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Respiratory chain of the alkalophilic bacterium, *Bacillus* sp. I-1

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The triphenyltetrazolium chloride (TTC) reduction activities of alkalophilic *Bacillus* sp. I-1 were higher than those of *B. subtilis* (Nishigami & Oshima, 1983). The component parts involved in membrane-bound respiratory chain and the mutual relationships between these components and TTC reduction activity of both strains were compared. The contents of cytochromes and quinones, and NADH dehydrogenase activities of *B.* sp. I-1 were higher than those of *B. subtilis*. In the cell-free experiment of *B.* sp. I-1, the reduction of TTC was only observed when NADH was used for the substrate. Further, inhibitory effects of 2-hepty1-4-hydroxyquinoline (HQNO), an inhibitor of respiratory chain, on the activity of TTC reduction were lower than those on NADH oxidase activity. Thus, NADH dehydrogenase may play an important role in TTC reduction of *B.* sp. I-1.

Introduction

Alkalophilic bacteria are able to grow at alkaline pH range. Their intracellular pH (acid inside) are maintained constantly even at external alkaline pH value (Booth, 1985). The chemical gradient of protons would be adverse, at alkaline pH range, with respect to the development of protonmotive force. In spite of this unfavorable condition, the growth yields of many alkalophilic bacillus were comperable to those obtained from neutrophilic bacillus (Lewis *et al.*, 1980). Thus, alkalophilus would possess the unique bioenergetic systems which function at alkaline pH range. It was found that the membranes of alkalophilic bacillus contain extraordinary high cytochrome hem contents (Lewis *et al.*, 1980).

We have isolated an alkalophilic *Bacillus* sp. I-1 from a part of solution of indigo-fermentation (Nishigami & Oshima, 1983). We found that the TTC reduction activity of *B.* sp. I-1 was very higher than that of *B. subtilis* at wide range of alkaline pH value. In this paper, the components of membrane-bound respiratory chain and the mutual relationships between the respiratory components and TTC reduction activity are discussed.

Meterials and Methods

Strain and growth conditions

Alkalophilic *Bacillus* sp. I-1, isolated from a part of solution of indigo-fermentation

(Nishigami & Oshima, 1983) and *Bacillus subtilis* strain, RIMD were used throughout this experiment. These strains were usually grown in NB-medium containing 10 *g* of Ehrlich meat extract, 10 *g* of peptone and 5 *g* of NaCl per liter. The pH value was abjusted with NaOH to 9.0 for *B.* sp. I-1 and 7.2 for *B. subtilis,* respectively. Cells were grown aerobically with shaking at 37°C.

Preparation of membrane vesicles

Cells in the mid-logarithmic phase of growth were harvested, and washed three times with 20 mM Na-phosphate buffer (pH 8) containing with 10 mM $MgCl_2$ and 0.2 M sucrose. Then, the membrane vesicles were prepared as described previously (Oshima *et al.,* 1987).

Assay procedures

TTC reduction activity and respiration activity were measured as previously reported (Nishigami & Oshima, 1983). NADH dehydrogenase (EC.1.6.99.3) and Succinate dehydrogenase (EC.1.3.99.1) activities were measured by using DCPIP and PMS as electron accepter, respectively (Takada *et al.,* 1981).

Measurement of cytochrome contents

Dithionite-reduced minus air-oxidized difference spectra were recorded at room temperature by Nihon Bunko Type UVIDEC-510 Double Beam Spectrophotometer. The following millimolar extinction coefficients and wavelength pairs were used for the measurement of the cytochrome content of the membrane vesicles of *B.* sp. I-1: cytochrome $(a+a_3)$, $\Delta A_{605-630}$, $\Delta\varepsilon=16.5$; cytochrome *b*, $\Delta A_{562-577}$, $\Delta\varepsilon=17.9$; and cytochrome *c*, $\Delta A_{554-538}$; $\Delta\varepsilon=19.1$, in the case of *B. subtilis,* wavelength pares, $\Delta A_{602-620}$, $\Delta A_{562-575}$ and $\Delta A_{557-510}$ were used, respectively.

Measurement of quinone contents

Quinones were extracted by etyl ether-ethanol (3:1) solution and purified by thin layer chromatography as described by Yamada *et al.,* (1969). Then, determination of quinones were carried out by using high performance liquid chromatography (HPLC)(Tamaoka *et al.,* 1983).

Protein detemination

Protein was determined by using Bio-Rad Protein Assay. Bovine albumin was used as a standard.

Chemicals

The sources of materials used in this work were as follows: Peptone and Ehrlich meat extract from Kyokuto Pharmaceutical Industrial Co., 2,6-dichlorophenol indophenol (DCIP) from Merk, triphenyltetrazolium chloride (TTC), 1-2′-thenoyl-3, 3,

3-trifluoroacetone-N-oxide (TTA) and phenazine methosulfate (PMS) from Nacalai tesqu, and NADH and 2-heptyl-4-hydroxyquinoline (HQNO) from Sigma. All other reagents used in this study were of analytical grade.

Results

Effects of pH on growth

Effects of pH on growth of *B.* sp. I-1 and *B. subtilis* were examined. The optimal pH on growth of both strains were pH 9 and pH 7, respectively (Fig. 1). At the optimal pH value, the growth rate of *B.* sp. I-1 is consistent with that of *B. subtilis*.

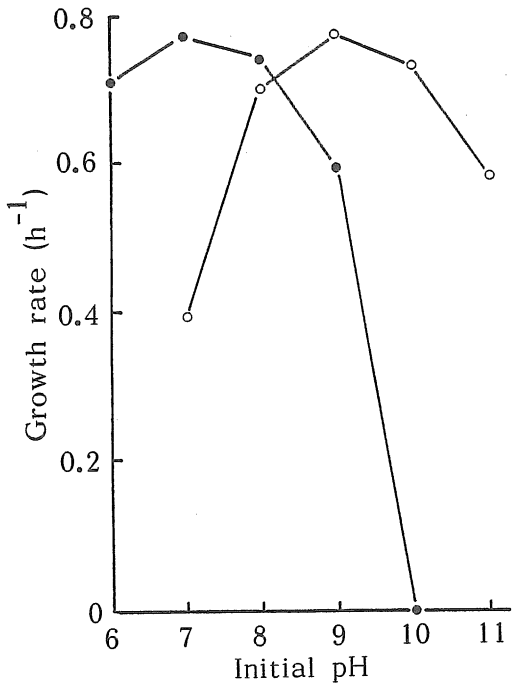

Fig. 1. Effect of pH on the growth rates of *B.* sp. I-1 and *B. subtilis*. pH of the medium was adjusted with HCl or NaOH. Symbols: ○—○; *B.* sp. I-1, ●—●; *B. subtilis*.

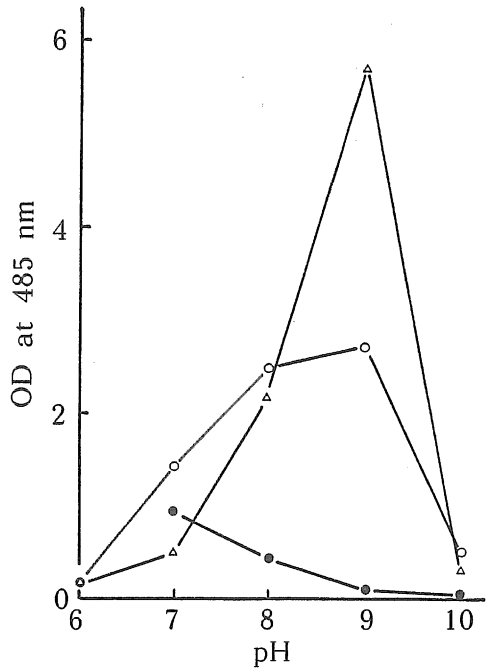

Fig. 2. Effect of pH on TTC reduction activities of *B.* sp. I-1 and *B. subtilis*. The activities were measured in the following buffer systems, 20 mM Na_2HPO_4-NaH_2PO_4 (pH 6, 7 and 8) and 20 mM $NaHCO_3$-Na_2CO_3 (pH 9 and 10). Symbols: ○—○; *B.* sp. I-1 was grown with shaking, △—△; *B.* sp. I-1 was grown without shaking, ●—●; *B. subtilis* grown with shaking.

Effects of pH on TTC reduction activity

In the alkaline medium, alkalophilic *B.* sp. I-1 can reduce the dye (indigo) from indigotin (oxidized form) to indigo white (reduced form). Indigotin is insoluble in water, so that, to determine of the enzymatic reduction activity, we used TTC which can be substituted for indigo (Takahara *et al.*, 1961). Fig. 2 shows the effects of pH on the reduction of TTC. *B.* sp, I-1 showed the maximal reduction at pH 9 which was the same pH value for the optimal growth. When *B.* sp. I-1 was grown without shaking, the reduction activity at pH 9 was increased about 2-fold, compared with shaking. In *B. subtilus,* no stimulated activity of TTC reduction was observed, even at the optimal pH value for growth.

Effects of pH on respiration activity

TTC is used for the screening of respiration-defect mutant of Yeast (Anne-Marie *et al.*, 1974) because TTC is known to an electron acceptor for cytochrome oxidase and fravoproteins (Nachlas *et al.*, 1960, Sato & Sato, 1965). Therefore, it was expected that the TTC reduction activities were dependent on a part of the respiratory activity. Fig. 3 shows that the optimum activities of *B.* sp. I-1 and *B. subtilis* were about pH 9 to 10 and 7, respectively.

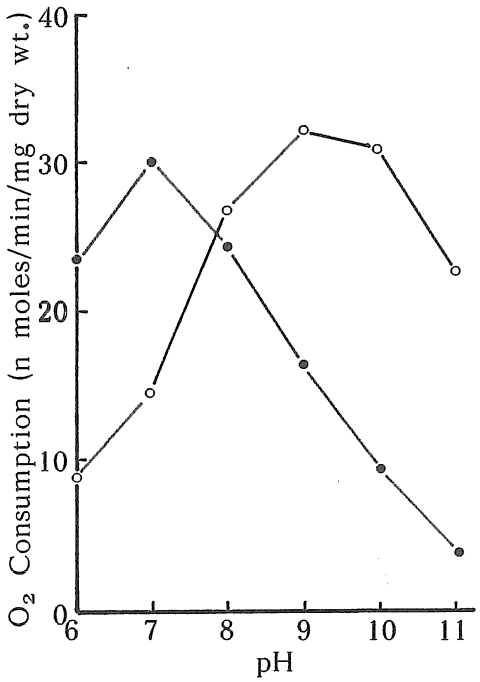

Fig. 3. Effect of pH on the respiration activities of *B.* sp. I-1 and *B. subtilis.* The activities were measured in the same buffer system as described in Fig. 2. Symbols: ○—○; *B.* sp. I-1, ●—●; *B. subtilis.*

Effects of pH on respiration enzymes

The TTC reduction activity of *B. subtilis* was very low than that of *B.* sp. I-1 at pH 8, while the respiration activity of *B. subtilis* was equal to that of *B.* sp. I-1. To elucidate the mutual relationship between the respiration activity and TTC reduction activity in the two strains, in the next place, we examined the activity of some enzymes involved in the respiratory chain. The NADH dehydrogenase of *B.* sp. I-1 showed the maximum activity at pH 9. On the other hand, in *B. subtilis,* a constant activity was always observed at pH value tested. At pH 9, the activity of this enzyme was about three-times higher than that of *B. subtilis.* (Fig. 4A). The activities of succinate dehydrogenase from *B.* sp. I-1 showed less pH-dependency, compared with those of NADH dehydrogenase, and the acitivities were lower than that of *B. subtilis* (Fig. 4B).

Cytochrome contents

It has been reported that the specific array of cytochromes and other respiratory components affect the efficiency of the energy transduction (Jones *et al.*, 1975). So,

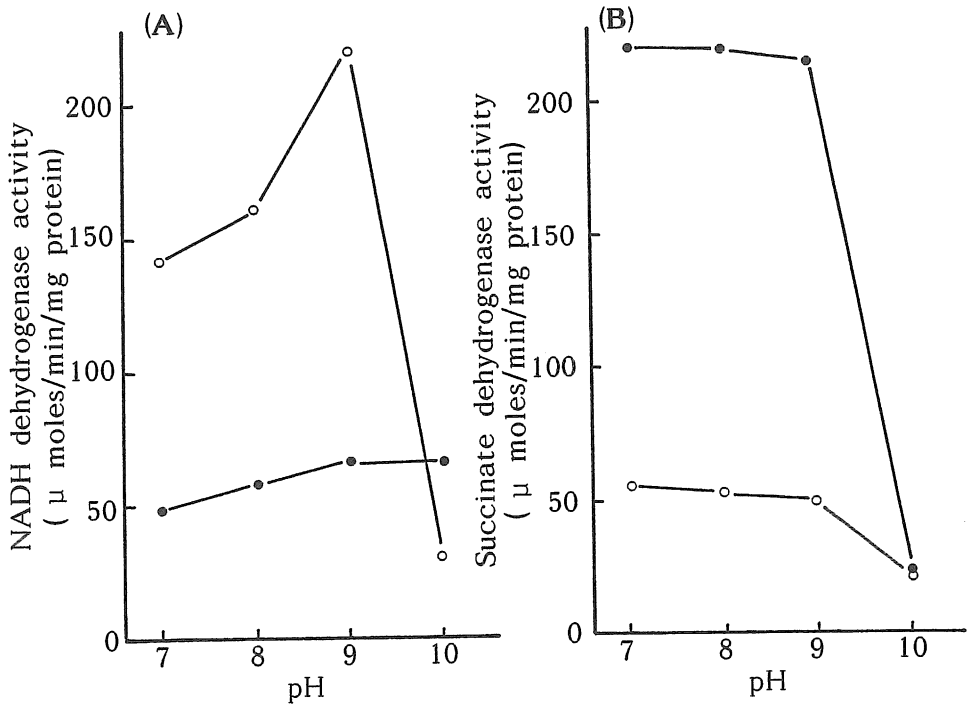

Fig. 4. Effect of pH on NADH dehydrogenase (A), and succinate dehydrogenase (B) activities. Membrane vesicles were prepared by the following buffer systems, 0.1 M Tris-HCl (pH 7, 8 and 9) and 0.1 M $NaHCO_3$-Na_2CO_3 (pH 10). Activities were measured out in 0.1 M Tris-HCl pH 7.4 as described in Materials and Methods.

we determined the contents of cytochromes of *B.* sp. I-1 and *B. subtilis*. Dithionite-reduced the everted vesicle preparations of *B.* sp. I-1 at pH 8 exhibited *a*-band peaks at 605 and 554 nm (Fig. 5A). Slight difference on cytochrome spectrum was observed between both the strains (Fig. 5B). Estimations of the cytochrome contents from difference spectra indicated a higher value in the *B.* sp. I-1 than in *B. subtilis* (Table 1); contents of *b*- and *c*-type cytochromes in *B.* sp. I-1 were higher than those of *B. subtilis*.

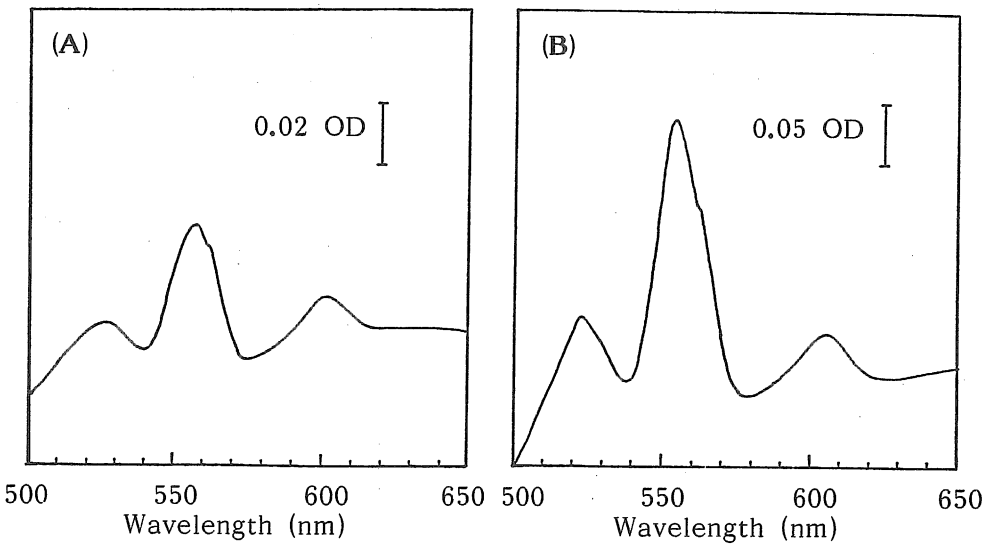

Fig. 5. Difference spectra of vesicles from *B. subtilis* (A), and *B.* sp. I-1 (B), at room temperature. Membrane proteins of *B. subtilis* and *B.* sp. I-1, used in this experiment, were 6.50 and 3.04 mg of proteins per ml, respectively.

A few grains of sodium dithionite were used for the reducing reagent.

Table 1. Cytochrome contents of membranes from *B.* sp. I-1 and *B. subtilis*

Strains	Cytochrome type		
	b	*c*	*a*
	n moles/mg protein		
B. sp. I-1	1.29	1.74	0.35
B. subtilis	0.69	0.70	0.21

Cytochrome contents were assayed by analysis of difference spectra as described in Materials and Methods.

Quinone contents

Quinones were extracted from the intact cells of *B.* sp. I-1 and *B. subtilis*. Spectral patterns of the quinones from both strains were same each other (Fig. 6) and the concentraions of quinone of *B.* sp. I-1 and *B. subtilis* were 9.8 and 4.9 μg/mg wet weight, respectively. It is known that *B. subtilis* contained menaquinone only (Bishop *et al.*, 1962). From analysis of the spectral pattern, it was shown that the membrane quinones of *B.* sp I-1 also consisted of menaquinone.

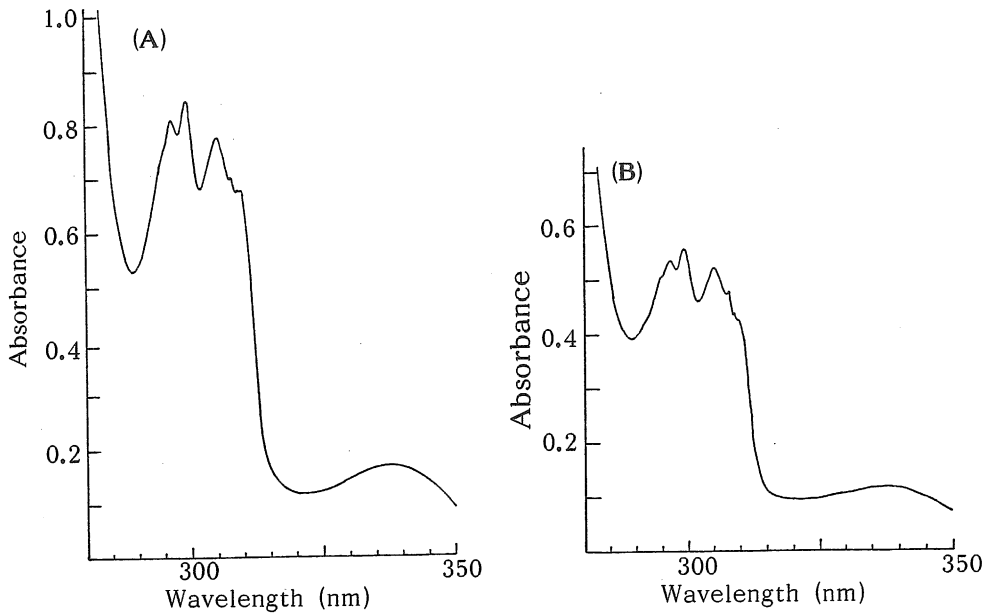

Fig. 6. Absorption spectra of quinones from *B.* sp. I-1 (A) and *B. subtilis* (B). Quinones from both the two strains were extracted and purified as described in Materials and Methods.

Table 2. Effect of respiration inhibitors on TTC reduction activity

	OD at 485 nm			
	Inhibitors			
Substrate	none	TTA	HQNO	KCN
None	0.004	ND*	ND	MD
NADH	1.155	1.208	0.840	0.770
Succinate	0.014	0.008	0.009	0.010

The concentratins of inhibitor used in this experiment were as follows: KCN, 10 mM; HQNO, 2μM; TTA, 1 mM.
*ND: not determined

Effect of the inhibitors on TTC reduction activity in cell-free systems.

TTC reduction activity by cell-free systems of *B.* sp. I-1 was measured by addition of some inhibitors on electron transport systems (Table 2). Reduction of TTC was only observed when NADH was used for the substrate. The inhibitory effects of HQNO, TTA and KCN on the activity of TTC reduction were lower than those on NADH oxidase activity (about 85–90% inhibition, data not shown).

Discussion

TTC is generally used as the indicators in redox reaction of biological systems. For example, the reduction activity of TTC is used as the indicator for screening of respiration activity deficient mutant, because cytochrome oxidase and flavoproteins reduce TTC immediately (Nachlas *et al.*, 1960, Sato & Sato, 1965). Thus, it is assumed that the activity of TTC reduction perhaps is dependent on a part of the respiration activity.

The TTC reduction activity of *B. subtilis* was very low than that of *B.* sp. I-1 at pH 8, while the respiration activity of *B. subtilis* was equal to that of *B.* sp. I-1. We propose a possibility that *B.* sp. I-1 may have the unique respiratory chain reducting TTC effectively which have the special array of respiratory chain components and/or high activity of enzymes. Lewis *et al.*, (1980, 1981) reported that the cytochrome content in membrane of alkalophiles were higher than other conventional neutrophyles. Further, they reported that the non-alkalophilic mutant, a derivative of *B. alcalphilus,* has lower quantities of membrane bounding cytochromes than its wild-type parent. We also found that the content of cytochromes of *B.* sp. I-1 was higher than that of *B. subtilis.* The respiratory chain components of *B.* sp. I-1 and *B. subtilis* were differenced in each other, while both the respiratory and growth rates of the two strains did not differ at the optimum pH values, respectively. This result suggests that there are no significant relation between contents of cytochrome (or quinone) and maximum rate of growth (or respiration). Jones *et al.*, (1975, 1977) have suggested that H^+/O ratios of bacteria are greatly depending upon the specific array of cytochromes and other respiratory chain components. High quantity of cytochromes and quinones in the alkalophilic membranes may suggest an adaptation, from evidence that it is able to grow at the alkaline pH.

In the cell-free systems, TTC reduction activity was accelerated by the addition of NADH. Further, the inhibitory effects of HQNO on the activity of TTC reduction were lower than those on NADH oxidase activity. Thus, it may be possible that the TTC reduction activity in *B.* sp. I-1 is depended on the activity of NADH dehydrogenase.

It is interesting that the reduction activity of TTC increases at alkaline pH range when *B.* sp. I-1 was grown without shaking. *B.* sp. I-1 may have the special site (coupling with NADH dehydrogenase) in respiratory chain to reduce TTC (or indigo)

and enable to grow at an anaerobic culture condition at alkaline pH range. To elucidate this possibility, further investigations are in progress.

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