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Identification of Respiratory Quinones from an Alkalophilic Bacterium, *Bacillus* sp. S1-3

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The respiratory quinones of an alkalophilic *Bacillus* sp. S1-3 were investigated. Quinones of the *B.* S1-3 were extracted and purified by reverse-phase partition high performance liquid chromatography (HPLC) following thin-layer chromatography. Similar to other members of the genus *Bacillus*, the *B.* S1-3 also contained menaquinones as a sole respiratory quinone. However, the main menaquinone type produced by *B.* S1-3 was the menaquinone with six isoprenoid units (MK-6) instead of MK-7, which is believed to be the main menaquinone type found in the genus *Bacillus*.

Introduction

The obligate alkalophilic bacteria grow at pH values above 10.0. At high alkaline pH values, the cytoplasmic pH is maintained at or below pH 8.6 owing to the Na^+/H^+ antiporter (Mandel *et al.*, 1980). The bioenergetic results of the antiporter function cause a decrement of the $\Delta\bar{\mu}_{H^+}$ and a simultaneous increment of the $\Delta\bar{\mu}_{Na^+}$. It is reasonable, therefore, that the alkalophilic bacteria use $\Delta\bar{\mu}_{Na^+}$ instead of $\Delta\bar{\mu}_{H^+}$ to operate solute uptake and flagellar rotation (Sugiyama *et al.*, 1986). Utilization of $\Delta\bar{\mu}_{Na^+}$ for bioenergetic works is also reported in alkalotolerant marine *Vibrio* which possesses a sodium cycle consisting of respiration-driven primary Na^+ pump and Na^+-coupled ATP synthesis mechanism (Skulachev, 1989; Unemoto *et al.*, 1990). In alkalophilic *Bacillus*, however, the sodium cycle is not demonstrated yet for the present.

Recently, we found that NADH oxidase activity of an alkalophilic *Bacillus* sp. S1-3 was stimulated by Na^+ (Oshima *et al.*, unpublished data). It is well known that the stimulation of the enzyme activity is one hallmark of the presence of respiration-driven primary Na^+ pump (Tokuda & Unemoto, 1984). Thus we expected that the alkalophilic *B.* S1-3 also has respiration-driven primary Na^+ pump. In some bacteria that has respiration-driven Na^+ pump, the site of Na^+ transport is mainly located in NADH-quinone reductase (NADH-QR) segment (Unemoto *et al.*, 1992). As for the electron acceptor of the enzyme reaction, some bacteria use ubiquinone (UQ), most others (mainly gram-positive ones) use menaquinone (MK) and a few others, such as *Escherichia coli* use both (Collins & Jones, 1981). Consequently, to investigate the characteristics of the NADH-QR from *B.* S1-3, identification of the native electron

acceptors for the enzyme reaction *in vivo* is required. Here we report the respiratory quinones from an alkalophilic bacterium, *B.* S1-3.

Materials and Methods

Strain and growth conditions

The alkalophilic *Bacillus* sp. S1-3 isolated from soil was used in this experiment. The growth medium used in this study was P-medium contained 0.5% of polypeptone, 20 mM glycerol and 1% of Na_2CO_3 (pH 10.5). Cells were grown aerobically with shaking at 37°C.

Extraction of quinones

Quinones were extracted by the method of Yamada *et al.* (1969) with minor modification. Cells in the logarithmic phase of growth were harvested and washed twice with 20 mM Tris-HCl (pH 7.8). The washed cells (fresh weight was about 2 g) were suspended in 20 ml of diethyl ether/ethanol (3 : 1, v/v) and sonic oscillated for 30 min. Then, liquid layer was collected after centrifugation and the residual pellet was reextracted with 20 ml of diethyl ether/ethanol (3 : 1, v/v). The combined liquid layer was diluted with equal volume of distilled water and the upper diethyl ether layer was collected. The diethyl ether layer was dried over anhydrous sodium sulfate and evaporated. The residues were dissolved in a small volume of acetone and stored at −70°C until use.

Purification of quinones

The extracted quinones were purified by thin-layer chromatography on a silica gel plate (Merk 60F254) with petroleum benzene (bp 60～80°C)/diethyl ether (9 : 1, v/v) as a developing solvent. After the development, spots of quinones on silica gel plate were detected by irradiation with UV light (254 mm) and then scraped off. The quinones were extracted from the silica gel with acetone, evapolated to dryness, and resuspended in a small volume of acetone.

Identification of quinones

The purified quinones were separated by reverse-phase partition high performance liquid chromatography (HPLC) with a pre-packed Shim-pack CLC-ODS column (6.0× 150 mm, Shimadzu Corp.) according to the method of Tamaoka *et al.* (1983). The samples were eluted with methanol/isopropyl-ether (9 : 1, v/v) at a flow rate of 1 ml/min. Quinones were detected with a UV monitor, Shmadzu Spectrophotometric Detector SPD-6AV, at 270 nm.

Chemicals

Vitamin K1 was purchased from Nacalai Tesque. Menaquinone-7 and menaquinone-8 were prepared from *Bacillus subtilis* and *Escherichia coli* K-12, respectively, according to the method of Yamada *et al.* (1969). All other reagents used were of analytical grade.

Results and Discussion

To analyze the respiratory quinones from *Bacillus* sp. S1-3, we extracted the quinones from the cells and purified by thin-layer chromatography. Figure 1 shows a thin-layer chromatogram of the quinones extracted from *B.* S1-3. One spot that had same mobility as vitamin K1 was detected. In UV absorption spectrum (Fig. 2), the spot represented characteristics of menaquinones (Dunphy & Brodie, 1971). Menaquinones extracted from silica gel were further purified by reverse-phase partition

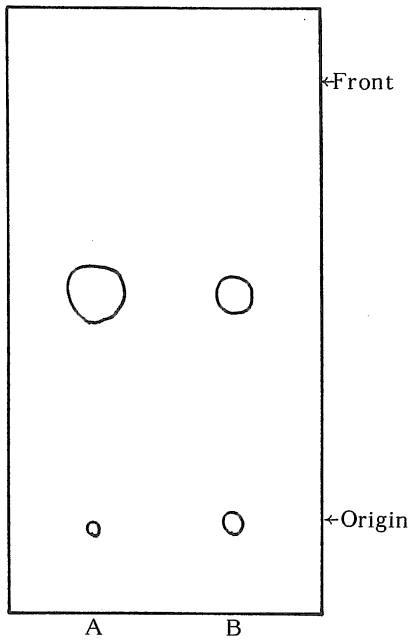

Fig. 1. Thin-layer chromatogram of quinones extracted from *Bacillus* sp. S1-3. A. vitamin K1; B. quinones extracted from *Bacillus* sp. S1-3.

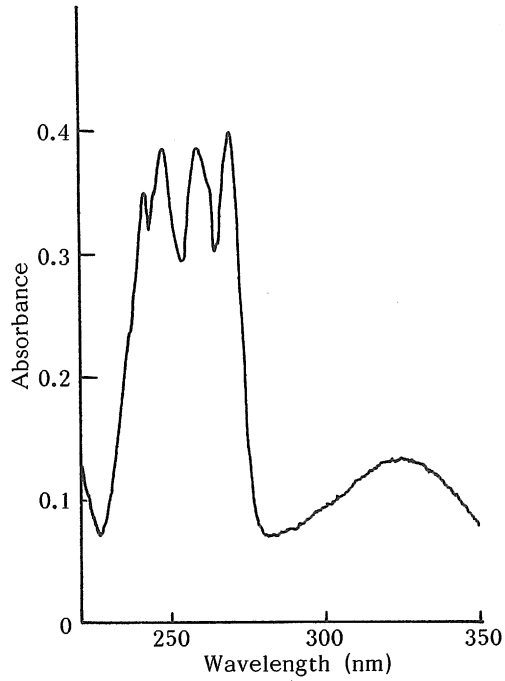

Fig. 2. UV absorption spectrum of the quinones. Quinones were extracted from silica gel and analyzed with UVIDEC-510 double beam spectrophotometer (Japan Spectroscopic Co., Ltd.). *n*-Hexane was used for the solvent.

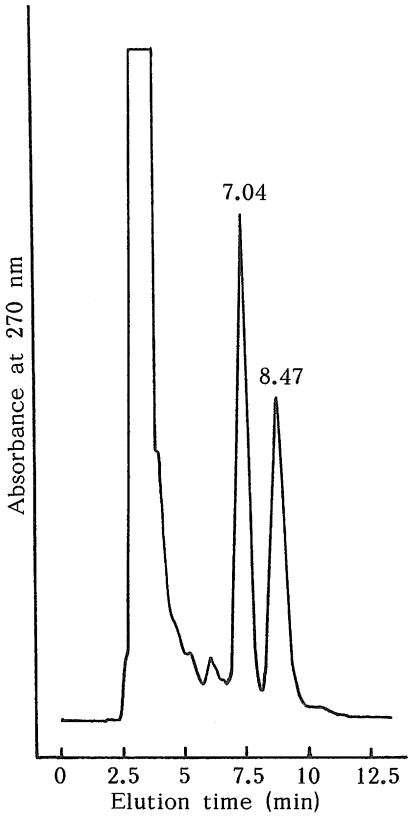

Fig. 3. Separation of menaquinones from *Bacillus* S1-3 by reverse-phase partition high performance liquid chromatography. The sample dissolved in acetone was injected at 0 time and arrowheads indicate the elution time.

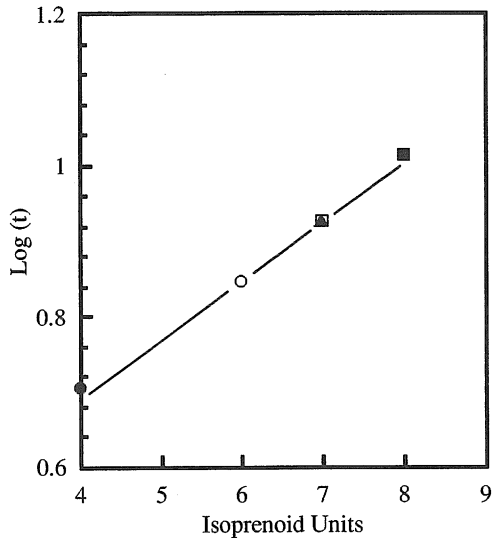

Fig. 4. The relationship between common logarithm of the elution time (t) and the number of isoprenoid units of menaquinones. The MK-4 (●), MK-7 (▲), MK-8 (■) and menaquinones from *Bacillus* S1-3 (○, □) were eluted under the same conditions as Fig. 3. Elution times of MK-4 (●), MK-7 (▲), MK-8 (■) and menaquinones from *Bacillus* S1-3 (○, □) were 5.10, 8.48, 10.34, 7.04 and 8.47 min, respectively.

HPLC. Major peaks were eluted at 7.04 and 8.47 min after injection of the sample, and the relative concentrations of the former and the latter peaks were 56% and 43%, respectively (Fig. 3). Tamaoka *et al.* (1983) reported that the common logarithm of the elution time vs. the number of isoprenoid units gives a near straight line. Then we tried to identify the menaquinone species isolated from *B.* S1-3 by the number of isoprenoid units using menaquinone-7 (MK-7), menaquinone-8 (MK-8) and vitamin K1 (MK-4) as standards. Figure 4 shows that the plot gives a near straight line, and then the peaks eluted at 7.04 min and 8.47 min were identified as menaquinone-6 and menaquinone-7, respectively.

While there have been a number of comparative studies projected to assess the

value of quinones in microbial taxonomy (Yamada *et al.*, 1976; Watanabe & Aida, 1972; Collins & Jones, 1981), our primary interest of quinones is in the bioenergetic function of these compounds in alkalophilic *Bacillus* and not in their value as taxonomic markers. Because quinones play an important role in the respiratory chain as the electron acceptor from NADH, that is, NADH-quinone reductase (NADH-QR) is an energy-transducing enzyme that produces $\Delta\bar{\mu}_{H^+}$ by electrogenic H^+ movement coupled with downhill redox reaction. Further, Tokuda & Unemoto (1984) reported that in *V. alginolyticus*, the NADH-QR segment in the NADH oxidase, which is stimulated by Na^+, functions as the electrogenic primary Na^+ pump and generates $\Delta\bar{\mu}_{Na^+}$ instead of $\Delta\bar{\mu}_{H^+}$. Recently, we also found that the NADH oxidase of *B.* S1-3 is also stimulated by Na^+ (Oshima *et al.*, unpublished data). Thus we expected the presence of respiration-dependent Na^+ pump in *B.* S1-3. From our results, it was confirmed that MK-6 and MK-7 are the native quinones in the respiratory chain of *B.* S1-3. Therefore, these quinones are the appropriate acceptor of NADH-QR from *B.* S1-3. However, it will be difficult to use these menaquinones for the acceptor of NADH-QR, because menaquinone with long isoprenoid units is too hydrophobic to dissolve in assay mixture. In *Vibrio alginolyticus*, ubiquinone-1 (Q-1) was used instead of their native quinones for the measurement of NADH-QR activity (Tokuda & Unemoto, 1984). Thus menaquinone with short isoprenoid units should be used for the measurement of the NADH-QR activity in *B.* S1-3.

As mentioned above, several works on quinones as taxonomically useful characters have shown the importance of the length and the hydrogenation of the side-chain (Yamada *et al.*, 1876; Collins *et al.*, 1981). From these works, it is accepted that menaquinone with seven isoprenoid units (MK-7) is the major quinone type and MK-6 and MK-3 are the minor quinone types found in representatives of the neutrophilic genus, *Bacillus*. In the present study, however, major quinone observed in alkalophilic *B.* S1-3 was MK-6 instead of MK-7. Since there are few reports concerning with the respiratory quinones of alkalophilic *Bacillus* (Lewis *et al.*, 1981; Oshima *et al.*, 1988), further study is required to determine whether the predomination of MK-6 is one of the characteristics of alkalophilic *Bacillus* or not.

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