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Requirement of Na^+ in flagellar rotation and amino-acid transport in a facultatively alkalophilic *Bacillus*

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A facultatively alkalophilic *Bacillus* strain YN-2000 grew well between pH 6.8 and 10, although the available size of the protonmotive force for the cells was high around neutral pH, but considerably low at alkaline pH. The energy source for the cellular functions of this bacterium grown at different pHs was analyzed to test if the cells switch the energy source between the protonmotive force and another source, depending upon the available size of the protonmotive force. The flagellar motors and the transport system of glutamine in this bacterium showed optimal activities at an alkaline pH and absolutely required the presence of Na^+ in the medium, irrespective of the growth pH. These results are consistent with the idea that, like obligate alkalophiles, the flagellar motors and an amino acid transport system of this bacterium are designed to utilize only the electrochemical potential gradient of Na^+ and have no switch of the energy source, irrespective of the available size of the protonmotive force during cell growth.

Introduction

It has been shown that bacteria living in moderate environment utilize the protonmotive force ($\Delta\tilde{\mu}_{H^+}$) as the energy source for their cellular functions such as many active transport systems and flagellar motors [1–3]. The $\Delta\tilde{\mu}_{H^+}$ is composed of the membrane potential ($\Delta\psi$) and the pH gradient across the cytoplasmic membrane (ΔpH). Since these bacteria have the ability to maintain the intracellular pH nearly neutral independent of the external pH [4], the value of the $\Delta\tilde{\mu}_{H^+}$ under alkaline conditions is calculated to be small. The cellular functions driven by the $\Delta\tilde{\mu}_{H^+}$ are, therefore, considered to be not fully active under alkaline conditions. This may be one of the reasons for the absence of cell growth of these neutrophilic bacteria under alkaline conditions.

Besides neutrophiles, there is a group of bacteria living only in alkaline environment [5]. The bacteria including obligately alkalophilic *Bacillus* have been reported to have a quite low $\Delta\tilde{\mu}_{H^+}$ at the optimal pH for their growth, since their intracellular pH is maintained lower than 9 [6–8]. For example, the intracellular pH of an alkalophilic *Bacillus* strain ATCC27647 was about 9 as its optimal growth pH of 11 so that the $\Delta\tilde{\mu}_{H^+}$ was estimated to be about -15 mV at this pH [6]. In order to survive such low $\Delta\tilde{\mu}_{H^+}$ conditions, these bacteria have developed a novel system for

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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energy coupling, which is driven not by the $\Delta\tilde{\mu}_{H^+}$, but by the electrochemical potential gradient of Na^+ ($\Delta\tilde{\mu}_{Na^+}$). For example, amino acid transport systems and flagellar motors of obligately alkalophilic *Bacillus* are driven by the $\Delta\tilde{\mu}_{Na^+}$ [6–12]. Thus, many cellular functions in neutrophiles are of the H^+-driven type, whereas those in alkalophiles are of the Na^+-driven type.

A facultatively alkalophilic *Bacillus*, which has the ability to grow well under both alkaline and neutral conditions, has been isolated and named as YN-2000 [13,14]. It is quite interesting, therefore, to investigate what kind of energy coupling mechanism is utilized by this bacterium for its cellular functions. There are many possibilities; e.g., the cells may have both the Na^+-driven and H^+-driven types at the same time or may have a mechanism to switch between these two types.

In this paper, we present the results that YN-2000 cells, irrespective of their growth pH, utilize the $\Delta\tilde{\mu}_{Na^+}$ for their flagellar rotation and glutamine transport. This result indicates that these cellular functions are fixed in Na^+-driven type and has no switching mechanism between H^+-driven type and has no switchings mechanism between H^+-driven and Na^+-driven type.

Materials and Methods

Bacterial strain and cell growth

The bacterial strain used was a facultatively alkalophilic *Bacillus* strain YN-2000 [13]. Cells were grown at 37°C with shaking in AB-4 medium at pH 7.5 or 9.5 as described previously [8,15]. The pH of the medium was adjusted by Na_2CO_3, and the final concentration of Na^+ in the medium was adjusted to 150 mM by the addition of NaCl. Cells were harvested by centrifugation at late logarithmic phase. Except otherwise noted, cells were washed 3 times with TG medium consisting of 25 mM Tris-HCl buffer (pH 7.5 or 9.5) and 5 mM glucose, and suspended in the same medium. In some experiments, CG medium consisting of 50 mM 2-(cyclohexylamino)ethane sulfonate buffer (pH 7.5 or 9.5) and 5 mM glucose was used instead of TG medium.

Measurement of bacterial motility

The swimming speed of the cells was measured by the photographic method as described previously [15]. When the effect of Na^+ concentration on cell motility was studied, the cells grown in AB-4 medium were concentrated by centrifugation and resuspended in the same medium without washing. The cells were then diluted 2000-fold or more with TG medium or CG medium containing 0.1 mM EDTA (K^+-salt) and various concentrations of NaCl. By this dilution, the amount of Na^+ contaminated from AB-4 medium became lower than 0.1 mM.

Measurement of glutamine transport and glucose transport

The transport of glutamine was measured by using radioactive L-glutamine. The cells ($8 \cdot 10^8$ cells or 0.2 mg proteins/ml) in TG medium containing various concentrations of NaCl or other cations and 125 μg/ml of chloramphenicol were incubated with shaking at 35°C for 2 min. Chloramphenicol was added to inhibit the protein synthesis during the assay. The cells were then mixed with 50 μM of [^{14}C]-L-glutamine (12 μCi/μmol, New England Nuclear, Boston, MA, U.S.A.) and incubated at 35°C. At intervals, samples (0.5 ml) were withdrawn and filtered by using a membrane filter (type 111, Sartorious Membrane Filter GmbH, Göttingen, F.R.G.). The filter was washed twice with 2 ml of TG medium and dried up. The radioactivity trapped on the filter was measured by a liquid scintillation spectrophotometer (Aloka model LSC-701N). As a control, the cells treated with 10 μM of gramicidin D were used.

The transport of glucose was measured as above by using ^{14}C-labeled glucose instead of [^{14}C]-glutamine. The cells ($8 \cdot 10^8$ cells/ml) in TG medium (pH 9.5) with or without 50 mM NaCl was mixed with 0.3 mM of [^{14}C]-D-glucose (0.5 μCi/μmol, New England Nuclear, Boston, MA, U.S.A.). After incubation for 1 min, 0.2 ml of the sample was withdrawn and filtered through a membrane filter. The radioactivity trapped on the filter was measured as described above. When necessary, the cells were added by 100 μM of carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and incubated for 2 min prior to the addition of [^{14}C]glucose.

Measurement of ATP content

The ATP content of the cells was measured as

described previously [12]. The cell concentration used for the measurement was about $5 \cdot 10^8$ cells/ml.

Measurement of membrane potential and pH difference

The $\Delta\psi$ of the cells was measured by using [^{3}H]triphenylmethylphosphonium ([^{3}H]TPMP$^+$) as described previously [12]. The cells (about $3 \cdot 10^8$ cells/ml) were mixed with 10 μM [^{3}H]TPMP$^+$ (0.19 mCi/μmol) and incubated for 5 min at 35°C. Gramicidin-treated cells were used as a control.

The intracellular pH was determined by using [^{14}C]methylamine (1.1 μM, 0.92 mCi/mmol) or [^{14}C]dimethyloxazolidine-2,4-dione (0.5 μM, 1 mCi/mmol) as described previously [8]. The cell concentration for the measurement was about $8 \cdot 10^9$ cells/ml.

All the radioactive materials were the products of New England Nuclear, Boston, MA, U.S.A.

Results

Growth rate and size of protonmotive force in YN-2000

In contrast to the obligately alkalophilic *Bacillus*, which only grow under alkaline conditions, a facultatively alkalophilic *Bacillus* strain YN-2000 can grow well between pH 6.8 and 10 (Fig. 1). The cell shape of the cells grown at either pH 6.8 and 10 looked similar. To understand what kind of energy source is utilized by this bacterium for the growth, the $\Delta\psi$ and ΔpH were measured at different medium pHs. As shown in Table I, with increasing the external pH, the $\Delta\psi$ was gradually increased whereas the intracellular pH stayed nearly constant at around 8.2. Hence, the protonmotive force calculated by these values was about -180 mV at pH 7.5 and decreased to be about -80 mV at pH 10.2. Thus, it is concluded that the growth rate of YN-2000 shown in Fig. 1 is not a direct reflection of the available size of the protonmotive force in the cells. Then, what is the energy source for the cellular functions of YN-2000?

Effects of external pH and Na^+ concentration on motility of YN-2000

It has been shown that flagellar motors of

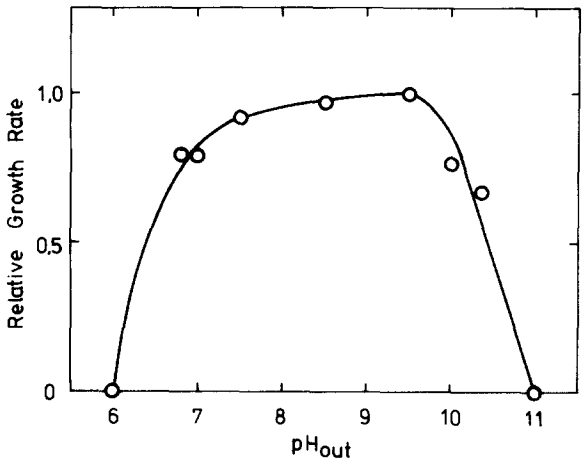

Fig. 1. Growth rate of YN-2000 in AB-4 medium with various pHs. The growth rate at pH 9.5 was 1.7 generation per h and designated as 1.0.

neutrophiles utilize the protonmotive force as the energy source whereas those of alkalophilies utilize the $\Delta\tilde{\mu}_{Na^+}$. Motility of YN-2000 cells was instantaneously and completely lost without any decrease in ATP content of the cells (about 7 mM) when the membrane potential of the cells was collapsed by the addition of 1 μM valinomycin in the presence of 100 mM KCl (data not shown). This indicates that, as well as the case of other bacteria, the flagellar motors of YN-2000 is driven by the influx of some ion. Since YN-2000 cells can grow well both neutral and alkaline pHs, it is quite interesting to ask if there is a possibility that the flagellar motors of this bacterium constructed at the growth pH of 7.5 utilize the protonmotive

TABLE I

EFFECT OF EXTERNAL pH ON THE BIOENERGETIC PARAMETERS OF YN-2000

The external pH (pH_{out}) of the cells in AB-4 medium was changed by the addition of KOH or HCl. The $\Delta\psi$ and the intracellular pH (pH_{in}) were measured as described in Materials and Methods. The $\Delta\tilde{\mu}_{H^+}$ was calculated from these values.

pH_{out}	pH_{in}	$\Delta\psi$ (mV)	$\Delta\tilde{\mu}_{H^+}$ (mV)
7.5	8.2	−130	−172
8.5	7.9	−159	−135
9.5	8.1	−181	−97
10.2	8.4	−188	−80

force while those constructed at pH 9.5 utilize the $\Delta\tilde{\mu}_{Na^+}$. The pH dependence and Na^+ dependence of motility of YN-2000 was studied to test this possibility.

As shown in Fig. 2, YN-2000 cells show optimal motility between pH 8 and 11 irrespective of their growth pH, although the available size of the protonmotive force under these pHs was clearly smaller than that at pH 7.5 (Table I). Thus, the flagellar motors of YN-2000 seem to be not affected by the available size of the protonmotive force at the time of the cell growth or of the motility measurement. This result suggests a similarity between the flagellar motors of this bacterium and those of obligately alkalophilic *Bacillus*. Consistent with this idea, YN-2000 cells showed no translational swimming in the absence of Na^+ in the medium irrespective of the pH for growth or motility measurement (Fig. 3). Motility was restored by the addition of Na^+ to the medium. Swimming speed was increased with increasing the Na^+ concentrations in the medium at either pH 7.5 or 9.5, and the speed reached maximum at about 10 mM Na^+. Motility was induced only by Na^+. K^+ and Li^+ up to 100 mM had no effect on the induction of motility. These results indicate that the flagellar motors of YN-2000 are fixed as the Na^+-driven type irrespective of the available size of the protonmotive force.

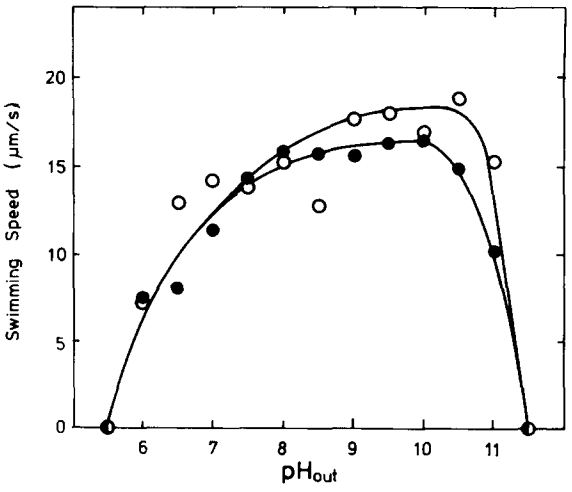

Fig. 2. pH dependence of swimming speed of pH 7.5-grown cells (●) and pH 9.5-grown cells (○). Motility was measured in TG medium containing 50 mM NaCl with various pHs as indicated.

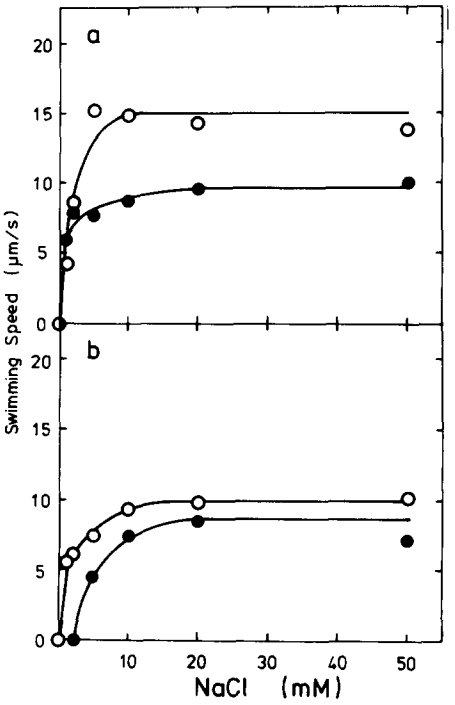

Fig. 3. Effect of Na^+ on swimming speed in pH 7.5-grown cells (●) and pH 9.5-grown cells (○). Swimming speed was measured in TG medium at pH 9.5 (a) or pH 7.5 (b) with various concentrations of NaCl.

Effect of external pH and Na^+ concentration on glutamine transport of YN-2000

In the case of flagellar motors, their structure is so complicated that it might be difficult to switch the driving force depending on the available size of the energy source. As a much simpler system, we took the amino acid transport system. Among the tested amino acids including leucine, proline, methionine, glutamine and α-aminoisobutylate, we chose the glutamine transport system, since it showed (5–10)-times more active than others (data not shown).

Fig. 4 shows that the pH-dependent curve of glutamine transport in YN-2000 cells grown at either pH 7.5 or 9.5. Irrespective of the growth pH, rate of the transport was low in neutral and high in alkaline. Thus, as well as the case of flagellar motors, YN-2000 cells might not switch the energy source for the glutamine transport based on the growth pH.

Similar to flagellar motors, Na^+ in the medium was found to be important for the glutamine

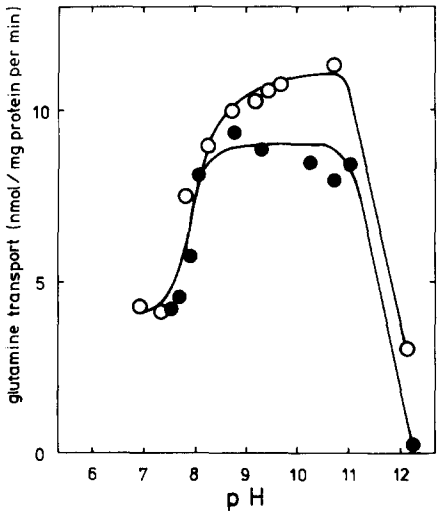

Fig. 4. pH dependence of glutamine transport in pH 7.5-grown cells (●) and pH 9.5-grown cells (○). The transport was measured in TG medium containing 50 mM NaCl with various pHs as indicated.

transport (Fig. 5). The rate of glutamine transport without the addition of Na^+ was quite low, but it was increased with increasing Na^+ concentrations irrespective of the growth pH. The addition of other cations did not stimulate the glutamine transport (Table II). Thus, the glutamine transport system of YN-2000 absolutely required the presence of Na^+ in medium. The low rate of glutamine transport observed without the addition of Na^+ is probably due to the presence of a small amount of Na^+ contaminated in the medium.

When Na^+ concentration was fixed at 50 mM and glutamine concentration was varied, the rate of glutamine transport in YN-2000 was increased with increasing glutamine concentrations and reached nearly maximum at about 20 μM. The K_m value for glutamine was estimated to be about 6 μM from the Lineweaver–Burk plot of the data irrespective of the growth and the assay pH (Fig. 6). The V_{max} of the transport was also only slightly affected by the growth pH. These results indicate that both the activity and the kinetic parameters of glutamine transport are not affected by the growth pH. Thus, it is concluded that the glutamine transport system of YN-2000 is fixed as the Na^+-driven type irrespective of the available size of the protonmotive force during cell growth.

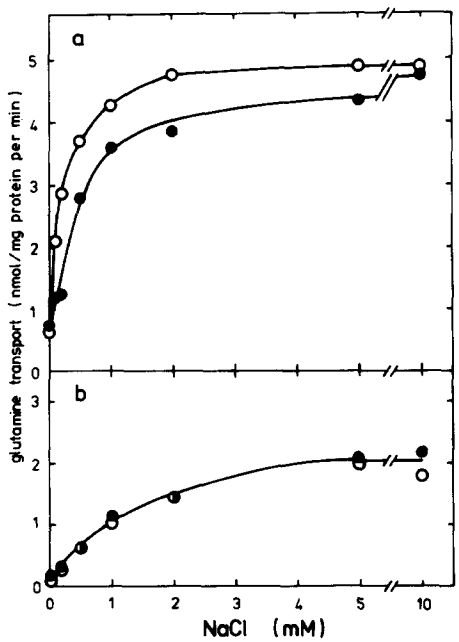

Fig. 5. Effect of Na^+ on glutamine transport in pH 7.5-grown cells (●) and pH 9.5-grown cells (○). The transport was measured in TG medium with various concentrations of NaCl at pH 9.5 (a) or pH 7.5 (b).

TABLE II

EFFECT OF VARIOUS CATIONS ON GLUTAMINE TRANSPORT IN YN-2000

Cells in TG medium (pH 9.5) were mixed with various cations as indicated. The glutamine uptake was measured after 5 min incubation at 35°C.

Cations	Concn. (mM)	Rate of glutamine uptake (nmol/mg protein per min)
None		0.8
NaCl	1	6.2
	50	7.4
KCl	1	1.1
	50	0.5
LiCl	1	1.2
	50	1.7
RbCl	1	1.2
	50	< 0.1
CsCl	1	0.9
	50	1.0
$MgCl_2$	1	1.1
	50	0.2
$CaCl_2$	1	< 0.1
	50	< 0.1
NH_4Cl	1	1.1
	50	0.5

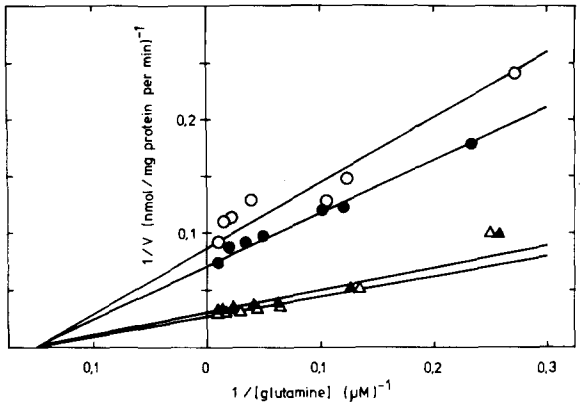

Fig. 6. Lineweaver–Burk plots of glutamine transport against glutamine concentration. The glutamine transport in pH 7.5-grown cells (●, ▲) or pH 9.5-grown cells (○, △) was measured in TG medium containing 50 mM NaCl at pH 9.5 (▲, △) or pH 7.5 (●, ○).

TABLE III

EFFECT OF ETHANOLAMINE ON THE CYTOPLASMIC pH HOMEOSTASIS, THE MEMBRANE POTENTIAL, MOTILITY, AND GLUTAMINE TRANSPORT IN YN-2000

Experiment I: the cells in AB-4 medium (pH 9.5) were used. Experiment II: the cells in TG medium (pH 9.5) containing 50 mM NaCl were used.

	Experiment I		Experiment II	
	pH_{in}	swimming speed (μm/s)	Δψ (mV)	glutamine uptake (nmol/mg protein per min)
No ethanolamine added	8.1	22	−189	7.2
150 mM ethanolamine added	9.5	18	−135	2.4

Role of Na^+ in the Δψ and ΔpH of YN-2000

There is a possibility that the Na^+ dependence of flagellar rotation and glutamine transport is the secondary effect of the Na^+ dependence of the energy production system or of the cytoplasmic pH homeostasis. We could eliminate this possibility by studying the effect of Na^+ and Li^+ on the Δψ and cytoplasmic pH. The Δψ of YN-2000 was about −180 mV and was not affected by the presence or absence of Na^+ in the medium, indicating that Na^+ is not essential for the energy production in YN-2000. The cytoplasmic pH at the external pH of 9.5 was 8.3 in the presence of Na^+, but increased to pH 9.3 by the omission of Na^+. It was found, however, that the cytoplasmic pH was decreased to 8.8 by the addition of 100 mM Li^+. This indicates that although the presence of Na^+ is essential for the cytoplasmic pH homeostasis, Li^+ is able to substitute for Na^+. Since Li^+ is not substitutable with Na^+ for the flagellar rotation and the glutamine transport in YN-2000 as shown before, the use of Li^+ can clearly separate the system for cytoplasmic pH homeostasis from the Na^+-dependent motility and glutamine transport.

We also found that the alkalinization of cytoplasm itself caused only a mild effect on motility and glutamine transport. When a membrane permeable weak base, ethanolamine, was added to YN-2000 cells at pH 9.5, the cytoplasmic pH homeostasis was completely collapsed. Under the condition, however, significant swimming and glutamine transport were observed (Table III).

In contrast to the requirement of Na^+ for motility and glutamine transport, glucose transport of YN-2000 was rather independent of the presence or the absence of Na^+ in the medium (Table IV). Furthermore, it was found that glucose transport was not affected by CCCP at the concentration where the membrane potential was collapsed and glutamine transport was inhibited. These results indicate that the omission of Na^+ from the medium did not result in a general

TABLE IV

EFFECT OF Na^+ AND CCCP ON GLUCOSE TRANSPORT IN YN-2000 CELLS

Cells in TG medium (pH 9.5) with or without 50 mM NaCl were incubated with shaking at 35°C for 2 min. When indicated, 100 μM of CCCP was also added. Glucose transport was then measured by the addition of [^{14}C]glucose. Samples were withdrawn after incubation for 1 min.

Addition	Glucose uptake (nmol/mg protein per min)
None	11
NaCl, 50 mM	16
CCCP, 100 μM	10
CCCP, 100 μM + NaCl, 50 mM	11

inhibition of the membrane functions in this bacterium. The glucose transport system may be powered by ATP, since the disruption of membrane potential did not affect the transport.

Discussion

It has been shown that many cellular functions located on the membrane of neutrophiles are H^+-driven whereas those of obligately alkalophiles are Na^+-driven. This change in the utilization of the energy source is quite reasonable because the available size of the protonmotive force is estimated to be high around neutral pH, but quite low at alkaline pH.

The facultatively alkalophilic *Bacillus* YN-2000 was found to have the ability to grow well under both neutral and alkaline conditions. Therefore, we expected in this strain a switch of the energy source for the membrane functions from the H^+-driven type around neutral to the Na^+-driven type in alkaline. Our results, however, showed that this was not the case. Irrespective of their growth pHs or assay pHs, the energy-coupling system for flagellar motors and glutamine transport in YN-2000 cells was fixed as the Na^+-driven type. Thus, a facultatively alkalophilic *Bacillus* YN-2000 has essentially the same energy coupling system as well-studied obligately alkalophilic *Bacillus* [5,6, 12].

It is interesting that the growth rate of YN-2000 was almost constant between pH 7 and pH 10, although the rate of flagellar rotation and glutamine uptake showed maximum at around pH 9. This may be an indication that this bacterium is evolved from an obligate alkalophile.

Although the energy coupling system for the membrane functions in YN-2000 is fixed as the Na^+-driven type irrespective of the growth pH, pH 9.5-grown cells showed slightly higher activities of flagellar rotation and glutamine transport than pH 7.5-grown cells. This may suggest that the energy supply system in pH 9.5-grown cells is slightly stronger than that in pH 7.5-grown cells. The finding that alkalophiles have more cytochrome contents than neutrophiles [16] may support this idea.

In the case of obligately alkalophilic *Bacillus*, the cytoplasmic pH homeostasis is maintained by the function of Na^+/H^+-antiporter [8,17]. Koyama and Nosoh [14] reported that YN-2000 cells at alkaline pHs utilized the Na^+/H^+-antiporter for the maintenance of the cytoplasmic pH homeostasis. However, around neutral pH, they suggested that the K^+/H^+-antiporter had an additional role in the pH homeostasis. Krulwich et al. [18] reported that an obligately alkalophile, *Bacillus firmus* RAB, had Na^+/H^+-antiporter but no K^+/H^+-antiporter. It is likely, therefore, that the presence of both K^+/H^+- and Na^+/H^+-antiporters, besides the Na^+-driven type of the energy coupling system, might be an essential factor for the cells which can grow under a wide pH range from neutral to alkaline.

Krulwich and co-workers [7,19] reported that the non-alkalophilic mutants were isolated from obligately alkalophiles at the frequency of a single mutational event. This suggested that the change in one component of the cells might be enough to change the alkalophiles to the neutrophiles, or vice versa. Recently, however, Krulwich [16] reported that the non-alkalophilic mutant, *B. firmus* RABN, exhibited only very poor growth at pH 7.0 and some leakyness of the membrane to Na^+. Thus, the non-alkalophilic mutants so far isolated from obligately alkalophiles seem to have a quite complex lesion. Therefore, the difference between obligate alkalophiles and neutrophiles is suggested to be not simple. It is quite likely that at least a few factors, besides Na^+/H^+- and K^+/H^+-antiporters, are required to strongly support the cell growth at alkaline pH.

Acknowledgments

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