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REVIEW

Cytochrome *c* and Bioenergetic Hypothetical Model for Alkaliphilic *Bacillus* spp.

Toshitaka Goto,[1,2] Toshihide Matsuno,[1,3] Megumi Hishinuma-Narisawa,[1]
Koji Yamazaki,[4] Hidetoshi Matsuyama,[3] Norio Inoue,[4] and Isao Yumoto[1,2]*

Research Institute of Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan,[1] Graduate School of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo 060-8589, Japan,[2] Department of Bioscience and Technology, School of Engineering, Hokkaido Tokai University, 5-1-1-1 Minaminosawa, Minami-ku, Sapporo 005-8601, Japan,[3] and Department of Marine Bioresources Chemistry, Faculty of Fisheries, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-0821, Japan[4]

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Although a bioenergetic parameter is unfavorable for production of ATP (ΔpH < 0), the growth rate and yield of alkaliphilic *Bacillus* strains are higher than those of neutralophilic *Bacillus subtilis*. This finding suggests that alkaliphiles possess a unique energy-producing machinery taking advantage of the alkaline environment. Expected bioenergetic parameters for the production of ATP (ΔpH and $\Delta\psi$) do not reflect the actual parameters for energy production. Certain strains of alkaliphilic *Bacillus* spp. possess large amounts of cytochrome *c* when grown at a high pH. The growth rate and yield are higher at pH 10 than at pH 7 in facultative alkaliphiles. These findings suggest that a large amount of cytochrome *c* at high pHs (*e.g.*, pH 10) may be advantageous for sustaining growth. To date, isolated cytochromes *c* of alkaliphiles have a very low midpoint redox potential (less than +100 mV) compared with those of neutralophiles (approximately +220 mV). On the other hand, the redox potential of the electron acceptor from cytochrome *c*, that is, cytochrome *c* oxidase, seems to be normal (redox potential of cytochrome *a*=+250 mV). This large difference in midpoint redox potential between cytochrome *c* and cytochrome *a* concomitant with the configuration (*e.g.*, a larger negative ion capacity at the inner surface membrane than at the outer surface for the attraction of H^+ to the intracellular membrane and a large amount of cyrochrome *c*) supporting H^+-coupled electron transfer of cytochrome *c* may have an important meaning in the adaptation of alkaliphiles at high pHs. This respiratory system includes a more rapid and efficient H^+ and e^- flow across the membrane in alkaliphiles than in neutralophiles.

[**Key words:** alkaline adaptation, alkaliphilic, cytochrome *c*, *Bacillus*, ion capacity, H^+-condenser, redox potential, membrane electrical potential]

Microorganisms exhibit a great diversity genetically as well as in physiological functions and are widely distributed in nature. There are microorganisms living in environments of extreme temperature, pH, salinity and hydropressure from the view-point of normal conditions for humans (1, 2). These microorganisms are called extremophiles. Extremophiles have brought us great benefits in terms of utility of their metabolic ability in extreme environments and their produced enzymes (2–4). Among such extremophiles, alkaliphilic *Bacillus* spp. have been isolated to investigate their physiological adaptation to high pHs and utilize their enzymes industrially owing to their heat stability and efficiency at room temperature (3). Although considered to be extremophiles, alkaliphilic *Bacillus* strains are distributed not only in unique places on Earth such as alkaline soda lakes (5), deep seas (6) and the intestinal tracts of certain insects (7), but also in ordinary soil (3), seawater, terrestrial and artificial environments (8).

The mechanisms underlying the adaptation of alkaliphilic microorganisms to alkaline environments have been studied (9–14). These involve the solute transport system that reduces intracellular pH and a cell wall structure that protects inner cell metabolisms and against bioenergetic problems to overcome a negative pH gradient across the membrane (ΔpH). Among several issues on the alkaline adaptation of alkaliphilic microorganisms, we focus on bioenergetic problems. On the basis of Peter Michell's chemiosmotic theory (15), the H^+ motive force (Δp) for driving ATPase consists of a transmembrane pH gradient (ΔpH) (inside pH – outside

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* Corresponding author. e-mail: i.yumoto@aist.go.jp
phone: +81-(0)11-857-8909 fax: +81-(0)11-857-8980

pH) and a membrane electrical potential (Δψ) (–, inside; +, outside). Surprisingly, alkaliphilic *Bacillus* spp. have an effective machinery for the production of energy in such unfavorable environments (16).

We have reported review articles describing the bioenergetics of alkaliphilic *Bacillus* spp. (13, 14). However, these articles did not mention the direct importance of the large difference in midpoint redox potential between cytochrome *c* and terminal oxidation and surrounding configurations (*e.g.*, a larger negative ion capacity on the inner surface of the membrane than at the outer surface for the attraction of H^+ to the intracellular membrane and a large amount of cytochromes *c*) in alkaliphilic *Bacillus* as a bioenergetic parameter. This is an important point particularly under high-aeration conditions in alkaliphiles. In this review, we discuss the alkaliphilic respiratory configuration that includes a more efficient H^+ and e^- flow across the membrane on the basis of the alkaline environment as the beneficial factor for the microorganism.

I. TAXONOMY

There are microorganisms that are able to grow at pH 9 but not at pH 10, and their optimal growth pH is lower than pH 9. Such microorganisms are categorized as alkali-tolerant microorganisms. Many strains of nonalkaliphilic *Bacillus* spp. are able to grow at pH 9 but not at pH 10 and their optimum growth pHs are around pH 7–8 (3). Alkaliphilic microorganisms can be defined as microorganisms that grow equally or better in terms of growth intensity or velocity at pHs higher than pH 9 than at neutral pH. Such alkaliphilic microorganisms are able to grow at pHs higher than pH 10. Alkaliphilic microorganisms can be further divided into facultative alkaliphiles, which can grow well at neutral pH, and obligate alkaliphiles, which cannot grow well at pHs lower than 8 (9). These facultative and obligate characteristics are reflected in the species characteristics and phylogenetic positions (17). However, a few species include both facultative and obligate alkaliphilic strains (18).

Numerous alkaliphilic *Bacillus* strains have been isolated (18–28). More than 20 alkaliphilic *Bacillus* spp. have become approved species. They form several clusters in phylogenetic trees based on their 16S rRNA gene sequences (17) (Fig. 1). Most of these alkaliphilic *Bacillus* spp. have been isolated from soil or soil-related samples (20). Although we presently do not know the ecological significance of the distribution of alkaliphiles, these microorganisms are widely distributed in nature. One of the reasons for the wide distribution of alkaliphilic *Bacillus* is the existence of alkaline environments in very small niche, *e.g.*, the intestines of insects. It has been reported that the gut of higher termites is a niche for alkaliphilic *Bacillus* spp. (7). On the other hand, many species of alkaliphilic strains not belonging to the genus *Bacillus* have been isolated from samples of environments other than soil and have been identified as approved species (13). These species also form clusters in certain positions in phylogenetic trees based on their 16S rRNA gene sequences (Fig. 1) (29–31). These findings may indicate that certain alkaliphilic bacteria have evolved in certain environmental niches and developed their own adaptation processes.

Strains used in bioenergetic studies are indicated in the phylogenetic tree (Fig. 1).

II. SOLUTE TRANSPORT SYSTEM

Solute transport systems for the Na^+ cycle instead of the H^+ cycle under low H^+ conditions have been studied (12, 32, 33). Solute uptake and flagella rotation occur d owing to a Na^+-based transmembrane potential. Recently, the stator-force generator (MotPS) that drives Na^+-dependent motility in alkaliphilic *Bacillus pseudofirmus* OF4 has been identified (34). A Na^+/H^+ antiporter, Mrp(Sha), was discovered as Δψ-driven and having an important role in *Bacillus halodurans* C-125 in alkaline environments (33). The amino acid sequence of Mrp(Sha) is similar to that of the membrane-impregnated hydrophobic domain of NADH-quinone oxidoreductase (Ndh-1). This antiporter has a crucial role in regulating intracellular pH. Na^+ efflux from strain C-125 is accelerated in the presence of electron donors of cytochrome *c* oxidase (ascorbate *plus N,N,N′,N′*-tetramethyl-*p*-phenylendiamine). The efflux is inhibited in the presence of 100 µM carboxyl cyanide *m*-chlorophenylhydrazone (CCCP). This observation suggests that the driving force of this antiporter is Δψ produced by the respiratory chain at a growth pH, *e.g.*, pH 10, and Na^+ efflux is not driven by the NADH-driven redox-coupled pumping, although the DNA sequence of the antiporter have similarity with that of Ndh-1 in the respiratory chain. In *B. pseudofirmus* OF4, at least two additional antiporters, including NhaC, have supporting roles in pH homeostasis (32).

It is considered that although alkaliphilic *Bacillus* strains are living under unfavorable conditions, they consume additional energy to regulate intracellular pH appropriately as compared with neutralophiles. However, alkaliphilic *Bacillus* strains exhibit relatively high growth rates as described below. This observation may be associated with a special energy-producing machinery existing in alkaliphilic *Bacillus* strains.

III. BACKGROUND ON BIOENERGETICS

The effect of external pH on the growth of facultatively alkaliphilic *B. pseudofirmus* strain OF4 has been studied in the steady state and pH-controlled culture at various pHs. The generation times of 54 and 38 min were observed at external pHs of 7.5 and 10.6, respectively (35). The molar growth yield of the strain is also 1.5 times higher when grown at pH 10.5 than at pH 7.5. We also observed the superior growth of alkaliphilic *Bacillus* spp. at pH 10 than at neutral pH. Furthermore, the growth rate and growth yield of alkaliphilic *Bacillus* spp. grown at pH 10 are higher than those of neutralophilic *Bacillus subtilis* (35; unpublished results). These results suggest that alkaliphilic *Bacillus* strains utilize alkaline conditions to their advantage for their metabolisms. When the Na^+/H^+ antiporter is required in the adjustment of intracellular pH for survival under high pH conditions, Δψ production via the respiratory chain decreases owing to Δψ consumption by the Na^+/H^+ antiporter. As a consequence, it is expected that a low growth rate and a low cell yield occur. We considered that alkaliphilic *Bacillus* spp.