cyclodextrins, Schardinger dextrins, are a group of homologous oligosaccharides obtained from starch by the action of cyclomaltodextrin glucanotransferase (CGTase). These compounds are well known by their unique properties as follows: (1) Cyclodextrins (CD) are homogeneous cyclic molecules composed of six or more glucose units linked α-1,4 as in amylose; (2) as a consequence of cyclic arrangement they have neither a reducing end-group nor a nonreducing end-group and are not decomposed by hot aqueous alkali; (3) they are rather resistant to acid hydrolysis and the common starch-splitting amylases; (4) they crystallize very well from water and from aqueous alcohols; (5) they form an abundance of crystalline complexes called inclusion compounds with organic or inorganic substances. The properties of cyclodextrins are shown in Table 8.5. Mass production of these unique compounds on an industrial scale has been attempted several times in the past. However, there were serious problems in the production processes. (1) CGTase from *Bacillus macerans* is not suitable for industrial use because the enzyme is not thermostable enough. (2) Yield of CD from starch is not high, usually 20% to 30% on an industrial scale. (3) Toxic or-

Table 8.5 Properties of cyclodextrins

	α-CD	β-CD	γ-CD
Number of glucose units	6	7	8
Molecular weight	973	1,135	1,297
Cavity diameter	5–6Å	7–8Å	9–10Å
Cavity depth	7–8Å	7–8Å	7–8Å
Crystal from (from H_2O)	Needle	Prism	Prism
$[\alpha]^{25}D(H_2O)$	+ 150.5°	+ 162.5°	+ 177.4°
Solubility (g/100 ml·H_2O, 25°C)	14.5	1.85	23.2

Table 8.6 Some properties of various CGTases

	Optimum pH	Optimum temp.	Molecular weight	p*I*	CDs composition from starch	Yield (%)
Bacillus macerans	5.0–5.7	55°C	65,000	4.6	α-CD>β-CD>γ-CD	50
Klebsiella pneumoniae	6-7	35–40	–	–	α-CD>β-CD>γ-CD	50–60
Bacillus stearothermophilus	6.0	70	68,000	4.5	β-CD≧α-CD>γ-CD	50
Bacillus megaterium	5.2–6.2	55	66,000	6.1, 6.8	β-CD>α-CD>γ-CD	62
Bacillus circulans	5.2–7.2	55	–	5.8, 6.6	β-CD>α-CD>γ-CD	–
Alkaliphilic *Bacillus*						
No. 38-2	5–9	65–70	75,160	5.4	β-CD>γ-CD>α-CD	75–85
No. 17-1	5–9	60	74,140	–	β-CD>γ-CD>γ-CD	70

ganic solvents such as trichloroethylene, bromobenzene and toluene. were used to precipitate CD due to the low conversion rate (Table 8.6). The use of such harmful organic solvents is strongly prohibited in various fields, especially food processing. Therefore, the development of large-volume use was quite limited. In 1968, author's group isolated several CGTases from alkaliphilic *Bacillus* strains. One of them, a CGTase produced by alkaliphilic *Bacillus* sp. No. 38-2, overcame all these weak points and we succeeded in mass-producing crystalline α-, β-, γ-CD and CD-mixture at low cost without using any organic solvents.

8.4.1 Analysis of CDs

CDs in the reaction fluid are easily separated from each other by high performance liquid chromatography (HPLC), and the contents of these CDs are calculated with a refractive index detector. However it takes a long time to screen γ-CD- producing enzymes from microorganisms. A simple colorimetric determination method for γ-CD was developed by Kato and Horikoshi (1984). One milliliter of sample solution (up to 700 μg as γ-CD) is mixed with 0.1 ml of Bromocresol green (BCG) (5 mM) and 2 ml of 0.2 M citrate buffer (pH 4.2), and the absorbance is measured at 630 nm. Addition of various carbohydrates, such as α-, β-CDs, glucose and soluble starch, did not cause significant error, as shown in Table 8.7.

Table 8.7 Effect of various carbohydrates on the BCG method

γ-CD	α-CD	β-CD	Glucose	Soluble starch	Amount found, μg	% Error †
440	0	0	0	0	440	
440	200	0	0	0	430	− 2.3
440	400	0	0	0	470	+ 6.8
440	4000	0	0	0	460	+ 4.5
440	0	200	0	0	440	0
440	0	400	0	0	460	+ 4.5
440	0	4000	0	0	450	+ 2.3
440	0	0	200	0	450	+ 2.3
440	0	0	400	0	440	0
440	0	0	4000	0	450	+ 2.3
440	0	0	0	200	440	0
440	0	0	0	400	450	+ 2.3
440	0	0	0	4000	460	+ 4.5
440	200	200	0	0	440	0
440	400	400	0	0	440	0
440	4000	4000	0	0	410	− 6.8
440	400	400	400	0	460	+ 4.5
440	200	200	200	200	430	− 2.3
440	400	400	400	400	460	+ 4.5
440	4000	4000	2000	2000	440	0

† Error (%) = (found/(γ-CD − 1)) × 100.

8.4.2 Purification and Properties of No. 38-2 and No. 17-1 Enzymes

Two strains, *Bacillus* sp. No. 38-2 and No. 17-1,were selected as the best enzyme producers from starch degrading-alkaliphilic strains (Nakamura and Horikoshi 1976a, b, c, d;). These bacteria were aerobic, spore-forming, motile and rod-shaped. All the strains belong to the genus *Bacillus*. Further properties on taxonomy are listed in Section 2.3. The organisms were aerobically cultivated for three days in Horikoshi-II medium at 37°C. The crude enzymes in the supernatant fluids were purified by starch adsorption followed by conventional DEAE cellulose column chromatography.

The crude enzyme in the culture broth of *Bacillus* sp. No. 38-2 was a mixture of three enzymes: acid CGTase having optimum pH for enzyme action at 4.6; neutral CGTase, pH 7.0 and alkaline CGTase, pH 8.5, as shown in Table 8.8. Southern blot hybridization experiment showed that only one band hybridized with *Bacillus* sp. No. 38-2 CGTase gene. The crude enzyme of *Bacillus* sp. No. 17-1 was a mixture of two enzymes: acid CGTase, optimum pH 4.5, and alkaline CGTase, optimum pH 9.5 (Horikoshi, unpublished data).

Since Nakamura and Horikoshi discovered and isolated the bacterium alkaliphilic *Bacillus* sp. No. 38-2, many alkaliphilic microorganisms producing CGTases have been reported. Nomoto et al. (1984a; 1986) found a CGTase produced by alkaliphilic *Bacillus* sp. No. HA3-3-2 isolated from soil from Taipei, Taiwan. The enzyme showed maximum CD-forming activity in the pH range of 6.5 to 8.0 and was stable between pH 6 to 11. Abelyan et al. (1994a, b) developed an isolation method for CGTase using cyclodextrin polymers and their derivatives. CGTases were directly purified from culture broth of mesophilic, thermophilic and alkaliphilic bacilli by affinity chromatography on a β-CD polymer. The enzymes were further purified by conventional methods such as gel filtration and DEAE cellulose column chromatography. The source of microorganisms has been expanded to include the deep sea from the surface of the earth. Georganta et al. (1993) isolated

Table 8.8 Properties of cyclodextrin glycosyltransferases from alkaliphilic *Bacillus* sp. No. 38-2

Property	Acid CGT	Neutral CGT	Alkaline CGT
Optimum pH	4.5–4.7	7.0	8.0–9.0
Optimum temperature(°C)	45	50	
Molecular weight	88,000	85,000–88,000	85,000–88,000
Isoelectric point	5.4		5.4
Stable pH [†1]	6.0–10.0	6.0–9.0	
Stable temperature [†2] (°C)	Up to 65	Up to 60	
Predominant product	β-Cyclodextrin	β-Cyclodextrin	β-Cyclodextrin

[†1] For 30 min at 60°C.
[†2] Treated for 30 min.
(Reproduced from K. Horikoshi and T. Akiba, *Alkalophilic Microorganisms*, p.107, Springer: Japan Scientific Societies Press (1982))

CGTase-producing psychrophilic alkaliphilic bacteria from deep-sea bottom mud samples. Isolates No. 3-22 and 1-7 were identified as an alkaliphilic *Bacillus* sp. The isolate No. 3-22 grew at 4℃, whereas No. 1-7 did not. The crude enzymes of both strains showed activity in both broad temperature and broad pH (5–9) ranges. No. 3-22 CGTase produced predominantly β-CD, and minor products were α- and γ-CDs. However, No. 1-7 enzyme produced β-CD as the main product and a small amount of α-CD. No α-CD was detected in the hydrolyzate (Table 8.9).

Hamamoto et al. (1987) and Kaneko et al. (1988) have characterized a CGTase of alkaliphilic *Bacillus* sp. No. 38-2. This is a unique enzyme, especially in having wide pH optimum, pH or thermal stability and high produc-

Table 8.9 Characteristics of isolates

Shape	No. 1-7 rod	No. 3-22 rod
Size (μm)	0.5–0.7 × 2.5–3.0	0.7–0.8 × 3.0–5.0
Spores	+	+
Ellipsoid	+	+
Round	–	–
Swelling of the sporangium	–	–
Gram reaction	+	+
Catalase	+	+
Anaerobic growth	–	+
VP reaction	–	–
Maximum temperature		
growth positive at (℃)	37	40
growth negative at (℃)	40	45
Growth in		
medium pH 5.7	–	–
NaCl 5%	+	–
7%	+	–
10%	+	–
Acid from		
glucose	–	+
L-arabinose	–	+
xylose	–	+
mannitol	–	+
Gas from glucose	–	–
Lecithinase	+	–
Hydrolysis of		
starch	+	+
gelatin	+	+
casein	–	n.d.
Use of		
citrate	+	–
propionate	–	–
Decomposition of tyrosine	–	–
Nitrate to nitrite	+	–
Indol	–	–
Phenylalanine deaminase	–	+
Arginine dihydrolase	–	–

n.d. : not done

tivity of CD from starch, compared with other microbial CGTases. Conventional purification methods showed that the crude enzyme preparation contained three enzymes: acid-, neutral- and alkaline-CGTase. Therefore, it is very interesting to investigate the genetic information of the enzyme(s) using gene cloning methods.

The CGTase gene of alkaliphilic *Bacillus* sp. No. 38-2 was cloned in *E. coli* and expressed (Accession Number D00129). A CGTase gene of *Bacillus* sp. No. 17-1 was cloned and sequenced. As shown in Fig. 8.9, chimeric experiments between No. 17-1 CGTase and No. 38-2 CGTase revealed that the pH-activity profile was affected by the C-terminal region of the protein molecule (Kaneko et al. 1989). Chimeric enzymes that contained the N -terminal and the C-terminal segments derived from CGTase 38-2 produced large amounts of CD and, in particular, a higher proportion of α-CD than other chimeric enzymes.

Why were many CGTases detected in the culture fluid of *Bacillus* sp. 38-2? We have not yet conducted any definitive experiment but the following hypothesis is highly possible. (1) During cultivation, the CGTase must be processed by protease(s) and processed protein molecules formed. (2) These protein molecules must have different thermal stabilities under specified pH values. (3) Therefore, these CGTases show different pH optima for

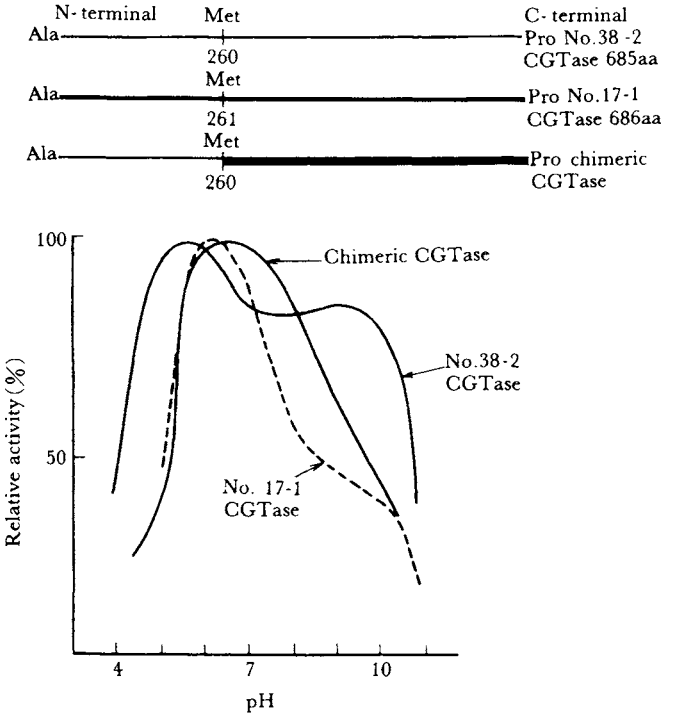

Fig. 8.9 pH activity curves No. 38-2, No. 17-1 and chimeric CGTases.

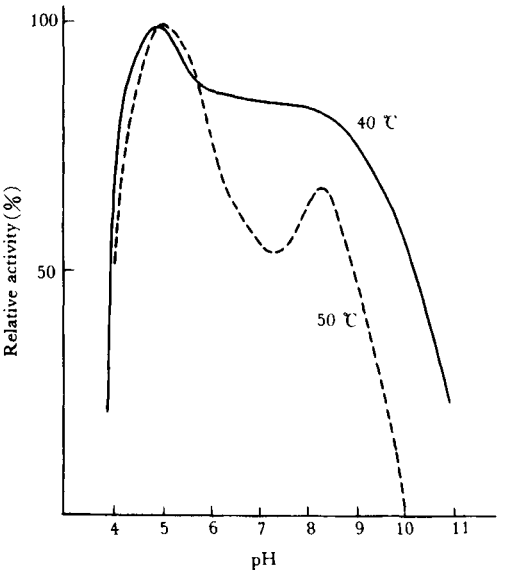

Fig. 8.10 pH activity curves at 40°C and 50°C.

enzyme action. Actually the CGTase produced by *E. coli* carrying pSC8 exhibited different pH activity curves under different incubation temperatures, as shown in Fig. 8.10 (Kaneko, unpublished data). These results strongly indicate that alkaliphilic *Bacillus* sp. No. 38-2 chromosomal DNA has one CGTase gene which produces one protein molecule. This protein molecule is probably processed during the cultivation or secretion process.

8.4.3 CGTase of *Bacillus* sp. No. 1011

Kimura et al. (1987a,b) cloned the gene (Accession Number M17366) for β-CGTase from an alkaliphilic bacterium, *Bacillus* sp. No. 1011, in an *E. coli* phage β-D69. This alkaliphilic bacterium produces extracellular β-CGTase. The structural gene for the β-CGTase consisted of 2,139 bp (713 amino acids with a total molecular weight of 78,339). The extracellular β-CGTase of *Bacillus* sp. No. 1011 had Ala-Pro-Asp at the N-terminal. These amino acids corresponded to nucleotides 82 to 90. Therefore, the first 27 amino acids, from the initiator methionine to alanine, constitute a signal peptide involved in the secretion of protein. The CGTase was composed of 686 amino acids (molecular weight 75,225), and the molecular weight estimated by SDS-PAGE was 66,000.

Upstream of the ATG initiation codon, the sequence for the ribosome-binding site was detected at nucleotide positions 12 to 6. The deduced amino acid sequence at the N-terminal side of the enzyme showed a high homology with the sequences of α-amylase in the three regions, which constitute the active centers of α-amylases.

In contrast, the COOH-terminal region of the β-CGTase was completely different from those of the α-amylases. Many α-amylases are composed of approximately 500 amino acids. Therefore, the COOH-terminal region of β-CGTase would contain an extra 200 to 250 amino acids in addition to the polypeptide exhibiting the amylase activity. Although such a hypothesis may be premature, it is suggested that β-CGTase may have two protein domains: the one on the N-terminal side cleaves the α-1,4-glycosidic bond in starch, and the other on the COOH-terminal side catalyzes other activities, such as cyclization, etc.

Nakamura et al. (1992b; 1993) studied extensively the functional relationships between cyclodextrin glucanotransferase from an alkaliphilic *Bacillus* and α-amylases by site-directed mutagenesis. Two Asp and one Glu residues, which are considered to be the catalytic residues in α-amylases, were also involved in CGTases. The three mutant CGTases, in which Asp Clu257 and Asp328 were individually replaced by Asn or Gln, completely lost both their starch-degrading and β-CD-forming activities. These three inactive enzymes retained the ability to be bound to starch (Nakamura et al. 1993). Furthermore, they revealed that three histidine residues in the active center are important in the stabilization. On the basis of the three-dimensional structures of CGTases, three histidine residues, which are conserved between CGTases and α-amylases, are located at the active center and are proposed to constitute the substrate binding sites. The three histidine residues (His-140, His-233, and His-327) of CGTase from alkaliphilic *Bacillus* sp. 1011 were individually replaced by site-directed mutagenesis to probe their roles in catalysis. Asparagine-replaced CGTases (H140N-, H233N-, and H327-CGTase) retained cyclization activity but had altered production ratios of α-, β-, and γ-cyclodextrin. Replacement of histidine by asparagine residues strongly affected the k(cat) for β-cyclodextrin-forming, coupling, and hydrolyzing activities, whereas it barely affected the K(m) values. The activation energies for α-cyclodextrin hydrolysis were increased to more than 12 kJ/mol by the replacement. Furthermore, the K(i) values of acarbose, which is thought to be a transition-state analogue of glycosidase catalysis, were 2–3 orders of magnitude larger in asparagine-replaced CGTases than that in wild-type CGTase. Therefore, the three histidine residues participate in the stabilization of the transition state, whereas they participate little in ground-state substrate binding. H327N-CGTase showed decreased activity over an alkaline pH range, indicating that His-327 is important for catalysis over an alkaline pH range.

Furthermore, Nakamura et al. (1993b) found that four aromatic residues, which are highly conserved among CGTases but not found in α-amylases, are located in the active center. To analyze the roles of these aromatic residues, Phe-183, Tyr-195, Phe-259 and Phe-283 of *Bacillus* sp. 1011 CGTase were replaced by site-directed mutagenesis and the effects of this procedure were examined. Taken together with the structural information of CGTase crystals soaked with substrates, the authors of the report pro-

posed that Tyr-195 plays an important role in the spiral binding of substrate. Replacing either Phe-183 or Phe-259 with leucine induced increased *Km* values for acceptors.

Ishii et al. (2000a, b) replaced histidine-233 of the CGTase (Accession Number M17366) to asparagine. Asparagine 233-replaced CGTase (H233N-CGTase) no longer produced α-cyclodextrin, while the wild-type CGTase produced a mixture of predominantly α-, β- and γ-cyclodextrins, catalyzing the conversion of starch into cyclic or linear α-1,4-linked glucopyranosyl chains. In order to better understand the protein engineering of H233N-CGTase, they studied the crystal structure of the mutant enzyme complexed with a maltotetraose analogue, acarbose. Taking a close look at the active site cleft in which the acarbose molecule is bound, the most probable reason for the improved specificity of H233N-CGTase is the removal of interactions needed to form a compact ring like α-cyclodextrin. Recently, Haga et al. (2003) reported the effects of essential carbohydrate/aromatic stacking interaction with Tyr100 and Phe259 on substrate binding of the CGTase. The stacking interaction between a tyrosine residue and the sugar ring at the catalytic subsite −1 is strictly conserved in the glycoside hydrolase family 13 enzymes. Replacing Tyr100 with leucine in the CGTase to prevent stacking significantly decreased all CGTase activities. The adjacent stacking interaction with both Phe183 and Phe259 onto the sugar ring at subsite +2 is essentially conserved among CGTases. These structural and biochemical data suggest that substrate binding in the active site of CGTase is critically affected by the carbohydrate/aromatic stacking interaction with Tyr100 at the catalytic subsite-1 and that this effect is likely a result of cooperation between Tyr100 and Phe259 through stacking interaction with substrate at subsite +2.

There are many CGTase papers reported besides those described above. therefore, all of them are not introduced. Some of them are reviewed bellow.

8.4.4 Other CGTase

Martins et al. (2001; 2002) isolated many alkaliphilic bacteria from soil and water samples obtained from Ethiopian soda lakes in the Rift Valley area-Lake Shala, Lake Abijata and Lake Arenguadi. Starch-hydrolyzing isolates were selected on the basis of their activity on starch agar plate assay. Sixteen isolates were chosen, characterized and subjected to 16S rRNA gene sequence analysis. All the isolates except one were motile endospore-forming rods and were found to be closely related to the *Bacillus* cluster, being grouped with *Bacillus pseudofirmus*, *Bacillus cohnii*, *Bacillus vedderi*, and *Bacillus agaradhaerens*. A new CGTase from an alkaliphilic *B. agaradhaerens* strain LS-3C was isolated from an Ethiopian soda lake. The purified enzyme was a monomer with an estimated molecular weight of 110 kDa, representing the largest *Bacillus* CGTase reported so far. The isoelectric point (p*I*) of

the enzyme was 6.9. The CGTase was stable over a very wide pH range, 5.0–11.4, at 25°C and was most active at pH 9.0. The enzyme exhibited an optimum temperature of 55°C and was stable up to 40°C for at least 1 h. Thermal stability could be improved in the presence of a substrate, $CaCl_2$, and to a lesser extent, by the product. The enzyme produced mainly β-CD (89% of the total cyclodextrin amount) with only α-CD as a minor product. The maximal conversion of maltodextrin to cyclodextrins varied between 10–15% depending on substrate concentration. The gene encoding CGTase was cloned and sequenced (Accession Number AY256412). It encodes a mature polypeptide of 679 amino acids with a molecular mass of 76488 Da. The deduced amino acid sequence of the mature CGTase revealed 99% and 95% identity to the CGTase sequences from the other *B. agaradhaerens* strains, DSM 8721(T) and 9948, respectively. Then they entrapped *B. agaradhaerens* cells in polyvinyl alcohol-cryogel beads and used it as an immobilized CGTase column. The cyclodextrin product from the immobilized cell bioreactor was continuously recovered by adsorption to Amberlite XAD-4 in a recycle batch mode. The product adsorption was facilitated at low temperature while hot water was used for elution.

A CGTase from an alkaliphilic *Bacillus* sp. A2-5a was purified by starch adsorption and Q-Sepharose chromatography (Kometani et al. 1994a). The purified enzyme had cyclizing activity, transglycosylating (coupling) activity, and starch-hydrolyzing activity, and their pH-activity curves had a single peak (pH 5.5 as the optimum pH) with a broad shoulder at alkaline pHs. Terada et al. (1997; 2001) analyzed the initial products in the hydroyzate. The reaction mixture was treated with glucoamylase and the resulting glucoamylase-resistant glucans were analyzed with high performance anion exchange chromatography; cyclic α-1,4-glucans, with degree of polymerization ranging from 9 to more than 60, in addition to well-known α-, β-, and γ-cyclodextrins (CDs), were detected. CGTase from *B. macerans* also produced large cyclic α-1, 4-glucans except that the final major product was α-CD. Based on these results, a new model for the action of CGTase on amylose was proposed, which may contradict the widely held view of the cyclization reaction of CGTase.

Ohdan et al. (2000) cloned the CGTase gene (Accession Number AB015670) and expressed in *Bacillus subtilis* ANA-1 as a host. The DNA region included an open reading frame encoding a 704-amino-acid polypeptide with a typical raw starch-binding motif in its C-terminal region. The CGTase purified from *Bacillus* sp. A2-5a bound to raw starch as strongly as porcine pancreas α-amylase, as expected from the sequence motif.

8.4.5 γ-CD-forming CGTase

Kato and Horikoshi (1986a) demonstrated that one strain of *B. subtilis* produced a γ-CD-forming CGTase in culture broth. The isolate, *B. subtilis* No. 313, was grown aerobically for five days at 37°C in a cultivation medium con-

taining 1% potato starch, 1% polypeptone, 0.1% yeast extract, 0.3% KH_2PO_4, 1% $MgSO_4$ and 0.02% $CaCO_3$. The γ-CD-forming activity in the crude broth was assayed by the BGC method (Kato and Horikoshi 1984). The crude enzyme preparation exhibited a relatively broad pH activity curve for CD formation with a pH optimum at 8.0, as shown in Fig. 8.11. Analysis of the enzymatic digest by HPLC showed the product to be only γ-CD.

The isoelectric point of the enzyme purified by column chromatography was 7.1 and its molecular weight was estimated to be 64,000 by the SDS-PAGE method. The NH_2-terminal sequence of the purified enzyme was NH_2-Ser-Val-Lys-Asn-Gly-Thr-Ile-Leu-His-Ala-Trp-Asn-Trp. The enzyme converted starch to γ-CD with an optimum pH 8.0 and was stable over the range pH 5.5–8.5 at 50°C (30-min incubation). Enzyme activity was stable up to 50°C and was inactivated at 70°C in the presence or absence of calcium ions.

This enzyme produced only γ-CD from starch, the yield being about 5%. In order to obtain further information on the formation of γ-CD from starch, the CGTase gene of *Bacillus subtilis* No. 313 was cloned and expressed in *E. coli* (Kato and Horikoshi 1986a,b). The CGTase expressed in *E. coli* produced only γ-CD from starch and neither α- nor β-CD was detected. At an early stage of the hydrolysis of starch the γ-CGTase produced γ-CD and acyclic dextrin at the same time. On the other hand, other CGTases producing predominantly CD and acyclic dextrin could be detected after long incubation. These results strongly suggest that the γ-CGTase may be an intermediate type of α-amylase and true CGTases.

Analysis of the nucleotide sequence showed that there was a single open

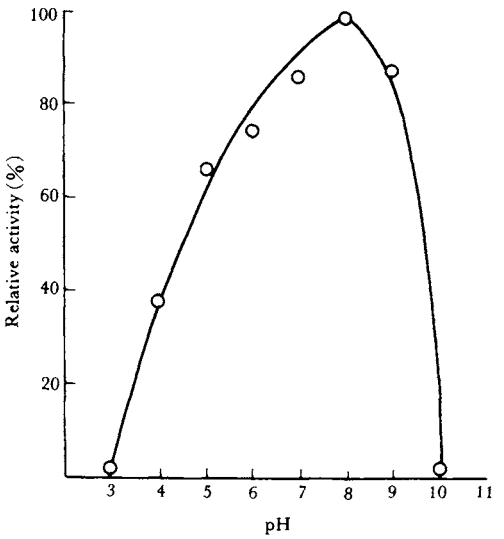

Fig. 8.11 Effect of pH on activity.

reading frame of 1,632 bp. The N-terminal amino acid sequence of the γ-CGTase from *Bacillus subtilis* No. 313 was NH_2-Ser-Val-Lys-Asn-Gly-Thr-Ile-His-Ala-Trp-Asn-Trp, which is identical to that deduced from the DNA sequence. Therefore, 45 amino acid residues (residues −45 to −1) are considered to represent the signal peptide which is removed during secretion of the enzyme.

Recently, Takada et al. (2003) isolated a novel alkaliphilic bacterium, *Bacillus clarkii* 7364. This strain produced a γ-CD-forming enzyme that exhibited a higher conversion rate from potato starch. The CGTase secreted into the culture medium from this bacterium was purified by affinity chromatography on a γ-CD-immobilized column, followed by chromatography on a gel filtration column. The γ-CD-forming activity was determined by the bromocresol green (BCG) method described above. As shown in Fig. 8.12, the enzyme converted 13.7% of pre-gelatinized potato starch (10% w/w per reaction mixture) into CDs at pH 10.0 and 50°C, and the majority (79%) of the product CDs was of the γ-form. The addition of 20% (v/v) ethanol to the reaction mixture gave 23% yield of γ-CD from gelatinized corn starch (1% w/v). This property is unique among the known CGTases. The γ-CGTase gene was sequenced (Accession Number AB082929) and the gene was introduced into *E. coli* and expressed. The recombinant enzyme retained biochemical properties quite similar to those of the original (Table 8.10). Although their data are not sufficient to give specific γ-CGTase, comparison of the deduced amino acid sequence of this γ-CGTase with those of

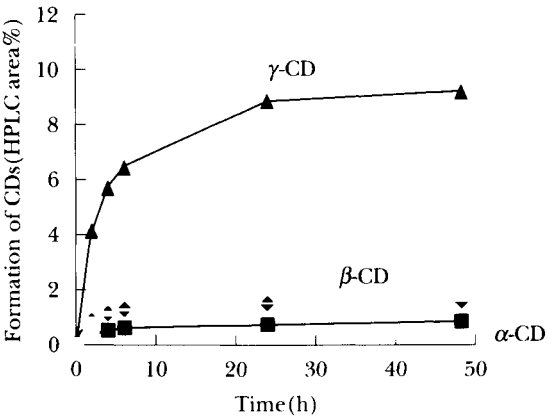

Fig. 8.12 Courses of formation of CDs from gelatinized potato starch by γ-CGTase. A reaction mixture containing 10% (w/w) gelatinized potato starch and crude γ-CGTase (0.75 U/g of dry starch) in 25 mM Gly-NaCl-NaOH buffer (pH 10.0) was incubated at 50°C. CDs formed in the mixture were determined by HPLC, as described under Materials and Methods. Closed triangles, γ-CD; closed diamonds, β-CD; closed squares, α-CD. From 100 ml of the reaction mixture, α-, β-CD and oligosaccharides were removed by crystallization and gel filtration. Finally, 175 mg of γ-CD was obtained. Its structure was confirmed by ^{13}C-and ^{1}H-NMR (data not shown).
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Table 8.10 Comparison of the properties of CGTases from various *Bacillus* sp.

	Bcla	Recombinant	Bmace	Bmega	Bf290	Brev
Isoelectric point	3.98	3.98	4.6	–	4.1	–
Optimum pH	10.5–11.0	10.5–11.0	5.2–5.7	5.0–5.7	6.0–8.0	10
Optimum temp. (°C)	60	60	55	55	50	45
pH stability	6.0–11.0	6.0–11.0	8.0–10.0	7.0–10.0	6.0–8.0	6.0–8.0
Thermal stability (°C)	40	40	55	55	–	30
Molecular weight (Da)	68,000	68,000	65,000	75,000	75,000	75,000
Main product	γ	γ	α	β	β/γ	β/γ

Bmace, Bmega, Bf290, Brev, and Bcla indicate α-CGTase from *B. macerans* (*7*), β-CGTase from *B. megaterium* (*29*), β/γ-CGTase from *Bacillus* sp. 290-3(*5*), *Brevibacterium* sp. (*28*), and *B. clarkii* 7364, respectively. "Recombinant" indicates the enzyme purified using a periplasmic fraction of *E. coli* JM109 cells harboring pGEFT-01.
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other known CGTases that have different product specificities would be useful for the construction of industrial γ-CGTase .

From the industrial point of view, γ-CD is an ideal compound for the production of pharmaceutical chemicals and food additives because of its large cavity diameter and biodegradability. Thus, enzymes having higher thermostability and higher yield of γ-CD from corn starch are desirable for industrial production.

8.4.6 Industrial Production of β-CD

The optimum concentration represents a compromise of several factors. Theoretically, the best yields are expected from the most diluted solutions. Since starch is a low-cost material, the use of high concentrations of starch has significant economic advantages for production on an industrial scale. As a result, the optimum substrate concentration in the author's plant was set at about 15%. Essential production process of CDs had been established in 1985 by Nippon-Shokuhin-Kako. One ton of potato starch suspension (15%) containing 5 mM $CaCl_2$ was liquefied by the No. 38-2 enzyme at 82°C at pH 8.5 then cooled to 65°C. The liquefaction was readjusted to pH 8.5 with $Ca(OH)_2$ and 1,500 g of the enzyme was added. CD formation was maintained at 65°C with continuous stirring for a further 30 h. After the reaction, the enzyme was inactivated by heating at 100 to 120°C. Then the pH of the reaction mixture was brought to 5.5 to 5.7, and an appreciable amount of bacterial α-amylase was added to hydrolyze the saccharides which were not converted to CD. The digest was decolorized with active charcoal and filtered off. The filtrate was passed through ion-exchange resin to remove ions. The hydrolyzate was concentrated to about 50% (w/v) under reduced pressure and crystallized by the addition of a small amount of mother crystalline β-CD lowering the temperature gradually. Crystalline material was separated by a basket type centrifuge and washed with a small amount of water. The cured β-CD was recrystallized by the conventional method

with hot water, if necessary. The filtrate contained α-, β-, γ-CDs, glucose, maltose and oligosaccharides. The filtrate thus obtained is able to form inclusion complexes with many organic substances because about 20% of the filtrate is a mixture of CDs (Fig. 8.13).

The production process of α- and γ-CDs from the filtrate was established at the same time. The filtrate was treated with bacterial amylase (saccharifying) and glucoamylase to hydrolyze β-, γ-CDs and maltooligosaccharides, decolorized, concentrated, and passed through a column of Diaion FRK 101 Na (Mitsubishi Chemicals Industrial Co., Tokyo) at 60–80°C to separate the α-CD and glucose. Fig. 8.14 shows an elution profile of α-CD, glu-

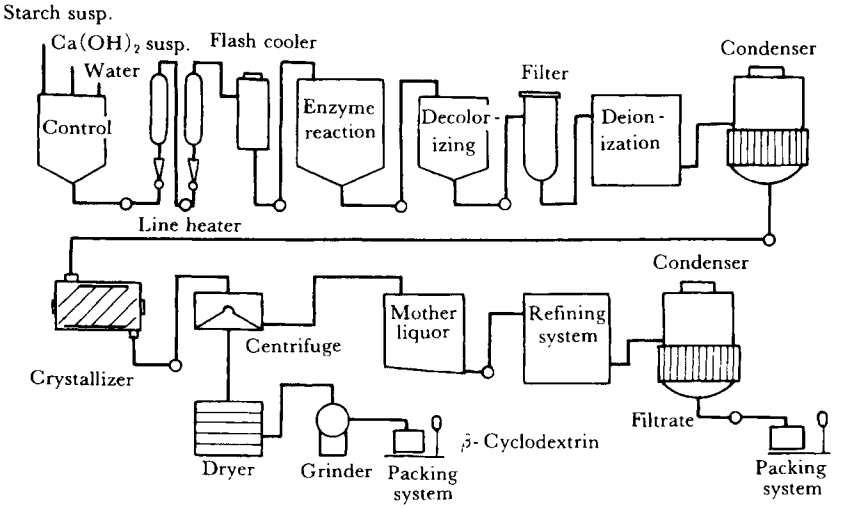

Fig. 8.13 Flow chart of production of β-cyclodextrin.

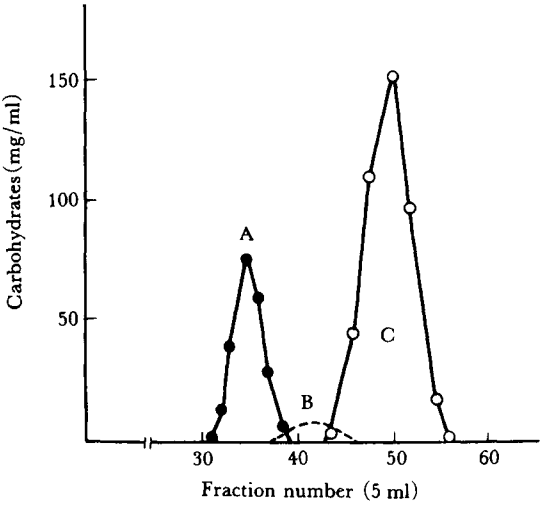

Fig. 8.14 Elution pattern of α-cyclodextrin, glucose and maltooligosaccharides from a Diaion FRK101 column: (A) α-CD; (B) maltooligosaccharides; (C) glucose.

cose and maltooligosaccharides on a laboratory scale. The α-CD solution was concentrated to up to 45–50% and α-CD crystallized. γ-CD was also crystallized from the filtrate as follows. The filtrate was incubated with glucoamylase at 50°C for 24 h to hydrolyze acyclic dextrins. The CD mixture and glucose were separated with a column of FRK 101, and γ-CD was isolated by passing it through a Toyopearl HW-40 column at 60–70°C. Then γ-CD fractions were collected and concentrated to about 60% (w/v) under reduced pressure. γ-CD in the concentrate was crystallized directly.

8.4.7 Uses of CD

The following uses are suggested. (1) Stabilization of volatile materials: a) conversion to dry form from toxic liquid chemicals, b) stabilization of flavors and spices, c) deodorization of medicines and foods. (2) Protection against oxidation and UV-degradation during storage or processing. (3) Modification of physical and chemical properties: a) to increase solubility of water-insoluble medicines, b) to masking of shifting of colors and fluorescences, c) to masking of bitterness in foods and medicines, d) to stabilization of deliquescent chemicals, e) to promotion of hydrolysis of some esters. (4) Emulsification of steroids, hydrocarbons, oils, fats and fatty acids. (5) Solidification of hydrocarbons, oils, fats and fatty acids. Using the above properties, many industrial applications of CD have been reported and some of them are now commercially available. Since we first succeeded in the industrial production of CD in 1978, commercial applications of these unique compounds have been investigated by numerous companies, and many of them are now in use in various fields. Table 8.11 summarizes the industrial application of CDs.

8.4.8 Novel Applications of CGTase

A novel application of CGTases as transglucosidase was reported by Kometani et al. (1994a, b; 1996a, b). The CGTase A2-5a exhibits strong transglycosylation activity especially at higher pH values. An alkaline pH was very effective for solubilizing neohesperidin, the amount of glycosides formed increased. As a result, its amount with β-CD at pH 10 was about seven times greater than that with soluble starch at pH 5. The enzyme from an alkaliphilic *Bacillus* sp. had wider acceptor specificity than that from *B. macerans*.

Among flavonoids, those containing rutinose (diosmin and hesperidin) were transglycosylated more effectively than those containing neohesperidose (naringin and neohesperidin). Furthermore, the CGTase produced hesperidin monoglucoside and a series of its oligoglucosides by the transglycosylation reaction with hesperidin as an acceptor and soluble starch as a donor. The formation of the glycosides was more effective at alkaline pH values than at neutral or acidic pH values because of the higher solubility of

Table 8.11 Industrial application of cyclodextrins

Functions	Guests	End Products
Foods		
Emulsification	Oils and fats	Margarine, Cake, Whipping cream, French dressing
Stabilization	Flavors, Spices, Colors and pigments	Horseradish paste, Mustard paste Cakes and cookies, Pickled vegetables,Dried vegetables
Masking of taste and odor		Juices, Soy milk, Bone powder, Boiled rice
Improvement of quality		Hard candy, Cheese, Soy sauce, Canned citrus fruits and juices
Reduce volatility	Ethanol	Food preservatives
Others		Breath mints
Cosmetics and toiletries		
Emulsification	Oils and fats	Face cream, Face lotion, Toothpaste
Stabilization	Flavors and fragrances	Bath refresher crystals
Agrochemicals		
Stabilization	Pyrolnitrin	Fungicide
	Pyrethroids	Insecticide
Reduce volatility	Organic phosphates (DDVP)	Insecticide
	Thiocarbamic acid	Herbicide
Reduce toxicity	2-Amino 4-methyl-phosphynobutyric acid	Fungicide

Functions	Guests and End Products
Pharmaceuticals	
Improve solubility	Prostaglandins, Steroids, Cardiac glycosids, Nonsteroidal antiinflammatory agents, Barbiturates, Phenytoin, Sulfornamides, Sulfonylureas, Benzodiazepines
Chemical stabilization	
Hydrolysis	Prostacylin, Cardiac glycosides, Aspirin, Atropine, Procaine
Oxidation	Aldehydes, Epinephrine, Phenothiazines
Photolysis	Phenothiazines, Ubiquinones, Vitamins
Dehydration	Prostaglandin E_1, ONO-802
Improve bioavailability	Aspirin, Phenytoin, Digoxine, Acetohexamide, Barbiturates, Nonsteroidal antiinflammatories
Powdering	ONO-802, Clofibrate, Benzaldehyde, Nitroglycerin, Vitamin K_1, K_2, Methylsalicylate
Reduce volatility	Iodine, Naphthalene, *d*-Camphor, *l*-Menthol, Methylcinnamate
Improve taste, smell	Prostaglandins, Alkylparabens
Reduce irritation to stomach	Nonsteroidal antiinflammatory agents
Reduce hemolysis	Phenothiazines, Flufenamic acid, Benzylalcohol, Antibiotics

the acceptor. The structure of the purified monoglucoside was identified as 4(G)- α-D-glucopyranosyl hesperidin. The solubility of both hesperidin mono- and diglucoside in water was about 300 times higher than that of hesperidin and they were found to have a stablizing effect on the yellow pigment crocin, from fruit of *Gardenia jasminoides*, against ultraviolet radiation. Therefore, CGTase can be used not only for the production of CDs but has other important potential applications.

10

Xylanases

The first paper of xylanase of alkaliphilic bacteria was published in 1973 by Horikoshi and Atsukawa (1973). The purified enzyme of *Bacillus* sp. No. C-59-2 exhibited a broad optimum pH ranging from 6.0 to 8.0 as shown in Tables 10.1 and 10.2. Later, Okazaki reported that four thermophilic alkaliphilic *Bacillus* stains (W1 (JCM2888), W2 (JCM2889), W3 and W4) produced xylanases (Okazaki et al. 1984; 1985). The pH optima for enzyme action of strains W1 and W3 was 6.0 and for strains W2 and W4 was between 6 and 7. The enzymes were stable between pH 4.5 and 10.5 at 45°C for 1 h. The optimum temperatures of xylanases of W1 and W3 were 65°C and those of W2 and W4 were 70°C. The degree of hydrolysis of xylan was about 70% after 24 h incubation.

10.1 Isolation of Alkaline Xylanase from *Bacillus halodurans* C-125 and Construction of Chimeric Xylanases

Subsequently, two xylanases were found in the culture broth of *Bacillus halodurans* C-125 (Honda et al. 1985a, b, c, d). Xylanase A had a molecular weight of 43,000 and that of xylanase N was 16,000. Xylanase N was most active at pH 6–7 and xylanase A was most active at a pH range of 6 to 10 and had some activity at pH 12 (Fig. 10.1). The xylanase A gene (Accession Number D00087) was cloned, sequenced and expressed in *E. coli* (Honda et al. 1985a, b, d; 1986a, b).

Nishimoto et al. (2002a) constructed chimeric xylanases form xylA(alkaline xylanase) of *B. halodurans* C-125 and XylB (neutral xylanase) of *Clostridium stercorarium*. As shown in Table 10.3, six chimeric xylanases were made by the selective substitution of the four fragments using an overlapping PCR technique. Two of the six xylanases, APnc and Apnc (regions originating from XynA are denoted by upper case letters and those from XynB are denoted by lower case letters), were produced in *Escherichia coli* while the other four xylanases were obtained only as inclusion bodies. The APnc and Apnc chimeric enzymes were purified by column chromatography using Ni-NTA agarose and DEAE-Toyopearl. The respective pH and temperature stabilities of the purified enzymes were observed from pH 5.6 to 11.6 and up to 45°C for APnc, and from pH 5.6 to 11.2 and up to 45°C for

Table 10.1 Morphological cultural and biochemical characteristics of strain No. C-59-2

1. Morphological characteristics		
Form	Rod	
Size	0.3–0.4 μ × 1.5–2.5 μ	
Motility	Motile	
Gram stain	Positive	
Sporangia	Slightly swollen	
Spore	0.5–0.7 μ × 1.0–1.2 μ	
2. Cultural characteristics		
	Growth at pH 7	Growth at pH 10.3
Nutrient broth	−	+
Nutrient agar slant	−	+
Glucose-nutrient broth	−	++
Glucose-nutrient agar slant	−	++
Potato	−	++
Horikoshi I-medium	−	++
Horikoshi II-medium	−	++
Glucose-nitrate agar slant	−	±
Glucose-asparagine agar slant	−	+
Anaerobic growth in glucose broth	−	+
Anaerobic production of gas from nitrate	−	−
Horikoshi I-medium containing 7% NaCl	−	−
3. Biochemical characteristics		
Hydrolysis of genatine and casein	Very weak	
Hydrolysis of starch	Positive	
Utilization of citrate	Utilized	
Utilization of ammonium salts	Utilized	
Reduction of nitrate to nitrite	Negative	
Voges-Proskauer test	Negative	
4. pH and temperature		
pH for growth in Horikoshi-I medium	pH 7.5 to pH 11	
Temperature for growth in Horikoshi-I medium	15–42°C at pH 10.3	

pH in the Horikoshi-I medium was adjusted by adding HCl of NaOH.
−, indicates no growth; ±, poor growth; +, normal growth; ++, abundant growth.
(Reproduced from K. Horikoshi and Y. Atsukawa, *Agric. Biol. Chem.*, **37**, 2098 (1973))

Apnc (Fig. 10.2). Thus, these enzymes were slightly less stable than the parental xylanases. An assessment of the pH-activity relationships for the chimeric xylanases employed *p*-nitrophenyl-β-D-xylobioside as the substrate in determinations of the *k*(cat) values. The p*K*(a1) values for the APnc and Apnc chimeric enzymes were 4.3 and 4.2, respectively, which were almost identical to those for the parental xylanases. In contrast, the p*K*(a2) values obtained for APnc and Apnc were 9.1 and 8.5, respectively; these values fall

Table 10.2 Properties of bacterial xylanases

Property	*Bacillus* sp. No. C-59-2	*B. subtilis* C-2	*Streptomyces xylophagus*	*Bacillus* sp. No. C-11
Optimum pH	6.0–8.0	6.0–6.2	6.2	7.0
Optimum temperature (°C)	60	37–40	55–60	–
Sedimentation constant	3.5			
Isoelectric point	6.3			
Stable pH	7.0–7.5	5.0–7.0	5.3–7.3	5.5–9.0
Stable temperature (°C)	Up to 60	Up to 45	Up to 40	
Predominant product	$X_2 > X_3 > X_4$	Ara, X_2, X_3	X, X_2	X, X_2, X_3
Max. hydrolysis rate (%)	40	38		

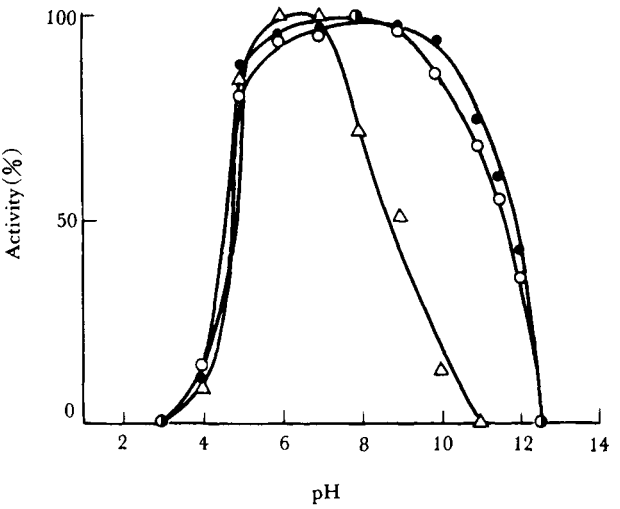

Fig. 10.1 Effect of pH on xylanase activity. Symbols: ○, Xylanase of *E. coli* HB101 carrying pCX311; ●, Xylanase A of alkaliphilic *Bacillus* sp. No. C-125; △, Xylanase N of alkaliphilic *Bacillus* sp. No. C-125.
(Reproduced with permission from H. Honda et al., *J. Bacteriol.*, **161**, 785 (1985))

between those for the parental xylanases, XynA (9.4) and XynB (7.8). These results indicate that the main regions necessary to maintain the high p*K*(a2) value of XynA are located in the A and P sections (Accession Number AY137373).

Gupta et al. (2000) isolated alkaliphilic *Bacillus* sp. NG-27, which produced a 42-kDa endoxylanase active at 70°C and at a pH of 8.4. The gene for this endoxylanase was cloned and sequenced (Accession Number AF015445). The gene contained one open reading frame of 1,215 bases. An active site characteristic of the family 10 β-glycanases was recognized between amino acids 303 and 313, with the active glutamate at position 310. The DNA sequence responsible for the xylanase did not exhibit high ho-

Table 10.3 Xylanase activity in culture supernatant and cell-free extract

	Xylanase activity (U/ml culture)			
	Small scale (2 ml)		Large scale (100 ml)	
	Supernatant	Cell-free extract	Supernatant	Cell-free extract
APNC (XynA)	0.60	0.07	1.45	0.32
APNc	0.01	0.01	ND	ND
APnc	0.56	0.07	0.28	1.21
Apnc	0.77	0.05	0.38	1.48
aPNC	0.01	0.01	ND	ND
apNC	0.01	0.01	ND	ND
apnC	0.02	0.02	ND	ND
apnc(XynB)	0.33	0.29	0.14	1.98

ND, No data.
(Reproduced with permission from M. Nishimoto et al., *J. Biosci. Bioeng.*, **94**, 395(2002))

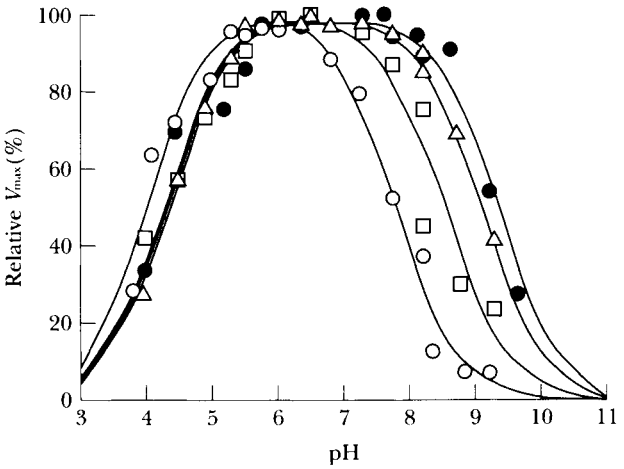

Fig. 10.2 Effects of pH on the activities of chimeric enzymes. Symbols are as follows: closed circles, XynA; open circles, XynB; squares, Apnc; triangles, APnc.
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mology but the decoded amino acid sequence showed very high homology with that of *B. halodurans* C-125.

Recently, Martinez et al. (2002) reported alkaliphilic xylanase isolated from *Bacillus halodurans* MIR32. *Bacillus* sp. MIR32 has been isolated using xylan as the only carbon source. 16S rDNA sequences, G + C content, and DNA-DNA hybridization showed that strain MIR32 should be classified as a member of the species *Bacillus halodurans*. The DNA sequence responsible for the xylanase isolated (Accession Number AF534180) exhibited 100% homology with that of *B. halodurans* C-125.

10.2 Thermostable Alkaline Xylanases

Nakamura et al. also reported that an alkaliphilic *Bacillus* sp. strain, 41M-1, isolated from soil produced multiple xylanases extracellularly (Nakamura et al. 1993a, b; 1995). One of these xylanases was purified to homogeneity by ammonium sulfate fractionation and anion-exchange chromatography. The molecular mass of this enzyme (xylanase J) was 36 kDa, and the isoelectric point was pH 5.3. Xylanase J was most active at pH 9.0. The optimum temperature for the activity at pH 9.0 was around 50°C. The enzyme was stable up to 55°C at pH 9.0 for 30 min. Xylanase J was completely inhibited by the Hg^{2+} ion and N-bromosuccinimide. The predominant products of xylan hydrolysate were xylobiose, xylotriose, and higher oligosaccharides, indicating that the enzyme was an endoxylanase. Nakai et al. (1994) reported that xylanase J showed high sequence homology (Accession Number AB029319) with the xylanase (Accession Number AAF32359) from *Bacillus pumilus* YC-335. Inami et al. (2003) found that the newly constructed mutant E177QΔJC had an acidic pH optimum and showed almost no activity at pH 8.0. However, the alkaliphily of the enzyme was restored by several mutations, such as, Y176S/E177QΔJC and G32V/Y176D/E177QΔJC as shown in Fig. 10.3. The optimum temperature for the activity at pH 9.0 was around 50°C

Alkaliphilic and thermophilic *Bacillusi* sp. strain TAR-1, isolated from soil, produced a xylanase extracellularly (Nakamura et al. 1994; Takahashi et al. 2000). The xylanase was most active over a pH range of 5.0 to 9.5 at 50°C. Optimum temperatures of the crude xylanase preparation were 75°C at pH 7.0 and 70°C at pH 9.0 (Figs. 10.4 and 10.5). Its molecular mass and isoelectric point were 23 kDa and pH 9.3, respectively. Analyses of xylan-degradation products and the N-terminal amino acid sequence revealed that the enzyme would be a family GH11 endoxylanase. Production of the xylanase was induced by xylan and xylose, but repressed in the presence of glucose.

Subsequently, many alkaline xylanases have been isolated from various alkaliphiles. Two alkali-tolerant thermophilic bacterial strains with xylanolytic activity were produced by continuous cultivation from samples collected near Bulgarian hot springs (Dimitrov et al. 1997). Xylanases produced by the two strains were similar with respect to temperature and pH optimum (70–75°C and pH 6.5–7.0) as well as their thermostability. The xylanases were thermostable at 70°C for 30 min. Lopez et al. (1998) reported xylanase production by a new alkali-tolerant isolate of alkali-tolerant *Bacillus*. Crude xylanase retained 72% of initial activity after 5 h at pH 9.0 and 45°C. Sunna et al. (1997) isolated three strictly aerobic strains (K-1, K-3d and K-4) from a hot-spring in Kobe, Japan, and a facultative anaerobic strain LB3A from sediments collected from the alkaline Lake Bogoria, Kenya. All strains were thermophilic and capable of growth on xylan. On the basis of morphological, physiological and phylogenetic studies the new

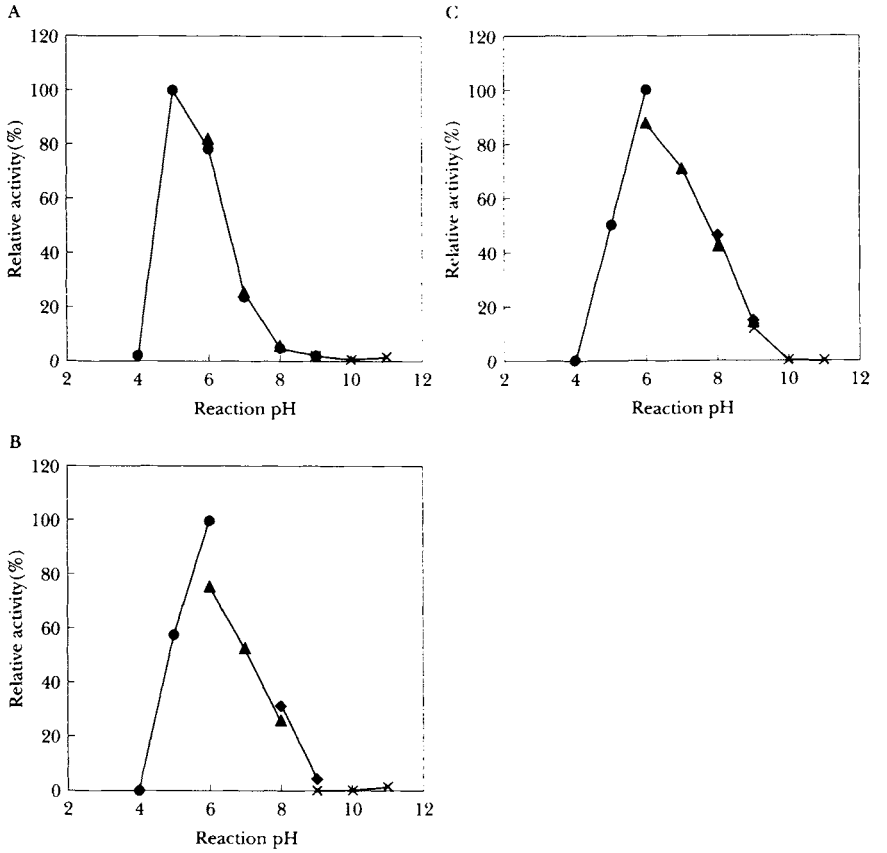

Fig. 10.3 Effect of reaction pH on activity of mutants E177QΔJC(A), Y176S/E177QΔJC(B) and G32V/Y176D/E177QΔJC(C). An activity of a cell free extract of each transformant was measured at 37℃. ●: citrate buffer (pH 4.0–6.0), ▲: phosphate buffer (pH 6.0–8.0), ◆: Tris buffer (pH 8.0–9.0), ×: carbonate buffer (pH 9.0–11.0). (Reproduced with permission from M. Inami et al., *Nucreic Acids Res., Suppl.* 315, (2003))

aerobic isolates resemble the thermophilic species *Bacillus thermoleovorans* while the facultative anaerobic isolate LB3A resembles the facultative anaerobic thermophilic species *Bacillus flavothermus.* Xylanases from strains K-3d and LB3A are active at temperatures between 40℃ and 90℃ and pH values between 5.0 and 9.0.

Gallardo et al. (2004) reported that the *xynA* gene encoding a xylanase from the recently isolated *Bacillus* sp. strain BP-7 has been cloned and expressed in *E. coli.* Recombinant xylanase A showed high activity on xylans from hardwoods and cereals, and exhibited maximum activity at pH 6 and 60℃. The enzyme remained stable after incubation at 50℃ and pH 7 for 3 h, and it was strongly inhibited by Mn^{2+}, Fe^{3+}, Pb^{2+} and Hg^{2+}. Analysis of xylanase A in zymograms showed an apparent molecular size of 24 kDa and a

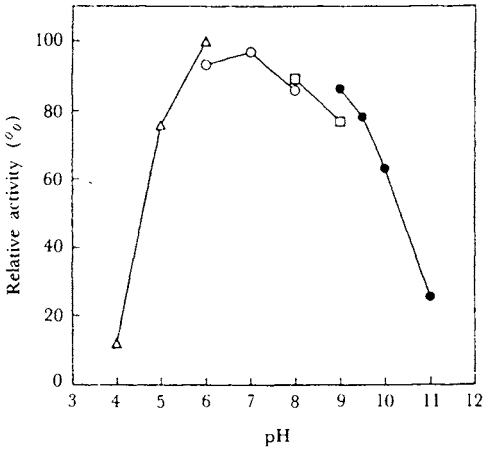

Fig. 10.4 Effect of reaction pH on activity.
(Reproduced with permission from S. Nakamura et al., *Biosci. Biotechnol. Biochem.*, **58**, 78(1994))

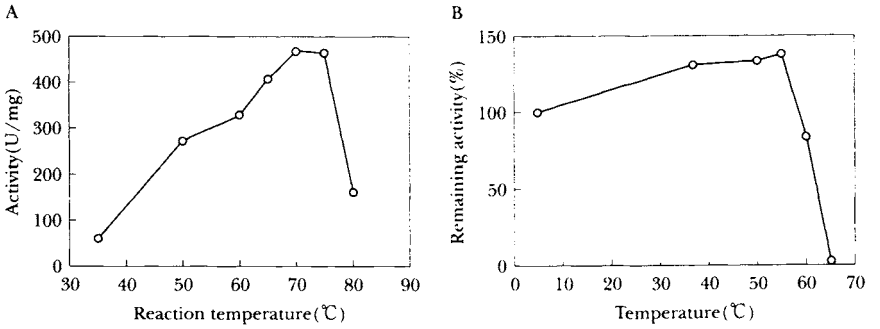

Fig. 10.5 Effects of temperature on activity (A) and stability (B) of XynT.
(A) Enzyme activity of the purified XynT was measured at pH 6.0 and different temperatures. (B) The purified XynT was incubated at various temperatures for 30 min, and then remaining activity was measured under standard assay conditions.
(Reproduced with permission from S. Nakamura et al., *Biosci. Biotechnol. Biochem.*, **58**, 78(1994))

p*I* of above 9. The amino acid sequence of xylanase A, as deduced from the *xynA* gene (Accession Number AJ536759), shows homology to alkaline pI-low molecular weight xylanases of family GH11 such as XynA from *Bacillus subtilis*.

10.3 Xylanases Isolated for Industrial Applications

Since our discovery of alkaline xylanase of alkaliphiles, many papers have been published for industrial applications. Ratanakhanokchai et al. (1999)

Table 10.4 Effects of metal ions and EDTA on xylanase activity [1]

Ion or EDTA(mM)	Relative activity (%)
None	100
EDTA(1)	42.2
EDTA(10)	22.2
EDTA(50)	0
$CaCl_2$	130.7
$CuCl_2$	51.2
$CoCl_2$	54.4
$FeCl_2$	261.6
$MgCl_2$	172.0
$MnCl_2$	16.5
$NiCl_2$	60.7
$ZnCl_2$	46.1
$CaSO_4$	191.6
$CuSO_4$	33.3
$CoSO_4$	76.3
$FeSO_4$	289.0
$MgSO_4$	174.0
$MnSO_4$	12.0

[1] For the case of metal ions, the enzyme solution was mixed with the final concentration of 50 mM EDTA and dialyzed with Tris-HCl buffer (pH 7.0). Then the EDTA-treated enzyme solutions were preincubated in a mixture containing various chemicals (1 mM) at 30°C for 1 h, after which the reactivated activity was measured under the standard assay conditions.
(Reproduced with permission from K. Ratanakhanokchai et al., *Appl. Environ. Microbiol.*, **65**, 694 (1999))

also isolated alkaline stable xylanase from alkaliphilic *Bacillus firmus* strain K-1. The molecular weight of the purified xylan-binding xylanase was estimated to be approximately 23 kDa. The enzyme was stable at alkaline pHs up to 12. The optimum temperature and optimum pH of the enzyme activity were 60°C and 5.5, respectively. As shown in Table 10.4, metal ions such as Fe^{2+}, Ca^{2+}, and Mg^{2+} greatly increased the xylanase activity, whereas Mn^{2+} strongly inhibited it. They also demonstrated that the enzyme could hydrolyze the raw lignocellulosic substances effectively. Rao's group (Rao et al. 1995; Kulkarni et al. 1999; Nath and Rao 2001) studied isolation and characterization of xylanase from alkaliphilic *Bacillus* sp. NCIM59. The molecular weight of the enzymes were estimated to be 35,000 (xylanase I) and 14,500 (xylanase II). The purified xylanases had similar temperatures (60°C) and pH 6 optima, and the isoelectric points were 4 and 8, respectively. The enzymes retained 100% activity at 50°C for 24 h. A 1.0-kilobase gene fragment from the genomic DNA of an alkaliphilic thermophilic *Bacillus* was found to code for a functional xylanase II (XynII). The complete nucleotide sequence including the structural gene and the 5' and 3' flanking sequences of the xylanase gene have been determined (Accession Number AF317713). An open reading frame starting from ATG initiator codon comprising 402 nucleotides gave a preprotein of 133 amino acids of calcu-

Table 10.5 Xylanases produced by alkaliphilic *Bacillus* strains

Source	Molecular mass(kDa)	p*I*	Optimum pH	Optimum temperature	Accession Number	Reference
Aeromonas sp. 212	145	-	7.0–8.0	50		(Ohkoshi et al. 1985)
	37	-	6.0–8.0	50		
	23	-	5.0–7.0	60		
Bacillus sp. C-59-2	-	6.3	5.5–9.0	60		(Horikoshi and Atsukawa 1973)
Bacillus halodurans C-125	43	-	5.0–10.0	70	D00087	(Honda 1985a)
	16	-	6.0–7.0	70		
Bacillus sp. W1	21.5	8.5	6.0	65		(Okazaki 1984; 1985)
Bacillus sp. W2	22.5	8.3	6.0	65		
	50	3.7	7.0–9.5	70		
Bacillus sp. W3	-	-	6.0	65		
Bacillus sp. W4	-	-	6.0–7.0	70		
Bacillus sp. NCL 87-6-10	45	5.3	6.0–10.0	40–60		(Balakrishnan et al. 1992)
	25	8.9	8.0	40–60		
Bacillus sp. YC-335	40	-	8.0–9.0	60		(Park et al. 1992)
Bacillus sp. NG-27	42	-	8.4	70	AF015445	(Gupta et al. 1992)
Bacillus sp. (VI-4)	-	9.1	6.0–7.0	55		(Yang et al. 1995)
Bacillus sp. SamIII	-	-	8.0	60		(Shah et al. 1999)
Bacillus sp. 41M	36	5.5	9.0	50	AB029319	(Nakamura et al. 1993)
Bacillus sp. NCIM59	35	4.0	6.0–8.0	50–60		(Dey et al. 1992)
	14.1	8.0	6.0–8.0	50	AF317713	(Shendye and Rao 1993)
Bacillus sp. TAR-1	40	4.1	5.0–9.5	75		(Nakamura et al. 1994)
Bacillus sp. MIR32	43	-	5.0–10.0	75	AF534180	(Martinez et al. 2002)
Bacillus sp. KK-1	40	-	6-8	55	AF045480	(Yoon et al. 1998)
Bacillus sp. N137	39	-	8.0	60	Z35497	(Tabernero et al. 1995)
Bacillus sp. BP23	32	9.3	5.5	55	AJ006645	(Blanco and Pastor 1993; Blanco et al. 1999)

lated molecular mass 14.090 kDa. The stop codon was followed by hairpin loop structures indicating the presence of transcription termination signals. The secondary structure analysis of XynII predicted that the polypeptide was primarily formed of β-sheets. XyaII appeared to be a member of the family GH11 of xylanases based on its molecular weight and basic p*I* (8.0). The conserved triad (Val-Val-Xaa, where Xaa is Asn or Asp) was identified only in the xylanases from alkaliphilic organisms. Their results implicate for the first time the concept of convergent evolution for XynII and provide a basis for research in evolutionary relationships among the xylanases from alkaliphilic and neutrophilic organisms.

The pH-induced conformational and structural changes of Xyl II have been investigated from the alkaliphilic thermophilic *Bacillus* sp. using kinetic, circular dichroism and fluorescence spectroscopy studies. Systematic studies on the folding and stability of cellulase-free xylanases are important, since their biotechnological applications require them to function under extremes of pH and temperature. Above pH 8, the enzyme exhibited unfolding transitions as revealed by a red shift in the emission maximum as well as decreases in the fluorescence intensity. Circular dichroism studies revealed a decrease in the CD ellipticity at 222 nm at pH 9 and 10. The sequence alignment studies of Xyl II, in combination with kinetic and chemical modification data provide strong evidence for the participation of Asp94 in the catalytic function. The Xyl II, produced from an alkalophilic source, was stable at pH 10. However, the enzyme exhibited pH optimum at near neutral values, which explained by the ionization and microenvironment of the active site residues, although they are not crucial.

Some properties of xylanases produced by alkaliphilic *Bacillus* strains are listed in Table 10.5.

10.4 Biobleaching

Since it was demonstrated that alkali-treated woodpulp could be biologically bleached by xylanases instead of by the usual environmentally damaging chemical process using chlorine, the search for thermostable alkaline xylanases has been extensive. Dey et al. (1992) isolated an alkaliphilic thermophilic *Bacillus* sp. (NCIM 59) that produced two types of cellulase-free xylanase at pH 10 and 50°C. Khasin et al. (1993) reported alkaliphilic *Bacillus stearothermophilus* T-6 produced an extracellular xylanase that was shown to optimally bleach pulp at pH 9 and 65°C.

Blanco et al. (1995) purified xylanase A (Accession Number AJ006645) from the recently isolated *Bacillus* sp. strain BP-23. The enzyme shows a molecular mass of 32 kDa and an isoelectric point of 9.3. Optimum temperature and pH for xylanase activity were 50°C and 5.5, respectively. Xylanase A was completely inhibited by *N*-bromosuccinimide. The main products of birchwood xylan hydrolysis were xylotetraose and xylobiose. The enzyme

was shown to facilitate chemical bleaching of pulp, generating savings of 38% in terms of chlorine dioxide consumption. The amino-terminal sequence of xylanase A has a conserved sequence of five amino acids found in xylanases from family GH10. Yang et al. (1995) isolated alkaline xylanase produced by an alkaliphilic *Bacillus* sp. from kraft pulp *Bacillus* sp. (V1-4) was isolated from hardwood kraft pulp. It was capable of growing in diluted kraft black liquor at pH 11.5 and produced xylanase when cultivated in alkaline medium at pH 9. Biobleaching studies showed that the enzyme would brighten both hardwood and softwood kraft pulp and release chromophores at pH 7 and 9. deJong et al. (1997) investigated the mechanism of xylanase prebleaching of kraft pulp. It was suggested that lignin-carbohydrate bonds are formed during the redeposition of lignin and xylan on the cellulose fibers and that xylanases can partly hydrolyze the lignin-carbohydrate complexes. This mechanism is proposed to be a major contribution to the bleach- boosting effect of these enzymes. Although the formation of chromophoric xylan was also observed during alkaline cooking, the results indicate that the hydrolysis of this class of compounds has a limited role in xylanase prebleaching .

An alkaliphilic *Bacillus* sp. Sam-3, producing high levels of cellulase-free xylanase active and stable at alkaline pH, was isolated from a soda lake (Shah et al. 1999). When grown in shake flasks for 48 h on agricultural residues such as wheat bran supplemented with yeast extract, optimum pH and temperature of the xylanase were 8 and 60°C, respectively. The enzyme retained approximately 75% of its activity at pH 8 and 60°C when incubated for 2 h. Enzymatic treatment of bagasse pulp carried out using 1.2 IU of enzyme per gram of pulp at 60°C and pH 8 for 2 h resulted in a 4 unit decrease in the κ number. Similar treatment of the pulp at pH 7 and 9 indicated that the Sam-3 xylanase was effective in reducing the kappa number of the pulp over a wide pH range.

Zheng et al. (2000; 2001) reported degumming of ramie fibers by alkaliphilic bacteria and their polysaccharide-degrading enzymes. Three strains of alkaliphilic bacteria, *Bacillus* sp. NT-39, NT-53 and NT-76, were selected for the degumming of ramie fibers and production of polysaccharide-degrading enzymes. After 48 h of incubation with the strains, the loss of the gum might amount to 5.0% or more of the fibers and a number of polysaccharide-degrading enzymes were secreted to the culture supernatants. The residual gum of the fibers decreased to 9.4% after 5 h of enzymatic degumming. Analysis of gum contents and enzyme activities revealed that pectate lyase and xylanase played an important role in the degradation of residual gum. Enzymatic degumming resulted in an increment of 5.4 ISO units in fiber brightness, whereas the reduction in bundle breaking tenacity of the fibers was less than 5%. The results confirmed that degumming of ramie fibers by alkaliphilic bacteria and their enzymes had substantial advantages.

11

Pectinases

Pectinolytic enzymes (pectinases), which degrade pectic polysaccharides such as pectin and pectic acid, are distributed in microorganisms and higher plants but are not found in higher animals. Pectinases from certain microorganisms are widely used in the fruit- and vegetable-processing industries. Recently, a novel field of application is envisaged for pectinases in the production of oligosaccharides as functional food components.

11.1 Early Studies on Pectin-degrading Enzymes

11.1.1 *Bacillus* sp. No. P- 4-N Polygalacturonase

The first paper on alkaline endo-polygalacturonase produced by alkaliphilic *Bacillus* sp. No. P-4-N was published in 1972 (Horikoshi 1972). *Bacillus* sp. No. P-4-N, could grow in the pH range from 7.0 to 11.0, but the most active growth was observed in a pH 10 medium containing 1% Na_2CO_3. The medium of pH 10.4 contained 1% Na_2CO_3, 0.005% $MnSO_4 \cdot 7H_2O$ and 3% pectin as essentials. The enzyme was most stable at pH 6.5 and up to 70°C in the presence of Ca^{2+}, but was inactivated completely at 80–90°C. Further details are given in a previous volume (Horikoshi and Akiba1982).

Recently, the author's group reidentified this strain by 16s RNA analysis and *Bacillus* sp. P-4-N was found to be very similar to or the same strain as *Bacillus halodurans* C-125.

11.1.2 *Bacillus* sp. No. RK9 Polygalacturonate Lyase

Kelly and Fogarty (1978) reported that *Bacillus* sp. No. RK9 isolated from garden soil grew well in alkaline media at pH 9.7 and produced endo-polygalacturonate lyase. The medium composition suitable for enzyme production was as follows (in grams per liter): sucrose, 10.0; bacteriological peptone, 3.0; yeast extract, 3.0; $Na_2CO_3 \cdot H_2O$, 10.0; $CaCl_2 \cdot 2H_2O$, 3.0; $MnSO_4 \cdot 4H_2O$, 0.04; $MgCl_2 \cdot 6H_2O$, 0.2; K_2HPO_4, 1.0; pH 9.7. Neither pectin nor pectic acid was required for the production of the enzyme, indicating that the enzyme is constitutive. The endo-polygalacturonate lyase of RK9 was purified 163-fold from culture fluid by precipitation with 50–90% saturated

$(NH_4)_2SO_4$ and DEAE cellulose column chromatography. The optimum pH of the enzyme was 10.0 towards acid-soluble pectic acid. The optimum temperature of the enzyme was 60°C. The enzyme retained 100% of its activity after incubation for 1 h at 37°C at pH 11.0. It was activated 2.9-fold by 0.4 mM $CaCl_2$ but inhibited by other divalent cations (Mg^{2+}, Sr^{2+}, Co^{2+}, etc.). The enzyme was completely inactivated by 1 mM EDTA, but the activity was recovered by dialysis, suggesting that EDTA had no direct effect on the enzyme itself.

11.2 Recent Studies on Pectin-degrading Enzymes

Recently, Ito and his colleagues have isolated many pectin-degrading enzymes from alkaliphilic *Bacillus* strains and tested for possible industrial application of the enzymes as a laundry detergent additive.

11.2.1 *Pel-4A* and *Pel-4B* from *Bacillus halodurans* C-125

Kobayashi et al. (2000) and Hatada et al. (2001) cloned two genes for alka-

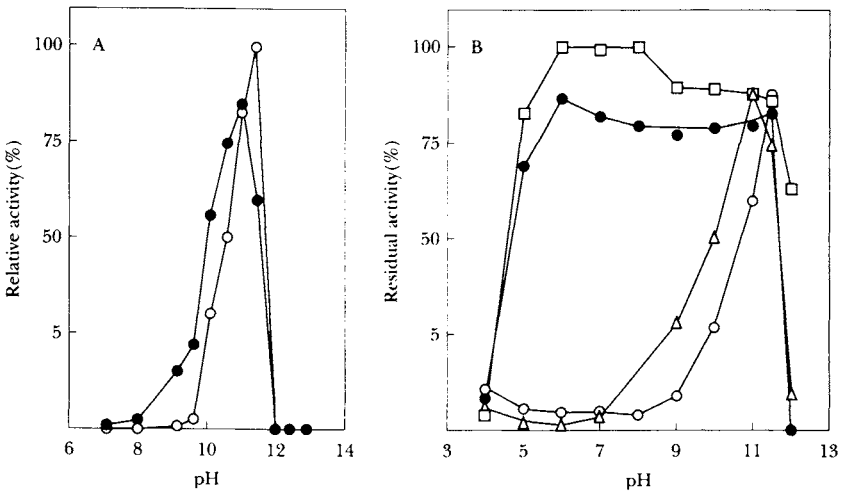

Fig. 11.1 Effects of pH and NaCl on activity and stability. **A** Effect on activity. Pel activity was assayed at 30°C and at the indicated pH either in 50 mM Tris-HCl buffer (pH 7–9.5), 50 mM glycine-NaOH buffer (pH 8–12), or in 50 mM KCl-NaOH buffer (pH 12–12.8), with 0.03 μg Pel-4A. *Open circles*, activity without additive; *solid circles*, activity with 100 mM NaCl. **B** Effect on stability. Pel-4A (0.4 μg) was incubated for 60 h at 5°C and at the indicated pH in 50 mM each of various buffers in the absence (*open circles*) or presence (*solid circles*) of 100 mM NaCl, 2.0 mM $CaCl_2$ (*open triangles*), or both additives (*open squares*). Portions of the solution were withdrawn, and the residual activity was measured under the standard conditions of the assay. The original activity at pH 10.5 (without preincubation) was taken as 100%. Buffers used: acetate, pH 4–6; MOPS, pH 6–8, Tris-HCl, pH 7–9; glycine-NaOH, pH 9–12. (Reproduced with permission from T. Kobayashi et al., *Extremophiles*, **4**, 377(2000))

line pectate lyase (*pel-4A* and *pel-4B*) from *Bacillus halodurans* C-125, sequenced and expressed in *Bacillus subtilis*. The deduced amino acid sequence of *pel-4A* (318 amino acids, 34805 Da, Accession Number AB041769) showed moderate homology to those of known pectate lyases. The purified recombinant enzyme had an isoelectric point of pH 9.7 and a molecular mass of 34 kDa, and exhibited a very high specific activity compared with known pectate lyases so far reported. The enzyme activity was stimulated 1.6-fold by the addition of NaCl at an optimum of 100 mM, and striking stabilization in the presence of 100 mM NaCl was observed in a pH range from 5 to 11.5 at 50°C for 60 h storage (Fig. 11.1).

Another alkaline pectate lyase pel-B was characterized by the same methods described above. The purified enzyme (Pel-4B) had an isoelectric point of pH 9.6 and a molecular mass of 35 kDa, values close to those of Pel-4A. The pH and temperature optima for activity were as high as 11.5 and 70°C, and showed 35.6% identity with Pel-4A on the amino acid level. The enzyme was kept at 40°C for 1 hour, the pH stability curve had two peaks around pH 10 and pH 8 (Fig. 11.2). Although they did not write any reason, in the presence of 2 mM $CaCl_2$ two such peaks shifted to one peak.

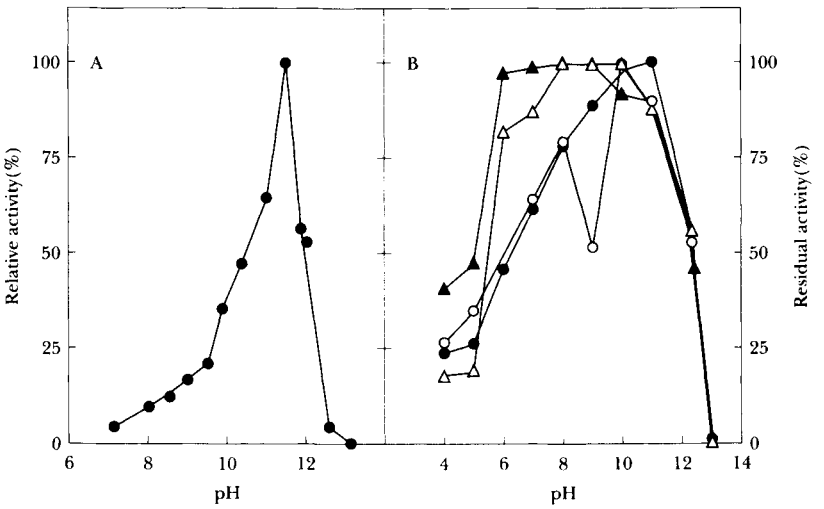

Fig. 11.2 Effects of pH. **A** Pel activity was assayed at 30°C and at the indicated pH either in 50 mM Tris-HCl buffer (pH 7–9.5), 50 mM glycine-NaOH buffer (pH 8–12), or in 50 mM KCl-NaOH buffer (pH 12–12.8) with 0.26 μg Pel-4B. **B** Effect on stability. Pel-4B (1.6 μg) was incubated for 1 h at 40°C and at the indicated pH in 20 mM each of various buffers in the absence (*open circles*) or presence (*solid triangles*) of 100 mM NaCl, 2 mM $CaCl_2$ (*solid circles*), or both additives (*open triangles*). Portions of the solution were withdrawn, and the residual activity was measured under the standard conditions of the assay. The original activity at pH 10.5 (without preincubation) was taken as 100%. Buffers used: acetate, pH 4–6; (*N-2-morpholino*) propane sulfonic acid (MOPS), pH 6–8; Tris-HCl, pH 7–9; glycine-NaOH, pH 8–11; KCl-NaOH, pH 12.4–13.
(Reproduced with permission from Y. Hatada et al., *Extremophiles*, **5**, 127(2001))

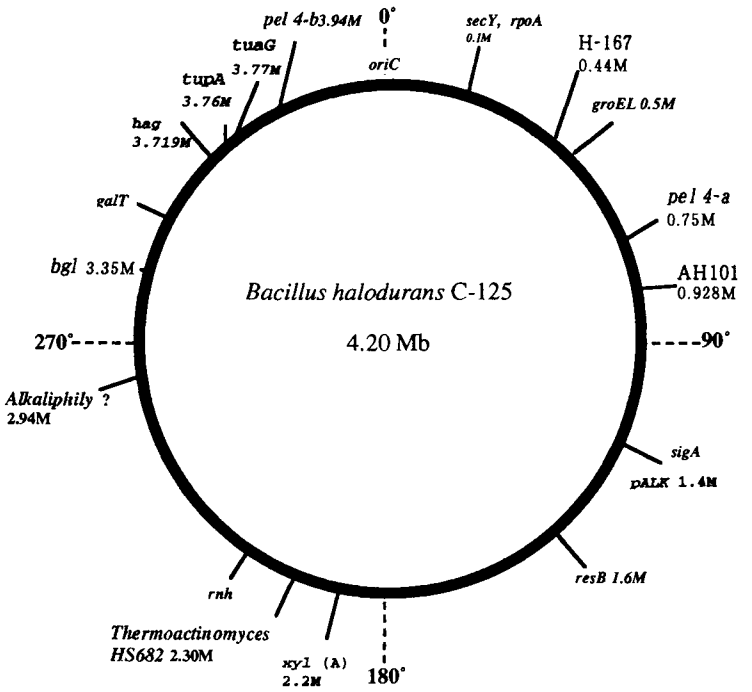

Fig. 11.3 Genetic map of the chromosome of *Bacillus halodurans* C-125. The locations of several genes are indicated on the map. A dashed line indicates the approximate position of the gene.

Addition of NaCl made temperature stability higher as shown in Pel-4A. It showed significant homology to other pectate lyases in the family 1, such as the enzymes from alkaliphilic *Bacillus* sp. strains KSM-P7 and KSM-P103 and the fungi *Aspergillus nidulans* and *Colletotrichum gloeosporioides* f. sp. *malvae*. These loci of these *Pel*-A and *Pel*-B(Accession Number AB042100) of *B. halodurans* C-125 are illustrated in Fig. 11.3.

11.2.2 Other Alkaline Pectin-degrading Enzymes

Kobayashi et al. (1999b) isolated a high-alkaline pectate lyase (pectate *trans*-eliminase, Pel-7) from alkaliphilic *Bacillus* sp. strain KSM-P7. The purified Pel-7 had a molecular mass of approximately 33 kDa as determined by SDS-polyacrylamide gel electrophoresis. The isoelectric point was about pH 10.5. In the presence of Ca^{2+} ions, Pel-7 *trans*-eliminated polygalacturonate in a random manner to produce oligogalacturonides; it exhibited optimal activity at pH 10.5 and at around 60 to 65°C in glycine-NaOH buffer. It also exhibited a protopectinase-like activity, liberating soluble pectin and/or oligogalacturonides from cotton fibers. The *pel* gene (Accession Number AB015043) was cloned and sequenced, and the deduced amino acid sequence of mature Pel-7 had 302 amino acids and 33, 355 Da. Furthermore,

Pel-7 appears to have a similar core structure of parallel β-helix and active site topology with other pectate lyase from conventional microorganisms. These results suggest that Pel-7 is basically grouped into the Pel superfamily although the enzymatic and molecular properties are different.

Furthermore, Hatada et al. (1999) isolated another high-alkaline pectate lyase (Pel-103) from alkaliphilic *Bacillus* sp. strain KSM-P103. Its enzymatic properties were quite similar to those of Pel-7. However, Pel-103 had a similar core and active site topology to the enzymes of known structure from *Erwinia chrysanthemi* and *Bacillus subtilis*. However, due to difficulties encounterd in the high production necessary for industrial scale systems, their interests focused on another pectate-lyase described below. The nucleotide sequence datum was submitted to the DDJB data base under Accession Number AB015044.

Kobayashi et al. (1999a) found a low-molecular-weight, high-alkaline pectate lyase (Pel-15) in an alkaline culture of *Bacillus* sp. strain KSM-P15, purified and crystallized. The enzyme had a relative molecular weight of approximately 20,300 as measured by sedimentation equilibrium, with a sedimentation coefficient (s20, w0) of 1.73 S. It was also a basic protein having an isoelectric point of pH 10.3, and the α-helical content was only 6.6%. In the presence of Ca^{2+} ions, the enzyme degraded polygalacturonic acid in a random fashion to yield 4,5-unsaturated oligo-galacturonides and showed optimal activity around pH 10.5 and 50–55°C (Fig. 11.4). It also had a protopectinase-like activity on cotton fibers. The amino acid sequences showed very low similarity with pectate lyases so far reported. These results strongly suggest that the pectate lyase of *Bacillus* sp. strain KSM-P15 may be a novel enzyme that belongs to a new family.

The nucleotide sequence of the gene for Pel-15 enzyme was determined by Hatada et al. (2000), and deposited it as the Accession Number AB028878. It harbored an open reading frame of 672 bp encoding the mature enzyme of 197 amino acids with a predicted molecular mass of 20 924 Da. The deduced amino-acid sequence of the mature enzyme showed very low homology (< 20.4% identity) to those of known pectinolytic enzymes in the large pectate lyase superfamily (the polysaccharide lyase family 1). In an integrally conserved region designated the BF domain, Pel-15 showed a high degree of identity (40.5% to 79.4%) with pectate lyases in the polysaccharide lyase family 3, such as PelA, PelB, PelC and PelD from *Fusarium solani* f. sp. pisi, PelB from *Erwinia carotovora* ssp. *carotovora*, PelI from *E. chrysanthemi*, and PelA from a *Bacillus* strain. By site-directed mutagenesis of the *pel-15* gene, they replaced Lys20 in the N-terminal region, Glu38, Lys41, Glu47, Asp63, His66, Trp78, Asp80, Glu83, Asp84, Lys89, Asp106, Lys107, Asp126, Lys129 and Arg132 in the BF domain, and Arg152, Tyr174, Lys182 and Lys185 in the C-terminal region of the enzyme individually with Ala and/or other amino acids. Consequently, some carboxylate and basic residues selected from Glu38, Asp63, Glu83, Asp106, Lys107, Lys129 and Arg132 were suggested to be involved in catalysis and/or calcium binding.

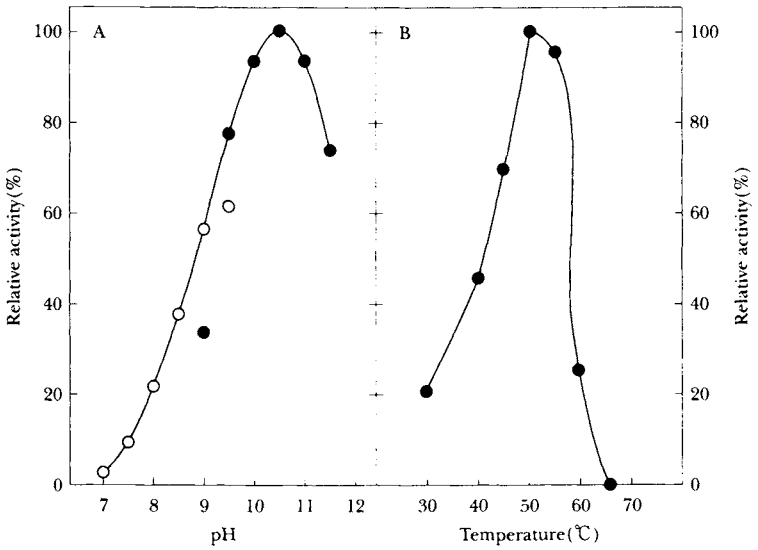

Fig. 11.4 Effects of pH and temperature on enzyme activity. (A) Effect of pH. Pel activity was measured at 30℃ and the indicated pH values in 50 mM Tris/HCl buffer (pH 7–9.5, ○) and glycine/NaOH buffer (pH 9–11.5, ●) with 0.19 μg Pel-15E. (B) Effect of temperature. Pel-15E (0.11 μg) was added to the reaction mixture and the reaction was carried out at the temperatures indicated. The maximum activity, which was observed at 50℃, was taken as 100%.
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A chimeric enzyme composed of Ala1 to Tyr105 of Pel-15 in the N-terminal regions, Asp133 to Arg159 of Fs PelB in the internal regions, and Gln133 to Tyr197 of Pel-15 in the C-terminal regions was constructed. The substituted PelB segment could also express β-elimination activity in the chimeric molecule, confirming that Pel-15 and PelB share a similar active-site topology.

Next, Akita et al. (2001) determined the crystal structure of a highly alkaline low molecular weight pectate lyase (Pel-15) at 1.5 Å resolution by the multiple isomorphous replacement (MIR) method. This is the first pectate lyase structure from the polysaccharide lyase family 3. The overall structure is a simple eight-turn right-handed parallel β-helix domain with one long loop protruding from one side of the β-helix. The low molecular weight of Pel-15 derives from the lack of N- and C-terminal extensions that are found in many β-helix proteins. Although the structure has one calcium ion at pH 6.7, raising the pH to 9.5 results in the binding of an additional calcium ion. The common calcium ion found in both the pH 6.5 and 9.5 structures seems to stabilize both the β-helix structure and the long protruding loop. The additional calcium ion only found in the pH 9.5 structure may neutralize the acidic substrate. The region around the additional calcium ion is thought to bind to the substrate, as this region is rich in charged amino-acid residues which are required in catalysis.

In a series of works, Sawada et al. (2000) found a minor, highly alkaline pectate lyase (Pel-15E, the nucleotide sequence data was submitted to the EMBL data bank under Accession Number AB028877) from the same culture fluid of alkaliphilic *Bacillus* sp. strain KSM-P15. The pectate lyase (pectate *trans*-eliminase), designated Pel-15E, was purified to homogeneity. The purified enzyme had a molecular mass of approximately 33 kDa, as determined by SDS/PAGE, and a pI of approximately pH 9.2. Pel-15E exhibited optimum activity at pH 10.5 and 50–55°C in glycine/NaOH buffer. Pel-15E had an absolute requirement of Ca^{2+} ions for manifestation of the enzymatic activity and *trans*-eliminated poly(galacturonic) acid, most likely by endo-type cleavage. A gene for the enzyme, which was cloned using the shotgun method and sequenced, contained a 960-bp ORF encoding 320 amino acids. The mature enzyme (286 amino acids, 32 085 Da) from the deduced amino-acid sequence showed quite low homology to known Pels from various microorganisms with 16.1–20.4% identity. Furthermore, no conserved regions in the sequences of other enzymes from the established Pel superfamily were found in the sequence of Pel-15E. Based on this amino-acid sequence homology, Pel-15E appears to belong to a new class of the Pel family.

Beside Pel-15 and Pel-15E, KSM-P15 produced another high-molecular-weight, high-alkaline and low-isoelectric-point enzyme (Pel-15H). Ogawa et al. (2000) purified it to homogeneity by sequential column chromatographies. The molecular weight of the enzyme determined by SDS-polyacrylamide gel electrophoresis was approximately 70,000 and the pI was around pH 4.6. Pel-15H randomly *trans*-eliminated polygalacturonate in the presence of Ca^{2+} ions, and the maximum activity was observed at pH 11.5 and at 55°C in glycine-NaOH buffer. The gene for Pel-15H was cloned and sequenced (Accession Number AB028878), and the structural gene contained a 2,031-bp open reading frame that encoded 677 amino acids including a possible 28-amino-acid signal sequence. The mature enzyme (649 amino acids, molecular weight 69,550) showed very low similarity to Pels from *Bacillus* with 12.7–18.2% identity.

Many pectate lyases have been found from alkaliphilic *Bacillus* strains for retting cotton fibers. There are a few examples of bifunctional enzymes possessing different active sites in a single polypeptide chain. Recently, a gene for a novel enzyme having pectate lyase (Pel) and pectin methylesterase (Pme) activities found in the genome of an alkaliphilic *Bacillus*, KSM-P358, was sequenced. The structural gene (Accession Number AB062879) contained a long open reading frame of 4314 bp corresponding to a 32-amino-acid signal peptide and a 1406-amino-acid mature enzyme with a molecular mass of 155,666. The mature enzyme contained two uncontiguous regions at amino acids 800–1051 and 1105–1406 exhibiting homology to a Pel from a *Bacillus* strain with 43.7% and a Pme from *Erwinia chrysanthemi* with 33.4% identity, respectively. The recombinant enzyme expressed in *Bacillus subtilis* cells had a molecular mass of 160 kDa and

exhibited pH and temperature optima for Pel activity of 10 and 40°C and those for the Pme activity of 8.5 and 45°C. The genes for the domains for the Pel and Pme could be separately expressed in *Escherichia coli* cells, and the catalytic properties of the respective protein fragments were essentially identical to those of the intact enzyme. This novel enzyme is "mosaic" in that some regions before the two domains exhibited limited but substantial similarity to some regions of carbohydrate-active enzymes. The regions contained parts of a gene for Pels from a *Bacillus* sp. and *Pseudomonas fluorescens*, a xylanase from *P. fluorescens* subsp. Cellulosa, a 1,4-β-mannanase from a *Pyromyces* sp., a putative Pel from a *Streptomyces coelicolor* cosmid, a (1,3-1,4)-β-glucanase from *Clostridium thermocellum*. Kobayashi et al. (2001) reported a high molecular weight alkaline exopolygalacturonase from *Bacillus* sp. strain KSM-P576 (Fig. 11.5; Fig. 11.6). The enzyme had a molecular weight of approximately 115,000 and maximum enzyme action at pH 8.0 at 55°C. Furthermore, they found an exopolygalacturonase extracellularly produced by *Bacillus* sp. strain KSM-P443. The enzyme had a molecular weight of ap-

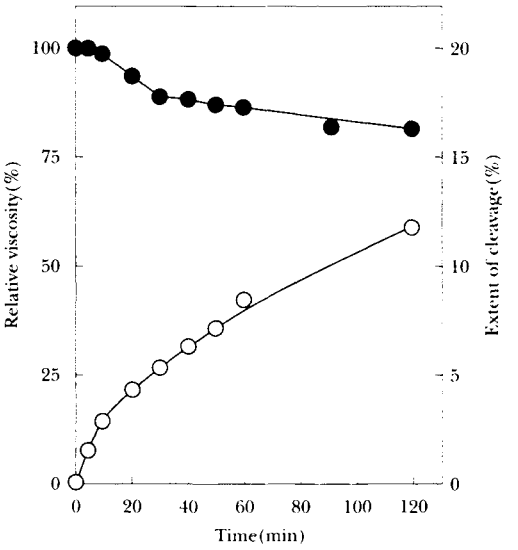

Fig. 11.5 Relationship between rates of viscosity reduction and galacturonosidic bond cleavage. The exo-PG(43 μg) was added to the reaction mixture in a total volume of 10 ml and, at intervals, viscosity was measured at 30°C. The initial viscosity was measured by adding 50 mM Tris-HCl buffer (pH 7.0) instead of the enzyme, which was taken as 100%. At the same time, aliquots (0.2 ml) were withdrawn, and the reducing sugars released were measured by DNS reagent. The percentage of cleavage was calculated on the basis of total galacturonic acid in the substrate. Closed circles, degree of viscosity reduction; open circles, the extent of cleavage of galacturonosidic bonds.

(Reproduced with permission from T. Kobayashi et al., *Enzyme Microb. Technol.*, **29**, 70(2001))

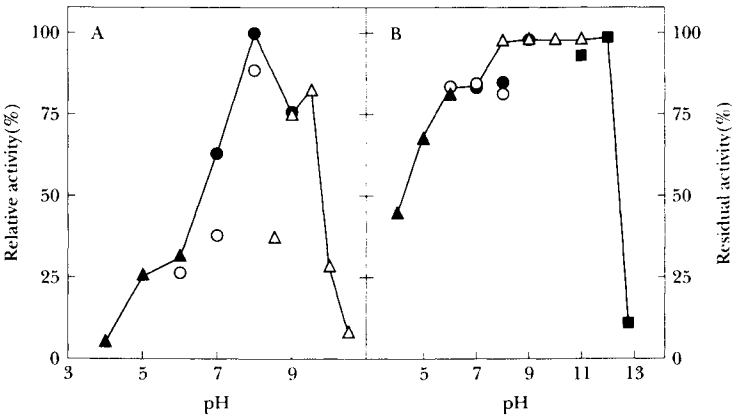

Fig. 11.6 Effects of pH on activity and stability. A: Effect on activity. Assay of hydrolyzing activity toward PGA was done at 30°C and at the indicated pHs in 100 mM of each buffer: acetate (pH 4–6, closed triangles), MOPS (pH 6–8, open circles), Tris-HCl (pH 7–9, closed circles) and glycine-NaOH (pH 8.5–10.5, open triangles) with 3.6 μg of the enzyme. B: Effect on stability. The exo-PG (5.6 μg) was incubated for 1 h at 30°C at the indicated pHs in 50 mM buffers, acetate (pH 4–6, closed triangles), MOPS (pH 6–8, open circles), Tris-HCl (pH 7–9, closed circles), glycine-NaOH (pH 8–11, open triangles) and KCl-NaOH (pH 11–12.7, closed squares). The original activity at pH 8.0 without preincubation was taken as 100%.
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proximately 45,000 and an isoelectric point of pH 5.8. The N-terminal sequence was Ser-Met-Gln-Lys-Ile-Lys-Asp-Glu-Ile-Leu-Lys-Thr-Leu-Lys-Val-Pro-Val-Phe and it had no sequence similarity to those of other pectinolytic enzymes so far reported. Maximum activity toward polygalacturonic acid was observed at 60°C and at pH 7.0 in 100 mM Tris-HCl buffer without requiring any metal ions. This is the first bacterial exo-PGase that releases exclusively mono-galacturonic acid from di-, tri-, tetra-, penta- and poly-galacturonic acids.

11.3 Industrial Applications

Hoondal et al. (2002) recently reviewed the microbial alkaline pectinases and their industrial applications. This review features the potential applications and uses of microbial alkaline pectinases, the nature of pectin, and the vast range of pectinolytic enzymes that function to mineralize pectic substances present in the environment. It also emphasizes the environmentally friendly applications of microbial alkaline pectinases thereby revealing their underestimated potential, although there are many difficulties that must be overcome.

11.3.1 Production of Japanese Paper (Washi)

The first application of alkaline pectinase-producing bacteria in the retting of Mitsumata bast was reported by Yoshihara and Kobayashi (1982). Alkaliphilic bacteria were isolated form soil, sewage and decomposed manure in Japan and Thailand. *Bacillus* sp. No. GIR 277 had strong macerating activity toward Mitsumata bast. The bacteria isolated were motile, aerobic, spore-forming rods and grew well on nutrient agar of pH 9.5 adjusted with Na_2CO_3. This bacterium produced pectate lyase which had an optimum pH for enzyme action at 9.5. Japanese paper was produced as follows. Four grams of Mitsumata bast was suspended in 100 ml of a culture medium containing 0.05% yeast extract, 0.05% casamino acids, 0.2% NH_4Cl, 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$. After sterilization, Na_2CO_3 was added to a concentration of 1.5%, and *Bacillus* sp. No. GIR 277 was inoculated into the medium. After 5 days of cultivation at 30°C with shaking, the bast retted was harvested and Japanese paper was prepared by the method described in Japanese Industrial Standard P8209. The overall yield of pulp was about 70%. The strength of the unbeaten pulp resulting from bacterial retting was higher than that obtained by the conventional soda ash-cooking method. The paper sheets were very uniform and soft to the touch. Yoshihara and Kobayashi also concluded that bacterial retting under alkaline condition is a potentially useful process for the production of pulp of excellent quality from non-woody pectocellulosic fibers.

11.3.2 Treatment of Pectic Wastewater with an Alkaliphilic *Bacillus* Strain

Wastewater from the citrus processing industry contains pectinaceous materials which are only slightly decomposed by microbes during activated-sludge treatment. To solve this problem the pretreatment of the wastewater with pectin-degrading microorganisms may be required. Some pectin-degrading organisms are known phytopathogens, making the application of these organisms to waste treatment very dangerous. However, alkaliphilic microorganisms may be one of the best candidates for this purpose because they can grow under limited conditions, i.e. an alkaline condition of pH 10. Tanabe et al. (1987) tried to develop a new process of waste treatment using alkaliphilic microorganisms. From soil in Thailand they isolated an alkaliphilic *Bacillus* sp. No. GIR 621, which produced an extracellular endo-pectate lyase in alkaline medium of pH 10.0. Strain GIR 621 was applied to the pretreatment of wastewater containing pectinaceous substrates from an orange canning factory. The strain was cultured on the wastewater supplemented with 0.25% polypeptone, 0.25% yeast extract, 0.05% soybean powder and 0.1% K_2HPO_4 at pH 10.0 (adjusted with 1% Na_2CO_3) at 27°C. The concentration of uronic acid in the waste water decreased by 69% at 12 h and by 91% at 36 h. The activity of endo-pectate lyase reached maximum

(3.3 units/ml) at 25 h. Furthermore, the enzyme yield was increased 20-fold (65 units/ml) by a mutant strain GIR 621-7 made by N-methyl-N-nitro-N-nitrosoguanidine (NTG) treatment (Tanabe et al. 1988). Strain GIR 621-7 grew faster in the wastewater and the uronic acid in it decreased by 93% even at 20 h. Treatment with strain GIR621-7 proved to be useful as a pretreatment to remove pectic substances. The pectate lyase from GIR 621-7 was purified and characterized under the following conditions: molecular weight, 33,000; isoelectric point, 8.8; optimum pH, 9.0; optimum temperature, 55–60°C; preferred concentration of Ca^{2+}, 0.4 mM.

11.3.3 Degumming Ramie Fibers

Cao et al. (1992) isolated four alkaliphilic bacteria, NT-2, NT-6, NT-33 and NT-82, producing pectinase and xylanase. The pH and temperature optima for the activity of PGase were 10.5 and 70°C, respectively. One strain, NT-33, had excellent capacity for degumming ramie fibers.

Zheng et al. (2001) selected three strains of alkaliphilic bacteria, *Bacillus* sp. NT-39, NT-53 and NT-76 for the degumming of ramie fibers and production of polysaccharide-degrading enzymes. After 48 h of incubation with these strains, the loss of the gum weight amount to 5.0% or more of the fibers and a number of polysaccharide-degrading enzymes were secreted into the culture supernatants. The residual gum of the fibers decreased to 9.4% after 5 h of enzymatic degumming. Analysis of gum contents and enzyme activities revealed that pectate lyase and xylanase played an important role in the degradation of residual gum. Enzymatic degumming resulted in an increment of 5.4 ISO units in fiber brightness, whereas the reduction in bundle breaking tenacity of the fibers was less than 5%. The results confirmed that degumming of ramie fibers by alkaliphilic bacteria and their enzymes had potential advantages.

12

Mannan-degrading Enzymes

β-Mannan is a kind of hemicellulose contained in higher plants such as konjac, guargum, locust bean and copra. It easily dissolves in alkaline water. Mannan-degrading enzymes of neutrophilic bacteria, actinomyces and fungi have been studied. However, no mannan-degrading enzyme that hydrolyzes under alkaline conditions had been found before its discovery by our research group.

12.1 Isolation and Properties of Mannan-degrading Microorganisms (Akino et al. 1987; 1988a,b)

A small amount of soil was spread on agar plates containing 1% β-mannan from larch wood, 1% polypeptone, 0.2% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.5% sodium carbonate. The plates were incubated at 37°C for 48–72 h. Strain AM-001 with a large clear zone around the colony was selected as the enzyme producer. The isolate grew at temperatures from 20°C to 45°C, with an optimum at 37°C in the medium described above. The pH range for growth was from pH 7.5 to 11.5 with the optimum at pH 8.5 to 9.5. The bacterium was aerobic, motile and gram-variable; the rod-shaped cells (0.6–0.8 μm × 3.0–6.0 μm) had peritrichous flagella and terminal swollen sporangia containing oval spores (1–1.2 μm × 1.5–2.0 μm). Recently, the taxonomical position of this alkaliphilic *Bacillus* strain was determined as *Bacillus mannanilyticus* by Nogi (2005).

Bacillus mannanilyticus AM-001 was cultivated aerobically under various conditions and activities of β-mannanase in the culture broth and β-mannosidase extracted from cells treated with 0.1% Triton X-100 were examined. Both enzymes formed when the bacterium was grown under alkaline conditions, and the optimum concentration was 0.5% Na_2CO_3 or 0.5–1.0% $NaHCO_3$. Various carbohydrates were also tested and the best carbohydrate for enzyme production was konjac powder (1% w/v). The optimum cultivation temperature for enzyme production was 31°C for β-mannosidase and 37°C for β-mannanase in the production medium composed of 1% konjac powder, 0.2% yeast extract, 2% polypeptone, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.5% sodium carbonate.

12.2 Properties of β-Mannanase

Three extracellular β-mannanases (M-1, M-II and M-III) were purified by ammonium sulfate precipitation (80% saturation) followed by DEAE-Toyopearl chromatography. The molecular weight estimated by SDS-PAGE was 58,500 for M-1, 59,500 for M-II and 42,000 for M-III. As shown in Fig. 12.1, β-mannanases, M-1 and M-II were most active at pH 9.0, and M-III demonstrated optimum enzyme action at pH 8.5. Although M-III enzyme was relatively more stable than the others, there were no significant differences among these three mannanases except for molecular weight. These enzymes hydrolyzed β-1,4-mannooligosaccharides larger than mannotriose, and the major components in the digest were di-, tri- and tetra-saccharides (Akino et al. 1988b).

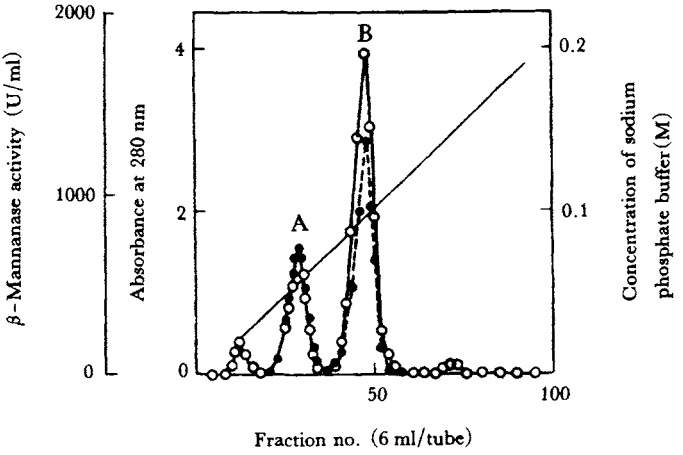

Fig. 12.1 Chromatography of the β-mannanases with hydroxyapatite column. Symbols: (●), β-mannanase activity; (○), A_{280} (Protein); ( ), sodium phosphate buffer concentration.
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12.3 β-Mannosidase

β-Mannosidase hydrolyzes the β-mannosidic linkage in various β-1,4-mannans and yields D-mannan. Although many studies have been performed on β-mannosidases of animal tissues, few microbial β-mannosidases have been investigated. Our alkaliphilic *Bacillus mannanilyticus.* AM-001 produces significant amounts of cell-associated β-mannosidase (Akino et al. 1988b)

The β-mannosidase was extracted from the cells in the presence of 0.1% (w/v) Triton X-100. After 2 h shaking the insoluble cells were centrifuged off and the supernatant fluid was used as the crude enzyme solution. The

Table 12.1 Relative hydrolysis rates of β-1,4-D-manno-oligosaccharides by β-mannosidase

Substrate	Activity (μmol/min·mg)	Relative activity (%)
Mannobiose	1.92	34
Mannotriose	3.88	68
Mannotetraose	5.68	100
Mannopentaose	4.48	79

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enzyme was electrophoretically homogeneous and its molecular weight was estimated to be 94,000. Isoelectric focusing showed the isoelectric point to be 5.5.

The purified enzyme was most active at pH 6.0 and stable at 40°C for 30 min in the pH range of 6.5 to 8.0. The enzymatic activity was strongly inhibited by the addition of metal ions and some chemicals but not by D-mannose. The enzyme hydrolyzed β-1,4-mannooligosaccharides and the best substrate was mannotetraose, as shown in Table 12.1, although long incubation resulted in weak transferase activity. The molecular weight (94,000) was smaller than that of other microbial β-mannosidases such as *Aspergillus oryzae* (120,000–135,000) and *Tremella fuciformis* (160,000–200,000). The final product from the oligosaccharides with the enzyme was mainly D-mannose, and the enzymatic activity was not inhibited by D-mannose or mannose-derivatives (D-mannosamine, D-mannonic acid and D-mannitol). These properties of the enzyme are good for the production of D-mannose from β-mannan in the presence of the β-mannanases described above.

12.4 Molecular Cloning of β-Mannanase Gene and Expression in *Escherichia coli*

As described in the previous section, this strain produced significant amounts of three extracellular β-mannanases and a cell-associated β-mannosidase. The three β-mannanases differed in several enzymatic properties, including optimum pH for enzyme action, optimum temperature, pH stability, thermal stability, isoelectric point and molecular weight. To elucidate the genetic basis for the production of multiple forms we cloned the β-mannanase genes of this strain into *E. coli* using pUC 19 as a vector (Akino et al. 1989). Transformants having mannanase activity could be detected directly on the LB-konjac plates containing ampicillin (50 μg/ml). A plasmid harbored by the transformant was designated pMAH3 containing a 2.5 kb DNA fragment of *Bacillus* sp. no. AM-001 was isolated. The nucleic acid sequence of gene responsible for β-mannanase was determined and deposited it as Accession Number A37219.

E. coli JM101 (pMAH3) was grown aerobically in LB broth for 24 h at 37°C. The β-mannanase activity was located mainly in the periplasmic (53%) and intracellular (43%) fractions. The periplasmic β-mannanase was purified by affinity chromatography and two active fractions (β-mannanase A and B) were separated. Molecular weight was 58,000 for A and 43,000 for B. As shown in Table 12.2, mannanase A has enzymatic properties similar to those of β-mannanase M-I and M-II of *Bacillus mannanilyticus* AM-001, and mannanase B properties are similar to those of β-mannanase M-III. The N-terminal amino acid sequence from amino acid I (Asn) to 9 (Gln) of *Bacillus mannanilyticus* AM-001 enzyme coincides with those from amino acid 4 (Asn) to 12 (Gln) of *E. coli* JM101 (pMAH3) enzyme, as shown in Table 12.3. This may reflect differences in the specificities of the signal peptidases of the two bacteria (*Bacillus mannanilyticus* AM-001and *E. coli*). There was a single open reading frame of 1,539 bp, which encoded a polypeptide of 513 amino acids. A putative ribosome binding site, a GGAG-GA sequence which highly complemented the 3' end of *B. subtilis* 16S ribosomal RNA, was observed upstream of the open reading frame. A signal peptide of 26 amino acids was cleaved during the secretion process of *E. coli* and 29 amino acids were removed in the case of *Bacillus mannanilyticus* AM-001. As both enzyme molecules had β-mannanase activity, Ser-Glu-Ala in the N-terminal fraction is not essential for the enzyme activity. As described above, *E. coli* JM101 harboring plasmid pMAH3 produced two β-mannanases, although it showed no capacity to encode two open reading frames. To elucidate this point C-terminal sequences of both enzymes were analyzed after tryptic digestion. The smaller enzyme (mannanase B) had a C -terminal fragment of Gly-Glu-Ile-Asp-Tyr-Gly-Gln-Ser-Asn-Pro-Ala-Thr-Val-COOH consisting of 339 amino acids. The larger was Leu-Asp-His-Val-Thr-Val-Arg-COOH consisting of 487 amino acids. Deletion derivatives having 1,098

Table 12.2 Comparison of some properties of the purified β-mannanases from alkaliphilic *Bacillus mannanilyticus* AM-001 and *E. coli* JM101 (pMAH3)

	AM-001			*E. coli* (pMAH3)	
	M-I	M-II	M-III	A	B
Optimum temperature (°C)	60	60	65	60	65
Optimum pH	9.0	9.0	8.5	9.0	8.5
Thermal stability (°C)	50	50	60	50	60
pH stability 8.0-9.0	8.0–9.0	8.0–9.0	7.0–9.0	8.0–9.0	7.0–9.0
Molecular weight (kDa)	58	59	42	58	43

The enzyme activities were measured by the method described in the text under various pH and temperature conditions. The buffers used were Carmody buffer and 50 mM of Good's buffer (CHES, pH 9.0). The enzyme solutions were incubated at various pHs in Carmody buffer for 30 min at 50 or 60°C, and at various temperatures for 30 min in 50 mM Good's buffer (CHES, pH 9.0). The residual activities were measured by the method reported previously.
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Table 12.3 N-Terminal sequences of β-mannanases

		1	2	3	4	5	6	7	8	9	10	11	12
Alkaliphilic *Bacillus mannanilyticus* AM-001													
β-mannanase	M-I	Asn	Gly	Ala	Ala	Leu	Ser	Asn	Pro	Asn	Ala	Asn	Gln
	M-II	Asn	Gly	Ala	Ala	Leu	Ser	Asn	Pro	Asn	Ala	Asn	Gln
	M-III	Asn	Gly	Ala	Ala	Leu	Ser	Asn	Pro	Asn	Ala	Asn	Gln
E. coli (pMAH3)													
		1	2	3	4	5	6	7	8	9	10	11	12
β-mannanase	A	Ser	Glu	Ala	Asn	Gly	Ala	Ala	Leu	Ser	Asn	Pro	Asn
	B	Ser	Glu	Ala	Asn	Gly	Ala	Ala	Leu	Ser	Asn	Pro	Asn

base pairs from the ATG star codon maintained the β-mannanase activity of the encoded polypeptide. However, clones harboring DNA fragments (1,051 bp) shorter than the gene which encoded β-mannanase B (1,095 bp) did not exhibit β-mannanase activity. Why were two protein molecules produced from one open reading frame? One possibility is processing by protease. However, the simultaneous production of both β-mannanases A and B in an *E. coli* transformant was demonstrated by the maxicell procedure. Another possibility is a difference in codon usage in the two microorganisms; however, no definitive experiment has been reported. Nucleotide sequence analysis indicated that the codon usage in *Bacillus mannanilyticus* AM-001 was different from that of *E. coli.*

Mendoza (1995) cloned mannanase gene and expressed in *E.coli* DH5α by using pUC 18 and a sequenced β-mannanase gene from *Bacillus subtilis* NM-39. The deduced amino acid sequence of the cloned mannanase showed sequence homology with mannanase from alkalophilic *Bacillus mannanilyticus* AM-001 (about 50%).

Then, George et al. (2001) isolated an alkalithermophilic *Thermomonospora* sp. producing high levels of xylanase from self-heating compost. The culture produced 125 IU/ml of xylanase when grown in shake flasks at pH 9 and 50°C for 96 h. The culture filtrate also contained cellulase (23 IU/ml), mannanase(1 IU/ml) and β-xylosidase (0.1 IU/ml) activities.

No further reports have so far been published.

13

Lipases

Lipases are an important group of biotechnologically relevant enzymes and they have many applications in the food, dairy, detergent and pharmaceutical industries. Some important lipase-producing bacterial genera include *Bacillus*, *Pseudomonas* and *Burkholderi.* Most lipases can act in a wide range of pH and temperature, though alkaline bacterial lipases are more common and not specific to alkaliphilic microrganisms. The latest trend in lipase research is the development of novel and improved lipases through molecular approaches such as directed evolution and exploring natural communities by the metagenomic approach.

Alkaline lipases have been isolated from many microorganisms as described in the previous book written by the present author (Horikoshi 1999a, b). However, few alkaliphilic *Bacillus* strains have been reported as alkaline lipase producers. Mostly non-alkaliphilic *Bacillus* such as *Bacillus stearothermophilus* have mainly been isolated and their lipases extensively reported.

13.1 Lipases from *Bacillus* Strains

Although the enzyme isolated was not pure lipase, Wang and Saha (1993) reported the purification and characterization of thermophilic and alkaliphilic tributyrin esterase from *Bacillus* strain A30-1 (ATCC 53841). The enzyme was purified 139-fold to homogeneity by sodium chloride (6 M) treatment, ammonium sulfate fractionation (30–80%) and phenyl-Sepharose CL-6B column chromatography. The native enzyme was a single polypeptide chain with a molecular weight of about 65,000 and an isoelectric point at pH 4.8. The optimum pH for esterase activity was 9.0, and its pH stability range was 5.0–10.5. The optimum temperature for activity was 60°C. The esterase had a half-life of 28 h at 50°C, 20 h at 60°C and 16 h at 65°C. It showed the highest activity on tributyrin, with little or no activity toward long-chain (12–20 carbon) fatty acid esters.

Wang et al. (1995), then produced thermostable alkaline lipase from a newly isolated thermophilic *Bacillus*, strain A30-1 (ATCC 53841). A thermophilic bacterium was isolated from a hot spring area of Yellowstone National Park, USA. The organism grew optimally at 60–65°C and in the pH range of

6–9. It was characterized as a *Bacillus* species. In the presence of corn or olive oil (1.0%) as the growth substrate, this *Bacillus* produced an extracellular lipolytic activity. The partially purified lipase preparation had an optimum temperature of 60°C at an optimum pH of 9.5. It retained 100% of its original activity after being heated at 75°C. The enzyme was active on triglycerides containing fatty acids having a carbon chain length of C16:0 to C22:0 as well as on natural fats and oils. The purified enzyme had an isoelectric point of 5.15 and an approximate molecular weight of 65,000.

Schmidtdannert et al. (1996) reported molecular cloning, nucleotide sequence, purification and some properties of the thermoalkalophilic lipase of *Bacillus thermocatenulatus*. An expression library was generated by partial *Sau*3A digestion of genomic DNA from the thermophile *B. thermocatenulatus* and cloning of DNA fragments in pUC18 in *E. coli* DH5α. Screening for lipase activity identified a 4.5 kb insert in pUC18 which directed the production of lipase in *E. coli* DH5α. A subclone with a 2.2-kb insert was sequenced. The lipase gene codes for a mature lipase of 388 amino acid residues, corresponding to a molecular weight of 43 kDa. The expressed lipase was isolated and purified 312-fold to homogeneity. N-terminal sequencing of the purified lipase revealed a correct cleavage of the preprotein in *E. coli* DH5α. Maximum activity was found at pH 8.0–9.0 with tributyrin and olive oil as substrates. The lipase showed high stability at pH 9.0–11.0 and towards various detergents and organic solvents. No report on industrial application such as laundry detergent additive has so far been published.

In 1998, Kim et al. (1998) cloned a gene coding for an extracellular lipase of *Bacillus stearothermophilus* L1 in *E. coli*. Sequence analysis showed an open reading frame of 1254 bp, which encodes a polypeptide of 417 amino acid residues. The polypeptide was composed of a signal sequence (29 amino acids) and a mature protein of 388 amino acids. An alanine replaces the first glycine in the conserved pentapeptide (Gly-X-Ser-X-Gly) around the active site serine. The expressed lipase was purified by hydrophobic interaction and ion exchange chromatography using buffers containing 0.02% (v/v) Triton X-100. The lipase was most active at 60–65°C and in alkaline conditions around pH 9–10. The lipase had the highest activity toward *p*-nitrophenyl caprylate among the synthetic substrates and tripropionin among the triglycerides. It hydrolyzed beef tallow and palm oil more rapidly than olive oil at 50°C.

Then, an extremophilic bacterium isolated from mangrove detritus produced an extracellular alkaline-thermostable lipase (Ghanem et al. 2000). The bacterium was identified by cell morphology, growth characteristics, G + C molar ratio and DNA/DNA hybridization as a strain of *Bacillus alcalophilus*. The bacterium grew optimally at pH 10.6, 60°C with NaCl tolerance up to 7.5% (w/v). Carbonates and/or bicarbonates enhanced lipase production, while NaCl had an inhibitory effect. Maximum lipase activity was at 60°C at pH 10.6, with approximately. 60% of its activity being re-

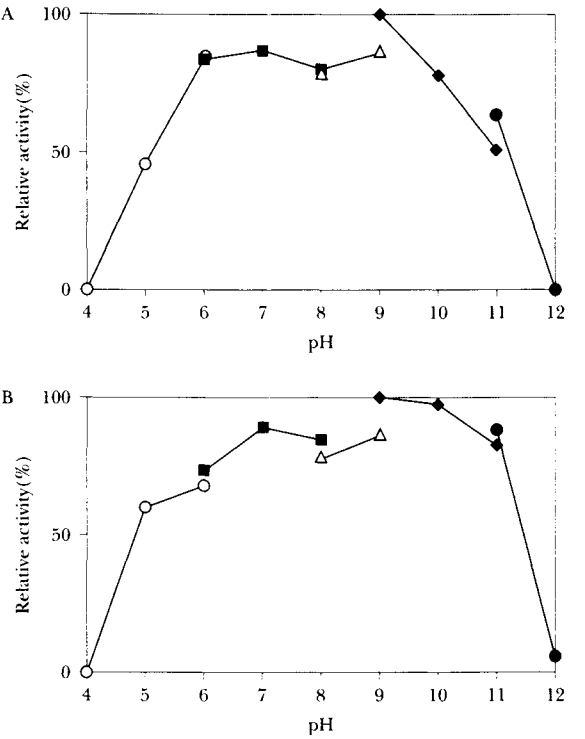

Fig. 13.1 Effect of pH on enzyme activity (A) and stability (B). T1 lipase was assayed at various pHs from pH 4 to pH 12 by a colorimetric method. Symbols used are: (○), Acetate buffer; (■), Potassium phosphate buffer; (△), Tris-HCl buffer; (◆), Glycine buffer; (●), Na_2HPO_4/NaOH buffer. For the stability test, the T1 lipase was assayed after incubation at various pHs (1:1, v/v) at 65°C for 30 min.
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tained at 80°C after 20 min and 80% of its activity was retained at pH 11 after incubation at 60°C.

Recently, Leow et al. (2004) reported that the gene of a thermostable extracellular lipase of *Geobacillus* sp. Strain T1 was cloned in *E. coli*. Sequence analysis revealed an open reading frame of 1,250 bp (Accession Number AY26074), which codes for polypeptide of 416 amino acid residues containing 28 amino acids as a singnal peptide. Although the microorganisms was not alkaliphilic, but it was stable up to 65°C at pH 7 and active over a wide pH range (pH 6–11), as shown in Fig. 13.1.

13.2 Lipases from *Pseudomonas* Strains

Lin et al. (1995; 1996) reported an extracellular alkaline lipase of alka-

liphilic *Pseudomonas pseudoalcaligenes* F-111 which was isolated from soil using a rhodamine B agar plate with Na_2CO_3 and purified to homogeneity. The apparent molecular weight determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 32,000, and the isoelectric point was 7.3. With *p*-nitrophenyl esters as substrates, the enzyme showed a preference for C-12 acyl and C-14 acyl groups. The enzyme was stable in the pH range of 6 to 10, which coincides with the optimum pH range.

Pseudomonas alcaligenes M-1 secretes an alkaline lipase which has excellent characteristics for the removal of fatty stains under modern washing conditions (Gerritse et al. 1998). A fed-batch fermentation process based on the secretion of alkaline lipase from *P. alcaligenes* was developed. The gene encoding the high-alkaline lipase from *P. alcaligenes* was isolated and characterized. Amplification of lipase gene copies in *P. alcaligenes* with the aid of low- and high-copy-number plasmids resulted in an increase of lipase expression that was apparently colinear with the gene copy number. It was found that over-expression of the lipase helper gene, *lipB*, produced a stimulating effect in strains with high copy numbers (>20) of the lipase structural gene, *lipA*.

13.3 Metagenomic Approach

Recently, Rees et al. (2003; 2004) constructed metagenomic libraries with DNA extracted directly from Lake Elmenteita in Kenya. From the Lake Elmenteita environmental library one positive clone for esterase/lipase activity was isolated (Table 13.1). The insert size was estimated to be 4.5 kb. Sequencing by primer walking showed that it comprised 4,313 bp, encoding two major putative ORFs. One encoding 402 amino acids gave highest homology of the translated protein sequence to a putative carboxylesterase from *Salmonella typhymurium* LT2 (NCBI entrez NP_460582.1), having 67% identity over 402 amino acids. It is therefore a strong candidate for the esterase/lipase activity. The sequence can be found at the EBI under Accession Number AJ537554. The second was 326 amino acids long, but not lipase. The authors then tried esterase screening from an enriched metagenomic library. A library of genomic DNA was made from a minimal olive-oil enrichment culture and yielded two positive clones after screening of 60,000 colonies for esterase/lipase activity on tributyrin agar. Agarose gel electrophoresis after restriction enzyme digestion showed that one, LIP1, was contained in a cloned insert of about 2.5 kb in size and the other, LIP2, in a cloned insert of about 3.5 kb.

Sequencing by primer walking gave an insert size of 2,285 bp for LIP1; the sequence can be found at the EBI under Accession Number AJ537555. Three putative ORFs were encoded. The most likely candidate for the lipase gene was a 792-bp region (263 amino acids) with the highest identity of 42% for the translated amino acid sequence over 252 amino acids to the

Table 13.1 Libraries, genes and encoded enzymes described in this section

Library	Incidence of positive clones	Activity	Clone name	Insert bp	Open reading frame information
Bacillus agaradhaerans genomic	1/3,000	CEL	BAGCEL	4,205	1,713 bp, 570 aa, 68% identity *B. halodurans* AP001509
Crater Lake environmental	1/36,000	CEL	CRATCEL	3,410	1,746 bp, 581 aa, 29% identity *Fusobacterium mortiferum* AAB49340
Lake Nakuru, minimal alkaline enrichment	1/60,000	CEL		Approx. 2,500	
Lake Nakuru, minimal carboxymethylcellulose enrichment	1/15,000	CEL	HKCEL	3,796	1,716 bp, 571 aa, 67% identity *B. halodurans* AP001509
Lake Elmenteita environmental	1/100,000	LIP	ELIP	4,313	1,209 bp, 402 aa, 67% identity *Salmonella typhymurium* NP_460582.1
Lake Elmenteita, minimal olive-oil enrichment	1/30,000	LIP	LIP1	2,285	792 bp. 263 aa, 42% identity *Vibrio cholerae* NP_232345
			LIP2	3,112	645 bp. 214 aa 43% identity *Escherichia coli* U82664

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hypothetical bioH protein of *Vibrio cholerae* (accession NP_232345, PID g15642712).

The LIP2 insert sequence was 3,112 bp in length; the sequence can be found at the EBI under Accession Number AJ537556. Three main ORFs were identified, with the most probable lipase/esterase encoding region being 645 bp, encoding a predicted protein 214 amino acids long. It had highest homology of 43% over 197 amino acids in a BLASTP search to an acyl-coA thioesterase I precursor of *E. coli*, U82664. Second and third putative ORFs may be not esterase. Although they did not report properties of the esterase/lipase isolated metagenome library, these libraries made directly from the environments are clearly a rich resource for the future identification of novel enzymes.

13.4 Industrial Application

A Japanese company has produced a laundry detergent containing a fungus lipase produced by recombinant DNA technology. The fungus itself is not alkaliphilic, but the enzyme is alkaline lipase. This is the only industrial application of lipase as a laundry detergent additive.

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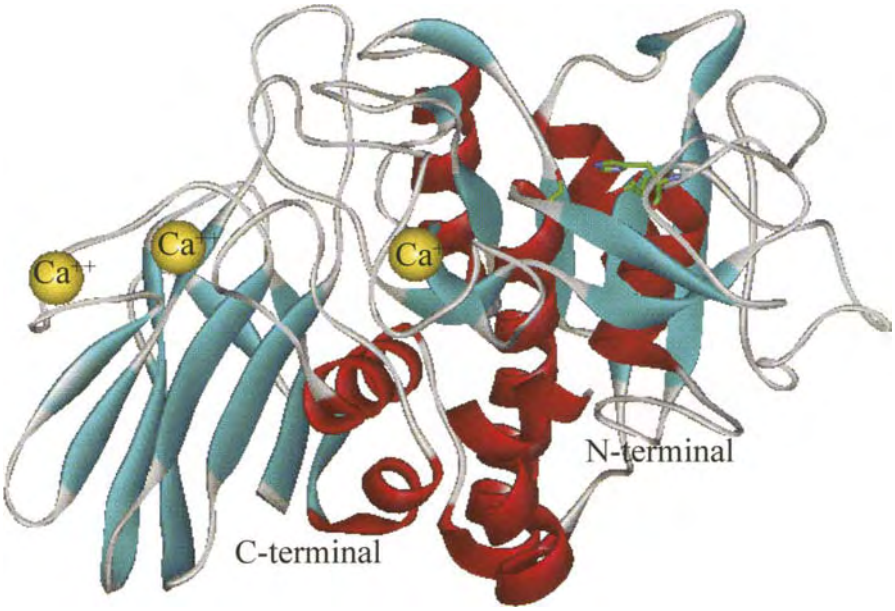

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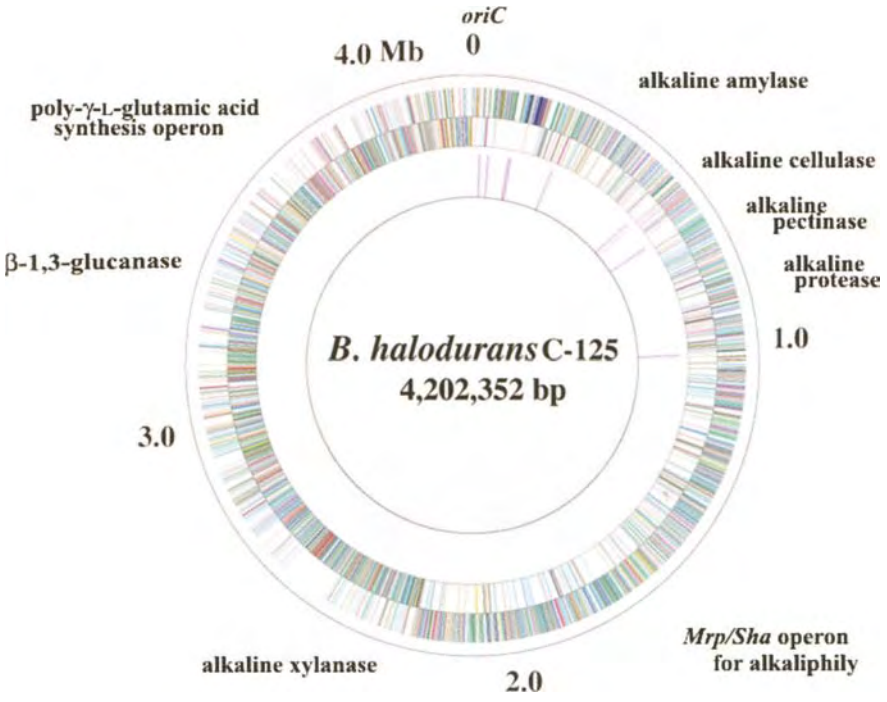

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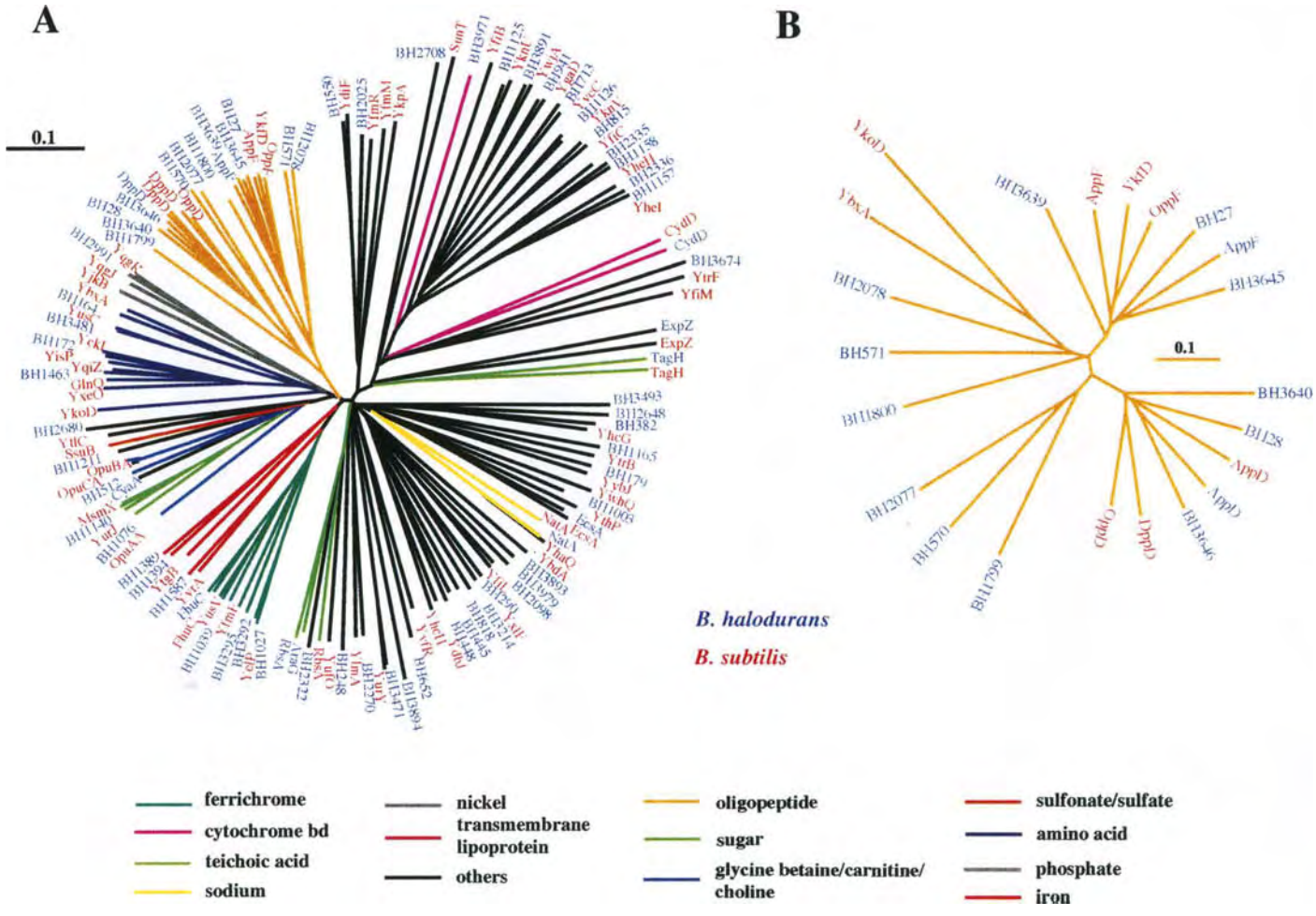

Dendrograms of members of the ABC transporter family of *Bacillus halodurans* and *Bacillus subtilis*