Menaquinone-4 Production by a Mutant of *Flavobacterium* sp. 238-7

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A considerable amount of menaquinone (MK)-4 was found in cells of a 1-hydroxy-2-naphthoate-resistant mutant, strain HNA 250-15, which was derived from *Flavobacterium* sp. 238-7, in which MK-6 is the major isoprenoid quinone. The MK-4 productivity was further improved by making the mutant resistant to usnic acid and menadione. The amount of MK produced by the resultant mutant, strain K₃-15, produced 125.4 mg/l of culture broth and 12.8 mg/g of dry cell weight, in the ratio of MK-4 and MK-6 of 6:1, under the optimal culture conditions in the presence of cedar wood oil.

Previously, we reported that *Flavobacterium meningosepticum*, which contains menaquinone (MK, vitamin K₂)-6¹⁾ as the component of isoprenoid quinone, newly acquired the ability to produce MK-5²⁾ by making it resistant to 1-hydroxy-2-naphthoate (HNA), an analog of the biosynthetic intermediate of MK. It was the first case of a change in the MK pattern in the same microorganism. During further study to develop a new fermentative process for MK production, an HNA-resistant mutant of *Flavobacterium* sp. 238-7, which produced MK-6 as the major component of respiratory quinone with some minor components, showed an increase in MK productivity due to the increased production of a minor component of MK. The present paper deals with the identification of the newly formed MK as MK-4, which is provided for clinical use by a chemical process, and the improvement of the MK-4 production by derivation of a mutant and optimization of the cultural conditions to improve the MK-4 production.

MATERIALS AND METHODS

Chemicals. Usnic acid, menadione (vitamin K₃), linoleol, tiglic acid (2-methylcrotonate) and cedar wood oil were purchased from Wako Pure Chemical Industries,

Ltd., and diphenylamine and α -pinene from Nakarai Chemicals, Ltd. All other chemicals were prepared or purchased as described previously.^{1~3)}

Microorganisms. *Flavobacterium* sp. 238-7, which was first isolated as a potent vitamin B-6 producer⁴⁾ and showed high MK productivity,³⁾ was used as the parent strain in this study.

Medium and cultivation. The glycerol-Polypepton (Daigo) medium used as the bacterial medium in a preceding study³⁾ was used as the basal medium. The addition of HNA and prenol alcohols to the medium, cultivation and harvesting of cells were carried out as described previously.^{1,3)}

Derivation of mutants. The *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and UV treatments for mutagenization were performed in principle in the same way as for *F. meningosepticum*.¹⁾

Analyses. MK was extracted with methanol from cells and determined by high performance liquid chromatography (HPLC).³⁾ Dry cell weight (DCW) was calculated from the optical density using a calibration curve. Physicochemical analysis of MK homologs was performed with measurement of UV and mass spectra.¹⁾

RESULTS

Production of MK-4 by an HNA-resistant mutant

HNA was found to inhibit the MK biosynthesis of *F. meningosepticum* as a possible ana-

log of 1,4-dihydroxy-2-naphthoate (DHNA), a biosynthetic intermediate of MK.³⁾ The MK content of *Flavobacterium* sp. 238-7 was also reduced by cultivation in the medium containing HNA. In the presence of 10^{-4} M HNA, the MK content decreased to 60% of that in the case of growth without HNA, but the growth was, however, hardly affected. Then, improvement of the MK productivity of *Flavobacterium* sp. 238-7 was attempted by deriving HNA-resistant mutants. During the mutation process, one strain, which could grow on a plate supplemented with HNA of 300 mg/l, was found to produce a considerable amount of another MK compound in addition to the major component of the major component of the wild type strain (MK-6). After mono-cell selection several times, a strain, HNA 250-15, which constantly produced the newly increased MK was finally obtained. Figure 1 shows the chromatographic patterns on HPLC of extracts from the wild type and mutant strains. The compound that increased in cells of the mutant strain showed a retention time (t_R) corresponding to that of MK-4.

The newly increased MK in strain HNA 250-15 was isolated according to the method described previously.¹⁾ Wet cells (45 g as DCW) obtained from 3 l of culture broth were subjected to the isolation process and 300 mg of MK homologs was obtained. About 50 mg of the yellow oil obtained was dissolved in a small volume of chloroform and then subjected to reversed-phase thin-layer chromatography.²⁾ The fraction showing an R_f value of 0.51 was collected and crystallized once from

ethanol. Yellow crystals (12 mg) were obtained.

The melting point of the yellow crystals was 34.0 to 35.5°C, corresponding to that of MK-4.⁵⁾ The UV spectrum of the isolate was typical for MK. The t_R on HPLC and the number of isoprenoid units of MK homologs show a quantitative relationship: a plot of $\ln t_R$ versus the number of isoprene units gives a straight line.²⁾ HPLC analysis of the authentic and isolated MK homologs showed that the t_R of the isolate corresponded exactly with that of MK-4. The mass spectrum showed a molecular ion peak at m/z 444, which corresponds to MK-4, and fragment ion peaks at m/z 376 ($M^+ - 68 \times 2$), m/z 308 ($M^+ - 68 \times 3$), m/z 240 ($M^+ - 68 \times 4$), m/z 225, m/z 198 and m/z 68. Fragments 376 to 240 were ascribed to compounds formed on the loss of the isoprene units one by one. The 3 intense peaks at m/z 225, 198 and 186 are characteristic of MK homologs. From these data, the isolated MK newly increased in strain HNA 250-15 was identified as MK-4.

MK of the wild type strain was also isolated in a similar manner. Physicochemical analyses of the isolated MK homologs indicated that the major component of MK was MK-6, other minor components being MK-3, MK-4 and MK-5, in the order of t_R on HPLC.

FIG. 1. HPLC of MK from the Wild Type Strain of *Flavobacterium* sp. 238-7 (A) and Strain HNA 250-15 (B).

FIG. 2. Production of MK by the Wild Type Strain of *Flavobacterium* sp. 238-7 (A) and Strain HNA 250-15 (B). The wild type strain of *Flavobacterium* 238-7 and strain HNA 250-15 were grown on the basal medium. The amounts of cell yield (○), MK-4 (▲) and MK-6 (●) were determined as described under MATERIALS AND METHODS.

Figure 2 shows the production of MK-6 by the wild type strain, and MK-4 and MK-6 by strain HNA 250-15. The amounts of MK-6 and MK-4 in cells of strain HNA 250-15 increased simultaneously during cultivation, becoming maximum at around 48 hr. The maximum yields of MK-6 and MK-4 were 19.7 mg/l of culture broth and 2.30 mg/g-DCW, and 6.1 mg/l and 0.71 mg/g-DCW, respectively.

Improvement of MK-4 productivity of strain HNA 250-15

Flavobacterium sp. 238-7 contains MK as the sole quinone in the respiratory chain. Among the uncouplers and respiratory inhibitors tested, an uncoupler, usnic acid, strongly inhibited the growth of strain HNA 250-15 at a low concentration. On subsequent mutagenization of the strain, a mutant strain, USN^r-2, which was resistant to 4 mg/l of usnic acid, was found to produce a much larger amount of MK-4 than strain HNA 250-15, whereas the MK-6 content was reduced. Strain USN^r-2 produced 18.7 mg/l and 2.26 mg/g-DCW of MK-4, and 13.1 mg/l and 1.59 mg/g-

DCW of MK-6, respectively. The productivity of MK-4 was increased more than three times compared with that of strain HNA 250-15.

Growth of *Flavobacterium* sp. 238-7 was strictly inhibited by the addition of 10^{-4} menadione, an artificial derivative of vitamin K, to the culture medium, and the content of MK was reduced in the presence of 10^{-5} M menadione. Derivation of menadione-resistant mutants was then carried out for enhancement of the MK productivity using strain USN^r-2 as the parent strain. Among the mutant strains obtained, strain K₃-15, which was resistant to 20 mg/l of menadione showed the highest contents of MK-4 and MK-6. The strain produced 23.6 mg/l and 3.3 mg/g-DCW of MK-4, and 14.2 mg/l and 2.0 mg/g-DCW of MK-6. The total MK productivity was increased about three times compared with that of the wild type strain.

Production of MK-4 by strain K₃-15

The cultural conditions for MK production was investigated with strain K₃-15 (HNA^r, usnic acid^r and menadione^r).

TABLE I. EFFECTS OF CARBON SOURCES ON MK PRODUCTION BY STRAIN K₃-15

Cultivation carried out for 48 hr on the basal medium containing each carbon source instead of glycerol.

Carbon source (1%, w/v)	Cell yield (g/l)	MK-n produced (mg/l)	
		MK-4	MK-6
Glycerol	8.19	14.7	11.0
Glucose	6.23	4.4	11.6
Fructose	5.09	2.0	6.7
Sorbose	2.64	0.2	3.6
Sucrose	2.80	0.5	4.8
Soluble starch	7.64	6.1	8.5
Ribose	3.42	1.2	5.2
Xylose	3.27	0.8	5.1
Gluconate (Na)	3.58	1.1	5.1
Malate (Na)	4.05	1.5	6.3
Succinate (Na)	3.50	1.6	5.8
Citrate (Na)	4.36	1.2	6.8
Acetate (Na)	4.20	5.9	5.7
Fumarate (Na)	3.90	1.7	6.0

TABLE II. EFFECTS OF NITROGEN SOURCES ON MK PRODUCTION BY STRAIN K₃-15

Cultivation was carried out for 48 hr on the basal medium containing each nitrogen source instead of Polypepton.

Nitrogen source (1.5%, w/v)	Cell yield (g/l)	MK-n produced (mg/l)	
		MK-4	MK-6
Polypepton (Daigo)	7.09	16.0	12.0
Peptone (Mikuni)	6.08	3.6	8.0
Bacto-tryptone	6.63	3.6	8.0
Peptone (BBL)	6.00	2.6	10.3
Proteose peptone (Difco)	6.08	0.4	7.1
Casein (from milk)	4.20	0.4	6.0
Casamino acids	3.42	0.6	4.2
Casamino acids (vitamin-free)	3.73	0.8	3.8
Corn steep liquor	4.83	0.6	6.1
Meat extract	2.64	0.1	2.5
Malt extract	1.01	trace	0.9
Yeast extract	5.61	1.4	8.1
Glutamate (Na)	5.61	1.5	5.6
Soybean hydrolyzate	5.69	0.2	7.1

Effects of carbon and nitrogen sources. The MK production of strain K₃-15 was compared with different carbon sources for cultivation times of at 24, 36 and 48 hr, and the maximum amount in each culture is shown in Table I. Glycerol was the most effective carbon source for both growth and MK production. The high amount of MK in glycerol-grown cells was mainly due to the increased amount of MK-4. The amounts of MK-4 in cells grown on other carbon sources except for acetate were lower than those of MK-6.

Table II shows the effect of organic nitrogen sources on the MK production. Polypepton was the best nitrogen source for both growth and MK production. The high productivity of MK-4 was also observed only for the culture with Polypepton as the nitrogen source as in the case of glycerol as the carbon source.

The MK production varied with the concentrations of glycerol and Polypepton. Among the combinations tested, the amounts of MK-4 and MK-6 were high in cells grown on medium consisting of 2.5% (w/v) glycerol and 2% Polypepton. The amount of MK-4 increased linearly in proportion to the glycerol concentration. Then, the effect of a high concentration of glycerol on the MK production was studied with medium containing 2% Polypepton (Fig. 3). Although the amount of MK-6 scarcely changed with increasing glycerol concentration, that of MK-4 increased

FIG. 3. Effect of the Glycerol Concentration on MK Production by Strain K₃-15.

The strain was grown on the basal medium containing 2% Polypepton and different concentrations of glycerol. ●, MK-4; ○, MK-6.

until 5% glycerol.

Further supplementation of inorganic nitrogen compounds and organic acids to the glycerol-Polypepton medium did not increase the MK production.

Effects of the concentrations of other components of the culture medium. The concentration of K₂HPO₄ strongly affected the MK production, the amount of MK being maximum at the concentration of 0.6%. The production of MK-4 was much more sensitive to a change in K₂HPO₄ concentration than that of MK-6.

An increase in the yeast extract concentration did not influence growth, but the amounts of MK-4 and MK-6 produced increased up to the concentration of 0.3%, and then abruptly decreased.

The effects of the concentrations of NaCl and MgSO₄·7H₂O on the MK production were also investigated. On the addition of 0.4% NaCl and 0.02% MgSO₄·7H₂O, the amount of total MK reached 95.1 mg/l and 9.22 mg/g-DCW.

FIG. 4. Time Course of Production of MK-4 and MK-6 by Strain K₃-15.

The modified basal medium containing 5% glycerol, 2% Polypepton, 0.3% yeast extract, 0.6% K₂HPO₄, 0.4% NaCl and 0.02% MgSO₄·7H₂O, pH 7.0, was used. The strain after preculturing in the basal medium for 24 hr was transferred to 50 ml of the modified basal medium in a 500-ml shaking flask and then cultivated at 28°C. ●, cell yield; ■, MK-4; □, MK-6; ▲, MK-4 + MK-6.

Effects of temperature, pH and aeration.

When the cultural temperature was varied between 25 and 37°C, the total amount of MK and cell yield were found to be maximum at 28°C. The accumulation of MK-4 was more sensitive to a change in the cultural temperature than that of MK-6.

A relatively high amount of MK was obtained on cultivation with an initial pH of 7.0, and also of pH 6.0 and 6.5.

Strain K₃-15 was cultured under different aeration conditions by changing the volume of the medium, between 25 and 150 ml, in a 500-ml shaking flask. The highest cell yield was obtained on cultivation in 25 ml medium, but the production of MK-4 was very low. The highest productivity of MK-4 was observed when the cultivation was carried out with 50 ml medium in the flask. On the other hand, the amount of MK-6 produced was almost the same under all aeration conditions.

Time course of MK production under the optimal culture conditions. Figure 4 shows the time course of the MK production and growth of strain K₃-15 under the optimal culture conditions established above. The cell concentration became maximum at 48-hr cultivation and then decreased gradually. The amount of MK-6 in the culture broth increased with increasing cultivation time and almost became maximum, 30.0 mg/l and 3.0 mg/g-DCW, at 48 hr. However, the accumulation of MK-4 still increased in the stationary and death phases, becoming maximum, 68.6 mg/l and 6.87 mg/g-DCW, at 6 to 7 days.

Effects of various additives. Diphenylamine, linalool and tiglic acid, which were effective for the CoQ₁₀ production by *Pseudomonas* spp.,⁶⁾ and α -pinene were added to the medium as ethanol solutions. α -Pinene and diphenylamine increased the MK-4 production. When α -pinene (0.2 g/l) was added, the total MK content reached 110 mg/l and 11.75 mg/g-DCW. The addition of diphenylamine (5 mg/l) increased the total MK content to 120 mg/l and 12.30 mg/g-DCW. Tiglic and linalool were more or less effective for the MK production.

FIG. 5. Effect of Cedar Wood Oil on MK Production by Strain K₃-15.

The strain was grown on the modified basal medium in Fig. 4 containing cedar wood oil. ●, MK-4; ○, MK-6; ▲, MK-4 + MK-6.

A variety of natural oils were supplemented to the culture medium directly before autoclaving at the final concentration of 0.05%. Among them, cedar wood oil decreased the growth but significantly increased the MK production. Figure 5 shows the effect of the concentration of cedar wood oil on the MK production. MK-4 production increased with increasing cedar wood oil concentration up to 0.04%, whereas the amount of MK-6 decreased. The total amount of MK reached 125.4 mg/l and 12.80 mg/g-DCW.

The effects of prenyl alcohols such as isopentyl alcohol, dimethylallyl alcohol, geraniol and farnesol on the MK production, which stimulated the production of MK by *F. meningosepticum*^{2,3)} and that of CoQ by *Pseudomonas schuylikillensis*,⁷⁾ and which possibly provided the polyprenyl side chain of MK, were investigated. None of the prenyl alcohols increased the MK production of strain K₃-15 at concentrations of 0.5 and 1 mM, in fact, the amount of MK-4 decreased.

Among L-tryptophan, L-phenylalanine, L-tyrosine, anthranilate and phenylpyruvate, only phenyl pyruvate somewhat increased the MK-4 production in contrast with in the case

of *F. meningosepticum*.³⁾

Precursors of the naphthoquinone nucleus and polyprenyl side chain of MK, such as shikimate, *o*-succinylbenzoate, DHNA and mevalonate, were added to the culture medium. These compounds slightly stimulated the MK production at a low concentration, 0.01 or 0.1 mM, but strongly inhibited it at a high concentration.

DISCUSSION

An MK-4 producer was obtained from *Flavobacterium* sp. 238-7, which contained MK-6 as the major component of the isoprenoid quinone system, as an HNA-resistant mutant. Such a change in the MK pattern was first observed with an HNA-resistant mutant of *F. meningosepticum*.²⁾ HNA was found to inhibit the MK biosynthesis³⁾ as a structural analog of DHNA, which is an intermediate of MK biosynthesis.⁸⁾ The MK-4 productivity was improved by further mutagenization leading to resistance to usnic acid and menadione. Usnic acid is an uncoupler isolated from *Usnea barbata*. Since *Flavobacterium* sp. 238-7 contains MK as the sole quinone, it is possible that the mutant might obtain the resistance through an increase in this component of the respiratory chain. Menadione is a synthetic naphthoquinone and exhibits vitamin K activity. But it inhibits mitochondrial phosphorylation by complexing with the functional sulfhydryl groups of enzymes.⁹⁾ The growth inhibition of *Flavobacterium* sp. 238-7 by menadione might be reversed with an increase in MK content on mutation.

The concentration of oxygen in the culture medium might be an important factor for MK production because MK is a component of the electron transport chain.^{2,10)} The MK-4 production required a relatively high oxygen supply but an excess supply led to reduced production. This was different from the case of MK-6 production by *F. meningosepticum*.³⁾

The improvement of MK production on mutagenization and optimization of the cultural conditions is shown in Fig. 6. The MK

FIG. 6. Improvement of MK Production by *Flavobacterium* sp. 238-7.

The left and right bars indicate the amount of MK in mg/l and mg/g-DCW, respectively, the open and shaded bars denoting MK-6 and MK-4, respectively. The amounts of MK produced by each strain indicated are the values when grown on the basal medium, except that K₃-15^a) was grown in the modified basal medium in Fig. 4 and K₃-15^b) in the modified basal medium supplemented with cedar wood oil.

productivity of strain K₃-15 was almost ten times that of the wild type strain. It is noteworthy that the increase MK was due to an increase in MK-4 but not MK-6. MK-4 was a minor component of the wild type strain. The physiological role of MK-4 in the mutant strain is uncertain. In the course of cultivation, the cellular content of MK-4 still increased after the growth had reached the stationary phase. This is similar to in the case of the accumulation of secondary metabolites such as antibiotics.

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