Production of Menaquinone (Vitamin K₂)-5 by a Hydroxynaphthoate-resistant Mutant Derived from Flavobacterium meningosepticum, a Menaquinone-6 Producer

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A 1-hydroxy-2-naphthoate-resistant mutant, strain HNA 12-D, which was derived from a menaquinone (MK)-6 producer, Flavobacterium meningosepticum IFO 12535, produced an increased amount of MK with the production of another type of MK in addition to MK-6. The newly formed MK was isolated and identified as MK-5. The total amount of MK reached 55.6 mg/liter of culture broth and 9.19 mg/g dry cells in the ratio of MK-6 and MK-5 of 1:1.7.

The diversity of the types of isoprenoid quinones in microorganisms has been extensively studied with the recent development of analytical tools. Different kinds of menaquinone (MK) homologs were detected in archaeabacteria, mycoplasmas, and gram-negative and gram-positive bacteria with or without minor component(s) in relation to taxonomic groupings. However, a change of the type of MK in the same organism has never been reported.

We have studied the improvement of the MK productivity of Flavobacterium meningosepticum, an MK-6 producer. During the mutation process, a mutant resistant to 1-hydroxy-2-naphthoate (HNA), which was found to be the analog of an intermediate of MK biosynthesis, was obtained. The MK productivity of the mutant was increased with the simultaneous production of another type of MK. The isolation and characterization of the newly formed MK and the MK production by the mutant will be described in the present paper.

MATERIALS AND METHODS

Chemicals. MK-8, MK-7 and MK-6 were prepared from cells of Escherichia coli IFO 3301,2) Bacillus subtilis IFO 30072) and F. meningosepticum IFO 125355) by extraction and thin-layer chromatography according to the methods described previously.5) N-Methyl-N'-nitro-N-nitrosoguanidine (NTG), isopentenol, dimethylallyl alcohol and farnesol were purchased from Aldrich Chemical Co., Ind. Geraniol was from Wako Pure Chemical Industries Ltd. Silica gel (Kiesel gel GF254 Type 60) was from Merek Japan Ltd. All other chemicals were usual commercial preparations and used without further purification.

Microorganism. An HNA-resistant mutant, F. meningosepticum HNA 250, derived from F. meningosepticum IFO 12535 in a preceding study,2) was used as the parent strain in this study.

Medium and cultivation. Glycerol-Polypepton medium used as the bacterial medium in a preceding study4) was used as the basal medium. The addition of prenyl alcohols in methanol solution to the medium, cultivation and harvesting of cells were carried out as described previously.4)

Derivation of mutants. F. meningosepticum HNA 250 was endowed with further resistance of HNA by NTG treatment as described previously.5)

Thin-layer and reversed-phase chromatography. A thin-layer plate of silica gel was prepared by the method described in a preceding paper.5) The thin-layer chromatography was performed with development with a solvent system of benzene-cyclohexane (1:1, v/v). For reversed-phase chromatography, the thin-layer plate was immersed gently in 5% liquid paraffin in n-hexane and stood hori-
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zontally in the air to allow evaporation of n-hexane. Reversed-phase chromatography was performed for the separation of individual MK homologs with development using a solvent system of acetone–water (95:5, v/v) to which a few drops of liquid paraffin had been added. The MK band was detected under UV light (3650 Å).

Analyses. MK was extracted with methanol from cells and determined by high performance liquid chromatography (HPLC).4) Dry cell weight was calculated from the optical density.5) The UV absorption and mass spectra were recorded as described in a preceding paper.5)

RESULTS

Isolation of strain HNA 12-D

A further mutation process using a plate containing HNA, 300 mg/liter, was carried out with a mutant, F. meningosepticum HNA 250, as the parent strain. One strain, which could grow on the plate, was found to produce a new MK compound in addition to MK-6. On HPLC as shown in Fig. 1, the compound showed a retention time (Rt) close to that of MK-6, and the intensity of its absorption at 248 nm was 2 times that of MK-6.

The MK productivity of the mutant was improved and stabilized by mono-cell selection for several times. A strain named F. meningosepticum HNA 12-D, which constantly produces the newly formed MK, was finally obtained.

Isolation of MK-6 and its homolog from strain HNA 12-D

The MK of strain HNA 12-D was isolated according to the method described previously.5) The cells from 14 liters of culture broth were harvested by centrifugation and a cell paste weighing 370 g (76 g as dry cell weight) was obtained. After the isolation process involving extraction with acetone–ethyl ether, partitioning with diethyl ether and thin-layer chromatography, 410.1 mg of MK homologs was finally obtained. About 50 mg of the yellow oil was dissolved in a small volume of acetone and subjected to reversed-phase chromatography. The fraction with the lower Rt value (=0.6) was identical to authentic MK-6. The fraction with the higher value (=0.7) was collected and crystallized once from ethanol. Yellow crystals (18.5 mg) were obtained.

Characterization of the isolated MK homolog

The melting point of the yellow crystals was 36 ~ 38°C. This corresponds to that to MK-5.6)

The UV spectrum of the isolated MK homolog was a typical MK absorption spectrum.5) The molar extinction coefficient (ε) was calculated to be 18,750 at 248 nm in n-hexane for MK-5, ε = 18,800 at 248 nm in n-hexane6).

Figure 2 illustrates HPLC of the authentic and isolated MK homologs. Tamaoka et al. reported that the Rt on HPLC and the number of isoprenoid units of MK homologs show a quantitative relationship: A plot of ln Rt vs. the number of isoprene units gives a straight line.7) Figure 3 shows such a plot. The Rt of the new MK homolog corresponded exactly to that of MK-5.

The mass spectrum in Fig. 4 shows a molecular ion peak at m/z 512, which corresponds to MK-5, and fragment ion peaks at m/z 444 (M+ - 68), m/z 376 (M+ - 68 × 2), m/z (M+ - 68 × 3), m/z 240 (M+ - 68 × 4), m/z 225, m/z 198, m/z 186 and m/z 68. Fragments from 444 to 240 are ascribed to compounds which lost the isoprene units one by one. Three intense peaks at m/z 225, 198 and 186 are characteristic of MK homologs.5)

From these data, the MK homolog newly
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Fig. 2. Chromatographic Behavior of Authentic MK and the Isolated MK Homolog on HPLC.

Fig. 3. (ln Rt vs. Number of Isoprene Units) Plot of Authentic MK and the Isolated MK Homolog.

Fig. 4. Mass Spectrum of the Isolated MK Homolog.

isolated from F. meningosepticum HNA12-D was identified as MK-5.

Production of MK-5 and -6 by strain HNA 12-D

Time course. The time courses of cell growth, and MK-5 and -6 productions of strain HNA 12-D are shown in Fig. 5. The cell concentration in the medium reached the maximum after 24-hr cultivation and then decreased gradually in a similar manner to the case of the wild type strain. The amounts of MK-5 and -6 in the culture broth increased simultaneously and reached the maximum levels at 60 hr. The levels were maintained during the following cultivation period. The maximum yields of MK homologs were 18.3 mg/liter of culture broth and 2.96 mg/g dry cells for MK-6, and 22.1 mg/liter and 3.58 mg/g dry cells for MK-5.

Effect of prenyl alcohols. The effect of prenyl alcohols on the formation of MK-5 and -6 was investigated. Isopentenol, dimethylallyl alcohol, geraniol and farnesol were tested. Among them, isopentenol was the most effective. Table I shows the results of isopentenol supplementation at various concentrations to
the medium. The concentration of isopentenol of $10^{-2} \sim 10^{-3}$ m was effective for the production of MK-5 and -6. This result was similar to that for MK-6 of the wild type strain.\(^4\)

**Effect of L-tyrosine supplementation.** The

| Table I. Effect of Isopentenol on Production of MK by F. meningosepticum HNA 12-D |
|---|---|---|---|---|---|
| Concentration (m) | MK formed |
| | mg/g dry cells | mg/liter |
| | MK-5 | MK-6 | Total | MK-5 | MK-6 | Total |
| 0 | 3.53 | 2.78 | 6.30 | 22.2 | 17.5 | 39.7 |
| $10^{-3}$ | 3.48 | 2.92 | 6.40 | 21.9 | 18.4 | 40.3 |
| $10^{-2}$ | 3.67 | 3.06 | 6.73 | 23.1 | 19.3 | 42.4 |

**Effect of simultaneous addition of isopentenol and L-tyrosine.** Based on the experimental results mentioned above and those obtained with the wild type strain,\(^4\) the effect of combined feeding of isopentenol and L-tyrosine on the increases of the MK-5 and -6 productions was investigated. L-Tyrosine and isopentenol were added at the beginning of the cultivation at concentrations of $0.4\%$ (2.2 $\times 10^{-2}$m) and $10^{-3}$m, respectively. Thereafter, isopentenol was fed at the concentration of $10^{-3}$m to the culture medium after 24- and 48-hr cultivation. The parent strain, HNA 250, was cultivated under the same conditions as a reference. As shown in Table III, the total amount of MK produced by strain HNA 12-D was 55.6 mg/liter of culture broth and 9.19 mg/g dry cells after 72-hr cultivation, whereas the parent strain produced 25.3 mg/liter and 4.14 mg/g dry cells of MK-6.

**DISCUSSION**

An HNA-resistant mutant of *F. meningosepticum*, HNA 12-D, was found to produce two types of MK, MK-5 and -6. The MK system of the wild type strain of the bacterium was
composed of only MK-6 without any minor components.\textsuperscript{4,5} The quantity of MK-5 produced by the mutant was 1-2 times as much as that of MK-6.

HNA is a structural analog of 1,4-dihydroxy-2-naphthoate, an intermediate of MK biosynthesis.\textsuperscript{8} A membrane-associated enzyme of \textit{E. coli} is known to catalyze the prenylation of the naphthalenic intermediate using both solanesyl pyrophosphate and octaprenyl pyrophosphate as substrates.\textsuperscript{9} 1,4-Dihydroxy-naphthoate: polyprenyl pyrophosphate transferase in the membrane fraction of \textit{Micrococcus luteus}, of which the MK system contains MK-6 as a major component, showed a broad substrate specificity with regard to prenyl-donating substrates.\textsuperscript{10} Prenyl pyrophosphates ranging in chain length from C\textsubscript{15} to C\textsubscript{45} were active as substrates. Thus, the spectrum of the chain length of MK in bacteria may not be controlled by the substrate specificity of the prenylation enzyme but may reflect the availability of prenyl pyrophosphates in cells. However, since all series of polyprenyl pyrophosphates, which are shorter in chain length than the intrinsic substrate for the enzyme, should always be present in cells, specificity of the enzyme as to chain length should exist. Polyprenyl pyrophosphate synthetase of a \textit{B. subtilis} that formed only MK-7 synthesized all-trans-heptaprenyl (C\textsubscript{35}) pyrophosphate exclusively.\textsuperscript{11} The enzyme of \textit{M. luteus} produced a variety of polyprenyl pyrophosphates ranging in carbon number from C\textsubscript{15} to C\textsubscript{45} in various ratios, depending on the concentration of Mg\textsuperscript{2+}, but the side chain of MK varied in length from C\textsubscript{35} to C\textsubscript{45} with a predominance of C\textsubscript{40}.\textsuperscript{12,13} Whether the change in the specificity of the enzyme or the supply of polyprenyl pyrophosphates in strain HNA 12-D extended the MK spectrum by the acquisition of HNA resistance remains to be determined.

On the other hand, the cultivation with the supplementation of MK-biosynthetic intermediates suggested the existence of strong regulation of the MK-6 biosynthesis in the wild type of \textit{F. meningosepticum}.\textsuperscript{4} Strain HNA 12-D might be a deregulatory mutant showing overproduction of MK-5 together with MK-6.

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\textbf{REFERENCES}