Menaquinone (Vitamin K₂)-6 Production by Mutants of Flavobacterium meningosepticum

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Summary Flavobacterium meningosepticum IFO 12535, a menaquinone (MK) producer, was mutagenized to improve productivity. A mutant, which was resistant to 1-hydroxy-2-naphthoate (HNA), was found to produce MK more abundantly: 34 mg/liter of culture broth and 5.5 mg/g of dry cells. The mutant was less sensitive to inhibition by HNA on MK biosynthesis than the wild-type strain. MK was isolated from cells of the mutant and identified as MK-6 based on its physicochemical characteristics. Mutants, which were given each KCN resistance, aromatic amino acid auxotrophy and no carotenoid productivity, did not show further increase of productivity.

Key Words Flavobacterium meningosepticum, 1-hydroxy-2-naphthoate, menaquinone, vitamin K₂

Mutational improvement of cells has contributed to the development of microbial processes for the industrial production of useful metabolites. These include a few water-insoluble compounds such as coenzyme Q (I).

In a preceding paper (2), we reported the screening of menaquinone (MK) producers among a variety of microorganisms and the optimization of culture conditions for the intracellular production of MK by Flavobacterium meningosepticum. MK production was repressed by the addition of compounds related to MK biosynthesis to the culture medium.

In the present study, the MK productivity of F. meningosepticum was improved by mutagenization. A mutant resistant to 1-hydroxy-2-naphthoate (HNA), which was found to be an inhibitor of MK biosynthesis but not of growth (2), was obtained as a high MK producer. Identification of MK in the mutant cells and attempts at further improvement of the mutant are also described.
EXPERIMENTAL

Chemicals. HNA was purchased from Tokyo Kasei Kogyo Co., Ltd. Isopentyl alcohol and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) were from Aldrich Chemical Co., Inc. Phylloquinone (vitamin K₇) was from Nakarai Chemicals, Ltd. MK-8 and demethyl MK-8, and MK-7 were prepared from cells of *Escherichia coli* IFO 3301 and *Bacillus subtilis* IFO 3007 (3), respectively, by the method described below. Silica gel used was Kieselgel GF₂₅₄ Type 60 from Merck Japan Ltd. All other chemicals were usual commercial preparations and used without further purification.

Microorganism. *F. meningosepticum* IFO 12535, which was selected as the highest MK producer in a preceding study (2), was used in this study.

Media and cultivation. Glycerol-Polypepton medium used for bacteria in a preceding paper (2) was used as the basal medium. The minimal medium contained 10 g of glycerol, 0.5 g of L-proline, 0.5 g of L-threonine, 2 g of NaCl, 5 g of K₂HPO₄, 2 g of KH₂PO₄, 0.1 g of MgSO₄·7H₂O, 0.002 g of Mn₅O₆·6H₂O and 0.001 g of FeSO₄·7H₂O in 1 liter of distilled water, pH 7.0. The medium for isolation of MK was the basal medium supplemented with 4 g/liter of L-tyrosine, to which isopentyl alcohol was added at a concentration of 10⁻³ M each at 0, 24 and 48 h of cultivation time. HNA and isopentyl alcohol in ethanol solution and KCN in aqueous solution were separately sterilized by filtration. The ethanol solution was added to the medium to within 1% by volume.

The cultivation and harvest of cells were carried out as described previously (2) unless otherwise noted. Cell yield was calculated by the formula: g dry cell weight/liter of culture broth = optical density at 610 nm × 0.63. This calculation could be used at optical density between 3 and 13.

Determination of MK in cells. MK was extracted with methanol from cells and determined by high performance liquid chromatography (HPLC) as described previously (2).

Derivation of mutant. Washed cells from the logarithmic growth phase in the basal medium were suspended in 0.1 M potassium phosphate buffer, pH 7.0, or physiological saline at a concentration of 10⁸ cells/ml.

HNA- and KCN-resistant mutants were derived by NTG treatment: Cells in phosphate buffer were shaken in the presence of NTG (100 mg/liter) for 30 min at 37°C, where the survival ratio was 1%. The cells were harvested and washed twice with the same buffer by centrifugation at 8,000 × g for 15 min, and then spread on an agar plate of the basal medium supplemented with HNA or KCN. Colonies which appeared during 5–6 days incubation at 28°C were picked up.

Aromatic amino acid auxotrophs were derived by UV treatment: Cells in physiological saline were irradiated with UV for 15–30 s at the lethal ratio of 99.9–99.99%. Surviving cells were cultivated in the dark on the basal medium for 36 h, harvested by centrifugation and then suspended in the minimal medium at a concentration of 10⁶ cells/ml. After 12-h starvation culture, ampicillin was added to
the medium at a concentration of 300 mg/liter. The cells were harvested and washed twice with physiological saline by centrifugation after 6-h cultivation, and then transferred to the minimal medium supplemented with 0.1 g/liter of L-tryptophan, L-phenylalanine and L-tyrosine. Further cultivation was carried out until full growth. The enrichment cycle was repeated twice. Aromatic amino acid auxotrophs were isolated by a replica plating method.

Carotenoid pigmentless mutants were obtained during the derivation of mutants resistant to HNA (350 mg/liter). F. meningosepticum loses its yellow color when grown in the dark, but pigment productivity is restored by a brief exposure to light. Colonies without carotenogenesis after 1-day exposure to light were picked up as carotenoidless mutant.

Isolation of MK. To a cell paste weighing about 100 g, 1 liter of acetone–ethyl ether (4:1, v/v) was added and agitated in a mini-jar fermentor (Mitsuwaseiki KMJ-2) for 2 h. Solid materials were filtered and extracted again in a similar manner. Combined extracts were evaporated under reduced pressure to less than 10% in volume. Each 10 ml of the concentrate was partitioned between 500 ml of diethyl ether and 500 ml of NaCl-saturated water. The nonpolar lipids fraction obtained was dried with anhydrous Na₂SO₄ and then evaporated. Resultant oily materials were dissolved in chloroform and applied on a thin-layer plate which was prepared by spreading a silica gel suspension (40 g/80 ml H₂O) on glass plates at a thickness of 0.75 mm, followed by activation at 110°C for 2 h after drying overnight at room temperature. Thin-layer chromatography was performed with development with a solvent system of cyclohexane–benzene (1:1, v/v). The MK band was detected under UV light (3650 Å) and scraped. MK was eluted with chloroform and crystallized twice from ethanol.

Measurements of UV, proton NMR, mass spectra and HPLC. UV absorption spectra were recorded with a Hitachi 200-10 double beam spectrophotometer; the sample was dissolved in n-hexane. Proton NMR spectra were measured with a JEOL-PMX 60 NMR spectrometer; the sample was dissolved in CDCl₃ and tetramethylsilane was used as the internal standard. Mass spectra were recorded with a Hitachi double focusing mass spectrometer M-80 at 70 eV ionizing potential; the sample was vaporized at the ion source with a heated direct inlet system operating at 180°C. HPLC was carried out as described previously (2).

RESULTS

Derivation of HNA-resistant mutants

Strain A-24-6, which was selected from F. meningosepticum IFO 12535 by mono-cell division according to its enhanced and stabilized productivity of MK (identified as MK-6 as described below), was used as the parent strain for the following experiments. When the cells (10⁷) were spread on the basal medium plate and incubated at 28°C for 5–6 days, growth was almost completely inhibited by addition of 200 mg/liter HNA.

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Table 1. Production of MK-6 by *F. meningosepticum*.
The basal medium was used for cultivation. Cultural conditions and the determinations of MK and cell mass are described in EXPERIMENTAL.

<table>
<thead>
<tr>
<th>Strain of <em>F. meningosepticum</em></th>
<th>MK-6 formed (mg/g dry cell)</th>
<th>MK-6 formed (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type IFO 12535</td>
<td>1.75</td>
<td>14.1</td>
</tr>
<tr>
<td>A-24-6</td>
<td>1.98</td>
<td>15.7</td>
</tr>
<tr>
<td>HNA 200</td>
<td>2.09</td>
<td>16.8</td>
</tr>
<tr>
<td>HNA 250</td>
<td>2.30</td>
<td>18.5</td>
</tr>
<tr>
<td>HNA 300</td>
<td>2.58</td>
<td>20.8</td>
</tr>
<tr>
<td>HNA 350-22</td>
<td>2.89</td>
<td>23.0</td>
</tr>
</tbody>
</table>

Fig. 1. Production of MK-6 by the wild-type strain and mutant HNA 350-22. Both strains were cultivated on the basal medium for 72 h. The cultivation and analysis are the same as in Table 1. Isopentyl alcohol (isopentenol) ($10^{-3}$ M) was added at 0, 24, 48 h of the cultivation time, and tyrosine (0.4%) at 0 h.

Thirty mutants resistant to 200 mg/liter HNA were obtained from strain A-24-6. Five of them showed higher productivity than the parent strain. One strain, HNA 200, was employed for the next mutation, where the concentration of HNA was increased to 250 mg/liter. In this manner, the concentration of HNA was next increased to 300 mg/liter and then to 350 mg/liter.

The MK productivity of mutants in each step is shown in Table 1. Mutant HNA 350-22, resistant to 350 mg/liter HNA, was used for the following experiments.

*Production of MK-6 by strain HNA 350-22*

Strain HNA 350-22 was cultivated in the basal medium with or without the addition of L-tyrosine and isopentenol, which were effective on MK production of the wild-type strain (2). As shown in Fig. 1, the MK-6 produced reached 34 mg/liter.
MICROBIAL PRODUCTION OF MENAQUINONE-6

Fig. 2. Effect of HNA on production of MK-6 by the wild-type strain (A) and mutant HNA 350-22 (B). Both strains were cultivated on the basal medium with (dotted lines) or without (solid lines) the addition of $10^{-4}$ M HNA. The cultivation and analysis are the same as in Table 1.

of culture broth and 5.5 mg/g of dry cells.

**Characterization of strain HNA 350-22**

Growth of the wild-type strain was not inhibited in the presence of $10^{-4}$ M HNA (Fig. 2). However, the cellular content of MK-6 decreased to about 40%. The content of strain HNA 350-22, which is resistant to $1.9 \times 10^{-3}$ M HNA, decreased to about 70% under the same culture conditions. This shows that the high MK productivity of the mutant might be due to the reduced sensitivity of MK biosynthesis to HNA.

**Identification of MK produced by strain HNA 350-22**

Crystalline MK (220.4 mg) was obtained from cells of strain HNA 350-22 (74 g dry cell weight) at a yield of 75%. MK loss occurred in the recrystallization step. The melting point of the yellow crystals was 49.0°C, corresponding to that of MK-6 (4).

The UV spectrum of the isolated MK showed $\lambda_{\text{max}} = 243, 248, 261, 270$ and 325–328 nm. The molar extinction coefficient ($\epsilon$) was calculated to be 18,600 at 248 nm in n-hexane. These coincide well with the properties of MK-6 (4).

The retention time of the isolated MK on HPLC was between phylloquinone, and MK-7, demethyl MK-8 and MK-8 (Fig. 3). This indicates that the MK isolated has a C-3 polyprenyl chain of the isoprenyl unit shorter than 7 and longer than 5 (5). The isolate appears to be chromatographically pure.

All bands observed in the proton NMR spectrum of the isolated MK ($\delta 7.85, 5.08, 3.37, 2.17, 2.00, 1.80, 1.67$ and $1.60$ ppm) accorded well with those reported for MK (4). No appearance of the band in the range of $\delta$ below 1.5 ppm shows the absence of the saturated hydrocarbon. The ratio of protons for the $-\text{CH}=\text{C}$ ($\delta 5.80$) and those for the $\text{CH}_3$-quinone ring ($\delta 2.17$) was 5.8:1, indicating the
Fig. 3. HPLC of methanol extract of *F. meningosepticum* HNA 350-22 cells. PK, phylloquinone; DMK, demethylmenaquinone.

Fig. 4. Mass spectrum of MK isolated from *F. meningosepticum* HNA 350-22.

The mass spectrum in Fig. 4 shows a molecular ion peak at $m/z$ 580 and fragment ion peaks at $m/z$ 512 ($M^+ - 68$), $m/z$ 444 ($M^+ - 68 \times 2$), $m/z$ 376 ($M^+ - 68 \times 3$), $m/z$ 308 ($M^+ - 68 \times 4$), $m/z$ 240 ($M^+ - 68 \times 5$), $m/z$ 225, $m/z$ 198, $m/z$ 186 and $m/z$ 68. Fragments from 512 to 240 are ascribed to compounds which have lost isoprene units one by one. Three intense peaks at $m/z$ 225, 198 and 186 are characteristic of MK homologs (6).

From these results, the MK of strain HNA 350-22 was identified as MK-6. The quinone system of *F. meningosepticum* ATCC 13253 is known to be MK-6 (7).

**Effect of KCN on MK production**

The MK-6 content of mutant HNA 300 increased when grown in basal medium supplemented with KCN, an electron transport inhibitor, and replaced...
glycerol with succinate, as the concentration of KCN increased. This led us to the derivation of KCN-resistant mutant. Growth of the mutant was almost completely inhibited on the basal medium plate containing 200 mg/liter of KCN. Some mutants resistant to 2 g/liter of KCN showed a slight increase in MK productivity. However, the addition of KCN (0.5 mg/liter) to the culture medium did not increase productivity.

**Derivation of aromatic amino acid auxotrophs**

Five mutants, which were derived from strain HNA 300 as tryptophan auxotroph, tyrosine auxotroph, tyrosine and phenylalanine auxotroph, and tryptophan, tyrosine and phenylalanine auxotroph, respectively, showed a slight increase in the cellular content of MK-6. Due to the decrease of growth, the amounts of MK in the culture of these mutants were almost similar that of the parent. The MK production of all mutants was somewhat increased by addition of L-tyrosine.

**Carotenogenesis and MK production**

The wild-type strain did not form carotenoid when grown in the dark. On the contrary, carotenoids were produced and the cellular content of MK-6 was low when grown with exposure to light. Carotenoidless mutants, which were derived from strain HNA 300, produced less MK-6 and fewer cells.

**DISCUSSION**

1,4-Dihydroxy-2-naphthoate (DHNA) is synthesized as the first naphthalenoid intermediate of MK biosynthesis (8) and then prenylated by catalysis of a membrane-associated transferase (9). In the way, MK is led to the membrane ruling as a respiratory quinone. HNA is a structural analog of DHNA and inhibits the MK production of F. meningosepticum (2). An HNA-resistant mutant was obtained as a high MK producer. The strain was less sensitive to the inhibition of HNA on MK biosynthesis. Whether the increase of the activity or the desensitization to feedback control of the enzyme in the biosynthesis contributed to the increased productivity remains to be determined.

Aerobic growth of *Escherichia coli* with an oxidizable substrate such as succinate in the presence of KCN led to a nine-fold increase in the MK content (10). Cyanide was reported to protect the cell viability and MK in cells against photodynamic action since the oxidized form of membrane-bound MK is more photo labile in visible light than the reduced form and cyanide ion changes the MK into the reduced form (11). No significant increase of the MK production was attained by cultivation of KCN-resistant mutants in the presence of a high concentration of KCN in the present study.

Biosyntheses of MK and aromatic amino acids share a common route until chorismate. Previously, it was found that the amount of MK increased with an
addition of L-tyrosine or \( p \)-hydroxyphenylpyruvate to the culture medium (2). No prominent increase of MK productivity was obtained by use of aromatic amino acid auxotrophs employed here.

Carotenoid pigments and the polyisoprenyl side chain of MK are biosynthesized the same way from mevalonate. The carotenogenesis and the decrease of MK content of \( F. \) meningosepticum when grown on exposure to light might be due to diversion of metabolic intermediates from carotenoid synthesis to the synthesis of the MK side chain. The decrease in MK productivity of carotenoidless mutants might be explained by the role of carotenoids protecting MK from breakdown by photodynamic action (12).

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REFERENCES


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