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REVIEW

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

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Although a bioenergetic parameter is unfavorable for production of ATP ($\Delta pH < 0$), 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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pH) and a membrane electrical potential ($\Delta\psi$) (−, 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 $\Delta\psi$-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 $\Delta\psi$ 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, $\Delta\psi$ production via the respiratory chain decreases owing to $\Delta\psi$ 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.

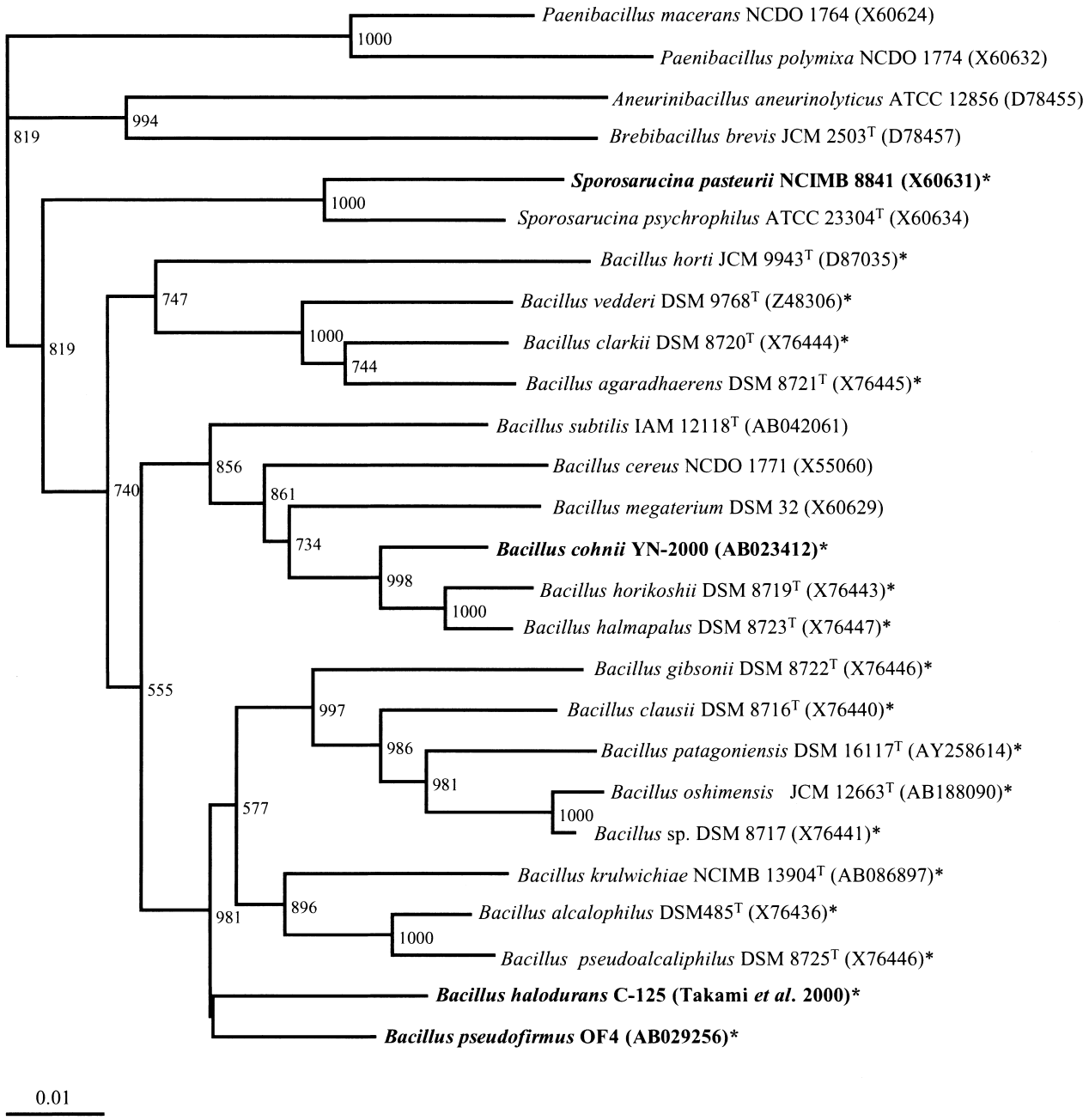

FIG. 1. Phylogenetic tree derived from 16S rRNA gene sequence data of alkaliphilic and neutralophilic *Bacillus* spp. and related strain. The phylogenetic positions of *B. cohnii* YN-2000, *B. pseudofirmus* OF4, *B. halodurans* C-125, *Sporosarucina pasteurii* are in bold. Asterisk indicates that the strain is an alkaliphile. Numbers indicate bootstrap values greater than 500. Bar: 0.01 K_{nuc}.

utilize alkaline-specific advantageous features for energy production. The discrepancy between experimental results and theoretically expected growth suggests that the observed bioenergetic parameters ΔpH and Δψ are not directly applicable to alkaliphiles.

Based on the Peter Mitchell's chemiosmotic theory (15), the electrochemical potential of H^+ across the membrane (Δp) is as follows.

$$\Delta p = \Delta\psi - Z\Delta pH \qquad Z = 2.3\ RT/F \cong 59\ mV$$

where Δψ is electrical potential of membrane (–, inside; +, outside), ΔpH is transmembrane pH gradient (inside pH – outside pH), R is gas constant, T is absolute temperature and F is Faraday constant. The theory is based on the following: (i) The respiratory chain pumps out H^+ from the inside to the outside of the membrane concomitant with the flow of electrons from NADH to O_2. (ii) ATPase produces ATP by translocating H^+ from the outside to the inside of the membrane. (iii) The membrane possesses a transporter system for obtaining substrates from the outside of the membrane. The system works utilizing the electrochemical potential of H^+ across the membrane. (iv) H^+ and OH^- are not

permeable across the membrane.

Guffanti and Krulwich reported ATP synthesis in ADP+P_i-loaded membrane vesicles of *B. pseudofirmus* strain OF4 grown at pHs 10.5 and 7.5 assayed at both pHs of 10.5 and 7.5 under energization with ascorbate ($E_{m,7}$=60 mV) *plus* phenazine methosulfate ($E_{m,7}$=70 mV) (16). Although the proton motive force (Δp) of vesicles of the strain grown at pH 10 is not higher than that at pH 7, ATP synthesis rate is higher in vesicles prepared from cells grown at pH 10 and measured at pH 10 than that in vesicles prepared from cells grown at pH 7.5 and measured at pH 7.5. The electrochemical potentials of H^+ across the membrane (Δp) calculated on the basis of bioenegetic parameters were −166 mV (pH_{out}= 7.5) in vesicles obtained from cells grown at pH 7.5 and −36 mV (pH_{out}=10.5) in vesicles obtained from cells grown at pH 10.5. These results indicate that as yet unknown effective energy production mechanisms are expressed in vesicles obtained from cells grown at pH 10 and measured at pH_{out}= 10.5. It may be considered that translocated H^+ via the respiratory chain is attracted by surface potential existing on the outer surface of the membrane to avoid losing H^+ to the bulk phase. The effect of 200 mM K_2SO_4, which was applied to diminish surface potential, on ATP synthesis in ADP+P_i-loaded membrane vesicles of strain OF4 grown at pH 10.5 was examined. The results showed that the rate of ATP synthesis was more rapid at pHs 7.5 and 10.5. The artificially introduced Δψ across the membrane by K^+ diffusion as the same intensity as ATP producing energized membrane on the basis of respiratory chain cannot produce ATP in membrane vesicles prepared from cells grown at pH 10 and measured at pH 10. Furthermore, a higher rate of ATP synthesis is observed in vesicles of cells grown at pH 10 and measured at pH 9.5 than at lower than pH 7.8 at the same Δψ produced by an energized respiratory chain. These experimental results (16) suggest the existence of alkaline-specific nonchemiosmotic energy factors. Krulwich *et al*. proposed a hypothetical model in which H^+ transfers directly from the respiratory chain complex to F_0 in ATPase in the intramembrane (16). However, we insist an another hypothetical model described in this review by the following reasons for the proposed model: (i) Proton extrusion to outside of the membrane has been observed in alkaliphilic *B. pseudofirmus* RAB (36). It is considered that H^+ transfers to the vicinity of the outer surface of the membrane. However, a certain amount of H^+ may come out to the bulk phase concomitant with the extrusion of H^+ via the respiratory complex. (ii) A structural basis has been suggested in ATPase (37) for the interaction of cytochrome *c* oxidase and ATPase, but there is no such structural basis in cytochrome *c* oxidase. Although there are reports on the interaction between cytochrome *c* oxidase and ATPase (38), there is no evidence of intramembrane H^+ transfer. (iii) Attractive forces of H^+ on the surface of the membrane are not only attributed to surface potential. The observation on the stimulation of ATP production caused by the reduction of surface potential may be a transient elevation of ATP production by the weakening of H^+ trapping force on the outer surface of the membrane. Therefore, the stimulation of ATP production caused by the reduction of surface potential is not a proof of intramembrane H^+ transfer. We consider that the most important point for ATP production may be the balance of attractive force of H^+ between the outer surface and the inner surface of the membrane concomitant with the redox reaction of the respiratory chain.

The H^+ transfer along the outer surface of the membrane under high pH conditions seems to be very difficult. There are many reports on the rapid movement of translocated H^+ by the respiratory chain along the membrane surface to the gate of the channel entrance under neutral conditions (39–45). The long-range H^+ transfer along the surface of black lipid bilayers between two integral membrane channels, one as a H^+ source, the other as a sink, has been observed by the patch-clamp technique. After traveling through the first channel, H^+ directly enters into the second channel (39). This traveling state disappears when the distance between the two channels is larger than the critical distance. This observation demonstrates that H^+ movement along the membrane surface is localized in the lipid bilayers. It was also reported that an outer surface phospholipid, *e.g*., cardiolipin, traps H^+ to prevent the equilibration of H^+ with the bulk phase (46). These reactions occur without an H^+-bond network of amino acids. On the other hand, there are several reports of the existence of a proton-collecting antenna consisting of carboxylate and histidine residues (42, 43). Protons are rapidly transported along the membrane surface to the entrance of H^+-conducting channels by the antenna. Mostly, the rapid transfer of H^+ has been demonstrated by experiments using a fluoresceine indicator specifically attached to the native cysteine residue. This transfer may be influenced by the p*K*a values of surface amino acids and the concentrations of H^+-transferable amino acids along the H^+ pathway. Another study showed that the turnover rate of the H^+ pump of cytochrome *c* oxidase is higher than the bulk diffusion limit (45). Very recently, a potential barrier has been determined to exist in the water phase approximately 0.5–1 nm away from the membrane surfaces by electrostatic calculations (47). It may be concluded that H^+-collecting factors, namely, amino acids and the head group of phospholipids collecting H^+ on the outer membrane surface, generate a potential barrier on the outer surface of membrane.

As described above, H^+ transfer along the outer surface of the membrane is obviously important for an effective energy production even under neutralophilic conditions. On the other hand, collecting H^+ in the inner surface of membrane of the H^+-gate of cytochrome *c* oxidase is also important for the efficient H^+ translocation from the inner to the outer surface of the membrane. The H^+-collecting function under the cytoplasmic surface (H^+ input side) has been studied using fluorescein-labeled cysteine as a H^+-sensitive marker (48). The protonation of fluorescein proceeds through multiple ways involving diffusion-controlled reactions and H^+ exchange among surface groups. Surface H^+ are efficiently collected by the antenna composed of closely connected carboxylate and histidine residues. The efficient H^+ collection of the inner gate of the H^+ translocation site in cytochrome *c* oxidase supports the physiological turnover of the enzyme. The enhancement of this system may be very important in sustaining the H^+ translocation of cytochrome *c* oxidase in alkaliphilic *Bacillus* spp., because the cytoplasmic pH of alkaliphiles is higher (around pH 8.3) than those

TABLE 1. Intracellular pH and $\Delta\psi$ in neutralophiles and alkaliphilic *Bacillus* strains at different external pHs

Microorganism	External pH	Internal pH	$\Delta\psi$	Ref.
B. subtilis (neutralophile)	7.5	7.5	−130	49
Bacillus lichemiformis (neutralophile)	7.5	8.7	ND	50
Lactococcus lactis (neutralophile)	7.3	7.6	ND	51
B. cohnii YN-2000 (facultative alkaliphile)	7.5	8.2	−130	52
	8.5	7.9	−159	
	9.5	8.1	−181	
	10.2	8.4	−188	
B. pseudofirmus OF4 (facultative alkaliphile)	7.5	7.5	−138	53
	8.5	7.7	−156	
	9.5	7.5	−177	
	10.5	8.3	−180	
B. pseudofirmus RAB (obligate alkaliphile)	9.0	7.7	−170	54
	10.5	8.3	−179	
B. alcalophilus DSM 485^T (obligate alkaliphile)	10.1	8.4	−206	55
B. alcalophilus ATCC 27647^T	10.3	8.4	−213	55

ND, No data.

of alkaliphiles grown at neutral pH and several neutralophiles (around pH 7.5) (Table 1). Although *Bacillus licheniformis* is a neutralophile, it exhibits a relatively high cytoplasmic pH (pH 8.7) (Table 1). In such a case, it is considered that H^+ translocation by the respiratory complex is difficult, while it is favorable firection for generating positive ΔpH. *B. licheniformis* may also have certain mechanisms for attracting H^+ at the vicinity of the inner surface of the membrane in the case of growth at higher than pH 7.5.

It is considered that these findings explain the ordinary H^+-coupling energy- producing phenomenon under neutral pH conditions. Therefore, these findings cannot fully explain the difficulty in energy-producing phenomenon among alkaliphilic *Bacillus* spp. Alkaline-condition-specific problems may exist such as the H^+ diffusion constants of the surface membrane and bulk under alkaline conditions versus the velocity of H^+ transfer along the outer surface of the membrane. Furthermore, H^+ transfer on the surface of membrane described above cannot directly explain differences in the phenomenon between alkaliphilic *Bacillus* spp. and neutralophilic *Bacillus* spp. (*e.g.*, *B. subtilis*) or between facultatively alkaliphilic *Bacillus* spp. grown at different pHs. However, alkaliphilic *Bacillus* spp. may have a specific configuration related H^+ transfer on the outer surface of membrane.

IV. RESPIRATORY COMPONENTS

Although respiratory complex III (bc_1 complex) has not been purified and characterized, several other complexes from alkaliphilic *Bacillus* spp. have been purified and characterized. These components were described previously. In this section, we only describe the components in terminal reaction and H^+-ATPase.

Cytochrome *c* oxidase Although caa_3-type cytochrome *c* oxidase from *B. pseudofirmus* OF4 has been purified and characterized (56), a novel type of cytochrome *c* oxidase, an aco_3 type, from *Bacillus cohnii* YN-2000 (57) has been purified and characterized (58–60). The same type of the cytochrome *c* oxidase from thermophilic *Bacillus* sp. PS3 has been purified (61). It has been reported that the content of cytochrome *a* containing cytochrome *c* oxidase of *B. pseudofirmus* OF4 increases when the microorganism is grown at around pH 10 compared with that grown at neutral pH. In the case of strain YN-2000, although the content of cytochrome *a* does not increase at pH 10 compared with that at pH 7 under air-limited conditions, the content is higher at pH 10 than at pH 7 under high-aeration conditions (unpublished result). Potentiometric titration indicated that the midpoint redox potential of heme *c* is 95 mV at pH 7.0 and suggested the presence of two forms of cytochrome *a* with midpoint redox potentials of +250 mV and +323 mV at pH 7.0 (59). The environment around the hemes and enzymatic properties suggest that cytochrome aco_3 (60) is very similar to cytochrome caa_3 (62) isolated from thermophilic *Bacillus* sp. PS3 except for the low midpoint redox potential of the cytochrome *c* moiety. When ascorbate *plus* TMPD-reduced cytochrome aco_3 is exposed to O_2, the reduced cytochrome *c* moiety is oxidized rapidly. On the other hand, the oxidation of the cytochrome *a* moiety is scarcely observed. The cytochrome *a* moiety seems to have no contributions to the oxidation reaction, but has a role as an e^- reservoir (59). However, we do not know the significance of cytochrome *a* as an e^- reservoir; a high affinity of e^- may have an important role in H^+-translocation reaction. The cytochrome *c* moiety may have an important role in the rapid electron flow to the cytochrome o_3-Cu_B reaction center concomitant with a rapid H^+-translocation across the membrane coupling reaction.

H^+-ATPase It has been reported that *B. pseudofirmus* OF4 (63) and *B. alcalophilus* (64) possess H^+-translocating ATPase. This finding corresponds to another finding that alkaliphilic *Bacillus* spp. possess the H^+-translocating respiratory chain. From the genomic sequences of ATPase in *B. pseudofirmus* OF4, *B. halodurans* C-125 and *B. alcaliphilus*, the proton pathway through the *a*- and *c*-subunits has alkaliphile–specific features. Site-directed mutational changes are introduced to such parts specific for alkaliphiles corresponding to those parts in neutralophilic *Bacillus megaterium* (65). Six out of eight mutants with substituted alkaliphile-specific amino acid residues show a markedly decreased yield of the cells when grown at pH 10 under nonfermentative growth conditions. This may indicate that alkaliphilic *Bacillus* spp. have specific features in ATPase that support effective H^+ translocation in taking advantage of alkaline-specific conditions.

Nonproteinaceous terminal oxidation In the respiratory system of obligately alkaliphilic *Bacillus* sp. YN-1, caa_3-type cytochrome *c* oxidase constitutes up to 10% of total oxygen-reducing activity, while 90% of the activity is due to cyanide-insensitive terminal respiratory component (66). Facultatively alkaliphilic *B. cohnii* YN-2000 has a higher content of the cyanide-insensitive terminal respiratory component when grown at pH 10 than at pH 8. The catalytic activity of the cyanide-insensitive substance is not as high as in strain YN-1. This difference is reflected in the higher cyanide insensitivity of strain YN-1 than strain YN-2000 when grown at pH 10. The catalytic activity of the isolated cyanide-insensitive terminal respiratory component is not inhibited by heat treatment (100°C for 5 min) in the presence of 1% SDS, suggesting that the component is very thermostable and a nonproteinaceous substance. The molecular mass of the purified terminal component itself was determined to be 662.0 by electron ionization mass spectrometry. These findings suggest that the component may not have H^+-translocation activity. The physiological function of the cyanide-insensitive component is unclear at present. It may have a function to consume residual e^- in the respiratory system to adjust suitable energy production at a high pH or may contribute to consuming e^- in the cytochrome *c* oxidase-independent energy production system, that is, the H^+-condenser system (will be described below). The cyanide-insensitive terminal respiratory component catalyzes the two-electron reduction of oxygen, resulting in the production of H_2O_2 as the by-product. This may indicate the necessity of H_2O_2 in the regulation of metabolism at a high pH. However, the high production of H_2O_2 at pH 10 corresponds to the high production of catalase-peroxidase in *B. cohnii* YN-2000 (67).

V. CYTOCHROME *c*

In the respiratory chain of aerobic organisms, cytochrome *c* (68–71) acts as an electron transporter between complex III (bc_1 complex) and complex IV (cytochrome *c* oxidase). A typical cytochrome *c* (class I) is defined as an electron-transfer protein having one low-spin heme *c* at the N-terminal side bound to the protein by two thioether bonds involving sulphydryl groups of cysteine residues. The fifth heme iron ligand is provided by a histidine residue and the sixth ligand by a methionine residue. This protein contains three conserved core helices that form a basket around the heme group with one heme at the edge exposed to the solvent. The molecular weight of cytochrome *c* is approximately 8000–14,000 (reviewed in Refs. 68–71).

There are numerous studies on soluble cytochrome *c* from various organisms including gram-negative bacteria (70). However, only a limited number of studies on membrane-bound cytochrome *c* from gram-positive bacteria are available. Gram-positive bacteria do not have a periplasmic space. Therefore, they do not have soluble cytochrome *c*. *B. subtilis* has two types of membrane-binding cytochromes *c*-550 and *c*-551 (72, 73). Cytochrome *c*-550 has a molecular weight of 13 kDa consisting of 120 amino acid residues with a membrane anchor domain consisting of a single α-helical transmembrane segment of a hydrophobic polypeptide consisting of 30 amino acid residues and a heme domain of about 74 residues. The latter domain is located on the outer surface of the cytoplasmic membrane (72). The midpoint redox potential of cytochrome *c*-550 is +178 mV (74). The function of this protein remains unknown. The other cytochrome *c*, *c*-551, has a molecular weight of 10 kDa consisting of 92 amino acid residues. This cytochrome *c* binds to the membrane via a diacyl-glyceryl-cysteine moiety. The midpoint redox potential of cytochrome *c*-551 is >100 mV and its p*I* is 3.8. The function of this cytochrome also remains unknown (73). Cytochromes *c* binds to the membrane via a diacyl-glyceryl-cysteine moiety are also present in thermophilic *Bacillus* sp. strain PS3 cytochrome *c*-551 (75), *Heliobacterium gestii* cytochrome *c*-553 (76) and *Sporosarucina* (*Bacillus*) *pasteurii* cytochrome *c*-553 (77). The two types of cytochrome *c* described above are examples of cytochrome *c* binding to the membrane via a different domain. The third type of membrane-binding cytochrome *c* fuses as an integral domain of a subunit in membrane-bound aa_3 (56, 61), bb_3 (78–80) or ao_3 (58, 60, 61)-type terminal oxidase.

It has been reported that a large content of membrane-bound cytochrome *c* is present in alkaliphilic *Bacillus* spp. (81–83) (Table 2). The amount increases with increasing initial pH of the culture medium. These findings suggest a relationship between a large amount of cytochrome *c* and environmental adaptation. The first purified and characterized cytochrome *c* from alkaliphilic *Bacillus* spp. is cytochrome *c*-552 from obligately alkaliphilic *B. pseudofirmus* RAB (84). Cytochrome *c*-552 has a molecular weight of 16.5 kDa and is acidic with p*I*=3.4. The midpoint redox potential of this cytochrome is +66 mV at pH 7 (Table 3), which decreases in a pH-dependent manner at pHs higher than 8.3. Resonance Raman spectroscopic studies of the purified cytochrome *c*-552 suggest that its low potential may be caused by a switch in the sixth ligand from methionine to histidine when the oxidized protein is reduced (85). This cytochrome is autooxidizable. The physiological function of

TABLE 2. Spectrophotometric estimation of cytochrome content of obligately alkaliphilic *Bacillus* sp. YN-1 and facultatively alkaliphilic *Bacillus cohnii* YN-2000 and neutralophilic *B. subtilis* IAM 1026 in cell extacts

Strain	Growth pH	Cytochrome content (nmol·mg protein^{-1})			Total
		c-type	*b*-type	*a*-type	
Bacillus sp. YN-1 (obligately alkaliphile)	10	0.510	0.610	0.029	1.149
	9	0.380	0.350	0.007	0.737
Bacillus cohnii YN-2000 (facultatively alkaliphile)	10	0.920	0.610	0.050	1.580
	9	0.420	0.350	0.053	0.823
	8	0.200	0.200	0.025	0.425
Bacillus subtilis IAM 1026 (neutralophile)	7	0.120	0.148	0.122	0.390

TABLE 3. Comparison of midpoint redox potentials ($E_{m,7}$) of cytochromes *c* from alkaliphilic *Bacillus* spp. with those of membrane-bound cytochromes *c* from neutrophilic *Bacillus* strains and other organisms

Organism	Alkaliphile or Neutrophile	Cytochrome *c*	Midoint redox potential	Ref.
B. cohnii YN-2000	Alkaliphile	*c*-553	+87 mV	99
		c-552	+92 mV	99
		cyt. *c* in cyt. aco_3	+95 mV	59
B. pseudofirmus RAB	Alkaliphile	*c*-552	+66 mV	84
B. pasteurii	Alkaliphile	*c*-553	+47 mV	86
B. subtilis	Neutralophile	*c*-551	>+100 mV	73
		c-550	+178 mV	74
Bacillus sp. PS3	Neutralophile	*c*-551	+225 mV	75
Heliobacterium gestii	Neutralophile	*c*-553	+215 mV	76

cyt., Cytochrome.

this cytochrome remains unclear.

The biochemical and physicochemical characteristics, primary structure and three-dimensional structure of membrane-bound cytochrome *c*-553 from *S. pasteurii* have been studied (77, 86–89). Cytochrome *c*-553 has a low molecular weight of 9.6 kDa with 92 amino acid residues and a low midpoint redox potential (+47 mV) (86), and is acidic with p*I*=3.3 (Table 3). Crystallized cytochrome *c*-553 contains only 71 of the 92 residues (88). Crystal structure analysis indicates that the protein is characterized by a highly asymmetric charge distribution. The surface of the heme group is devoid of net charges. The protein contains a hydrophobic region. On the other hand, most charges are located on the opposite side of the exposed heme edge. The amino acid sequence of *S. pasteurii* cytochrome *c* exhibits very high similarities with those of cytochromes *c* from gram-positive bacteria such as *Bacillus licheniformis* cytochrome *c*-552 (53% similarity) (90), thermophilic *Bacillus* sp. strain PS3 cytochrome *c*-551 (45%) (91) and *B. subtilis* cytochrome *c*-551 (41%) (73). However, *S. pasteurii* cytochrome *c*-553 exhibits a high 3D-structural similarity with *Pseudomonas aeruginosa* cytochrome *c*-551 (92, 93), followed by *Monoraphidium braunii* cytochrome c_6 (94, 95), *Desulfovibrio vulgaris* cytochrome *c*-553 (96), *Thermus thermophilus* cytochrome *c*-552 (97) and *Saccharomyces cereviseae* cytochrome *c* (98). The possible relationship of heme solvent accessibility in terms of structure with thermodynamic parameters of electrochemical reduction and a low midpoint redox potential has been discussed as follows. The structural parameter suggests a low and high midpoint redox potentials correspond roughly to small and large solvent accessibilities for the heme, respectively. The analysis of previously reported data revealed that heme solvent accessibility has a higher correlation with the entropy rather than with the enthalpy of reduction. The reason for the low midpoint redox potential is considered to be the decrease in the entropy of reduction by the extrusion of water molecules from the protein hydration shell, which occurs upon the reduction of heme (88). The solution structure of reduced *S. pasteurii* cytochrome *c* has been determined by NMR. The structure is very similar to that in the oxidized state, with only Try87 and propionate showing significant differences. The mobility of the molecule was also investigated. The results showed that the oxidized form is generally more mobile than the reduced form (89).

Solubilized *B. cohnii* YN-2000 membranes prepared from cells grown at pH 10 contain a larger amount of cytochrome *c*-553 than those grown at pH 7 (99). The native molecular weight of cytochrome *c*-553 was determined to be 127 kDa in the presence of 0.5% Triton X-1000. On the other hand, the molecular weight of the cytochrome was estimated to be 10,500 Da by SDS–PAGE and the heme *c* content was determined to be 89.35 nmol mg protein^{-1}. This suggests that one molecule of cytochrome *c* contains one heme *c*. The combined results of SDS–PAGE and gel filtration indicate that the native cytochrome *c* is oligomeric in structure consisting of 10 of the 10,500 Da units. Although the molecular weight of micelles of Triton X-100 is about 100 kDa, it is considered that cytochrome *c* exhibits an oligomeric feature because another membrane-bound protein purified in our laboratory in the presence of Triton X-100 does not exhibit an increase of 100 kDa as determind by gel filtration or does not reflect the molecular weight of micells of the detergent used. The p*I* of cytochrome *c*-553 is 3.9 and the midpoint redox potential is +87 mV in the pH range from 6 to 8. The estimated midpoint redox potentials of cytochromes *c* from alkaliphilic *Bacillus* spp. are always lower than that of the typical cytochrome *c* (approximately +250 mV) (Fig. 2) (Table 3). There is a large midpoint redox potential differ-

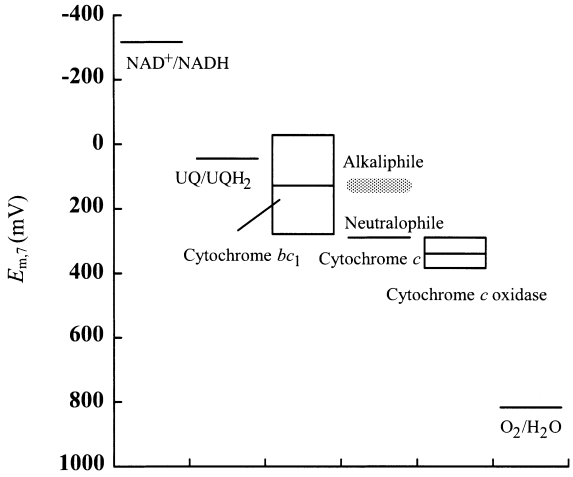

FIG. 2. Midpoint redox potential of cytochrome *c*-553 of alkaliphilic *Bacillus cohnii* YN-2000. Midpoint redox potentials of other components are indicated in this figure on the basis of mitochondrial respiratory chains. The terminal redox components of strain YN-2000 are equivalent to midpoint redox potential corresponding to mitochondrial cytochrome aa_3. A large difference in midpoint redox potential can be identified between cytochrome *c* and terminal reaction in alkaliphilic *Bacillus* spp.

ence between cytochrome *c* and cytochrome *a* in terminal oxidase. We previously reported that this may be attributed to the electron flow from cytochrome *c* to a terminal enzyme across the membrane that should be overcome under a large $\Delta\psi$. A low p*I* is also a common feature among cytochromes *c* from alkaliphilic *Bacillus* spp. This may be due to the attraction of H^+ at the vicinity of the membrane surface upon H^+ transfer on the outer surface of the membrane under high-pH conditions. Cytochrome *c*-553 reacts with a native cytochrome *c* oxidase, cytochrome aco_3, and the reaction is greatly accelerated in the presence of poly-L-lysine. Poly-L-lysine exhibiting a positive charge may accumulate two negatively charged molecules, cytochrome *c*-553 and cytochrome aco_3, which assist in the appropriate reaction. The electron flow from ascorbate to oxygen through cytochrome *c*-553 and cytochrome aco_3 is five times faster at pH 8 than at pH 6. This finding suggests that the reaction is accelerated at high pHs. However, it is quite difficult to measure the reaction at pHs higher than pH 9 due to the autoxidation of ascorbate.

The other-membrane-bound cytochrome *c* in *B. cohnii* YN-2000 is cytochrome *c*-552 composed of six subunits with different molecular weights of 40, 32, 19, 17, 14 and 12 kDa and a heme *c* bound to 14 and 12 kDa subunits (99). The molecular weight of the native enzyme was estimated to be 143 kDa. Taking the subunits and native molecular weight together indicates that the cytochrome molecule is composed of one molecule of each of the six subunits. The heme *c* content of cytochrome *c*-552 is 13.7 nmol mg protein^{-1}. This finding supports the idea that the cytochrome molecule consisting of the six subunits contains two heme *c* molecules. The cytochrome has an p*I* of 4.0 and its midpoint redox potential is +91 mV at pH 7. Cytochrome *c*-552 does not react with the native cytochrome *c* oxidase, cytochrome aco_3, even in the presence or absence of poly-L-lysine. The physiological function of the cytochrome *c* remains unknown.

The cytochrome *c* moiety of cytochrome aco_3 also has a low midpoint redox potential of +95 mV as described above. On the basis of genetic analysis of this moiety, a marked paucity of basic amino acid residues is observed in subunit II-binding cytochrome *c*, which may produce a negative charge on the outer surface of the membrane to attract H^+ in the vicinity of the membrane (100). The same aspect of cytochrome *c* binding to cytochrome caa_3 in *B. pseudofirmus* OF4 is also observed (56). These results show that terminal-oxidase-binding cytochrome *c* also has a low midpoint redox potential and is an acidic protein. These characteristics may play an important role in supporting the extremely high-pH-adapted respiratory chain in rapid electron flow as well as support of H^+-transfer at the vicinity of the outer surface of the membrane.

VI. PROTON (H^+)-COUPLED ELECTRON (e^-) TRANSFER FUNCTION OF CYTOCHROME *c*

It has been considered that the redox potential of cytochrome *c* is largely influenced by the fifth and sixth iron ligands and by the nature of the polypeptide environment surrounding the heme (101). For example, the ionization of polypeptide groups close to the heme may influence the redox potential of cytochrome *c* (102). In such a case, the redox state of heme may impose a difference in the p*K*(a) values of the residues surrounding the heme. Indeed, the case of *P. aeruginosa* cytochrome *c*-551 exhibiting a redox potential change depending on the surrounding pH has been interpreted as being caused by the ionization of a heme propionic acid substituent with p*K*(a) values of 6.3 in the oxidized form and 7.2 in the reduced form (101). In addition, it has been proposed that the deprotonation of His-47 in cytochromes *c* of *Pseudomonas* spp. causes the observed fall in redox potential depending on the increase in pH (103). The changes in p*K*(a) in the residue surrounding the heme depending on the redox state and the deprotonation of residues depending on the pH indicate the H^+-transfer-coupled e^- transfer of cytochrome *c*.

The tetraheme cytochrome c_3 is found in *Desulfovibrionaceae* spp. as well as in other sulfur-reducing bacteria in large quantities (104). The cytochrome is a soluble protein with heme *c* covalently bound to the polypeptide chain through the thioether bridges of cysteine and axially coordinated by two histidines. The redox potential of cytochrome is very negative and changes depending on the pH (105, 106). These properties provide the characteristic function of cytochrome c_3, that is, to couple the transfer of e^- and H^+ (107). The thermodynamically coupled transfer of e^- and H^+ observed in soluble cytochrome *c* has been termed H^+ thrusting because it parallels the linkage between redox and proton-transfer mechanisms of larger, membrane-embedded protein complexes, the electron transfer complex and ATPase (108).

Horse heart cytochrome *c* is electrostatically bound to a self-assembled monolayer (SAM) on an Ag electrode, which is formed by ω-carboxyl alkanethiols of different chain lengths (109). The kinetic H/D effect can be observed at a short electron transfer distance between cytochrome *c* and the electrode. This effect is not observed for electron-transferring protein in solution. Therefore, it is considered that the effect arises from the electric field at the Ag/SAM interface. The electric field effects on heterogeneous electron transport may not happen only on the electrodes but also on the biological plasma membrane. The transmembrane electrical or H^+ potential may control interprotein e^- transport reactions under a certain physiological state.

These observations indicate that cytochrome *c* functions not only in e^- transfer but also in H^+ transfer in biological energy production. There are several possibilities in the transfer of H^+ by cytochrome *c*. The first possibility is that reduced cytochrome *c* attracts H^+ via e^- and H^+ dissociates from cytochrome *c* upon oxidation. The second possibility is that the oxidized state of cytochrome *c* exhibits a higher affinity for H^+ than a reduced cytochrome *c* state and H^+ dissociates upon the reduction of cytochrome *c*. The third possibility is that transferable H^+ exists in both oxidized and reduced cytochrome *c* and H^+ exchange occurs via protein network on the surface of the membrane between cytochrome *c* and H^+ acceptor, that is, ATPase concomitant with the redox reaction of cytochrome *c*. We considered that the third possibility may be correct. If cytochrome *c* exhibiting

a low midpoint redox potential possesses electrically attracted H^+, H^+ flow at the vicinity of the outer surface of the membrane may become rapid in oxidized state, because cytochrome *c* exhibiting a low midpoint redox potential *i.e.*, easily dissociate e^- and possessing large amount of transferable H^+, that is, flexible feature along with H^+ and e^- transfer in the molecule compared with ordinary cytochrome *c*. This idea also consistent with the fact oxidized cytochrome *c* exhibits more flexible molecular feature than that reduced form (89).

VII. FEATURE OF BIOENERGETICS AND POSSIBLE MODEL

Although it has been reported that the NADH:quinone oxidoreductase segment translocates Na^+ instead of H^+ in marine microorganisms (110, 111), Na^+ does not stimulate NADH oxidation or respiration in any alkaliphilic *Bacillus* spp. (82). F_1F_0-ATPase of alkaliphilic *Bacillus* spp. does not translocate Na^+ (63, 64). On the other hand, it has been reported that facultatively alkaliphilic *Bacillus* spp. exhibit certain specific changes under high-pH growth conditions such as changes in cell wall components (112–115), the contents of cytochromes (81–83), and the expression of nonproteinaceous terminal components (66). A high H^+ translocation activity has been demonstrated by the ratio of H^+ translocation per O_2 consumption (36). These reports suggest that alkaliphilic *Bacillus* spp. generally use the H^+ translocation system for energy production with a certain alkaline-condition-specific system. To date, we have certain knowledge on the energy production system in neutralophiles and alkaliphiles. We are able to compare respiratory components and bioenergetic parameters between neutralophiles and alkaliphiles. Furthermore, we can also apply the knowledge obtained from studies on neutralophiles and the mitochondrial system to the consideration of the bioenergetic problem of alkaliphiles. A certain part of the respiratory system in alkaliphiles is similar to that in neutralophiles. However, several characteristics are very specific to alkaliphiles. For example, alkaliphiles possess large amounts of cytochrome *c* that are acidic and have a low midpoint redox potential (Tables 2 and 3, Fig. 2) and existence of a nonproteinaceous terminal component. If homeostatic adaptation for lowering intracellular pH were important, alkaliphiles may consume additional $\Delta\psi$ compared with neutralophiles. However, several alkaliphilic *Bacillus* spp. grow even more rapidly than neutralophilic *B. subtilis*. Furthermore, the final cell mass of alkaliphiles is higher than that of *B. subtilis*. These findings suggest that the bioenergetics of alkaliphiles is based on the ability to take advantage of an alkaline environment. In this review, we would like to discuss the important property of bioenergetics of alkaliphiles from the viewpoints described below.

Large redox potential difference between outer surface of the membrane redox components and inner membrane redox components As described above, all the cytochromes *c* in alkaliphilic *Bacillus* spp. have low midpoint redox potentials (Fig. 2 and Table 3). On the other hand, the midpoint redox potential of cytochrome *a* is almost the same as those of neutralophiles such as *B. subtilis* (59, 116). The difference in redox potential from cytochrome *c* to O_2 reduction is approximately 1.3 times higher in alkaliphiles than that in neutralophiles. This larger difference in redox potential may reflect a more rapid H^+ translocation from inside to outside the membrane by a terminal oxidase, cytochrome aco_3. The more rapid H^+ translocation by the terminal oxidase may be reflected in the higher $\Delta\psi$ in alkaliphiles (Table 1) and higher H^+/O ratio as shown by experimental data (36). Consequently, a rapid H^+ translocation may reflect a rapid or effective ATP production. These findings may reflect an effective H^+ translocation pathway both in cytochrome *c* oxidase and H^+-ATPase. Compared with the terminal oxidation machinery of neutralophiles, that of alkaliphiles is bigger in scale and more rapidly produces energy potential. Because of the high-energy production system as described above and the presence of the H^+ condenser system (will be described below) (14), alkaliphiles may have a nonproteinaceous terminal oxidation substance for the adjustment of an appropriate energy production and e^- flow corresponding to the physiological state of a group of cells.

Higher intracellular pH than neutralophiles It was reported that the intracellular pH of alkaliphilic *Bacillus* spp. is almost 2 units lower than the extracellar pH (Table 1). The estimated intracellular pHs are neither as high as the pH (pH 10) of the culture medium nor as neutral as in neutralophiles (pH 7–7.5). It was reported that the cytoplasmic pH of alkaliphilic *Bacillus* spp. is 8.3–8.4, when grown at pH 10.2–10.5. These values are higher than those of neutralophilic *B. subtilis* (pH 7.5) and other netralophiles. Intracellular pH increases with extracellular pH. Presumably, alkaliphiles may possess a mechanism that prevents the occurrence of a large difference between intracellular pH and extracellular pH. A high intracellular pH may have a negative effect on energy production because the translocation of H^+ by the respiratory complex becomes difficult owing to a low concentration of H^+ inside the cell (14, 117). Each respiratory complex should have an ability to accumulate H^+ at the vicinity of the H^+ gate of the complex located on the inner surface of the membrane. This ability has been demonstrated in the neutralophilic system (49). However, it should be difficult to accumulate H^+ in the alkaliphilic system because of the even higher intracellular pH than that of neutralophiles. It may be considered that there is a certain mechanism for the accumulation of H^+ in the vicinity of the inner surface of the membrane.

Bioenergetic parameters Although we are able to estimate the bioenergetic parameters $\Delta\psi$ and ΔpH in the bulk phase, these values may not reflect real values in the vicinity of the outer surface of membrane. It may be considered that these bioenergetic parameters do not take into consideration ion localization, environmental parameters (*i.e.*, parameters in the vicinity of the membrane) and the cycle of reactions depending on the reaction stage. As described above, it has been reported that artificial electrical transmembrane potential ($\Delta\psi$) induced by K^+ diffusion produces the same intensity as the energized respiratory chain, which cannot produce ATP in membrane vesicles prepared from cells grown at pH 10 and measured at pH 10 whereas natural potential by respiration can produce ATP (16). This can

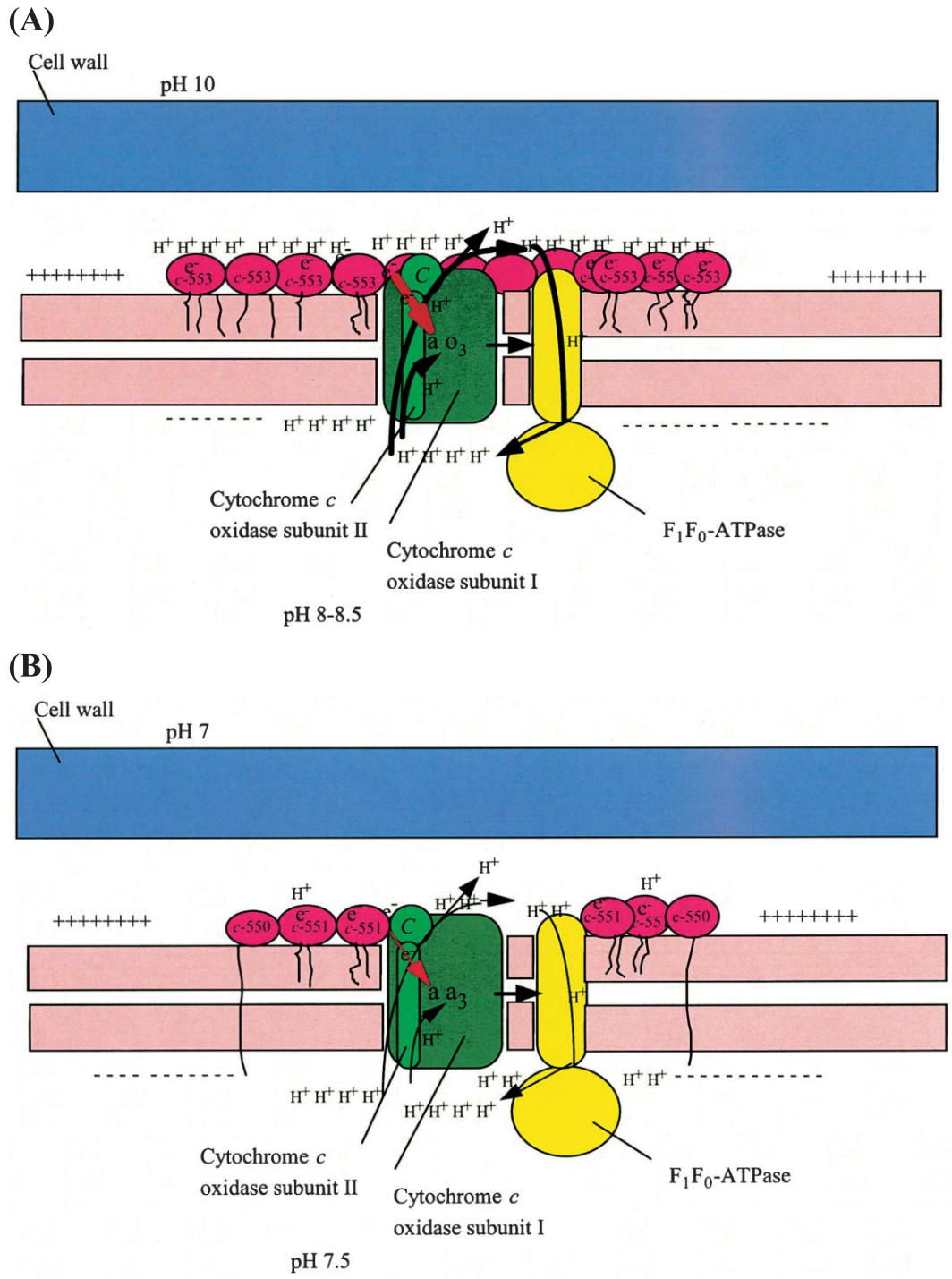

FIG. 3. Hypothetical models of (A) alkaliphilic and (B) neutralophilic terminal oxidations. Arrow between cytochrome *c* oxidase and ATPase indicates interaction between the two complexes (*e.g.*, Ref. 36). (A) A large difference in midpoint redox potential exists between cytochrome *c* and cytochrome *a*. A rapid electron electron flow between cytochrome *c* and terminal reaction produces a rapid H^+ pumping from the inner to the outer surface of the membrane. The frequency of H^+ translocation from the inner to the outer surface of the membrane, the H^+ transfer in the outer surface of the membrane and the H^+-trapping structure of cytcochrome *c* and other factors (*e.g.*, a larger negative ion capacity at inner surface membrane than at the outer surface membrane) sustain energy production. The surface structure and reduced cytochrome *c* may facilitate the trapping of H^+ in the vicinity of the outer surface of the membrane. This model is constructed on the basis of the data obtained from *Bacillus cohnii* YN-2000. Generally, it is considered that the important factors are as follows: (i) a protective acidic cell wall structure against outside environment, (ii) a proton-trapping nature at the outer surface of the membrane, (iii) a proton-trapping and -transferring background at the inner surface of the membrane, (iv) a large difference in midpoint redox potential between cytochrome *c* and terminal reaction, and (v) the frequency of H^+ pumping from the inner to the outer surface of the membrane. These factors would be related to each other or certain factors compensate for other factors. The balance of these factors is very important depending on the growth conditions. (B) There is no large difference in midpoint redox potential between cytochrome *c* and terminal reaction. H^+ transfer can easily occur on the outer surface of the membrane. This model is constructed on the basis of *B. subtilis*.

be explained by the finding that natural potential includes localized ΔpH and a reaction cycle, whereas artificial potential does not include such parameters. It has also been reported that a more rapid ATP production is observed in vesicles obtained from cells grown at pH 10 and measured at pH 9.5 than in those grown at pH 10 and measured at pH 7.8 with the same intensity of $\Delta\psi$ produced by the energized respiratory chain (16). This indicates that the membrane of alkaliphiles has suitable conditions for energy production at a high pH and the membrane utilizes alkaline conditions to its advantage.

H^+ condenser depending on aeration and strain

Obligately alkaliphilic *Bacillus* sp. YN-1 has a very low content of a terminal oxidase component, cytochrome *a*, and the strain showed KCN-resistant growth at a high pH (66). An NADH dehydrogenase has been purified from strain YN-1 and characterized (118, 119). Its biochemical characteristics suggest that this protein is a non-energy-coupling type. This indicates that the strain does not markedly depend on the function of the ordinary H^+-translocating and energy-coupling respiratory complex. On the other hand, the strain possesses large amounts of cytochromes *b* and *c* in the membrane (81–83). This suggests that the membrane of this strain has a certain capacity for electron storage. The electron storage capacity and acidic nature of these proteins in the outer surface of membrane may produce an electron and an H^+ condenser across the membrane. The e^- and H^+ condencer state will have a more important role under high-pH and/or air-limited conditions because of a low H^+ concentration in the outer surface of the membrane environment and/or low frequency of H^+ extrusion through the respiratory complex. *B. cohnii* YN-2000 produces a larger amount of terminal oxidase than strain YN-1 and lesser amount of nonproteinaceous terminal substance. However, strain YN-2000 is associated more with the nonproteinaceous substance at pH 10 than at pH 7 (66). This finding may also be associated with the accumulation of electrons at cytcohrome *c* and the partial independence of H^+ translocation coupling energy production by respiratory complexes. The electron accumulation and acidic nature of cytochrome *c* may support the accumulation of H^+ in the vicinity of the outer surface of the membrane. These characteristics will be quite effective because the electron flow will decrease under air-limited conditions or at a growth phase later than the late exponential phase. In contrast, when electrons flow quite rapidly, H^+ translocation consequently becomes very rapid and H^+ transportation sufficiently effective; therefore the effect of the H^+ condenser may not be as significant as that under air-limited conditions.

VIII. CONCLUSION AND PERSPECTIVE

As described above, alkaliphilic *Bacillus* spp. utilize alkaline-environment events for energy production to compensate for the disadvantages of high-pH conditions (Fig. 3). The significant advantages are the ability to utilize a large difference in redox potential between cytochrome *c* and terminal oxidation (factor I), a large attractive force for H^+ in the inner (factor II) and outer surfaces of membrane (factor III). The latter two factors become stronger at high pHs. In the presence of a high concentration of O_2, alkaliphilic *Bacillus* spp. can utilize the large difference in redox potential as the driving force of ATPase. A high frequency of H^+ translocation (factor IV) and a higher negative ion capacity in the inner membrane surface (factor II) than outer membrane surface are important under the high-aeration conditions. The extent of factor III is not as large as that of factor II in the case of respiration-dependent energy production. However, factor II supports a rapid H^+ transfer in the outer surface of the membrane and H^+ transfer along the outer surface of the membrane may occur locally (factor V). Under high-aeration conditions, certain alkaliphilic *Bacillus* spp. possess a larger scale of energy-producing machinery (Table 4). In the case of air-limited conditions, alkaliphilic *Bacillus* spp. accumulate H^+ and e^- at redox proteins, *e.g.*, cytochromes *c* existing in the outer surface of the membrane and concentrate H^+ and e^- for efficient energy production at high pHs (factor VI). In this case, a large attractive force for H^+ in the inner membrane surface and H^+-translocation by respiratory complexes become less important. The importance of the factors described above may be different depending on the species. The balances of these factors are very important depending on growth conditions. The factors may be related to each other or certain factors may have stronger effects than other factors in each specific strain and under various growth conditions. Alternatively, other factors not described above can compensate for the six factors mentioned above. We described our hypothetical model of bioenergetics in alkaliphilic *Bacillus* strains based on *B. cohnii* YN-2000. However, we consider that these six factors cannot be applicable to all alkaliphilic *Bacillus* spp., because these groups of microorganisms have a certain diversity, and the degree of their adaptation and their strategies in alkaline environments may be highly diversified. We only have certain knowledge on a quite limited number of alkaliphilic *Bacillus* strains among identified alkaliphilic *Bacillus* spp. To date, only a limited number of alkaliphilic *Bacillus* spp. have been isolated and identified. The alkaline-adaptation physiology of isolated alkaliphilic species other than *Bacil-*

TABLE 4. Comparison of several bioenergetic and growth factors between *Bacillus chonii* YN-2000 and *Bacillus subtilis* IAM 1026 under high-aeration conditions

Bioenergitic factors	*B. chonii* YN-2000 (pH 10)	*B. subtilis* IAM 1026 (pH 7)
Cytochrome *c* content	High	Low
Difference in negative ion capacity inner>outer surface of membrane	Large	Small
Difference in $E_{m,7}$ between cytochrome *c* and cytchrome *c* oxidase	Large	Small
O_2 consumption rate	High	Low
Membrane electrical potential ($\Delta\psi$)	Large	Small
Maximum growth yield at stationary phase of growth	High	Low

lus spp. (30, 31, 120–133) is as yet unclarified. We only have very limited knowledge of alkaline adaptation in a quite limited number of alkaliphilic *Bacillus* spp. However, the obtained knowledge may have significance with respect to the bioenergetic properties of not only other alkaliphilic microorganisms but also the fundamental understanding of bioenergetics in neutralophilic microorganisms.

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